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SUMMARY

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SECONDARY CARDIAC INVOLVEMENT OF MEDIASTINAL T-CELL LYMPHOMA IN A YOUNG SCOTTISH FOLD CAT - CASE REPORT

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Abstract

Mediastinal lymphoma in cats affects the lymph nodes in the cranial mediastinum and/or the thymus. The tumor comprises 10-20% of feline lymphomas. Males appear to be overrepresented. A 2-year-old neutered male Scottish Fold cat with hypertrophic cardiomyopathy treated according to the results of the medical investigation was presented for a second opinion consult. X-ray examination showed a gigantic neoplastic mediastinal mass, and the ultrasound examination revealed a right-sided deviation of the heart axis. Due to the poor prognosis, the cat was euthanized, and the necropsy was performed. Mediastinal T-cell lymphoma with neoplastic invasion into the pericardium and discreetly into the epicardium was diagnosed based on gross pathology, histopathology, and immunohistochemistry. The left atrium presented endocardial fibroelastosis, while the ventricular myocardium showed intercellular edema, myocardocyte laceration, vacuolar and hyaline degeneration, and necrosis in small, isolated areas. Additionally, the lung displayed diffuse collapse, edema, and congestion.

Key words: *fibroelastosis, T- lymphocytes, heart metastasis, CD3, immunohistochemistry.*

INTRODUCTION

Lymphoma (lymphosarcoma) is an aggressive malignant tumor of a group of lymphoreticular cells and often is originated in lymphoid tissues but may also occur in non-lymphoid organs (Shih, Brenn, & Schrope, 2014). The incidence is 0.2%, which accounts for 90% of all diagnosed neoplasms in cats (Burgess, 2020). It often grows as a solitaire tumor, but could be multicentric, non-visceral, or presents visceral involvement.

According to the literature, feline lymphoma is divided by anatomic classifications in mediastinal, leukemic, gastrointestinal, multicentric, and miscellaneous sites, like the kidneys, nasal, laryngeal or tracheal, central nervous system, and skin (Mori et al., 2019; Shih et al., 2014).

Clinical signs are associated with anatomical localization of the primary tumor and its metastases, including dyspnea, lethargy, anorexia, and emesis, or may not show clinical signs until the last stage. In cats, the location of the tumor is a determinant of clinical signs and prognostic (Burgess, 2020).

For feline lymphoma exists a bimodal age pattern distribution. In the young-age group, in addition to the lymph nodes, lymphoma can also affect the thymus in the cranial mediastinum (Alejandro et al., 2019; Sunpongsri et al., 2022). Retrovirus−positive cats present the neoplasm at a young age (1-3 years), and virus−negative cats present at an older age (10-13 years). The median age at tumor development is approximately of 11 years. There is no gender nor breed predilection, but Siamese and Siamese-related breed cats are overrepresented, suggesting a genetic susceptibility to the disease (Alejandro et al., 2019; Burgess, 2020; Seo et al., 2006). However, in some studies, the male had a greater ratio than the female, with a ratio 2.1: 1 (Sunpongsri et al., 2022).

Like in the presented case, mediastinal lymphoma is assumed to appear frequently in young cats (Burgess, 2020; Twomey & Compendium, 2005).

MATERIALS AND METHODS

A Scottish Fold neutered male cat, aged two years, weighing 3.6 kg, with hypertrophic cardiomyopathy treated according to the results of the medical investigation, was presented for a second opinion consult. In its medical records, the cat was diagnosed with hypertrophic cardiomyopathy class C and a small quantity of pleural effusion. Also, a month ago, the blood examination results revealed a high level of aspartataminotransferase, creatin kinase, triglycerides, calcium, and magnesium. The NT-proBNP was 50/pmol/L, and the lymphocytes were under the physiological level. Treatment with amlodipine, torasemide, and clopidogrel was prescribed. At the present consult, the gingival mucosa was pink, and the temperature was 38.6°C. Auscultation revealed diminished pulmonary and cardiac sounds.

Thoracic X-rays were performed with the patient in latero-lateral recumbency, and ultrasound with the patient in latero-lateral left and right recumbency.

Based on imagistic results, a gigantic intrathoracic mass, possibly originating in the thymus or mediastinal or sternal lymph nodes, was suspected. Due to the poor prognosis, the cat was euthanized, and the necropsy was performed.

The post-mortem examination was performed, and samples were collected for histopathological and immunohistochemical examination from all the modified organs. Cytological impression smears of the mediastinal mass were performed, air-dried, and stained with May Grunwald Giemsa (MGG) for light microscopy evaluation. The selected specimens from the free and involved organs were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax, cut into 3 µm sections, and stained with hematoxylin and eosin (HE). For immunohistochemistry, primary antibodies for B-lymphocyte proliferation and differentiation PAX-5 (SP34) Rabbit Monoclonal Antibody were used, and to qualitatively identify T-lymphocytes, CONFIRM anti-CD3 (2GV6) Rabbit Monoclonal Primary Antibody were used. The samples were automatically processed using Ventana BenchMark ULTRA system.

RESULTS AND DISCUSSIONS

Cardio-respiratory diseases often require radiographic and echocardiographic examination for diagnostic approach. In this case, the chest x-ray examination of latero-lateral incidence revealed a severe, diffuse, increased radio-opacity of the cranial and cardiac thoracic region with soft tissue density, in 81 x 57 mm dimension, suggestive of a gigantic mediastinal mass (Figure 1*)*. The trachea was partial collapse in the middle and posterior third.

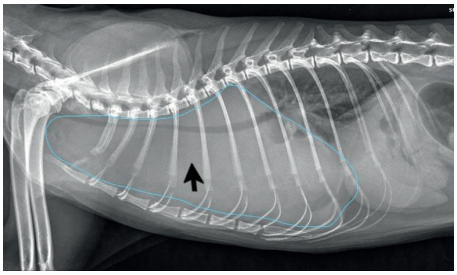

Figure 1. Right lateral thoracic radiograph depicting a large cranial and cardiac mediastinal mass (encircled), partial tracheal collapse (arrow), and lung displacement

Subsequently, cardiac ultrasound examination revealed a right-sided deviation of the heart axis, cardiac frequency of 120 beats per minute (BPM), and regular sinusal rhythm. In B-mode interventricular septum in diastole (IVDS) measured 3.0 mm, the left ventricular chamber diameter in diastole (LVDd) 15.0 mm, the left ventricular wall in diastole (LVWD) 3.0 mm, left ventricular chamber diameter in systole (LVDs) 8.0 mm and fractional shortening (FS) 46%. Blood pressure was 110 mmHg Doppler. All those measurements are consistent with no pathological findings.

The post-mortem examination revealed a large, smooth, tan, multinodular mass, extended into the mediastinum and enveloping the heart (Figure 2), involving the parietal surface of the pericardium. The mass also surrounded the proximal descending aorta and the esophageal adventitia. The surface of the myocardium presented a 0.3 cm dark-red area near the apex. Additionally, the lung displayed diffuse collapse, edema, and congestion.

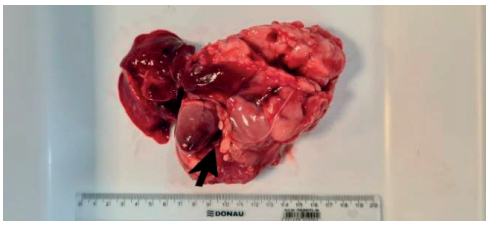

Figure 2. The mediastinal neoplastic mass surrounding the pericardium (arrow). Lung with diffuse collapse and congestion

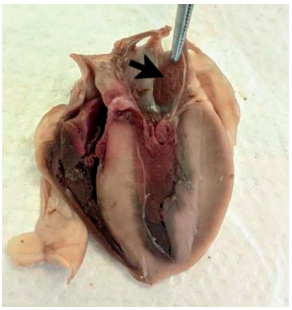

Figure 3. Four-chamber cross-section view of the formalin-fixed heart showed focal 0.55 x 0.4 cm area of endocardiosis (arrow) in the left free wall atrium

The four-chamber cross-section view of the formalin-fixed heart showed an oval 0.55 x 0.4 cm severe focally extensive area with endocardiosis in the left free wall atrium (Figure 3).

Cytological evaluation of the mass revealed a predominance of the medium-sized lymphocytes, with round eccentrically placed nuclei, centrally-placed prominent nucleoli, moderate anisocytosis, and anisokaryosis and atypical mitosis supportive of lymphoma (Figure 4).

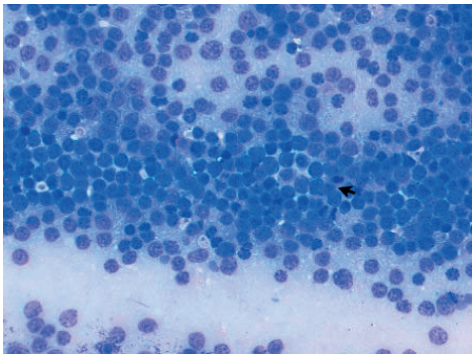

Figure 4. Medium-sized lymphocytes with round eccentrically placed nuclei, centrally-placed prominent nucleoli, moderate anisocytosis, anisokaryosis, and atypical mitosis (arrow) (MGG, ob. 40)

The histopathological evaluation of the proliferation showed a homogeneous population of round small lymphocytic cells with scant cytoplasm, and round nuclei. The nuclear size was generally 1 to 1.5 times the diameter of an erythrocyte. The number of mitoses per high-power field (400x, 0.237 mm^2 area) ranged from 2 to 10, classifying the tumor as high grade, with a total mitotic count of 54 in 2.37 mm^2 (Figure 5 A). Immunohistochemically, the neoplastic cells from tumoral mass showed an intense and diffuse reaction for CD3 monoclonal antibody, confirmed T-cell lymphoma (Figure 5 B), but no immunoreactivity with PAX-5 antibody. Based on the cytological, histological, and immunohistochemical results, the tumor was diagnosed as T-cell lymphoma, with neoplastic invasion into the pericardium and discreetly into the epicardium, anatomically classified as thymic lymphoma.

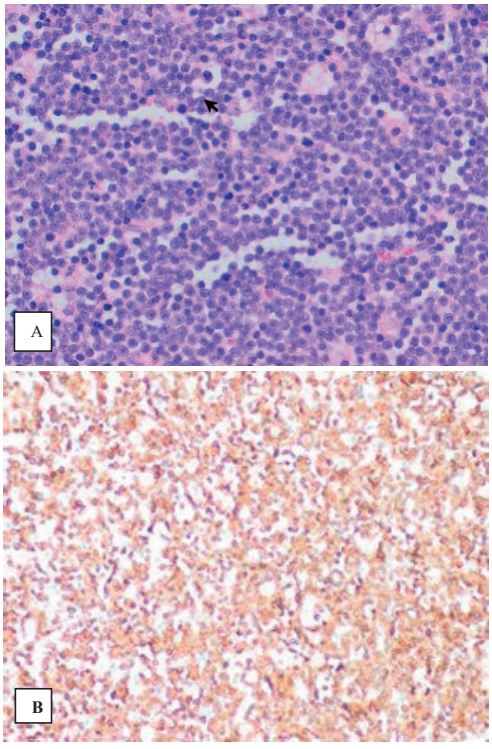

Figure 5. Histological section of feline mediastinal lymphoma, with atypical mitosis (arrow) (A) (H & E stain, ob. 10), and immunohistochemical staining for CD3 (B) (ob. 10)

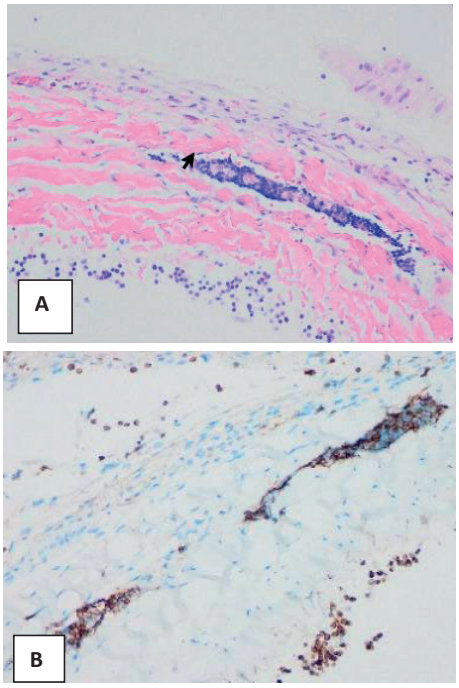

Figure 6. Pericardium with tumoral cells infiltrate (arrow) (A), showing a diffuse cytoplasmic positivity for CD3 (B) (H & E stain, ob. 20).

The pericardium showed multifocal invasion with small lymphocytic cells, with neoplastic cells diffusely expressed CD3 (Figure 6). Left atrial endocardium presented subendothelial focal thickening of connective tissue composed of dense elongated or stellate fibroblasts with elongated nuclei and pale enlarged intercellular spaces (edema) (Figure 7). At the left ventricular wall epicardium were observed discrete scattered small lymphocytic cells, among of neutrophils and degenated mesothelial cells (Figure 8 A), CD3 immunopositive (Figure 8 B), while the ventricular myocardium showed intercellular edema, cellular dilaceration, vacuolar and hyalin degeneration, and necrosis in small, isolated areas. The presence of granulation tissue, with neoformation capillaries, infiltration with small lymphocytic cells, plasma cells, and macrophages has been also identified. Additionally, the lung displayed collapse, edema and congestion.

The feline heart is a rare location for neoplasia, and less than 1% of 51,322 cats diagnosed with cancer had cardiac involvement (Kharbush, Hohenhaus, Donovan, & Fox, 2021).

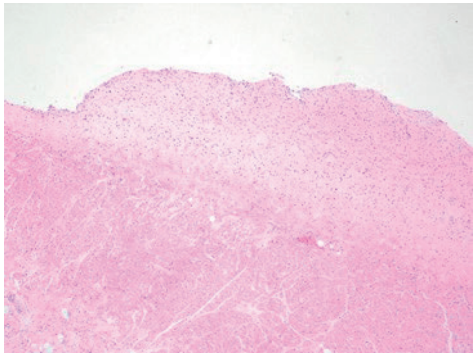

Figure 7. Endocardial fibroelastosis (H & E stain, ob. 10)

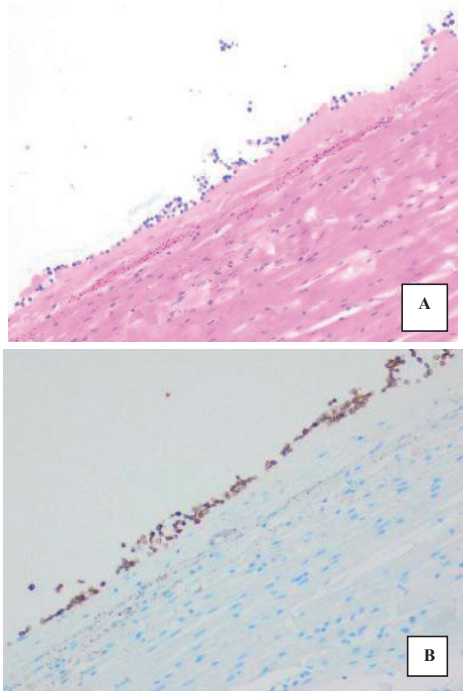

Figure 8. Epicardium with discrete tumoral cells infiltrate (A) (H & E stain, ob. 20), and neoplastic cells diffpositive for CD3 (T-cell marker) (B) (ob. 20)

Secondary cardiac involvement of lymphoma occurs more frequently than primary cardiac lymphoma, the incidence of the last being 0.03% in cats (Burgess, 2020). Metastatic tumors of the heart and pericardium appear by displacement or spread from other thoracic organs with neoplasia (Tilley, Bond, & Patnaik, 1981). In contrast with primary tumors, most metastatic neoplastic lesions reported by post-mortem (75%) are found in the inner third of the left ventricular free wall, in the

interventricular septum, or both (E. Treggiari, Pedro, Dukes-McEwan, Gelzer, & Blackwood, 2017).

According to the literature, lymphoma is the most common neoplasm in cats, occurring in 90% of feline tumors with hematopoietic origin (Sunpongsri et al., 2022; Treggiari, Pedro, Dukes−McEwan, Gelzer, & Blackwood, 2017; Twomey & Compendium, 2005). The point-of-care testing for feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) of this cat was unknown. The FeLV infection increases the relative risk 60-fold or greater; approximately 25% of cats with this virus association can develop lymphoma. FIV infection increases the relative risk of lymphoma 5-6-fold (Burgess, 2020). Cats with FeLV and FIV co-infection have a relative risk increased 75-fold (Alejandro et al., 2019).

Like in this case report, most young cats with lymphoma have T-cell positive lymphoma (Seo et al., 2006), although, in cats, B-cell lymphoma is more common than of T-cell origin (Twomey & Compendium, 2005). The age range of cats with B-cell lymphoma varied from 6 to 204 months (median of 60) and from 10 to 240 months (median 120) for cats with T-cell lymphoma (Leite-Filho et al., 2020).

In dogs, immunophenotyping is commonly used for prognostication purposes, but in cats is frequently used to confirm the presence of a phenotypically identical population of lymphoid cells (Burgess, 2020). A prognostic factor parameter is age. The cats aged under four years had shorter survival time than those over four years of age (Sunpongsri et al., 2022). Cell morphology (large cell vs. small cell) is used more commonly than a phenotypic distinction to determine treatment and prognosis.

Mediastinal high-grade lymphoma progresses rapidly; even with chemotherapy, the prognostic is poor (2-3 months) (Burgess, 2020).

The criteria of the initial diagnosis of hypertrophic cardiomyopathy (HCM) phenotype of this case report are not known. Pathological examination reveals only discreet tumoral cell infiltration at the pericardium and did not show other myocardial damage such as extended myocardial necrosis. However, neoplastic diseases such as lymphoma, myocarditis, and toxoplasma infection have been reported as myocardial diseases for differentiating hypertrophic myocardium in cats (Tanaka, Suzuki, Hirata, Kagawa, & Koyama, 2022).

Endocardial fibroelastosis (EFE) is an anomaly characterized by fibrous and elastic endocardial thickening and can be diffuse or localized. Diffuse EFE can be primary, in the absence of other important cardiac lesions, or secondary, associated with congenital cardiovascular malformations, viral myocarditis, cardiomyopathy, myocardiosis, myocardial necrosis. Localized endocardial thickening can be secondary to a multitude of myocardial injuries. Like in this case, the left side of the heart is nearly always involved (Zook & Paasch, 1982).

CONCLUSIONS

In this case, mediastinal lymphoma with pericardial invasion was diagnosed post-mortem. Based on the results, mediastinal lymphoma with cardiac involvement should be considered in young cats with clinical respiratory and cardiac signs. Mediastinal masses may be suspected basis on the physical and radiographic findings, but the final diagnosis is based on cytological, histopathological, and immuno-histochemically examination.

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RESEARCH ON THE VALUES OF SOME ELECTROCARDIOGRAPHIC PARAMETERS IN GOAT RECORDED USING DUBOIS LEADS

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Abstract

The aim of the present research paper was to evaluate the main electrocardiogram parameters in goat. The electrocardiograms were performed on a batch of 20 Carpathian goats, using the Dubois lead system. By analysing the results obtained, we concluded that, using the Dubois lead system, the best method of performing the electrocardiogram in goats is using lead II, the amplitude values being 0.117 mV for the P wave, 0.310 mV for the ventricular complex and 0.386 mV for the T wave, while lead I is not suitable for the EKG exam in this species, due to the extremely low waves' amplitude, resulting in an almost obscure recording. Regarding the duration of the electrocardiographic waves, the values obtained were 0.048±0.016 seconds for the P wave, 0.046±0.014 seconds for the QRS complex and 0.088±0.016 seconds for the T wave. As for the segments and intervals, the durations obtained were 0.062±0.020 seconds for the P-R segment, 0.246±0.014 seconds for the Q-T interval, 0.384±0.037 seconds for the P-T interval and 0.096±0.020 seconds for the T-P segment. The heart rate, electrographically calculated, was 128.5 bpm ± 12.215.

Key words*: goat, leads, Dubios lead system.*

INTRODUCTION

The electrocardiogram represents the graphic recording of the heart's electrical activity through repeated cardiac cycles, exploring the heart using a non-invasive method. Clinically, the ECG is used to investigate the cardiac conduction system's integrity, to determine the duration and frequency of the atrial and ventricular systoles and also to evaluate the heart's topography and the relative size of the cardiac compartments (Codreanu, 2018; Ghiță, et al., 2007; Șerdean, 2010). The ECG also allows the diagnosis of rythm disorders and several other heart conditions (Pourjafar et al., 2012; Cotor et al, 2020). In the research field, the ECG is used to detect the effects of some substances on cardiac activity (Brăslașu et al., 2015; Ghiță et al., 2008; Codreanu, 2018).

The proper execution of the ECG exam has a great importance in the clinical field, being recommended in the calculation of duration (for waves, segments and intervals) and amplitude (for waves), as well as in determining the electrical cardiac axis (Ghiță, et al., 2007).

Currently, the electrocardiogram recording represents one of the most common used methods of investigating the cardiac activity, in the speciality literature the information about goat-performed ECGs is poor, this subject not being commonly studied (Ghiță, M. et al., 2022). In this study, we recorded the ECG in goats using Dubois leads, the aim being to determine the electrocardiographic parameter values in goats. Studying the specialty literature, we found a relatively reduced number of studies regarding the subject, most of them made by Asian authors (Mohan et al., 2005; Jafrin et al., 2016; Fakour et al., 2013; Atmaca et al., 2014; Ghiță et al., 2008; Mohapatra et al., 2018).

The main objective of our study was to determine the electrocardiographic waves amplitude registered using the help of Dubois leads, tracking their aspects and the recorded values (mV) for each individual lead. The resulted values were then compared between

each other to discover and reveal which lead allows optimal recordings.
Regarding the ECG components duration, in our research we wanted to discover the duration length of the main event which take place in the heart during a cardiac cycle. Therefore, we determined the heart frequency (calculated based on the P-R interval) and the durations of the: P wave, P-R (P-Q) segment, P-R (P-Q) interval, the ventricular complex (QRS interval), Q-T interval, T wave, P-T interval, T-P segment, S-T segment and R-R interval. The resulted values were subsequently compared with other author's communicated values.

MATERIALS AND METHODS

The research was carried out on an experimental batch consisting of 20 goats from the Carpathian breed, aged between 3 and 5 years, at the end of the lactation period.
The ECG recording's in goats involved using a electrocardiography device (we used a portable device with 10 channels which allowed the recording for each individual lead), metal electrodes (we used „alligator" - like shaped electrodes because they are easy to attach to the goat's skin without having to trim its fur), and electrically conductive medium (we used rubbing alcohol - isopropyl alcohol, being easy to apply through pulverisation and non-irritating to the skin).
In order to record the ECG using the Dubois leads, the electrodes are placed on the body as follows: the red electrode in front of the right shoulder, the yellow electrode in front of the left shoulder, the green electrode between the xiphoid appendix and the umbilical scar, the black electrode anywhere, except for the triangle formed by the active electrodes. This lead system differentiates itself from the limbic lead system by the fact that the triangle is smaller, therefore allowing the recording of higher amplitudes.
The recordings were performed at the speed of 25 mm/s and the miniVolt amplitude of 10 mm.

RESULTS AND DISCUSSIONS

Using the Dubois leads system we can record electrocardiograms in 3 bipolar leads (marked as LI, LII, LIII) and in 3 augmented unipolar leads (marked as aVR, aVL, aVF). Analysing the resulted electrocardiograms, we observed that they presented an easy to interpret graph, without artifacts and the electrocardiographic wave amplitudes fairly high.
As far as the electrocardiographic wave amplitude values, the results we obtained are presented in the Tables 1, 2 and 3 and in the graphs form Figures 1, 2 and 3.

Table 1. The amplitude values of the P wave (mV) recorded using the Dubois leads system in goats

No.	I	II	III	aVR	aVL	aVF
1	0.1	0.15	0.1	0	0.05	0.05
2	0	0	0.05	0.1	0	0.1
3	0.05	0.15	0.1	0	0.05	0.05
4	0	0.1	0.1	0.1	0	0
5	0.1	0.1	0.15	0.05	0	0.1
6	0	0.15	0.1	0	0.1	0.05
7	0.05	0.1	0.05	0.1	0	0.1
8	0	0.15	0.1	0	0.1	0.05
9	0.05	0.1	0.05	0.1	0	0.1
10	0.05	0.15	0.1	0.1	0.05	0.1
11	0	0.1	0	0.1	0	0.05
12	0	0	0.1	0.05	0	0.1
13	0.1	0.15	0	0.1	0.05	0.1
14	0	0.1	0.05	0.1	0.05	0.15
15	0	0.15	0.1	0	0.1	0.05
16	0.05	0.1	0.05	0.1	0	0.1
17	0.1	0.15	0.1	0	0.05	0.05
18	0	0.15	0.1	0	0.1	0.05
19	0.1	0.15	0.05	0	0.05	0.05
20	0	0.15	0.1	0	0.1	0.05
MEAN	**0.037**	**0.117**	**0.077**	**0.05**	**0.042**	**0.072**
Standard error	0.009	0.010	0.008	0.010	0.009	0.007
Standard deviation	**0.045**	**0.046**	**0.037**	**0.048**	**0.040**	**0.034**
Variant	0.001	0.002	0.001	0.002	0.001	0.001

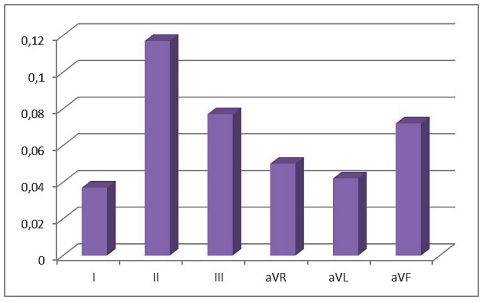

Figure 1. The average amplitude of the P wave (mV) recorded using the Dubois leads system in goats

Table 1 and Figure 1 show that the highest amplitude of the P wave is recorded in lead II, with an average value of 0.117 mV, the variability being minor (variant of 0.002 and standard deviation of 0.046). High amplitudes of the P wave can also be observed in lead III - 0.077 mV, and in aVF, with 0.072 mV. The

lowest amplitude is recorded in lead I (0.037 mV), which concludes that in this type of lead, the P wave is very hard to observe.

Table 2. The amplitude values of the QRS complex (mV) recorded using the Dubois leads system in goats

No.	I	II	III	aVR	aVL	aVF
1	0.15	0.25	0.3	0.2	0.25	0.25
2	0.15	0.3	0.25	0.25	0.2	0.2
3	0.3	0.35	0.2	0.2	0.25	0.15
4	0.15	0.3	0.3	0.15	0.2	0.25
5	0.15	0.25	0.35	0.15	0.15	0.3
6	0.15	0.2	0.25	0.15	0.2	0.25
7	0.3	0.45	0.45	0.25	0.15	0.3
8	0.25	0.2	0.35	0.2	0.15	0.25
9	0.1	0.3	0.25	0.2	0.2	0.2
10	0.3	0.3	0.2	0.2	0.25	0.15
11	0.15	0.35	0.3	0.15	0.2	0.25
12	0.15	0.3	0.35	0.2	0.15	0.35
13	0.15	0.2	0.25	0.15	0.2	0.25
14	0.1	0.35	0.3	0.2	0.15	0.3
15	0.3	0.35	0.2	0.2	0.25	0.15
16	0.3	0.35	0.2	0.2	0.25	0.15
17	0.15	0.25	0.35	0.15	0.15	0.3
18	0.15	0.35	0.3	0.15	0.2	0.25
19	0.25	0.45	0.45	0.25	0.15	0.35
20	0.3	0.4	0.4	0.25	0.15	0.35
MEAN	0.194	0.310	0.305	0.192	0.189	0.255
Standard error	0.017	0.017	0.017	0.008	0.009	0.014
Standard deviation	0.074	0.075	0.076	0.038	0.039	0.064
Variant	**0.005**	**0.005**	**0.005**	**0.001**	**0.001**	**0.004**

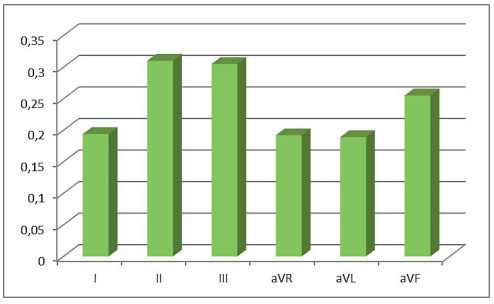

Figure 2. The average amplitude of the QRS complex (mV) recorded using the Dubois leads system in goats

Table 2 and Figure 2 show that the highest amplitude of the ventricular complex is recorded using the IInd lead, with a value of 0.310 mV (variant of 0.005 and standard deviation of 0.075). High amplitudes of the ventricular complex can also be seen in lead III, with the value of 0.305 mV (variant of 0.004 and standard deviation of 0.076), in aVF, with a value of 0.255 mV (variant of 0.004 and standard deviation of 0.064). The lowest amplitudes of the ventricular complex are recorded in aVL (0.189 mV), aVR (0.192 mV) and lead I (0.194 mV), leads which, in our opinion, can't be used to gather data regarding the electrical ventricular activity.

Table 3. The amplitude values of the T wave (mV) recorded using the Dubois leads system in goats

No.	I	II	III	aVR	aVL	aVF
1	0.15	0.45	0.4	0.25	0.2	0.4
2	0.1	0.4	0.35	0.2	0.15	0.25
3	0.15	0.25	0.3	0.15	0.2	0.2
4	0.1	0.3	0.25	0.1	0.15	0.25
5	0.15	0.5	0.45	0.25	0.25	0.4
6	0.1	0.35	0.3	0.2	0.15	0.35
7	0.1	0.5	0.35	0.25	0.1	0.25
8	0.15	0.45	0.4	0.3	0.25	0.4
9	0.1	0.4	0.3	0.2	0.15	0.25
10	0.25	0.25	0.3	0.1	0.25	0.15
11	0.1	0.35	0.25	0.15	0.15	0.2
12	0	0.4	0.45	0.3	0.25	0.4
13	0.1	0.35	0.3	0.25	0.1	0.35
14	0.15	0.5	0.3	0.25	0.15	0.3
15	0.15	0.45	0.45	0.25	0.2	0.45
16	0.20	0.25	0.35	0.1	0.25	0.35
17	0.1	0.4	0.45	0.3	0.25	0.45
18	0.15	0.35	0.3	0.2	0.15	0.35
19	0.1	0.45	0.35	0.25	0.15	0.35
20	0.1	0.5	0.45	0.25	0.1	0.35
MEAN	**0.126**	**0.386**	**0.347**	**0.213**	**0.184**	**0.321**
Standard error	0.011	0.019	0.015	0.015	0.012	0.020
Standard deviation	**0.051**	**0.083**	**0.067**	**0.066**	**0.052**	**0.088**
Variant	0.002	0.006	0.004	0.004	0.002	0.007

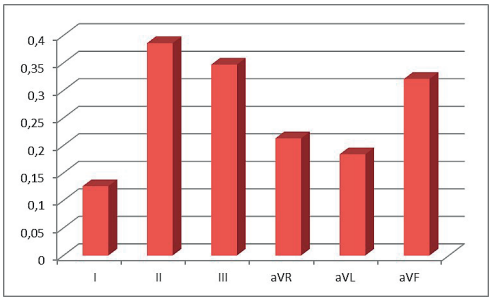

Figure 3. The average amplitude of the T wave (mV) recorded using the Dubois leads system in goats

As concerning the T wave's amplitude (Table 3 and Figure 3), the highest value was recorded using the IInd lead, with a value of 0.386 mV with high variability (variant 0.006 and standard deviation 0.083). A high amplitude of T wave is also recorded using the IIIrd lead, with a value of 0.347 mV with low variability (variant 0.004 and standard deviation 0.067), but also in aVF, with 0.321 mV and low variability (variant 0.007 and standard deviation 0.088). For all the other leads, the average values of the T wave are lower than 0.3 mV, therefore we consider that they should not

be used to gather information regarding the ventricular repolarisation.

Comparing our results with the communicated results of other authors, we notice that they obtained: the highest amplitude of the P wave in leads I, II and aVF (Atmaca, N. et al., 2014), in lead I (Jafrin, A. et al., 2016; Kumar, V. et al., 2016) and in leads II and aVF (Mohan, N. et al., 2005), the highest amplitude of the QRS complex in lead II (Pogliani, F. et al., 2013), in leads III and aVR (Atmaca, N. et al., 2014) and in leads II and III (Fakour, S. et al., 2013) and the highest amplitude of the T wave, in lead II (Pogliani, F. et al., 2013; Pradhan, R. et al., 2017), in leads II and III (Atmaca, N. et al., 2014) and in lead III (Mohan, N. et al., 2005). These results obtained by several authors are similar with the results we obtained during this study. To determine the duration of some electrocardiogram components, we used lead II, due to its easy to interpret graph, being the lead that offers the highest amplitude for most of the electrocardiographic waves, as proven previously.

We also determined the cardiac frequency (based on the R-R interval) and the durations of the: P-wave, P-R (P-Q) segment, P-R (P-Q) interval, ventricular complex (QRS), Q-T interval, T wave, P-T interval, T-P segment, S-T segment and R-R interval. In Table 4, we present the values of the electrocardiogram components in goat, resulted following the analysis of our recordings.

Table 4. The duration (seconds) of the electrocardiogram's main components in goats

No.	P	P-R segment	P-R interval	QRS	Q-T	T	P-T	T-P	S-T	R-R	H.R.
1	0.04	0.04	0.08	0.04	0.24	0.12	0.4	0.08	0.2	0.4	150
2	0.04	0.08	0.12	0.04	0.4	0.08	0.4	0.12	0.16	0.52	115
3	0.04	0.08	0.12	0.04	0.2	0.08	0.4	0.08	0.12	0.48	125
4	0.08	0.04	0.12	0.08	0.16	0.08	0.4	0.08	0.12	0.44	136
5	0.04	0.04	0.08	0.04	0.24	0.12	0.36	0.08	0.08	0.4	150
6	0.04	0.04	0.08	0.04	0.2	0.08	0.28	0.12	0.08	0.44	136
7	0.08	0.04	0.12	0.04	0.12	0.12	0.4	0.12	0.12	0.52	115
8	0.04	0.04	0.08	0.04	0.24	0.12	0.36	0.08	0.08	0.4	150
9	0.04	0.08	0.12	0.04	0.2	0.08	0.4	0.08	0.12	0.48	125
10	0.04	0.04	0.08	0.04	0.2	0.08	0.28	0.12	0.08	0.44	136
11	0.04	0.08	0.12	0.04	0.4	0.08	0.4	0.12	0.16	0.52	115
12	0.04	0.08	0.12	0.04	0.2	0.08	0.4	0.08	0.12	0.48	125
13	0.04	0.08	0.12	0.04	0.4	0.08	0.4	0.12	0.16	0.52	115
14	0.04	0.08	0.12	0.04	0.2	0.08	0.4	0.08	0.12	0.48	125
15	0.08	0.04	0.12	0.08	0.16	0.08	0.4	0.08	0.12	0.44	136
16	0.04	0.08	0.12	0.04	0.4	0.08	0.4	0.12	0.16	0.52	115
17	0.04	0.08	0.12	0.04	0.2	0.08	0.4	0.08	0.12	0.48	125
18	0.04	0.08	0.12	0.04	0.4	0.08	0.4	0.12	0.16	0.52	115
19	0.08	0.04	0.12	0.08	0.16	0.08	0.4	0.08	0.12	0.44	136
20	0.04	0.08	0.12	0.04	0.2	0.08	0.4	0.08	0.12	0.48	125
MEAN	**0.048**	**0.062**	**0.110**	**0.046**	**0.246**	**0.088**	**0.384**	**0.096**	**0.126**	**0.470**	**128.5**
Standard error	0.003	0.004	0.003	0.003	0.021	0.003	0.008	0.004	0.007	0.009	2.731
Standard deviation	**0.016**	**0.020**	**0.017**	**0.014**	**0.095**	**0.016**	**0.037**	**0.020**	**0.032**	**0.042**	**12.215**

The waves and the ventricular complex had the following durations, as presented in Table 4: the P wave duration was 0.048±0.016 seconds (average and standard deviation), the ventricular complex duration was 0.046±0.014 seconds and the T wave duration was 0.088±0.016 seconds. Regarding other segments and intervals durations: the P-R segment duration was 0.062±0.02 seconds, the P-R interval duration (which represents the atrial systole and diastole) was 0.110±0.017 seconds, the Q-T interval duration (which represents the ventricular systole and diastole) was 0.246±0.014 seconds, the P-T interval (which represents one cardiac cycle duration) was 0.384±0.037 seconds, the T-P segment duration (which represents the general diastole duration) was 0.096±0.02 seconds, the S-T segment duration was 0.126±0.032 seconds and the R-R interval duration (which represents the interval between two cardiac cycles) was 0.470±0.042. The heart rate calculated

electrocardiographically based on the R-R interval was 128.5±12.215. Studying the data presented in the speciality literature, the values obtained by different authors were between 0.02 s (Atmaca et al., 2014) and 0.06 s (Jafrin et al., 2016) for the P wave; 0.08 s (Pogliani et al., 2013) and 0.12 s (Jafrin et al., 2016) for the P-R interval; 0.03 s (Mohan et al., 2005) and 0.063 s (Fakour, et al., 2013) for the ventricular complex; 0.23 s (Mohan, et al., 2005) and 0.267 s (Fakour et al., 2013) for the Q-T interval; 0.06 s (Atmaca et al., 2014) and 0.113 s (Mohan, et al., 2005) for the T wave and 0.03 s (Pogliani, et al., 2013) and 0.48 s (Mohan, et al., 2005) for the R-R interval. Comparing the results obtained during this study with the data presented above, it is clearly proven that our research values are in direct accordance with the values mentioned in the speciality literature.

CONCLUSIONS

In goats, using the Dubios lead system, the lead that provides the highest amplitude to the electrocardiographic waves is lead II, the values being: 0.117 mV for the P wave, 0.310 mV for the ventricular complex and 0.386 mV for the T wave.

The values of the electrical events that take place in the cardiac compartments were: 0.048±0.016 seconds for the atrial depolarization, 0.046±0.014 seconds for the ventricular depolarization and 0.088±0.016 seconds for the ventricular repolarization.

The main components of the electrocardiogram had the following values: 0.110±0.017 seconds for atrial systole and diastole, 0.246±0.014 seconds for the ventricular systole and diastole, and 0.384±0.037 seconds for a cardiac cycle.

The average value for the heart rate in goats was 128.5±12.215, calculated based on the R-R interval.

Based on the results obtained, for the electrocardiogram recording in goats using the Dubois leads system, the II, III and aVF leads for the ventricular complex and T wave, and the II and aVF leads for the P wave, can successfully be used. The aVR and aVL leads both present, in our opinion, a restricted recommendation, meanwhile lead I cannot be used for recording the electrocardiogram because it records extremely low amplitude electrocardiographic waves, therefore making the ECG extremely difficult to interpret.

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STUDY REGARDING THE DISTRIBUTION OF THE CELIAC ARTERY IN NEWBORN CALVES

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Abstract

Ruminants are characterized by the presence of four separate gastric compartments. The volumetric ratio between the proper stomach and the compartments preceding it is not the same throughout the life of the individual. At the early stage of a calf life, the proventriculus is reduced and is not involved in the digestive process. In the specialized literature, there are insufficient data on the distribution of vascular formations in early life and the following evolution of these structures. Our study aims to identify some characteristics of the branches of the celiac artery at an age when the only functional compartment is the abomasum. Following the dissection of the arteries, the classic distribution of some formations was found, but also specific elements: the poorly development of the reticular artery, the presence of a collateral from the left ruminal artery destined for the cardia and the presence of three hepatic branches. Surgery in young cattle requires a thorough knowledge of possible individual arterial variations.

Key words*: vascularization, celiac artery, calf, digestive system.*

INTRODUCTION

A well-functioning digestive system in ruminants has a significant impact on growth, but also on future production (i.e. meat and milk). In the first days of life, calves undergo major changes, both in terms of environment and nutrition (Diao et al., 2019). These stressors can lead to pathologies of the digestive tract, the treatment of which can often include surgery (Davis & Drackley, 1998).

Researches on the vascularization of gastric compartments in ruminants began as early as the 20th century, with authors such as Montané and Bourdelle (1917) and Martin and Schauder (1938) addressing this subject. To understand variations regarding the courses of certain arteries and their origin, is important for any surgery concerning the viscera from the abdominal cavity (Alsafy, 2009; Mohamed, 2020). If these surgeries regard the compartments of the stomach or the intestine (displacement of the abomasum, enterotomy and rumenotomy), a good knowledge of the vascularization of the organs prevents the occurrence of hemorrhages, helps to identify the sources when they occur especially following accidental trauma (loss of integrity of the abdominal cavity associated with external aggressions) and to perform timely suturing of the blood-vessel or blood-vessels involved (Mohamed, 2017).

MATERIALS AND METHODS

The study material consisted of 5 bovine carcasses, aged between 2 and 6 days. The animals used for the study came from breeding farms and were destined for dissection and research in the Comparative Anatomy Laboratory of the Faculty of Veterinary Medicine, Bucharest.

Immediately after slaughtering/exitus of the animals, a longitudinal incision in the linea alba was made between the xiphoid appendix and the pubis, followed by injection of a low-viscosity epoxy resin into the aortic artery. The injection was performed anterior to the celiomesenteric common trunk in a centrifugal direction (Figure 1). In this way, the contrast substance reached through the celiomesenteric trunk and into the branches of the celiac and cranial mesenteric arteries. Hardening of the bicomponent epoxy resin was achieved within 24 hours, during which time the gastrointestinal tract was refrigerated, the substance used

solidified, in the arteries and in this way the blood-vessels became easy to trace and highlight. The arteries were then carefully dissected in order, before or after the conservation in formalin of the visceral tract thus prepared.

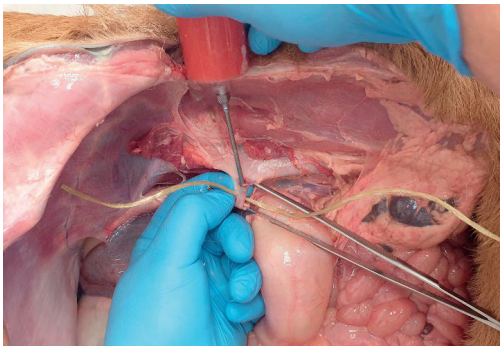

Figure 1. Injection of contrast substance immediately anterior to the celiacomesenteric trunk (original)

To study the morphology of the viscera in two specimens, they were carefully removed from the cavity and emptied of their contents. A moderate amount of polyurethane foam was introduced into each compartment so that the viscera would regain their approximate shape and volume, while avoiding excessive wall tension.

The description, identification and homologation of the formations presented was carried out according to the Veterinary Anatomical Nomination - 2017.

RESULTS AND DISCUSSIONS

In the first week of life, the digestive system of ruminants is not fully developed. The abomasum in the newborn calf is the only one of the four chambers actively involved in digestion, with nutrients coming from the milk. As the calf gets older, it begins to diversify its diet, consuming solid feed, and consequently the gastric compartments increase in volume, with the rumen becoming the most developed of them (Sisson & Grossman, 1969).

The figures below clearly demonstrate the abovementioned elements, showing the relationship between the forestomach and the proper stomach, the abomasum being the only functional compartment (Figures 2 and 3).

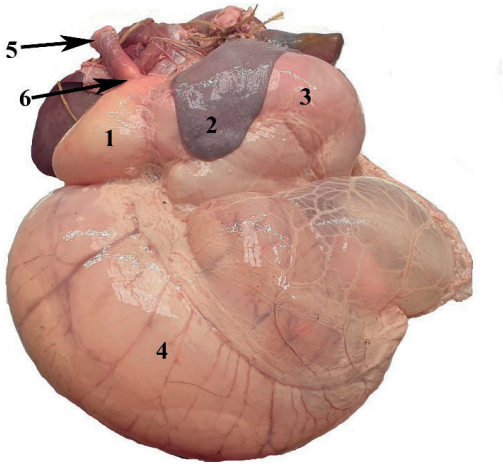

Figure 2. Gastric compartments in the ruminant - left side (original):
1 - reticulum; 2 - spleen; 3 - rumen; 4 - abomasum; 5 - oesophagus; 6 - cardiac orifice

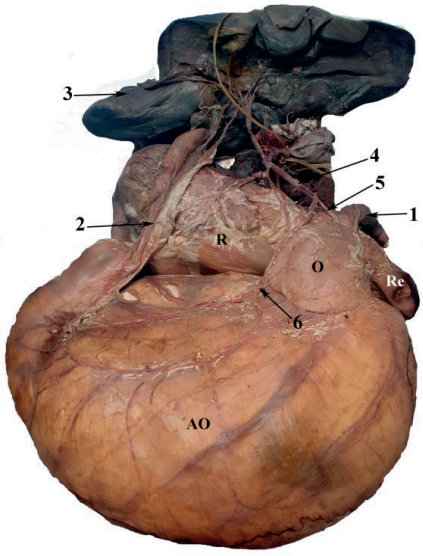

Figure 3. Gastric compartments in the ruminant - right side (original):
R - rumen; Re - network; O - omasum; AO - abomasum
1 - oesophagus; 2 - duodenum; 3 - liver; 4 - left gastric artery; 5 - left gastroepiploic artery; 6 - the place where left gastric artery divides into two branches

Following the measurement of the gastric compartments at moderate fullness, relative values were obtained and used to determine the proportion of each compartment. The values obtained are represented in the chart below (Figure 4).

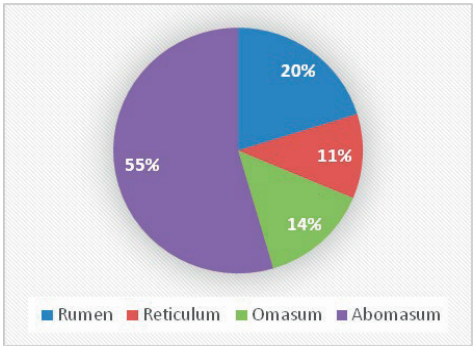

Figure 4. Ratio of gastric compartment proportions in the newborn calf

Other studies have shown similar values, Davis et al, 1998: rumen and reticulum - 38%, omasum - 13%, abomasum - 49%, and in the research by Heinrichs and Jones, 2003: rumen - 25%, reticulum - 5%, omasum - 10% and abomasum - 60%.

In the following are presented the results on the origin and distribution of the arteries of these compartments in the specimens analyzed. In all dissected cases the celiac artery split in common with the cranial mesenteric artery, thus forming celiacomesenteric trunk (Figure 5).

The splenic artery (*A. lienalis*) runs caudo-ventrally and to the left, with no notable characteristics, except that in all cases the left ruminal artery forms a common trunk with the lienal artery (Figure 5).

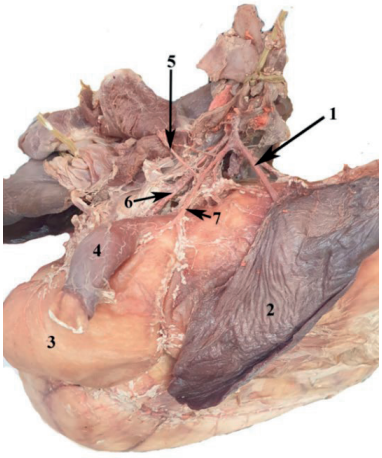

Figure 5. The origin of the main branches of the celiac artery - dorsal view (original):
1 - the splenic artery; 2 - the spleen; 3 - the reticulum; 4 - the abdominal part of the oesophagus; 5 - phrenic branches; 6 - the left gastric artery; 7 - the reticular artery

The left ruminal artery (*A. ruminalis sinistra*) emits at 0.5 cm from the origin a phrenic branch that approaches the diaphragm near the dorsal border of the liver. Immediately after this, the left ruminal artery emits the reticular artery. After the arising of the reticular artery, the left ruminal artery passes to the right of the ruminal atrium behind the cardiac orifice, with a ventro-cranial oblique course towards the cranial ruminal groove. At about 1 cm from the origin, it emits a relatively thin branch about 5 cm long and with a flexuous course that branches off to the right side at the cardia. After crossing the cranial ruminal groove from right to left, it will engage the left ruminal groove, level where it emits collaterals both to the upper margin and ventral branches for to the ventral sac of the rumen. The left ruminal artery is smaller than the right homologous artery and is distributed mainly to the first two thirds of the parietal surface of the rumen. In the left caudal third, the rumen is irrigated by terminal branches of the right ruminal artery, which cross the caudal groove of the rumen and follow the dorsal and ventral coronary grooves on the left side of this compartment.

The right ruminal artery (*A. ruminalis dextra*) didn't present any particular features compared to those found in the literature, being the main source of rumen irrigation.

The reticular artery (*A. reticularis*) intersects the medial surface of the right gastric artery and sends at 1 cm from its origin a phrenic branch which is distributed to the diaphragmatic pillars. Subsequently, the artery passes superior to the ruminal atrium, to the right of the cardiac orifice then continues to the superior margin of the reticulum, below the reticulo-omasic junction. Along its course it gives off a variable number of branches (8-10) of varying caliber, collaterals that are mostly directed towards the visceral side of the reticulum.

The left gastric artery arises in all cases at the the same level as the hepatic artery. This artery is the most developed branch of the celiac artery, distributing to the abomasum, which is the only functional gastric compartment in newborn ruminants. It passes to the right face of the omasum and continues its course towards the abomasum, specifically at the level of the lesser curvature. Being included in the structure

of the lesser omentum after a trajectory of about 2 cm along the above-mentioned curvature, it doubles over a portion of the course of about 12-13 cm, before it anastomoses with the right homologous artery in the vicinity of the angular notch. The distance between the two collaterals of the left gastric artery is about 4 cm near the middle of the lesser curvature. No accessory reticular artery was present in any of the specimens in the study.

On the course, the left gastric artery gives off the left gastroepiploic artery (*A. gastroepiploica sinistra*), forming an acute angle between them.

After a 4 cm path**, the left gastroepiploic artery** emits 1-2 thin branches to the ruminal atrium. The next two collaterals distribute to the right side of the omasum. To the left are emitted the reticular branches which correspond to the accessory reticular artery that is present in small ruminants (Figure 6).

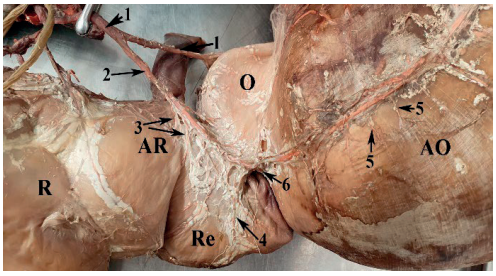

Figure 6. The distribution of the left gastroepiploic artery (original):
R - rumen; RA - ruminal atrium; Re - reticulum; O - omasum; AO - abomasum;
1 - left gastric artery; 2 - left gastroepiploic artery; 3 - branches for the ruminal atrium; 4- reticular branches; 5 - abomasal and epiploic branches; 6 - first branch destined for the *fundus* of the abomasum.

The hepatic artery (*Artera hepatica*) runs ventro-laterally and to the right. The hepatic artery has two terminals: the right branch and the left branch, in one specimen a middle branch is present.

The left branch (*Ramus sinister*) extends approximately horizontally to the deep surface of the left portion of the caudate lobe, where it emits two thin dorsally oriented branches that enter the parenchyma at the base of this lobe. When it emerges from under the caudate lobe, it is visible for about 1 cm, after which it enters deep into the left lobe. This branch usually gives off the right gastric artery.

The right branch (*Ramus dexter*) is poorly developed and has a smaller calibre. It follows a descending course on the surface of the portal vein and enters the hepatic parenchyma at its ventral edge, to the right side of the cystic duct (Figure 7)**.**

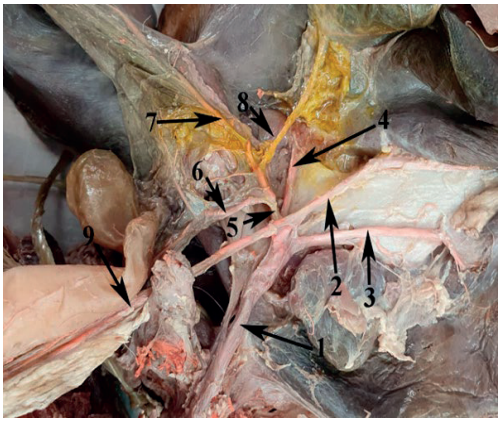

Figure 7. The terminals of the hepatic artery (original):
1 - hepatic artery; 2 - middle branch; 3 - left branch; 4 - right branch; 5 - gastro-duodenal artery; 6 - the right gastro-epiploic artery; 7 - the cranial pancreatico-duodenal artery; 8 - the cystic artery; 9 - the right gastric artery

The middle branch was present in 20% of cases studied. When it was present, the right gastric artery was a collateral of the middle branch. The middle branch had approximately the same diameter as the left branch and a descending path in the superficial plane to the portal vein. At the superior margin of the quadrate lobe, it makes a leftward bend after which it enters deep into the hepatic parenchyma. In its path it emits at its inferior margin 7-8 collaterals that progressively decrease in length and diameter and enter the parenchyma at the ventral margin of the portal vein.

The right gastric artery (*A. gastrica dextra*) generally has its origin in the left hepatic branch. A special case was encountered in the specimen presenting the middle branch of the hepatic artery, in this situation the right gastric artery is a first collateral of this branch (Figure 8).

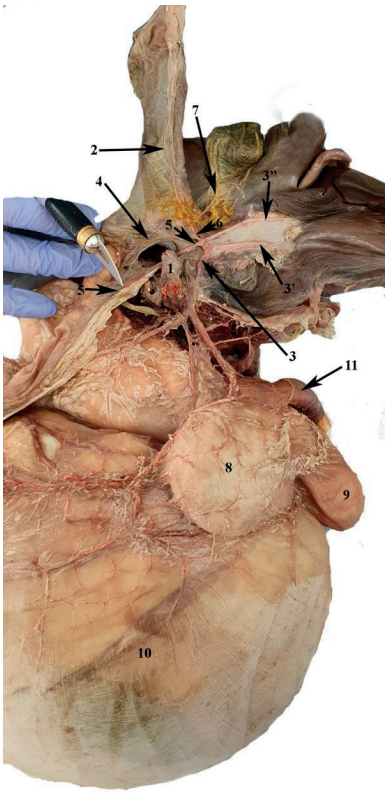

Figure 8. The gastric compartments and the origin of the main branches of the celiac artery - craniodorsal view: 1 - the celiac trunk; 2 - the cranial pancreatico-duodenal artery; 3 - the lienal artery; 3' - the middle branch of hepatic artery; 3" - the left branch of hepatic artery; 4 - the right gastroepiploic artery; 5 - the right gastric artery; 6 - gastro-duodenal artery; 7 - cystic artery; 8 - omasum; 9 - reticulum; 10 - abomasum

The gastroduodenal artery (*A. gastroduodenalis)* has about 1 cm and seems to be an extension of the hepatic artery, dividing at an acute angle into a cranial branch (representing the right gastroepiploic artery) and a caudal branch (representing the cranial pancreatoduodenal artery). **The right gastroepiploic artery** (*A. gastroepiploica dextra*) orients towards liver, level where it also emits pancreatic branches (towards the liver, towards the hilum), after which it crosses the great curvature of the duodenum in intimate relation to it. At about 4-5 cm from the pylorus, it emits a pyloric branch which ends in the area of this orifice, after which the gastro-epiploic artery is engaged in the thickness of the greater omentum at 2- 3 cm distance from the greater curvature of the cecum. The cranial pancreaticoduodenal artery has its course recorded in the literature. In contrast to the data in the literature which mentions the cystic artery as a collateral of the gastro-duodenal artery, we identified in one specimen a particularity, the origin of the artery being in the cranial pancreatico-duodenal artery. The cystic artery follows the cystic duct along the gallbladder wall, giving branches on the anterior side of the gallbladder wall (2 branches) and one on the posterior side.

CONCLUSIONS

Following dissection of the arteries previously injected with a low-viscosity epoxy resin, it was observed that in general the main trunks were distributed according to the common norm, but there were also specific elements, including: a poor development of the reticular artery associated with difficulty in the identification its oesophageal branches, the presence of a collateral branch from the left ruminal artery irrigating the right surface of the cardiac orifice, the presence in one calf of three hepatic branches, an aspect which is not reported in the literature.

A particular case was the origin of the cystic artery, which in 20% of specimens originated from the cranial pancreaticoduodenal artery.

Finally, we can conclude that the research should be extended to a larger number of animals of different ages in order to certify the correlations between the development of the gastric compartments and the morphological particularities of the gastric arteries at different stages.

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THE AGE IMPACT ON THE URINARY BEHAVIOUR IN CATS - COMPARATIVE CASE STUDY

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Abstract

The urinary behaviour in cats can be influenced by a series of physiological factors like age, physiological status and water intake. The present study highlights the age impact on the urinary behaviour, in terms of frequency and behavioural manifestation duration in different age groups. The study was carried out on 3 groups of clinically healthy cats, each consisting of 10 individuals grouped according to age: group 1 - youth, aged 3 months - 2 years old; group 2 - adults, aged 2-10 years old; group 3 - seniors, aged above 10 years old. The urinary behaviour was studied by performing individual ethograms, based on 24 hours/day video recordings, 5 consecutive days. The results showed that the highest urinating frequency/24 hours was recorded in youth, 62% higher than in adults, while in seniors the average frequency was 48.5% higher than in adults. Concerning the average length of the urinating session, the lowest mean value was recorded in youth, 27.9 seconds, 32.6% lower than adults, while in seniors the average value was 53.2 seconds, 28.5%, respectively 90.7% higher than adults, respectively youth.

Key words*: urinary behaviour, domestic cats, age.*

INTRODUCTION

The main physiological factors that have an impact on the urinary behaviour in felines are: age, weight, level of physical activity, as well as the physiological status - pregnancy/lactation.

The urinary behaviour is, from a physiological point of view, directly correlated with the dipsic behaviour in all animal species (Codreanu, 2022; Cunningham, 2019). Thus, an increased water intake will, invariably, leads to an increase of the urinary behaviour's manifestation, translated by the elimination of a larger volume of urine as well as by an increase of the frequency and duration of the urinating sessions (Codreanu, 2022).

From a physiological point of view, we can observe a sensitive diminution/accentuation of the urinary behaviour's manifestation depending on the environmental conditions, physiological status and, invariably, water intake (Batges & Polzin, 2011; Codreanu, 2017; Neilson, 2004), anuria, dysuria or polyuria being pathophysiological conditions.

As presented in the specialty literature, the age has a major impact on water consumption, therefore on the urinary behaviour as a direct consequence, being also correlated with the level of physical activity (Deng et al., 2011).

The first stage of the ethological research is establishing the inventory of all the activities that form the behavioural repertoire of a species, this inventory being known as the individual's ethogram (Stanton et al, 2015; Codreanu, 2022). Therefore, in our study we performed individual ethograms, following the effect of age, as a main physiological factor with direct impact on the urinary behaviour in domestic cats, the two main coordinates used in order to characterise the urinary behaviour were the urinating frequency (times per 24 hours) and the average length of the urinating session (in seconds).

MATERIALS AND METHODS

In order to perform this study we formed, according to age, 3 groups of healthy individuals, as follows: Group no. 1 (generically named: the youth group) - consisted of 10 individuals aged between 3 months and 2 years old; Group no. 2 (generically named: the adults group) - consisted of 10 individuals aged between 2 and 10 years old; Group no. 3 (generically named:

the seniors group) - consisted of 10 individuals 10 + years.
The gender was not a selection criterion, all 3 groups consisting of both males and females.
In order to create the groups, all individuals were subjected to both clinical and paraclinical examinations, namely: blood test - hemogram, biochemistry and blood smear, urine test - urine summary and sediment and also ultrasonography.
The study was performed in the time span August 2022 - December 2022, being an "on the field" study, the data was gathered by analysing the behaviour of each patient in its normal living conditions and performing ethograms. The data required for the ethograms was gathered by video recordings. The area of the litter box was recorded using the Xiaomi Smart Camera C300, the data being stored and processed using the Xiaomi Home mobile app, Android version and the Huawei MatePad 11 tablet. Each individual's litter box was monitored for 5 days, 24 hours a day.
The owners were required to sign an agreement for the video recording and also agreed to let us know if they observed the patient urinating in other place than their own litter box, as this aspect could influence the results of our study. None of the owners observed signs of urination outside the litterbox during our study.

RESULTS AND DISCUSSIONS

The preliminary results were gathered in the form of group ethograms performed for each of the 5 days of monitoring, and that were used for the synthetic data. In Tables 1, 2 and, respectively, 3 are presented the ethograms performed for the individuals from the 3 studied groups on day one of monitoring.

Table 1. Ethogram of the urinary behaviour - day no. 1, in cats from the youth group (3 months - 2 years old)

TIME INTERVAL	INDIVIDUAL FROM THE YOUTH GROUP									
	1	2	3	4	5	6	7	8	9	10
0 A.M. - 1. A.M.				35 sec.		26 sec			38 sec.	
1 A.M. - 2. A.M.		29 sec.					34 sec.			
2 A.M. - 3. A.M.	21 sec.		31 sec.					18 sec.		
3 A.M. - 4. A.M.										27 sec
4 A.M. - 5. A.M.					38 sec.					
5 A.M. - 6. A.M.		26 sec.				23 sec.		24 sec.		
6 A.M. - 7. A.M.	23 sec.						42 sec.		32 sec.	
7 A.M. - 8. A.M.			27 sec.							
8 A.M. - 9. A.M.				38 sec.						
9 A.M. - 10. A.M.					33 sec.			27 sec.		
10 A.M. - 11. A.M.			21 sec.			30 sec.				35 sec
11 A.M. - 12. P.M.	19 sec.									
12 P.M. - 13 P.M.							29 sec.			
13 P.M. - 14 P.M.								19 sec.		
14 P.M. - 15 P.M.		33 sec.	19 sec.							
15 P.M. - 16 P.M.	29 sec.			29 sec.		26 sec.			44 sec.	
16 P.M. - 17 P.M.										28 sec
17 P.M. - 18 P.M.			22 sec.		41 sec.		32 sec.			
18 P.M. - 19 P.M.		21 sec.				18 sec.		33 sec.		
19 P.M. - 20 P.M.										
20 P.M. - 21 P.M.								17 sec.		
21 P.M. - 22 P.M.	20 sec.						25 sec.		29 sec.	
22 P.M. - 23 P.M.			29 sec.	33 sec.		32 sec.				43 sec
23 P.M. - 0 A.M.					27 sec.			21 sec.		
Number of urinating sessions/24 hours	5	4	6	4	4	6	5	7	4	4
The average number of urinating sessions/24 hours for the youth group	4.9 times									
Total time of the urinating sessions/24 hours	112 sec.	109 sec.	149 sec.	135 sec.	149 sec.	155 sec.	162 sec.	159 sec.	143 sec.	133 sec.
The mean time/urinating session	22.4 sec.	27.2 sec.	24.8 sec.	33.7 sec.	37.2 sec.	25.8 sec.	32.4 sec.	22.7 sec.	35.7 sec.	33.2 sec.
The mean time / urinating session for the youth group	29.5 seconds									

The manifestation of the urinary behavior

Approaching the litter box (without actually urinating/defecating)

Table 1 shows the ethogram for the 10 individuals monitored in day no. 1. There are specified the time intervals when the urinary behaviour occurred in each individual (red colour), the time of each urinating session, the number of urinating sessions per 24 hours per individual, the average frequency and length of the urinating sessions per 24 hours per group, and, additionally, with green colour, the times the individuals approached the litterbox (as captured on video camera) without actually urinating or defecating, but, most likely with the intent of doing so. We did not statistically follow those periods as they do no present interest for our study, but, it can be easily observed in Table 3 that geriatric patients have the tendency to visit the litterbox more often than the individuals from the other two age categories. This finding, although doesn't have specific significance for our study is in accordance with the data in the specialty literature (Landsberg et al., 2010; Davies, 2001).

We proceeded similarly for all 5 days of monitoring, performing each day ethograms like the one presented in Table 1. Then we corroborated the data from all 5 ethograms and obtained the synthetic results in the youth group.

The procedure was applied also for the other two groups. As well as in the youth group, the other two groups were monitored for 5 days, each day performing ethograms that gathered information used for the synthetic results in adult, respectively, senior patients.

Table 2. Ethogram of the urinary behaviour - day no. 1, in cats from the adult group (2 - 10 years old)

TIME INTERVAL	INDIVIDUAL FROM THE ADULT GROUP									
	1	2	3	4	5	6	7	8	9	10
0 A.M. – 1. A.M.		48 sec.						51 sec.		
1 A.M. – 2. A.M.			50 sec.		33 sec.					
2 A.M. – 3. A.M.							49 sec.			
3 A.M. – 4. A.M.						46 sec.			43 sec.	
4 A.M. – 5. A.M.	45 sec.			51 sec.						
5 A.M. – 6. A.M.										41 sec.
6 A.M. – 7. A.M.			42 sec.							
7 A.M. – 8. A.M.					31 sec.					
8 A.M. – 9. A.M.										
9 A.M. – 10. A.M.										
10 A.M. – 11. A.M.										
11 A.M. – 12. P.M.							31 sec.			
12 P.M. – 13 P.M.	39 sec.									
13 P.M. – 14 P.M.					42 sec.					
14 P.M. – 15 P.M.		53 sec.						37 sec.		
15 P.M. – 16 P.M.										
16 P.M. – 17 P.M.			38 sec.						50 sec.	
17 P.M. – 18 P.M.						39 sec.				
18 P.M. – 19 P.M.	41 sec.									
19 P.M. – 20 P.M.							33 sec.			45 sec.
20 P.M. – 21 P.M.					39 sec.					
21 P.M. – 22 P.M.				42 sec.						
22 P.M. – 23 P.M.			33 sec.							29 sec.
23 P.M. – 0 A.M.									32 sec.	
Number of urinating sessions/ 24 hours	3	2	4	2	4	2	3	2	3	3
The average number of urinating sessions/ 24 hours for the adult group	**2.8 times**									
Total time of the urinating sessions/ 24 hours	125 sec.	101 sec.	163 sec.	93 sec.	145 sec.	85 sec.	113 sec.	88 sec.	125 sec.	115 sec.
The mean time / urinating session	41.6 sec.	50.5 sec.	40.7 sec.	46.5 sec.	36.2 sec.	42.5 sec.	37.6 sec.	44 sec.	41.6 sec.	38.3 sec.
The mean time / urinating session for the adult group	**41.9 seconds**									

The manifestation of the urinary behavior

Approaching the litter box (without actually urinating/defecating)

Table 3. Ethogram of the urinary behaviour - day no 1, in cats from the seniors group (> 10 years old)

TIME INTERVAL	INDIVIDUAL FROM THE SENIORS GROUP									
	1	2	3	4	5	6	7	8	9	10
0 A.M. – 1. A.M.		47 sec.					51 sec.			
1 A.M. – 2. A.M.			64 sec.						53 sec.	
2 A.M. – 3. A.M.				56 sec.						49 sec.
3 A.M. – 4. A.M.	61 sec.					67 sec.				
4 A.M. – 5. A.M.				42 sec.				54 sec.		
5 A.M. – 6. A.M.		53 sec.			71 sec.					
6 A.M. – 7. A.M.									46 sec.	
7 A.M. – 8. A.M.			49 sec.			51 sec.				
8 A.M. – 9. A.M.	44 sec.									
9 A.M. – 10. A.M.		42 sec.		61 sec.			58 sec.		42 sec.	
10 A.M. – 11. A.M.					53 sec.			57 sec.		
11 A.M. – 12. P.M.			57 sec.							
12 P.M. – 13 P.M.										61 sec.
13 P.M. – 14 P.M.										
14 P.M. – 15 P.M.	53 sec.			51 sec.						
15 P.M. – 16 P.M.					61 sec.				51 sec.	
16 P.M. – 17 P.M.										
17 P.M. – 18 P.M.			44 sec.			61 sec.				
18 P.M. – 19 P.M.		56 sec.		43 sec.						
19 P.M. – 20 P.M.								59 sec.		
20 P.M. – 21 P.M.	49 sec.						61 sec.		47 sec.	
21 P.M. – 22 P.M.				38 sec.						
22 P.M. – 23 P.M.		51 sec.				42 sec.				57 sec.
23 P.M. – 0 A.M.					46 sec.			41 sec.		
Number of urinating sessions/ 24 hours	4	5	4	6	4	4	3	4	5	3
The average number of urinating sessions/ 24 hours for the seniors group	**4.2 times**									
Total time of the urinating sessions/ 24 hours	207 sec.	249 sec.	214 sec.	291 sec.	231 sec.	221 sec.	170 sec.	211 sec.	239 sec.	167 sec.
The mean time / urinating session	51.7 sec.	49.8 sec.	53.5 sec.	48.5 sec.	57.7 sec.	55.2 sec.	56.6 sec.	52.7 sec.	47.8 sec.	55.6 sec.
The mean time / urinating session for the seniors group	**52.9 seconds**									

The manifestation of urinary behavior

Approaching the litter box (without actually urinating/defecating)

The synthetic data regarding the urinating behaviour in the three groups of cats, during the study period (5 days), is comparatively presented in Table 4 and Figure 1.

The results obtained showed that the highest urinating frequency/24 hours was recorded in youth, being 62% higher than in adults, while in seniors the average frequency was 48.5% higher than in adults. Therefore, we can observe a decrease of the urinating frequency in adults compared to youth and an increase in seniors compared to adults.

Concerning the average length of the urinating session, the lowest mean value was recorded in youth, 27.9 seconds, 32.6% lower than adults, while in seniors the average value was 53.2 seconds, 28.5%, respectively 90.7% higher than adults, respectively youth.

Therefore, there can be observed a gradual increase of the average time spent at the litterbox as the individuals get older. The results obtained are similar to the ones found in speciality literature (Churchill & Firmann, 2021; Davies, 2001).

In youth, the increased urination frequency (62% higher than in adults) can be justified by the high level of physical activity that translates into less sleep, a longer awake period and an increase of the dipsic behaviour manifestation (Deng et al., 2011).

The speciality literature also mentions that cubs and youth show a higher level of physical activity than adults and geriatric individuals, therefore, they consume larger amounts of water, and the frequency of watering and, consequently the frequency of the urination sessions is also higher in these age categories (Neilson, 2004; Bartges & Polzin, 2011).

Although the urination frequency is high, the time spent at the litter box is the shortest in all

3 groups, due to, most likely, their agility and the small capacity of the bladder. The increase of the urinating behaviour manifestation in senior cats (with 48.5% compared to adults) and also the concomitant increase of the time spent at the litter box (28.5% more than adults and 90.7% more than youth) are most likely correlated with the degeneration of the excretory system, a tendency for polyuria as the kidneys age (Codreanu, 2017; Neilson, 2004; Bartges & Polzin, 2011).

Also, studies show that in seniors and geriatric patients the urinating sessions' frequency is higher due to a decrease of the bladder's sphincter's tonus, this being also a cause of periuria in these patients (Cline, 2011; Fortney, 2012; Davies, 2001).

The speciality literature mentions a separate category of pathophysiological factors related to age, that have resonance, tangentially, on the urinary behaviour in this species, namely the locomotory system disorders, with a direct impact on the patients' mobility and locomotion capacity (Gunn-Moore et al., 2007; Landsberg et al., 2010). Thus, in geriatric patients, the impairment of locomotion will lead to the modification of a wide range of behavioural manifestations (Bradshaw, 2018; Landsberg et al., 2010), in the case of the urinating behaviour, periuria is most often recorded as well as difficulties in reaching and properly using the litterbox (Fortney, 2012).

In our study, the increased length of the urinating session in senior cats is in accordance with the results and studies in the specialty literature (Churchill & Firmann, 2021; Fortney, 2012), as we can assess that the long time spent at the litter box can be caused by the decreased mobility that comes with older age and that leads to slower movements and difficulties in reaching, entering and leaving the litter box.

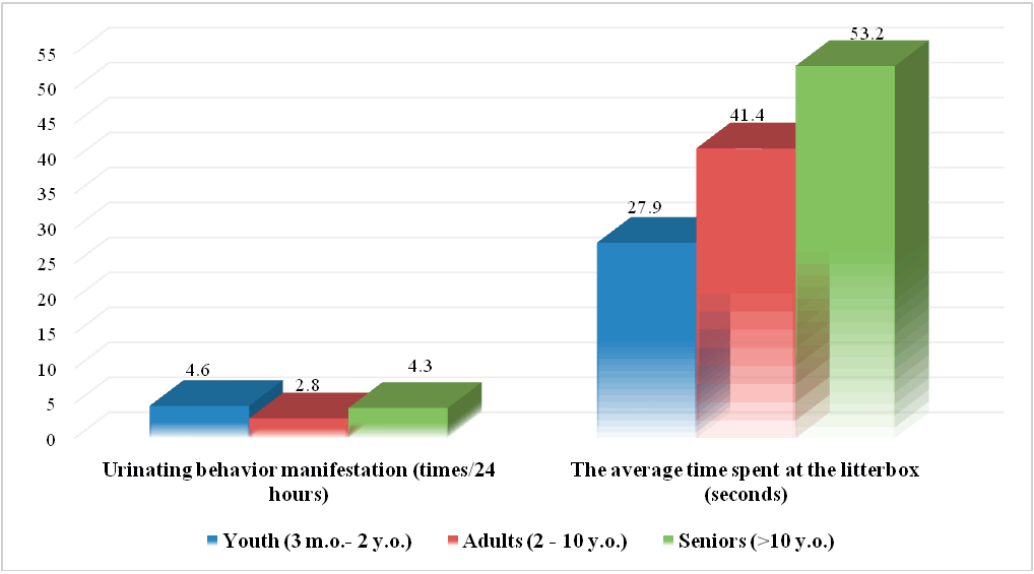

Figure 1. The comparative average data regarding the urination frequency and urination session time for the 3 studied groups of cats

Table 4. The average urination frequency and urination session time for the 3 studied groups of cats

Age group	The average frequency of urination/24 hours	The average time spent at the litter box
Youth (3 months - 2 years old)	4.7 times	27.9 seconds
Adults (2 - 10 years old)	2.9 times	41.4 seconds
Seniors/Geriatrics (> 10 years old)	4.3 times	53.2 seconds

CONCLUSIONS

The age has an important influence on both the manifestation of the urinary behaviour and the time length of the urinating sessions.
In youth, the increased level of physical activity has an impact on the urinary behaviour in this species, with an increased number of urinating sessions, each session lasting approximately 30% less than in adults.
In senior patients, the advanced age effects on the urinary behaviour translate into more urinating sessions per 24 hours, the urination frequency being approximately 60% higher than in adults and into the highest urinating frequency in all three age groups – approximately 30% higher than adults and 90% higher than youth. All these changes observed being, most likely, the consequence of the degeneration of the kidney function, the urinary incontinence and the low physical activity and mobility that come with the advanced age.

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This study is part of the research carried out during the doctoral studies of PhD student Călin (Nicolae) Simona and will be widely presented in the doctoral thesis "*Research on the physiological and pathophysiological factors involved in the modification of dipsic and urinating behavior in cat*".

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PCR PROTOCOLS FOR MOLECULAR SEXING IN MONOMORPHIC BIRDS

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Abstract

More than a half of bird species around the world are monomorphic, therefore they do not show distinct sexual dimorphic traits. The paper aims to present three molecular methods for sex identification of monomorphic companion birds. Samples of feathers, oral swabs and blood were collected from Psittaciformes (Ara ararauna, Psittacus erithacus and Psittacula krameri) and Columbiformes (Columba livia domestica). All samples were tested by three different PCR protocols in order to identify the chromo-helicase-DNA-binding (CHD1)-W gene and CHD1-Z genes in females and the CHD1-Z gene in males. Two protocols were conventional PCR, using P2 and P8, respectively P2 and NP primers, while the third protocol was a multiplex PCR using P0, P2 and P8 primers. As a conclusion, all three PCR protocols can be used for molecular sexing of monomorphic Psittaciformes and Columbiformes companion birds. Feather, oral swab and blood samples provided adequate DNA templates for the sex identification of birds.

Key words*: monomorphic birds, feathers, oral swab, blood, PCR.*

INTRODUCTION

Over half of all bird species globally lack distinct sexual dimorphism (Griffiths et al., 1998; O'Malley, 2005). Among the most popular pet birds are the *Psittaciformes*, commonly known as parrots, which consist of roughly 400 species (Curro, 1998; Forshaw, 2010). Most parrots, including the African Gray Parrot (*Psittacus erithacus*) and the Yellow-breasted Macaw (*Ara ararauna*), are sexually monomorphic (Forshaw, 2010). However, certain parrot species display sexual dimorphism after they reach sexual maturity. For instance, male parakeets (*Melopsittacus undulatus*) typically have blue ceres, whereas females have pinkish-brown ceres. Unfortunately, these sexually dimorphic traits only become apparent after the birds have reached sexual maturity (Forshaw, 2010; O'Malley, 2005). In Rose Ringed Parakeets (*Psittacula krameri*) only adult males display a black neck ring, while females and both sexes' sexually immature birds lack one. The Rose-Ringed Parakeet fails to exhibit sexual dimorphism until reaches the age of 3 years, when it achieves sexual maturity (Forshaw, 2010; O'Malley, 2005).

Columbiformes are considered monogamous. Domestic Pigeons do not exhibit sexual dimorphism, but can sometimes be sexed using traditional sexing techniques. Traditional methods of pigeon sexing involve observing of the characteristic secondary sex features, such as male neck plumage, bird size and head shape (males are larger and have larger beaks) and observing behaviour (the male's characteristic song and dance during the ritual of mating and ponta, a late method, but which has 100% accuracy in females) (Tudor, 1991; O'Malley, 2005).

The male birds are homogametic (ZZ), while the females have two different sex chromosomes (ZW). The amplification of CHD1 (chromo-helicase-DNA binding protein) gene, located in both sex chromosomes of all birds, allowed the sex identification in most avian species (Griffiths et al., 1998). More precisely, sex identification is achieved by amplifying the homologous sections of the two genes CHD1-Z and CHD1-W, including introns, which normally vary in size.

There were described three molecular sexing methods for identification of the two genes: PCR-RFLP (restriction fragment length polymorphism) (Griffiths and Tiwari, 1995; Griffiths et al., 1996), PCR-SSCP (single-strand conformational polymorphism) (Ellegren, 1996; Cortés et al., 1999) and conventional PCR based on intronic size variation (Griffiths et al., 1998). The last one is more rapid and simple, but is not a universal method.

PCR-based bird sexing methods have broad applications in studying reproductive and conservation biology, ecology, and evolution (Morinha et al., 2012). Given that pet birds are highly social and require pairing, molecular sexing is critical in ensuring their early and appropriate welfare needs (Peng and Broom, 2021).

MATERIALS AND METHODS

Sample collection

Samples of contour feathers with intact calamus, oral swabs and blood (dried blood spots) were collected from 4 individuals from order *Psittaciformes* as followed: two Yellow-breasted Macaws (*Ara ararauna*), one African Gray Parrot (*Psittacus erithacus*) and one Rose Ringed Parakeet (*Psittacula krameri*) and from four Domestic Pigeons *(Columba livia domestica)*. Oral swabs were collected using sterile cotton swabs (Prima, Taizhou Honod Medical Co., Ltd., Zhejiang, China). One drop of blood from each bird was collected on filter paper (dried blood spot).

The samples were collected with the consent of the owners, who voluntarily participated in the study. All samples were labelled individually and stored at -20°C until processing.

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from all samples of feathers (n = 8), oral swabs (n = 8) and dried blood spots (n = 8) collected from birds. The calamus of each feather was sectioned in small pieces (2-3 mm) with the help of a sterile scalpel and then was subjected to mechanical destruction by high-speed shaking together with steel beads, using TissueLyserII (Qiagen, US). Oral swabs were transferred to 1.5 ml Eppendorf tubes using sterile scissors. The dried blood spots were subjected to DNA extraction. DNA extraction was performed using a commercial kit (Isolate II Genomic DNA kit; Meridian Bioscience, USA) following the manufacturer`s protocol. For all types of tissues, the same protocol was used.

All the samples were tested for the presence of specific genes CHD1-W and CHD1-Z by three PCR protocols. PCR was carried out in a 25 µl reaction mixture consisting of 12.5 µl of MyTaq Red HS Mix (Meridian Bioscience, USA) and 25 pM of each primer. The volume of DNA template was 4 µl.

Protocol 1. The identification of the CHD1 gene was performed according to the protocol described by Griffiths et al. (1998), using the primers P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and P8 (5′-CTC CCA AGG ATG AGR AAY TG-3′) (Generi-Biotech, Hradec Králove, Czech Republic). Cycling conditions were: 95°C for 5 min for initial denaturation, followed by 48°C for 45 sec, 72°C for 45 sec and 95°C for 30 sec (35 cycles); 48°C for 1 min and final extension at 72°C for 5 min.

Protocol 2. The identification of the CHD1 gene was performed according to the protocol described by Ito et al. (2003), using the primers P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and NP (5'-GAG AAA CTG TGC AAA ACAG-3') (Generi-Biotech, Hradec Králove, Czech Republic). Cycling conditions were: 95°C for 5 min for initial denaturation, followed by 52°C for 45 sec, 72°C for 45 sec and 95°C for 30 sec (35 cycles); 50°C for 1 min and final extension at 72°C for 5 min.

Protocol 3. The identification of the CHD1 gene was performed according to the protocol described by Han et al. (2009), using the primers P0 (5′-ATT GAG TTG GAA CCA GAI CA-3′), P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and P8 (5′-CTC CCA AGG ATG AGR AAY TG-3′) (Generi-Biotech, Hradec Králove, Czech Republic). Cycling conditions were: 95°C for 5 min for initial denaturation, followed by 95°C for 30 sec, 53°C for 30 sec and 72°C for 45 sec (35 cycles) and 72°C for 5 min for final extension.

For all the three protocols the amplification was performed in Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules,

California). Aliquots of each PCR product were electrophoresed on 3% agarose gel stained with RedSafe Nucleic Acid Staining Solution 20.000x (iNtRON Biotechnology), and examined for the presence of the specific fragment under UV light (Bio-Rad BioDoc-ItTM Imagine System). DNA fragment size was compared with a standard molecular weight, 100 bp DNA ladder (Fermentas; Thermo Fisher Scientific, Waltham, Massachusetts). Females are characterized by obtaining two bands corresponding to the CHD1W and CHD1Z genes, while males present only one band corresponding to the CHD1Z gene.

RESULTS AND DISCUSSIONS

All three PCR protocols tested allowed sex determination in both, *Psittaciformes* and *Columbiformes* bird species. The results of molecular sexing of monomorphic companion birds from oral swabs, feathers and dried blood spot are presented in Table 1.

Table 1. Results of molecular sexing monomorphic companion birds included in this study

Birds	Protocol 1	Protocol 2	Protocol 3
Psittaciformes			
Yellow-breasted Macaw 1 (*Ara ararauna*)	M	M	M
Yellow-breasted Macaw 2 (*Ara ararauna*)	M	M	M
African Gray Parrot (*Psittacus erithacus*)	M	M	M
Rose Ringed Parakeet (*Psittacula krameri*)	F	F	F
Columbiformes			
Pigeon 1 (*Columba livia domestica*)	M	M	M
Pigeon 2 (*Columba livia domestica*)	M	M	M
Pigeon 3 (*Columba livia domestica*)	M	M	M
Pigeon 4 (*Columba livia domestica*)	F	F	F

The PCR products showed two bands in females (corresponding to CHD1-W and CHD1-Z genes) and a single band in male (corresponding to CHD1-Z gene). The PCR electrophoresis gel for sex identification in *Columbiformes* using protocol 2 was presented in the Figure 1.

All three tested PCR protocols targeted to identify the CHD1, gene that has been conserved in the avian W chromosome and which allows sex identification in most species.

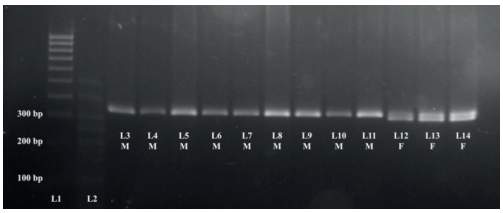

Figure 1. PCR gel from *Columbiformes* using protocol 2. Legend: L1 - size standard (100-bp DNA ladder); L2 - size standard (25-bp DNA ladder); L3, L6, L9, L12 - oral swab; L4, L7, L10, L13 - feathers; L5, L8, L11, L14 - dried blood spots; M - male; F - female

The most used and cited method of molecular sexing of birds is the P2/P8 sexing test described by Griffiths et al. (1998). The test involves a simple conventional PCR performed with a single pair of primers to amplify an intron in CHD-W and CHD-Z genes. The result shows a band in males and two bands in females. The P2/P8 test was successful used to sex identification in 27 bird species from the class Aves. *Psittaciformes* and *Columbiformes* birds species can be can be sexed by this molecular method (Turcu et al., 2022; Turcu et al., 2023). Because is based on intronic length polymorphism size variation, the method is not universal and cannot be used for sex identification in birds that not present or have a small size variation (Han et al., 2009).

The second protocol presented here was described by Ito et al. (2003) and it is used for DNA sexing. This method is based on the intronic length variation between CHD1-W and CHD1-Z. This method was developed for sex identification in *Falconiformes*, but in the present paper we demonstrated that it can be use with success for molecular sex identification in *Psittaciformes* and *Columbiformes.* Our results showed that P2/NP can offer the strong bands in both tested species.

Han et al. (2009) described a multiplex PCR for DNA sexing in birds. In addition to P2/P8 primer pair described by Griffiths et al (2009), a new P0 primer is used. P0 is a specific primer that can amplify a sequence present only on CHD1-W gene. The advantage of the multiplex PCR method is that it can determine the sex of the birds regardless of the intron size variation. In some species, the females can display three different bands, the extra-band being amplified by the primers P0/P2. In the present paper we

have identified an extra band in the female Rose Ringed Parakeet (*Psittacula krameri*) (Figure 2).

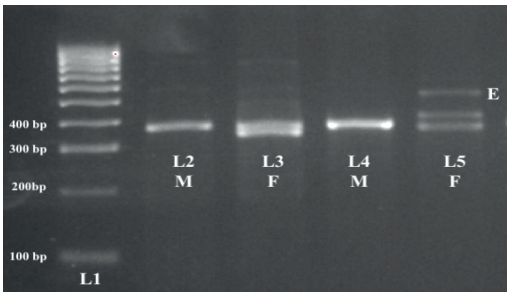

Figure 2. PCR gel from *Columbiformes* and *Psittaciformes* using protocol 3.
Legend: L1 - size standard (100-bp DNA ladder); L2, L3 - *Columba livia domestica*; L4; L5 - *Psittacula krameri*; M - male; F - female; E - additional band

CONCLUSIONS

Feather, oral swab and dried blood spot samples provided DNA templates suitable for sexing the monomorphic birds. All three PCR protocols can be used for molecular sexing of the following birds orders: *Psittaciformes* and *Columbiformes*. We recommend protocol which uses the P2/NP primers pair, described by Ito et al. (2003), which gives the strongest bands in both tested species.

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MORPHOLOGICAL PARTICULARITIES OF THE ZONOSKELETON, STYLOPODIUM, AND ZEUGOPODIUM OF THE THORACIC LIMB IN THE EURASIAN BROWN BEAR (*Ursus arctos arctos*) - CASE STUDY

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Abstract

This study aims to analyze and describe the morphological features of the bones that comprise the pectoral girdle (zonoskeleton), stylopodium, and zeugopodium of the thoracic limb of the Eurasian brown bear (Ursus arctos arctos) from Romania. The bones of a brown bear specimen, belonging to the collection of the discipline of Anatomy, were used. This species is protected and hunting is restricted. These particularities play an important role in differentiating this species from other carnivores. The data in the specialized literature is limited, to bone pathology, mechanics of the forelimb joints, and skull particularities. The analysis of the bones` morphological particularities leads to the following conclusions. The ratio of the supraspinous fossa and the infraspinous fossa is 1: 1, as seen in most carnivores. In this species, the infraspinous fossa is limited caudally by a straight and high thoracic edge (supplementary spine), smaller compared to the scapular spine. Posterior to the additional spine is another surface, intended to insert the teres major muscle. The humeral tubercles are short, and the lateral epicondyle crest is high, sharp, and drawn craniolaterally.

Key words*: bear, infraspinous fossa, humeral tubercles, lateral epicondyle crest.*

INTRODUCTION

Eurasian brown bear (*Ursus arctos arctos*) (Linné, 1758) belongs to the Family Ursidae, Genus Ursus, Order Fissipeda. It is a carnivorous species whose habitat extends throughout Europe, and the number of specimens differs from country to country. In Romania, the habitat increases as the species is protected by law.

In the scientific literature, there are studies about the morphology and morphometry of the skull of this species (Mihaylov et al., 2013; Yousefi, 2016; Roșu et al., 2022) but also comparative studies between bones of the appendicular skeleton, between the vertebral skeleton of the bear and different fossils of the Family Carnivora. These studies have highlighted similarities between bones, joints, muscles, and dental morphology (Sorkin, 2006; Argot, 2010; Siliceo et al., 2014). Also, general studies on the morphology of the species or comparative studies on the morphology of the locomotor system at different species of bears were published (Galateanu et al., 2013; Vonk & Shackelford, 2019).

The descriptions of the morphological particularities of the zonoskeleton, stylopodium, and zeugopodium in the scientific literature are in a small number regarding the species Eurasian brown bear *(Ursus arctos arctos)*. However, there are much data on the morphological aspects of the appendicular skeleton in other carnivore species (Getty et al., 1975; König & Liebich, 2020).

MATERIALS AND METHODS

The bones representing the studied material form the zonoskeleton, stylopodium, and thoracic zeugopodium of Eurasian brown bear (*Ursus arctos arctos*) belong to the collection of the Anatomy discipline.

Knowing there are similarities between the proximal extremity of the brown bear's humerus and the human species' humerus, two human humeri belonging to the discipline's collection were used for comparison. The most

interesting aspects were described and photographed. The terminology used for the description and identification complies with *Nomina Anatomica Veterinaria* (N.A.V. 2017).

RESULTS AND DISCUSSIONS

Only the scapula represents the thoracic zonoskeleton in the Eurasian brown bear (*Ursus arctos arctos*). On the lateral face of the scapula, there are two fossae, supraspinous and infraspinous, in a ratio of 1: 1, separated by an oblique and straight scapular spine. At the distal end, the scapular spine ends with a thickened and widened acromion, which exceeds the ventral (articular) angle. A reduced suprahamatus process flanks the acromion (Figure 1).

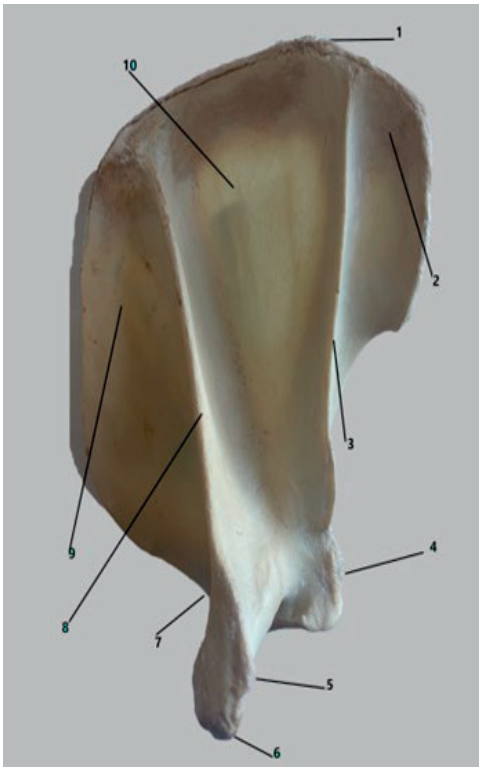

Figure 1. Scapula of the brown bear (*Ursus arctos arctos*) (original) - left limb, lateral face -
1. Epiphyseal lip; 2. Postscapular fossa; 3. Reduced additional spine; 4. The postglenoid tubercle;
5. Suprahamatus process; 6. Acromion; 7. Scapular neck;
8. Scapular spine; 9. Supraspinous fossa;
10. Infraspinous fossa

The cranial margin, slightly thickened in the middle third, presents at the distal extremity a noticeable scapular notch.

The surface of the infraspinous fossa is slightly wavy, and at the distal extremity, towards the base of the spine, a first-order vascular foramen can be observed (Figure 2). The infraspinous fossa is delimited in the thoracic part by a small additional spine, relatively rectilinear, slightly bent over a small postscapular fossa, which presents a rough surface.

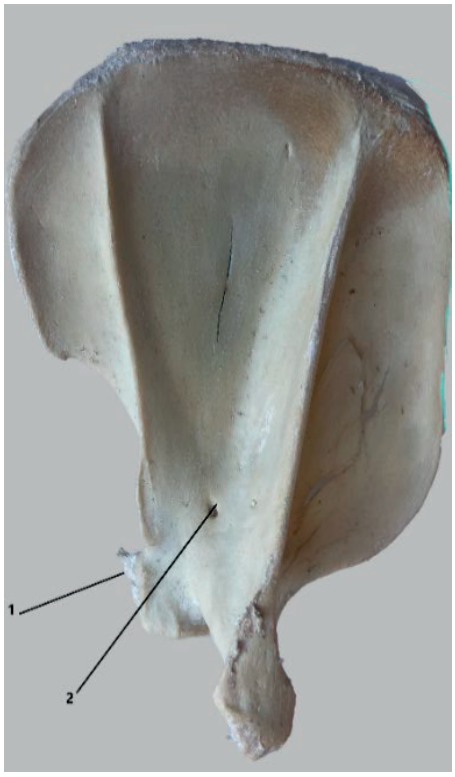

Figure 2. Brown bear scapula (*Ursus arctos arctos*) (original) - right limb, lateral face -
1. Postglenoid tubercle; 2. First-order vascular foramen

The dorsal margin does not present suprascapular cartilage, but a rough epiphyseal lip replaces it.

The caudal margin presents a well-marked postglenoid tubercle at the distal end.

The medial face shows numerous lines of muscle insertion, and the serrate surface has a comma-like appearance. Also, on this face, a reduced medial ridge can be observed, detached precisely on the line that corresponds laterally to the scapular spine (Figure 3).

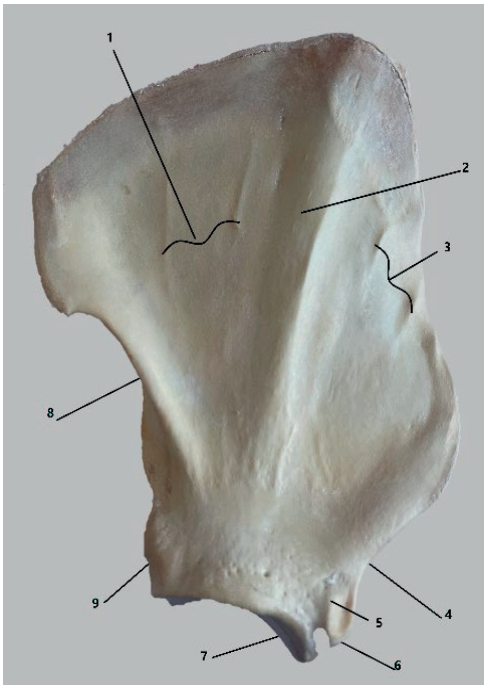

Figure 3. Brown bear scapula (*Ursus arctos arctos*) (original) - left limb - medial face -
1-3. Rough lines of muscle insertion; 2. Medial ridge; 4. Scapular neck; 5. Coracoid process; 6. Supraglenoid tuberosity; 7. Glenoid cavity; 8. Thoracic margin; 9. Postglenoid tubercle

The articular angle is elongated and oval in appearance. From near the cranial edge of the glenoid cavity, the supraglenoid tuberosity emerges cranially, ornamented anteromedially by a reduced coracoid process. At the distal extremity of the tuberosity, a reduced articular surface with an oval appearance is observed (Figure 4).

The humerus represents the thoracic stylopod, which has at the proximal extremity an articular head arranged caudally and slightly elongated distally. Laterally, there is the greater tubercle, and medially, the lesser tubercle, both undivided. The two tubercles are reduced in height and do not exceed the articular surface of the humeral head (Figure 5).

Under the articular head, caudolateral, a pronounced crest is observed. It is directed ventral and craniomedially and reaches the distal third of the bone, creating continuity between the ancone line, the deltoid crest, and the humeral crest (Figure 7).

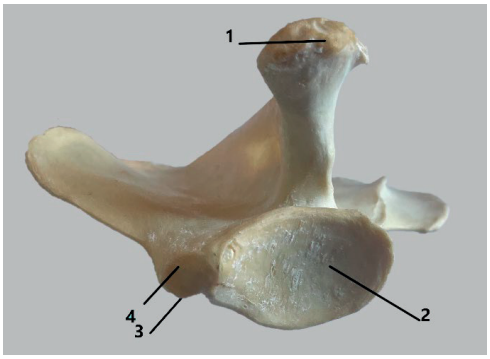

Figure 4. Brown bear scapula (*Ursus arctos arctos*) (original) - left limb, distal extremity - 1. Acromion; 2. Glenoid cavity; 3. Coracoid process; 4. Supraglenoid tuberosity

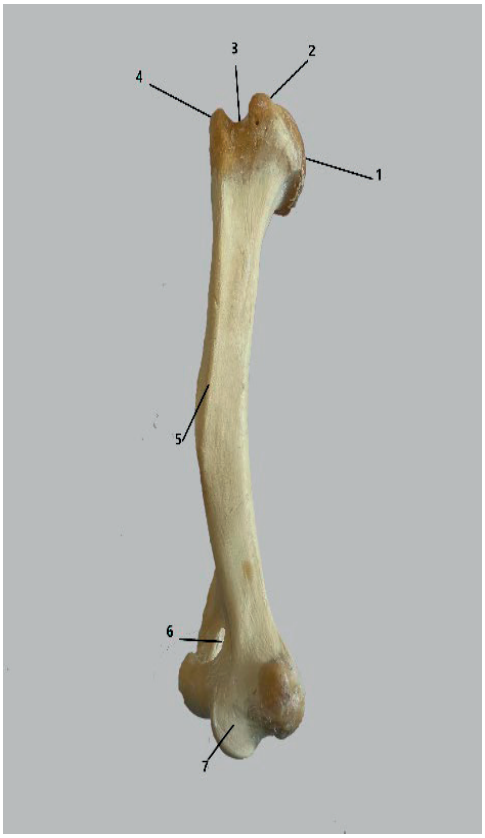

Figure 5. The left humerus of the brown bear (*Ursus arctos arctos*) (original) - medial face -
1. Articular head; 2. The lesser tubercle; 3. Intertubercular groove; 4. The greater tubercle; 5. Crest of the greater tubercle; 6. Supratrochlear foramen; 7. The medial lip of the trochlea

The proximal extremity of the diaphysis in the brown bear is highly developed and thick

compared to that of humans, which is thin and cylindroid. However, the proximal epiphysis of the humerus has similar characteristics in both species (stretched articular head surface, reduced humeral tubercles) (Figure 6).
The oval infraspinous surface and a conspicuous tubercle for the teres minor muscle are visible on the lateral side of the proximal extremity.
On the cranial face, the crest of the greater tubercle joins the humeral crest in the distal third of the body.
The brachial groove is weakly highlighted.
The tubercle for the teres major and latissimus dorsi insertion is in the upper third of the medial face.

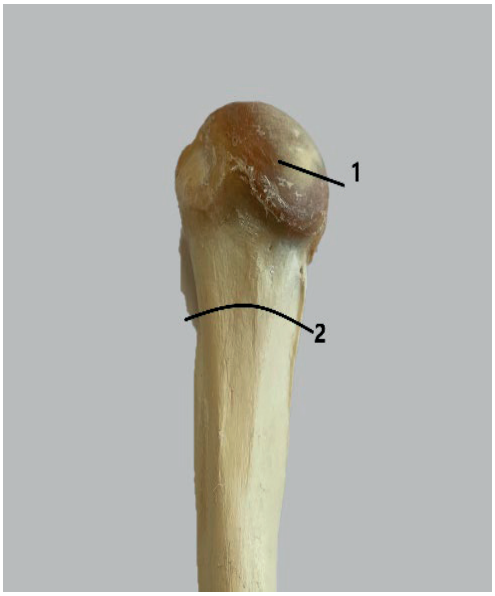

Figure 6. The right humerus of the brown bear (*Ursus arctos arctos*) (original) - lateral, cranial view - 1. The greater tubercle; 2. The proximal extremity of the diaphysis

Distally, the lateral epicondyle's crest is highly developed, an essential differential character compared to the humerus of any large European species (Figure 7).
Although, in the vast majority of studies on the bear humerus, the absence of the supratrochlear foramen is specified, in the studied specimen, this foramen appears on the right limb, allowing the communication of the olecranon and coronoid fossae (Figure 9). In the humerus on the left side, a thin bony blade covers the foramen, so fossae mentioned above are separated (Figure 7)
The distal articular surface presents a condyle laterally and a trochlea with unequal lips medially, the medial one being higher and with a slightly sharp edge.
The humeral epicondyles do not extend beyond the distal articular surface.
Dorso-cranial from the distal articulation surface, the coronoid and radial fossae are present, separated by a small ridge, and caudally, at the same level as the two, is the olecranon fossa (Figure 8).

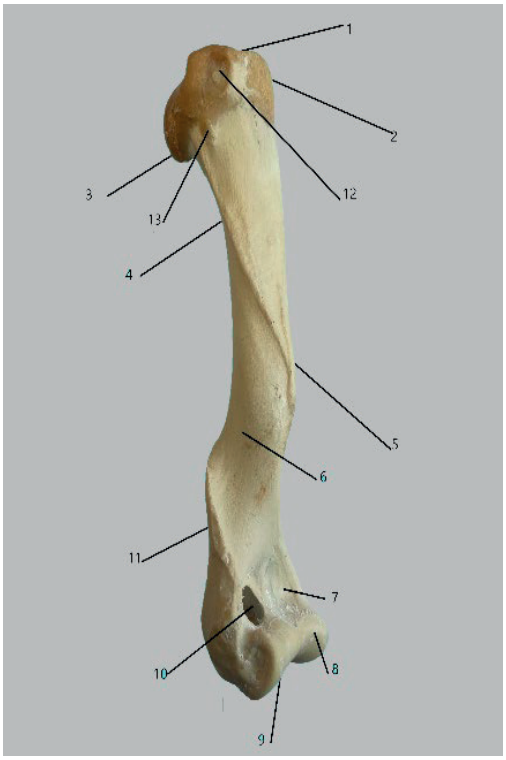

Figure 7. The right humerus of the brown bear (*Ursus arctos arctos*) (original) - lateral-cranial face - 1.The greater tubercle; 2. The lesser tubercle tubercle; 3. Articular head; 4. Deltoid crest; 5. Humeral crest; 6. Brachial groove; 7. Radial fossa; 8. The medial lip of the trochlea; 9. Humeral condyle; 10. Supratrochlear foramen; 11. Crest of the lateral epicondyle; 12. Facies infraspinatus; 13. Teres minor tubercle

Two bones, the radius and the ulna represent the thoracic zeugopodium and are articulated at the extremities.
The radius presents at its proximal extremity a single glenoid cavity elongated, slightly

oblique mediolaterally, and relatively oval (Figure 10).
The radial notch is on the cranial edge of the glenoid cavity, with a single articulation surface for the ulna. The cranio-medial edge of the glenoid cavity shows a small prominence.
A rough surface of muscle insertion occurs at the proximal extremity of the body of the radius, on the medial side.
In the middle third, the body of the radius is slightly curved laterally. On the medial edge of the body, from the middle third to the distal extremity, there is a ridge, very evident in the distal portion.

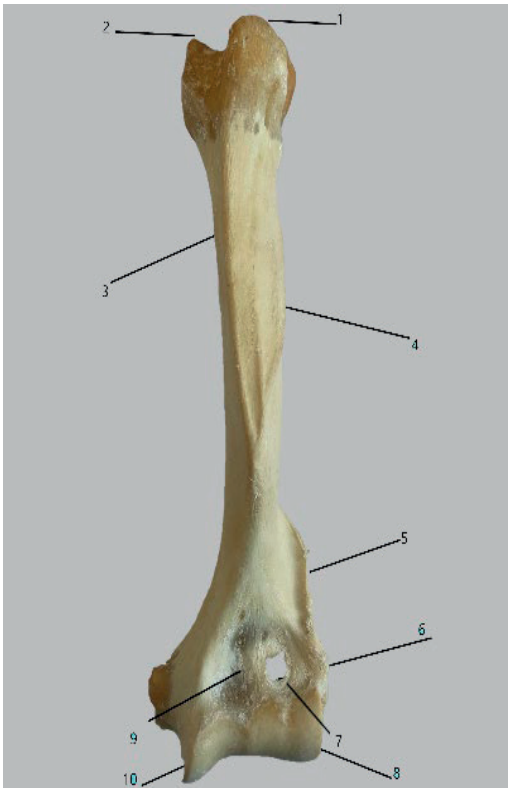

Figure 8. Right humerus of brown bear (*Ursus arctos arctos*) (original) - cranial face - 1. The lesser tubercle tubercle; 2. The greater tubercle; 3. The crest of the greater tubercle; 4. Deltoid crest; 5. Crest of the lateral epicondyle; 6. Supratrochlear foramen; 7. Coronoid fossa; 8. Humeral condyle; 9. Radial fossa; 10. The medial lip of the trochlea

A rugose surface is observed from the proximal to the middle third, placed on the caudomedial side.

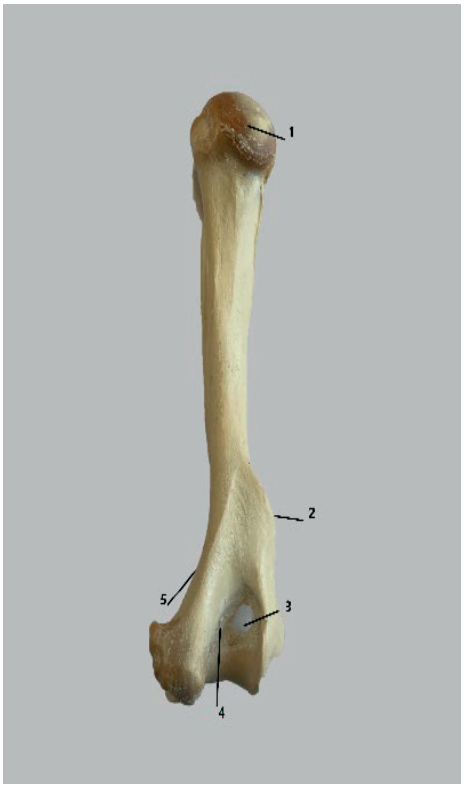

Figure 9. Right humerus in the brown bear (*Ursus arctos arctos*) (original) - caudal face - 1. Articular head; 2. Crest of the lateral epicondyle; 3. Supratrochlear foramen; 4. Olecranon fossa; 5. Crest of the medial epicondyle

The distal articular surface is elongated mediolaterally, being wider medially and narrower laterally.
The ulna has a body slightly curved medially, and a rough surface is on the medial face in the middle third.
On the cranial face at the level of the distal extremity, three tendinous grooves are visible, two arranged longitudinally and one obliquely cranio-distal. On the lateral side of the distal extremity, there is an obvious radial styloid apophysis, and medially, there is an articulation surface for the ulna (Figure 11).
At the proximal extremity of the ulna is the olecranon with a relatively rectangular appearance, which presents an obvious olecranon tuberosity of a relatively triangular appearance, decorated in the central part with a prominent tubercle (Figure 13).

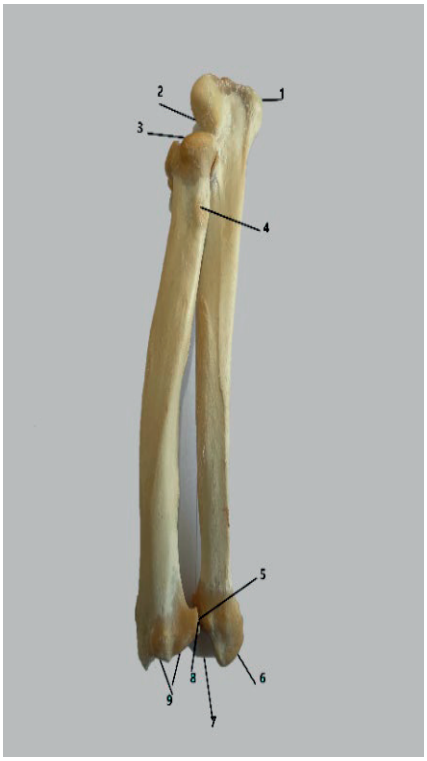

Figure 10. Radius and ulna of the left limb of the brown bear (*Ursus arctos arctos*) (original) - lateral view - 1. Olecranon tuberosity; 2. Trochlear notch; 3. Glenoid cavity; 4. Lateral tuberosity; 5. The articular surface for the ulna; 6. Ulnar styloid apophysis; 7. Carpal joint surface; 8. The articular surface for the radius; 9. Tendon grooves

The anconeal process is drawn cranially, and from its level descends a large trochlear notch, with an articular surface arranged obliquely cranio-laterally and slightly twisted (Fig. 12).
At the proximal extremity, the ulna has a single articulation surface for the radius.
A small muscular crest is at the distal extremity of the body, on the lateral side.
The distal extremity presents an articulation surface for the radius, arranged medially, and an oval articulation surface for the carpal bones.

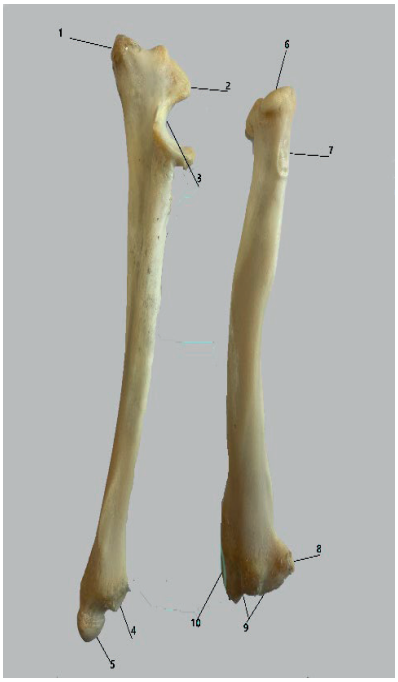

Figure 11. Radius and ulna in the brown bear (*Ursus arctos arctos*) (original) - left side - 1. Olecranon tuberosity; 2. Anconeal process; 3. Trochlear notch; 4. The articular surface for the radius; 5. Ulnar styloid apophysis; 6. Glenoid cavity; 7. Lateral tuberosity; 8. The articular surface for the ulna; 9, 10. Tendon grooves

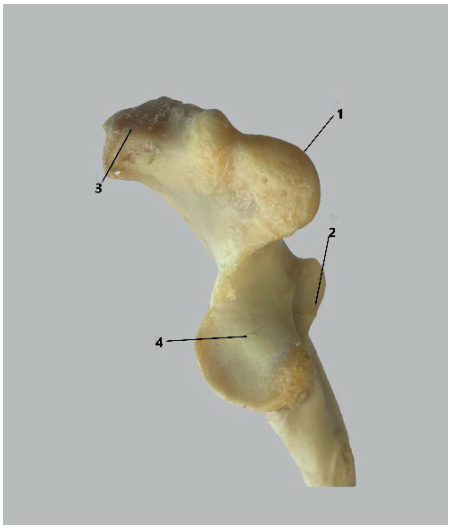

Figure 12. Left ulna in the brown bear (*Ursus arctos arctos*) (original) - dorsal view of the proximal extremity - 1. Anconeal process; 2. Radial incision; 3. Olecranon tuberosity; 4. Trochlear notch

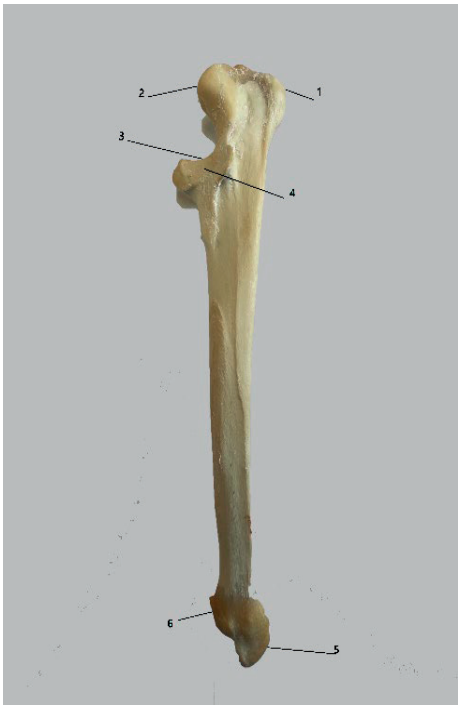

Figure 13. Left ulna in the brown bear (*Ursus arctos arctos*) (original) - lateral view - 1. Olecranon tuberosity; 2. Anconeal process; 3. Trochlear notch; 4. Radial incision; 5. Ulnar styloid apophysis; 6. The articular surface for the radius

CONCLUSIONS

1. The supraspinous fossa and infraspinous fossa are divided by an oblique, rectilinear scapular spine, having a ratio of 1: 1.
2. The acromion is thick and wide and exceeds the articular angle of the scapula.
3. The presence of an additionally reduced spine in the thoracic part of the infraspinous fossa delimits a reduced postscapular fossa.
4. The presence of a postglenoid tubercle at the distal end of the thoracic edge of the scapula.
5. There is a reduced, oval-shaped articular surface at the distal extremity of the supraglenoid tuberosity.
6. The humeral tubercles are undivided, very low in height, and do not exceed the articular surface of the humeral head.
7. The infraspinous surface has an oval appearance, and the tubercle for the small round is well highlighted.
8. Although the proximal epiphysis of the bear humerus is very similar to that of the human species, the proximal third of the diaphysis is thick in the bear and progressively connects to the humeral head. In humans, the bulky proximal epiphysis connects to a much-reduced epiphysis.
9. The anconeal, deltoid, and humeral crests, of equal height, form a continuous line without a clear demarcation line between them.
10. The crest of the lateral epicondyle is very high, a differential character from the humerus of any large domestic or wild European species.
11. The supratrochlear foramen, well highlighted in the humerus on the right side, is covered by a thin bony membrane in the humerus on the left side.
12. The olecranon presents a cranially drawn anconeal process and a sizeable trochlear notch. The articular surface is arranged obliquely cranio-laterally and slightly twisted.

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EFFECT OF LONG-THERM VITAMIN A AND E DIET SUPPLEMENT ON SOME SEMEN TRAITS IN ROOSTER

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Abstract

The paper presents the effects of long-term dietary supplementation with vitamin A (600 IU or 180 μg/kg diet), vitamin E (600 IU or 270 mg)/kg diet) or vitamin A+E (same values) on some biological traits of sperm in Cornish hybrid roosters from 40 to 57 weeks of age versus control. Ejaculate volume, sperm density, and motility were analyzed weekly from 47 to 57 weeks of age of the roosters. The analyzed traits decreased from week to week reaching the levels of the control two or three weeks later than control, showing an effect of improving of the analyzed properties of the sperm and prolonging the reproductive capacity of the roosters at least up to the age of 57 weeks of the roosters. These effects were recorded for both A and E investigated vitamins. Vitamin A better (significantly) improved ejaculate volume and sperm density, while vitamin E predominantly improved sperm motility. The association of the two vitamins in dietary supplements did not lead to potentiating, or mutual inhibition phenomena of the biological traits of sperm in any aged roosters.

Key words*: roosters, sperm traits, vitamin A, vitamin E.*

INTRODUCTION

The aging process of breeding cocks is one of the main economic problems of poultry farming. The damage produced by the physiological aging process with direct effects on reproductive performance drew in the attention of many researchers in the field (Khang et al., 2022; Rengaraj & Hong, 2015; Bensoussan et al., 1998). Vitamin deficiencies are one of the causes that accelerate the aging of roosters and lead to their reformation. Vitamins with an antioxidant effect, as vitaminA, vitamin E, vitamin C and so are among the most likely to be in insufficient amounts in the diets or to by impaired in their biological actions.

Diets contain such vitamins in their composition, but specialists have not reached a consensus regarding the level of supplementing diets with such vitamins (Rengaraj & Hong, 2015; Khang et al., 2022). An explanation would be the great variability of situations and requirements, variability generated by the peculiarities of the breed, age, production level, diet composition, microclimatic conditions, physiological state of the birds, etc. Many studies are focused on the effects of antioxidant vitamins on the characteristics of the semen only (Khang et al., 2022; Kolb, 1997; Kolb, 1998; Mehranjani & Taefi, 2012). The morphological and functional effects of different levels of vitamin supplements in the diet on the reproductive system, mainly the testis, epididymis and deferens duct are not known in detail. On the other hand, breeding roosters have the particularity of aging before the reformation of hens of the same age, raising the issue of extending their reproductive capacity. The present work tests the effects of two antioxidant vitamins (A and E) administered separately and combined on the sperm properties (ejaculate volume, sperm viability, and density) in Cornish hybrid roosters during the aging physiological process.

MATERIALS AND METHODS

Animals and experimental design

40-week-old Cornish hybrid roosters were used in this experiment. Three experimental groups (noted: group A, group E and group A+E) and a control group were constituted, 16 animals each one. Each group was housed in its own 2.2/1.8/0.6 cm cage. All groups were fed on the same commercial diet based on maize 35.6%, wheat 27.4%, soy extruded 21.3% and containing 15.43% crude protein, 3.89% calcium, 0.39% phosphorus, and 2,880 kcal/kg ME (calculated values). The basal commercial diet already contained 120 IU (36 μg) vitamin A/kg and 100 IU (45 mg) vitamin E/kg. Group A diet was supplemented with 600 IU (180 μg) vitamin A (as retinal)/kg diet. Group E diet was supplemented with 600 IU (or 270 mg) vitamin E (as vDL-α-tocopherol acetate vitamer, Hoffmann La Roche, Basel, Switzerland)/kg diet and group A+E diet was supplemented with both vitamins, in the same amounts as groups A and E, respectively. The feeding by the diets enriched in the respective vitamins started at the age of 40 weeks of roosters and continued until the age of 57 weeks. The animals were fed restrictively (220 g/capita/day) and had free access to water. The shelter temperature was maintained between 18° and 23°C. The light schedule was 15.5 hours, from 5:00 a.m. to 8:30 p.m. The experiment was approved by the ethic committee of the institution.

Semen sampling

Starting by 47 weeks of age, sperm samples were taken weekly according to the method described by Bunaciu et al. (1989).

Semen analysis

The ejaculates were collected in transparent glass graduated collection tubes. Volumes were directly recorded in the tube immediately after collection at the lower margin of the semen meniscus and were expressed in microliter (μL). Sperm motility was assessed as previously described by Bălăceanu et al. (2022), by a wet preparation technique using a Nihon Kohden optical microscope (Sapaco 2000, Bucharest, Romania) on a warmed plate. Briefly, the semen sample was diluted (1: 200) in 37°C Ringer's solution immediately after collection. One drop of the diluted semen (no more than 20 μL) was placed on a slide and covered with a 20 × 20 mm glass coverslip. Motility was estimated by direct observation of spermatozoa in at least five fields under 400× magnification and a lowered condenser to disperse the light. Motility was expressed as the percentage of all spermatozoa showing progressive movements, either linearly or in a large circle, regardless of speed (rapid or slow). Nonprogressive spermatozoa with other patterns of movement were not considered in this category. Sperm count was determined using a hemocytometer with a Nihon Kohden optical microscope. Fresh semen samples were diluted (1: 200) and fixed using neutral Hancock's solution (62.5 mL of 37% formaldehyde, 150 mL of 1% saline, 150 mL of sodium phosphate buffer, and 500 mL of double-distilled water) and a Potain pipette, as previously described Bălăceanu et al. (2022). The results are expressed as the number of spermatozoa per milliliter (mL).

Statistics

The obtained values were statistically processed using a Microsoft Excel 2019 software. The differences between the vitamin supplemented groups and the control were considered significant when the probability of the null hypothesis was below 5% ($P < 0.05$).

RESULTS AND DISCUSSION

The effect of the vitamin supplementation on the ejaculate volume are presented in the Table 1. According to the date presented in the Table 1, the ejaculate volume was significantly higher in the groups supplemented in vitamin A (groups A and A+E) versus control, following the first seven weeks of vitamin administration, but not in the group supplemented in vitamin E. The evolution was downward in the next ten weeks for all groups, including the control. Ejaculate volume levels in the groups supplemented in vitamin A decreased to the level of the control with a delay of about three weeks (Figure 1). The level of differences decreased progressively during the next ten weeks of monitoring. The development suggests a moderate ejaculate volume-stimulating effect of vitamin A.

Table 1. Evolution of ejaculate volume (in µL) in Cornish hybrid roosters fed on vitamin A and/or in vitamin E supplemented diets from 40 to 57 weeks of age

Group	Week of age						Mean	SD	P*
	47	49	51	53	55	57			
Control	390$^{a;\ b}$±76	387±60	345±54	274±76	265±110	265$^{a;\ b}$±44	321.2$^{a;b}$± 33.3	25.1	0.005
Group A	451^{a}±59	441±44	396±37	396±77	276±32	303^{a}±56	377.6^{a}±54.5	14.4	0.004
Group E	370±54	380±36	370±26	367±45	228±54	271±22	331.3±27.9	14.2	0.001
Group A+E	466^{b}±32	443±13	414±43	40133	267±38	287^{b}±11	379.6^{b}±30.0	15.2	0.000

The administration of vitamin supplements started at the age of 40 weeks of the roosters
Values are expressed as mean ± standard error of mean
SD = standard deviation
* P by Tukey test
Values in the same column with the same superscript differ significantly (P < 0.05)

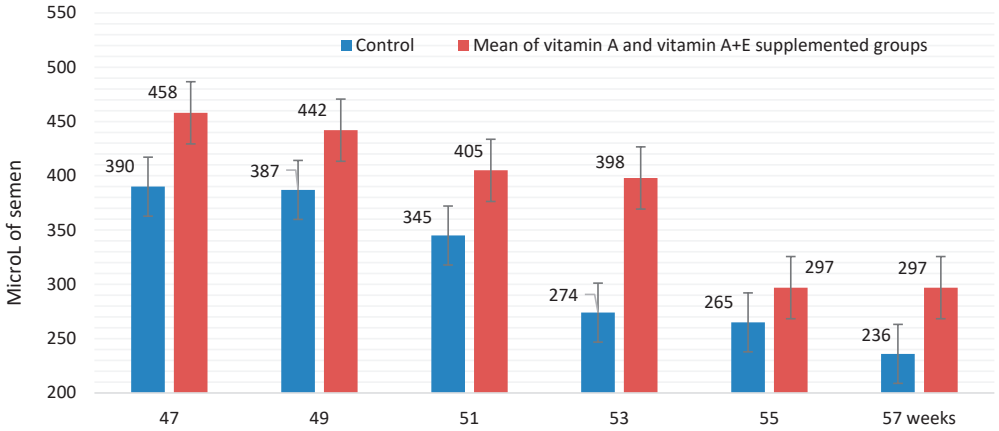

Figure 1. Weekly comparativ evolution of ejaculate volume in vitamin A supplemented Cornish hybrid roosters versus control from 40 to 57 weeks of ge (expressed in microL)

The levels of the ejaculate volume in the vitamin E supplemented group did not differ significantly from those of the control, thus limiting the stimulating effect of this vitamin on the secretory capacity of the rooster genital system. A relatively small difference between the ejaculate volume averages of the three experimental groups and the control, of only 41.2 µL, was to expect considering the particularities of the reproduc-tive system in the rooster: the lack of accessory sexual glands. This makes it difficult to show significant differences in seminal plasma volume. The high values of the standard deviation illustrate the heterogeneity of the samples of biological material from both the control and the vitamin-supplemented groups. Regarding the data from the literature on this topic, we mention that Sima et al. (2020) determined sperm traits in Cornish roosters during the physiological aging process and they found a decrease of 115 µL from 53 to 63 weeks of age. In our study, the decrease in ejaculate volume was 125 µL in the control, 148 µL, 99 µL and 179 µL in the experimental groups, respectively, which makes the results compatible. In a meta-analysis of the effect of vitamin E supplementation on rooster sperm, Hayanti et al. (2022) state that vitamin E does not significantly influence ejaculate volume, which

is in agreement with our results on Cornish hybrid roosters.

Mean sperm motility (Table 2) was higher in all vitamin-supplemented groups (with a three-group mean of 62.6% versus 56.5% in the control) at 47 weeks of age. The downward trend during the next 10 weeks of age was, however, much slower in groups supplemented in vitamin E and A+E, respectively, maintaining significant differences ($P < 0.05$) from the control until 57 weeks of roosters (Table 2). The decrease in motility from 47 to 57 weeks was 9.9% in the control group, 13.5% in group A, 6% in group E and 7.6% in group A+E. This aspect points to an effect of sperm motility stimulation of vitamin E and prolonging the level of this parameter beyond the age of 57 weeks in roosters, thanks to the antioxidant properties of this vitamin. Bunaciu et al. (1989) showed that motility in Cornish, Plymouth Rock and Sussex roosters does not change significantly throughout the period of technological exploitation live. This reveals breed differences considering that in our research sperm motility decreased significantly with the age of the roosters, too. Khan (2011) provides interesting theoretical explanations about the particularities of action of vitamins A and E on sperm motility in birds: the author states that avian spermatozoa have a particular sensitivity to the action of oxidative factors, being characterized by high proportions of polyunsaturated fatty acids, which are associated with increased susceptibility to reactive oxygen species and lipid peroxidation. Antioxidants of vitaminic type studied in this paper, especially vitamin E, are compounds that suppress the formation of reactive oxygen species. Yan et al. (2017) reported positive results in increasing sperm motility in roosters by using vegetable plants or plant parts in their feed. Turmeric (*Curcuma longa*) is one of them.

Table 2. Evolution of sperm motility (in %) in Cornish hybrid roosters fed on vitamin A and/or in vitamin E supplemented diets from 40 to 57 weeks of age

Group	Week of age						Mean	SD	P*
	47	49	51	53	55	57			
Control	56.5$^{\#;@}$	55.5	52.3	50.5	47.3	46.4$^{b;c}$±11.1	51.4	3.33	0.002
Group A	60.5	60.1	57.0	55.7	50.2	47.1± 6.5	55.1	11.0	0.050
Group E	62.2$^{\#}$	62.2	60.0	58.5	56.0	56.0^{c}± 4.5	59.2	3.03	0.069
Group A+E	65.5$^{@}$	65.5	63.0	60.6	57.2	57.9^{b}± 3.9	61.6	9.11	0.053

The values as percentage of spermatozoa with forward movements were calculated from the total spermatozoa examined. The administration of vitamin supplements started at the age of 40 weeks of roosters;

Values are presented as mean ± standard error of mean;

SD = standard deviation; * P by Tukey test;

Values in the same column with the same superscript differ significantly ($P < 0.05$).

Regarding the third sperm trait analyzed in the experiment, sperm count (or sperm density), at the age of 47 weeks of roosters, the density values were higher only by $0.3x10^9$/mL in groups whose diet was supplemented in vitamin A and only by $0.1x10^9$/mL in the group whose diet was supplemented in vitamin E These values of sperm density, however, were no significantly higher compared to the control both in groups A and A+E and in group E. The analysis of the Figure 2 on the evolution of the total number of spermatozoa *per ejaculate* reveals at least two aspects: 1) at 47 weeks, the group fed on the vitamin A-supplemented diet shows a density of spermatozoa significantly improved versus control. A similar situation is presented by group A+E, revealing the predominant stimulating effect of vitamin A on

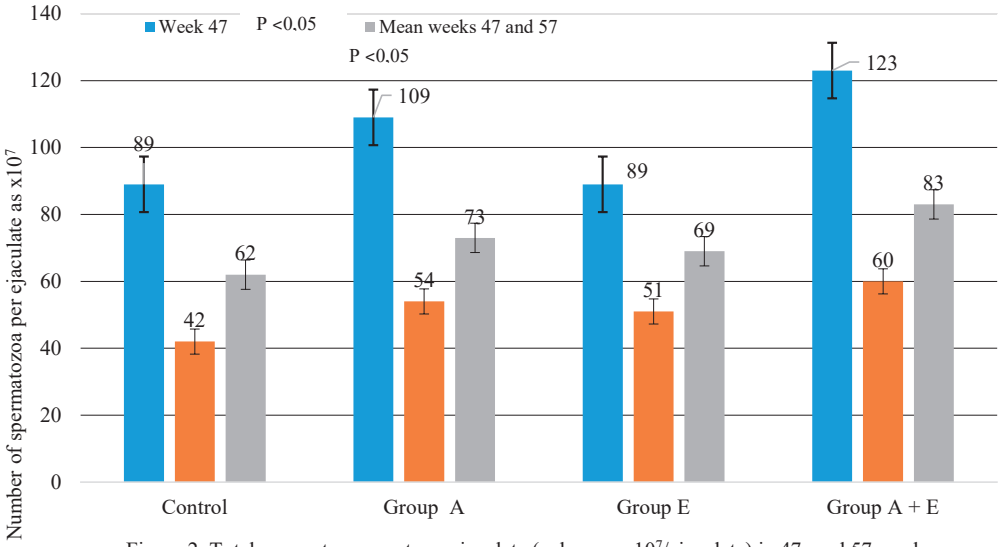

Figure 2. Total spermatozoa cunt per ejaculate (values as x10^7/ejaculate) in 47- and 57-week-old Cornish hybrid roosters fed on vitamin A (group A), vitamin E (group E) or vitamin A+E (group A+E) enriched diets from 40 to 57 weeks of age (values calculate)

spermatogenesis; 2) sperm production values per ejaculate of these groups drop to values with no significant differences versus the control in the next three weeks (meaning three weeks later), suggesting the usefulness of the vitamin A supplement for extending the duration of the technological exploitation life of these birds. Our results are in agreement with those published by Khang et al. (2022) who performed experiments on the supplementation of rooster diet with different amounts of vitamin E (75 and 125 mg/kg diet) but they did not report stimulation effects on the division rate of the spermatogenic line, so sperm density remained unchanged seven weeks from experimental feeding. Very rarely vitamin A may not influence or even decrease sperm density: Yokota et al. (2019) administered excess vitamin A to mice (enormous amounts: 1000 IU/g feed!) finding a 3% decrease in sperm density.

CONCLUSIONS

Long-term dietary supplementation with vitamin A and vitamin E improves sperm biological traits in roosters, extending the reproductive capacity to at least 57 weeks of age, about three weeks longer versus unsupplemented roosters. Vitamin A mainly maintains ejaculate volume and sperm density. Vitamin E mainly stimulates sperm motility. The association of the two vitamins in dietary supplements does not lead to aspects indicating mutual potentiation or inhibition phenomena in their actions on the biological properties of the semen in Cornish hybrid roosters.

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CLINICAL SCIENCES

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PARTIAL INTRAVENOUS ANESTHESIA WITH ISOFLURANE AND ALFAXALONE FOR AN ADULT SHEEP UNDERGOING SOFT TISSUE SURGERY

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Abstract

A 6-year-old Tsurcana male sheep was anesthetized in hospital conditions for a soft tissue surgery- bilateral orchidectomy. After intramuscular premedication with midazolam (0.2 mg/kg), butorphanol (0.1 mg/kg), and ketamine (5 mg/kg), anesthesia was induced intravenously with alfaxalone (1.6 mg/kg IV). The subject was intubated with an 8.5 mm endotracheal tube and anesthesia was maintained with isoflurane (1.5-2.5 vol % in O_2) and 3 boluses of alfaxalone (1.0 mg/kg) delivered intravenously every 10 minutes. The total surgical time was 40 minutes and the whole inhalation anesthesia time was 78 minutes, including the preparation of the subject at the beginning and after the surgical procedure. The mean arterial blood pressure was maintained at 80 mmHg throughout anesthesia, the average end-tidal CO_2 at 51 mm Hg, the mean oxygen saturation of haemoglobin was 99.5%, the mean heart rate was 130 beats per minute, respectively 31 respirations per minute. In conclusion, this anesthetic protocol may be clinically applicable for a male sheep undergoing invasive soft surgery, especially if there is a particular case involving a risk patient.

Key words*: alfaxalone, isoflurane, sheep, surgery.*

INTRODUCTION

In veterinary practice, especially for small animals, soft tissue surgeries are usually performed in hospital conditions under closely monitored inhalation anesthesia. While sheep (*Ovis aries)* are widely used as large animal models (Costea et al., 2022), transferring this technique to farm animals such as sheep, requires adapted techniques and protocols.

Isoflurane anesthesia in small ruminants provides a shorter and safer recovery with a decreased impact over the cardiovascular and respiratory functions (Chowdhury, 2020). Balanced anesthetic techniques can include for sheep premedication the use of a benzodiazepine, an opioid and ketamine for their potent sedative and analgesic effects. In one of our previous studies (Costea et al., 2022), the premedication protocol used (midazolam, butorphanol, ketamine) provided a good sedation and analgesia, enabling painful surgical procedures, reducing the stress of the patients and assuring a smooth recovery.

Alfaxalone is a synthetic neuroactive steroid that acts non-reversible on the gamma aminobutyric acid receptors in the central nervous system. It may be utilized from sedation to maintenance and produces dose-dependent unconsciousness and muscle relaxation with minimal effect on cardiovascular variables (Andaluz et al., 2012).

MATERIALS AND METHODS

An adult male, Tsurcana sheep (6-year-old), weighing 50 kg (Figure 1) was premedicated intramuscularly (IM) with 0.2 mg/kg midazolam (Midazolam®, Aguettant 5 mg/ml), 0.1 mg/kg butorphanol (Butormidor®, Richter Pharma AG 10 mg/ml) and 0.5 mg/kg ketamine (Ketamine® Richter Pharma AG Richter Pharma AG, 100 mg/ml). The subject was induced using the IV route, 10 minutes later, with 1.6 mg/kg alfaxalone (Alfaxan®, Orion Pharma 10 mg/ml) administered slow, over approximately 1.5 minutes. Alfaxan® is a non-irritant product composed of ethanol (150

mg/ml), chlorocresol (1.0 mg/ml) and benzethonium chloride (0.2 mg/ml).

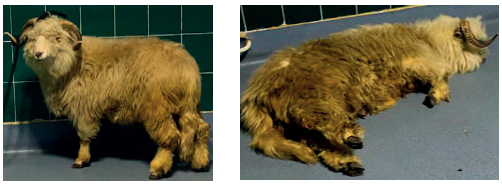

Figure 1. Tsurcana sheep before premedication and 7 minutes after premedication

For intubation 10 mg lidocaine (Xilina®, Zentiva, 10 mg/ml) was topically administrated onto the arytenoid cartilages and endotracheal intubation was performed with the sheep positioned in sternal recumbency, on a padded surgical table using an 8.5 mm diameter cuffed endotracheal tube. The endotracheal intubation was followed by the placement of an oro-ruminal rubber tube.

The subject was maintained using isoflurane (Vetflurane®, Virbac, 1000 mg/g) in 100% oxygen, delivered connected to a rebreathing anesthesia circuit. The protocol included the possibility of using the controlled mechanical ventilation anesthesia machine (AEON 7200®) set to deliver a peak inspiratory pressure (PIP) of 10 cm H_2O and an inspiratory time of one second.

Physiological variables were continuously monitored: pulse oximetry, capnography, electrocardiography, oscillometer and Doppler arterial blood pressure and nasopharyngeal temperature via a vital sign monitor adapted for veterinary medicine (COMEN C80-V®).

Ringer Lactate solution was provided via an infusion pump set at 5 mL/kg/hr.

The subject also received 10 mg/kg Amoxiciline (Amoxicilina FP 20%®, Pasteur) and 1 mg/kg meloxicam (Meloxidyl®, Ceva 5 mg/ml), subcutaneous at the end of the surgery.

For this subject withholding food and water was performed for 12 hours before the procedure.

RESULTS AND DISCUSSIONS

The main challenge of this case was represented by the choice of an anesthetic protocol, which would allow the management of a surgical intervention on soft tissues, for a farm animal raised as a companion animal and operated in hospital conditions, with maximum reduction of the anesthetic risk.

Lower doses of drugs should be administered when combinations protocols are used or high-risk animals are involved, preferable to use inhalational anesthetics only for maintenance of anesthesia and injectable anesthetic drugs for the premedication and induction (Galatos, 2011).

Preanesthetic evaluation was performed in low stress conditions with minimum contention. The subject was assigned to ASA 2 risk group classification adapted for veterinary subjects (Costea et al., 2022): a healthy patient, with no organic disease. Inhalational anesthesia, with the subject intubated with a cuffed endotracheal tube, should be considered for risk animals or prolonged and complicated surgical procedures, giving the advantages of avoiding aspiration of ruminal contents and saliva in the same time with an efficient ventilation and oxygenation.

We did not decide to use a locoregional anesthesia technique, as we wanted to evaluate the effectiveness of the protocol used for anesthesia and pain management, independent of any locoregional anesthesia technique.

The choice of alfaxalone as an induction agent allowed easy intubation in sternal recumbency, with the patient relaxed, without producing any significant respiratory and cardiovascular effects, the patient remaining stable. The use of alfaxalone for a premedicated (sedated) sheep, as an induction agent to be followed by inhalant anesthesia and completed with alfaxalone boluses, was decided since the literature offered similar protocols for unsedated sheep or protocols in which alfaxalone was used in different combinations with alpha 2 agonists (Riebold, 2015).

Intubation was done quickly, under direct visual control, 10 minutes after premedication and pre-oxygenation trough flow by with 100% O_2, with the subject adequately in sternal recumbency, with the head and neck aligned, fully extended, using a long laryngoscope blade.

Since the rumen cannot be emptied completely after withholding food and water, we maintained the subject during surgery in right lateral recumbency, head lowered in order to minimize the aspiration the risk to allow drainage of fluids (Figure 2). The subject's

position was changed after induction and left for the entire maintenance in right lateral recumbency.

During the maintenance of anesthesia, the depth of anesthesia and vital signs were carefully monitored and the anesthetic depth was adjusted in order to maintain an adequate level for the surgical procedure. Starting from these considerations, during the 40 minutes of surgical procedure, 3 consecutives boluses of alphaxalone (1 mg/kg, IV) were administrated intravenously (IV) every 10 minutes in order to maintain the surgical plan of anesthesia.

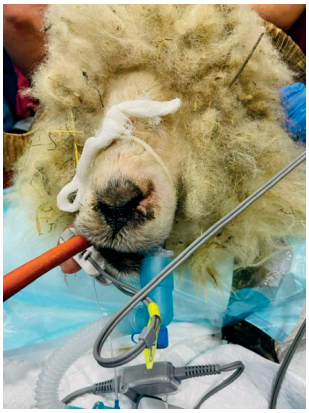

Figure 2. Subjected during maintenance phase

Heart rate rhythm (HR) was stable and maintained between 101 and 135 bpm, with a mean of 130 bpm. The same trend was respected for the respiratory frequency, respectively a mean of 31 rpm (30-33 rpm), with the subject breathing with spontaneous autonomy during the entire anesthesia. As a matter of fact, the controlled mechanical ventilation protocol was not used for this case. As a result, the recovery was extremely quick and the subject was well oxygenated during the entire length of anesthesia (SpO_2 values registered 98-100%).

The average end-tidal CO_2 ($EtCO_2$) level remained high during the maintenance phase, with a mean value of 51 mm Hg, while we targeted $ETCO_2$ value between 30-45 mmHg (Chandrasekharan et al., 2016). The higher values where corelated with the sequences of lower repiratory frequency, after alphaxalone bolus administration (1 mg/kg, IV).

MAP measured oscillometrical was maintained above 80 mmHg throughout anesthesia, but that measuring method required many attempts to measure it, since the cuffed where difficult to position. From this point of view, we recommend the introduction in sheep anesthesia monitoring techniques of higher sensitivity techniques, respectively the invasive arterial blood measuring.

The evolution of body temperature was monitored during the entire anesthesia, from premedication to induction, with no major impact on its trend, since the subject remained normothermic during the procedure. In this sense, we used two methods in comparison, respectively monitoring with the help of an esophageal temperature probe respectively with an electronic thermometer, from the level of the nasal cavity. We did not register significant differences between the two measurement methods. The technique used at the level of the nasal cavity, however, being easier, in the context in which the subject had an endotracheal and esophageal tube, can be recommended.

The total surgical duration was 40 min and the total inhalation anesthesia time was 78 min, including the preparation of the subject between premedication and induction and the post-surgery cleaning procedures.

Respiration was assisted until adequate gaging reflexes developed, 10 min after the end of gas anesthesia and then after another 2 minutes, the endotracheal tube was safely removed.

The patient was able to adopt the quadrupedal position 8 minutes after extubation (Figure 3), with complete control over posture and walking, 17 minutes after extubation.

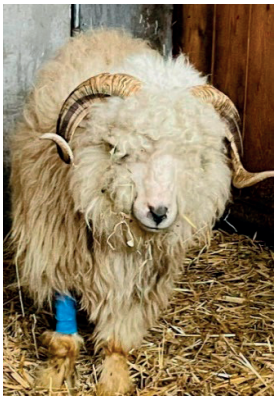

Figure 3. Patient in quadrupedal position, 8 minutes after extubation

CONCLUSIONS

The anesthesia of farm and production animals must be regarded with the same seriousness as that of companion animals. When a sheep is not raised for production, but as a companion animal, the challenge of ensuring a safe protocol is extremely important.

For the presented case, we used a protocol based exclusively on a partial intravenous anesthesia technique, thus ensuring the anesthesia and analgesia necessary to perform the bilateral orchidectomy, without other additional locoregional anesthesia techniques.

The clinical monitoring of the depth of anesthesia, vital signs, as well as the surgical comfort were maintained within the specific physiological limits, so that there were no syncopes during the anesthesia that would determine the change of the initial anesthetic plan or the stabilization of the patient.

Maintaining anesthesia using isoflurane and repeated boluses of alfaxalone was an optimal solution for the patient, assuring stability and an awakening, respectively a quick recovery, without complications.

The use of alfaxalone as a unique agent for induction and, respectively as a maintenance agent along with isoflurane, was an optimal choice for this patient, as it allowed easy intubation, as well as maintaining the patient in stable and safe conditions.

The monitoring of the patient during the anesthesia was possible without any complications, the only difficulties being represented by the positioning of the cuff for measuring the non-invasive pressure, so we recommend the use of the invasive technique if this is available.

The awakening phase was a stage managed very easily, as the patient returned from anesthesia gradually, stably, without showing any adjacent complications, subsequently his evolution was perfect.

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STUDY ON TICK INFESTATIONS OF SMALL RUMINANTS, IN SOUTHERN ROMANIA

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Abstract

Ticks (Acari: Ixodidae) are the most important vectors of zoonotic diseases, presenting a high risk of causing diseases in animals and humans. The purpose of this study was to investigate the prevalence of ticks and the identification of tick species infesting small ruminants in Southern Romania. The study was undertaken from March 2019 to October 2020, including nine sheep and goats flocks in four Prahova County premises. A total number of 2463 sheep and 1202 goats were examined and 76.53% of sheep and 81.2% of goats respectively were infested. Overall, from the infested animals 2233 ticks were collected and the following species were morphologically identified: Haemaphysalis punctata, with an overall prevalence of 66.5%, followed by Dermacentor marginatus (24.5%) and Ixodes ricinus (9.0%). The annual dynamics of tick infesting small ruminants in the investigated areas showed maximum tick abundance in April, for H. punctata and Dermacentor marginatus, and May for I. ricinus, respectively. To reduce the high prevalence of ticks and their impact on productivity in small ruminants, immediate attention is required as control interventions.

Key words*: ticks, infestations, small ruminants, southern Romania.*

INTRODUCTION

Ticks (Acari: Ixodidae) are the most important vectors worldwide, since they transmit numerous diseases to animals and humans such as tularemia, brucellosis, Q fever, rubella, tick-borne encephalitis (Sonenshine, 1991; Mitrea, 2011; Ioniță & Mitrea, 2017). It is reported that about 10% of the known tick species are vectors for a variety of pathogens (viruses, bacteria, protozoa) of animals and humans. Of them, a high number are causative agents of tick-borne diseases that nowadays are re-emerging in many European countries and worldwide (Jongegan and Uilenberg, 2004; Estrada-Pena et al., 2018).

Tick infestations in small ruminant represent one of the most important condition affecting livestock industry; ticks are a major constraint on ruminant production, directly, as hema-topahgous parasites, but also indirectly, due their vectorial role for pathogens, such as *Anaplasma* spp., *Babesia* spp., *Theileria* spp. etc, that impact sheep health and their production (Jongejan, 1999; Genchi & Manfredi, 1999).

Over several generations, the tick has the ability to maintain the pathogen in nature through transstadial and transovarial passages. Biological factors contributing to their high vector potential are their lifestyle, salivary secretions, and blood digestion (Sonenshine, 1991; Márquez-Jimenez et al., 2005; Dantas-Torres et al., 2012).

In Romania, the tick fauna is very diverse, including about 25 species that have been reported infesting animals and/or humans (Feider, 1965; Bădescu, 1967; Mihalca et al., 2012a; Ioniță et al., 2003). In recent years, there has been a significant increase in the abundance of tick infestations on animals (Mitrea et al, 2004; Ioniță et al., 2010). Among the tick species reported in Romania, the most widespread is *Ixodes ricinus* and the most abundant is *Dermacentor marginatus* (Mihalca et al., 2012). However, other species belonging to the following genera, *Haemaphysalis, Hyalomma, Boophilus,* or *Rhipicephalus* have been also reported (Ioniță et al., 2003; Ioniță et al., 2010; Mihalca et al., 2012; Sandor et al., 2017; Ioniță & Mitrea, 2017).

Studies on the prevalence of ticks in Romania, as well as their geographical distribution and seasonal dynamics were carried out in several areas of our country, in North-Eastern, South-

Eastern, or Western (allowing a good assessment of the tick density in the studied areas as well as the seasonal dynamics and/or risks for tick-borne pathogens of relevance for for the animal and public health (Ioniță et al., 2003; Mitrea et al., 2004; Ionita et al., 2010; Chițimia, 2007; Mihalca et al., 2012; Raileanu et al., 2015). In addition, the increased abundance of tick populations associated with climate change and socio-economic conditions is a high potential risk for tick-borne diseases in Romania, as recently reported including by molecular methods (Ioniță et al., 2012, 2013, 2016, 2018; Ioniță & Mitrea, 2017).

Sheep and goat breeding is an important activity in many areas of Romania, particularly for production of meat, milk, and cheese. However, epidemiological surveys on ticks infesting small ruminants in Romania are still limited. Therefore, the purpose of this study was to determine the tick species infesting sheep in goats in some areas in Southern Romania, and the associated potential risks for tick-borne diseases.

MATERIALS AND METHODS

In order to characterize the tick species infesting small ruminants in Southern Romania, a study was carried out, from March 2019 to October 2020, including nine sheep and goats flocks in four districts of Prahova County.

Ticks were collected from animals during of the clinical examination. The entire animal was examined for ticks, with special attention at the head, udder, perineum, lower abdominal area, and the internal parts of the legs. When present, ticks were removed and stored into vials containing 70% ethanol. The vials were labelled with the date of collection, location, and host species and were transported to the Parasitology laboratory of the Veterinary Faculty of Bucharest, for morphological identification.

The tick identification was performed under a stereomicroscope, by using morphological keys (Estrada et al., 2004).

RESULTS AND DISCUSSIONS

The infestation with ticks on small ruminants was investigated in nine localities belonging to four districts of the Prahova County, Romania. For this, there were examined nine flocks of sheep and goats, from which three flocks in Valea Călugărească (44°57′40″North, 26°9′20″ East), four in Urlați (44°59′28″North, 26°13′50″East), one in Iordăcheanu (45°02′45″Nord 26°14′20″ East) and one in Drăgănești (44°49′41″Nord, 6°17′14″East). A total number of 2463 sheep and 1202 goats were examined for tick infestation and 76.53% of sheep and 81.2% of goats, respectively were infested.

From the infested animals, altogether, 2233 ticks were collected and three tick species were identified, namely: *Haemaphysalis punctata*, with an overall prevalence of 66.5% (731 females and 101 males at goats; 562 females and 90 males at sheep), followed by *Dermacentor marginatus* (24.5%) (144 males, 102 females, collected from goats and 185 males, 115 female collected from sheep), and *Ixodes ricinus* (9.0%) 93 females, 24 males at goats; 72 females and 14 males at sheep) (Tables 1, 2, 3).

According to the host species, the frequency of the tick species identified was as following: *D. marginatus,* 546 (24.5%), of which 300 in sheep and 246 in goats; *H. punctata,* 1484 (66.5%) of which in sheep 652 and goats 832, and *I. ricinus,* 203 (9.0%), of which 86 in sheep and 117 in goats. The prevalence of the tick species identified is shown in Figures 1, 2.

Table 1. Prevalence of tick species infesting sheep and goats in Southern Romania (Prahova county)

Tick species/ Host	*Haemaphysalis punctata*	*Dermacentor marginatus*	*Ixodes ricinus*
Total (n = 2233)	**1484 (66.5%)**	**546 (24.5%)**	**203 (9.0%)**
Sheep (n = 1038)	652 (62.8%)	300 (28.9%)	86 (8.3%)
Goats (n = 1195)	832 (69.6%)	246 (20.6%)	117 (9.8%)

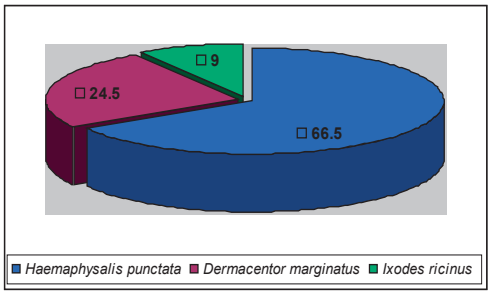

Figure 1. Proportion of Ixodidae tick species populations in small ruminants in Prahova County

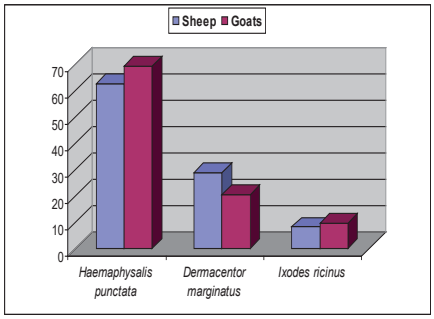

Figure 2. Prevalence and specific structure of Ixodide tick populations in sheep and goats, in Prahova County

Of the examined sheep 76.5% (n=1885) were infested with ticks; from them 1038 ticks were collected, of which 62.8% (n=652) were identified as *Haemaphysalis punctata,* 28.9% (n=300) as *Dermacentor marginatus,* and 8.3% (n=86) were *Ixodes ricinus* (table 2).

Out of the 1202 examined goats, 81.2% (n=976) were infested with ticks; of them a number of 1195 ticks were collected; of them, 69.6% (n=832) were *Haemaphysalis punctata,* 20.6% (n=246) *Dermacentor marginatus*, and 9.8% (n=117) were *Ixodes ricinus* (table 3).

With regards to sex ratio characteristics, among the collected specimens, the proportion of females was higher for two of the identified species, namely *Haemaphysalis* and *Ixodes.* Therefore, the highest abundance was registered for females of *H. punctata* (87.8%) in goats and 86.2% in sheep, higher than in males. For *D. marginatus* the abundance of males was slightly higher than of females, representing 61.7% in sheep and 58.5% in goats. The overall lowest abundance was found in *I. ricinus*, in the both, sheep and goats, but also females predominated, comparative with males (tables 2, 3).

There is known that sex ratio is influenced also by mating strategies, as reported in questing tick (Hornok, 2009). For instance, *Ixodes* ticks are known to mate also on the vegetation (Kiszewski et al., 2001). Also, this may indicate a transition in the relevant period towards higher number of males questing (to fertilize females on animals) (Randolph et al., 2002).

Table 2. Number and prevalence (%) of ticks by category (species, male, female) collected from domestic goats, in nine study areas in Prahova County

Tick species	Number of collected female ticks (%)	Number of collected male ticks (%)	Number of collected total ticks (%)
Haemaphysalis punctata	731 (87.8%)	101 (12.2%)	832 (69.6%)
Dermacentor marginatus	102 (41.5 %)	144 (58.5%)	246 (20.6%)
Ixodes ricinus	93 (79.5 %)	24 (20.5%)	117 (9.8%)
Total No ticks (%)	926 (77.5%)	269 (22.5%)	1195

Table 3. Number and prevalence (%) of ticks by category (species, male, female) collected from domestic sheep in nine study areas in Prahova County

Ticks species	Number female ticks (%)	Number male ticks (%)	Number of collected total ticks (%)
Haemaphysalis punctata	562 (86.2%)	90 (13.8%)	652 (62.8%)
Dermacentor marginatus	115 (38.3 %)	185 (61.7%)	300 (28.9%)
Ixodes ricinus	72 (83.7%)	14 (16.3 %)	86 (8.3 %)
Total No. ticks (%)	749 (72.2%)	289 (27.8%)	1038

Elective places for attaching of ticks were the inner face of the thigh, the sternal region, the lower abdominal region, and the cervical region. The tick infestation was higher in animals with poor body conditions.

The frequency of tick species and their distribution by host species and originating locality are detailed in Tables 4 and 5.

Table 4. The distribution and the specific structure of the Ixodidae tick population in sheep, in nine study areas of the Prahova County (Southern Romania)

Zones /No. hosts infested by tick (n=1885)	Pantazi/ 272	Racheri/ 249	Varfurile/ 100	Valea Mieilor/ 268	Cherba/ 170	Valea Nucetului /305	Valea Pietrei/ 200	Iordacheanu/ 199	Draganesti/ 122
Ticks species (n=1038)	Number ticks; % (n=152)	Number ticks; % (n=133)	Number ticks; % (n=104)	Number ticks; % (n=123)	Number ticks; % (n=97)	Number ticks; % (n=182)	Number ticks; % (n=99)	Number ticks; % (n=96)	Number ticks; % (n=52)
Haemaphysalis punctata (n=652)	81 [53.3%]	61 (45.9%)	58 (55.7%)	87 (70.7%)	73 (75.2%)	129 (70.9%)	65 (65.7%)	70 (72.9%)	28 (53.8%)
Dermacentor marginatus (n=300)	61 (40.1%)	56 (42.1%)	40 38.5%)	25 (20.4%)	16 (16.5%)	42 (23.1%)	26 (26.3%)	16 (16.7%)	18 (34.6%)
Ixodes ricinus (n=86)	10 (6.6%)	16 (12.0%)	6 (5.9%)	11 (8.9%)	8 (8.3%)	11 6.0%)	8 (8.0%)	10 (10.4%)	6 (11.6%)

Table 5. The distribution and the specific structure of the Ixodidae tick population in goats in nine study areas of the Prahova County (Southern Romania)

Zones / No. hosts infested by tick (n=976)	Pantazi/ 155	Racheri/ 150	Varfurile/ 80	Valea Mieilor/ 95	Cherba/ 60	Valea Nucetului/ 198	Valea Pietrei/ 99	Iordacheanu/ 89	Draganesti/ 50
Ticks Species (n=1195)	Number ticks; % (n=192)	Number ticks; % (n=165)	Number ticks; % (n=127)	Number ticks; % (n=113)	Number ticks; % (n=98)	Number ticks; % (n=199)	Number ticks; % (n=126	Number ticks; % (n=109)	Number ticks; % (n=66)
Haemaphysalis punctata (n=832)	121 (63.0%)	105 (63.7%)	93 (73.2%)	85 (75.2%)	72 (73.5%)	135 (67.8%)	89 (70.6%)	88 (80.7%)	44 (66.7%)
Dermacentor marginatus (n=246)	56 (29.2%)	47 (28.5%)	25 (19.7%)	15 (13.3%)	11 (11.2%)	38 (19.1%)	19 (15.1%)	18 (16.5%)	17 (25.8%)
Ixodes ricinus (n=117)	15 (7.8%)	13 (7.8%)	9 (7.1%)	13 (11.5%)	15 (15.3%)	26 (13.1%)	18 (14.3%)	3 (2.8%)	5 (7.5%)

The annual dynamics of the tick population recorded in the study area registered their peak in spring, due to the environmental and vegetation conditions. The maximum population abundance for *D. marginatus* and *H. punctata* was in April, while for *I. ricinus*, the maximum was in May followed by a sudden decrease in June due to low humidity.

Interspecific variations of the annual dynamics were observed. The appearance of ticks was observed for *D. marginatus* in March, unlike *I. ricinus* where the beginning of the invasion was in May. The maximum value of the invasion indices was for in April, for *H. punctata* and *D. marginatus* and in May for *I. ricinus*. The dynamic curve decreased in the summer months for all three species and started to re-increase in the autumn, this being about 25% of the spring maximum. These differences were found both in sheep and goats.

The abundance of Ixodidae ticks is influenced by the ecological conditions (Ioniță, 2004). In the plain area, the temperature is higher and the humidity is low, compared to the hill area, where the temperature is lower and the humidity is higher.

Based on the registered data during the study period (2019-2020), diagrams of the annual dynamics of the tick populations identified for each species are presented in fig. 3 and 4.

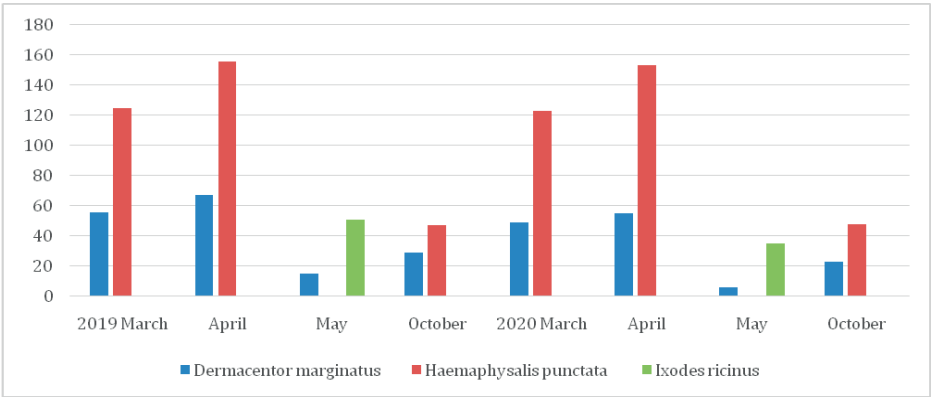

Figure 3. Annual dynamics of the recorded ixodid tick species in sheep, in the studied

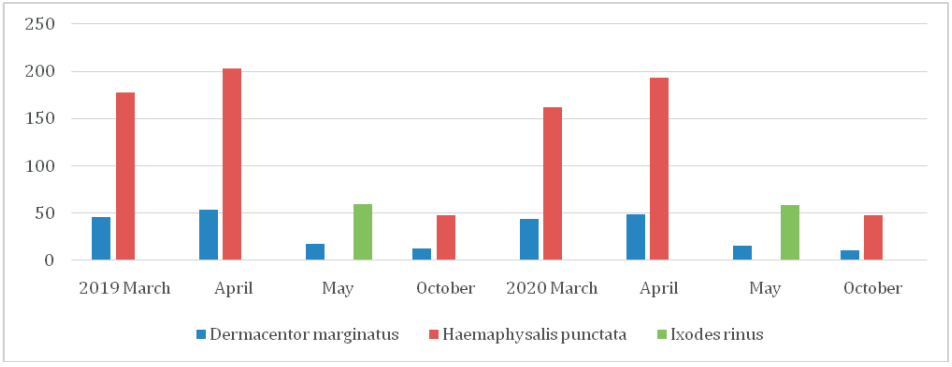

Figure 4. Annual dynamics of the recorded ixodid tick species by months in goats, in the studied area

The findings of this study add new data to update epidemiological knowledge regarding tick populations composition in goats and sheep from southern areas of Romania, particularly Prahova county. Therefore, in our study, the most prevalent tick species were *H. punctata* and *D. marginatus,* and in lower frequency and abundance *I. ricinus*. The bio-ecological features of these tick species are congruent with the characteristics of the environment and biotypes were from tick's host originated. All the three species identified in the present survey have been previously reported in different areas of Romania, including in areas from Southern Romania. For instance, some study reported higher prevalence values than the current study on *I. ricinus*, of 34.24%, in the South-East and North-East areas of Romania (Ioniță et al., (2003, 2010). It is recognized as the most widespread tick species in Romania, with the highest range of host species, including small ruminants; its prevalence varies form 41.6% to 86.9%, according to the geographical area (Ionita et al., 2003, 2010; Dumitrache et al., 2012; Mihalca et al., 2012b).

D. marginatus is also widespread in Romania, with prevalence values from 9.5% to 34.2% (Ionita et al., 2010; Chitimia, 2007; Mihalca et al., 2012a). *H. punctata* in the current study is higher showed the highest prevalence in both sheep and goats, followed by *D. marginatus*. These findings are in line with similar studies in small ruminants from Italy (Rinaldi et al., 2004).

Tick infestation was compared with the global average prevalence in Europe. Therefore, a similar study in sheep in the southern Italian Apennines, reported the most frequent species, at the farm level, *D. marginatus* (37.6%), followed by *H. punctata* (29.4%), and with lower % *I. ricinus* (0.5%).

Our findings are different than those reported in neighboring countries, such as Serbia. For

instance, in a study investigating tick infestation in sheep and goats from Belgrade area revealed that 60.14% of examined sheep and 34.42% of goats were infested. The relative abundance analysis showed that in both sheep and goats *I. ricinus* was dominant (41.91%, in sheep, 64.42%, in goats). Other tick species recorded were as follows: in sheep - *D. marginatus* (32.91%), *Rhipicephalus bursa* (17.22%), *R. sanguineus* (6.72%), *H. punctata* (2.21%) and *D. reticulatus* (1.17%); while, in goats, were registered *Rhipicephalus bursa* (17.22%), *Rhipicephalus sanguineus* (6.72%), *H. punctata* (4.22%) and *D. marginatus* (2.91%) (Pavlović et al., (2013).

In a similar study in sheep and goats, in Bulgaria, the predominant species was found *Rhipicephalus bursa,* parasitizing on both goats and sheep in Plovdiv region (Arnaudov et al., 2014; Arnaudov & Arnaudov, 2017).

All these findings emphasize on the differences on the tick species composition of sheep and goats accordingly to the local geo-climatic characteristics that significantly impact the biology of ticks and their ecological niche, and consequently the ecology of tick-borne diseases (Márquez-Jiménez et al., 2005; Walter et al., 2016; Krčmar, 2019).

The knowledge on the tick species occurring in a particular area is of high relevance for the both, animal health and public health, as numerous tick-borne pathogens have also zoonotic potential, as recent molecular studies reported also on Romanian ticks (Ionita et al., 2013, 2016). Therefore, it is requiring additional in-depth studies on ticks to focus develop tick control strategies based on their spatiotemporal distribution, host preferences, and climatic niches, which could provide opportunities to assess the risk of infection at the local level through prevention and surveillance of critical points (Mitrea, 2002; Dantes-Torres et al., 2012).

CONCLUSIONS

This study provides new data on the species composition and epidemiology of ticks infesting sheep and goats in Southern Romania. The results confirmed the presence of *Dermacentor marginatus, Haemaphysalis punctata,* and *Ixodes ricinus* in the investigated area and emphasize on the high potential risks for tick-borne diseases in animals and humans. Therefore, to reduce the high prevalence of ticks and their impact on productivity in small ruminants and the associated risks for tick-borne pathogens, immediate attention is required as control interventions.

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EFFICIENCY OF SURFACE DISINFECTION BY NEBULIZATION USING CUBE ATOMIZERS IN A VETERINARY UNIT – PRELIMINARY STUDY

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Abstract

Maintaining proper asepsis and hygiene conditions in spaces intended for veterinary surgery remains a paramount for compliance with professional ethics. Moreover, environmental surfaces can contribute to the spread of cross-infections, and therefore constitute a likely transitory site for the accumulation of microorganisms. The aim of the current study was to evaluate, implement and measure the efficacy of a novel nebulization technique method for surface disinfection. The procedure was carried out in ten enclosed spaces used as surgery rooms within veterinary clinics. Disinfection was performed using Cube Atomizers, a nebulizer with a revolutionary spraying system, which transforms the biocide substance into microparticles that persist in the air for a long period of time, ensuring decontamination of the treated volume (air and all types of surfaces). Thus, using an approved biocide, the device ensured a successful disinfection of spaces, eliminating bacteria, and other biological pathogens. The microbiological tests were carried out before and after disinfection on different growth mediums (Agar for the total bacteria count, Chapmann for Staphylococcus, Holmes for Streptococcus, Levine for Gram-negative Cocci and Sabouraud for fungi). An increased efficiency of disinfection was observed, with a significant decrease in total bacteria count of almost 90-97% and the value of colony-forming units reaching 0 after nebulization in some cases; for Staphylococcus (Chapmann) there was a significant decrease, between 85-95%; for Streptococcus (Holmes) the decrease was almost 90-99%; for Gram-negative Cocci (Levine) the decrease was almost 92-99%; and for fungi (Sabouraud) the decrease was around 50%. The Cube Atomizers device is easy to use, can be fitted anywhere and guarantees safety for the user, environment, and all treated materials. Its revolutionary system ensures decomposition of the biocide into microparticles, leaving no residues.

Key words*: disinfection, Cube Atomizer, veterinary unit, nebulization.*

INTRODUCTION

Maintaining appropriate aseptic and hygienic conditions in spaces intended for veterinary surgery remains a paramount in the desire for success and respect for professional ethics. Animal surgery requires the same precautions regarding infections as human surgery. (Andercou, 1993) In addition to the antisepsis used for disinfection of hands, instruments and materials, rigorous disinfection of the surgical/obstetrical area is also necessary. (Bogdan et al., 2009; Boitor et al., 1986; Bolte S., 1988; Cenariu M. et al., 2020; Groza I. et al., 1998) The occurrence of infection as a result of wound contamination is one of the greatest risks of surgery (Mateș, 2001; Mitrănescu Elena, 2014; Oană L. et al., 2012; Răpuntean S. et al., 2017) Preventing this is done by the correct execution of asepsis, antisepsis and disinfection measures, by removing all possibilities of contamination of the surgical wound. (Leau et al., 2014) Although data related to nosocomial infections in veterinary medicine are limited, they are present and lately their frequency is increasing. (Ruple-Czerniak et. al, 2013; Ruple-Czerniak et al., 2014; Stull et al., 2015). In veterinary clinics, the fact that a large proportion of the pathogens involved in causing nosocomial infections are zoonotic is a big problem for both humans and animals, considering that many zoonoses can have a serious, sometimes fatal, course (Bîrțoiu A. et al., 2004; Gonciarov Magda, 2014; Igna C., 2001; Savu et al., 2000). Unlike human medicine, in veterinary medicine no hygiene protocols are developed for veterinary clinics. In a veterinary hospital, where faeces and different types of secretions are always present, the susceptibility of harbouring pathogens is much higher and represents an increased risk for contamination with nosocomial and zoonotic diseases, so that

rigorous disinfection is crucial. Veterinarians and ancillary staff need to be educated in this regard and disinfection protocols need to be implemented in every veterinary medical unit. (Traverse et al., 2015) The aim of the current study was to evaluate, implement and measure the efficacy of a novel nebulization technique method for surface disinfection.

MATERIALS AND METHODS

For this study, disinfection by nebulization was carried out in closed spaces intended for surgery rooms in ten veterinary clinics in Romania, Cluj County. The device used was Cube Atomizers, a nebulizer with a revolutionary spraying system that transforms the biocide into microparticles that remain in the air for a long time, ensuring total decontamination of the treated volume (air and all types of surfaces). Thus, using an authorised biocide, the machine successfully disinfects premises, eliminating bacteria, fungi, viruses and other biological pathogens of any kind from all surfaces in just a few minutes. Its revolutionary system breaks down the biocide into micro-particles, leaving no residue. Compared to other means of disinfection used in current practice (e.g. steam generators or ultraviolet lamps) where more manpower is required, or the time of use is long, the Cube Atomizers disinfects the entire surface in just a few minutes, is easy to use, can be installed anywhere and requires only one person to handle it. Unlike the technique of disinfecting premises with ultraviolet lamps, the Cube nebuliser guarantees safety for the user, the environment and all treated materials. Ultraviolet lamps require a longer period of time to disinfect the space and can cause skin and eye damage, degrade treated surfaces over time, and cause respiratory tract damage through the generation of ozone (FDA, 2021). Sanitation samples were taken 2-3 hours before the start of the nebulization operation (BEFORE test), and 1-2 hours after the nebulization was performed (AFTER test).

A. Materials needed

The following materials were required (Figure 2): non sterile disposable gloves, protective equipment (disposable gown), protective goggles, utensils and measuring devices for preparing the quantities of needed disinfectant product, cleaning materials (detergent and wipes), disinfectant, waste collector, a device for measuring room size and volume (laser telemeter), sterile swabs with culture medium, sterile test tubes specially prepared for sampling, Petri dishes and other utensils needed for sampling for bacteriological analysis, the Cube Atomizers nebulizer, a power supply (grounded socket) and a timer. Biosan Steridet disinfectant solution, 20 g/l water, was used for this study. Biosan Steridet (Figure 1) is a pink powder with strong disinfectant action, containing Pentapotassium Bis (peroxymonosulphate) Bis (sulphate) as active substance.

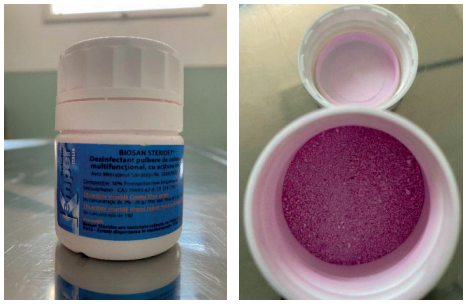


Figure 1. Biosan Steridet solution (Original)

Figure 2. Materials needed: cube atomizers, Petri dishes, sterile swabs with culture medium, disinfectant solution, timer, graduated beakers, laser telemeter (Original)

B. Preparing the space for disinfection and actual nebulization

Mechanical cleaning of the space and surfaces was carried out prior to the commencement of nebulisation, according to general cleaning protocols. Coarse dirt was removed prior to disinfection and the floor was mopped and surfaces (tables, lamps, and equipment in the surgical rooms) were wiped with a washcloth dipped in cleaning solution (detergent). After preparing the space, the room volume was calculated using the laser telemeter to determine the time required to operate the machine and the amount of disinfectant material required for use. Finally, the space was closed as tightly as possible. Protective equipment was put on, and the machine was prepared and supplied with the required amount of disinfectant (Figure 3).

Figure 3. Positioning and preparing the machine (Original)

Then, using an earthed socket, the Cube Atomizers was placed in a corner of the room, as close as possible to the outlet and with the nozzle oriented diagonally across the room. In order to start the nebulisation process, the windows and doors of the room were closed, it was ensured that no other person was in the room, and access was prevented until the room was ventilated after the cycle (nebulisation/ activation/aeration) was completed. The button was placed in the ON position and the room was left, closing the door. The appliance was left to operate and waited until nebulisation was complete (completion of the process is identified by the cessation of the specific sound made by the appliance at the time of operation). The switch was set to the OFF position, the machine was removed from the room and the room was kept closed for the contact time and then the room was ventilated. After use, the machine is wiped with a cloth and stored in a place protected from the bad weather and out of direct sunlight.

C. Sample collection and analysis

For bacteriological analysis of air, the sedimentation method was used, thus collecting the microorganisms using Petri dishes. The sedimentation method consists of gravity deposition of airborne germs on the surface of solid culture media in Petri dishes. In the rooms where the determinations were carried out, the Petri dishes were placed horizontally in the middle of the room, their covers were removed and the exposure time was timed. Before misting for 2-3 hours, for sampling with Petri dishes the exposure time of the culture media was 15 minutes, and 1-2 hours after misting and venting, the exposure time of the Petri dishes was 30 minutes. Another method used for the determination of microorganisms was sampling by swabbing with sterile culture media swabs. Both before and after nebulisation, samples were collected with these swabs from various surfaces in the room (tables, floors, surgical lights, inhalers, cabinets and other appliances) (Figure 4). Samples collected in this way were seeded on solid culture media in Petri dishes. The plates were then incubated and after incubation the developed colonies were counted. Simple agarose (nutrient agar) known as agar is used to determine the total number of germs (NTG). By frequency in air and on all surfaces, as well as implications for pathology, in addition to the total number of germs, the following pathological agents were determined in particular: staphylococci on Chapmann medium, streptococci on blood agar, sodium azide and crystal violet (Holmes medium), gram-negative germs on Levine medium, and fungi on Sabouraud medium.

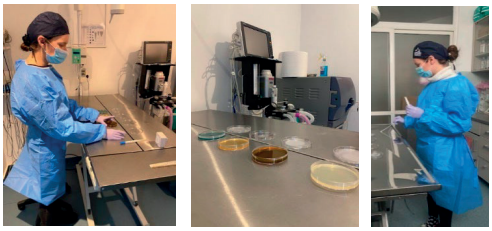


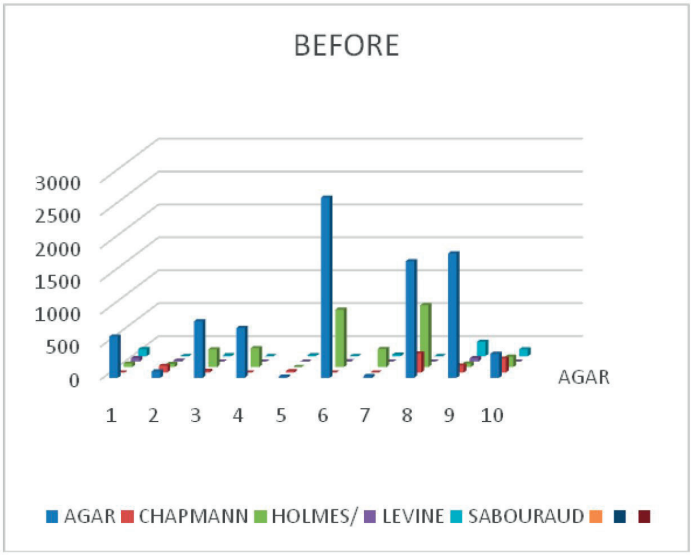
Figure 4. Sampling procedure (Original)

RESULTS AND DISCUSSIONS

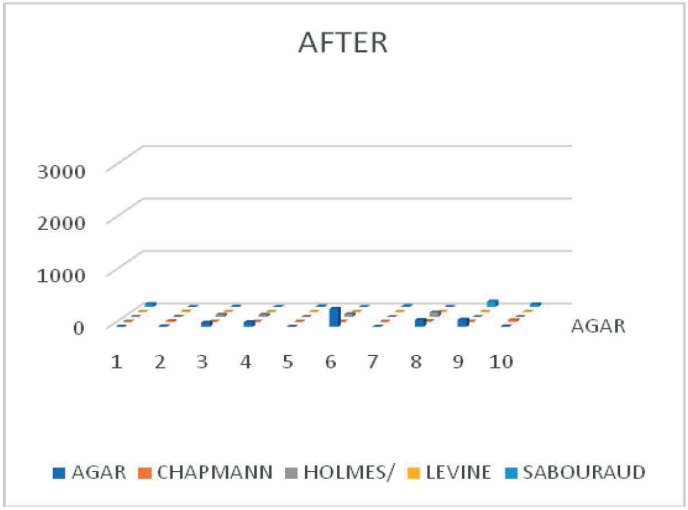
The results of the sanitation tests before the start of the disinfection procedure (Before test) and after mechanical cleaning and disinfection (After test) in the 10 premises used as surgery rooms in the veterinary clinics can be seen in the table (Table 1) and graphs (Graph 1 and Graph 2) below.

Table 1 - Results of sanitation tests

SAMPLE	Exposure time (minutes)/ METHOD	AGAR (Total bacteria count) (ufc/m^3)	CHAPMANN (Staphylococcus) (ufc/m^3)	HOLMES (Streptococcus) (ufc/m^3)	LEVINE (Gram-negative Cocci) (ufc/m^3)	SABOURAUD (fungi) (ufc/m^3)
1-Before disinfection	15'/Sedimentation	628	0	52	52	105
1-After disinfection	30'/Sedimentation	0	0	0	0	52
2-Before disinfection	15'/Sedimentation	98	100	47	12	2
2-After disinfection	30'/Sedimentation	8	11	4	1	1
3-Before disinfection	15'/Sedimentation	859	25	269	0	12
3-After disinfection	30'/Sedimentation	78	2	39	0	4
4-Before disinfection	15'/Sedimentation	759	0	287	0	0
4-After disinfection	30'/Sedimentation	87	0	38	0	0
5-Before disinfection	15'/Sedimentation	21	20	0	0	12
5-After disinfection	30'/Sedimentation	0	0	0	0	7
6-Before disinfection	-/Sterile swab	2740	0	871	3	2
6-After disinfection	-/Sterile swab	342	0	49	0	0
7-Before disinfection	-/Sterile swab	27	0	274	0	22
7-After disinfection	-/Sterile swab	0	0	0	0	12
8-Before disinfection	-/Sterile swab	1772	290	940	0	0
8-After disinfection	-/Sterile swab	131	12	82	0	0
9-Before disinfection	-/Sterile swab	1891	105	52	52	219
9-After disinfection	-/Sterile swab	135	0	0	0	98
10-Before disinfection	-/Sterile swab	367	210	157	0	104
10-After disinfection	-/Sterile swab	6	29	0	0	49



Graph 1. Sanitation sample results before nebulization (before test)



Graph 2. Sanitation sample results after nebulization (after test)

After keeping the samples in the thermostat at the appropriate temperature and conditions, the results were interpreted (Figure 5).

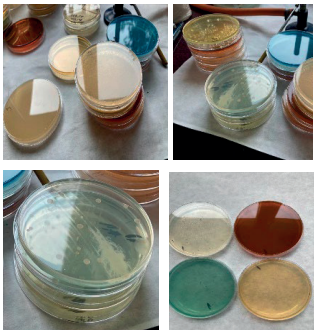


Figure 5. Sanitation samples - Petri dishes (Original)

Thus, in the samples cultivated on Agar culture medium, a significant decrease in the total number of germs was observed with an average of 94.26% after disinfection by the method chosen by us for this research, in some cases the success rate was 100%, reaching a value of 0 after disinfection. When using Chapmann medium in some cases we did not find the presence of staphylococci either at the beginning of the experiment or 30 minutes after the application of disinfection, but in the cases where we found the presence of staphylococci, the number of staphylococci was reduced to 93.84% after disinfection and in some cases the value reached 0 (100%) after disinfection. In the case of Holmes culture media, the number of streptococci was observed to decrease by an average of 94.36%, and in some cases the number reached 0 (100%) following disinfection. The same was true for the Levine medium, specific for the isolation of gram negative bacteria, with a decrease of 97.9% on average and 100% efficiency in some cases. In the case of Sabouraud medium a decrease of 57.79% on average was observed. After processing the samples, we found a 30-40% higher efficacy of the machine used in this study than in the case of using the ultraviolet lamp or the steam generator machine as a disinfection method, where the same disinfectant was used. Due to the speed, dispersion and reflux of microparticles throughout the space, the use of Cube Atomizers ensures disinfection of all surfaces (appliances, furniture, ceiling, floor, windows, as well as hidden parts of furniture and appliances etc.) unlike the steam generator or UV lamp which achieve partial disinfection of surfaces.

CONCLUSIONS

Following the application of the new method of disinfection in veterinary medical premises dedicated to surgical interventions, the following were observed:

- In many cases, for most categories of germs, the value of colony-forming units reached 0 after

nebulisation (efficiency was 90-97% for total germ count, 85-95% for staphylococci, 90-99% for streptococci, 92-99% for Gram-negative bacteria and around 50% for fungi), which is of particular importance in view of the obligation of a high degree of hygiene in these premises;
- Efficiency is 30-40% higher for the method proposed for this study than for other disinfection methods used in current practice (e.g. steam generator or UV lamp);
- The Cube Atomizer is more efficient as it does not require a long time for use, does not leave residues, uses a small amount of disinfectant, is easy to handle and does not require changing the disinfectant used by the unit;
- Due to the short disinfection application time, the easy handling, its efficiency, on average 80-90%, compared to the other methods used, where the results were 30-40% lower;
- The small volume of disinfectant used, the short application time and the low labour involved lead to lower costs and a short payback of the initial investment;
- The method used for disinfecting veterinary medical premises has the advantage of being able to disinfect surfaces (floors, ceilings, appliances, windows, furniture, etc.) on all sides, including the back, areas underneath, edges, pipes, etc.), which would take much longer or be impossible to achieve with conventional decontamination methods, and would result in partial disinfection of surfaces compared to the above;
- Periodic disinfection with the Cube Atomizers in surgical wards on the basis of a planned schedule can ensure a high level of hygiene at all times and prevent nosocomial infections;
- In case of epizootiologic risk it should be carried out whenever necessary;
- It is necessary to educate veterinary staff on the obligation to maintain a high level of hygiene in veterinary hospitals.

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USE OF DIODE LASER IN OPHTHALMOLOGY SURGERIES IN DOGS AND CATS: 161 CASES (2019-2022)

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Abstract

Recently used in veterinary medicine, the diode laser represents the surgical option for the entire eye pathology in dogs and cats. It is indicated in: eyelid tumours, trichiasis, distichiasis, conjunctival tumours, iris melanosis, uveal cysts, iris tumours, intraocular tumours, retrobulbar tumours and glaucoma. The diode laser for veterinary use has programs set for each surgical option, which provides intraoperative comfort for surgeon. The study was conducted over a period of 3 years (November 2019-November 2022) in 161 patients. The favourable postoperative evolution with the absence of complications was highlighted in the case of tumours, uveal cysts, and iris melanosis. In glaucoma cases, transscleral cyclophotocoagulation is not effective, 85% of the cases remained blind. The use of the diode laser in the case of symblepharon highlighted the rapid recurrence accompanied by neovascularization. Experimental using of diode laser in pigmentary keratitis revealed a short period of time with clear cornea after removing the pigmentation and the neovascularization was abundant.

Key words*: diode laser surgery, glaucoma, ocular tumours, symblepharon.*

INTRODUCTION

The diode laser in veterinary ophthalmology (transcleral cyclophotocoagulation) has been used for glaucoma surgical treatment in: dogs (Cook et al., 1997; Hardman et al., 2001; Spiess, 2012; Sapienza et al., 2018; Story et al., 2021) and horses (Annear et al., 2010; Cavens et al., 2012, Gellat et al., 2007, Wilkie, 2010).

For deflation and coagulation of uveal cysts in dogs, cats and horses, Gemensky et al., reported in 2004 that semiconductor diode laser coagulation of anterior uveal cysts is safe, effective and noninvasive. Stas et al., in 2022 used diode laser in iris cysts in horses with good results. In 2002, Cook et al., treated iris melanoma using diode laser photocoagulation in 23 dogs. The conclusion of the study was the method is safe and effective for isolated and pigmented iris masses in dogs.

In 1996, Sullivan et al., used photocoagulation of limbal melanoma in dogs and cats and in 2016, Andreani et al., evaluated effectiveness and safety of debulking and diode laser photocoagulation (DPC) for the treatment of limbal melanoma (LM). Transpupillary diode laser retinopexy had good results (Pizzirani et al., 2003).

Since 1996, ARC-Laser produced and developed the medical laser systems in human surgery. In 2011 an important step in the development of diode laser ocular surgery in veterinary medicine was the adaptation of the ARC laser device from human medicine.

User-friendly and with the work parameters preset in the software and at the same time adjustable during the operation, ARC remains the easiest laser system to use.

To the authors' knowledge, this is the first report of use of diode laser in retrobulbar tumours in dog and cats, corneal tumour in dog, symblepharon in cats and pigmentary keratitis in dog evaluating the long-term postoperative outcome.

MATERIALS AND METHODS

Medical records of 161 cases (98 dogs and 63 cats) underwent laser diode surgery from November 2019 to November 2022, were reviewed. All cases underwent complete ophthalmic and physical examination. Prior to surgery the additional diagnostic tests, such as complete blood count and serum biochemistry were performed.

Ocular ultrasound was performed to all patient. MRI or CT were performed to the patients diagnosed with conjunctival, corneal, limbal, third eyelid, intraocular and retrobulbar tumors. The ERG was performed in cats diagnosed with symblepharon and the surgery was performed if the retina had normal function.

Patients were placed under general anesthesia. The patients were pre-medicated with dexmedetomidine (Dexdomitor 0.1 mg/ml, Orion Pharma) 15 mcg/kg, ketamine (Ketamidor 100 mg/ml, Richter Pharma, Austria) 5 mg/kg and butorphanol (Butomidor 10 mg/ml, Richter Pharma, Austria) 0.2 mg/kg IM. Anesthesia was induced with propofol (Propofol Lipuro 10 mg/ml, Braun Germany) IV 2-4 mg/kg. The patients were intubated, maintained on oxygen and isoflurane 1.5-2% (Anesteran, Rompharm S.A., Romania).

The diode laser protocol was selected for each eye pathology. After the diode laser surgery, the Elizabethan collar is not mandatory.

Postoperative medications local antibiotic and anti-inflammatory eyedrops (DexaTobrom®, SC Rompharm Company SRL, Ilfov, Romania) for 21 days, (kanamycin ointment BID on the sutures Kanamicina®, SC Antibiotice SA, Iasi, Romania), hyaluronic acid (Diferion®, Micromed, Austria and an-Hypro® (an-Vision GmbH, Hennigsdorf, Germany) for 21 days.

After the surgery the cases were re-evaluated at 14 days, 1 month and 6 months.

RESULTS AND DISCUSSIONS

In the study were included 161 cases, 98 dogs and 63 cats (Table 1), and the selection criteria were the following:

- ERG within normal parameters for symblepharon in cats;
- complete mydriasis in iris melanosis;
- absence of osteolysis in retrobulbar tumour and third eyelid tumour;
- no response to local treatment in glaucoma patients.

All the surgeries were performed by the same clinician (Iuliana Ionașcu) at the Ophthalmology Department of the Faculty of Veterinary Medicine, Bucharest. The median age was 4 years, with a range between 6 months and 14 years.

Eyelid tumors

Of all cases (n=161), included in the study, 18 dogs and 7 cats presented eyelid tumours. In dogs the meibomian adenoma (Figure 1) was confirmed after histopathological exam.

The work parameters varied between 300 mW-2 Watts (Figure 2) and it developed an average energy of 450 J.

The bleeding was minor (Figure 3), the conjunctival oedema was present, no collar was needed and the depigmentation of the free edge of the eyelid was recorded.

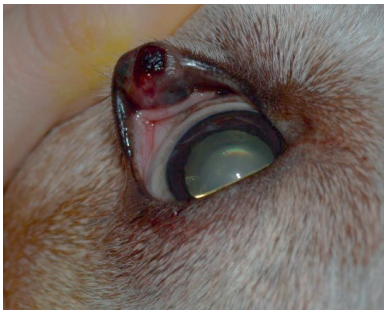

Figure 1. OD Eyelid tumour in a 9 year-old Labrador

Figure 2. The parameters of the diode laser for eyelid surgery

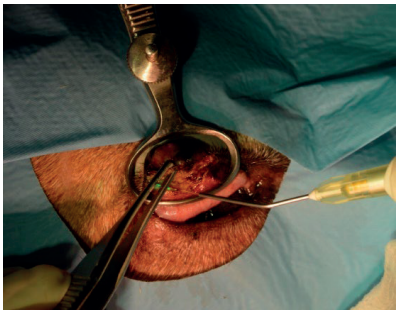

Figure 3. Case from Figure 1, clinical appearance into the surgery. (9 year-old Labrador)

Table 1. Patients' eye pathology included in the study

	Ocular pathology	Dogs	Cats	Total	Observation
1	Eyelid tumour	18	7	25 (15.52%)	No relapse/no collar/HP
2	Trichiasis/Distichiasis	11	0	11 (6.83%)	Relapse/ Stade Technique's
3	Conjunctival tumour	6	8	14 (8.69%)	No relapse
4	Symblepharon	0	11	11 (6.83%)	Relapse
5	Third eyelid tumour	7	5	12 (7.45%)	CT/MRI/ablation of the third eyelid/enucleation of the eyeglobe/HP
6	Iris melanosis	0	4	4 (2.48%)	Dyscoria
7	Anterior Synechia and PPM	0	6	6 (3.72%)	Effective in the presence of pigment
8	Uveal cysts	5	0	5 (3.10%)	No relapse
9	Iris tumour	2	0	2 (1.24%)	Relapse/enucleation of the eyeglobe/HP
10	Intraocular tumour	7	7	14 (8.69%)	Enucleation of the eyeglobe/HP
11	Retrobulbar tumour	14	8	22 (13.66%)	Minor bleeding/Enucleation of the eyeglobe/HP
12	Pigmentary keratitis	4	0	4 (2.48%)	Relapse/vascularization/no collar
13	Glaucoma	18	2	20 (12.42%)	2 visual/18 blind
14	Ablation of remaining tissues from the orbit after eye trauma	3	4	7 (4.34%)	Minor bleeding/no collar
15	Corneal tumour	3	0	3 (1.86%)	Scar/ no relapse/no collar
16	Limbal tumor	0	1	1 (0.62%)	No relapse/no collar
		98	63	161	

One Persian cat was diagnosed with multiple hydrocystoma on the eyelids and periocular skin (Figure 4 and Figure 5). After ablation of the big cyst the suture was performed, while for small one no suture needed (Figure 6).

Figure 4. Hydrocystomas in a 7 year-old Persian Cat

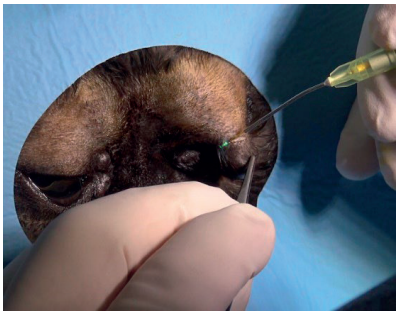

Figure 5. Case from Figure 4, clinical appearance into the surgery. (7 year-old Persian Cat)

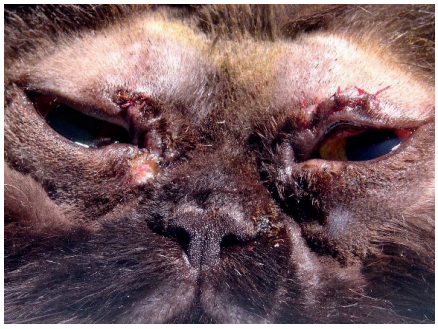

Figure 6. Case from Figure 4, clinical appearance after the surgery. (7 year-old Persian Cat)

Trichiasis/Distichiasis

11/98 dogs represented 6.83% diagnosed with trichiasis/distichiasis (Figure 7) underwent diode laser surgery. 3/98 dogs presented secondary corneal ulcer (Figure 8).

The laser parameters (Figure 9) varied between 500 mW - 1 Watts and it developed an average energy of 175 J.

The conjunctival oedema was present, no collar was needed and the depigmentation of the free edge of the eyelid was recorded.

After the surgery the dogs present mild pruritus and in 4 dogs the relapse occur and the Stade's technique was performed.

Figure 7. OD Trichiasis, districhiasis in a 2 year-old Shih Tzu

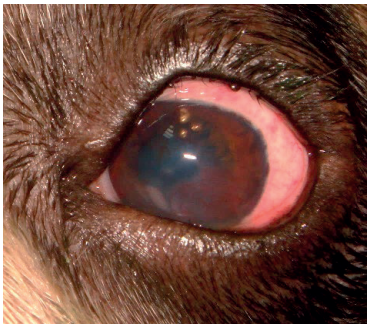

Figure 8. OS Secondary corneal ulcer due to trichiasis, districhiasis in a 9 month-old English Bulldog

Figure 9. The parameters of the diode laser for trichiasis/distichiasis surgery

Conjunctival tumours

Of all cases (n=161), included in the study, 6 dogs (Figure 10) and 8 cats presented conjunctival tumours (Figure 12) and underwent diode laser surgery. The bleeding and conjunctival oedema (Figure 11) were not to be considered. The laser parameters varied between 1 Watt – 3 Watts and it developed an average energy of 760 J.

Relapses occur in one dog and two cats (Figure 13) and underwent eye globe enucleation.

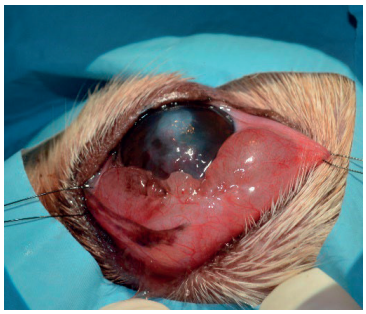

Figure 10. OD Conjunctival tumour in a 14 year-old Crossbred

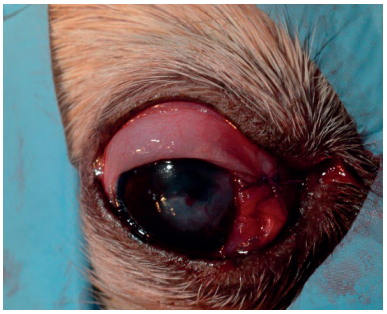

Figure 11. Case from Figure 10, clinical appearance after surgery revealing conjunctival oedema (14 year-old Crossbred)

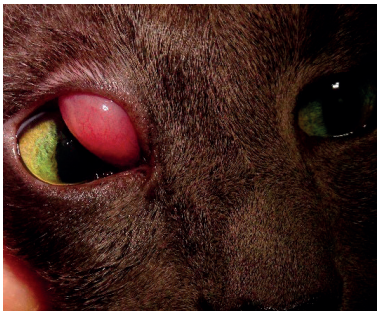

Figure 12. OD Conjunctival tumour (adenocarcinoma) in a 10 year-old DSH

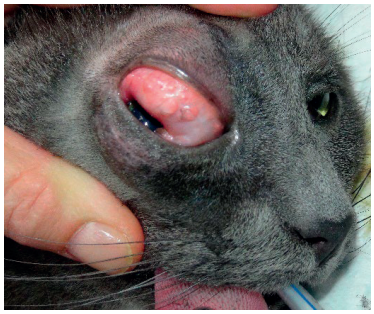

Figure 13. Case from Figure 12, relapse 6 months after the first surgery. (10 year-old DSH)

Symblepharon

11/63 cats with symblepharon (Figure 14) were included in the study had ocular ultrasound without retinal detachment and normal ERG (Figure 15).

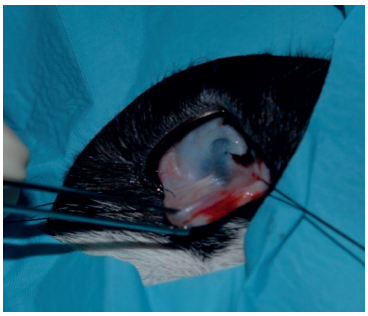

Figure 14. OD Symblepharon in a 7 month-old DSH. The cat is blind secondary to attached conjunctiva on to cornea

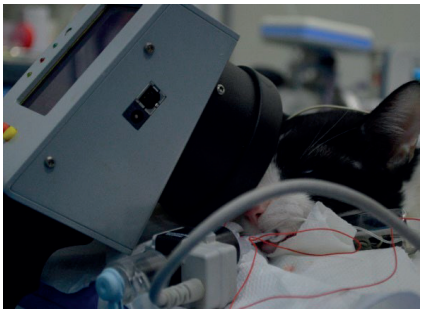

Figure 15. Performing the ERG before symblepharon surgery using laser diode

For section of the conjunctival synechia from the surface of the cornea, between the conjunctival fornix and the third eyelid (Figure 16) we used 2-3 Watts laser parameters with an average energy of 1.5 kJ.

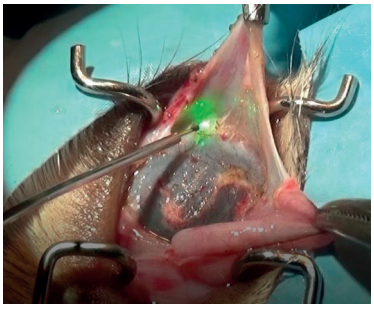

Figure 16. Case from Figure 14, clinical appearance into the surgery (7 month-old DSH)

In all patients the ablation of free edge of the third eyelid with preserving the gland was performed, so that the third eyelid does not cover the cornea (Figure 17).

After the surgery the cornea had opacization but the cats regained vision (Figures 18 and 19).

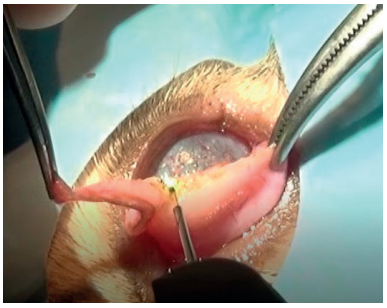

Figure 17. Ablation of the free edge of the third eyelid, appearance during surgery

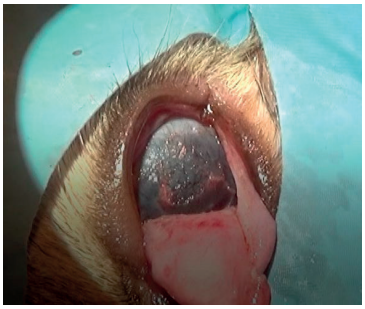

Figure 18. Case from Figure 16, clinical apearance after the surgery

Figure 19. Case from Figure 14, clinical apearance after the surgery

After the surgery, relapses occurred in 2-4 weeks and the cornea had neovascularization and 6/11 cats lost the vision.

Third eyelid tumours

Of all cases (n=161), included in the study, 7 dogs (Figure 20) and 5 cats presented third eyelid tumours (Figure 21) and underwent eye globe enucleation and tumour ablation using diode laser surgery (Figure 22). The laser parameters varied between 5 Watts - 7 Watts and it developed an average energy of 3.3 kJ. The histopathological exam of the tumour was performed for all patients.

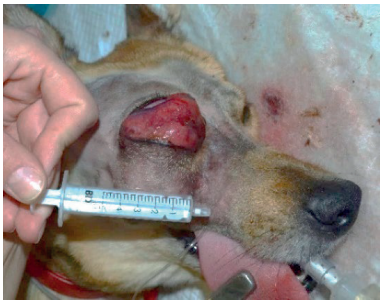

Figure 20. OD Third eyelid tumour in a 3 year- old Crossbred

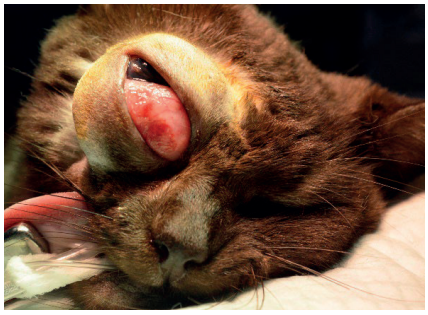

Figure 21. OD Third eyelid tumour in a 13 year- old DSH

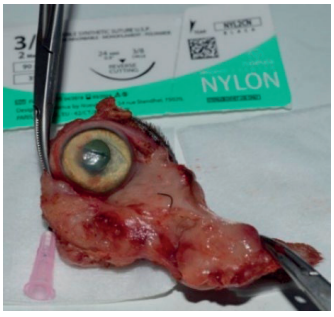

Figure 22. Case from Figure 21, appearence of the tumour after the surgery. (13 year-old DSH)

Iris melanosis

4/63 cats with iris melanosis (Figures 23, 25 and 27) were selected if the drug induced mydriasis was uniform (Figure 24) thus excluding the iris tumour in which dyscoria is present.

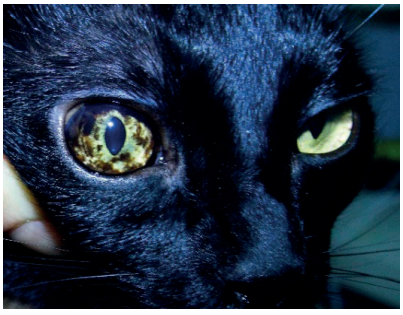

Figure 23. OD Iris melanosis in a 6 year-old DSH

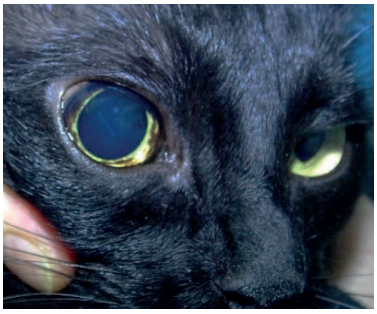

Figure 24. Case from Figure 23, clinical appearence after drug induced mydriasis (6 year-old DSH)

The laser parameters varied between 800 mW - 1.2 Watts and it developed an average energy of 175 J (Figure 26).

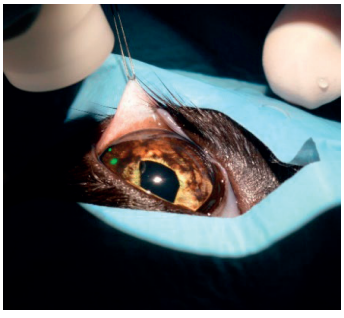

Figure 25. Case from Figure 23, intraoperative appearence. The focused green is on the melanosis area and the lighter spot is the reflection of the cornea

Figure 26. The parameters of the diode laser for iris melanosis surgery

During the surgery, after the carbonization of the area of iris melanosis, fine brown particles (Figure 28) can be observed in the aqueous humour, which disappear within 5 days under local anti-inflammatory treatment.

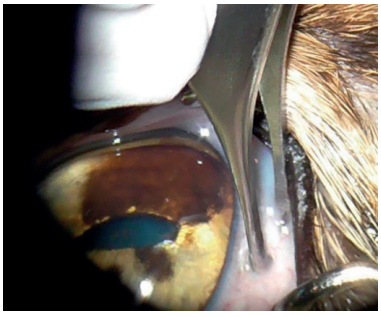

Figure 27. OD Iris melanosis, clinical appearence before diode laser surgery, in a 8 year-old DSH

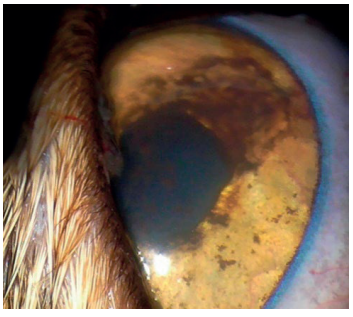

Figure 28. Case from Figure 2, Iris melanosis, clinical appearence after diode laser surgery, in a 8 year-old DSH

In the surgery obvious shrinking of the tissue is present and therefore the deformation of the pupil. After the surgery the inflammation was mild but the dyscoria occur secondary to the iris scar (Figure 29, Figure 30).

Figure 29. Case from Figure 28, dyscoria 2 months after diode laser surgery (8 year-old DSH)

Figure 30. Case from Figure 23, dyscoria 1 month after diode laser surgery. (6 year-old DSH)

Anterior synechia and PPM

In 6 cats with anterior synechia and pigmentary PPM after diode laser the field of sight increased due to the sectioning of the tissue clamps and the pupil is mobile. The corneal opacification does not disappear but it only reduces as a surface in time. The diode laser is not effective when the synechiae are unpigmented.

Uveal cysts

5/98 dogs diagnosed with uveal cysts (Figure 31) underwent deflation using diode laser. The surgery is not painful and miotic pupil is mandatory intraoperative (Figure 32). The laser parameters varied between 300 mW - 800 mW and it developed an average energy of 150 J (Figure 33). After the deflation of the uveal cysts fine brown particles can be observed in the aqueous humour, which disappear within 3-5 days under local anti-inflammatory treatment. Systemic anti-inflammatory is not mandatory.

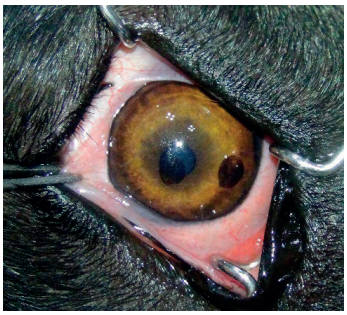

Figure 31. OD Uveal cysts
in a 2 year-old Cane Corso

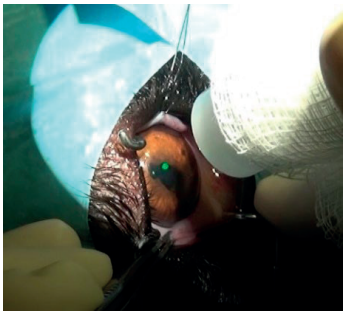

Figure 32. Case from Figure 31, intraoperative appearance of the uveal cysts
in a 2 year-old Cane Corso

Figure 33. The parameters of the diode laser for uveal cysts surgery

Iris tumours

Of all cases (n=161), included in the study, 2 dogs (Figure 34) presented iris tumours and underwent tumour reduction using diode laser surgery (Figure 35). The laser parameters varied between 2 Watts - 5 Watts and it developed an average energy of 1.3 kJ. The diode laser is effective on pigmented iris tumours, but a constant reduction of its size was observed 2 months after the surgery.

Figure 34. OS Iris tumour
in a 10 year- old Crossbred

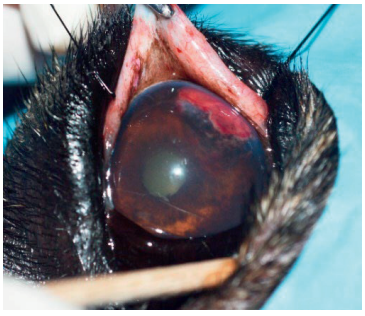

Figure 35. Case from Figure 34, intraoperative appearance after the diode laser surgery
in a 10 year-old Crossbred

Intraocular tumours

Of all cases (n=161), included in the study, 7 dogs (Figure 36) and 7 cats presented intraocular tumours and underwent eye globe enucleation using diode laser surgery (Figure 37). The laser parameters varied between 4 Watts - 7 Watts and it developed an average energy of 2.9 kJ. The bleeding was minor and mild oedema of the skin after the surgery was noticed.
The histopathological exam of the tumour was performed for all patients.

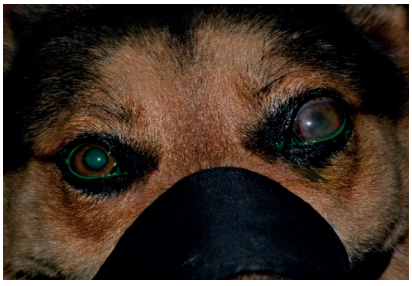

Figure 36. OS Intraocular tumour, secondary glaucoma in a 11 year-old German Shepherd

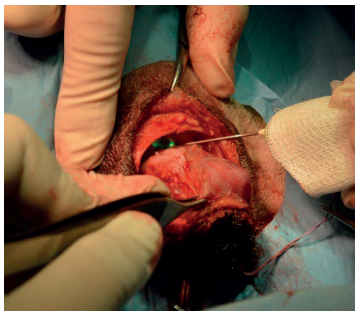

Figure 37. Case from Figure 36, intraoperative appearance in a 11 year-old German Shepherd

Retrobulbar tumours

For all the patients with retrobulbar tumours, CT and/or MRI were performed and in the study were included the cases without osteolysis of the orbit or invasion of the frontal and maxillary sinuses.

Of all cases (n=161), included in the study, 14 dogs and 8 cats diagnosed with retrobulbar tumours presented exophthalmos (Figure 38) and strabismus. All the patients underwent eye globe enucleation and retrobulbar tumour ablation using diode laser surgery.

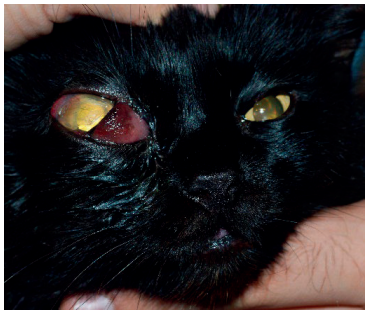

Figure 38. OD Retrobulbar tumour, in a 12 year-old DSH

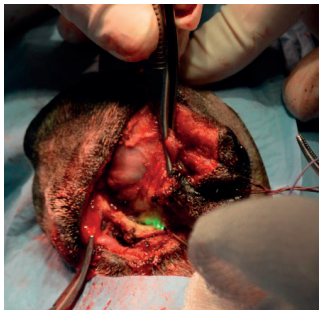

Figure 39. Intraoperative appearance in retrobulbar tumour ablation using diode laser

The enucleation of the eyeball and the ablation of the retrobulbar tumour was performed transpalpebral (Figure 39) and the diode laser parameters varied between 5 Watts - 7 Watts and it developed an average energy of 3.6 kJ. The bleeding was minor and mild oedema of the skin after the surgery was noticed. The histopathological exam of the tumour was performed for all patients.

Pigmentary keratitis

Four blind patients due to pigmentary keratitis (Figure 40) in which all local treatments failed were included in the study.

The diode laser very easily removes pigmentation from the cornea (Figure 41), without pain, without discomfort. We used the iris melanosis laser parameters, between 800 mW - 1.2 Watts and the energy developed had an average of 90 J.

The patients saw immediately after the operation. Three weeks after de surgery, the vascularization starts from the limbus and cornea regained the pigmentation in 4 months after the surgery.

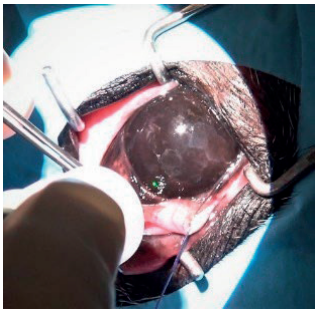

Figure 40. OS Pigmentary keratitis, in a 6 year-old French bulldog

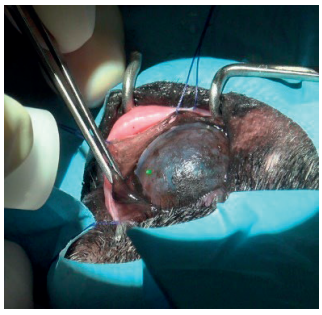

Figure 41. Case from Figure 40, intraoperative appearance after removing the corneal pigmentation in a 6 year-old French bulldog

Glaucoma

Of all cases (n=161), included in the study, 18 dogs (Figure 42) and 2 cats with refractory glaucoma underwent diode laser surgery (Figure 43). The goal is to partially destroy the ciliary epithelium to reduce aqueous humour secretion and decreased the IOP. The laser parameters varied between 800 mW - 1500 mW (Figure 44) and it developed an average energy of 120 J with 20% of the spots with popping sound. The conjunctival oedema was present (Figure 45) and the mild uveitis occur.

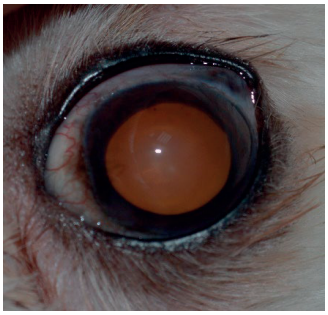

Figure 42. OS Glaucoma refractory to treatment in a 7 year-old Crossbred

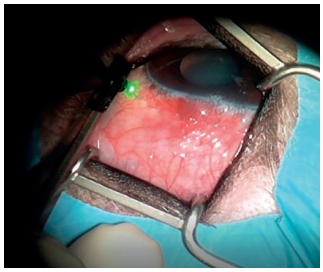

Figure 43. Intraoperative appearance in transcleral diode laser surgery for glaucoma

Figure 44. The parameters of the diode laser in transscleral surgery for glaucoma

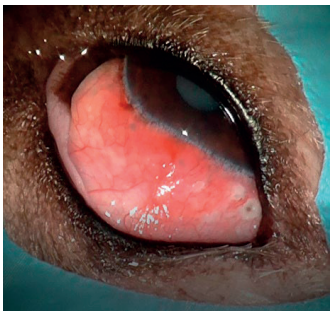

Figure 45. Case from Figure 43, intraoperative appearance after transcleral cyclophotocoagulation using diode laser for glaucoma

After the surgery, 0.5 ml of aqueous humour was removed from the anterior chamber of the eye (Figure 46). Three dogs preserved the vision after the surgery and 85% of the cases, 2 cats and 15 dogs lost the vision due to retinal detachment, high IOP or phtisis bulbi. Patients underwent intrascleral prosthesis due to painful eye.

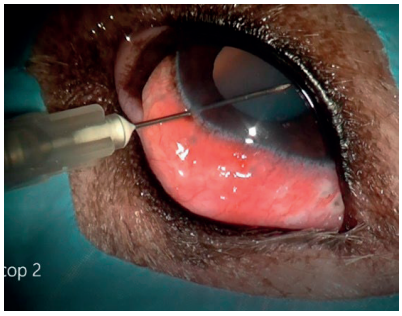

Figure 46. Case from Figure 45, intraoperative appearance, removing the aqueous humour

Ablation of remaining tissues from the orbit after eye trauma

Of all cases (n=161), included in the study, 3 dogs and 4 cats with phtisis bulbi and remaining tissues into the orbit after eye trauma underwent diode laser surgery.

The ablation of the remaining tissues from the orbit was performed transpalpebral (Figure 47) and the diode laser parameters varied between 4 Watts - 5 Watts and it developed an average energy of 2.9 kJ. The bleeding was minor and mild oedema of the skin after the surgery was noticed. The blepharorrhaphy wound heals with suppuration under the crust, with minimal oedema (Figure 48).

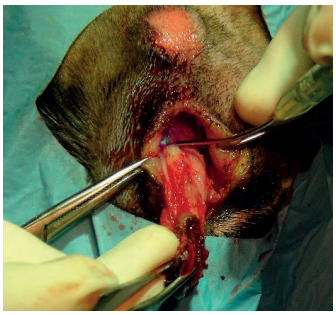

Figure 47. Intraoperative appearance in diode laser surgery for abation of the remaining tissues into the orbit after eye trauma

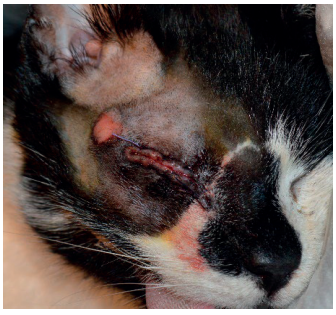

Figure 48. Case from Figure 47, clinical appearance, after diode laser surgery

Corneal tumours

In the study were included 3 cases with corneal tumours (Figures 49 and 50).

The diode laser very easily removes the tumour from the cornea (Figure 51). The laser parameters were between 3 Watts - 5.5 Watts and the energy developed had an average of 1.9 kJ (Figure 52). The histopathological exam of the tumour was performed for all patients. No relapses were noticed in the period of study.

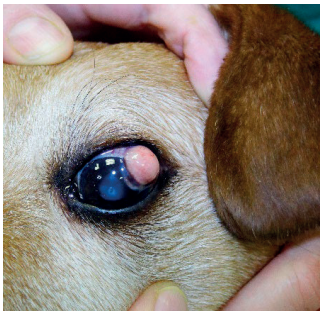

Figure 49. OS Corneal tumour in a 11 year-old Beagle

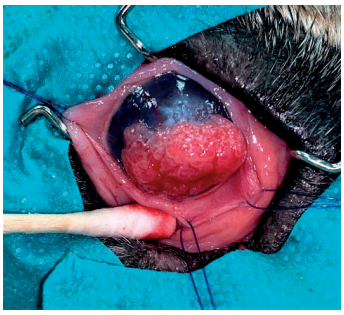

Figure 50. OS Corneal tumour (adenocarcinoma) in a 8 year-old English Bulldog

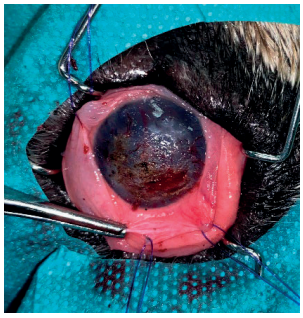

Figure 51. Case from Figure 50, intraoperative appearance of the cornea, after ablation of the tumour

Figure 52. The parameters of the diode laser for ablation of the corneal tumour

Limbal tumour

In the study one cat with limbal melanoma was included. Surgical debulking of limbal melanomas (Figure 53) followed by diode laser photocoagulation (Figure 54), The diode laser parameter was 3 Watts and the energy developed was 1.2 kJ. The cat's eye was visual after the surgery and no relapse occurred (Figure 55).

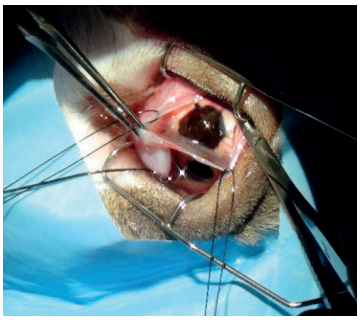

Figure 53. OS Limbal melanoma in a 4 year-old DSH

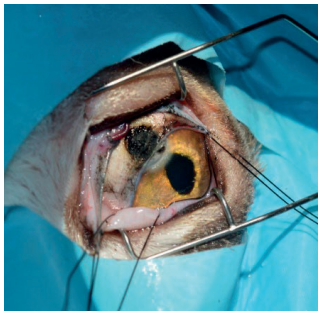

Figure 54. Case from Figure 53, clinical appearance, after photocoagulation using diode laser surgery

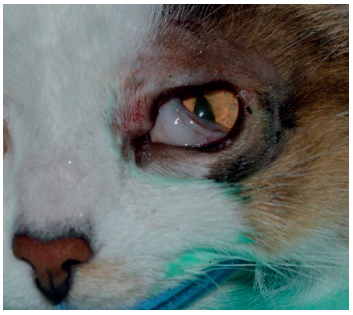

Figure 55. Case from Figure 54, clinical appearance, 14 days after the surgery

ARC-Laser used in veterinary ophthalmology surgery had power calibration at fiber distal tip, with the patented fiber connector, fiber change is quick and easy without compromising the performance. It has ergonomically designed hand pieces and delivery systems enables surgeons multiple treatments options.

The diode laser for veterinary use has programs set for each surgical option, which provides intraoperative comfort for surgeon. The green aiming beam is highly visible at all treatment tissues and the touch-screen permit the selection and changing of treatment parameters very easily. The preset settings depending on the pathology are very helpful and as a general rule, the surgical intervention should be initiated with the minimum parameters.

The most complex studies using the diode laser (TSCP) were done in normal equine eye (Morreale et al., 2007; Harrington et al., 2012) to revealed the histologic effect (retinal detachment and hemorrhage) and to demonstrated the histological lesions of the ciliary body that result in a significant and sustained decrease in IOP therefore may be an effective management for equine glaucoma (Cavens et al., 2012; Bădicu et al., 2015).

Comparative studies endoscopic and transscleral cyclophotocoagulation for the treatment of refractory glaucoma were performed by Lin S., 2008; Lin et al., in 2006 and Lutz et al., 2008 and 2009. Sapienza et al., in 2005 and 2009 had good results in primary glaucoma in dogs using combined TSPC and Ahmed gonioimplantation and used TSPC in 2009 for pseudophakic and aphakic dogs with secondary glaucoma.

A retrospective study in 18 dogs with primary glaucoma (Hardman et al., 2001) revealed after diode laser transscleral cyclophotocoagulation (TSCP) that low energy, higher power laser was effective, with 50% of potentially visual eyes regaining vision, but may cause an increased incidence of secondary cataracts and postoperative complications of hyphema and phthisis bulbi were not seen in this series.

Micropulse transscleral cyclophotocoagulation (MP-TSCP) for refractory glaucoma was successful in controlling IOP in 30 dogs as well as to reduce postoperative medications with minimal resultant intraocular inflammation and complications and the micropulse procedure also can be repeated (Sapienza et al., 2018; Story et al., 2021). In our study using TSPC had a small rate of success, in 85% of the case phtisis bulbi occur.

The use of diode laser for deflation and coagulation of anterior uveal cysts in dogs, cats and horses was reported in 2004 (Gemensky et al., 2004) Semiconductor diode laser coagulation of anterior uveal cysts is safe, effective and noninvasive.

Stass et al, in 2022 used noninvasive diode laser for iris cysts in horses. Iris cysts in horses are often asymptomatic and noticed

incidentally. However, cysts can cause local corneal oedema and erratic behavior like shying, decreased performance and head-shaking.

Both short- and long-term results indicate diode laser treatment is a useful and safe option for iris cyst size reduction, with a low risk of recurrence. Presurgical ultrasonography is recommended to assess the feasibility of treatment and to allow for better surgical planning (Stas et al., 2022).

Our study highlighted a rapid recovery after uveal cyst surgery using TSPC. Dogs did not show corneal edema or eye pain.

Sullivan et al., in 1996, performed photocoagulation of limbal melanoma in dogs and cats (15 cases) and Andreani et al., in 2016 combined surgical debulking and diode laser photocoagulation for limbal melanoma treatment in 21 dogs. Debulking, in addition to TSPC was technically straightforward to perform, minimally invasive, well tolerated, and highly successful in this case series.

In 2002 Cook et al., used TSPC for the treatment of presumed iris melanoma in 23 dogs.

Minor complications related to laser treatment were seen, including: dyscoria, iris hyperpigmentation, and corneal edema due to collateral hyperthermia. Glaucoma and cataract formation were not observed. Non-invasive diode laser photocoagulation appears to be a safe and effective method of treatment for isolated, pigmented iris masses in dogs.

In our study the diode laser used in iris melanosis in cats had good outcome and only mild dyscoria was present.

CONCLUSIONS

The diode laser surgery is used successfully in: eyelid tumours, trichiasis, distichiasis, conjunctival tumours, iris melanosis, uveal cysts, iris tumours, intraocular tumours, retrobulbar tumours and glaucoma. The favourable postoperative evolution with the absence of complications was highlighted in the case of eyelid tumours, uveal cysts, and iris melanosis. Due to the chronic evolution in refractory glaucoma, the rate of blindness was 85%. The use of diode laser for eye globe enucleation ensures the comfort of the surgeon following minimal hemorrhage. The use of the diode laser in the case of symblepharon highlighted the rapid recurrence accompanied by neovascularization. Experimental using of diode laser in pigmentary keratitis revealed a short period of time with clear cornea after removing the pigmentation and the neovascularization was abundant.

To the authors' knowledge, this is the first report of use of diode laser in third eyelid tumours and retrobulbar tumours in dog and cats; corneal tumour in dog; symblepharon in cats and pigmentary keratitis in dog evaluating the long-term postoperative outcome.

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CLINICAL MANIFESTATIONS OF ACUTE PANCREATITIS IN DOGS - DIAGNOSTIC AND PROGNOSTIC VALUE

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Abstract

The clinical signs of acute pancreatitis depend largely on the severity of the disease, which can range from subclinical to life-threatening. The more common clinical signs are a direct result of inflammation of the pancreas or of the systemic effects of inflammation. The present study was performed in 83 dogs with spontaneous acute pancreatitis and 12 dogs with experimentally induced acute pancreatitis. The indicators general condition, appetite, vomiting, defecation, pain, mobility and reactivity were assessed. Both the frequency and the degree of manifestation of the individual clinical signs were taken into account. The most common symptoms were lethargy, anorexia, vomiting, diarrhea, and abdominal pain. There was a statistically significant difference between the experimental groups in the degree of manifestation of some of the signs, but not in the frequency of their manifestation.

Key words*: pancreatitis, dog, clinical signs, prognostic value.*

INTRODUCTION

Numerous studies have been conducted worldwide describing clinical or clinico-pathological abnormalities in dogs with acute pancreatitis (AP) (Thordal-Cristensen and Coffin, 1956; Anderson and Low, 1965; Williams, 1995; Morita et al., 1998; Krstic et al., 2001; Williams, 2005; Morozov, 2006; Watson et al., 2007). Most of the specialists working in this field agree that the clinical manifestation of AP is extremely diverse and non-specific (Thordal-Cristensen and Coffin, 1956; Schaer, 1979; Westermarck and Rimaila-Paranen, 1983; Watson et al., 2007). Acute pancreatitis, even as an independent disease, leads to a variety of functional and humoral, primary and secondary pathological effects in the patient's body, and its clinical manifestation depends a lot on the changes in the gland and on the involvement of other organs and systems in the inflammatory process (Morozov, 2006).

The main clinical signs are concentrated in Mondor's triad - pain, vomiting and flatulence (Morita et al., 1998; Krstic et al., 2001; Pápa et al., 2011). Usually, in human medicine, the first and most significant sign of AP is pain localized in the epigastrium, which can also cover other parts of the abdomen and even the chest girdle. In veterinary medicine, pain is not always recognizable and we have to judge it by indirect signs. One of them is vomiting, which in the absence of another etiology is taken as a manifestation of visceral pain.

Since the pancreas is located in the abdominal cavity, its examination is an indispensable part of the process of diagnosis of AP. The abdomen in severe cases is most often soft, pasty with flatulence in the initial part of the small intestine. Sometimes a defense in the epigastrium can also be found, known in human medicine as "Keurte's symptom" (Takov et al., 2004).

Clinical manifestations of organ failure, such as respiratory (acute respiratory distress syndrome), renal, cardio-circulatory, or severe metabolic or hemostatic disorders are indicative of rapid disease progression (Freudiger, 1991; Hofbauer and Saluja, 1998; Kondratenko et al., 2008; Mansfield et al., 2008; Sato et al., 2017; Lazarov, 2021).

MATERIALS AND METHODS

The study included 83 dogs with spontaneous acute pancreatitis divided into two groups (A and B), depending on the outcome of the disease, and 12 clinically healthy dogs in which experimental acute pancreatitis was reproduced. In group A, 22 animals (with a fatal outcome) were included. In group B, 61 animals (with a favorable outcome) were

included. Primary clinical examinations of the spontaneous cases were performed at the time of admission of the animals to the clinic. A total of 12 clinically healthy dogs, provided by the Stara Zagora municipality shelter, were used for experimental reproduction of acute pancreatitis. They were divided into two experimental groups (C and D) with 6 dogs each. In experimental group C, we induced acute pancreatitis by placing ligatures of the ductus pancreaticus, and in group D, acute pancreatitis was reproduced by introducing oleic acid into the ductus pancreaticus minor.

Before the start of the experiments, all animals were vaccinated and treated against endo- and ectoparasites. Daily clinical and periodic paraclinical (blood and urine) examinations were conducted for 14 days, which confirmed their good health. They were fed with dry pelleted food according to their type and weight, and they were provided with unlimited access to drinking water.

At the 0th, 24th, 48th, 72nd, and 96th hours from the start of the experiments, hematological, ultrasonographic, and clinical examinations were performed in all animals.

Figure 1. Ordinal rank scale for assessment of clinical signs

The complete clinical examination, including the parameters of Status Praesens (general and special part), as well as indicators such as: appetite, general behavior, mobility, sensitivity, reactivity, vomiting, pain, defecation, etc., was performed according to the routine methods of clinical diagnostics. The results of the study were recorded in an ethogram prepared for the purpose. The degree of manifestation of the individual clinical signs was transformed into a numerical expression using an ordinal rating scale from 0 to 4 (Figure 1). According to this scale, score 0 means no manifestation of the corresponding parameter, 1 - weak manifestation, 2 - moderate, 3 - strong, and 4 - extreme manifestation.

RESULTS AND DISCUSSIONS

As can be seen from the tables (Table 1 and Table 2), our studies in dogs with spontaneous and experimental AP showed extremely categorical values of the anorexia index. Bearing in mind Williams' statement (Williams and Steiner, 2005) that pain in AP worsens after feeding and/or supine and is relieved by fasting, we can hypothesize that anorexia in AP is partly caused by the presence of pain in the abdominal area. High-grade anorexia in AP, reaching anorexia, has been reported by many other authors (Van den Bossche, 2010; De Causmacker, 2009; Mansfield, 2008; Mix, 2006). In a review of the prevalence of clinical signs in AP in dogs and cats, Van den Bossche placed anorexia in the leading position with 91% in dogs and 63-100% in cats (Van den Bossche, 2010). Our studies in dogs with spontaneous and experimental AP showed even more definite values of this indicator. In all experimental groups, we reported anorexia in 100% of the animals. The difference was in the degree of manifestation in the different groups. Anorexia was the highest in experimental group A (non-survivors), which we attribute to multi-organ dysfunction and more serious damage in the structure of the pancreas.

In experimental group A, we observed vomiting in 91% of the dogs, in group B - 88%, and in groups C and D, all animals demonstrated vomiting. This largely confirms the literature sources, according to most of which vomiting is observed in about 85% of cases of AP. Vomiting in AP is usually painful and persistent and does not lead to relief of the patient's condition. Unlike intestinal obstruction in AP, there is never vomiting of fecal matter. It is mainly due to the increased excitability of the stomach and duodenum as a consequence of the irritation of the vagus by the inflamed pancreas (Filipenko, 2004; Filipenko, 2004 -1; Rau, 2004). According to the same authors, repeated and prolonged vomiting should be considered as an unfavorable prognostic sign.

Another important diagnostic and prognostic indicator in AP is the general condition of the individual. In most cases it is severely affected. The percentage of dogs with AP demonstrating weakness and/or lethargy varies around 80% (Van den Bossche, 2010). Most commonly, animals are lethargic, but in severe cases anxiety, fear and inappropriate behavior are present (Watson, 2003; Gruys, 1998). All these signs were present in the animals we studied, with the predominant manifestations being adynamia and lethargy. Our survey showed 100% occurrence of this indicator. Especially clearly, with a statistically significant difference in the severity of the manifestation $p<0.001$, the depressive states were expressed in the animals of group A. In a large part of the dogs of this group, severe apathy was observed, sometimes reaching somnolence, signs that completely coincide with the literature data. Takov (Takov et al., 2004) points out those neurological disorders in acute pancreatitis in humans are very diverse and are a sign of toxic encephalopathy and deepening metabolic disorders (metabolic acidosis, hyperglycemia, hypocalcemia). The correlation we found between high blood sugar levels and a high frequency of neurological abnormalities in experimental group A fully corresponds to this statement.

Pain localized in the epigastrium is usually indicated as the first and most significant sign of AP. However, the localization of the pain can cover other parts of the abdomen and even the chest girdle.

According to Dent (1991), abdominal pain syndrome is the leading clinical manifestation of most diseases of the digestive organs. Precisely the pain reaction was one of the first signs established by us, both in the course of the conducted experiments and during the examinations of the animals with spontaneous pancreatitis.

The characteristics of the pain syndrome observed by us, namely: not always defined localization and irregular rhythm (of indefinite duration and unrelated to food intake, time of day, the act of defecation or mechanical provocation), completely coincide with the literary description of visceral pain, characteristic of acute pancreatitis (Simpson, 2006). The immediate cause of visceral pain is the activation of pancreatic proteases and tissue necrosis leading to the release of inflammatory mediators (Nathens, 2004; Mayerle, 2004). They not only exacerbate the inflammatory process, but also affect the sensory fibers of the intestinal plexus (T5-T9), which leads to visceral pain. It usually occurs suddenly and can be very severe, sometimes leading to ileus or even loss of consciousness (Nathens, 2004; Mayerle, 2004). A similar nature of pain during the development of toxemic syndrome is explained by A.P. Popov (2001), and E.M. Drapeza (2002) through the impact of hydrolytic, proteolytic enzymes, kinins and other biologically active substances released in the bloodstream on organs remote from the pancreas. However, cases of AP have been described (and we have also observed such), which were asymptomatic and the diagnosis was established after performing an autopsy (Geyer, 1968). In the course of our study, a manifestation of visceral pain was found in 60% of the animals in group A, and in 67% of the animals in groups B and C. When quoting these figures, we should not overlook the fact that the determination of pain symptoms in the veterinary medicine is largely subjective. In this regard, it is perhaps more correct to define all animals with vomiting as demonstrating a pain response.

Table 1. Clinical signs in dogs with spontaneous acute pancreatitis from experimental groups A (non-survivors) and B (survivors)

Symptom	**Group A (n = 22)**		**Group B (n = 61)**	
	n	**%**	**n**	**%**
Anorexia ($p<0.001$)	22	**100 (3.7)**	61	**100 (2.3)**
Vomiting	20	**91**	54	**88**
Lethargy ($p<0.001$)	22	**100 (3.2)**	61	**100 (1.9)**
Pain	15	**68**	42	**67**
Diarrhea	10	**45**	15	**24**

Table 2. Clinical signs - percentage of involvement, degree of manifestation and statistical reliability compared to the 0-th hour in dogs with experimental AP from experimental groups C (ligatures) and D (oleic acid).

Symptom	0 h		24 h		48 h		72 h		96 h	
Group	C	D	C	D	C	D	C	D	C	D
Anorexia	0	0	83/**1**^{c}	100/**3**^{c}	100/**2**^{c}	100/**3**^{c}	100/**2**^{c}	100/**3**^{c}	100/**2**^{c}	100/**3**^{c}
Vomiting	0	0	50/**1**^{a}	50/**1**^{b}	100/**2**^{c}	100/**2**^{c}	100/**2**^{c}	100/**1**^{c}	100/**2**^{c}	100/**2**^{c}
Lethargy	0	0	100/**2**^{c}	83/**2**^{c}	100/**2**^{c}	100/**2**^{c}	100/**2**^{c}	100/**2**^{c}	100/**2**^{c}	100/**2**^{c}
Pain	0	0	50/**1**^{a}	67/**1**^{b}	100/**2**^{c}	67/**2**^{b}	100/**2**^{c}	100/**2**^{b}	83/**1**^{c}	100/**1**^{b}
Diarrhea	0	0	50/**1**	67/**1**^{a}	83/**2**^{b}	83/**1**^{b}	67/**1**^{a}	83/**1**^{b}	17/**2**	50/**1**

$^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$

The percentage of dogs with AP demonstrating diarrhea typically ranges from 20 to 50 percent (R D Eddine et al., 2018; T. Sato et al., 2017; Watson P.J. et al., 2007). Our research on spontaneous cases of AP fully confirmed this statement. However, the results of the experimental setups showed that this indicator is too dynamic and unstable. In experimental groups C and D, the percentage of animals with diarrhea reached a peak of 83% at 48 hours from the start of the experiment. This high percentage is most likely also due to the mechanical trauma of the operative intervention on the wall of the duodenum, but fully confirms Simpson's opinion that diarrhea is a much more common sign in dogs with experimental pancreatitis (Simpson, 2006). Exocrine insufficiency of the pancreas as a result of destructive processes in the parenchyma or mechanical disorders in the outflow of pancreatic juice is indicated as the main cause of diarrhea in AP (Lazarov, 2021; Morozov, 2006; Dragiša, 1999). The ligatures placed by us on the pancreatic duct prevent not only the outflow of pancreatic juice, but also create conditions for biliary reflux in the pancreatic duct system. Regarding the type of diarrhea, there is complete agreement that the presence of bloody stools and melena are poor prognostic indicators and suggest a severe development of the disease (Sato et al., 2017; Mansfield et al., 2008).

CONCLUSIONS

In spontaneous acute pancreatitis, the severity of the course and the outcome of the disease depend to a large extent on the structural and functional changes both in the pancreas itself and in indirectly related organs and systems (heart, kidneys, liver, and central nervous system). Relying on clinical signs alone is not sufficient to make an accurate diagnosis, but could be useful in predicting the development of AP. For greater accuracy, it is necessary to make a complex assessment of the results of the patient's clinical, paraclinical and imaging examination.

Based on the results obtained in the course of our research, the following conclusions can be drawn:

1. Acute pancreatitis in dogs (induced and spontaneous) presents with lethargy, anorexia, vomiting, diarrhoea and abdominal pain.
2. In acute pancreatitis, structural and functional changes in cellular and tissue elements are of a phase nature, and the leading morphological component in its development is the appearance of necrotic foci. The degree of destructive phenomena in the parenchyma and stroma of the organ corresponds to the severity of the clinical picture.

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STUDY OF AN EPISODE OF SUBCLINICAL KETOSIS IN A SHEEP FARM IN SOUTHERN ROMANIA

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Abstract

Subclinical ketosis in sheep is considered a disorder of carbohydrate metabolism, which causes the mobilization of lipids, disturbance of fatty acid metabolism and the formation of ketone bodies. The main objective of this work is to identify local and general factors that contribute to the occurrence of subclinical ketosis in a sheep farm in Dolj County (Southern Romania) and to highlight by laboratory analysis the disturbances of energy metabolism, as well as other conditions closely related to them. A good nutritional management of the ewes before and after lambing is essential knowing that their nutritional requirements increase in this period and the peak will be reached in the first two-three weeks of lactation. Careful monitoring of the ewes in this period: trained personal to identify early signs of disease, diagnostic tests and quick intervention is also needed to prevent these episodes and other complications, such as pregnancy toxemia, which can lead to severe nervous manifestations (secondary to hypoglycemia) and significant economic losses.

Key words*: subclinical ketosis, sheep, ketonemia, anemia, ketonoruria.*

INTRODUCTION

Subclinical ketosis (SK) is very common in ruminants, but in sheep herds is often underdiagnosed because of the absence of clinical signs. Identification of this condition is possible through its main dominant: hyperketonemia (Duffield et al., 2009). The pathophysiological mechanism is based on the increase of the ewe's nutritional needs, while the ability to store feed decreases because of the abdominal expansion of the pregnant uterus, which will result in a negative energy balance. SK is characterized also by imbalance of carbohydrate and fat metabolism (Bergman, 1971).

In general, preventive measures (proper nutrition and energy suppliers) are taken to avoid this condition, but routine testing for SK should be done at least in the last four weeks of pregnancy, knowing the increased risk of complications that may appear during this period. The gold standard for SK diagnosis is analysis of blood β-Hydroxybutyric acid (BHBA), which according to Bostedt et al., 1990 is the most stable ketone, being significant for approximately 85% of the total ketones in sheep with pregnancy toxemia, but it requires specialized testing and increased prices. Identification of ketone bodies in the urine is also used, but it is not a very relevant test, knowing that ketonuria may occur in other conditions too. Determination of elevated BHBA is possible now on the farm by using a ketone meter. This method is very affordable and with great diagnostic value according to various authors. (Jones et al., 2018; Pichler et al., 2014). SK should be differentiated using liver enzymes activity tests from other liver disease that induce incorrect glucose metabolism (Braun, 2010).

Subclinical ketosis may rapidly degenerate into clinical ketosis, in case of inappropriate environmental conditions, inadequate nutrition, transport stress. In practice, nutrition is one of the factors of sensitization of sheep and associated with other stressors, may be the trigger for the disease (Marutsova et al., 2018).

Ketosis is associated with pregnancy toxemia in sheep, being a metabolic disease that often occurs in the last 3 weeks of gestation and is considered more aggressive in twin or triplet-bearing ewes (Fthenakis et al., 2012).

Symptomatology includes low food intake or selective anorexia, ketone breathing odor, weight loss, or, in decompensated cases (pregnancy toxemia): muscle tremors, opisthotonos 1-3 weeks before lambing. In a few days, blindness, ataxia, prolonged sternal decubitus, comatose state or death may occur. (Van Saun, 2000; Schlumbohm et al., 2008)

MATERIALS AND METHODS

The study was carried out in a privately-owned farm of Tsurcana breed with 122 heads (12 males and 110 females), located in the South of Romania, approximately 20 km away from Danube River (Figure 1).

Figure 1. View from the sheep herd in Southern Romania

The analysed population included 84 ewes (in second or third gestation), 5 with twin pregnancies (diagnosed by ultrasound by the farm physician). Anamnesis showed that in the last years, 6 of these sheep aborted, without suffering from an infectious disease.

Table 1. The flock effective in the period of our study (December 2021-February 2022)

	110 EWES	Previous Abortion	Previous Twin lambings
Flock N=122	32 in 3rd gestation	3	4
	3 twin lambings		
	52 in 2nd gestation	2	2
	2 twin lambings		
	26 in 1st gestation	-	-
	12 RAMS		

All animals were up to date with preventive actions (vaccinations, internal-external deworming). They were monitored during their lambing period (December 2021 - February 2022) (Table 1) in the last three weeks of gestation for early signs of Ketosis (low food intake or selective anorexia, weight loss) and for high levels of blood BHBA, by using The Nova Vet Meter (UK). Diagnosis of SK was possible in 12 ewes with BHBA values: 0.8-1.6 mmol/l (group A). Also, a control group of healthy ewes (n=5) was established according to the absence of clinical signs and to the BHBA values (under 0.8 mmol/l: group B)
Blood samples were collected from both groups via jugular vein puncture, using 21 G needles, heparin and K3EDTA vacutainers. Hematological and biochemical tests were performed in order to assess the metabolic status of the SK group compared to the control group, using the hematology analyser Abacus Junior Vet 5 (SUA) and the biochemistry analyser Arkray Spotchem EZ SP4430 (Japan). Results were compared to the available relevant literature data, using the keywords: subclinical ketosis, sheep, ketonemia, anemia, ketonuria. The following sections will emphasize the obtained results.

RESULTS AND DISCUSSIONS

A very important role in this survey was taken by the clinical examination of the animals, their environmental conditions and their welfare. Their rations were in concordance with their physiological needs (late pregnancy), but we considered that the protein component was of low quality. Ewes were reared in facilities in compliance with the welfare standard for the species. At the group examination we concluded that the flock was in a good maintenance condition. The clinical exam of the individuals was performed and a special attention was paid to palpation of each sheep in order to establish the Body Condition Score (BCS), using the five-point system (1.0-5.0) at 0.5-point intervals between 2.0 and 4.0, the appearance of the skin, the colour of the mucous membranes. BCS of the group A was 3 and BCS of the group B was 2.5, except the twin bearing ewes which had the BCS 3, which means that the BCS is mild decreased in the SK

group, probably associated with the lower food intake and its quality of the fodder. Also the ability to consume the entire quantity of food was appreciated as good in both groups, according to the low litter size observed in the exploitation.

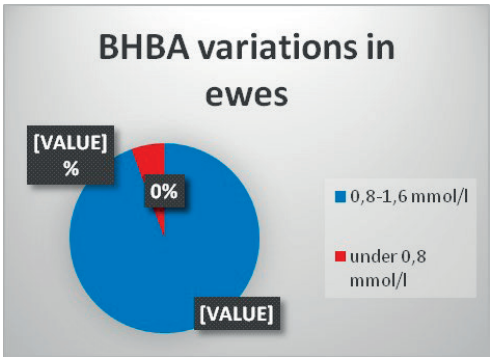

Figure 2. Values of BHBA in our study

The prevalence of SK in late pregnancy was 14.28%, which is similar to what Gupta et al. reported in their study from 2008 (14.86%), performed in pregnant ewes. We think that this percentage is quite significant and it can be directly associated to the occurrence of clinical ketosis and its economic implications, taking into account that all ewes with twin pregnancy were included. On the other hand, there are also studies with higher incidence of SK: during the last four weeks of gestation, 46.0% of ewes exhibited persistent BHBA >0.8 mmol/L, as Jones et al. (2018) report in their paper.

We believe that in appropriate environmental conditions, but with unbalanced rations, each breeder should pay more attention to his flock and more investigations should be carried out to assess the metabolic state of the ewes. Imbalances should be corrected in order to prevent other pathologies such as: fetal death, mammitis. In this sense, we implemented a minimal set of analyses for a more efficient monitoring of sheep during the peripartum period at a low cost. This panel included a hemoleucogram and some suggestive biochemistry parameters: glucose (GLU), total protein (TP), urea (BUN), γ-glutamyltransferase (GGT), calcium (Ca). Hematological parameters included: Red Blood Cells (RBC), White Blood Cells (WBC), Hemoglobin concentration (Hb), Hematocrit (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Platelet Volume (MPV). The variation of the main indices of the red blood cells will be presented in Table 2 for group A and in Table 3 for group B. Also, the average of the values was calculated and compared to the reference interval (RI) to emphasize the differences between the 2 groups.

Table 2. Variation of the main erythrocyte indices, in pregnant sheep from group A

No	RBC mil/mm^3	Hct %	Hgb g/dl	MCV μ^3	HEM	MCHC g/dl Er.
RI	**12**	**36**	**11**	**33**	**10**	**32**
1	12.5	36.7	11.4	35	10.5	33
2	12.4	37.8	11.2	33	10.1	32.3
3	13.7	38.5	12.0	34.3	11.4	33.8
4	13.4	37.5	11.7	36.4	11	33.5
5	12.8	39.0	12.8	33.0	11.6	37.6
Average	**12.96**	**37.9**	**11.82**	**34**	**10.92**	**35.3**

Clinically healthy sheep had normal values in HLG, while those with SK had mild microcytic hypochromic anemia. This type of anemia has been reported due to some deficiency (Iron). In this exploitation, this pattern associated with mild decreased BCS sustain the assumption that the sheep with SK consume insufficient feed. On the other hand, this type of anemia can also occur as a result of the increase in the need of the fetus, a fact supported by the lower values of red blood cells in ewes with twin pregnancies (red colour in the Table 3).

Table 3. Variation of the main erythrocyte indices, in pregnant sheep from group B (Sk group)

No	RBC mil/mm^3	Hct %	Hgb g/dl	VEM μ^3	HEM	CHEM g/dl Er.
RI	**12**	**36**	**11**	**33**	**10**	**32**
1	11.1	34.2	10.4	34	9.8	30.1
2	10.9	33.8	10.2	33	10.1	31.3
3	10.7	33.5	10.0	31.3	9.3	29.8
4	12.3	32.5	9.7	26.4	7.8	29.8
5	9.0	34.0	8.8	31.7	9.7	25.8
6	9.3	33.6	9.5	31.1	10.2	28.2
7	9.7	36.1	9.0	31.4	9.2	29.5
8	9.3	39.0	10.8	41.0	11.6	27.6
9	10.0	33.6	9.6	33.6	9.6	28.5
10	10.7	38.8	10.2	36.2	9.5	26.2
11	10.0	34.5	9.7	32.5	10.7	31.0
12	10.5	33.9	9.5	32.2	9.0	28.0
Average	**9.30**	**34.85**	**8.8**	**33.5**	**9.70**	**27.6**

During periods of under-nutrition, tissue reserves are mobilized resulting in increases in plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate that may alter insulin responsiveness. (Brown et al., 2010). Because NEFA are not available in the area of the exploitation under study, energy metabolism was assessed using: total protein, glucose and BHBA. To differentiate the SK from hepatic disease, GGT was evaluated, knowing that in sheep it is specific for hepatobiliary damage, while AST, ALT are poorly specific to the liver. (Braun et al., 2010).

Table 4. Variation of the main biochemistry parameters, in pregnant sheep from group A

No	GLU mg/dl	TP g/DL	BHBA mmol/l	GGT UI/L	Ca mg/Dl
RI	**30-60**	**6.2-8.3**	**< 0.8**	**13-40**	**8.4-10**
1	54	7.8	0.5	22	9.9
2	53	8.1	0.6	28	10.1
3	62	7.1	0.6	32	8,7
4	58	6.9	0.4	32	9.0
5	55	6.4	0.4	26	8.5
Average	**56.4**	**7.38**	**0.5**	**28**	**9.24**

The values of GGT have been normal in the control group, while almost normal (borderline elevated) in SK group, which reveals that preventive actions (deworming) were done rigorously and there is no evidence of other concurrent liver damage.

Generally, serum concentrations of BHBA have been used to determine hyperketonemic status and subclinical ketosis. This rise in serum BHBA is a compensatory mechanism and a reflectionary response to carbohydrate deficiency and inhibition of Kreb's cycle, according to Anoushepour et al., 2014.

Plasma calcium concentration, as indicative of mineral metabolism has been tested too. About Calcium it is known that it is influenced by food supply, and is reported to be lower in twin than in single pregnant ewes, but no difference was observed between twin and triplet-pregnancies (Corner et al., 2008). In our study Calcium levels were within reference ranges in both groups.

Upon a closer analysis of the data obtained among group B, it is observed that sheep with twin pregnancy have lower blood glucose and total protein values than those with normal pregnancy, a fact that once again emphasizes the need to adjust the rations according to the needs of each individual. Also, the GGT values were higher in the twin bearing ewes (red colour in the Table 5) than in the rest of the ewes.

Table 5. Variation of the main biochemistry parameters, in pregnant sheep from group B

No	GLU (mg/dl)	TP (g/dl)	BHBA mmol/l	GGT UI/L	Ca mg/dl
RI	**30-60**	**6.2-8.3**	**< 0.8**	**13-40**	**8.4-10.3**
1	31	7.7	1.3	34	10.3
2	35	7.3	1.1	33	9.9
3	34	7.5	1.2	28	13.4
4	40	7.3	0.9	31	10.1
5	26	6.4	1.3	44	9.7
6	28	6.8	1.6	43	9.0
7	44	7.9	1.1	32	10.4
8	26	6.9	1.5	48	9.5
9	34	8.2	1.4	34	9.7
10	54	8.1	1.2	36	9.2
11	30	6.4	1.5	40	10.2
12	29	6.4	1.4	52	9.7
Average	**34.25**	**7.24**	**1.3**	**37.91**	**9**

Comparing the results of both groups, we can state that the Sk group has low levels of glucose, total protein and GGT at the upper limit, a fact that can lead to the clinical form of the disease at any time (Figure 3).

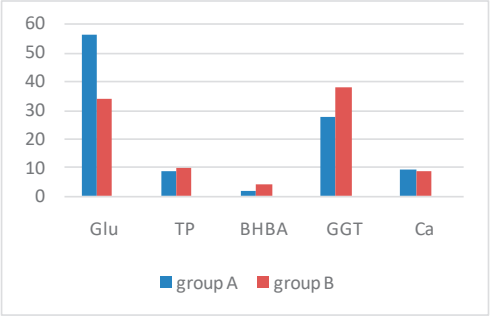

Figure 3. Variation of the main biochemistry parameters between the 2 groups

Once the clinical signs of ketosis are installed, there is only one more step (inadequate management) to pregnancy toxemia, which leads to significant productive and reproductive

losses. Knowing that sheep also have a tendency to develop anemia, that their BCS tends to decrease, we recommend re-evaluating the rations and improving the quality of the forage, as well as its nutritional values.

CONCLUSIONS

A good management of sheep flocks during lambing period should include: good quality feed, optimized according to individual needs, periodic measurement of BHBA levels, monitoring of the herd through blood tests. Ketonemia has been recognized as an essential characteristic of SK, the main predisposing cause was inadequate long-term feeding, sustained also by the decreased BCS and mild anemia found in group B.

We consider that a minimum database of blood tests, as those presented previously may be useful in evaluating the health status of the ewes, helping us take the necessary measures before the development of any clinical conditions. Also, in the case of subclinical ketosis, identifying and correcting imbalances as early as possible will mean a healthier herd, with fewer problems and higher productions.

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THE LONG TERM USE OF ENFLICOXIB IN DOGS WITH OSTEOARTHRITIS: CLINICAL SAFETY AND EFFICACY

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Abstract

Osteoarthritis (OA) is a pathologic condition characterized by progressive destruction of various components of synovial joints. The OA is generally associated with pain and inflammation and therefore lameness, which are capable to decrease the quality of dog life for a long period of time. Unfortunately, there is no treatment for solving OA, but it is possible to slow down its progression through a correct therapeutic approach which could relieve pain and improve the quality of life of the dog and, consequently, of the owner. The objective of the present study was to evaluate the efficacy and safety of enflicoxib for the treatment of naturally occurring canine OA. Fourteen dogs were treated for 13 weeks with enflicoxib (Daxocox®, Ecuphar NV, Italy) administered once a week at 4 mg/kg, with an initial loading dose of 8 mg/kg. From day 0 to day 90 efficacy was assessed by the veterinarian by using clinical pain and lameness scores, and by the owners using the Canine Brief Pain Inventory. At day 0 and 90 a complete blood count and a biochemistry profile were performed in all treated animals. From the first weeks of treatment, a meaningful improvement in the clinical and owner scores was noticed. In conclusion, long term weekly administration of enflicoxib at the proposed dosage, resulted in great benefit for the quality of life of the dog affected by OA.

Key words*: osteoarthritis, enflicoxib, COX-2, NSAID, dog.*

INTRODUCTION

Osteoarthritis (OA), also known as degenerative joint disease (DJD), is a chronic and progressive inflammatory disease characterized by cartilage degeneration, osteophyte formation and bone remodeling, changes in the synovial membrane and periarticular tissues. This musculoskeletal disease results in lameness, chronic pain, loss of joint function and mobility and reduced quality of life (Henrotin et al., 2005). It is highly prevalent in dogs (Paster et al., 2005; Smith et al., 2006) with 20% of the canine population over the age of 1 year old affected by the disease (Johnston 1997; Moreau et al., 2011). There is no cure for OA, and the treatment involves long-term management of the symptoms by treating inflammation and pain, improving mobility and hence the quality of life (Mlacnik et al., 2006; Aragon et al., 2007; Vandeweerd et al., 2012; Bhathal et al., 2017). This is achievable through a multimodal approach that provides nutritional supplementation, physiotherapy, and weight management but non-steroidal anti-inflammatory drugs (NSAIDs) are still considered the medical foundation for the management of canine OA (Sanderson et al., 2009; Bound et al., 2011).

Given the chronic pain caused by OA, its treatment requires long-term continuous administration. It seems that long-term NSAIDs treatment does not significantly increase side effects (Innes et al., 2010). However, gastrointestinal (GI), hepatic and renal side effects may occur and should be monitored (Luna et al., 2007; Monteiro-Steagall et al., 2013; Moreau et al., 2003; Walton et al., 2014).

Preferential and selective cyclooxygenase-2 (COX-2) inhibitors have been developed to potentially reduce the risk of unwanted side effects caused by the inhibition of COX-1 (Kukanich et al., 2012; Toutain et al., 2018).

Enflicoxib (also known by its research acronym E-6087) is a new pyrazoline derivative COX-2 inhibitor with long-lasting activity that has been developed for veterinary use in dogs. The pharmacokinetic characteristics of enflicoxib allow it to be administered once a week

ensuring constant blood availability of the drug during the treatment period (Homedes et al., 2021). Weekly administration would allow to reduce fluctuations in blood concentrations as for daily administered NSAIDs. Moreover, the weekly treatment interval would improve owner compliance as well as provide a better pain control and decrease reluctance of dogs to be medicated.

Previous studies suggested that a dosage of 4 mg/kg of enflicoxib, once a week, with an initial loading dose of 8 mg/kg, could be safe and efficacious for the treatment of canine OA (Cendros et al., 2021; Salichs et al., 2021; Salichs et al., 2022).

The objective of the present study was to evaluate the efficacy and safety of 90 days enflicoxib (Daxocox®, Ecuphar NV, Italy) administration in clinical cases of dogs with naturally occurring OA.

MATERIALS AND METHODS

A prospective and uncontrolled study was conducted. Informed consent was obtained from all dog owners prior to enrolment. The observation period lasted for 10 months.

Animal selection

Client-owned dogs of both sexes and any breed presented to the Veterinary Teaching Hospital of the University of Perugia as veterinary patients showing clinical signs of OA such as pain and lameness for at least 3 weeks were evaluated and scored for possible inclusion in the study. OA had to be confirmed through a radiographic investigation showing signs compatible with the pathology such as subchondral bone sclerosis, bone remodelling, osteophytes, irregular or diminished joint space. If more than one joint was affected by OA, to evaluate the efficacy of the drug, the most affected joint was taken into consideration.

All dogs were required to be in good general health as assessed based on a complete physical examination and the results of routine blood tests (haematology and biochemical profile) within normal ranges and performed within 7 days prior to enrolment in the study. For haematology, minimum data required were haematocrit (Hct), haemoglobin concentration (Hb), red blood cell (RBC) count, absolute reticulocyte count (RetC), mean cellular haemoglobin (MCH), mean cellular haemoglobin concentration (MCHC), mean cell volume (MCV), red cell distribution width (RDW), count total leukocyte count (WBC), differential leukocyte count: neutrophils (N), lymphocytes (L), eosinophils (E), basophiles (B), monocyte (M) and platelet count (Plt). For biochemistry, minimum data required were alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine (Creat), total protein (Total Prot) and albumin (Alb). All dogs were required to be microchipped and to have a minimum age of 6 months and a minimum weight of 3 kg. Body condition score (BCS) through a nine-point system (WSAWA, 2020) was recorded.

In the days prior to inclusion, dogs must not have been treated with short-acting NSAIDs, corticosteroids or any other medications that could affect inflammation, pain or joint well-being as indicated by Salichs et al. (2021; 2022).

Dogs were not enrolled if they were suffering from concomitant kidney, liver, GI tract, haemorrhagic disorders or other diseases that could interfere with the evaluation of treatment effect. Dogs with hypersensitivity to enflicoxib, to any of the excipients or to sulphonamides were excluded. Recent joint surgery or axial skeletal disease were considered exclusion criteria as were comorbidities affecting the joint object of study assessments (Salichs et al., 2021; Salichs et al., 2022).

Animals intended for breeding were not included.

Concomitant treatment with NSAIDs, systemic corticosteroids, anticoagulants or other therapies with potential nephrotoxic effect was not permitted during the study. Physiotherapy, laser therapy and massage were avoided during the study. Administration of other concomitant medications was permitted but had to be recorded. Administration of food supplements was permitted if these products had been administered at a constant dosage for at least one month before the start of the present study.

The day of inclusion, before first treatment administration, both the veterinarian and the owner scored the severity of clinical signs of

OA. The same veterinarian throughout the study assessed pain and lameness using a numerical rating scale (NRS), as described by Salichs et al. (2021; 2022), that included the assessment of four parameters in the following order: posture while the dog was standing, lameness at walk, lameness at trot and pain at palpation/manipulation of the affected joint. The sum of scores for these four parameters represented the clinical sum score (CSS) and it ranged from 0 to 18.

As previously described (Pollmeier et al., 2006; Musco et al., 2019; Autefage et al., 2011; Salichs et al., 2021; Salichs et al., 2022), a factor of two was applied to place more weight on lameness at walk and at trot as part of the clinical picture (Table 1). Dogs selected for inclusion in the study had to have a CSS≥6 on day 0 (D0), prior to treatment. For a better classification of lameness, in addition to the CSS lameness assessment, the veterinarian used also a NRS with a score ranging from 0 (no lameness) to 5 (no support of the limb) as previously described (Impellizeri et al., 2000; Quinn et al., 2007) (Table 2). To confirm OA and rule out conditions that precluded subjects' inclusion, radiographic evaluation of the affected joint was performed at D0, and based on the radiographic findings, each dog was assigned a score from 1 to 4 to define the severity of OA. See Table 3 for the description of the classification criteria extrapolated from Canine OA Staging Tool (COAST) by Cachon et al. (2018).

The owner evaluation was performed using the Canine Brief Pain Inventory (CBPI) (Brown et al., 2007; Brown et al., 2008), a two-part instrument: the pain severity score (PSS) is the arithmetic mean of four items scored on an 11-point (0-10) numerical scale, and the pain interference score (PIS) is the mean of 6 items scored similarly (0 = no pain or interference and 10 = severe pain or interference). For this assessment, a value of PSS and PIS scores ≥ 2 on D0, prior to treatment, was required for a dog to be included. Furthermore, in the CPBI the owner was asked to also rate his or her overall impression of the dog's quality of life (QoL), which was graded as Poor, Fair, Good, Very Good or Excellent.

Owners were instructed not to change home management and daily exercise routine of their dogs during the study in order not to have an impact on the evaluation of the efficacy of the test product. The reasons for the withdrawal of the dogs from the study are the same previously described in the papers by Salichs et al. (2021; 2022).

Table 1. Clinical Sum Score (CSS).
Table from Salichs et al. (2021)

1. Posture (dog standing)	
Score	**Description**
0	Normal stance
1	Slightly abnormal stance: partial weight bearing of limb, but paw remains firmly in contact with floor
2	Markedly abnormal stance: partial weight bearing of limb with minimal contact between paw and the floor
3	Severely abnormal stance: no weight bearing
2. Lameness at Walk	
Score	**Description**
0	No lameness: normal weight bearing on all limbs
2	Mild lameness with partial weight bearing
4	Obvious lameness with partial weight bearing
6	Marked lameness with no weight bearing
3. Lameness at Trot	
Score	**Description**
0	No lameness: normal weight bearing on all limbs
2	Mild lameness with partial weight bearing
4	Obvious lameness with partial weight bearing
6	Marked lameness with no weight bearing
4. Pain on Palpation/ Manipulation	
Score	**Description**
0	No pain on palpation/manipulation of effected joint
1	Mild pain (e.g. turns head in recognition)
2	Moderate pain (e.g. pulls limb away)
3	Severe pain (e.g. vocalizes or becomes aggressive or will not allow veterinarians to palpate/manipulate the joint due to pain)

Table 2. Lameness NRS. *The main distinction between NRS 4 and NRS 5 was for a score of 4 the dog might be weight bearing when standing or walking, but not when trotting whereas NRS 5 was only used when a limb was never weight bearing when standing or moving

Lameness Severity	Score
Clinically sound	0
Barely detectable lameness	1
Mild lameness	2
Moderate lameness	3
Severe lameness	4*
Could not be lamer	5*

Table 3. Radiographic OA classification criteria extrapolated from COAST by Cachon et al. (2018)

Radiographic signs of OA	OA Grade
No radiographic signs of OA	**1**
Mildly abnormal with subtle changes (early signs of OA, minimal osteophytes)	**2**
Moderately abnormal with obvious changes (obvious osteophytes)	**3**
Severely abnormal with very obvious changes (advanced osteophytes and remodelling)	**4**

Treatment

Dogs that met the inclusion criteria were enrolled by the veterinarian and received an initial loading dose of 8 mg/kg with a subsequent weekly maintenance dose of 4 mg/kg, for 13 additional weeks. Dose calculations for study treatments were performed using the body weight determined on D0, which was defined as the day of inclusion and the first day of treatment.

Following label indications, tablets were administered immediately before or with food, as food increases its absorption.

Assessments

Following the protocol developed by Salichs et al. (2021; 2022), general physical examinations and clinical assessments of pain and lameness were performed by the veterinarian on D0, prior to treatment and thereafter at each study visit on days 7, 14, 28, 56 and 90 (± 2 days) using the CSS and the lameness score. Furthermore, during these checks, the investigator proceeded to score lameness through a 6-grade NRS (Impellizeri et al., 2000; Quinn et al., 2007) and make videos of the animal as it got up from its reclining position, in quadrupedal station, while walking and trotting in a straight line. For each type of activity performed, four videos had to be made by filming the animal respectively from the front, from the back and on both sides. In addition, still following Salichs et al. (2021; 2022) protocol, during each clinical assessment day and also on days 21, 35, 42, 49, 63, 70, 77 and 84 (± 2 days) through a telephone call, the veterinarian interviewed the owner to record their assessments using the CBPI. The owner was not aware of the required threshold level for PSS and PIS scores for inclusion in the study and did not have access to the scores of previous assessments when completing each CBPI. On D90 haematology and biochemical profile were repeated on each animal.

Efficacy outcome measures

As previously described by Salichs et al., 2021 and 2022, a predefined criterion of treatment response was used. For the veterinary assessment, a dog was classified as "responder" if the CSS score was < 6 in any of the follow-up visits. For the owner assessment, a dog was classified as "responder" if it had a decrease ≥1 in PSS, and ≥2 in PIS in any of the follow-up visit compared to basal scores (Brown et al., 2013). Efficacy was evaluated as the percentage of CSS and CBPI responders at any time point. To better characterize the efficacy of the drug, the evolution of PSS and PIS scores, the QoL parameter and the degree of lameness according to the 6-grade NRS classification were also taken into consideration.

Safety outcome measures

Safety was evaluated by recording adverse effects (AEs) that occurred throughout the study. Owners were instructed to detect any suspected AE related to NSAID treatment such as anorexia, vomiting, diarrhoea, melena and to report them to the veterinarian. Any alterations in the laboratory values recorded at the end of the study were considered as AEs.

Statistical analysis

Statistical analysis was performed using a dedicated statistical software package (JASP, Version 0.16.1, University of Amsterdam, Amsterdam, Netherlands).

Continuous and ordinal data were summarized by mean and standard deviation or by median and range, as appropriate. Categorical variables were presented as frequencies and percentages.

Descriptive statistics of the study group was performed overall including age, sex, spay status, weight, breed, affected limb (forelimb, hindlimb), affected joint (hip, stifle, elbow, shoulder, metacarpus, intervertebral), unilateral, CBPI score or bilateral disease. The continuous variables (age, weight, OA grading, BCS, lameness score, CSS, PSS, PIS, QoL and the hematobiochemical parameters) were assessed for homoscedasticity using Shapiro-Wilk's test for normality and Levene's test for homogeneity of the variance; subsequent tests were applied as appropriate.

Differences between the repeated measurements of BCS, hematobiochemical parameters between T0 and T90 were tested with the paired sample Student-t test or Wilcoxon test as appropriate. Differences between the repeated measurements of lameness score, CSS at T0, T7, T14, T28, T56 and T90 and PSS, PIS, QoL at T0, T7, T14, T21, T28, T35, T42, T49, T56, T63, T70, T77, T84 and T90 were tested with repeated measurement ANOVA or Friedmann test as appropriate. To apply Friedmann test, QoL categories were given the score of 0=Poor, 1=Fair, 2=Good, 3=Very good, 4=Excellent. Post-hoc analyses were applied using Bonferroni or Conover's tests for multiple comparisons, as appropriate. Significance was set at $p < 0.05$.

RESULTS

Fourteen dogs were enrolled and included in this study. They represented an heterogenous population in which 9 (64%) were intact males and 5 (36%) were females, 3 (21%) of which were neutered. Breed represented were for 21% Labrador retriever (n=3), 14% German Shepherd (n=2), 14% mixed-breed (n=2), 7% Border Collie (n=1), 7% Pinscher (n=1), 7% English Bulldog (n=1), 7% Chow-Chow (n=1), 7% Corso (n=1), 7% Lagotto Romagnolo (n=1), 7% Newfoundland (n=1). Median age of animals was 6 years, ranging from 1 to 12 and the median weight was 27,5 kg, ranging from 3 to 68 kg. Median BCS was 5/9, ranging from 4/9 to 7/9. Forelimb was affected in 43% of dogs (n=6), hindlimb in 50% (n=7). In 21% of cases both forelimbs (n=3) and in 7% both hindlimbs were affected (n=1). Affected joints were elbow in 36% of cases (n=5), stifle in 29% (n=4), hip in 14% (n=2); shoulder, metacarpus and intervertebral were each affected in 7% of cases. 43% (n=6) of dogs were diagnosed with dysplastic arthropathy and 57% of dogs (n=8) with acquired degenerative arthropathy. Based on radiographic evidence, OA was graded as 1 in 3 dogs (21%), 2 in 2 dogs (14%), 3 in 2 dogs (14%) and 4 in 7 dogs (50%). At the time of inclusion, 79% (n=11) of dogs presented severe clinical signs of OA, having a CSS ≥8 (Salichs et al., 2021; Salichs et al., 2022). No dogs were withdrawn prior to completion of the study. Three dogs received concurrent antibiotic treatments during part of the study. In one case, a combination of amoxicillin/clavulanic acid and enrofloxacin was used by an external colleague to treat a soft tissue injury in the hind limb (not involved in OA assessment). One dog received amoxicillin/clavulanic acid to treat a pyoderma of the cheek. One dog was treated with clindamycin hydrochloride for sternal osteomyelitis due to a previous foreign body migration from the pleural space.

Efficacy evaluation

For the veterinary assessment: percentages of CSS responders on D7, D14, D28, D56 and D90 were 29%, 50%, 57%, 71% and 57%, respectively. CBPI percentage of responders increased progressively reaching values of 93% except for day 90 when it settled down to 79 (Table 4).

Table 4. CSS and CBPI percentage of responders throughout the study

% of responders/day of study	CSS	CBPI
D7	29	50
D14	50	71
D21	-	86
D28	57	86
D35	-	86
D42	-	86
D49	-	93
D56	71	93
D63	-	93
D70	-	93
D77	-	93
D84	-	93
D90	57	79

The analysis of the CSS total scores compared to the basal values showed high significance

(P<0.01) at all time points. Comparisons between CBPI components (PIS and PSS) basal scores and those recorded during the weekly CBPI assessments also showed high significance (p<0,01) at all time points.
Figures 1 and 2 show the evolution of CSS and CBPI (PIS and PSS) scores respectively at different time points during the study.
On D0 QoL was recorded as Poor in one dog (7%), Fair in 6 dogs (43%), and Good in 7 dogs (50%) while, on D90 no dogs were recorded as Poor (0%), 2 dogs were recorded as Fair (14%), and no dogs as Good (0%), observing a shift towards Very good and Excellent, with 8 dogs (57%) and 4 dogs (29%), respectively (Figure 3).

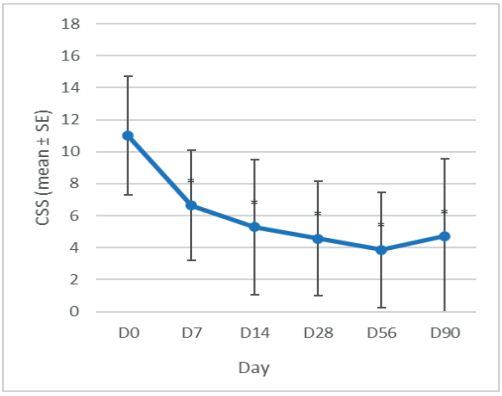

Figure 1. Average CSS (mean ± Standard Error) for each time point. Asterisks indicate significance vs basal scores (p<0.01)

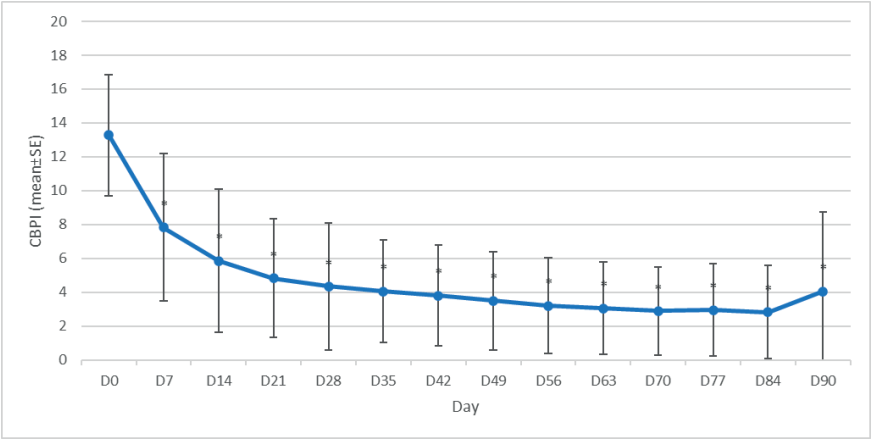

Figure 2. Average CSS (mean±Standard Error) for each time point. Asterisks indicate significance vs basal scores (p<0.01)

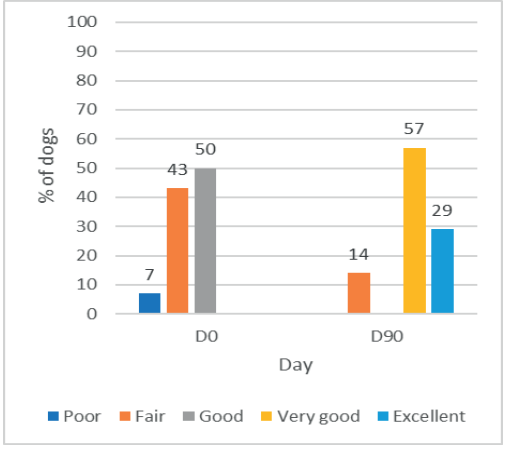

Figure 3. Percentages of dogs in QoL categories on D0 and D90

Statistical analysis showed significance (p<0.01) in the difference between QoL recorded on D0 and that registered in the weekly CBPI compilation. Lameness using the 5 grades NRS was classified at D0 as 5 in 7% of dogs, as 4 in 21%, as 3 in 43%, and as 2 in 21%. On D90 no dogs showed 5 or 4 grade lameness. 14% of caseswere recorded as grade 3.43% as grade 2 and 21% as grade 1.21% of dogs were free from lameness. The statistical analysis showed significance (p<0.01) in the difference between the degree of lameness recorded on D0 and that registered on D7, D14, D28, D56 and D90 veterinary assessments.

Safety evaluation

No symptoms related to the GI tract or haemorrhagic disorders were reported throughout the study. Significance was found in the difference between blood urea and Creat values obtained at the beginning (basal) and at the end of the study ($p<0.05$). Taking as reference the ranges of minimum and maximum values suggested by the laboratory machine used for blood tests, at the end of the study 11 patients (79%) showed a significant increase in urea values compared to the basal. Among these, only 4 dogs (29%) exceeded the indicated threshold limit. An increase in Creat values was observed in 11 dogs (79%), but only 1 dog exceeded the threshold limit. See Table 5 for a complete individual description of urea and Creat values on D0 and D90. Despite the observed changes in laboratory values of urea and Creat, no case experienced clinical symptoms related to renal failure. No other AEs were reported.

Table 5. D0 and D90 urea and Creat values. In Bold parameter increase between D0 and D90 without exceeding the maximum limit. In red: parameter increase between D0 and D90 exceeding the maximum limit

N	NAME	UREA D0	CREAT D0	UREA D90	CREAT D90
1	CANDY	38	1.24	38	1.24
2	MEA	12	0.84	**41**	**1.08**
3	DARCO	10	0.7	**22**	1
4	BRUNO	33	1.48	88	**1.65**
5	NAMI	48	1.44	191	2.27
6	LUCKY	26	0.95	162	**1.57**
7	EROS	38	0.95	**46**	**1.03**
8	ROMEO	34	1.03	**45**	**1.17**
9	CHARLIE	33	1.03	67	**1.45**
10	RIUK	49	1.64	45	1.64
11	DOC	27	1.13	**37**	**1.33**
12	SEBASTIAN	27	0.65	**32**	**1.29**
13	CRISTINA	38	1.14	**50**	**1.3**
14	NALA	52	1.54	50	**1.68**

DISCUSSIONS

The results of this study showed that oral enflicoxib (Daxocox®, Ecuphar NV, Italy) administered at once weekly dose of 4 mg/kg with a loading dose of 8 mg/kg for 13 weeks reduced significantly the CSS and CBPI (PSS and PIS) scores. Statistical analysis of CSS and lameness scores throughout the study shows that there was a strong clinical improvement from the first week of treatment which remained almost unchanged until the end of the study.

To assess lameness and pain during the study, the veterinarian used the CSS, a NRS which was considered one of the main tools to evaluate the effectiveness of enflicoxib treatment in this study. Although a NRS could be considered subjective if compared to other objective assessments like force gait plate analysis (Quinn et al., 2000), CSS relies on the parameters described in several publications to construct an NRS for the veterinary assessment of the efficacy of NSAIDs in the treatment of canine osteoarthritis in multicentre studies (Pollmeier et al., 2006; Autefage et al., 2011; Edamura et al., 2012; Payne-Johnson et al., 2015; Musco et al., 2019), therefore it can be considered a valid method to assess pain and lameness in dogs with OA.

CSS scores obtained in this study are satisfactory and, in comparison to other clinical trials on the use of enflicoxib and mavacoxib for treatment of canine OA (Salichs et al., 2021; Salichs et al., 2022), it is possible to note a similar efficacy even if the data could not be fully compared due to the small number of cases of this trial and the difference in design between the studies.

Furthermore, the achievement of a score of CSS<6 could represent a very strict standard of efficacy: indeed, some of the dogs that entered the study with a high CSS (≥8) had a strong clinical improvement and a significant reduction in CSS and lameness, but they could not be considered as responders according to the established efficacy evaluation criteria.

As can be seen from the CSS trend, there was a reduction in the percentage of responders at the end of the study (from 71% on D56 to 57% on D90). This is probably related to the concomitant development of complications not linked to the joint object of study assessments. In particular, around one week before the end of the study, dog n°7 presented knee cranial cruciate rupture in a limb other than the one object of study assessment. Another dog (n°6), which presented severe bilateral elbow OA (radiographically scored as 4), was a CSS responder from D28 onwards but on D90 it presented a worsening of pain and lameness, reaching a score of 6 in CSS assessment. For lack of compliance of the owner we could not deepen the issue and we have no clinical

evidence to determine the cause of the worsening of the symptoms.
Furthermore, according to the established criterion for CSS, four dogs were unresponsive to treatment throughout the observation period. Dog no 2 presented severe bilateral hip dysplasia (Figure 4) considered most severe in the right coxo-femoral joint. Although it cannot be considered a responder for the CSS as indicated above, it presented a significant decrease in the CSS value, going from a score of 17 on D0 to 7 on D90. Additionally, despite veterinary warnings regarding weight control, the dog experienced a significant increase in BCS from an initial grade of 4 to a grade of 9 at the end of the study. This may have had a negative impact since weight gain represents a predisposing and aggravating factor for OA (Impellizeri et al., 2000). Notwithstanding that, the dog was a CBPI responder from the second week of treatment onwards and from D0 to D90 had a 3-degree reduction in lameness score, and a change in QoL from Fair to Very good. Therefore, its clinical improvement was considered satisfactory.

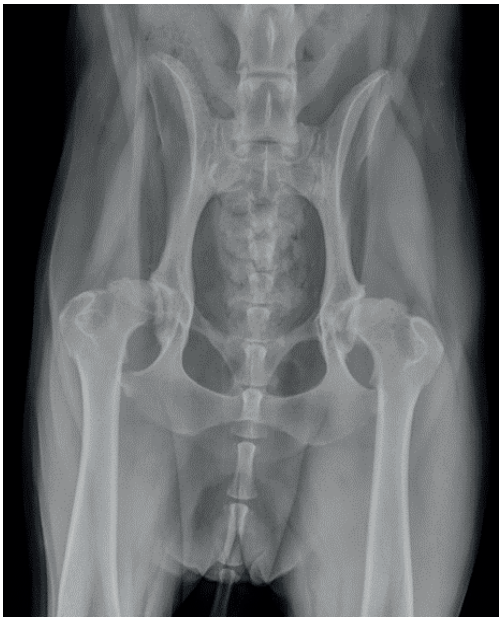

Figure 4. Ventro-dorsal projection of the pelvis of "Mea" (dog n°2) showing severe bilateral osteoarthritis of the hips on a dysplastic basis

Dog n°5 entered the study with grade 2 knee OA due to partial rupture of the cranial cruciate ligament (CrCL). Also in this case, the dog was a CBPI responder from D28 onwards, CSS gradually improved from a score of 10 on D0 to 6 on D90, lameness reduced by one grade (from 3 to 2) and QoL improved from Good to Very good. Regarding this case, veterinary and owner assessments on D14 revealed a worsening of the scores compared to previous visits. Based on the clinical examination, complete CrCL rupture had occurred. However, as previously anticipated, the dog gradually improved throughout the study; despite that, it could not be considered a responder to treatment under the stablished criteria.
Dog n°3 suffered from very severe dysplastic elbow OA, classified as 4 according to D0 radiographic findings (Figure 5).

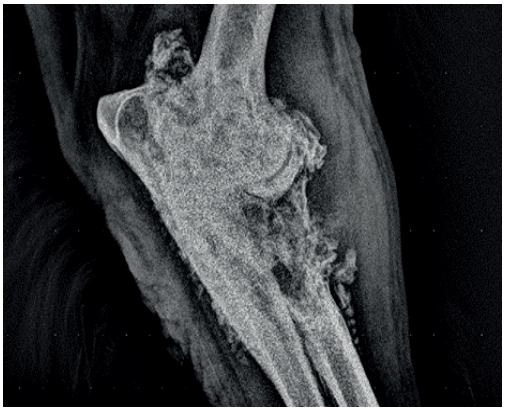

Figure 5. Mediolateral projection of the elbow of "Darco" (dog n°3) showing very severe arthrosis. Note the advanced osteophytes and remodeling of the bony heads forming the humeral-radio-ulnar joint

It never was a responder under the CSS criterion, but it was for the CBPI from D7 onwards. On day 90 (D90) it presented a sharp worsening of either CSS or CBPI, which based on the clinical examination, it was attributed to the rupture of the CrCL of the right hind limb which probably occurred in the previous 7 days. The last dog (n°12) presented 4 grade hip OA due to dysplasia. According to the veterinary assessment it never experienced a clinical improvement. Based on the owner's opinion, the dog still experienced a one-point improvement in QoL (from Good to Very good) and was a CBPI responder only on D14 and D21. Owner evaluation was carried out through the CBPI, a validated instrument

(Brown et al., 2007; Brown et al., 2008) with a previously established inclusion criterion and definition of treatment success (Brown et al., 2013), of which the owners were not aware in order not to influence their assessments. CBPI trend confirmed a positive response to the drug, as shown by the gradual increase in CBPI responders throughout the study.

Confronting CBPI efficacy data obtained in this study with other published clinical trials on the use of NSAIDs in the treatment of canine OA, percentages recorded are very good if compared to CBPI scores obtained for the same enflicoxib or other NSAIDs like mavacoxib (Salichs et al., 2021; Salichs et al., 2022), carprofen (Brown et al., 2013) or firocoxib (Vijarnsorn et al., 2019). CBPI numbers are excellent especially considering that most of the dogs in this study were affected by severe osteoarthritis on the basis of their baseline CSS values (≥8) (Salichs et al., 2021; Salichs et al., 2022).

Furthermore, based on owners' opinion, it is possible to state that the treatment contributed to the improvement of the QoL of dogs from the beginning and throughout the study. Regarding CBPI, there was a reduction in responders (from 93% from D49 onwards to 71% on D90) at the end of the study: three dogs did not respond, which were some of the ones related to the decline for the CSS (dogs n°3, 6 and 12). Moreover, dogs n° 3 and 6 were the only ones maintaining Fair classification for the QoL on D90.

It must be considered that, although small, the dog population included in this study was characterized by a high heterogeneity as regards of breed, weight and age and also the underlying pathology. Although some dogs could not be considered responders based on the predefined criteria of the study, a clear improvement in symptoms, as well as in QoL as assessed by their owners was observed. Therefore, enflicoxib proved to be a versatile treatment in the management of different OA conditions in various type of patients, leading to clinical improvement in almost the entire population of this study.

Regarding AEs, NSAID-associated renal adverse effects are the main consequence of reduced prostaglandin production. In the present study an increase in both urea and Creat was observed in some subjects compared to baseline values before treatment started, with a significant difference (Table 5). However, this increase in values exceeding the limits in one dog for both urea and Creat, and in 3 dogs for urea only, did not translate in any of them experiencing clinical signs related to renal failure. These results are in contrast to those of previous studies such as the 7-month safety laboratory study by Homedes et al. (2021), in which drug administration up to five times the therapeutic dose for 3 months did not induce a significant increase in urea values at the end of the study. Probably the inclusion of healthy young Beagle dogs in the previous study, the limited number and the heterogeneity of the clinical cases in this study, may have influenced these results. Therefore, it would be useful to expand the population under study in long-term treatments. In order to confirm actual renal damage, it would have been appropriate to carry out additional investigations as urea and Creat are not described as highly sensitive indicators of reduced renal function (Raekallio et al., 2006), due to the fact that they are affected by extra-kidney factors including muscle mass, liver function and diet (O'Connell et al., 1962; Balint and Visy, 1965; Braun et al., 2003). For a better assessment of renal function, it would have been more adequate to perform further investigations such as urinalysis or evaluation of glomerular filtration rate (GFR) (Raekallio et al., 2006).

Furthermore, it would have been advisable to perform follow up blood tests after the end of treatment to evaluate whether the renal parameters were back within normal limits.

Despite GI tract is the main site of organ toxicity of NSAIDs in humans and companion animals (Monteiro-Steagall et al., 2019), in the present study, the repeated administration of enflicoxib did not cause any toxic effects at the GI level and no AEs such as diarrhoea or vomiting occurred, often associated with the use of NSAIDs. Similar results, demonstrating the safety of the treatment, were obtained in the previous overdose long term safety study with Beagle dogs by Homedes et al. (2021) or in the clinical studies in the target population of old dogs naturally affected with OA (Salichs et al., 2021); according to Homedes et al. (2021) some episodes of soft stools and emesis in

some dogs occurred but no faecal occult blood was detected. In any case, AEs were those expected after treatment with this class of compounds and tended to occur early in therapy. This was confirmed by Lascelles et al. (2005) who demonstrated that most cases of NSAID-associated GI toxicity occur within 48-72 hours of starting treatment. Moreover, long-term treatment with NSAIDs is not associated with an increase in the incidence of AEs (Innes et al., 2010).

The good gastrointestinal tolerability reported in this study could be associated, as extensively described by Homedes et al. (2021), with the fact that enflicoxib belongs to COX-2 selective inhibitors and needs a weekly administration, which would reduce the local effects on the gastric mucosa.

Lastly, blood chemistry evaluations did not reveal significant differences in the plasma concentrations of liver enzymes (ALT, AST, ALP), therefore no signs of liver toxicity were observed, and enflicoxib administration did not show potential for interference with blood haemostasis.

The results of this study support the good efficacy and safety of enflicoxib treatment as previously described in several studies (Homedes et al., 2021; Salichs et al., 2021; Salichs et al., 2022). However, limitations of the study must be considered, such as the small number of cases included and the analysis being conducted without a control group. The absence of a control group did not allowed a randomised study design with blind evaluation of the cases.

CONCLUSIONS

The results of this study support that enflicoxib (Daxocox®, Ecuphar NV, Italy) administered orally at an initial loading dose of 8 mg/kg and weekly maintenance doses of 4 mg/kg for a total period of 90 days could be consistently effective with an adequate level of safety in the treatment of dogs with naturally occurring OA. To fully validate these results, it would be appropriate to carry out further field studies that include a greater number of animals, and designed as controlled, randomised and blind studies.

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HUMAN LACTOFERRIN CAN ENHANCE THE OSTEOGENIC DIFFERENTIATION OF EQUINE MESENCHYMAL STEM CELLS?

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Abstract

Mesenchymal stem cells (MSCs) are harvested after birth; they are adult stem cells and, due to their unique potential, are considered very valuable tools for equine regenerative medicine. MSCs have self-renewal capacity and multilinear differentiation potential. Multiple protocols are used to induce the directed differentiation of these cells. The aim of our study was to evaluate the osteoinductive potential of a glycoprotein from the transferrin family, lactoferrin (Lf) on MSCs isolated from equine synovial fluid. The cell line (syMSCs) used for this study was obtained from synovial fluid samples from a healthy horse. The isolated cells were characterized morphologically, immunophenotypically and functionally respecting the standards of the International Society for Cell Therapy which were originally drawn up for human MSCs (cellular plastic adherence, expression of specific surface markers and trilinear differentiation capacity). The cells were cultivated in normal propagation medium for MSCs. For osteogenic differentiation, syMSCs were seeded at a concentration of 1 × 10^5 cells/3 mm well, and cultured in osteogenic induction medium with (3 different concentration: 20, 50, 100 µg/mL) and without Lf. The proliferation potential of the cells were assessed using CCK8 assay and the markers of osteogenic differentiation (alkaline phosphatase, ALP) were detected using fluorimetric assay. Our results demonstrate the osteogenic potentiation capacity of human lactoferrin correlated with concentration, thus our future studies will try to elucidate the osteoinductive mechanism of lactoferrin by applying genomics and proteomics techniques.

Key words*: mesenchymal stem cells, equine, differentiation, osteogenic, lactoferrin.*

INTRODUCTION

Mesenchymal stem cells (MSCs) are cells of mesodermal origin; they are multipotent adult cells with important biological properties for regenerative medicine (Cequier et al., 2021). These cells can be harvested from different tissues, are self-renewal capacity and multilinear differentiation potential (Pall et al., 2016). Equine mesenchymal stem cells have been isolated from multiple tissues, namely bone marrow, peripheral blood, umbilical cord blood, adipose tissue, placenta, (Prado et al., 2015), synovial membrane (Sakaguchi et al., 2005) and synovial fluid (Crecan et al., 2019). MSCs have fusiform morphology, with adhesion capacity and high proliferation potential. According to the International Society of Cellular Therapy, there is a set of standards to define human MSCs: adhesion on the cultivation surface, the expression of specific surface molecules (CD105, CD73, CD90, lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR) and trilinear differentiation capacity (Dominici et al., 2006). *In vivo* MSCs have the ability to differentiate in addition to the chondrogenic and adipogenic lineages to the osteogenic lineges. Osteogenic differentiation can be induced *in vitro* using specific osteogenic induction medium. Different substances such as multi-bioactive mesoporous silica nanoparticles, curcumin, quercetin, kaempferol, naringin and other flavonoids, can also induce this ability (Tavares et al., 2020, Xiong et al., 2020; Zhang et al., 2022). Multiple studies have shown that lactoferrin (Lf) is an anabolic factor (Ying et al., 2012). Lf is a bioactive iron binding globular protein, representative of the transferrin family (Pall & Roman 2020, Pall et al., 2023). In addition to the antioxidant potential, Lf also has anti-inflammatory, immunomodulatory, analgesic antibacterial, antiviral, antifungal and anticancer potential (Hao et la., 2019; Pall & Roman 2020).

Lf is present in various secretions and tissues (Atsushi, et al., 2000), including saliva, milk, colostrum, saliva, uterine secretions, semen,

gastrointestinal fluid (Pall & Roman 2020). In the body, Lf circulates approximately at a concentration of 2–7 μg/mL, being a promoter of bone growth, acting as a growth factor for the proliferation of osteoblasts and having an inhibitory effect on osteoclasts (Amini & Nair 2011, Yin et al., 2020).
In this context, the aim of present study was to evaluate the osteoinductive potential of Lf on MSCs isolated from equine synovial fluid.

MATERIALS AND METHODS

Synovial fluid derived MSCs isolation

This research was approved by the Bioethics Committee of the Faculty of Veterinary Medicine Cluj-Napoca. Synovial fluid was harvested from the metacarpophalangeal joints during arthroscopic procedure from a healthy horse.

MSCs cells isolation

Synovial fluid samples was mixed with propagation medium DMEM/F12 (Gibco Life Technologies, Paisley, UK),) supplemented with 10 % of fetal bovine serum (FBS, Sigma-Aldrich, St.Louis, MO, USA) and 1 % antibiotic-antimycotic (Sigma-Aldrich, St.Louis, MO, USA). Culture plates were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. After 72 h, non-adherent cells were removed, and the culture medium was replaced. The cultures were monitored daily, the medium was changed every 2 days, and at a confluence of 70-80% the cells were passaged. After 6 successive passages the cells were evaluated immunophenotypically using flow cytometry.

Immunophenotype characterization

For the evaluation of specific surface markers, the cells were removed from the cultivation surface, and the cell suspension was labeled with anti-human CD44, anti-human CD105, anti-human CD90, anti-human CD45, anti-human CD73 and anti-human CD 34. The specific antibodies were purchased from Becton Dickinson, USA. A concentration of 1×10^5 labeled cells were acquired with flow cytometry (FACS Canto™II, Becton Dickinson, USA). The results were analyzed using FACSDiva Software (Becton Dickinson, USA).

CCK-8 assay

The synovial fluid derived MSCs were cultured in normal propagation medium supplemented with 3 different concentrations of Lf (20, 50, 100 μg/mL). To evaluate the proliferation potential of synovial fluid derived MSCs a concentration of $1x10^5$ cells were seeded in 96-well tissue culture plates in normal propagation medium. After 24 and 48h of incubation, the cells were treated with 3 different concentrations of Lf and incubated at 37°C in a humidified atmosphere supplemented with 5% CO2. Negative control was represented by untreated cells (cells maintained in normal propagation medium). Cell viability was measured using CCK-8 reagent and incubating for 4h at 37°C. For the interpretation of the results the optical density was determined at 450 nm by using a BioTek Synergy 2 microplate reader (Winooski, VT, USA). The obtained results were expressed as relative viability percentage to the negative control (untreated cells). All experiments were performed in triplicate.

Osteogenic differentiation of isolated cells

To evaluate the osteoinductive potential of lactoferrin, MSCs derived from the synovial fluid were cultured at a concentration of 3×10^3 cells/cm^2 in normal propagation medium. After 24 h the culture medium was removed and the specific osteogenic medium (100 nM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid (all from Sigma-Aldrich, St.Louis, MO, USA) supplemented with three different concentration of Lf (20, 50, 100 μg/mL) was added. The culture medium was changed every three days, and after 7 and 14 days of culture, the concentration of alkaline phosphatase was evaluated.

Alkaline phosphatase evaluation - fluorimetric assay

Quantitative analysis of ALP was assessed using fluorimetric Alkaline Phosphatase detection kit (Sigma-Aldrich, St.Louis, MO, USA). For evaluation, the samples were collected at 7 and 14 days of culture, respectively. The samples (20 μl) together with the negative control (cells maintained in osteoinductive medium without lactoferrin and

cells maintained in simple propagation medium) were added in microplates with 96 wells. The plates were incubated for 20 minutes at 65°C, after which they were cooled followed by the addition of the diluted fluorescent mixture (1:8). The results were evaluated using Biotek Synergy 2 reader (360 nm excitation and 440 nm emission).

Statistical Analysis

The one-way ANOVA and t-test (GraphPad Prism 8) were used for statistical analysis. The results were expressed as mean ± standard deviation (SD); $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Synovial fluid derived MSCs cells isolation

Mesenchymal stem cells were derived from synovial fluid from a healthy horse presented for routine analysis. The synovial fluid was incubated in specific propagation medium. After 72 hours the cultures were examined, and the culture medium was changed. The cells attached to the cultivation surface showed spindle morphology along with stellate and round cells. A high percentage of the cells were in suspension. After 5 days, the culture showed a high degree of heterogeneity, and by changing the culture medium, the non-attached cells were removed (Figure 1).

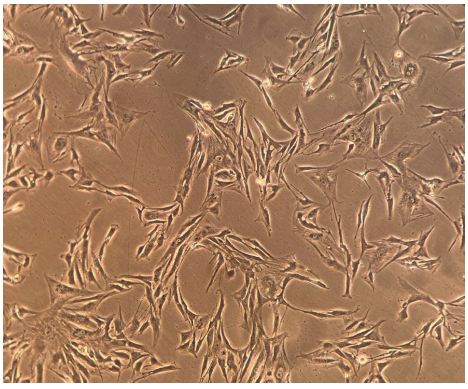

Figure 1. Morphology of cells isolated from equine synovial fluid

At 10 days, the cultures showed a confluency of 80% when the first passage was performed. After the third passage, the culture became homogeneous, the cells showing spindle morphology with pronounced bipolarity, and after the sixth passage, the cells were immunophenotypically characterized.

Immunophenotype characterization

Characterization of cells isolated from synovial fluid was performed using flow cytometry.

The evaluation was carried out according to the standards of the International Society for Cellular Therapy. The isolated cells showed positivity for CD105 (96.5%), CD90 (93.4%), CD44 (99.0%), low expression for CD73 (12.7%) and negativity for CD34, CD45 (Figure 2).

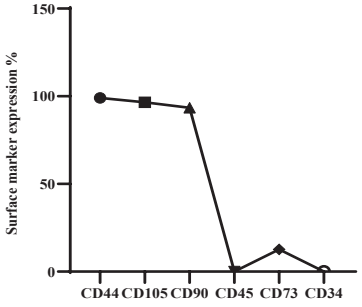

Figure 2. Results of immunophenotype evaluation

CCK-8 assay

To evaluate the proliferative potential of lactoferrin, the propagation medium of MSCs was supplemented with three different concentrations of Lf. Cell viability and the degree of cell proliferation was evaluated by the CCK8 assay. The results suggest that lactoferrin has a stimulating effect on cell proliferation. After 24 hours, the average cell viability of treated cultures was 105.53%±0.41. The highest proliferation rate was obtained at the highest concentration (100 μg/mL) of Lf, the average proliferation rate was 108.47%±4.02 (Figure 3).

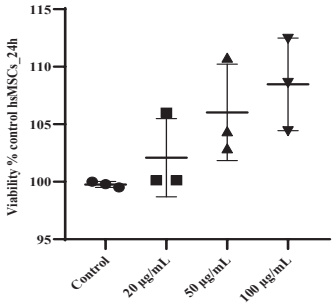

Figure 3. Cell viability rate after 24h of treatment with three different concentrations of human lactoferrin

The increasing tendency of the proliferation rate was maintained even after 48 hours of evaluation. The average of the proliferation rates were 107.30±0.060. Supplementing the propagation medium with a concentration of 100 µg/mL Lf led to an average viability of 111.94%±1.14. The lowest proliferation rate was observed in the cultures treated with 20 µg/mL of Lf. All results were compared with the control culture (Figure 4).

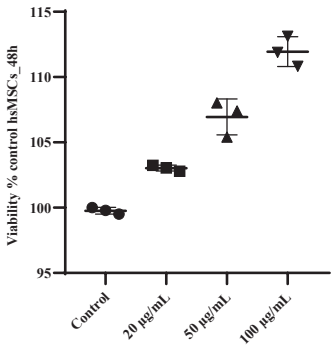

Figura 4. Cell viability rate after 48h of treatment with three different concentrations of human lactoferrin

Through the global analysis of the results, it can be concluded that lactoferrin has a stimulating effect on cell proliferation, and it is dose dependent.

Osteogenic differentiation of isolated cells

To demonstrate the osteoinductive potential of lactoferrin, cells derived from equine synovial fluid were treated with the simple osteogenic medium and the osteogenic medium supplemented with lactoferrin in three different concentrations. At 7 and 14 days, respectively, the cell lysates were tested for the evaluation of ALP activity. The results showed higher levels of ALP in cultures treated with lactoferrin compared to the osteogenic medium and simple propagation medium. 7 days after differentiation, the highest level of ALP was recorded in cultures treated with 100 µg/mL of lactoferrin, followed by those treated with 50 µg/mL of lactoferrin (Figure 5).

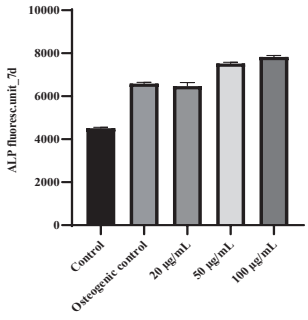

Figure 5. Alkaline phosphatase level evaluated after 7 days of treatment of MSCs with osteogenic medium and lactoferrin

Analyzing the results after 14 days, the ALP levels showed a slight increase, but these increases were not statistically significant compared to the previous evaluations. In this evaluation, similar to the previous ones, the highest values of ALP in the evaluated cell lysates were recorded in the case of cultures treated with the highest concentration of lactoferrin (Figure 6).

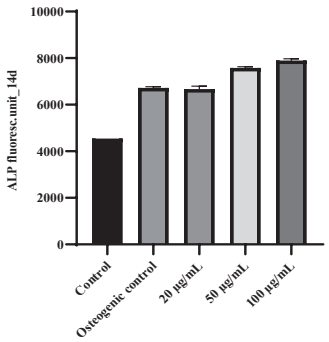

Figure 6. Alkaline phosphatase level evaluated after 14 days of treatment of MSCs with osteogenic medium and lactoferrin

Our results demonstrate the potential of lactoferrin to increase the cells viability of MSCs. Similar studies demonstrate this potential; Ryu et al (2017) indicates that lactoferrin potentiates cell proliferation, which is dose-dependent and occurs through the MAPK signal pathway (Ryu et al., 2017). An important characteristic of MSCs is their multilineage differentiation potential. In the body, this differentiation potential is controlled by several mechanisms and is dependent on the intervention of specific growth factors. *In vitro*, this potential can be demonstrated through various substances, either synthetic or natural. In our experiment we chose lactoferrin because our preliminary studies demonstrate the multiple capacity of this bioactive globular protein (Pall et al., 2023). Multiple studies demonstrate that osteoblast differentiation is a crucial stage in osteogenesis (Spi et al., 2020). To demonstrate osteogenesis, the evaluation of ALP activity is an important indicator (Liu et al., 2015) and is expressed at the beginning of the mineralization process. In the present study, ALP activity was evaluated in cell lysate in two stages of directional differentiation (early and intermediar stage). The studies of Zhang et al. (2014) demonstrated that the oral administration of Lf in ovariectomized rats acted preventively on the reduction of bone loss due to estrogen deficiency and contributed to the improvement of bone microarchitecture. Similar *in vitro* studies demonstrated that Lf promotes osteoblast proliferation and survival, but simultaneously has an inhibitory effect on osteoclastogenesis (Cornish et al., 2004).

CONCLUSIONS

Lf is considered an anabolic factor with a demonstrated role in osteogenesis. Our study demonstrated its differentiation potential on mesenchymal stem cells derived from the synovial fluid. But further studies are needed to demonstrate the potential mechanisms involved. Due to its antimicrobial, antibiofilm, antitumor, anti-inflammatory potential, Lf from different sources can play an important role in veterinary medicine, for the treatment of diseases with multiple etiologies.

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PRELIMINARY DATA ON DETECTION OF ANTIBODIES AGAINST *Anaplasma* spp. IN CATTLE, ROMANIA

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Abstract

Anaplasmosis, a tick-borne disease affecting cattle worldwide caused by Anaplasma spp. (Rickettsiales: Anaplasmataceae), is associated with considerable economic losses to dairy and beef industries. Data about exposure to Anaplasma spp. infection of cattle in Romania is limited. Therefore, a cross-sectional serological study was conducted to preliminary investigate the serological status on Anaplasma spp. infection in Romanian catlle. For this, 182 catlle originated from three counties in Northeastern and Southeastern Romania were included in the study. Cattle serum samples were tested with a competitive enzyme linked immunosorbent assay (cELISA) detecting an epitope of major surface protein 5 (MSP5) of Anaplasma spp. The results were expressed as percentage (%) of inhibition (I) calculated according to the indications of the manufactures; samples with I ≥30% were considered positive. Subsequently, the overall seropositivity was 42.3% (95% CI: 35.03 - 49.84); significant difference of seroprevalence values (p< 0.05) were registered according to the cattle originating county, ranging from 22.2% (95% CI: 13.26-33.57) to 72.0% (95% CI: 57.50-83.77). These findings emphasize on the need for further studies to identify the species and associated risk factors for Anaplasma inffection of cattle in Romania, as base for developing control strategies.

Key words*: Anaplasma spp., serological investigation, cELISA, cattle, Romania.*

INTRODUCTION

Anaplasmosis is a tick-borne disease affecting cattle worldwide caused by *Anaplasma* spp. (Rickettsiales: Anaplasmataceae). It is associated with considerable economic losses to dairy and beef industries worldwide (Kocan et al., 2010). The species of the genus *Anaplasma* are Gram-negative, immobile, obligatory intracellular bacteria that cause diseases in humans and animals, displaying specific cell tropism in the vertebrate hosts (erythrocytes, granulocytes, monocytes/ macrophages, or platelets), depending on the species (Dumler et al., 2001).

The main species affecting domestic ruminants of socio-economic impact are *Anaplasma marginale, Anaplasma centrale, Anaplasma bovis, Anaplasma phagocytophilum, Anaplasma ovis,* and *Anaplasma capra* (Kocan et al., 2010).

Of them, *A. phagocytophilum,* a multi-host pathogen also with zoonotic potential, causes the tick-borne fever in domestic ruminants, while *A. marginale* and *A. centrale* are responsible for bovine anaplasmosis (Dumler et al., 2001; de la Fuente et al., 2005). Ticks are referred as main biological vectors of *Anaplasma*, but also mechanical transmission by biting flies or fomites are described (Kocanet al., 2010; Aubry and Geale, 2011; DaSilva et al., 2014).

Anaplasma spp. causes persistence infection in the host population, which assures more spreading and occurring of new outbreaks.

Bovine anaplasmosis is clinically characterized by anaemia, fever, weight loss, jaundice, abortion, and death (Kocan et al., 2010).

The disease is endemic in tropical and sub-tropical regions of the world, causing significant economic losses to dairy and beef industries worldwide. It is being reported more frequent also in Europe, in Switzerland, Italy (Sicily), Hungary, Spain (Hofmann-Lehmann et al., 2004; de la Fuente et al., 2005; Hornok et al., 2007). Recently, fatal cases of bovine anaplasmosis have been reported in southwestern Spain (Moraga Fernadez et al., 2022).

Different diagnostic tests have been developed and are available for diagnostic; of them, light microscopy of Giemsa-stained blood smears is useful during of the disease's acute phase, while serological tests and molecular-biology based techniques are able to detect subclinical

infected animals and/or low-level infection in carrier animals (Aubry and Geale, 2011).

Several enzyme-linked immunosorbent assay (ELISA)-based tests have been developed and are currently used, of them competitive inhibition enzyme-linked immunosorbent assay (cELISA) and card agglutination test (CAT) are recommend for field, epidemiological studies (O.I.E., 2015).

In Romania data about *Anaplasma* spp. infection in cattle is very limited and no recent studies about bovine anaplasmosis in Romania are available. Therefore, a cross-sectional study was conducted to investigate the serological status on *Anaplasma* spp. infection in Romanian cattle.

MATERIALS AND METHODS

The study was conducted in three counties located in North-eastern and South-eastern Romania (Figure 1). In all three counties there were reported tick infesting cattle.

A number of 182 dairy cattle originated from two different areas in Northeastern (Suceava - SV county; n=60) and Southeastern (Buzau-Bz; n=50; Teleorman- Tr; n=72) Romania, were included in the study.

From cattle, blood samples were collected without anticoagulant, from which serum samples were obtained for serological testing. All samples were stored at -20ºC until testing.

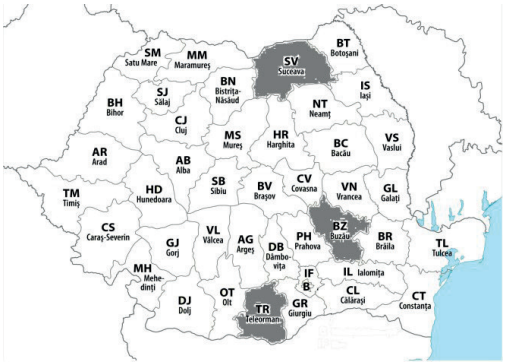

Figure 1. Map of Romania: the study area (cattle originating counties are shown)

The serum samples were tested for detecting IgG antibodies against the MSP5 protein of *Anaplasma* spp. by using a competitive ELISA (cELISA, VMRD v2, Inc.,Pullman, WA, USA) test. The kit is approved for detection of antibodies against *A. marginale, A. ovis,* and *A. centrale*, being reliable for identifying persistently infected cattle (Knowles et al., 1996; Dreher et al., 2005).

The assay was performed in accordance with the manufacturer's instructions. Briefly, 50 µl of the controls and serum samples were transferred to the corresponding wells of the *Anaplasma* Antigen-Coated Plate and incubated for 1 h. After two washes with wash solution, 50 µl of conjugate-peroxidase was added and incubated for 20 min. After washing the plate four times, 50 µl of substrate solution was added to each well and incubated in darkness for 20 min. Finally, 50 µl of stop solution was added and the optical density of the plate was read by a microplate reader Epoch at 630 nm. Results were calculated according to the formula provided by the kit's manual and expressed as percentage of inhibition (%I). Samples with %I! ≥ 30 were considered positive.

RESULTS AND DISCUSSIONS

To investigate the serological status on infection with or exposure to *Anaplasma* spp. infection, cattle reared in two geographical areas in Romania (northeastern and southeastern) were tested by using a cELISA technique. Of the 182 tested cattle, 77 were seropositive and 105 were seronegative.

The overall observed apparent seroprevalence of antibodies against *Anaplasma* spp. was of 42.3% (95% CI: 35.03 - 49.84).

Significant diferences of seroprevalence ($p<0.05$) were registered according to the originating county, ranging from 22.2% (95% CI: 13.26 - 33.57) to 72.0% (95% CI: 57. 50 - 83.77) (Table 1).

Table 1. Seroprevalence of *Anaplasma* spp. in cattle in two different regions of Romania as revealed by cELISA results, stratified by originating area/county

Region/ county	No. tested	No. positive	Prevalence (%)	95% Confidence Interval	P-value
Northeastern					<0.05
Sv	60	25	41.7	29.06-55.12	
Southeastern					
Bz	50	36	72.0	57.50 - 83.77	
Tr	72	16	22.2	13.26 -33.57	
Total	182	77	42.3	35.03- 49.84	

In the present study, using the cELISA (VMRD v2) test an overall seroprevalence of 42.3% of antibodies against *Anaplasma* spp. was recorded, with presence of positive cattle in all the studied counties, suggesting the presence of *Anaplasma* spp. infection in Romanian cattle.

Several recent studies has reported *Anaplasma* spp. infection in Romania by ELISA-based test but primarily in horses (Bogdan et al., 2021) and dogs (Ionita et al., 2012; 2023; Leica et al., 2019), either as single or concurrent infection with other tick-borne pathogens. Moreover, molecular studies have reported the presence of *A. phagocytophilum* in ticks infesting various species, cattle, dog (Ionita et al., 2013; 2016).

Additionally, should be mentioned that in Romania it is a divers tick fauna, comprising up to 25 species belonging to the genera: *Ixodes, Dermacentor*, *Haemaphysalis, Hyalomma, Boophilus & Rhipicephalus,* of them *Ixodes ricinus and Dermacentor marginatus* is the most widespread and the most abundant, respectively (Ionita et al., 2010; Mihalca et al., 2012; Pavel et al., 2020).

Up to date, DNA of *A. marginale* have been detected in various tick species, such as *I. ricinus, D. marginatus, D. reticulatus,* R. *bursa, R. sanguineus, H. marginatum* (Antunes et al., 2016; de la Fuente et al., 2005; 2004; Hornok et al., 2012), all these species are reported in Romania (Ionita et al., 2010; 2013).

Moreover, in the last decades, an increasing abundance of tick population is being reported, and subsequently tick-borne diseases are being more frequently detected, including in cattle (Ionita and Mitrea, 2017; Ionita et al., 2012; 2018; Pavel al., 2020).

Therefore, considering the diverse and sympatric occurrence of tick fauna in Romania, cattle are at are high risks for tick-borne diseases, including bovine anaplasmosis.

The cELISA method used in the present study is recommended and largely applied for serological screening of bovine anaplasmosis (OIE, 2015), with high sensitivity (96.0-98.0%) and specificity (95.2-100%) (VMRDv2; (Knowles et al., 1996; Aubry and Geale, 2011).

Analysing the results obtained with other similar studies on cattle in Europe reveals variations according to the country/region and investigation test used.

Bovine anaplasmosis has been recorded in many countries in Central and South America, Africa and Asia, in the United States, and in European countries (Kocan et al., 2010).

Oh the cattle infecting *Anaplasma* species, *A. marginale* is regarded as the most pathogenic, causing acute disease, which can be also fatal, depending on various factors, including susceptibility of the host, virulence of the strain, and concurrent infection (Hofmann-Lehmann et al., 2004; Jurković et al., 2020).

Fatal disease has been recently reported in Spain and northern Europe, suggesting the risks when infection occurs within a herd (Moraga Fernandez et al., 2022).

Moreover, animals recovered from acute disease are of high epidemiological significance since they remain lifelong persistently infected carriers and serve as natural reservoir (Kocan et al., 2010).

A.centrale is a closely related to *A. marginale* and considered less pathogenic, causing usually benign diseases characterized by mild anemia; it is used as live vaccine against *A.marginale* - South America, Africa, Israel, so (de la Fuente et al., 2005b; Bellezze et al., 2023).

In Europe, *A. marginale* is endemic in some Mediterranean countries, such as in Spain (de la Fuente et al., 2004; Calleja-Bueno et al., 2022), Italy (de la Fuente et al., 2005a) but it is being reported also in other European countries, such as in Italy (de la Fuente et al, 2005), Portugal (Pereira et al., 2016), Hungary (Hornok et al., 2007; 2008; 2012), including in the northern areas, the Alpine region (Dreher et al., 2005a; Hofmann-Lehmann et al., 2004).

A. marginale is endemic also in the island of Sicily (Italy), where high prevalence (78.0%) of seropositivity of *Anaplasma* spp. was detected by cELISA in 50 tested cattle (de la Fuente et al., 2005). A similar serological cELISA-based study peformed on 26 cattle in northern Hungary reported a seropositivity of 80.8% among the tested animals; in the positive animals, *A. marginale* was confirmed by PCR (Hornok et al., 2007).

In Belgium, a sero-prevalence of 15.6% (53/339) was reported in cattle from all regions from Belgium, with higher seropositivity for cattle in Liege (44.4%) than in Flanders (8%) (Adjadj et al., 2023).

The seroprevalence value differences between the originating counties in our study are similar to those described by Spare et al. (2019), in Kansas, reporting an overall seroprevalence of the 52.5% at the herd level; by region the seroprevalence varied from 19.1% (in Western Kansas) up to 87.3% (in Eastern Kansas) and selected management practices have been found associated with herd infection status (Spare et al., 2019). However, multiple factors can be involved in spreading of the disease.

Higher prevalence values are reported in cattle from Egypt, of 54.8% by serology and 68.3% by qPCR (Al-Hosary et al., 2020). Similar high infection rates were reported in cattle from Mozambique, varing from 76.5% in southern region up to 86.3% in Manputo (Tembue et al., 2011).

Nevertheless, the serological surveys provide valuable data of epidemiological relevance to evaluate the distribution and the risk associated factors, in a particular area.

CONCLUSIONS

This study revealed the presence of *Anaplasma* spp. antibodies in cattle in Romania. As the economic impact of anaplasmosis in cattle is major and of increasing importance, further research are need to assess the distribution and risk factors for this pathogen in Romanian cattle. This study was the first to screen for the presence of antibodies to *Anaplasma* spp. in cattle in Romania. Additionally, the findings emphasize on the need for further studies to identify the species and associated risk factors for *Anaplasma* infection of cattle in Romania, as base for developing appropriate control strategies.

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NEGATIVE PRESSURE WOUND THERAPY AND MEDICAL-GRADE HONEY COMBINATION SWIFTLY HEALS A LOWER EXTREMITY INJURY WITH BONE EXPOSURE IN A CAT

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Abstract

Lower extremity injuries in cats can present significant challenges by a lack of tissue, bone exposure, and bacterial contamination, requesting effective treatment methods. This case report describes the use of a combination of negative pressure wound therapy (NPWT) and medical-grade honey (MGH) dressing in the treatment of a complex lower extremity injury in a cat. An old injury in the metatarsal area of a 3-year-old male cat resulted in an open fracture of all the metatarsal bones. The wound was left open after osteosynthesis via intramedullary pin insertion. A combination of NPWT and MGH dressing was used to promote healing and prevent bacterial contamination. The combination of NPWT and MGH dressing was effective in promoting the healing and full closure of the soft tissue injury in this case. NPWT supported granulation tissue formation, maintained a moist wound environment, and prevented infection. The MGH dressing helped prevent further bacterial contamination and promoted wound healing. This case highlights the benefits of using a bimodal staged approach in the management of challenging lower extremity injuries in cats.

Key words*: negative pressure wound therapy, medical grade honey, lower extremity wound, secondary intention healing.*

INTRODUCTION

A significant portion of the recent literature on wound management discusses the challenges faced by veterinary practitioners in managing wounds, particularly those in the lower extremities that involve bone or tendon exposure and are highly contaminated (Erwin et al., 2021; Nolff, 2021). Effective wound management requires an understanding of the healing process and the use of appropriate techniques to promote healing. While many options are available, factors such as healing time, patient comfort, and cost must be considered. One critical period is the inflammatory phase until granulation tissue formation, during which removing necrotic tissue, controlling infection, and managing exudate are essential to promote healing. Negative pressure wound therapy (NPWT) is one technique that can be used during this period to target the post-wounding period and promote healing (Agarwal et al., 2019; Lee et al., 2009).

The use of suction devices or vacuum properties as an adjuvant in wound therapy has been quoted since antiquity and has been improved during the last century, leading to the development of NPWT technology in the past two decades (Miller, 2012). NPWT, also referred to as vacuum-assisted closure, involves the application of sub-atmospheric pressure to a wound. This is achieved through a closed system usually consisting of a porous foam, an adhesive drape used as a wound sealer, and intermittent or continuous vacuum applied through tubing connected to the foam via a small fenestration. Exudate control is provided as excess exudate is aspirated through the tubing into a canister, keeping the system entirely closed.

With increasing research results, the utilization of NPWT has expanded to a wider range of applications in human medicine (Seidel et al., 2020; Xie et al., 2010). In the veterinary field, the main focus has been on the open management of large contaminated or infected wounds, septic peritonitis, and skin graft

augmentation (Stanley et al., 2013). Most studies have shown that NPWT results in reduced overall healing time, early granulation tissue formation, prevention of thermal and oncotic fluctuations, decreased edema, maintenance of optimal environmental conditions to keep the wound moist, three-dimensional wound contraction, modulation of wound healing mediators, increased tissue perfusion, and neovascularization (Ben-Amotz et al., 2007). While these properties have been proven in experimental settings on animal models such as rats or porcine models, the same results associated with small animals, particularly cats, are still under discussion and require further research (Jacobs et al., 2009; Morykwas et al., 2001). NPWT does not support epithelialization, thus the technique is limited to the stage when granulation tissue is formed (Demaria et al., 2011; Guille et al., 2007).

Medical grade honey (MGH) follows strict criteria to ensure the quality, safety, and efficacy of honey for medical applications (Hermanns et al., 2020). MGH has antimicrobial and wound healing activities, both are based on multiple mechanisms. The antimicrobial activities include acidic pH, osmotic activity, and the presence and formation of antimicrobial molecules make it effective against a broad-spectrum of microorganisms, without the risk of developing resistance (Pleeging et al., 2020). Wound healing is enhanced by creating a moist wound environment, its anti-inflammatory and anti-oxidative activities, and stimulation of autolytic debridement, angiogenesis, granulation tissue formation and reepithelialization (Smaropoulos et al., 2020; Pleeging et al., 2022a).

Once the wound bed transforms into healthy and well-vascularized tissue, there are two primary categories of wound closure options: secondary intention healing through the use of dressings and bandages, or surgical reconstruction via skin grafts or flaps. The market offers an immense range of dressings and topical products with evidence-based healing properties. However, choosing the right product or combination of products can be challenging due to several factors, including availability, price, previous experience, estimated time for healing, bandage change frequency, and individual factors. This case report highlights the successful combination of NPWT and MGH dressings for a lower extremity injury in a cat, demonstrating promising results.

MATERIALS AND METHODS

A 3-year-old, 3.2 kg intact male mixed-breed cat was presented to the University Veterinary Hospital of the Faculty of Veterinary Medicine of Bucharest with a one-week old open metatarsal fracture that was referred for limb amputation. The patient was normothermic, normotensive, and normopneic, with non-weight bearing lameness in the right hind limb. The surgical consultation revealed an open fracture in the metatarsal area with displacement and exposure of the fracture site. The patient is an outdoor cat, and the exact time of the traumatic event was unknown but estimated by the owner to be between 5-7 days prior to the examination. A limb-sparing approach was proposed, with intramedullary pin osteosynthesis of the fractured metatarsal bones, debridement, and secondary or tertiary intention healing of the wound. The initial surgery was performed by another surgical team, and the case was presented to the authors for wound healing. The initial wound evaluation was performed after surgery when intramedullary pinning was performed on the 2nd, 3rd, and 5th metatarsal bones of the right hind limb, and clinically nonviable tissue was surgically debrided. A deep wound affecting most of the anterior profile of the metatarsal area with bone and metal implant exposure, extensive muscle and tendon injuries, and severe tissue loss was observed. The perilesional tissue appeared viable at the initial evaluation, since surgical debridement had already been performed, and the wound was thoroughly irrigated with Ringer's lactate. A bacterial swab was taken for aerobic culture and antibiotic sensitivity testing. An aseptic technique was used to apply a bandage, which consisted of a MGH-based product (L-Mesitran Soft-gel, www.mesitran.com) as the contact layer, covered by sterile non-woven gauze pads, synthetic cast padding, and self-adhesive cohesive wrap. General intravenous antibiotics had already been initiated by the first surgical

team and continued until the antibiotic sensitivity test results were available (clindamycin 10 mg/kg/day IV). Postoperative pain was managed by administering buprenorphine at a dose of 20 micrograms/kg every 8 hours for 5 days.

The decision was made to use a NPWT device. However, since the clinic did not have this equipment on-site, it took 48 hours to initiate the treatment. Due to the high exudation process, the initial bandage was changed twice more, as previously stated. The only difference was that the MGH gel was covered by a secondary dressing of polyurethane foam, which had a larger absorbent capacity to increase the interval between bandage changes. Within the first 24 hours, extensive oedema was noted in the perilesional tissue, with a colour change in the wound, which had a mostly ischemic aspect associated with areas of questionable viability (such as purple-dark-read muscle fibers and loose tendons). During pressure lavage (approximately 250 ml sterile buffered solution lactated Ringers using a pressure bag at 300 mm Hg) and bandage changes, no active haemorrhage was observed. Under sedation, the wound was irrigated with Ringer lactate and carefully dried using sterile gauze pads to prepare for the application of NPWT dressings. A silver-containing barrier dressing (Atrauman Ag 5 x 5 cm) was placed as the contact layer, and a fine-pored grey foam (VivanoMed Foam 10 x 7.5 x 3.3 cm) was precisely cut to fit the wound surface. The dressings were secured in place by two adhesive Hydrofilms that provided excellent impermeability and sealed the entire system. Due to the complex shape of the wound and its location on the lower extremity, a sandwich technique was employed to create an airtight environment by having the two Hydrofilms adhere to each other rather than to the perilesional skin.

To ensure proper functioning, the dressings were connected to a sterile canister and pump via port and plug connectors. A small window was carefully cut from the Hydrofilm covering the foam in the middle part to enable the port to contact the foam. The adhesive film covering the port was firmly sealed to maintain a closed environment and prevent any leaks.

Considering the bone exposure and the small size of the cat's paw, a continuous mode (VivanoTec Pro) with a pressure of -70 mm Hg was chosen for the NPWT system (Figure1). To prevent patient interference with the system, a covering bandage was applied using synthetic cast padding and a self-adhesive wrap.

The dressings were changed every three days for a total of three changes, allowing the NPWT to be used continuously for a period of nine days. The continuous vacuum was turned off only during bandage changes. During each dressing change, sterility was maintained, and new products were used to replace the entire system, with the only exception being the vacuum pump, which was reused.

At the conclusion of the NPWT treatment, healthy granulation tissue was observed, although moderate edema and exudative processes remained. To continue the wound healing process, a primary dressing of L-Mesitran foam, a polyurethane foam coated with a layer of MGH, was applied. To secure the dressing in place, a non-woven gauze pad and synthetic cast padding served as a secondary layer, with a self-adhesive cohesive wrap as an external layer, providing complete coverage of the lower extremity. Initially, the bandage was changed every two days, a total of three times, followed by every three days until day 49. Thereafter, the bandage was changed every 2-3 days, with L-Mesitran Soft Gel, a MGH gel, used as a contact layer, and the foam being replaced while keeping the additional layers consistent (Figure 2).

By day 61, the wound had fully healed, with continuous monitoring of limb mobility and pain since the initial injury involved metatarsal bone fractures. After the soft tissue had healed, a second surgery was performed to remove the metal implant, which was responsible for a small fistulous-like lesion with a tendency to reoccur. The cutaneous lesion was treated by applying a soft gel intralesionally. However, despite the formation of new epithelium, its integrity was compromised by the exudate produced due to the inflammatory process caused by the metal implant. At the end of the entire process (day 88), the animal had regained appropriate mobility and the limb was spared, with an extra rotation of the lower extremity.

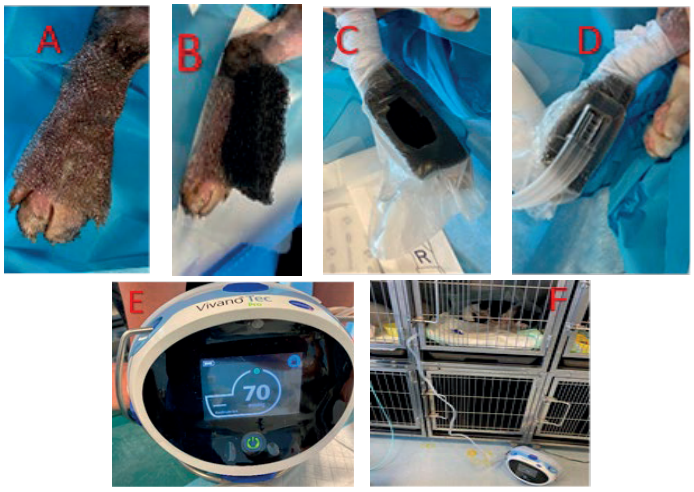

Figure 1. Negative pressure wound therapy dressing application

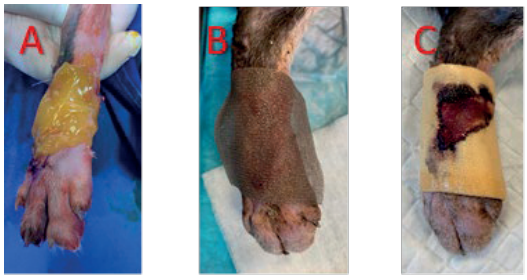

Figure 2. Dressings used for wound management
A. MGH gel; B. Silver dressing; C. MGH Foam

On day 5, the bacterial culture came back positive for *Enterococcus cecorum* and *Staphylococcus* spp., both of which were found to be resistant to Clindamycin, which had been prescribed previously. To address the bacterial infection, Clindamycin was discontinued and IV Ceftriaxone was initiated at a dose of 25 mg/kg twice daily for a total of 14 days. Throughout the entire wound healing process, the patient maintained a good appetite and did not exhibit any other symptoms. However, slight to moderate lameness was present when the cat used the bandaged limb. During the bandage change after the end of the NPWT, the cat did not require sedation and only exhibited minimal pain, which was associated with the bone lesion rather than the wound itself. No signs of pain were exhibited during wound lavage, cleaning, and redressing. After the initial course of opioid (Buprenorphine), no further targeted pain management was required. Additionally, for the first 5 days, a nonsteroidal anti-inflammatory drug (Meloxicam 0.2 mg/kg/day) was administered.

RESULTS AND DISCUSSIONS

Injuries to the lower extremities have been known to pose a challenge for small animals. When the severity of the wound is higher due to muscle, tendon, and bone injuries with bone exposure, the challenge is even greater.In addition, poor healing response in cats compared to dogs is a frequently mentioned aspect in research with exclusive feline patients, supported by specific studies such as those conducted by Bohling and his collaborators (Bohling et al., 2006; Bohling & Henderson, 2006). Furthermore, cats are more easily affected by stress and long hospitalization periods. Therefore, a method that would shorten the overall healing time, lower the manipulation frequency, and achieve good outcomes sounds promising. Although

most case reports, case series, and comparative studies conducted on cats mention large skin defects, the severity of a wound has multiple factors of determination besides the extent of injury. The initial description of this wound makes it a good candidate for NPWT. The clinical aspect of the wound is presented in Figure 3.

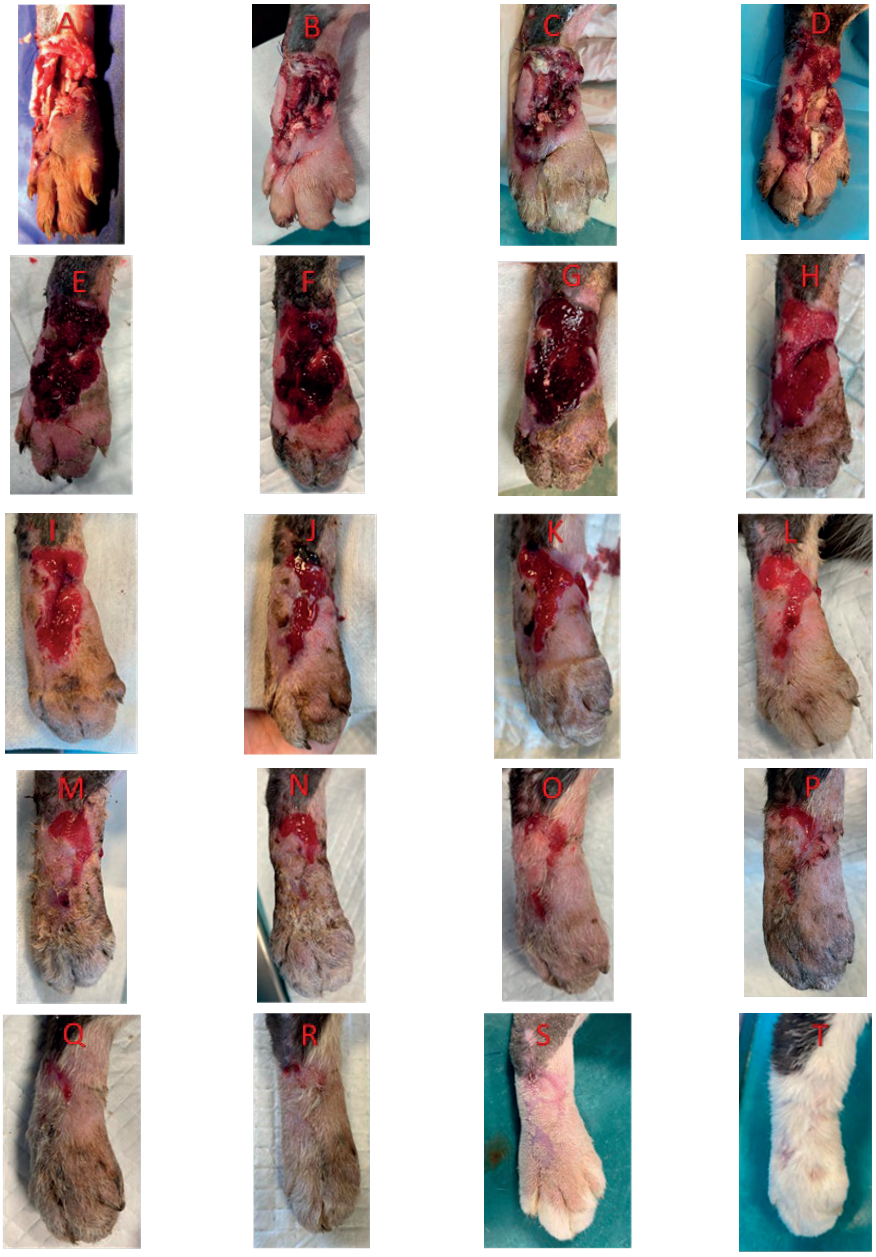

Figure 3. Wound healing timeline.
A. Day 1; B. Day 2; C. Day 4; D. Day 7; E. Day 10; F. Day 13; G. Day 16; H. Day 18;
I. Day 21; J. Day 24; K. Day 27; L. Day 34; M. Day 40; N. Day 46; O. Day 50; P. Day 53; Q. Day 58;
R. Full closure on Day 61; S. Follow-up on Day 74; T. Follow-up on Day 100.
Figure A-D: treatment with NPWT+ MGH, Figure E-R: only MGH treatment

At the conclusion of the NPWT, granulation tissue had covered almost the entire wound, reaching the skin level except for the central area. Although some edema was still present, it was significantly reduced compared to the initial presentation. The level of exudate also decreased, as depicted in Figure 4, which shows a clear difference between day 3 and day 13. The first image shows the foam dressing fully soaked with wound exudate after 12 hours, whereas the second image shows only partial saturation of the foam dressing with exudate after 48 hours.

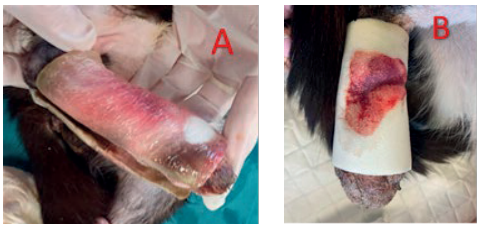

Figure 4. Exudate level. A. Day 2; B. Day 15

The main effect of the NPWT in this situation is the formation of granulation tissue, which is critical for the healing process as it covers the wound bed with healthy tissue. The increased perfusion facilitated by NPWT also plays a significant role in infection control by enhancing antibiotic delivery. Although the systemic antibiotics given are primarily responsible for infection control, previous research has suggested that the effect is potentiated by NPWT through various mechanisms. NPWT and MGH offer advantages over antibiotics, such as avoiding resistance, penetrating biofilms, and localized application. This aligns with the concept of antibiotics stewardship, aiming to reduce antibiotic use. Non-invasive or minimally invasive alternatives in wound management, like NPWT and MGH, can help achieve this goal. However, it's important to consider limitations such as the need for specialized equipment with NPWT and potential limitations of MGH against drug-resistant bacteria or severe infections requiring systemic treatment. Emphasizing the direction towards reducing antibiotic use through alternative products and systems is crucial. The dressings used in this case were according to the manufacturer's recommendations. The use of a non-adherent silver-containing barrier dressing is an additional layer not described in other protocols, justified by its ability to prevent maceration and adherence. The efficacy of silver ion-based dressings, known as antimicrobials is controversial, but when used in combination with vacuum-assisted closure as a primary layer, it has shown promising outcomes (Leaper, 2011; 2012). While using continuous vacuum for prolonged periods, granulation tissue may grow or become drawn into the grey foam, causing pain when removed and leading to minor setbacks when the tissue is disrupted during bandage changes. In this case, this aspect was observed during the last bandage change when the granulation tissue also developed a purple-dark red color. This color change may be associated with the high negative pressure used, but since the value was already considerably lower than pressure values in other studies, it was left unchanged.

Many publications provide guidelines for the duration and pressure values used in NPWT. However, there is still no consensus regarding the optimal parameters for different species, body locations, and specific characteristics. In this case, a value of -70 mm Hg was used, as advised by the manufacturer who had previous experience just with human subjects, taking into consideration the bone exposure and the small diameter of the lower extremity. The use of -70 mm Hg led to satisfactory results in promoting granulation tissue formation and controlling the infection after three dressing changes. These outcomes are consistent with previous literature and highlight the importance of individualizing treatment based on the patient's needs and response to therapy. Two approaches are available for lower extremity injuries after obtaining granulation tissue: skin grafting and secondary intention healing. The decision depends on factors such as the wound's extent and depth, presence of infection or inflammation, and patient's health. Skin grafting is preferred for faster and complete closure, but it is costly and invasive compared to secondary intention healing, which allows natural wound healing. In this case, secondary intention healing was chosen due to incomplete granulation tissue coverage, metatarsal instability, and potential complications with skin grafting caused by inflammation and exudate production.

The use of MGH products in wound management is a well-established practice, supported by ample research in both human and veterinary medicine, demonstrating the positive effects of MGH dressings on acute and chronic wounds (Aziz & Abdul Rasool Hassan, 2017; Lukanc et al., 2018; Mandel et al., 2019; Peteoaca et al., 2019; Pleeging et al., 2022a; Pleeging et al., 2022b; Vogt et al., 2021; Yilmaz & Aygin, 2020). Studies have reported that combining MGH with vitamins C and E, such as in L-Mesitran Soft, offers higher antimicrobial activity (Bocoum et al., 2023; Nair et al., 2020; Smaropoulos & Cremers, 2020). However, in highly exudative wounds, MGH alone may not meet all requirements for an ideal healing environment. While MGH is known to draw fluid out and reduce edema as a hyperosmotic product, it does not provide the best absorbent properties.

Polyurethane foam dressings are a good option for managing large amounts of exudate. Commercially available products come in a variety of combinations, such as silicon and hydrogel layers or adhesive margins covered by hydrofilms. One newer option is polyurethane foam coated with a MGH gel, such as L-Mesitran Foam (Mthanti et al., 2022). This product provides the benefits of MGH's healing properties while offering an absorbent layer that can maintain an optimal moisture level. Moist wound therapy is currently the main direction of wound healing guidelines. Using foam dressings reduces the frequency of bandage changes and provides a pain-free removal experience with no disruption of granulation tissue.

Combining L-Mesitran Soft gel with L-Mesitran Foam can be an adequate option for various types of wounds depending on the healing phase and wound characteristics. By preserving the antimicrobial and neovascular-rization-promoting properties of MGH and offering proper exudate control, this combination provides a competitive dressing. Additionally, having a versatile product line like this can significantly lower the number of dressing types needed in stock, providing a great advantage.

To achieve a more accurate assessment of the wound size at specific time points, the ImageJ analyzer software was utilized (Schneider et al., 2012). The measurements were calibrated using different markers in the image, and the area of the wound was calculated by tracking its contour. Although the wounds were measured in triplicates, it should be noted that small errors may be associated with the wound contouring performed by the human user. Furthermore, the three-dimensional aspect of the wound, such as its depth, was not taken into account, which may have introduced some bias in the measurements.

To obtain a more comprehensive evaluation of the wound healing process, consideration of other factors beyond the wound area, such as granulation tissue formation rate and quality, control of infection, exudate level, and other factors that may be more difficult to quantify, is essential. This approach could yield a more accurate overall healing score. Nonetheless, even without a depth correction factor, a visible decrease in the wound area was observed during the treatment until it achieved full closure. The results presented in Table 1 indicate that after 16 days, the wound size had reduced by almost half of its initial size and remained under one-quarter of its original size at day 34 (18%).

Table 1. Wound surface area corelated with the healing timeline

Day (healing timeline)	Area in cm	Percentage from initial area	Treatment
Day 1	4.274		Before NPWT
Day 4	4.978	100%	Start of NPWT
Day 7	4.305	86.48%	-
Day 10	3.518	70.67%	-
Day 13	3.467	69.646%	End of NPWT
Day 16	2.773	55.70%	MGH
Day 21	2.015	40.47%	MGH
Day 34	0.914	18.36%	MGH
Day 46	0.638	12.81%	MGH
Day 53	0.259	5.20%	MGH
Day 58	0.032	0.642%	MGH
Day 61	0.001	0.0200%	Healing complete

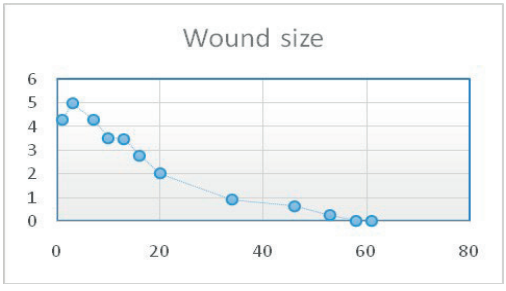

Figure 5. Wound size in cm^2 correlated to the healing timeline

These results are comparable to other similar cases and are considered satisfactory (Fehr et al., 2015; Nolff & Meyer-Lindenberg, 2014; Nolff et al., 2015; 2016; Pitt & Stanley, 2014).

At the one-year follow-up, a subtle scar was observed without any recurrence. The scar was mostly covered by hair, but there was a reduced range of motion in the metatarsal-phalangeal joint with a slight external rotation of the lower extremity. This case report cannot prove the superiority of the techniques used, but the results presented are consistent with recent literature regarding its efficacy.

Clinical studies on spontaneous injuries, rather than experimental wounding, present many biases and challenges in standardization. These challenges include unique patient characteristics, etiology, adjunctive treatments (such as orthopedic repairs, systemic antibiotics, medication for concurrent pathologies), the presence of infection and the type of pathogen present, time passed since the wounding, previous treatments, wound location, animal temper, species, and many others.

Although the standard of care often dictates surgical reconstruction as the optimal closure after healthy tissue formation, it is not always the best route for all patients, as previously presented. Open wound management remains a good alternative if an optimal healing environment is provided via interactive wound dressings.

CONCLUSIONS

Injuries to small animals' lower extremities with bone exposure pose a challenge, especially for cats, which have a poor healing response and are easily stressed by long hospital stays. NPWT can reduce healing time and manipulation frequency, as demonstrated in this case study. NPWT promotes granulation tissue formation and controls infection, although the optimal parameters for different species, body locations, and specific characteristics are not yet clear. The decision to use skin grafting or secondary intention healing after obtaining granulation tissue depends on the wound's extent, depth, the patient's health, and costs. MGH products and polyurethane foam dressings are viable options for wound management, and combining them may offer better outcomes. The use of NPWT and MGH may be an adequate protocol for challenging wounds, such as the one presented in this case report.

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CANINE RHABDOMYOSARCOMA - LITERATURE REVIEW

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Abstract

Rhabdomyosarcoma (RMS) is a rare malignant neoplasm arising from skeletal muscle, occurring predominately in young individuals. In dogs, is most commonly located in the urogenital tract, followed by head, neck, face, limbs and skin, mammary gland included. This article reviews the microscopic patterns, diagnostic and prognostic aspects of RMS in dogs. In veterinary medicine, the classification of RMS into subtypes is based only on histologic characteristics, with no relevance in regard of prognosis. The prognosis depends on the severity and extent of invasiveness, as well as the presence of metastases. Macroscopic aspects are variable, as well as cellular morphology and histological patterns. Immunohistochemistry is used to confirm the diagnosis, RMS being positive for vimentin, desmin, muscle and sarcomeric actin, myoglobin, myogenin and negative for cytokeratin and α-smooth muscle actin. Further investigations are needed to better understand the biological behaviour and outcomes of this tumour.

Key words*: rhabdomyosarcoma, canine, immunohistochemistry, prognosis, histopathology.*

INTRODUCTION

Rhabdomyosarcoma (RMS) is a rare malignant neoplasm arising from skeletal muscle, occurring predominately in young individuals. Due to its rarity and highly variable macroscopic and microscopic patterns, it is considered a diagnostic challenge in both human and veterinary medicine (Caserto, 2013).

Human RMS is diagnosed by various techniques, such as histopathology, immune-histochemistry (IHC), electron microscopy and molecular techniques (Watchtel et al., 2006; Caserto, 2013), the first two being the most commonly used in veterinary medicine. Although in humans the prognosis is established by different histologic patterns of RMS, the prognostic significance of RMS subtypes in dogs still remains undetermined (Tuohy et al., 2021). In veterinary medicine, rhabdomyosarcomas are categorised as embryonal, botryoid, alveolar and pleomorphic. The purpose of this paper it to review the cytological and histological aspects of rhabdomyosarcoma in dogs, with emphasis on current diagnostic and prognostic aspects of this tumour. Being rarely diagnosed in veterinary medicine and having a vast morphology, we present its main characteristics, for a better understanding and approach for both veterinary clinicians and pathologists.

To review the literature describing RMAs in canine patients, we collected the necessary material from the current database, using the following key-words: rhabdomyosarcoma, canine, case report and literature review, immunohistochemistry, prognosis. Selected articles were chosen based on addressing the most important diagnostic and prognostic features of this tumour, referring primarily to dogs and often with comparison to human medicine for a better understanding of the process. The provided information was compared with available data in the reference books of veterinary pathology and oncology. Often, comparison with human rhabdomyo-sarcoma is provided, for a better understanding of the subjects. The common points and important differences which have resulted from this analysis are presented in the following sections.

BIOLOGY, AND CURRENT DIAGNOSTIC AND PROGNOSTIC ASPECTS OF RMS

Origins of RMS

Rhabdomyosarcoma is a primitive malignant soft tissue sarcoma of skeletal muscle

phenotype. Its etiology and risk factors remain largely unknown in both human and veterinary medicine; it originates from a primitive mesenchymal cell (Leiner, 2020).

Currently, very few aspects related to the origin of RMS are known in domestic animals. In human medicine literature, there are different hypotheses regarding the histogenesis of rhabdomyomas/rhabdomyosarcomas in all species.

Some authors suggest it may develop from pluripotent stem cells from the primitive urogenital ridge remnants, suggestive of botryoid rhabdomyosarcoma.

Another possibility would be the development from the mesenchymal progenitor cells invading the Müllerian and Wolffian ducts. This process is described as being either local or through the circulation coming from the bone marrow. Mesenchymal stem cells capable of myogenic differentiation identified in the bone marrow and in other locations could explain why rhabdomyosarcomas can be found in tissues which don't have skeletal muscle (Hettmer, 2010).

Clinical aspects of RMS

Canine RMS is reported to appear mainly in young dogs, of 2 years or younger. Taking into account the age of all dogs diagnosed with rhabdomyosarcoma from the total cases reported so far, over 80% were under 10 years old of age (Caserto, 2013; Murakami et al., 2010). Most affected breeds reported in the literature are Saint Bernards and Retrievers (Gerber et al., 2009; Bae et al., 2007). Different subtypes of RMS are reported in adult or old dogs (Brockus et al., 2004, McDonald et al., 2017, Avallone et al., 2010; Dagher et al., 2017). This may be an indicator that age predilection in dogs is not as accurate as in people, where RMS occurs in children younger than 15 years (Hettmer, S. and Wagers, A.J., 2010). Caserto mentions in his review from 2013 that in dogs, the most common location for RMS is in the urogenital tract (49%). Following this type of location are the head, neck and face (37%), with limbs (8%) and skin, mammary gland included (3%) being the less common.

Cases of orbital RMS have been described recently (McDonald et al., 2017; Scott et al., 2016), but remain rarely reported in dogs. Da Roza et al. (2010) identified in an 11-month-old male boxer dog an uncommon spindle cell variant of RMS that affected the frontal region of the skull.

A primary meningeal spindle cell variant of rhabdomyosarcoma has been described for the first time within the T9 - T11 spinal cord of a 7-week-old male black Labrador retriever, who also presented many cutaneous neurofibromas (Hoon-Hanks et al., 2018). This case marks a novel differential diagnosis for spinal tumours, especially in young dogs.

Mammary RMA are rare in dogs. Dagher et al. (2017) described for the first time a mammary localisation of this type of tumour with pulmonary metastasis in a 10-year-old female mixed-breed dog. The diagnosis was established by histology, IHC and electron microscopy. Unique for both the type of tumour and localisation, the authors describe areas where large lobules and trabeculae of cartilage and immature bone were present among the neoplastic cells.

Laryngeal RMS has been described only in dogs and in the vast majority of the cases, in those over 2 years of age (Cooper, 2017; Yamate, 2011).

On gross examination, RMS is usually described as solitary mass. Gombert et al. (2020) reported in a five-year-old male Labrador Retriever two concurrent embryonal rhabdomyosarcomas, located in the oesophageal and perilaryngeal regions. The first oesophageal RMS case was reported in a 15-month-old great Dane dog and characterised as embryonal type RMS (Devriendt et al., 2017).

Cutaneous RMS are rarely reported. One of the main causes for the small number of cases could be misdiagnosis as poorly differentiated soft tissue sarcoma or anaplastic neoplasias in absence of immunohistochemical investigations (Caserto, 2013; Avallone et al., 2010).

A first report of a cutaneous multifocal form of alveolar type RMS in veterinary medicine has been described in an 8.5-month-old Labrador Retriever. The animal presented a cutaneous mass in the right maxillofacial region and swelling of the right maxillary gingiva. Based on cell morphology, this case shows that alveolar RMS could be included in the

differential diagnosis of cutaneous round cell tumours in dogs (Otrocka-Domagala et al., 2015).

Cardiac RMS is reported in the literature among malignant cardiac muscle tumors. The few cases reported up to present were in dogs of 7-year-old. Right atrium and right ventricle appear to be the most frequently involved (Treggiari et al., 2017; Perez et al., 1998). Akkoç et al. (2006) reported the first case of cardiac metastasized rhabdomyosarcoma in a 7-year-old great Dane, with multiple metastases in the heart, lungs, diaphragm, liver, kidney and omentum, thus confirming that this type of tumour with this localization has the potential to metastasize.

Taking into account the complexity of the diagnosis and the need to make a differential diagnosis with mesenchymal tumours with round and fusiform cells, in the recent literature a third situation appears, when myoid differentiation may morphologically resemble rhabdomyosarcoma. Recently, a case of unusual myoid differentiation has been reported in a 13-year-old crossbreed female dog diagnosed with benign mixed mammary tumour (Brunetti et al., 2021). In this case, the authors describe the mesenchymal components identified in the tumour (smooth and striated muscle, cartilage and bone) as well-differentiated, with no signs of cellular atypia, therefore of metaplastic origin. On immunohistochemistry, the mesenchymal cells were positive for all the markers of the IHC panel for rhabdomyosarcoma. The cell morphology and the benign biological behaviour ruled out a possible malignant neoplasia of striated or smooth muscle origin. This case is similar to the of Dagher et al. (2017), where the mammary tumour had areas of cartilaginous and osseous metaplasia, but the cell atypias, IHC results and presence of pulmonary metastasis confirmed the presence of a RMS.

Cytology of RMS

Cytological examination proved not reliable in diagnosing RMS in dogs, because of the vast appearance of neoplastic cells, from mature myoblasts and rhabdomyoblasts to undifferentiated round cells (Tuohy et al., 2021). Cytologically, smears from both rhabdomyomas and rhabdomyosarcomas consist of individualized, round to polygonal cells with low nuclear-to-cytoplasmic (N:C) ratio. Neoplastic cells have large amount of eosinophilic to sometimes basophilic granular cytoplasm. Cells with a high N:C ratio and indistinct cytoplasm can also typically be encountered.

In order to distinguish between a rhabdomyoma and a rhabdomyosarcoma on cytology, sufficient criterias of malignancy should be present, most of the time a correct diagnosis being difficult. RMS in most cases display increased pleomorphism, bizarre mitotic figures and marked anisocytosis and anisokaryosis (Valenciano, 2020).

Embryonal form of RMS consists of three variants, distinguished also in cytology based on a predominant cell morphology: the myotubular variant (characterised by the presence of multinucleated elongated tubular cells), the rhabdomyoblastic variant (characterised by presence of large round cells containing abundant cytoplasm) and the spindle cell variant (characterised by fusiform, elongated cells placed in streams). It is typical for embryonal RMS to see all three variants together, making the cytological diagnosis of thsese tumours very difficult. Very typical but uncommon, the multinucleated cells have nuclei arranged in a row (*straplike cells*). Rarely, striations could be observed within the cytoplasm. These multinucleated cells result from the fusion of rhabdomyoblasts. The rhabdomyoblasts typically appear as round cells and display high to moderate N:C ratios (Raskin et al., 2023).

The alveolar RMS is cytologically characterized by highly cellular smears. Numerous atypical round cells are identified. These cells are similar to lymphoid cells. Mitotc figures are also frequent (Raskin et al., 2023).

In a case report of an alveolar RMS in a 7-month-old Labrador Retriever, Snyder et al. (2011) describe the cytological appearance of this tumour. The smears contained numerous round/oval cells, had variably distinct cell margins and basophilic cytoplasm. The anisocytosis and anisokaryosis were reported as moderate. Interestingly, on the backround there were many tiny basophilic cytoplasmic fragments.

Pleomorphic RMS exhibits plump splindle cells haphazardly arranged. The tumoral cells display marked anisocytosis and anisokaryosis. The mitotic figures are described as bizarre (Raskin et al., 2023).

Histology and patterns of RMS

In veterinary medicine, four variants of rhabdomyosarcoma are described - embryonal, botryoid, alveolar and pleomorphic.

According to data collected from case reports in veterinary medicine, botryoid RMS is the most diagnosed subtype, followed by embryonal, alveolar and least frequently, the pleomorphic subtype (Gombert et al., 2020).

Embryonal RMS includes three variants, described previously on cytological patterns: myotubular, rhabdomyoblastic and spindyloid. Histology of myotubular variant consists of presence of characteristic multinucleated "strap cells", which form myutubes (Caserto, 2013). The myotubes frequently contain cross-striations which can only be identified using special staining (phosphotungstic acid hematoxylin). On this staining, the striations appear dark blue or purple, while the myofibers appear as paler blue or purple.

The rhabdomyoblastic variant consist on histology of frequent round to polygonal cells with abundant eosinophilic cytoplasm. Striations are rarely positive for phosphotungstic acid hematoxylin staining, being a main feature in differentiating the two variants of embryonal RMS (Parham, 2001). In human medicine, this variant is difficult to diagnose and commonly needs molecular biology which is unavailable for canine rhabdomyosarcoma. No prognostic significance of these two variants has been demonstrated in either human or canine RMS (Tobar et al., 2000).

The spindle-cell variant of RMS is rare and a relatively new category. Histological aspect consists of thin spindyloid myoblast cells, usually with formation of bundles and myxoid stroma (Cooper, 2017). It was reported only three times in veterinary medicine: mass arising from the skull of an 11-month-old Boxer dog (da Roza et al., 2010), meningeal mass in a 7-week-old Labrador Retriever (Hoon-Hanks et al., 2018) and left hindlimb mass in a 3.5-year-old Bulldog (Shi et al., 2023). In human medicine it is considered the variant with less aggressiveness (Caroll, 2013).

Botryoid RMS is considered a variant of embryonal RMS in both human and veterinary medicine. Macroscopically, it appears as a polypoid, grape-like mass and is encountered most commonly in the urinary bladder, where it can be seen protruding from the mucosa. Histological examination reveals many undifferentiated rhabdomyoblasts and/or strap cells suspended in a myxoid matrix, these being characteristic (Kobayashi et al., 2004). The densely cellular *cambrium layer* located beneath the mucosa is considered a strong diagnostic feature (Caserto, 2013).

Alveolar RMS is histologically subdivided in classic and solid variants.

The classic variant is characterized by aggregates of small, poorly differentiated, round cells. The cells have scant cytoplasm and are supported by a fibrovascular stroma. The tendency of centrally located cells to become degenerated and get separated one from each other due to a poor cohesivity gives shape to the so called "alveolar pattern". Differentiated rhabdomyoblasts are uncommon, with cross-striations rarely present (Cooper, 2017). The solid variant in dogs consists of sheets of small round neoplastic cells divided by thin fibrous septa. This pattern is not always present, making the histologic architecture similar to rhabdomyoblastic embryonal RMS, thus the diagnosis is very difficult. Molecular genetic analysis has been proven efficient in this matter (Caserto, 2013).

Kimura et al. (2013) report a gingival alveolar RMS in a 3-year-old Shih Tzu, composed of anaplastic cells arranged in typical alveolar pattern, numerous mitotic figures and multinucleated cells. The cross-striations and glycogen accumulation were absent.

Pleomorphic RMS marks the least common variant in human medicine (Parham, 2001). In dogs, like in humans, is diagnosed typically in adults and is extremely rare in young patients. The tumour rises almost exclusively within skeletal muscle of the limbs. Histologically, the architecture consists of exclusively spindle cells with abundant eosinophilic cytoplasm, arranged in a haphazard manner. Rare multinucleated cells may be present. The bizarre or multipolar mitotic figures, lack of

any embryonal or alveolar pattern, general lack of cross-striations and the high degree of pleomorphism are the most characteristic features of this tumour. Glycogen content of the neoplastic cells is commonly seen. The accurate diagnosis always needs immunohistochemistry for confirmation (Cooper, 2017; Caserto, 2013). Yamate et al. (2011) describe a low-grade pleomorphic RMS located in the larynx of a 6-year-old dog.

Unclassified RMS (RMS NOS) has been noted in various articles, as its complex morphological patterns could not fall into a specific histologic subtype/variant (Caserto, 2013).

Immunohistochemistry of RMS

IHC is the preferred diagnostic technique for confirmation canine rhabdomyosarcoma.

Commonly used immunohistochemical indicators are characterised by positive labeling with vimentin, desmin and myoglobin, and negative labeling for α-smooth muscle actin (α-SMA).

A panel of antibodies is strongly recommended for IHC characterization of these tumors, as various antigens are expressed at different times during cell development. Vimentin, desmin and sarcomeric actin are expressed early, but later vimentin is lost during the muscle fibers development. Myoglobin is expressed later than desmin (Cooper, 2017).

Vimentin is a type III intermediate filament protein. Its expression in IHC demonstrates the mesenchymal origin of the neoplastic cells, making it indispensable in the IHC panel of RMS.

Desmin has also been proven useful in human and veterinary medicine, especially when it comes to prove the myogenic differentiation in alveolar or embryonal RMS. It is not a marker exclusively only to skeletal muscle, being common also in cardiac and smooth muscle, as well as in myofibroblasts. It may raise difficulties in the diagnostic procedure due to its high degree of variability in regard to the staining process and low specificity. Because of these matters, newer methods use myoblast determination protein 1 (MyoD1) and myogenin to identify undifferentiated myoblasts. Myogenin and MyoD1 are early embryological transcription factors. These proteins are involved in the differentiation of mesodermal cells into myoblast cells and also proliferation and differentiation of myoblast cells into multinucleated myotubes (Caserto, 2013).

Immature rhabdomyoblasts with high proliferative capacity will express MyoD1. In human medicine, undifferentiated RMS have been shown to express more MyoD1 and myogenin and less actin, myoglobin, myosin and desmin (Sebire, 2003).

Studies show that diffuse expression of myogenin in the nuclei of tumour cells could indicate an alveolar or embryonal RMS (uniform expression for alveolar, heterogenous for embryonal), while for myogenin there is insufficient data to conclude this characteristic. Also, human embryonal RMS exhibits few cells with expression of either myogenin or MyoD1 (Caserto, 2013). However, absence of immunostaining with MyoD and myogenin doesn't exclude a myogenic origin. The expression depends on the degree of differentiation (Kobayashi et al., 2004).

There is no exact correlation between human and canine RMS using myogenin or MyoD1 immunohistochemical staining, as the use of these antibodies is new and rare in veterinary medicine (Tuohy, 2021).

Kobayashi et al. (2004) described the immunohistochemical pattern of a canine botryoid RMS as myogenin staining strap cells preferentially and MyoD1 being limited to the nuclei of numerous small and round myoblast cells, while other studies didn't find such connection between cellular component and staining. Similar to humans, muscle actin antibody can identify α-actin isoforms in all three types of muscles. More specific to skeletal muscle is the expression of α-actin isoform, which can be identified by the sarcomeric actin antibody.

Cases of RMS reported in dogs are consistently immunohistochemically positive using for vimentin, muscle actin, sarcomeric actin and desmin. The lack of cellular differentiation of RMS makes myoglobin expression variable. In dogs, both embryonal and botryoid rhabdomyosarcomas are commonly positive for myoglobin, as the large rhabdomyoblasts or strap cells show immunopositivity (Kobayashi et al., 2004).

Prognosis of RMS

A prognosis and accurate diagnosis of canine RMS are difficult to reach in veterinary medicine due to the rarity and often misdiagnosis of this type of tumour. Frequently, they fall under the high-grade soft tissue sarcoma category (Avallone et al., 2010). Absence of follow-up / survival data, election of euthanasia, lack of post-mortem examination and unknown presence of possible metastasis make prognostic value of canine RMS still a challenge for veterinary specialists.

Data gathered up to present in veterinary medicine show that the most aggressive rhabdomyosarcomas are the alveolar and embryonal variants. Very young dogs, under 2 years of age, develop the most aggressive behaviour. Subclassification into the rhabdomyoblastic or myotubular variant in case of embryonal RMS has no prognostic significance (Caserto, 2013; Wachtel et al., 2006).

In humans, clear differentiation between alveolar and embryonal RMS is very important, as the alveolar variant is more locally aggressive, with a higher metastatic rate, thus bearing a poorer prognosis. In veterinary medicine, this prognostic significance is still unavailable, due to lack of sufficient data. Based on the small number of cases reported in dogs, metastatic rate seemed to be the highest in unclassified RMS, followed closely by embryonal and alveolar types, bothryoid RMS reportedly having the lowest rate and better prognosis (Caserto, 2013; Shi et al., 2023).

At present, in canine rhabdomyosarcoma, the extent and severity of invasiveness and the presence of metastasis are used to establish the prognosis (Caserto, 2013).

CONCLUSIONS

In veterinary medicine, histopathological and immunohistochemical examinations are crucial in diagnosing rhabdomyosarcomas and excluding other types of neoplasia.

In dogs, the prognosis depends on the severity and extent of invasiveness, as well as the presence of metastases, morphological subtypes having no prognostic value.

The vast variability of this tumour, the lack of prognostic significance of its histologic subtypes and the small number of cases reported in animals makes RMS a challenge for both pathologists and clinicians.

Further investigations are needed to better understand the biological behaviour and outcomes of this tumour and to reach an effective way to classify and characterise them.

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SPECIFIC THERAPEUTIC MANAGEMENT IMPLICATIONS IN NEONATAL LAMBS MORTALITY

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Abstract

Curative interventions, expensive and long, are often followed by clinical recovery, but with compromise and reduction of weight dynamics and productive growth. In this context, the precocity of the therapeutic approach, together with the consistency and dynamic modulation of the treatment of peri- and neonatal diarrhea syndromes in lambs conditions the recovery of such affected patients (with minimal productive consequences), contributes to ensuring the well-being of the animals and reducing the risk of inducing antibiotic resistance. The research took place between 2022-2023 on 196 lambs of different breed and age, within the county of Tulcea, the locality of Baia. The animals included in the study were divided into 2 groups, namely study group 1 consisting of 98 lambs in which the diagnosis was established clinically and study group 2 consisting of 98 lambs that benefited from a definite etiological diagnosis (rapid Rainbow test Bio K) along with an etiologically oriented and specific therapeutic protocol. The percentage analysis of the mortality rate in the diarrheal syndrome in lambs shows an increased value in lambs treated with non-specific medication compared to lambs in which the therapeutic protocol approached was of an etiological nature where the percentage value is reduced. The calculated productive difference in body weight between etiologically diagnosed and related treated group 4 versus group 1 without etiotropic approach resulted in a 15% greater weight gain at day 60 in the group 4 member compared to the group 1 member.

Key words: *lambs, mortality, therapy.*

INTRODUCTION

In the intensive breeding of farm animals, the peri- and neonatal period is a critical window of maximum vulnerability due to the functional lack of an own immunological system and the dependence on passive immunity - acquired through the absorption of immunoglobulins from colostrum, in the first 24 hours of life.

Curative interventions, expensive and long, are often followed by clinical recovery, but with compromise and reduction of weight dynamics and productive growth. In this context, the precocity of the therapeutic approach, together with the consistency and dynamic modulation of the treatment of peri- and neonatal diarrhea syndromes in young sheep conditions the recovery of such affected patients (with minimal productive consequences), contributes to ensuring the well-being of the animals and reducing the risk of inducing antibiotic resistance.

MATERIALS AND METHODS

The research took place between 2022-2023 on 196 lambs of different breed and age, within the county of Tulcea, the locality of Baia.

The animals included in the study were divided into 2 groups, namely study group 1 consisting of 98 lambs in which the diagnosis was established clinically (inappetence, anorexia, moderate dehydration, hair loss, prolonged sternal recumbency and inert to environmental stimuli, all patients presented enteric syndrome, diarrhea with a pasty-fluid consistency, ihorous odor and reduced quantity) (Figure 1) in the absence of additional diagnostic methods, and the treatment implemented was non-specific (Table 1) (Enrofloxacin single dose of 7 mg/kg orally/ Amoxicillin dose of 10 mg/kg GC every 24 hours, 3 days, Buscopan compositum 0.15-0.2 ml intramuscularly, per os rehydration with a solution composed of 1l water, 7 g NaCl, 1.5 g KCl, 0.5 g $CaCl_2$, with 150 ml/individual)

and the group of study 2 composed of 98 lambs that benefited from an etiological diagnosis of certainty (Rainbow Bio K rapid test) (Figure 2) alongside an etiologically oriented and specific therapeutic protocol (Table 2) - Paromomycin dose of 100 mg/kg GC, per os, administration per day, for 11 days, to 50 individuals and Ceftiofur to 28 lambs and Halofuginone to 20 animals, with a classification based on the therapy carried out (6 batches).

Figure 1. Lamb diarrhea Syndrome

Figure 2. Rainbow rapid test

Table 1. Non-specific therapy in group 1 lambs

Batch 1 n=34	Batch 2 n=28	Batch 3 n=36
Enrofloxacin	Amoxicillin	Enrofloxacin

Table 2. Specific therapy in group 2 lambs

Batch 4 n=30	Batch 5 n=28	Batch 6 n=40
Paromomycin	Ceftiofur	Halofuginone

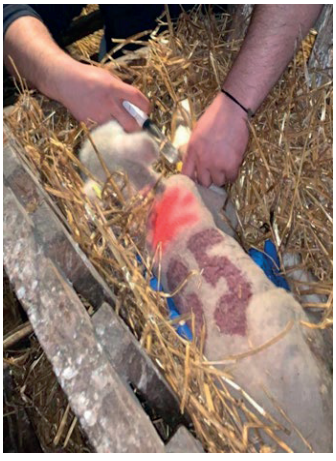


Figure 5. Specific teraphy administration in lambs

RESULTS AND DISCUSSIONS

Percentage analysis of the mortality rate in the diarrheal syndrome in lambs shows an increased value in lambs treated with non-specific medication compared to lambs in which the therapeutic protocol addressed was of an etiological nature where the percentage value is reduced.

The clinical approach of the first three batches proved that the establishment of a non-specific symptomatic therapy is not judicious, which, in addition to the failure to correct the disease, risks creating favorable conditions for the creation of chemotherapy resistance or antibiotic resistance. The result being an unsatisfactory one, which brings unjustified expenses to the operation.

Table 3. Mortality rate in group 1

Batch 1	Batch 2	Batch 3
50%	50%	40%

Diagnosing the etiological agent through paraclinical examinations, in a specialized laboratory, provides clear and certain information, however, the average term of 5-10 days imposed for receiving the result, helps us to form an idea of the characteristics of the microbiome of the farm more than to quickly establish an etiologically related treatment.

On the other hand, the diagnosis by the method of rapid antigen coupling tests from feces is a simple, pragmatic and easy method to perform in the field (Figures 3, 4).

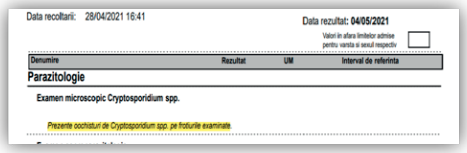

Data recoltarii: 28/04/2021 16:41 Data rezultat: 04/05/2021
Valori in afara limitelor admise pentru varsta si sexul respectiv
Denumire Rezultat UM Interval de referinta
Parazitologie
Examen microscopic Cryptosporidium spp.
Prezente ochisturi de Cryptosporidium spp. pe frotiurile examinate.

Figure 3. Test pozitiv - *Cryptosporidium* spp.

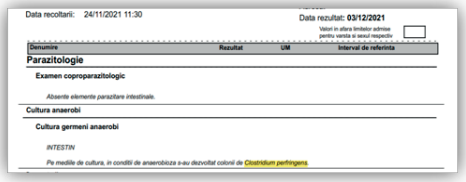

Data recoltarii: 24/11/2021 11:30 Data rezultat: 03/12/2021
Valori in afara limitelor admise pentru varsta si sexul respectiv
Denumire Rezultat UM Interval de referinta
Parazitologie
Examen coproparazitologic
Absente elemente parazitare intestinale.
Cultura anaerobi
Cultura germeni anaerobi
INTESTIN
Pe mediile de cultura, in conditii de anaerobioza s-au dezvoltat colonii de Clostridium perfringens.

Figure 4. Test pozitiv - *Clostridium perfringens*.

With a high sensitivity and sensitivity, it shortens the response time and helps to establish an optimized treatment scheme in correlation with the etiological agent.
Plus the value that the etiological diagnosis brings in the choice of treatment, is proven by the differences in mortality between groups 1, 2, 3 (approximately 50%) and groups 4, 5, 6 (approximately 10%). The fatality rate dropped by up to 80%. Mortality rates by batch are found in the Tables 3 and 4.

Table 4. Mortality rate in group 2

Batch 4	Batch 5	Batch 6
11%	12.5%	38.3%

The evolution of weight during the pediatric period represents both an individual and a group factor that highlights the well-being of the animal (as well as hygienic-dietary conditions, along with the absence of infectious pathologies. So, in order to observe the evolution of individuals from the same environment, we resorted when measuring their weights. Individuals' weight is similar at the start of the study (Figure 6, Tables 5 and 6).

Figure 6. Weight determination in a lamb

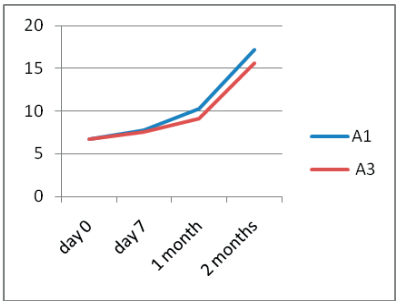
Table 5. Evolution of body weight in group 1 (kg)

Body weight	Batch 1		Batch 2	
	M1	M2	M3	M4
day 0	6.3	6.1	6	6.1
day 7	7.4	7.1	7.2	7
1 month	9.2	9	8.6	8.4
2 months	15.4	15.2	14.8	14.9

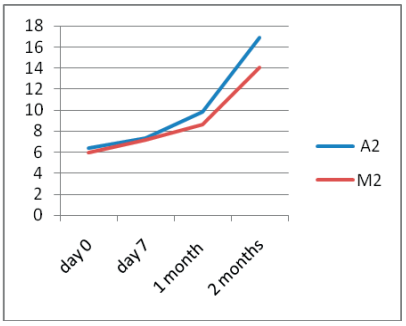
The calculated productive difference (Graphics 1 and 2) in body weight between etiologically diagnosed and related treated group 4 versus group 1 without etiotropic approach resulted in a 15% greater weight gain at day 60 in the group 4 member compared to the group 1 member.

Table 6. Evolution of body weight in group 2 (kg)

Body weight	Batch 4		Batch 5	
	6.7	6.4	6.7	6.4
day 0	7.8	7.3	7.6	7.2
day 7	10.25	9.8	9.1	8.6
1 month	17.15	16.85	15.64	15.34
2 months	6.7	6.4	6.7	6.4



Graphic 1. Batch 4-batch 5 productive differences



Graphic 2. Batch 4-batch 1 productive differences

CONCLUSIONS

The approach of a correlated etiological treatment, after the identification of the primary pathogen responsible and the implementation of a modular therapy, individually adapted, allowed the reduction of mortality in patients with peri- and neonatal diarrhea syndrome in young sheep.
The use of etiotropic treatment schemes induced a decrease in the percentage of mortality in the herd by 75% and an increase in weight at 60 days by 15%, comparing the results of batch 1 and 4.

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DIAGNOSTIC APPROACH TO BRAINSTEM DYSFUNCTION IN DOGS AND CATS - A CASE SERIES REPORT

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Abstract

Neurological pathology has known a marked expansion in recent years in veterinary practice in Romania, the diversity and complexity of cases representing a constant challenge for clinicians. Consequently, the diagnostic methodology was in a continuous dynamic, being influenced by the particularities of each patient and the accuracy of the available diagnostic methods. However, identifying the localisation of the lesion according to the correspondence of the neurological deficits with the functional segment of the brain (forebrain, brainstem, cerebellum, vestibular apparatus) remained an essential stage. Decerebrate rigidity, a comatose mental status accompanied by a decrease in the activity of the vital centres, and multiple deficits of the cranial nerves are cardinal signs of a brain stem lesion. This paper aims to present the clinical, neurological, and imaging features of 15 patients (dogs and cats) diagnosed with brainstem deficits in the Faculty of Veterinary Medicine of Bucharest, in 2021. Each case was conducted according to a standard protocol and the results were analysed to observe the population dynamics and possible predisposing factors.

Key words*: neurological examination, brainstem, decerebrate rigidity, nervous system, MRI.*

INTRODUCTION

Brainstem dysfunctions resulting from trauma, anomaly or central extension of infections are life-threatening conditions, which have been rarely described in the scientific articles related to the neuropathology of domestic carnivores (Sturges et al., 2016). An explanation for the absence of this data could be given by the fact that, in most cases, lesions that affect the brainstem are associated with a significant rate of mortality, which prevents the dynamic study of patients and a gap in relevant analytical data.
From a clinical point of view, brainstem diseases are expressed in the form of a syndrome characterized by the presence of a markedly depressed mental status (stupor or coma), a decerebrate posture (opisthotonos and extension of all four limbs), ipsilateral hemiparesis/hemiplegia or tetraparesis/tetraplegia, several cranial nerves (CN) deficits, delayed proprioception responses that could affect all limbs or only the ipsilateral limbs, with normal or exaggerated spinal reflexes (associated with muscle hypertonicity). Lesions affecting the cranial nerves or their nuclei can manifest in different clinical forms, such as ventrolateral strabismus, bilateral non-reactive mydriasis and ptosis of the lower eyelid (CN III), mandibular paralysis and facial hypoesthesia (CN V), a diminished palpebral reflex (CN V and VII), facial paralysis (CN VII), nystagmus, head tilt, rolling (CN VIII), pharynx, oesophagus or larynx paralysis (CN IX and X), lingual paralysis (CN XII) (Thomas, 2010).
Knowing that the respiratory centres are in the brainstem, along with the neurological symptoms, clinical assessment of the patient will reveal modified respiration, which may take the form of neurogenic central hyperventilation, apneustic breathing, or central alveolar hypoventilation (Braund, 1994; Kornegay, 1997). In addition to breathing problems, the animal can also show cardiovascular changes, manifested as bradycardia or arrhythmias.
All these cardinal symptoms should lead to a precise differentiation between lesions that affect the brainstem and lesions located at the level of the other intracranial structures, such as the forebrain, cerebellum, or vestibular

apparatus. However, if the neurological deficits cannot be attributed to a single lesion, then the disease should be considered multifocal or diffuse, with inflammatory, congenital, metabolic, or degenerative causes.
The differential diagnosis will be made according to the acronym "VITAMIND", as it is shown in Table 1 (Jaggy & Spiess, 2010; Garosi, 2019), and the confirmation will be made using advanced imagistic techniques given that the development and availability of magnetic resonance imaging (MRI) in veterinary medicine have greatly improved structural evaluation of the brain.

Table 1. Brainstem main diseases according to "VITAMIND" acronym (Jaggy & Spiess, 2010)

VASCULAR	Infarction, cerebral haemorrhage
INFLAMATION/ INFECTION	Abscess, Babesiosis, Distemper, Feline Infectious Peritonitis (FIP), Rabies, Toxoplasmosis, Neosporosis, Parasitic / Rickettsia Meningoencephalitis, Mycoses
TRAUMA	Compressions, haemorrhages
ANOMALY	Chiari Malformation, Hydrocephalus, Intraarachnoid cyst
METABOLIC	Encephalopathies, Metronidazole Intoxication
NEOPLASTIC	Brain tumours
DEGENERATIVE	Storage diseases

This article presents the main aspects of the clinical, neurological, and imaging features of a series of 15 patients who were diagnosed with brainstem deficits in the Faculty of Veterinary Medicine of Bucharest.

MATERIALS AND METHODS

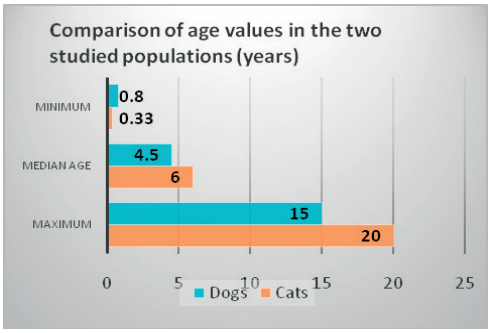
The study was conducted between January 2021 and December 2021 on 15 domestic carnivores (eight dogs and seven cats) diagnosed with brainstem pathologies at the Clinic of the Faculty of Veterinary Medicine in Bucharest. Each case was conducted according to the protocol already implemented in our clinic, in which medical history, clinical and neurological examination were indispensable stages, which preceded the neurolocalisation. For this study, the inclusion criteria were the localization of the lesion within the brainstem, both in unifocal and diffuse form. Differential diagnosis and choice of paraclinical investigations were made using the acronym "VITAMIND" (vascular/inflammatory/trauma/ anomaly/metabolic/idiopathic/neoplastic/degenerative) (Dewey & da Costa, 2016). All 15 selected cases were submitted to magnetic resonance investigation MRI (performed with the VET MR GRANDE machine from ESAOTE with a power of 0.3 Tesla using T1 Spin Eco (SE) and T2 Fast Spin Eco (FSE) protocols in three sequences - sagittal, transverse, and dorsal) which allowed confirmation of etiological diagnosis.
The obtained data were manually collected and revised from the Consultation Register. The analysis regarding significant information about signalment, clinical abnormalities and neurological deficits will be discussed in the following sections.

RESULTS AND DISCUSSIONS

Signalment information like species, breed, age, and sex was compared to describe the epidemiological features of the studied population.
Regarding the species impact on the obtained results, the number of affected dogs (53.33%, n=8) was almost equal to the number of affected cats (46.66%, n=7), which suggests that brainstem pathology evolved almost with the same morbidity in both populations of domestic carnivores.
The two populations were characterized by different breed structures. Thus, within the dog population, purebred specimens predominated (87.5 %, n=7), as follows: Bichon (n=2), Yorkshire (n=1), French Bulldog (n=1), Husky (n=1), German Wirehaired Pointer (n=1) and Poodle (12.5%, n=1) breeds. Only one investigated dog was crossbred.
In contrast, the European breed predominated in the feline population (85.71%, n=6) and only one specimen belonged to a purebred - Burmese (14.21%, n=1).
The average age in the canine population was 4.5 years, with patients belonging to an age range of 8 months - 15 years. The two dogs younger than 1-year-old (German Wirehaired Pointer - 10 months, Husky - 11 months) were diagnosed with congenital anomalies at the level of the brainstem, which confirms the prevalence of this aetiology in juvenile animals (Schrauwen et al., 2014; Turbatu et al., 2019). On the other hand, patients belonging to small breeds (Bichon, Yorkshire, French Bulldog)

had an age limit of 3.5 years and a diagnosis of encephalitis, which also emphasises the high incidence of this type of inflammation in young dogs belonging to breeds with a genetic predisposition. Compared to the data obtained in the canine population (Chart 1), the average age in the feline population was slightly higher, up to a value of 6 years, and the age limits belonged to a wider range, from 4 months to 20 years. As in the canine population, age under 1 year was associated with congenital anomalies (n=1), and geriatric cats were diagnosed with neoplastic pathologies (n=2).

Chart 1. Comparison of age values in the two studied populations (years)



Analysis of gender distribution showed that males were better represented in both species, with a percentage of 62.5% in dogs (n=5) and 71.42% (n=5) in the feline population.
Considering that the examined patients presented brainstem dysfunctions caused by various etiologies, the anamnesis revealed different aspects, as follows:

- There were three cases (two cats, one dog) of traumatic etiologies precisely described by the owners (two falls from a height, one household accident);
- For nine cases (five dogs, four cats) the symptoms observed by the owners were behaviour changes (depression, vocalizations), circling, instability, and epileptiform seizures. Clinical signs started, on average, 3-10 days before presentation and had a progressive evolution. In addition, non-specific clinical signs were observed in some patients, like progressive emaciation, decreased appetite, and apathy.
- In patients under the age of 1 year (n=3, two dogs, one cat), the owners noticed a delay in the growth process, accompanied by apathy, incoordination, visual or auditory deficiencies and the appearance of epileptiform seizures.

Clinical examination revealed the presence of permanent decubitus, changes in breathing, such as bradypnea, dyspnoea or cardiovascular abnormalities (bradycardia and murmurs).
The neurological examination was carried out according to the protocol already implemented in the clinic to our protocol and revealed a severe depressed mental status (stupor), abnormal posture - the presence of decerebrate rigidity (flexion of all limbs, sometimes opisthotonos - Figure 1) and permanent decubitus, several cranial nerves deficits, like bilateral non-reactive mydriasis and anisocoria (Figure 2), facial hypoesthesia, tongue protrusion (Figure 3), and delayed or absent proprioceptive responses (Figure 4).

Figure 1. Decerebrate rigidity in two dogs with brainstem inflammatory lesions

Figure 2. Non-responsive mydriasis and anisocoria in two feline patients with brainstem lesions

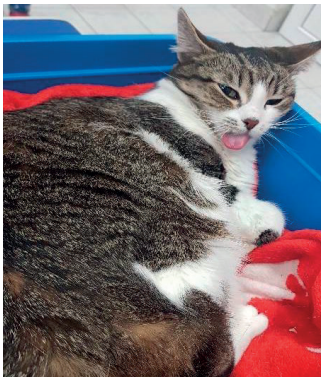


Figure 3. Permanent decubitus, tongue protrusion and facial hypoesthesia in a feline patient with neoplastic brainstem lesion

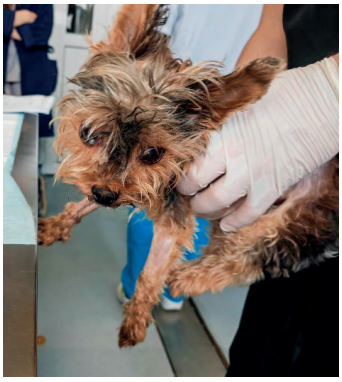


Figure 4. Head tilt on the left side, strabismus, and absent proprioceptive reaction in a dog with brainstem traumatic lesion

The correlation of the data from the anamnesis with the deficits recorded in the neurological examination and the impairment of the respiratory and cardiovascular functions led to a neurolocalisation compatible with brainstem lesions. For 11 cases, the neurological deficits could not be associated with a single location, so the disease was classified as multifocal, affecting the brainstem and the forebrain (n=9) or the brainstem and the vestibular apparatus (n=2). The differential diagnosis was made based on the acronym "VITAMIND", the highest weight being registered by inflammation (n=6), followed by trauma, anomaly, and neoplasia, each diagnosed in 3 cases, as shown in Chart 2.

For all cases, confirmation of the neurolocalisation established in the previous stages was performed using the imagistic technique (MRI) (Figure 5 A, B).

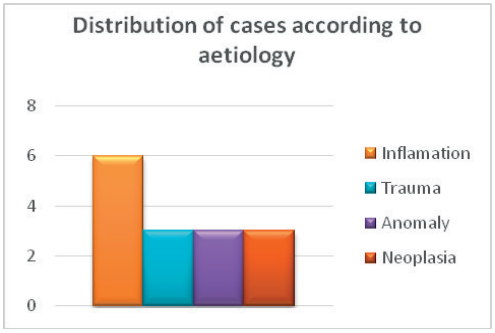


Chart 2. Distribution of cases according to aetiology

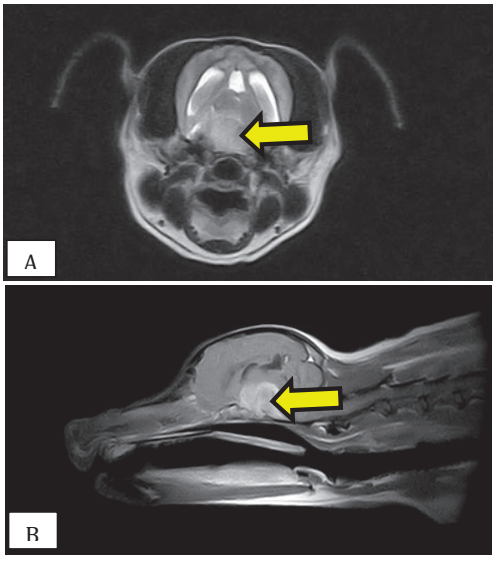


Figure 5. French Bulldog, M, 7 years old. [A] T2 and [B] T1 contrast enhancement transversal and sagittal MRI sections. Delimitated mass (arrows) with soft tissue density and signal, approximately 25 x 27 x 15 mm, with T2/FLAIR hypersignal and T1 contrast enhancement, having an invasive pituitary localization at the level of the right hemisphere, with compression on the adjacent structures, including the brainstem and cerebellum

CONCLUSIONS

Brain stem disorders were diagnosed with an equal share in both studied populations, the differences being related to breed - in the canine population, purebred animals predominated, and in the feline population, European cats predominated.

In both populations, young animals were diagnosed with brainstem anomalies and old animals with neoplastic diseases.

Recognition of the specific deficits (by a proper approach of the patient) and the corroboration of anamnesis, clinical and neurological examination findings (breathing abnormalities, stupor, decerebrate rigidity, multiple cranial nerves deficits) were essential stages that lead to a correct localisation of brainstem lesions.
The imaging technique remains a valuable tool in confirming the etiological diagnosis when structural changes in the brain are suspected.
Complications of brainstem pathologies can occur in domestic carnivores, resulting in life-threatening conditions.

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THORACIC WALL RECONSTRUCTION WITH POLYPROLENE MESH IN A DOG WITH SEVERE FLAIL CHEST

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Abstract

Blunt force traumas that are caused by hit-by cars, animal abuse and bite wounds on the thoracic region can end up with flail chest in dogs. Flail chest, is one of the serious injuries with presence of two or more continuous ribs fractured. As a result, a portion of the thoracic wall with fractured ribs moves paradoxically in inspiration and expiration. According to many previous case reports, mortality rate is high in this life-threatening situation. Patients present in emergency with thoracic pain and shortness of breath (SOB).
9 years old, female Shih-Tzu breed dog clinically presented with severe dyspnea, pain and paradoxical movement of the chest on the left side of the thorax. 24 hours after the patient's stabilization, CT scans revealed loss of the intercostal muscle integrity and 5 ribs fractures on the left thoracic region. After the imagistic certain diagnosis, the dog was taken to reconstructive thoracic surgery.

Key words*: blunt force trauma, flail chest, mesh, reconstructive surgery.*

INTRODUCTION

Bite wounds commonly seen in small dogs and are mostly crushing the tissues. Even some bite wounds cause small dehiscence or lacerations, those bites can end up with severe crushing, avulsion and devitalization of the tissues underneath the skin. The severity of the situation may vary depending on the bitten area. This blunt force trauma can be life-threatening when the bites crush the airways or thoracic region (Sharar et al., 1997; Holt and Thawley, 2015).
Flail chest manifests paradoxical movement during respiration with fractured two or more ribs (Olsen et al., 2002; Orton, 2003). However, the severity of the flail chest also depends on the tearing of the intercostal tissues and the deterioration of their integrity.
Besides the degree of the thoracic damage can easily misdiagnosed while physical examination. Therefore, it is mandatory to provide thoracic radiographs and/or computed tomography to evaluate the severity once the patient stabilized. Various emergency procedures and methods can be used in a combination of supplemental oxygen, thoracentesis, mechanical ventilation, pain management, antibiotic therapy and cage rest. Surgical management of flail chest requires external splint or internal fixation with the combination of latissimus dorsi muscle flap.
In human medicine many surgical techniques has introduced in past years including intramedullary fixation (Moore, 1975), Judet strut (Judet, 1973), using plates with combination of cerclage wires (Moore, 1975).
Even more recent reports the use of absorbable plates (Mayberry et al., 2003).
Yet in veterinary medicine the reports are less than human medicine due to the breed size limitation (Anh et al., 2016).
This case report describes the clinical presentation and successful surgical stabilization of flail chest with cerclage wire and polypropylene mesh, Lene - Vetsuture (Noévia SAS, France).

CASE PRESENTATION

A 9 years old, 5 kg neutered female Shih-Tzu breed dog was brought to the University Veterinary Emergency Hospital of USAMV Bucharest, ≈30 minutes after bitten by a crossbreed large dog. On the physical examination no puncture wounds were observed. Paradoxical respiratory movement on the left thoracic wall diagnosed clinically with severe respiratory destress.

Moderate subcutaneous emphysema also clinically diagnosed. Peripheral oxygen saturation (SpO2) levels measured between 89 and 90%. Mucus membranes (MM) were pale and capillary refill time (CRT) was ≥ 2 sec. For stabilization dog directly placed on sternal position and supplemental oxygen was provided via mask. For local pain management, intercostal nerve block provided on the level of 4^{th}, 7^{th}, 9^{th} and 11^{th} ribs with Lidocaine, (Zentiva S.A. Bucharest/Romania) injection and 0.33 ml IV Buprenorphine (Richter Pharma AG Austria) was administered.

Fast abdominal (A-FAST) and fast thoracic (T-FAST) ultrasounds were performed and no free liquid diagnosed in both thoracic and abdominal cavities but pneumothorax was diagnosed and thoracentesis procedure was performed. Via thoracentesis 150 ml of free air retrieved. Simple Ringer solution (Fresenius Kabi Germany) with the dose of 15 ml/h was given, and antibiotic therapy also provided. The dog kept in oxygen cabin for 24 h with monitoring the vitals.

COMPUTERIZED TOMOGRAPHY (CT) SCAN

After stabilization CT scans were provided. On the left thoracic region 5^{th}, 6^{th}, 7^{th}, 8^{th} and 9^{th} broken ribs were confirmed with mild pneumothorax and pulmonary contusions as well as subcutaneous emphysema. (Figures 1a, 1b; Figures 2a, 2b; Figures 3a, 3b). After CT scans 3D construction of the thoracic cage also provided (Figure 4).

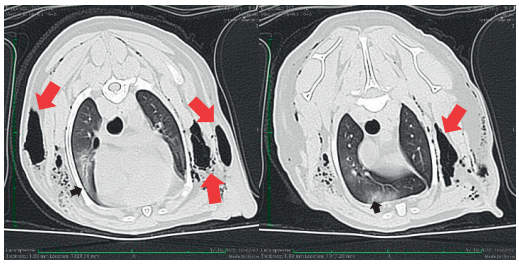

Figure 1a. Red arrows are showing subcutaneous emphysema bilaterally. Black arrow is showing atelectasis on the ventral part of the cranial right lobe

Figure 1b. Red arrow is showing subcutaneous emphysema and black arrow is showing pulmonary contusion on the cranial right lobe.

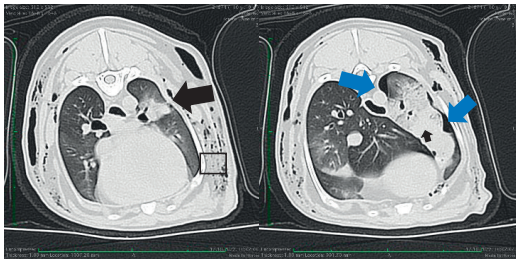

Figure 2a. Black arrow is showing pulmonary contusion and the square is howing the rib fracture

Figure 2b. Black arrow is showing pulmonary contusion on the left cranial caudal lobe and blue arrows are showing the pneumothorax

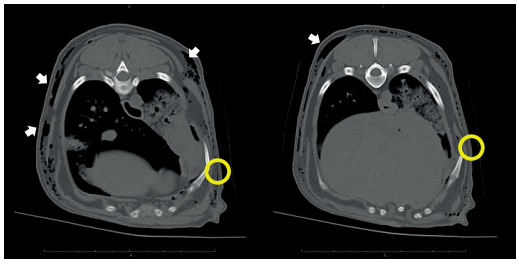

Figure 3a. White arrows are showing the subcutaneous emphysema and yellow circle is showing the fracture on the 6^{th} rib.

Figure 3b. White arrow is showing the subcutaneous emphysema and yellow circle is showing the fracture on the 7^{th} rib.

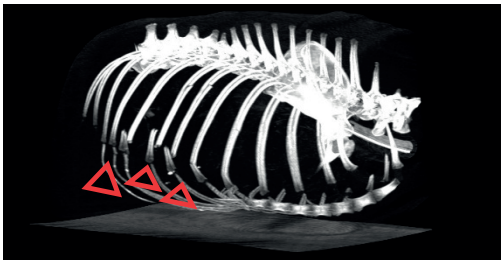

Figure 4. Red arrows are showing the fractures on the 5^{th}, 6^{th}, 7^{th}, 8^{th} and 9^{th} ribs

SURGERY

After the CT scans, the patient prepared for left thoracotomy. Patient was premedicated with butorphanol (Butomidor 10 mg/ml, Richter Pharma, Austria) 0.3 mg/kg and Diazepam (Terapia S.A. Cluj-Napoca/Romania) 0.2 mg/kg. For induction Propofol (Propofol Lipuro 10 mg/ml, Braun Germany) 2 mg/kg was given IV and after intubation with 5 mm et tube, for the maintenance isoflurane 1.5-2% (Anesteran, Rompharm S.A., Romania), O_2 and lidocaine (Zentiva S.A. Bucharest/Romania) Continuous rate infusions (CRI) are used. After second skin preparation with aseptic technique, skin marked on the level of 7^{th}, 8^{th} and 9^{th} ribs with sterile surgical marker (Figure 5). On the level of hearth, last rib and abdominal region several skin contusions were observed in detail which reflects the severity of the trauma.

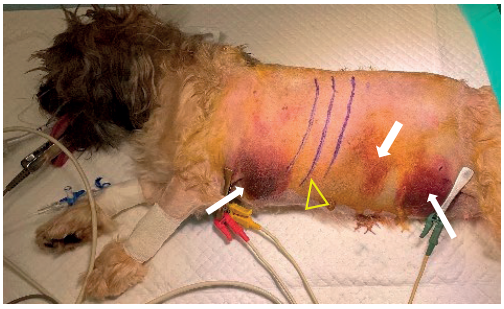

Figure 5. White arrows show the contusions and yellow triangle shows the marking which was drawn via sterile surgical marker

The skin incision was made on the level of 8^{th} rib with n:10 scalpel blade.

After the skin incision subdermal multiple traumas revealed, including *Latissimus dorsi* muscle tears, disruption of integrity of intercostal muscles as well as devitalized muscle parts created by the crushing bites (Figures 6 and 7).

Pulmonary contusion and adherent parts of caudal left lobe were observed during the surgery. However, lung lobectomy was not performed due to lobe regained normal color and expansion during the mechanical ventilation.

After debridement of the soft tissues, subcutaneous tissues, parts of *Latissimus dorsi*, intercostal muscle the fracture line on the 8^{th} rib revealed. Normally the fractures of the ribs can be fixed with small titanium plates like in the humans, or with cerclage wire by performing "X" shape (Orton and Monet, 2018) to have better fixation. Unfortunately, this was not feasible due to patients rib width.

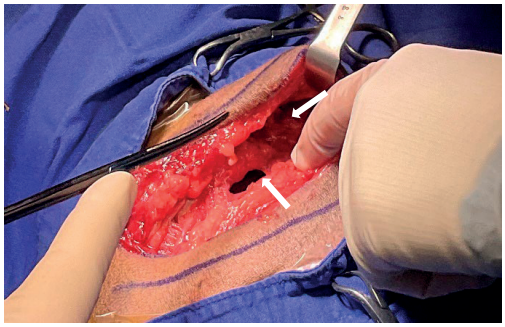

Figure 6. Intraoperative appearance. White arrows are showing the ruptures of the intercostal muscles

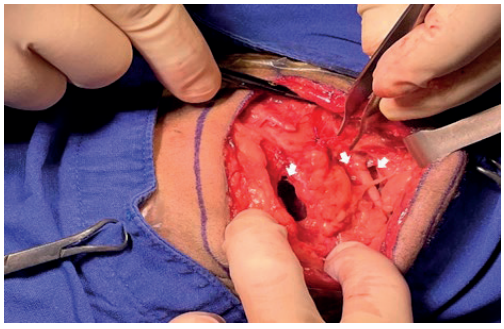

Figure 7. Intraoperative appearance. White arrows are showing the ruptures and disruption of integrity of intercostal muscles between 7^{th} - 6^{th} - 5^{th} ribs and also between 9^{th} and 8^{th} ribs

The dog's rib width was 0.5 cm. For this reason, only two holes drilled approximately 3-4 mm away from the fracture line and 0.4 mm diameter cerclage wire was introduced through the holes and secured with twist knot. After repairing the 8^{th} rib, 7^{th} and 9^{th} rib fractures aligned in their natural position. For this reason, the same technique was not required for fixation. Those rib fractures' alignment were stabilized with the help of sutures.

After orthopedic fixation of the rib, the surgery continued with 12 gauge x 30 cm (12 inch) Mila Catheter (MILA International Inc., USA) chest tube placement. The catheter was introduced to thoracic cavity between 9^{th} and 10^{th} intercostal space. This type of chest tube

has a guiding wire. After introducing the catheter guide wire was inserted and through the guide wire, the chest tube was introduced into the thoracic cavity and secured (Figure 8).
The polypropylene mesh placed in place of the intercostal tissues which integrity was impaired. As polypropylene mesh 7.6 cm x 15 cm (3" x 6") Lene Vetsuture® was used. The mesh was measured in the surgery on the thoracic wall and shaped with Metzenbaum scissors to ensure proper closure. The mesh was secured with 4/0 monofilament Nylon suture, Vetsuture® from four corners first, to maintain the tension.

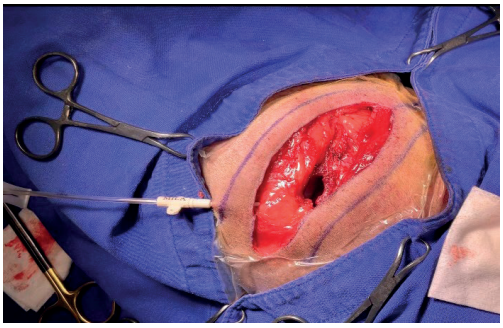

Figure 8. Placing the chest tube

After securing the mesh from the corners, another eight sutures were placed on the several points of the mesh (Figure 9).
With the mesh all the gaps between the intercostal spaces from 6^{th} to 10^{th} ribs were successfully closed.

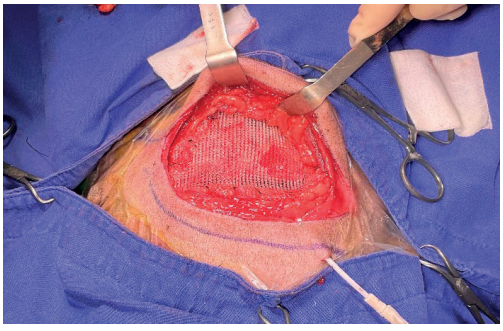

Figure 9. Intraoperative appearance after the suture of the mesh

The polypropylene mesh was covered with latissimus dorsi muscle flap (Figure 10) and before the closure of the skin, free air in the thoracic cavity was evacuated via chest tube until $ETCO_2$ 42% was provided. Afterwards, Heimlich valve attached to the tube.

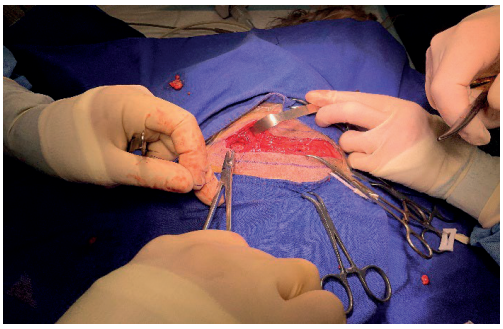

Figure 10. Intraoperative appearance before closing the skin

Skin closure was made with 3/0 monofilament PDX suture material, Vetsuture® with routine fashion technique. After the surgery, Thoracic bandage was performed and the patient placed in the Intensive Care Unit (ICU) unit with supplemental oxygen and monitor.

POST-OP. MANAGEMENT

In the first 24h, each 8 hours the chest tube was controlled and care provided. For the first 72 hours Methadone (Richter Pharma AG, Austria) 0.3 mg/kg in each 8 hours and for five days Meloxicam (Dopharma Resaerch B.V. Raamsdonksveer/NL) 0.2 mg/kg was given to the patient.
The dog was kept in oxygen cabin for the first 24 h and monitored for possible arrhythmias. Vitals checked in every 8 hours no remarkable changes were observed.
24 hours after the surgery control radiographies were provided (Figure 11). In the control radiographies pneumothorax not observed and the rib fractures were aligned. 72 hours after the surgery chest tube was removed without any local or general anesthesia needed. Thoracic bandages were changed each 48 hours and the patient discharged home after 6 days with the recommendation of antibiotics and anti-inflammatories.
The sutures were removed 14 days after the surgery. No respiratory distress was observed during the consultation and removal of the sutures. 60 days after the surgery the patient called back for control radiographies (Figure

12). After the control radiographies, the patient was fully discharged.

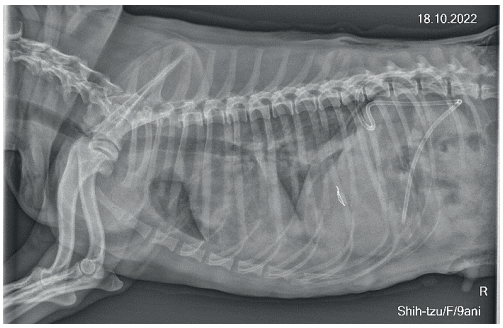

Figure 11. Radiographic image 24 hours after the surgery

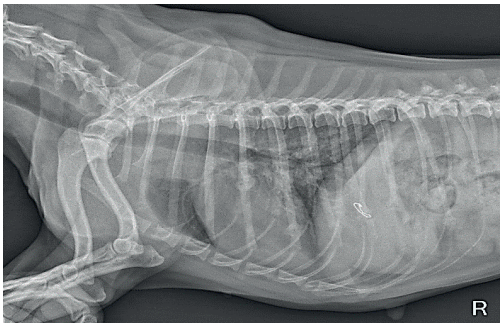

Figure 12. Radiographic image 24 hours after the surgery

DISCUSSIONS

Thoracic bite wounds can result with severe crushing and penetrating injury, both of which can tear subdermal tissues, muscles and internal organs. Those bite wounds even can result with life threatening injuries, yet remarkable skin defects may not be observed in many of them (Davidson, 1998; Shamir et al., 2002). Flail chest in dogs, mostly caused by bite wounds (Olsen *et al*., 2002). With the existence of internal organ damage, patients can have severe respiratory distress (McKierman et al., 1984). In this case report, the dog showed severe respiratory distress with peripheral oxygen saturation (SpO_2) levels were measured between 89 and 90% after being bitten by another dog. It is well known that paradoxical respiratory movement of thorax is also seen in pseudo-flail chest. Pseudo-flail chest manifests paradoxical respiratory movement due to complete tear of the intercostal muscles with only one or without any rib fracture (Scheepens et al., 2006). It is crucial to determine if the patients' present paradoxical respiratory movement due to flail chest or pseudo-flail chest. Because the conservative and surgical treatments can show difference. In this case, the dog had flail chest with five rib fractures (5^{th}, 6^{th}, 7^{th}, 8^{th} and 9^{th} left ribs).

As an addition, diagnostic techniques are also important to define all the traumatic injuries of the tissues and organs.

CT scans are more sensible and effective. It's detailed impact on radiographic diagnosis cannot be indisputable. In this case, once the patient was stabilized, CT scans preferred to have fast surgical planning as well as to determine all the traumatic injuries that the dog knowledged.

Flail chest is considered life threatening emergency situation in human medicine and regarded as a marker of significant injuries (Ciraulo et al., 1994). Most of the therapies for flail chest in veterinary medicine are based on the methods developed in years in human medicine.

The treatment for flail chest can be conservative or surgical. Yet the studies in veterinary medicine associated with flail chest are rare.

Methods of thoracic reconstruction can be classified as: internal fixation, external fixation, or *Latissimus dorsi* muscle flaps. Internal fixations involves intramedullary pins, cerclage wires or plates.

In this case intramedullary pinning or using plates was not possible due to the size of the dog and the diameter of the ribs. Choosing internal fixation via wiring has been used as a surgical method for the rib fractures in dogs of many sizes (Shahar et al., 1997; Olsen et al., 2002).

Thoracic wall reconstruction techniques mostly involves with *Latissimus dorsi* flaps (Byong-Su Min et al., 2016). However, some other authors also reported surgical mesh usage in their cases. Xiong Qin used degradable polymer, collagen coated polydioxanone (CCP) mesh and chitin fiber reinforced polycaprolactone (CFRP) strut. (Xiong et al., 2008) and pointed that the CFRP material kept its tensile strength for a long time after surgery and had an advantage of lower reactivity than other short-term re-absorbable copolymer such as poly-L-

lactide (PLA). Some other authors focused on usage of polypropylene mesh usage after oncologic surgeries especially involving thoracic wall and pointed the use of flexible synthetic polypropylene mesh especially in cases where there is excision of several ribs (Castro et al., 2015.

In this case for the reconstruction, polypropylene mesh was chosen due to huge defects were observed in intercostal muscles. The polypropylene mesh was covered with *Latissimus dorsi* muscle flap. The second reason of choosing polypropylene mesh was also related to reduce the tension on the thoracic wall after the surgery.

Although there are some advantages and disadvantages of polypropylene mesh usage. The most advantages reported in human medicine as: durability, low infection risk and being comfortable for the patient while disadvantages reported as; lack of flexibility, high adhesion risk and shrinking. The other consideration of the polypropylene mesh usage is the result in rejection as a foreign body after implantation (Anderson, 2001). Yet those studies are regarding polypropylene mesh implantation in hernia surgeries in human medicine. In our case, not any side-effects or rejection was observed in hospitalization period after the surgery as well as in re-controls after 30, 60 and 90 days.

However, we need more studies in veterinary medicine so that we can have more information about the short and long-term effects of the polypropylene meshes.

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THORACIC TRAUMA UPDATES IN FELINE HIGH-RISE SYNDROME. WHAT CHANGED IN 30 YEARS? 50 CASES IN ONE YEAR

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Abstract

High-rise syndrome is a general definition of multiple traumatic injuries that cats experience after falling from a height of 2 or more floors of high-rise buildings in urban areas. This falling generally results with multiple injuries including thoracic, abdominal, orthopedic and craniomandibular or craniomaxillofacial trauma. The combination of multiple traumatic injuries can be life threatening. 50 cats diagnosed with high-rise syndrome between period December 2021 - December 2022 in Veterinary Emergency Hospital, University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine. Thoracic trauma was diagnosed in 82% of cats. Pneumothorax was diagnosed in 54% of cats and Pulmonary contusions diagnosed in 50% of cats. Some cats diagnosed both pneumothorax and pulmonary contusion. Past 30 years, some authors pointed and rated the injuries they diagnosed in their articles and case reports. The aim of this study is to point increased thoracic trauma after high-rise syndrome related with hitting more hard grounds than past years in urban areas.

Key words: *high-rise syndrome, pneumothorax, pulmonary contusion, thoracic trauma.*

INTRODUCTION

Feline high-rise syndrome (FHRS) refers to combination of some traumatic injuries that cats experience after falling from a certain height (8 meters or 24 feet or 2 floors) generally in urban areas. High-rise syndrome, mostly reported and described in cats by several authors in the past. (Robinson 1976, Dupre et al., 1995; Flagstad et al., 1998; Papazoglou et al., 2001). This syndrome is also been reported in dogs (Gordon et al., 1993), but it is rare than in cats. In humans the terms “high-flyer syndrome” or “jumpers syndrome” were also used by some authors (Reynolds et al., 1971; Smith et al., 1975).

Some authors reported the relationship between the height of the fall and the severity of the injuries feline high-rise syndrome the severity of the injuries (Flagstad et al., 1998; Papazoglou et al., 2001) while some others focused severity of the traumatic injuries linearly with the height of the fall (Dupre et al., 1995).

According to popular belief, falls from a height usually happen while chasing a bird or an insect on the balcony or losing the balance and slipping on the edge of the balcony railing as well as window.

The aim of this study is statistically evaluating 50 cats which were diagnosed with FHRS between December 2021 and December 2022 in the Veterinary Emergency Hospital of USAMVB and focus on increased thoracic trauma percentages than previous reports and articles.

All kind of injuries were documented, likewise the timing of the fall (night or day time), the ground they hit (soft soil garden or concrete) to understand the severity of the injuries with thoracic trauma and also to understand if there is a relationship with height.

MATERIALS AND METHODS

In the period between December 1, 2021 and December 1, 2022 50 cats were treated after high-rise syndrome in the Veterinary Emergency Hospital of University of Agronomic Sciences and Veterinary Medicine, Bucharest (USAMVB). Only the cats which felt from second and higher floors included in this study. Trauma triage was performed to all the cases. Depending on the triage further diagnostic tools were performed such as biochemical and hematological analysis. Fast thoracic (T-FAST) and fast abdominal (A-FAST) ultrasounds were performed to all patients as a general trauma approach protocol. X-rays or computed tomography (CT) performed only after patients were stabilized.

To evaluate the traumatic injuries, we used following criteria:

- Thoracic trauma, pulmonary contusions, pneumothorax, hemothorax and traumatic diaphragmatic hernia (TDH) - score 3;
- Epistaxis, craniomaxillofacial fractures, colon vertebrae fractures and dislocations, hard palate fractures and abdominal trauma with bladder rupture or urethral rupture - score 2;
- Orthopedic fractures and luxations - score 1

Based on this scoring system, each kind of injuries summed up. As an example, if a cat diagnosed with pneumothorax and pulmonary contusions with a fractured femur scored as; 3+3+1 = 7 whereas if a cat had two metatarsal fractures with fractured radius and ulna scored as; 1+1+1+1 = 4. Each fracture scored with 1 point. Thus, if a cat had more than one metacarpal or metatarsal fractures each counted as 1.

All cats were evaluated with impact force they exposed after hitting the ground to understand the relationship between the weight of the cats and the height they felt from. At the same time, to find out the minimum impact force that can cause thoracic trauma, pneumothorax and/or pulmonary contusion. To calculate the impact force "Newton's second law of motion" is needed. This law basically describes of the changes that a force can produce on the motion of a body.

Second law of motion states that the time rate of change of the momentum of a body is equal in both magnitude and direction to the force imposed on it. The momentum of a body is equal to the product of its mass and its velocity and the equation is $F = ma$, where F (force), m (mass) and a (acceleration) are both vector quantities. Secondly the "conservation of energy" must be considered. Conservation of energy manifests that energy isn't created or destroyed, just transformed from one form into another.

The conservation of energy is needed to calculate how much kinetic energy an object has just before the point of impact. This is the energy which the object has all come from the gravitational potential it has before falling and the equation is $E=mgh$, where E is the energy, m is the mass of the object, g is the acceleration due to gravity constant ($9.81\ m\ s^{-2}$ *or 9.81 meters per second squared*), and h is the height the object falls from.

The other information that we know from the impact force calculation is the penetration. As an example, if an object penetrates into the ground after the impact, the impact force that it will be exposed is smaller. In another words, greater penetration implies smaller impact force while higher impact force will be exposed by the object if it hits to harder ground because hard ground means less penetration. The other information is that if the object bounces back, the impact force will be even greater because of greater chance in momentum and many cats bounce back little or more after falling and hitting the ground.

With all those information the formula will be;

$$\text{average impact force} = \frac{\text{kg} \times 9.81\ \text{m}/s^2 \times \text{m}}{\text{d}}$$

d is the average distance that the object bounces after the impact. For our study we assumed d as 0.1 m = 10 cm for all the cats hit the ground.

In modern building constructions the average height of the second floors are between 4.7 and 5.8 meters depending on the construction materials used.

In this study the average of the second floor considered as 5.25 meters and each floor after second floor considered as 3 meters.

According to the average impact force formula and all the data, if a cat with 3.5 kg body weight falls from 5th floor (14.25 meters height), the average impact force that the cat exposed will be 4887.7 Newton(s) N.

RESULTS

During the defined period age ranges of the cats with HRS were between 3 months and 11 years old. It was determined that the majority of cats felt from a height were one year old or below, 44% (22/50). The oldest cat was 11 years old (Figure 1).

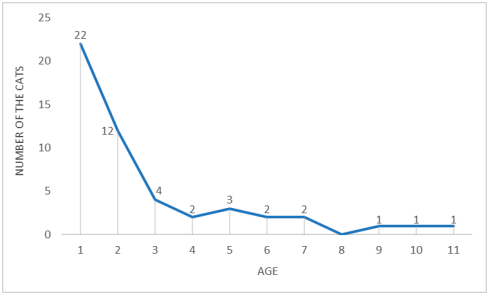

Figure 1. Age dispersion of the cats with HRS

Maximum number of the falls happened in July (13 cats), while the minimum was in January (1 cat). In March and April, we didn't receive any cats felt from balcony or window.

Remarkably 13 cats were brought to the hospital between October and January which mostly owners said that cats jumped from the window or felt from the edge of the balconies right after they arrived home and opened the windows to have fresh air in the house (Figure 2).

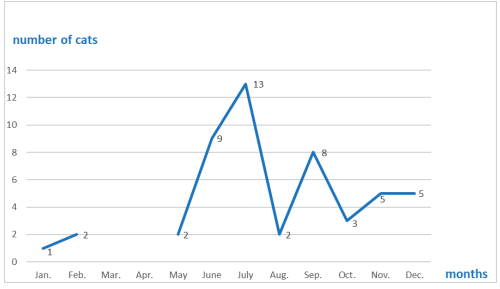

Figure 2. Number of cats and months they felt from balconies or window

46% of the cats were female (23/50), 24% were male (12/50), 10% of the cats were spayed females (5/50) and 20% of the cats were neutered males (10/50) (Figure 3).

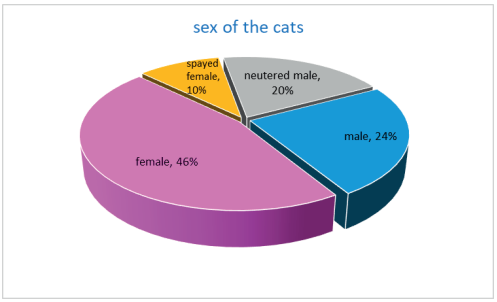

Figure 3. Sex dispersion of the cats with HRS

31 cats fell at night time while 19 cats felt during the day time. 5 neutered males felt at the night time and 5 neutered males felt in the day time. 2 male cats felt in the day time, while 10 non-neutered males felt at the night time. Similar results are also found in female cats. 3 spayed females felt in day time, while 2 spayed felt at night. 9 females felt in the day time and remarkably 14 non-spayed females felt at night (Figure 4). This valuable information helped us to understand cats' behavior. We all know that cats are night hunters by nature. Therefore, high-rise syndrome were observed mostly at night period. The other finding was that spayed and neutered cats probably were less active at night time than the non-spayed and non-neutered cats.

This also might be evidence of behavioral changes after spaying or neutralizing. Besides we learned 2 female and 1 male cats knowledge high-rise syndrome 1 year before approximately the same period.

Thoracic trauma was diagnosed in 82% (41/50) of cats. All those cats received supplemental O_2 via flow-by, mask or O_2 cabin technique due to respiratory distress, dyspnea, or cyanosis. Pneumothorax was present in 65.8% (27/41) of these cats with thoracic trauma. Thoracentesis carried out bilaterally to all those 27 cats without rushing the radiography to avoid extra stress and as well as not to worsen the respiratory situation.

Thoracentesis is our hospital's protocol for dyspneic, thoracic trauma patients due to its diagnostic and therapeutic purposes.

12h to 24h hours later, when the cats stabilized, radiographic images confirmed also the existence of pulmonary contusions in 55.5% (15/27) of the cats which were also had pneumothorax. 24.3% (10/41) of the cats with thoracic trauma presented only pulmonary contusions. All the cats with pneumothorax treated with thoracentesis or chest tube placement.

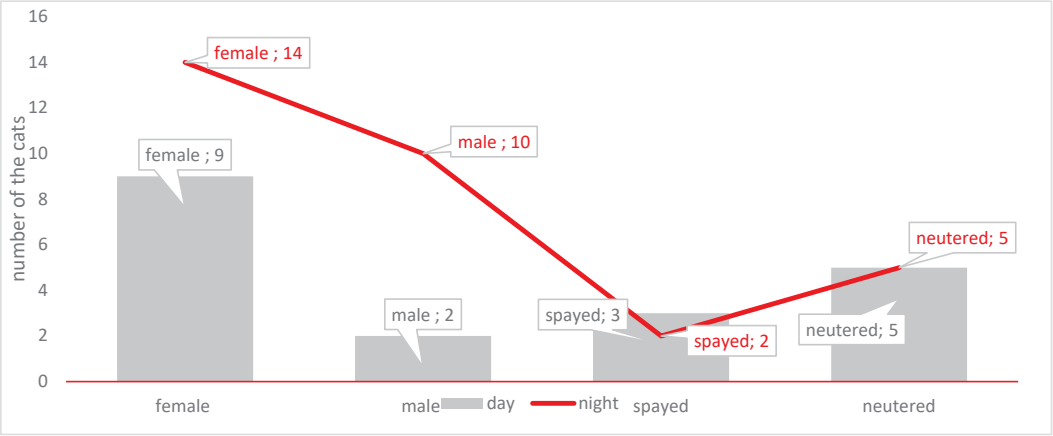

Figure 4. Cats sex dispersion and falling period relations

Only one 9 years old 4.2 kg one female cat which felt from 8th floor was diagnosed with spontaneous pneumothorax. For this cat thoracentesis performed in each 8 h for the first 36 h and in each 12 h for the following 24 h. Atelectasis, collapsed lung lubes on the right side diagnosed via computed tomography (CT) and the lung lobectomy surgery performed via sternotomy. Unfortunately, this patient passed away one hour later the surgery in ICU. The other 1 year old, 3 kg body weight, cat which felt from 6th floor died in 30 minutes after entering to emergency room due to hemothorax. Nevertheless, the survival rate was 96% (48/50).

4% (2/50) cats were diagnosed with traumatic diaphragmatic hernia and both cats were taken to surgery 24 h to 36 h later after they came to hospital.

4% (2/50) cats had rib fractures and 1 cat clinically diagnosed with flail chest.

Thoracic trauma was diagnosed regardless of floor difference. While pneumothorax and pulmonary contusion were diagnosed together in 33.3% of the cats which felt from the 5th floor. Pulmonary contusion and pneumothorax were diagnosed together in 44.4% of the same number of the cats which felt from the 6th floor. One cat which felt from the highest floor (10th floor), diagnosed with only pneumothorax and the oldest cat (11 years old) which felt from 5th floor also diagnosed with only pneumothorax (Table 1).

Incidence of thoracic trauma in female cats were 86.2% (25/29) while the incidence of thoracic trauma in male cats were 76.1% (16/21).

Incidence of pneumothorax diagnosed together with pulmonary contusions in the majority of cats at 1 year old and below was 31.8% (7/22). This value was even greater than the pneumothorax and pulmonary contusion component diagnosed together in all cats in other age groups 25% (7/28).

Impact force calculated for each cat to understand the relationship between impact force and thoracic trauma.

Cats diagnosed with thoracic trauma had an average body weight of 5.7 kg and the average impact force they experienced was 2919.75 N, felt from the 2nd floor.

Table 1. Thoracic trauma relation with the height

	Pneumothorax	Pulmonary contusions	Pneumothorax & Pulmonary contusions	Hemothorax	Flail chest	Traumatic diaphragmatic hernia	Without thoracic trauma
2nd floor	3		2				2
3rd floor		2	1				1
4th floor	2	1	2				
5th floor	2	1	3	1			2
6th floor	3		4		1		1
7th floor	1	3					3
8th floor		3	3			2	
10th floor	1						

The average body weight of the cats which felt from 3rd floor was 4.5 kg and the average impact force they experienced was 3638.25 N. The average body weight of the cats which felt from 4th floor was 3.2 kg and the average impact force they experienced was 3555.55 N. The average body weight of the cats which felt from 5th floor was 4 kg and the average impact force they experienced was 5638.3 N. The average body weight of the cats which felt from 6th floor was 3.3 kg and the average impact force they experienced was 5530.32 N. The average body weight of the cats which felt from 7th floor was 3.8 kg and the average impact force they experienced was 7441.85 N. The average body weight of the cats which felt from 8th floor was 3.9 kg and the average impact force they experienced was 8848.15 N.

All cats evaluated with a special traumatic injuries score system. Each injury added to main thoracic trauma scores. Only 4 cats diagnosed with thoracic trauma did not present any other injuries. Rest of the cats with thoracic trauma also presented with multiple other injuries. 12% (6/50) of the cats diagnosed with hematuria. Other than hematuria, abdominal injuries not observed in 50 cats.

11 cats were hypothermic, body temperatures were between 35.1°C-36.3°C. Those cats were kept in oxygen incubators with heating pads until their body temperature turn back to normal range.

Tibia was the most frequently fractured bone (11 cats). Tibia followed by radius bone fractures (9 cats). 8 of the cats also presented with ulna fractures.

One cat had radius and ulna fractures in both legs. 8 cats diagnosed with metacarpal fractures. Those cats had more than one metacarpal bone fracture. In total 21 metacarpal fractures were diagnosed. Fractured femur diagnosed in 6 cats. 42% (21/50) of the cats diagnosed with single one bone fracture. 58% (29/50) of the cats diagnosed with multiple fractures (Table 2).

3 cats clinically diagnosed with hard palate fracture. 2 cats were diagnosed with craniomaxillofacial fractures and 1 cat with mandibular fracture. Esophagostomy feeding tubes placed 3 of those 6 cats.

Coxofemoral luxations diagnosed in 5 cats. femoral head ostectomy (FHO) performed to those cats.

Titanium implants used in all radius, tibia, femur and pelvic fractures. Only in one cat intramedullary pin used due to economic reasons of the owner.

Cats with the rib fractures treated with cage rest.

Spinal fusion surgery performed to a cat with colon vertebrae fracture. This cat hospitalized 21 days and discharged home after.

Table 2. Fractures and luxations dispersions in cats after HRS

Fractured bones and injuries	Number of cats
Metacarpal fractures	8
Radius	10
Ulna	9
Scapula	1
Craniomaxillofacial	2
Mandibula	1
Hard palate fractures	3
Rib fractures	2
Vertebral fractures	2
Pelvic fractures	4
Sacroiliac fractures /luxations	3
Coxofemoral luxations	5
Femur	6
Tibia	11
Metatarsal fractures	1

According to special traumatic injuries score system (TIS), Female cats were diagnosed with more severe injuries than male cats. Average traumatic injuries score of female cats were higher than male cats. Even though, interestingly the average TIS were greater in 1 year old and younger males than the same age range female cats (Average TIS = 6.3 male cats, age 1 and below/Average TIS = 5.13 female cats age 1 and below). No remarkable difference found between young and old cats with TIS (Average TIS = 5.7 in cats, 1 year old and below/Average TIS = 5.4 in 2 years and older cats).

DISCUSSIONS

High-rise syndrome is seen mostly younger cats. Younger cats fall from balconies and windows whilst playing, chasing a bird or butterfly (Vnuk et al., 2004). The mean age of the cats in our study was 2.76 years. 44% (22/50) of the cats were 1 year old and below. Whitney and Mehlhaff (1987) reported that 65% of the cats were under age 3. The mean age of the cats in our study were higher then that reported by Dupre et al. (1995) - 2.5 years, Flagstad et al. (1998) - 2.3 years, Vnuk et al. (2004) - 1.8 years and even older than the mean age of the cats reported by Papazoglou et al. (2001) - 1.2 years.

Papazoglou et al. (2001) reported 51% males, 46% females, 1% neutered males, 1% spayed females and 1% unrecorded gender status. Whitney and Mehlhaff (1987) reported 48% females and 48 % males and 4% unrecorded. In that study also mentioned that 23% males were neutered and 27% females were spayed. Vnuk et al. (2004) reported 53.8% were females, 42% males, neutered males 3.4% and undetermined 0.8% in their study. In our study 46% of the cats were females, 24% males, 10% spayed females and 20% neutered males. The study of Papazoglou et al, were covering the years between 1988-1998 in Greece with the greatest percentage of males experienced high-rise were in their study (51%). The study of Vnuk et al, were covering the years between 1998-2001 in Croatia which male percentage were 24%. Our study covers one year between December 2021 and December 2022 in Bucharest, Romania with a percentage of the males 24%. In our study neutered males were 20% which were higher than the studies of Papazoglou et al. and Vnuk et al.

The decreasing percentage of males experienced high-rise syndrome may be due to increasing numbers of neutralized males in years.

Yet, we do not know the percentage of cats fell from balconies or windows in day time and/or at night from many other authors' studies. Papazoglou et al. (2001) reported the majority of the cats fell during day time in their study, while Whitney and Mehlhaff (1987) reported %40 fell at night. In our study 38% of the cats fell from high buildings in the day time and 62%, were fell at night. The nighttime fall rate of non-spayed female cats 28% (14/50) was significantly higher than the daytime fall rate of non-spayed female cats in our study 18% (9/50). The result we found was even more interesting when we made the same comparison between non-neutered male cats. (4% (2/50) day time fall - 20% (10/50) night time). This differences and the higher value of night time falls, inevitably brings to mind the question of whether cats are jumping from a balcony or a window with the instinct of hunting rather than a game or sexual urge.

Thoracic trauma was diagnosed in 82% of the cats. Only pneumothorax was diagnosed in 12 cats. Only pulmonary contusion was diagnosed in 10 cats. However, 15 other cats diagnosed with pulmonary contusion and pneumothorax together. This was clear finding that the incidence of pneumothorax were 54% and 50%

pulmonary contusion. Whitney and Mehlhaff (1987) diagnosed 90% of cats with thoracic trauma, pneumothorax in 63% of cats and pulmonary contusions in 68% of cats in their study. Papazoglou et al. (2001) reported thoracic trauma in only 13% of the cats, pneumothorax 4% and pulmonary contusions as 6.8% in 207 cats between 1988 and 1998 in Greece. Flagstad et al. (1998) diagnosed pneumothorax only in 7.1% of the cats in Denmark. Vnuk et al. (2004) reported 33.6% pneumothorax and 20% pulmonary contusions in 119 cases between 1998 and 2001 in Croatia. Merbl et al. (2013) reported 21.5% pneumothorax and 18.7% pulmonary contusions in 107 between 1999 and 2009 in Israel.

The percentage of pulmonary contusions were extremely higher than other authors' reports except study of Whitney and Mehlhaff (1987). Whitney and Mehlhaff recommended thoracic radiographies in all cats and thoracic radiography carried out in 69% of the cats in their study. Papazoglou et al. (2001) reported thoracic radiographies were carried out in all cats. Vnuk et al. (2004) reported thoracic radiographies carried out only in cats which were showing the abnormal respiration. In our study thoracic radiographies carried out on all cats but only after they were clinically stabilized. Besides all the pneumothorax cases diagnosed with thoracentesis in the emergency room before radiographs. Thoracentesis is our protocol before thoracic radiographs or CT scans not to worsen the situation in dyspneic patients. Besides timing of radiographs are seriously important for diagnosis in pulmonary contusions. Because pulmonary contusions may not appear up to 6-12 h post trauma. The low appearance of pulmonary contusions in other reports may due to timing of the radiographs or due to some animals with thoracic trauma may have minimal clinical signs or even none (Aron and Roberts, 1993). In those animals, possible thoracic trauma was not diagnosed.

If we look closely to the cat numbers and the years of the reports, we can think more cats will be observed with thoracic trauma in the future, due to the change in living style, moving to higher buildings. We think that concretization in cities and the risk of cats falling on concrete grounds more frequently increased.

In addition, 27 cats diagnosed with pneumothorax in our study and 15 of those cats were also diagnosed with pulmonary contusion which the percentage were 55.5%. This strong finding reinforcing the fact that it would not be strange to suspect the presence of pulmonary contusion in cats which are clinically diagnosed with pneumothorax in HRS.

Pneumothorax and pulmonary contusions and other thoracic traumas were diagnosed in all floors that cats fell from. This clinical finding disproves the thesis that cats would have less thoracic trauma if they fall from lower floors.

Vnuk et al. (2004) reported 38.5% cats with forelimb fractures. Papazoglou et al. reported 68% of the limb fractures were forelimb fractures. Merbl et al. (2013) reported 16.8% forelimb fractures. Zaghloul and Samy (2018) reported 20% out of 45 cases between 2015 and 2018 in Egypt but not reported any thoracic trauma. Catalkaya et al. (2022) reported 22.2% forelimb fractures in 72 cases in 2019 in Turkey but also not reported any thoracic trauma, pneumothorax or pulmonary contusions. This difference may be due to their reports mostly focused on orthopedic injuries. In our study forelimb fractures percentage was 68% in total bone fractures. Unlikely the cats with hindlimb fractures, all the cats diagnosed with forelimb fractures also diagnosed with thoracic trauma, pneumothorax and/or pulmonary contusions. This interesting clinical finding makes us believe that there can be high incidence of thoracic trauma existence in the cats with forelimb fractures after high-rise syndrome.

Unlike the studies of other authors, in our study the impact force calculations were made to understand if there is a relation between thoracic trauma and the impact force that cats are experiencing while hitting the ground.

All average impact force and the average body weight, which play a role in the formation of pneumothorax and pulmonary contusion, are listed floor by floor in our study.

One of the interesting findings regarding the effect of impact force on thoracic trauma was that 2 cats with the same body weight (3.3 kg) also both felt from 8th floor in different times. Both cats clinically diagnosed with

pneumothorax and pulmonary contusions. The impact force they faced was 7519 N. Another interesting finding was with the cat 4.4 kg body weight. This cat felt from 10th floor and experienced 12612.6 N impact force while hitting the ground. However, the cat diagnosed with only pneumothorax and without any orthopedic injuries. Next surprising clinical finding was the cat with 6,5kg body weight fell from 6th floor. This cat also experienced extremely high impact force, 10988.2 N and diagnosed with only fractured tibia.

Instead of these two exception cases, we do believe there might be strong effect of the impact force on thoracic trauma. But yet to prove this we need to study on many more cases.

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DETERMINATION OF CHEMICAL-ANALYTICAL QUALITY CRITERIA OF KING SCALLOP (*Pecten maxima*), ATLANTIC SEA SCALLOP (*Placopecten magellanicus)* AND QUEEN SCALLOP (*Aequipecten opercularis*) SAMPLES FROM NORTH ATLANTIC OCEAN, ENGLISH CHANNEL AND FROM THE TRADE MARKET

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Abstract

King scallops (Pecten maximus), Atlantic sea scallops (Placopecten magellanicus) and Queen scallops (Aequipecten opercularis) were were directly caught from natural environment of North Atlantic Ocean and English Channel Bay of Biscay during four of Walther Herwig's III (WH III) expeditions and compared with an enlarged range of frozen scallop purchased on the German market. Proximate composition, was examined in the muscle to identify changes as a result of freezing and processing. Investigations took place on-board and at the Max Rubner Institute Hamburg. In the investigated purchased bivalve molluscs samples, in two cases (Pecten spp., Placopecten magellanicus) perfect positive correlations (R2 = 1) were observed between the amount of phosphate and the pH values, while in the other 5 samples, the correlations were negative (R2 = −1). A negative but weak correlation was established between protein percentage and TVB-N % (R2 = − 0.26). There were calculated reasonable positive correlations between phosphate and ice glaze (R2 = 0.48).

Key words*: phosphates; water content; scallops; proximate composition; additives.*

INTRODUCTION

Shellfish quality refers to aspects of consumer preference such as freshness, nutrition and the sensory attributes such as color, texture and flavour (Boulter, 1996). Storage and handling practices during *rigor mortis*, resolution of *rigor mortis*, nucleotide degradation (autolysis) and microbial growth affect freshness, flavour and texture. Nucleotide degradation and microbial growth specifically affect the freshness and flavour of fish and shellfish (Tudor, 2013). Tenderness (texture) is an important aspect of scallop quality and can be measured by both instrumental and sensory methods (Ocano-Higuera et al., 2006). Autolysis, microbial and textural changes in scallops under various storage and handling practices have been widely reported (Ocano-Higuera et al., 2006; De Vido & de Mattio et al., 2009; Makri, 2009). Quality can also refer to the physiological condition of an animal and its ability to respond to its environment. A combination of physiological, biochemical and behavioural tests, including condition indices and carbohydrate content was used in order to assess the response of scallops to stress.

Indices mainly measured the physiological activity of an animal at a given time or environmental condition and were computed for specific tissues (adductor muscle) in relation to other tissues after taking morphometric measurements (Beltran-Lugo et al., 2006).

Biochemical composition is an important quality indicator and is closely associated with above mentioned condition indices. Storage of nutrient reserves in the adductor muscle contributes to the nutritional value of the scallops (King et al., 1990; Ocano-Higuera et al., 2006).

MATERIALS AND METHODS

Established methods were used in the laboratory of Max Rubner Institute (Hamburg, Germnay) to analyse the contents of water, protein (according to Dumas), ash, total phosphate (photometric) and to measure the pH values. The qualitative detection of condensed phosphates was carried out using the photometric reference method, a quantitative analysis.

The percentage of dry matter and water respectively were determined after drying over night at 105ºC aliquots of the homogenized samples. The ash content was measured according to Antonacopoulos (1973) as previously described (Boițeanu & Neacsu, 2022). Samples were mineralised over night into a mufle furnace at 550°C, than cooled in a desiccator and weighed.

In order to measure the total phosphorus content the photometric reference method was carried out. The homogenized samples were dried and calcined, the ash was then hydrolysed with nitric acid. After the addition of ammonium metavanadate and ammonium heptamolybdate to an aliquot part of dilute nitric acid (~20%), a yellow coloration resulted, whose extinction was photometrically measured. Extinction was directly proportional to the phosphorus content.

The percentage of fats from scallop samples was exactly measured by gravimetric method Smedes (1999) as amended by Karl et al., (2012) and as previously described (Boițeanu & Neacsu, 2022). The method is based on the extraction of fat with cyclohexane and 2-propanol followed by the transfer of the fat to the cyclohexane phase by addition of water. Phase separation is made by centrifugation. Finally, the gravimetric determination of fat takes place, after separation and concentration of the cyclohexane phase.

Determination of the percentage of protein substances in fish was based on the principles of the Dumas combustion method (Miller et al., 2007) as previously described (Boițeanu et al., 2014). After well-homogenized samples were obtained, they were heated in a high-temperature furnace where the combustion took place at over 1000°C in the presence of pure oxygen. This heat treatment produced (among others) nitrogen as NyOx (oxides). The gas mixture was passed through a reduction chamber containing copper heated to 650°C in which the nitrogen oxides were converted into elemental nitrogen and the excessive oxygen was collected. Finally, total nitrogen content was measured by a thermal conductivity detector. The entire process took less than 4 minutes. In the end, total protein was measured multiplying the N results by 6.25.

Salt percentage was measured by titration with 0.1N silver nitrate solution using an automatic titrator (Karl et al. 2002) as already described (Boițeanu et al., 2014). The principle of the method is based on the extraction of salt (by means of dissolving into water) from the pre-weighed sample (the sample is suspended in water and acidified with nitric acid). After protein precipitation, the chloride concentration is determined by titration (up to the final potentiometric point), with an aliquot part of the standardized silver nitrate solution (0.1N).

RESULTS AND DISCUSSIONS

Water in fresh and frozen scallops

Marketed amount of quick-frozen scallops (*Pectinidae*) has significantly increased in the recent years and has been extended from the king scallop (*Pecten maximus*) to a variety of species. Incorrect labelling of frozen scallops and excessively high water addition are the two associated and hard-to-solve problems.

Due to their tasty large sphincter (adductor muscle), scallops are traded as high-priced seafood compared to many other molluscs. Their taxonomy could be confusing due to the similar appearance and because of assigning names in an unscientific way.

It is not uncommon for other low quality scallops to be incorrectly labelled and to pass as more valuable scallops (*Pecten maximus* or *Pecten jacobaeus*) in order to increase commercial profits (Näumann et al., 2012). Difficulty resides in their differentiation because most of the deep-frozen scallops are available on the european market without the shell, with or without the orange-yellow roe.

Scallops are caught in both the Atlantic and Pacific regions and marketed. Besides wild scallops catches, there is a targeted mussel culture economy that allows for faster and more

reliable harvesting. Processing techniques for commercial purposes vary by species, region and country. In US and Canada trap fisheries targeting sea scallops (*Placopecten magellanicus)*, use the technique consisting in the on-board removal of shells and viscera (to minimize risk of biotoxins formation). The catch is then stored in sacks on ice or in ice and seawater mixture. Catches can be frozen for short fishing trips, if necessary. The shells are also removed after landing.

Different processing techniques are adopted from catching or harvesting to the deep-frozen scallop product and have a significant impact on their quality. The current use of water to separate the innards and cleanse the adductor muscle is a necessary treatment step and inevitably leads to water uptake. Significant amounts of added water could get into the scallops' meat also by prolonged immersion. The use of additives such as polyphosphates increases the water-binding capacity, and leads to high water retention during thawing and preparation (Flick, 2012; Manthey-Karl et al., 2015).

Criteria for assessing the water content in scallops

Studies that dealt with the natural proximate composition of scallops and the influence of the various processing technologies on the product quality are to be mentioned. In the '70s and '80s, the US and Canada, both of which have traditionally been important fisheries for scallops, numerous investigations were carried out regarding the biology of these species (Rosseker & McKay, 2010). Untreated fresh scallops of known origin and with exact taxonomy assignation were subjected to studies that underlined the importance of the ripening cycle and seasonal dependence on the composition of the flesh. Interest was given also to the influence of the various processing steps on the end product, which is mainly sold on the market as deep-frozen muscle and the industrial scallops processing methods (Shumway & Parsons, 2006).

There is a considerable range in water content depending on season and food supply. Botta and Cahill (1992) determined between 74.6% and 80.2% in *Placopecten magellanicus*; a Canadian study (DuPaul et al., 1996) comes to similar results with 74.2-80.9%.

Comparable contents were also determined by other authors. Table 1 gives an overview of the composition of the raw materials and the differently treated meat from the literature. No difference was found between wild and cultivated forms of *P. magellanicus* (Naidu, 1978).

The interim storage of the muscle meat, which has already been freed from the shells, in ice/ ice water can cause the water content to rise. In doing so, DuPaul et al. (1996) found an initial natural content in *P. magellanicus* of 73.9-78.9%, whereas 74.2-82.6% was determined for the meat at the time of landing. At the time in North America there was an 80% limit for water content and exceeding this value was considered as added water. Rippen et al. (1998) stated, however, that this limit value is not practicable in connection with the strong fluctuations in the natural water content in the raw material.

In France, scallops (king scallops, *Pecten maximus*) are traditionally traded, most of which come onto the market fresh but also deep-frozen. The global trade in scallops of other origins and species did not stop at the French market either. As early as the late 1980s, research institutes, such as Institut Français de Recherche pour l'Exploitation de la Mer, were trying to find suitable criteria to detect unacceptable additives in foreign water. They examined freshly caught and frozen samples of *Pecten maximus* and *Mizuhopecten yessoensis* (Japanese scallops), which are imported to France in significant quantities from Japan. As a result, France introduced the determination of the water to protein ratio (W/P) as the most appropriate criterion for determining extraneous water. The untreated *Pecten maximus* muscle showed a ratio between 3.9 and 4.7. It was thus comparable to that for *Mizuhopecten yessoensis* (Loreal, 1990). Immersion in water for 15 minutes increased the (W/P) ratio to 5.2. In the case of frozen goods, de-glazing proved to be a critical point and should be carried out according to a standard procedure. Overall, a W/P < 5 is derived from the test results, taking into account a 5% increase in weight during

processing such as transport and washing, for *Pecten maximus* and *Mizuhopecten yessoensis*.

Additives in scallops

The muscle meat composition and the water to protein ratio (W/P) was determined by Manthey et al. (2015) in samples from the German retail. The results showed that a considerable number of samples had very high moisture contents and W/P ratios > 5. These facts were mainly true for *Placopecten magellanicus* and *Mizuhopecten yessoensis*. There was no conformity with the prescribed declaration of food additives.

Phosphates

Scallops are treated with water-binding additives, such as phosphates, in countries where it is permitted. In particular, phosphates (di- and higher condensed phosphates) are used to reduce the so-called "drip loss" that occurs as a result of freezing and thawing. They increase the water-binding ability of the muscles by supporting the dissociation of proteins and thus water retention. In the US, phosphates are classified as GRAS (Generally Recognized As Safe; FDA, 2012) food additives. They can be used in accordance with good manufacturing practice to create the impression of 'natural' succulence in the meat. There is no limit value for phosphorus-based additives in the end product. Significantly higher values are only considered critical in the USA in connection with increased water content (Kennedy, 2011).

According to the EU regulation 1333/2008 (EU, 2008) and the still valid directive 95/2/EG (EU, 1995) fresh molluscs must not be treated with phosphates. They are only allowed for frozen products. However, the added content in the product must not exceed 5 g/kg phosphate (calculated as P_2O_5). The Codex Alimentarius draft standard for scallops does not deviate from these restrictions on phosphates. No additives are allowed in the fresh raw product either.

Sidwell et al. (1977, review of the literature) gives an average natural phosphorus value of 0.27 ± 0.038 g P/100 g (≙ 0.62 ± 0.087 g P_2O_5/100 g) for scallop species (*Pectinidae* spp.) that are not specified in more detail, with a range of 0.21-0.34 g P /100 g (≙ 0.48-0.78 g P_2O_5/ 100 g).

Phosphates get into the product to be processed mainly by dipping. The concentrations of the phosphate baths vary from 2% to 10%, with the necessary exposure times depending on the type of fishery product (Schnee, 2004). Added table salt (NaCl) in concentrations of about 1.0% in a sodium polyphosphate brine can support the water binding capacity of the phosphates (Fisher *et al.*, 1996). Only at higher salt concentrations of over 6%, as is the case in the brines of salt fish, the water is no longer retained in the muscle meat, and dehydration processes occurs (Schröder, 2010). In contrast to the various phosphate compounds, table salt is not an additive, but an ingredient that serves to add flavour and can also increase the water content in the scallop. It must be declared without specifying the exact quantity.

Investigations in North Sea and North Atlantic

The sampling of untreated scallops took place on board the FFS "Walther Herwig III" during the voyages to the North Sea and the North Atlantic (232nd, 256th, 304th and 310th , between 2001 and 2007. Objectives of cruises as resumed were investigations on commercially used fish and molluscs species in respect to food safety and food quality.

Plattform Platform	Reise-Nr. Cruise-No.	Zeitraum Period	Projekt Project	Arbeitsgebiet Working area
Walther Herwig III	232	14.09.2001 - 04.10.2001		North Atlantic Ocean

Fahrtleiter Chief Scientist	Institut Institute	Auslaufhafen Port of departure	Einlaufhafen Port of return	Stationskarte Station map	Schiffsroute Trackchart
Oehlenschlager Jorg	Institut fur Biochemie u. Technologie der BFA Hamburg	Bremerhaven	Bremerhaven		

Ziel der Reise / Objectives of Cruise:

Investigation on commercially used fish species in respect of food saftey and food quality.

Messungen / Measurements

Institut Institute	Wissenschaftler Scientist	Anzahl Number	Einheit Unit	Typ der Messungen Type of measurements	Kommentar Comments	Daten im DOD Data in DOD
IBT	Oehlenschlager Jorg	49	hauls	B90 Other biological / fishery measurements	Inorganic analysis	no

Figure 1. Objectives and areal of Walther Herwig's III (WH III) 232nd trip

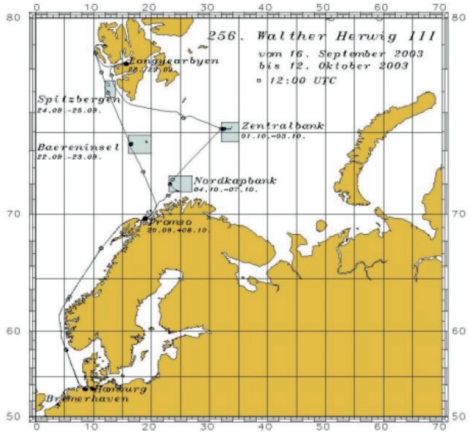

Figure 2. Explored waters
during Walther Herwig's III 256th expedition

I. On-bord investigations

Sensory assessment

The sensory assessment of scallops' quality included the on-board assessment of the samples after cooking for 8 minutes in the cooking bag. Inspectors experienced in assessing fishery products, each of whom had to assess all samples, assessed the quality characteristics of smell, appearance, taste and consistency on Torry rating scale of 0 to 5. The better the rating, the higher the number of points awarded.

As expected, the longer the scallops were stored in ice, the less fresh they becomed. The shelf life of scallops in ice (1.5°C) was compared to that under super-chilled conditions (-0.9°C), the latter increasing the shelf life from 9 to 16-17 days. If the pieces were additionally packed in a modified atmosphere, the shelf life in ice increased from 9 to 14 days, while under "super-chilled" conditions a further increase in shelf life to 21 days was observed. However, when the latter combination was applied, texture changes towards "meaty" were noted, possibly caused by increased drip.

At the time of sensory spoilage of ice-stored scallops after 10 days, *Photobacterium phosphoreum* dominated the spoilage flora. Ketones (33%) followed by amines (29%), alcohols (15%), acids (4%), aldehydes (3%) and a small amount of esters (<1 %) were detected as volatile components at the time of spoilage.

In addition to the sensory analysis, the total content of volatile N-bases (TVB-N value) was determined. The preparation of the perchloric acid extract, was made from 20 g of minced sample which were homogenized with 180 ml of 6% (w/v) perchloric acid for 30 s using an UltraTurrax, than the mixture was filtered and the clear perchlorate acid extract was frozen at −28°C until future analysis.

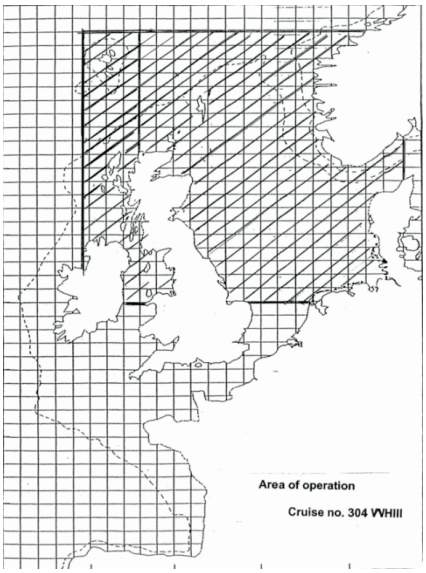

Figure 3. Operational area of Walther Herwig III
during the 304th cruise

Figure 4. Variable stations of fishing and area of
investigation during the 310th WH III cruise

TVB-N, cooking loss, pH, color and fish tester

The TVB-N value as an objectively determinable variable, which according to European standards should always be used when sensory findings indicate deviations in quality, showed that during the investigations the total content of volatile basic N-compounds were subjected to only minor changes (figure 7).

The determination of the pH value (using the laboratory pH meter 761, Knick Electronic Measuring Devices, Berlin) was carried out using a puncture electrode at different locations of the molluscs belonging to the test group (n = 4) accordingly.

The pH values are not subject to any significant changes during ice storage, as can be seen from the small standard deviations of the mean values over the entire storage period. However, as expected, the pH value was influenced by the measuring location.

The results of the instrumental color measurements showed only insignificant changes in lightness. The values determined with the Fish Tester Intellectron VI showed a largely linear relationship to the storage time. There was detected a significant decrease along with increasing storage time due to the decrease in the resistance of the samples along with reduced freshness. The values measured on freshly caught molluscs agree well with those previously described.

Modifications of the electrical properties of the muscle tissue were also measured with a torrymeter. Torrymeter values also decreased with decreasing freshness. The correlation of the Torrymeter values with the storage time or with sensory properties was significantly low.

Figure 5. Torrymeter

Measuring the electrical properties of the meat (resistance, conductivity and capacitance) transversely through the adductor muscle with a Fish Tester Intellectron VI is a reliable method with regard to the storage time in melting water ice. The tester value was determined for each scallop (n=5) in a test group measurement, determined at the level of the sphincter.

Figure 6. Wild scallops samples preparation before proximate analysis

II. Investigations at the Max Rubner Institute

Results showed large differences in quality of frozen scallop products and the problem of added water in frozen seafood in general and in frozen scallops in particular is remaining practically unchanged during.

The lack of quality and the affordable prices of frozen scallops is a fact due to the large range of products sold under the generic name “Frozen scallops” that are not correctly labelled and identified as species and regarding their added water percent. A too flexible regional

and global legislation led to the apparition of aquaculture assortments such as *Pangasius* spp. usually sold with very high amount of added water and at very cheep prices.
Water and additives contents of seafood were subjects largely discussed in many publications, in the past 20 years, and some laws were adopted by countries (France and USA) to protect consumers from purchasing practically just water instead of seafood they desire.

Figure 7. TVB-N (mg %) in analysed *Pectinidae*: R (refrigerated) and C (congelated) samples

All nutrient-rich seafood products are highly perishable. Large variety of additives and preservatives are used to maintain/ augment aspects regarding the quality, safety, wholesomeness, and consumer acceptance. Analytical methods have been developed to detect and monitor the residues of many additives and preservatives for ensuring consumer health and safety in compliance with regulatory EU standards. Because the current analytical efforts are considered, in their majority, time-consuming more advancement is needed and most important is to ensure rapid and simultaneous detection of multiple ingredients and additives (Surendran Nair et al., 2020).
Water absorption during processing cannot be technologically avoided. Therefore, intakes of 0.5 g/kg are tolerable. Taking into account that raw seafood originally contents 80% water, the final water added product would contain 81%. For a theoretical increase to 85%, percent that in real life is found in many marketed seafood products, is needed > 33% of added water although the labels are showing clean products.
A more serious fact is that phosphates content rarely withstands analytical testing being mostly eliminated before analysis during thawing.

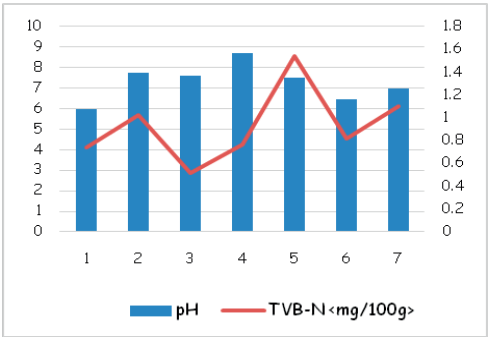

Figure 8. Variation of pH and TVB-N (mg %) in the analyzed *Pectinidae* samples

The consumer is therefore often cheated when buying scallops and/ or other seafood if the composition of the product does not correspond truly to the labelling.

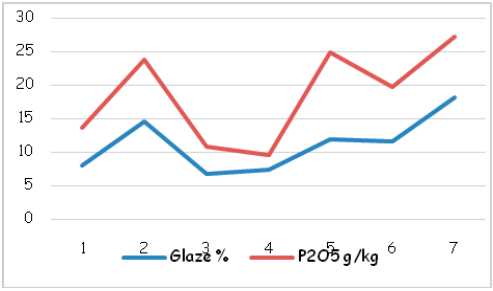

Figure 9. The variation of phosphorus content depending on the proportion of glaze in the analyzed Pectinidae samples

Salutary efforts were made by the CAC in order to develop standards and codes of good practices. Suitable information for the buyers and complete labelling in the food chain, respected both by the producer and the trader is a must for the scallops' and seafood market generally. Question are to be addressed regarding the form in which the manufacturer's information can be checked for traceability reasons (Tudor et al., 2022).
To establish reliable values for litigation purposes, firstly reliable values for the initial proximate composition of the raw material should be settled and published. As an exemple, measuring the water/protein (W/P) ratio offers valuable assessment perspectives.
Summarized in Figure 10 are the most important analysis W/P ratio results of the various samples. The pH values measured were

between 5.98 and 8.71 while fresh scallops have usually pH-values situated around 6.0. Values of pH above 7.0 were previously associated with the use of additives.

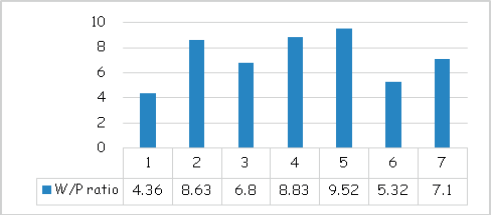

Figure 10. W/P ratio in marketed *Pectinidae*.

All other samples showed a clear relationship between water content and W/P ratio. With a water content of over 80%, the W/P ratio was > 6. As regards W/P ratio calculated, only one product had results in the borderline range with 77.1% water and a W/P of 4.4 (Table 1).

Table 1. Water (W) and protein (P) content of Wild *Pectinidae* in %

Sample no.	1	2	3	4	5	6	7
W%	77.12	86.53	84.49	86.7	88.03	79.21	84.35
P%	17.67	10.03	12.43	9.82	9.25	14.88	11.9

Phosphate amount largely varied between the different samples from 2.2 g P_2O_5/kg in *Placopecten magellanicus* and 12.9 g P_2O_5/kg in *Pecten yessoensis*. This result should also take into account the results obtained using total phosphate determination methods in fishery products which showed very often large natural fluctuations.

Different qualities of *Pecten maximus* were previously found on the european market . None of these marketed scallops declared the additives in the list of ingredients. The calculated W/P ratios were between 4.3 and 8.6. Research on fresh scallops confirmed a water to protein ratio in fresh scallops below 5 (Manthey-Karl et al., 2015).

Table 2. Investigated *Pectinidae* samples from the German market

Species	Glaze < % >	pH	TVB-N <mg/100g>	DM < g/kg >	Ash < g/kg >	Fat <g/kg>	Protein <g/kg>	P_2O_5 < g/kg>	NaCl <g/kg>
Pecten spp.	8	5.98	0.74	228.8	12.5	7.1	176.7	5.64	3.8
Pecten spp.	14.5	7.75	1.02	134.7	20.5	4.1	100.3	9.33	3.1
Placopecten magellanicus	6.7	7.62	0.52	155.1	17.4	4.1	124.3	4.15	5
Placopecten magellanicus	7.3	8.71	0.77	133	30.3	3.3	98.2	2.2	1.1
Placopecten magellanicus	11.6	6.45	0.82	207.9	17.3	5.9	148.8	8.07	3.8
Pecten yessoensis	11.9	7.52	1.54	119.7	25.2	4.5	92.5	12.94	0.9
Pecten maximus	18.1	6.99	1.1	156.5	20.6	8.3	119	9.11	1.8

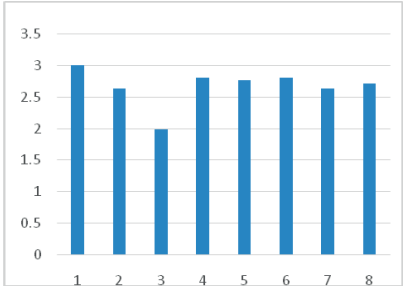

Figure 11. P_2O_5 content (oy) as <g/kg> in 8 wild scallops samples (ox)

Investigated purchased from the German market bivalve mollusc samples, showed (cf. Table 3), in two different species (*Pecten* spp., *Placopecten magellanicus*) perfect positive correlations (R2=1) between the amount of phosphate and the pH values, while in the other 5 samples, the correlations were negative (R2 = –1). A negative but weak correlation was established between protein percentage and TVB-N % (R2 = –0.26).

There were reasonable positive correlations between phosphate and ice glaze (R2 = 0.48), a fact that was expected (Figure 12). The highest amount of glaze (18%) overpassing the 10% limit, was found in marketed *P. maximus* percent which was correlated with a high

quantity of phosphates (9 g P_2O_5/kg) situated next to the doubled maximal value permitted (5 g P_2O_5/kg).

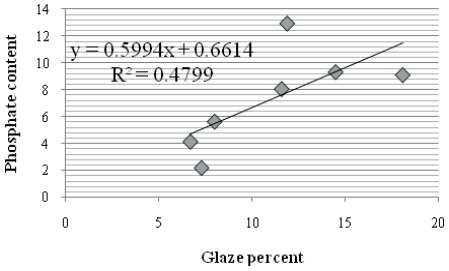

Figure 12. Scatter plot showing a reasonable correlation (R^2 = 0.48) between the amount of phosphate (in g P_2O_5 /kg) and the proportion of glaze ice (%) in scallops samples from German market

The percentage of water found in *P. magellanicus* was between 79.2 and 86.7%, higher values than those previously reported in the literature (74.2-82.5%) by Du Paul et al. (1996) for untreated scallops. The same parameter, measured in *P.maximus*, was 77.1-86.5%, the values being clearly higher than those reported by Sidwell (1977) in the case of raw scallops. The marketed *P. yessoensis* reached the water percent of 88%, a value clearly higher than those specified for wild scallops by Loreal (1990), namely 77.6-79.2% . The total phosphate content in marketed Pectinidae was in 5 samples perfect negatively correlated with the pH, while in 2 investigated samples the same correlation found was perfectly positive (Table 3).

Table 3. Regression equations for phosphate (y) versus pH in purchased scallops samples

Species	Regression equations	Pearson correlation coefficient
Pecten spp.	Y= - 4.6471 x + 35.539	R^2=−1
Pecten spp.	Y = 10.206 x – 53.411	R^2=1
Placopecten magellanicus	Y = 19.147 x - 105.79	R^2=1
Placopecten magellanicus	Y = - 15.941 x + 102.85	R^2=−1
Placopecten magellanicus	Y = - 4.7647 x + 34.943	R^2=−1
Pecten yessoensis	Y= - 6.2353 x + 44.277	R^2=−1
Pecten maximus	Y = - 2.12 x + 11.2	R^2=−1

Investigated samples caught in the wild during 4 of WH's III cruises showed a small variability of natural phosphates content in the range of 1.98 to 3 g P_2O_5/kg (Table 4).

Table 4. Total Phosphates content of Wild *Pectinidae* in g P_2O_5/kg

Species	g P_2O_5/kg
Pecten maximus (Walter Herwigs III 232nd Trip)	*3.01*
Pecten maximus (Walter Herwigs III 232nd Trip)	*2.64*
Pecten spp. (Walter Herwigs III 256th Trip)	*1.98*
Aequipecten opercularis (Walter Herwigs III 310th Trip)	*2.81*
Aequipecten opercularis (Walter Herwigs III 310th Trip)	*2.77*
Aequipecten opercularis (Walter Herwigs III 310th Trip)	*2.80*
Pecten maximus (Walter Herwigs III 304th Trip)	*2.63*
Pecten maximus (Walter Herwigs III 304th Trip)	*2.72*

Litigation concerns

Although, there are specific roles for additives in foods (e.g., direct additives such as phosphates added in seafood to retain moisture and protect flavour), the chances of minute amounts of indirect food additives (for instance, packaging substances) finding their way into foods during processing and storage cannot be neglected. Therefore the competent authorities require that all materials coming in contact with food should be certified as safe before they are used in the indicated manner.

Food additives are studied, regulated, and monitored to ensure that consumers feel safe about the foods they eat and are protected from hazards. The entire responsibility rests with the manufacturers which are entitled to ensure that the ingredients are of a food-grade purity and comply with specifications and restrictions.

All international trade risk assessments are conducted by an independent, international expert scientific group, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (WHO, 2018), ensuring that there are monitoring steps in place at various levels of global food production.

Phosphates are added to seafood to increase water-holding capacity (Sebranek, 2015). Sodium tripolyphosphates are additives of the phosphate family used in the seafood industry with a humectant function, i.e., those substances maintain the moisture of the product, being more used in scallops, shrimp and lobsters processing.

Sodium tripolyphosphates in scallops, added in concentrations from 2.5% to 10% (1 min) until the moisture percent was increased to 82-86%, respectively, in conjunction with 1% of NaCl, was shown efficient to prevent the drip loss during defrosting and after cooking, and inhibited the microbial growth. Generally, a long exposure to tripolyphosphates demonstrated no other notable advantage, while a short exposure of scallops to these additives produced desirable functional effects, generally without exceeding 83% of moisture in the final product (Rippen et al. 1993; Gonçalves & Duarte Ribeiro, 2008).

Health concerns and safety assessment regarding the chronic exposure to food additives and the associated adverse health impacts are growing concerns globally (Maffini et al., 2017). With the evidence unveiled through epidemiological studies linking the consumption of phosphate food additives and increased cardiovascular risk, the European Food Safety Authority is currently pursuing a high-priority re-evaluation of added phosphates in seafood and meat products (Foley et al., 2009; Cancela et al., 2012; EFSA, 2013).
(Surendran Nair et al., 2020)

A maximum water content in scallop meat is not internationally established by food laws. Some authors (Gonçalves & Duarte Ribeiro, 2008) indicate the 5 g P_2O_5/kg maximal value adopted in the EU. Codex Alimentarius Committee (CAC) for Fish and Fishery Products, discussed the topic of adding water to frozen scallops, and this subject has been controversial for a long period of time (Schröder & Siegert-Clemens, 2012). At the CAC's 32nd meeting that took place in 2012, the member states (MS) established the labelling options, that were more transparent and consumer-protecting: "Scallop (name…) with added water" or "Product from scallop (name…) with added water" or another labelling announce which fully informes the buyer. Draft standard for frozen scallops was in 2015 at 6/8 stage process, pending acceptance by the CAC and was finally amended in 2016 and revised in 2017. Recommendations regarding the added water were made for "Quick frozen Scallop Meat" and "Quick Frozen Roe-on Scallop Meat" that shall have the mention "added water" as a part of products' name.

CONCLUSIONS

The very high water content, corroborated with the large amounts of total phosphate (up to 13 g P_2O_5/kg) detected in the corresponding samples from the German market, entitle us to say that large amounts of phosphate-based additives (well over the legal limits) were used for traded scallops, intended to increase the water-binding capacity.

On the other hand, samples caught in the wild during 4 of WH's III cruises showed a small variability of natural phosphates content in the range of 1.98 to 3 g P_2O_5/kg.

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IDENTIFICATION OF ANTIBIOTIC RESISTANCE PATTERNS IN *Escherichia coli* BACTERIA FROM CLOACAL SWAB SAMPLES OF BROILER CHICKENS FROM FARM THAT USE PROBIOTIC *Lactobacillus* sp.

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Abstract

This study aims to determine the resistance pattern of Escherichia coli in broiler chickens given Lactobacillus sp. during the maintenance period. A total of 48 chicken cloacal swab samples given Lactobacillus sp. and 48 samples of untreated chicken cloacal swabs were taken from farms in Cimarigi Village, Sukadana District, Ciamis Regency. E. coli was isolated and identified, followed by an antimicrobial susceptibility test using the disc diffusion method according to the Kirby Bauer method against the antibiotics amoxicillin (20 µg), erythromycin (15 µg), and ciprofloxacin (5 µg). Data on the diameter of the antibiotic inhibition zone were compared with standard bacterial sensitivity and classified as sensitive, intermediate, and resistant. The results showed that E.coli from both sample groups were 100% resistant to amoxicillin and erythromycin. The pattern of resistance to ciprofloxacin in the sample group given probiotics was 76% intermediate and 24% resistant, while the sample group that was not given probiotics was 96% resistant, 2% intermediate, and 2% sensitive.

Key words*: Escherichia coli, Lactobacillus sp., resistance pattern of antibiotics, broiler chicken*

INTRODUCTION

The use of AGP (Antibiotic Growth Promotor) in broiler chicken feed can result in the formation of resistant bacteria in the body of broiler chickens and continue in humans (Prasetyo, 2020). The formation of bacteria that are resistant to antibiotics due to high exposure to antibiotics so bacteria form a defense mechanism against antibiotics (Besung et al, 2018). Among the microorganisms carrying antibiotic-resistance genes with the highest clinical relevance are Extended Spectrum *β-lactamase*(ESBL)-producing *Enterobacteria*, especially *Escherichia coli* which has been listed among the twelve serious threats that are drug-resistant by the Centers for Disease Control and Prevention (CDC) (CDC, 2017). *Eschericia coli* is a classic indicator of fecal contamination that is routinely used to assess the microbiological quality of water and food and plays a major role in the spread of antibiotic resistance (Szmolka & Nagy, 2013). *Escherichia coli* is a bacterium that is an opportunistic pathogen that is commensal in the digestive tract of both humans and animals and is spread in the environment (Loncaric et al., 2013). The bacteria Escherichia coli, which originates from poultry farms, has the potential to disseminate into the environment, primarily through manure, serving as a means for the transmission of resistance from poultry farms (Wegener, 2012). Currently, the incidence of antibiotic resistance has become a global problem, based on data obtained in 2009, Indonesia is a country with the title of multi-drug resistance ranked 8th out of 27 countries with the highest rating in the world (Supriyantoro, 2011). An alternative solution of antibiotics is needed that can be used to prevent poultry disease and also improve the performance of chickens during rearing but does not have a negative impact on its use. One of them is the use of probiotics. Giving

Lactobacillus spp. to broiler chickens as a treatment can reduce the production of toxins by harmful microorganisms and minimize the negative effects caused by pathogenic bacteria. This can improve feed absorption by repairing the digestive organs, particularly the small intestine, boosting the production of digestive enzymes, increasing antibody production in the digestive tract, and generating vitamins and antimicrobial substances. These actions help achieve optimal digestive organ health (Sumarsih et al., 2012).The positive effects arising from the use of *Lactobacillus* spp. and maintaining the stability of the gut microbiota is also a mechanism by which probiotics can influence the spread of antibiotic resistance. A study conducted by Ouwehand (2016) showed that lactic acid produced by *lactobacilli* strains can increase the susceptibility of Gram-negative bacteria to antimicrobial agents. Lactic acid produced by *Lactobacillus* spp. can work as a permeabilizer on gram-negative bacterial cells. Permeabilizers do not need to possess bactericidal or bacteriostatic properties against gram-negative bacterial cells. Instead, their function is to facilitate the penetration of other compounds, thereby enhancing susceptibility to hydrophobic antibiotics, detergents, lysozyme, or bacteriocins (Alakomi et al., 2005 as cited in Hongmei et al., 2021). The mechanism of action of probiotics involves competition between probiotics and pathogenic microorganisms. The antagonistic competition mechanism among bacteria in the digestive tract serves as an ecological balance, preventing excessive growth of any specific species within the digestive tract.

MATERIALS AND METHODS

Research materials

The sample used in this study was a cloacal swab sample taken from 48 Cobb chickens given the probiotic Lactobacillus sp. and 48 broiler chickens that were not given probiotics according to the program from the broiler farm owned by PT. YAM. In this study, no intervention was performed on the sample population during maintenance. The sampling location was carried out in Cimarigi Village, Sukadana District, Ciamis Regency in June - July 2022.

Sampling method

To collect cloacal swab samples, chickens that met the research criteria were captured. A sterile cotton swab (Nesco) was then gently inserted into the cloaca, rotating it slowly to a depth of 1.5 to 2.5 cm. The swab was rotated 360° inside the cloaca before being carefully removed. Any excess sample (feces > 0.5 cm) was discarded. The swab sample was placed into a transport medium by opening the tube and inserting the swab tip until it reached about ¾ of the bottom of the tube. The excess swab tip was cut using sterilized scissors soaked in 70% alcohol, and the tube was tightly closed. A number label was assigned to the tube containing the sample. The sample was then placed in a cool box at a temperature of 2-8°C and sent to the laboratory within one day of sampling (BAVET Semarang, 2018).

Isolation, identification, and bacterial sensitivity test of *Eschericia coli*

All samples are then sent to the laboratory of the West Java Animal Health and Veterinary Public Health Center for sensitivity testing against antimicrobials. Each sample from both groups was grown on Nutrient Agar and Eosin Methylene Blue Agar (EMBA) media. Bacterial colonies that are metallic green in color with a dark center are suspected as *E. coli* colonies which will be followed by the identification of the bacteria. Identification was carried out by Gram staining and biochemical tests, using Tripple Sugar Iron Agar (TSIA), Simmons citrate Agar (SCA), Sulphide Indole Motility (SIM), and Methyl Red Voges Proskauer (MRPV) methods (Oxoid, Basingstoke, UK).

Escherichia coli bacteria that have been identified are followed by a sensitivity test to antibiotics Amoxicillin (20 µg), Erythromycin (10 µg), and Ciprofloxacin (10 µg). The sensitivity test was carried out by agar diffusion using the Kirby-Bauer method. Colonies of *E. coli* were then grown in liquid Mueller Hinton medium and incubated for 2 hours at 37ºC until a turbidity equivalent to 0.5 Mc Farland was obtained (containing 106 cells/ml). Then 0.5 ml of the culture was planted on Mueller Hinton Agar (MHA) media and spread evenly and incubated for about 30 minutes.

Data analysis

The data obtained from positive *Escherichia coli* cloacal swab samples will be subsequently analyzed both descriptively and quantitatively. This analysis will involve calculating the percentages of bacteria that exhibit sensitivity, intermediate resistance, and full resistance to antibiotics. The test results will be presented in tabular form. Furthermore, statistical analysis of the data will be conducted using the Mann-Whitney test, which is utilized for comparative analysis of two independent samples containing ordinal data (Siregar, 2013).

RESULTS AND DISCUSSIONS

Identification of bacteria

Identification results of broiler chicken cloacal swab samples from PT. YAM which shows positive isolates of *Eschericia coli* bacteria is shown in the Table 1.

Table 1. *Eschericia coli* identification

No.	Sample	Total Sample	*Eschericia coli* positive	*Eschericia coli* negative
1.	Non-probiotic Group	48 Samples	48 Samples	(-)
2.	Probiotic Group	48 Samples	48 Samples	(-)

The results in this study were obtained from two different groups, namely the group of chickens that were treated with antibiotics as many as 48 samples, and the group of chickens that were given antibiotics and probiotics as many as 48 samples.

Based on the data in Table 1. with a total sample of 48 samples from each group, positive results were obtained from the probiotic and non-probiotic sample groups, each of which was 48 positive samples of *Eschericia coli*.

Table 2. Zone of Inhibition Interpretation Standard

Antibiotics	Bacteria	Zone of Inhibition Interpretation Standard (mm)		
		Resistent	Intermediate	Sensitive
Amoxicillin	*E. coli*	≤ 13 mm	14-17 mm	≥ 18 mm
Ciprofloxacyn	*E. coli*	≤ 15 mm	16-20 mm	≥ 21 mm
Erythromycin	*E. coli*	≤ 12 mm	14-22 mm	≥ 23 mm

Resistance pattern of Eschericia coli

Antimicrobial susceptibility test (AST) is used to determine the pattern of resistance of bacteria to antibiotics. The potency of an antibiotic that was tested for sensitivity to *Escherichia coli* bacteria was classified into three criteria according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI), shown in the Table 2.

Resistance pattern to amoxicillin

The results of sample testing for amoxicillin antibiotics are presented in the Tables 3 and 4.

Table 3. Pattern of amoxicillin resistance from probiotic group samples

Amoxicillin			
Probiotic Group	Diameter of Inhibition Zone		
	0-13 mm	14-17 mm	≥ 18 mm
Interpretation	Resistent	Intermediate	Sensitive
Jumlah	48 Samples	0 Sample	0 Sample

Table 4. Amoxicillin resistance patterns from non-probiotic group samples

Amoxicillin			
Non-Probiotic Group	Diameter of Inhibition Zone		
	0-13 mm	14-17 mm	≥ 18 mm
Interpretation	Resistent	Intermediate	Sensitive
Jumlah	48 Samples	0 Sample	0 Sample

Table 5. Mann-Whitney U Test for amoxicillin resistance

Statistic	Value	Information
Mann-Whitney U	1152,000	H_0 accepted
Sig. (2-tailed)	1,000	

Based on the data above, it can be seen the distribution of data for the Probiotic and Non-Probiotic groups in testing the antibiotic amoxicillin. The results of testing the samples from the group of chickens that were given probiotics showed resistant results in all samples. AST testing on samples from chickens that were not given probiotics also showed resistant results in all samples. Next, hypothesis testing is conducted to examine the difference between the probiotic and non-probiotic groups in the testing of erythromycin as follows:

H0: ($\eta 1 = \eta 2$), there is no difference between the group of chickens given *Lactobacillus* sp. probiotics and the group of chickens not given *Lactobacillus* sp. probiotics.

H1: ($\eta 1 \neq \eta 2$), there is a difference between the group of chickens given *Lactobacillus* sp. probiotics and the group of chickens not given *Lactobacillus* sp. probiotics.

With $\alpha = 5\%$, the results of the analysis are as follows. Statistical test results were not significantly different ($P > 0.05$) between cloacal swab samples of chickens given probiotics and not given probiotics (Table 5).

Resistance pattern to erythromycin

The results of sample testing for erythromycin antibiotics are presented in the Table 6.

Table 6. Patterns of erythromycin resistance from probiotic group samples

Erythromycin			
Probiotic Group	Diameter of Inhibition Zone		
	0-13 mm	14-22 mm	≥ 23 mm
Interpretation	Resistant	Intermediate	Sensitive
Total	48 Samples (100%)	0 Sample	0 Sample

Table 7. Patterns of erythromycin resistance from non-probiotic group samples

Eritromisin			
Non-Probiotic Group	Diameter of Inhibition Zone		
	0-13 mm	14-22 mm	≥ 23 mm
Interpretation	Resistant	Intermediate	Sensitive
Total	48 Samples (100%)	0 Sample	0 Sample

Table 8. Mann-Whitney U test for erythromycin resistance

Statistic	Value	Information
Mann-Whitney U	1152,000	H_0 accepted
Sig. (2-tailed)	1,000	

The table above shows the results of the distribution of AST data for the antibiotic erythromycin in both groups. The probiotic and non-probiotic groups had the same results, that is, all samples obtained resistant results with a percentage of 100%. Statistical test results were not significantly different ($P > 0.05$) between cloacal swab samples of chickens given probiotics and not. The pattern of *Escherichia coli* resistance did not change from the cloacal swab samples of chickens given *Lactobacillus* sp. to erythromycin can also be due to the antibacterial properties of the lactic acid component produced by *Lactobacillus* sp. not persistent in *Eschericia coli* that has been cultured from the sample.

Resistance pattern to ciprofloxacin

The results of sample testing for ciprofloxacin antibiotics are presented in the following tables.

Table 9. Pattern of Ciprofloxacin Resistance from Probiotic Group Samples

Ciprofloxacin			
Probiotic Group	Diameter of Inhibition Zone		
	0-15 mm	16-20 mm	≥ 21 mm
Interpretation	Resistant	Intermediate	Sensitive
Total	13 Samples (27%)	35 Samples (73%)	0 Sample

Table 10. Pattern of ciprofloxacin resistance from non-probiotic group samples

Ciprofloxacin			
Non-Probiotik Group	Diameter of Inhibition Zone		
	0-15 mm	16-20 mm	≥ 21 mm
Interpretation	Resistant	Intermediate	Sensitive
Total	46 Samples (95%)	1 Sample (2%)	1 Sample (2%)

Table 11. Mann-Whitney U Test for ciprofloxacin resistance

Statistic	Value	Information
Mann-Whitney U	377,500	H_0 denied
Sig. (2-tailed)	0,000	

All samples from the probiotic group showed an intermediate resistance pattern with a percentage of 73%, 27% of the samples showed resistant results, and none of the samples had sensitive results. Furthermore, for the non-probiotic group, sample testing obtained resistant results with a percentage of 96%, intermediate by 2%, and sensitive by 2%. The statistical test results showed a significant difference ($P < 0.05$). This shows that there is a significant difference between the resistance patterns of *Eschericia coli* from the sample groups that were not given probiotics and those that were given probiotics, whereas in the samples given probiotics, 35 out of 48 samples had intermediate resistance patterns.

DISCUSSIONS

Amoxicillin and erythromycin resistance patterns

There was no difference in the pattern of resistance between chicken samples given *Lactobacillus* and not given *Lactobacillus* to the pattern of amoxicillin resistance. Dust scattered in chicken coops can contain 105-106 *Eschericia coli* cells/gram and may spread into the cloaca of chickens (De Carli et al., 2015; Sayad et al., 2018). In addition, the more aerobic condition of the cloaca can also cause changes in the metabolic pathways of *Lactobacillus* sp. Research conducted by Quatravaux et al. (2006) in Zotta et al. (2017) found that under aerobic conditions, the presence of oxygen can interfere with the transcription of the nLDH operon and that NADH-dependent oxidase NOX can compete with nLDH for the NADH pool, diverting pyruvate from lactate production. Acetate accumulation has been found in aerobically grown cultures of homofermentative and heterofermentative *Lactobacillus* species. This allows *Eschericia coli* from the environment and digestive tract to grow better in the cloaca compared to *Lactobacillus* sp. Based on this, it can be concluded that the use of *Lactobacillus* sp. did not change the resistance pattern of *Eschericia coli* strains taken from cloacal swab samples of broiler chickens to amoxicillin.

Based on the findings by Alakomi et al. (2005), lactic acid works as a strong disintegrating agent against the outer membrane of gram-negative bacteria which can cause the release of lipopolysaccharide bonds, making bacteria more susceptible to antimicrobial agents when interacting directly with bacteria. Lactic acid which interacts directly with lipopolysaccharide cell membranes can

increase the susceptibility of gram-negative bacteria to hydrophobic antibiotics, one of which is erythromycin. The effect of taking samples from a cloacal swab also affects the test results because, under aerobic conditions, *Lactobacillus* sp. diverts the pathway of pyruvate metabolism away from lactate production. The results of this study indicate that Lactobacillus sp. did not affect the susceptibility of *Eschericia coli* taken from cloacal swabs of broiler chickens to the genetic stage so the pattern of resistance to erythromycin did not change.

Ciprofloxacin resistance pattern

All samples from the probiotic group showed an intermediate resistance pattern with a percentage of 73%, 27% of the samples showed resistant results, and none of the samples had sensitive results. Furthermore, for the non-probiotic group, sample testing obtained resistant results with a percentage of 96%, intermediate by 2%, and sensitive by 2%. The statistical test results showed a significant difference ($P < 0.05$). This shows that there is a significant difference between the resistance patterns of *Eschericia coli* from the sample groups that were not given probiotics and those that were given probiotics, whereas in the samples given probiotics, 35 out of 48 samples had intermediate resistance patterns. The difference in the pattern of resistance from testing the two samples is thought to be due to the probiotic *Lactobacillus* sp. has an anti-adhesion effect that can prevent the adhesion of 80% of *ciprofloxacin-resistant Eschericia coli* strains studied in Caco-2 cell cultures (Abedi et al., 2013). The result indicate that *Lactobacillus* sp. supplementation can increase *Escherichia coli* sensitivity to ciprofloxacin.

Research conducted by Abedi et al. (2013) on Caco-2 cell culture, *Lactobacillus* sp. is more successful at binding to cellular receptors and preventing the adhesion of pathogenic bacteria by pre-attaching to those sites. The anti-adhesion effect produced by *Lactobacillus* sp. through the mechanism of production of antimicrobial materials including bacteriocins, lactic acid, and biosurfactants can effectively prevent the formation of biofilms from *Eschericia coli*. The biofilm produced by *Eschericia coli* can make it resistant to many antibiotics compared to *Eschericia coli* in a free (planktonic) state and almost resistant to ciprofloxacin, carbenicillin, cloxacillin, cephaloridine, novobiocin, and vancomycin (Yeganeh et al., 2017). Based on the findings from a study conducted by Yeganeh et al. (2017), it was found that *Lactobacillus* sp. has an inhibitory effect on Ciprofloxacin Resistant Uropathogenic *Eschericia coli* (UPEC) strains in body tissues. The results of this study confirm the hypothesis of Yeganeh et al. (2017) that *Lactobacillus* sp. can inhibit the growth of *Eschericia coli* strains that are resistant to ciprofloxacin by preventing the formation of biofilms so that different patterns of resistance are obtained in the research results. The decrease in the percentage of resistant *Eschericia coli* strains in samples originating from chickens given probiotics compared to the unexpected was due to *Eschericia coli* strains that were resistant to ciprofloxacin derived from breeding so that after being given *Lactobacillus* sp., the growth of the *Eschericia coli* strain was inhibited. Further research is needed to determine the *Eschericia coli* strain obtained from cloacal swabs of broiler chickens given *Lactobacillus* sp. to confirm the findings of this study.

CONCLUSIONS

Based on the findings in this study, it can be concluded that the administration of *Lactobacillus sp.* in broiler chickens affected the resistance pattern of *Eschericia coli* to ciprofloxacin from 96% resistant, 2% sensitive and 2% intermediate to 27% resistant, 96% intermediate, and 0% sensitive. There was no change in the pattern of resistance to amoxicillin and erythromycin.

SUGGESTIONS

Further research is needed to see the effect of *Lactobacillus* sp. on the pattern of resistance of *Eschericia coli* in other segments of the digestive tract of broiler chickens to the class of antibiotics commonly used for broiler therapy.

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THE USE OF HISTOLOGICAL METHODS IN MEAT AND MEAT PRODUCTS FOR FRESHNESS DETERMINATION - A POSSIBLE FUTURE TREND?

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Abstract

Food products preservation was and will be of general interest, especially due to the current challenges faced by the industry. In this regard, data collection methods diversity represents an opportunity for extending the current knowledge in meat preservation. Meat and meat products microscopical imaging leads to obtaining some extremely useful information that cannot be substituted by any numerical equivalents of other comparative methods. This fact has been noted in many countries worldwide and was adopted as a complementary method for assessing the integrity, quality and shelf life of food products. In this regard, the aim of this literature review was to assess the benefits of using the histological method for freshness determination in meat and meat products, along with the documented procedure and interpretation.

Key words*: meat, meat products, histological assessment, meat freshness.*

INTRODUCTION

The histological method is a valuable tool for assessing the meat and meat products freshness (Avinee et al., 2010). This method involves the examination of tissue samples under a microscope to identify changes in tissue structure and composition that occur as the meat ages. These changes can provide important insights into the quality and safety of meat, and can be used to optimize processing and packaging techniques to ensure the production of fresh, high-quality meat products (Abbasy-Fasarani et al., 2012; Abdel Hafeez et al., 2016).

One of the key advantages of the histological method is its ability to detect changes in muscle tissue that occur at the cellular level. As meat ages, the structure of muscle fibres begins to break down, resulting in changes in the appearance and texture of the meat. These changes can be observed and quantified using histological techniques, allowing researchers and food producers to determine the optimal time frame for processing and packaging meat products (Kalyuzhnaya, 2020).

Histological analysis can also provide important information about the meat tissue composition. For example, the presence of connective tissue or fat can affect the tenderness and flavour of meat, and changes in the distribution of these tissues can indicate changes in the quality of the meat.

Histological techniques can be used to identify the types of bacteria (bacilli or cocci) present in meat samples, allowing food producers to respond appropriately in order to prevent contamination and ensure the safety of their products. In this regard, the aim of this paper is to identify the histological assessment main advantages and disadvantages along with the current perspectives of use in meat and meat products freshness determination.

HISTORICAL PERSPECTIVE AND CURRENT TRENDS

The invention of the optical microscope at the end of the 16th century represented a significant opportunity for food researchers, as it was the first instrument that could magnify the images of samples 20 to 30 times their original size. The resolution of contemporary optical microscopes is 103 times that of the human eye, resulting in a 4 to 1500 times magnification. In addition, they are adaptable

and can be used in a variety of configurations, including bright field, phase contrast, differential interference contrast, polarised light, and fluorescence (Toldrá et al., 2009).

Since 1850, it has been acknowledged that microscopic food evaluation technologies provide a comprehensive, inexpensive, and rapid alternative for getting essential data regarding the control and quality of food products. Since 1920, meat and meat products have been evaluated using histological techniques. Currently, 75% of European studies on food analysis are based on meat and meat products, with 25% of these researches focusing on histological evaluation (Guelmamene et al., 2018).

Acquiring a microscopic image of the meat and meat products structure results in the import of incredibly relevant data that cannot be replaced by the numerical equivalents of other comparative methods for assessing the food quality or safety (Řezáčová et al., 2011). This fact has been recognised in numerous countries, where it has been used as a complementary method for evaluating the food safety, quality, and preservation (Isaconi et al., 2020).

In addition, food products microscopic examination enables the determination of content and types of unauthorised animal or vegetable tissues, the labelling accuracy by histomorphometric analysis, raw materials specificity (origin), tenderness analysis, the detection and evaluation of mechanically separated meat, and meat quality prediction under freezing and thawing conditions, ante-mortem animal handling effects evaluation, parasites or microbial cells detection. Under these circumstances, the histological evaluation for meat and meat products quality and preservability control can be rapid, sensible, and accurate (Guelmamene et al., 2018).

From the evaluation methodologies point of view, current microscopic technologies have surpassed the spatial resolution limitations of conventional optical microscopy, which is based on a light beam and objectives.

Nonetheless, conventional optical microscopy is still utilised for numerous reasons. One of these is the chromatic capability of distinguishing different structures or substances by staining, which facilitates interpretation and imaging visualisation. In addition to optical microscopy, other types of microscopy (e.g., electron and X-ray microscopy) use stains, albeit in considerably less numbers than in optical microscopy (Toldrá et al., 2009).

In terms of costs, optical microscopy is far less expensive than other methods of microscopy. Moreover, no particular environmental conditions are necessary (Toldrá et al., 2009).

ASSOCIATED CHALLENGES

One of the primary challenges associated with the histological method is the need for specialized equipment and expertise (GOST 23392-2016, 2016). The method requires the use of a microscope and specialized staining techniques to prepare tissue samples for examination. Additionally, interpreting the results of the histological analysis requires specific expertise, which can be challenging to acquire. As a result, many small-scale meat producers may not have access to the necessary equipment or expertise, limiting their ability to utilize this method for freshness determination.

Another challenge associated with the histological method is the time and cost required for analysis. Preparing tissue samples for examination can be time-consuming, and the process requires specialized staining techniques, which can be costly.

Accuracy is another challenge associated with the histological method. While the method can provide valuable information about the quality and freshness of meat and meat products, the accuracy of the results can be influenced by several factors, including the quality of the tissue samples, the expertise of the technician performing the analysis, and the staining techniques used (GOST 23392-2016, 2016). Any inaccuracies in the analysis can affect the reliability of the results and potentially lead to incorrect conclusions about the freshness of the product.

The histological method is also limited in its ability to assess other important attributes of meat quality, such as flavour, aroma, and texture. While the method can provide information about the structure and composition of the tissue, it does not provide information about other sensory attributes that can influence the overall quality and freshness of the product. As a result, the histological

method may need to be used in conjunction with other methods for assessing meat quality and freshness.
Moreover, the optical microscopy has a limited depth of focus, difficulties in excluding optical defects, and limited magnification possibilities (Toldrá et al., 2009).
In contrast, considering the fact that none of the freshness assessment methods used in the food industry is perfect in terms of execution, costs or precision, optical microscopy is still a practical, simple, and cost-effective approach compared with others.

WORLDWIDE PERSPECTIVE

The histological method has been adopted in the meat industry by several countries worldwide, including the European Union (EU), United States, Canada (Canadian Food Inspection Agency, 2018), Russian Federation and Australia. In the EU, histological assessment is used as part of the post-mortem inspection of meat from some species. In some particular situations, the EU has strict regulations in place to ensure that the meat from these animals is subject to histological examination to determine its fitness for human consumption. For example, according to Regulation (EC) no. 999/2001, the histological method can be used to diagnose Bovine Spongiform Encephalopathy in bovines. The EU also requires that any imported meat must meet the same standards (European Commission, 2005; European Commission, 2004, European Commission, 2001).
Outside of the EU, other countries have also adopted histological assessment for meat and meat products. In the United States, for example, the USDA Food Safety and Inspection Service has approved the use of histological analysis as a means of evaluating the quality and freshness of meat. The method is used primarily for detecting and preventing cases of meat adulteration, particularly in ground beef products (USDA Food Safety and Inspection Service, 2021).
Other countries that have adopted histological assessment methods for meat and meat products include Japan, South Korea, and Australia. In the Russian Federations' constituent republics, the GOST standards describe the histological method of meat and meat products assessment by optical microscopy (GOST, 2013; GOST, 2016).

THE MEAT AND MEAT PRODUCTS HISTOLOGICAL ASSESSMENT METHOD

General steps

The following are the general steps involved in the meat and meat products histological assessment (Figure 1):

1. Tissue sampling: A meat or meat product sample of approx. 1 cm^3 is collected for analysis.
2. Tissue fixation: The tissue is fixed using a solution such as formalin. This helps to preserve the tissue structure and prevent degradation.
3. Dehydration: The tissue is dehydrated using a series of alcohol washes, which remove water from the tissue.
4. Embedding: The tissue is embedded in a medium such as paraffin wax. This helps to support the tissue and make it easier to cut thin sections.
5. Sectioning: The embedded tissue is cut into thin sections using a microtome. The thickness of the sections depends on the type of analysis required.
6. Staining: Depending on the coordinates of the research or examination, the routine staining is haematoxylin eosin, in tissues of animal origin, the nuclei of the cells having a dark blue colour, and the cytoplasm red tones varying in intensity and shade. Other stains such as toluidine blue can also be used to identify the origin and evaluate the quality (vegetable and animal tissues). Additionally, PAS (Periodic Acid Schiff) Calleja with Trichromic blue or green (collagen evaluation) or Alizarin Red can be used for the detection of bone fragments (Petrášová et al., 2016).
7. Microscopic examination: The stained sections are examined under a microscope, and images are captured for analysis. The images can be analysed using various software programs to quantify features such as muscle fibre size, fat content, and connective tissue content (Lukášková et al., 2011).

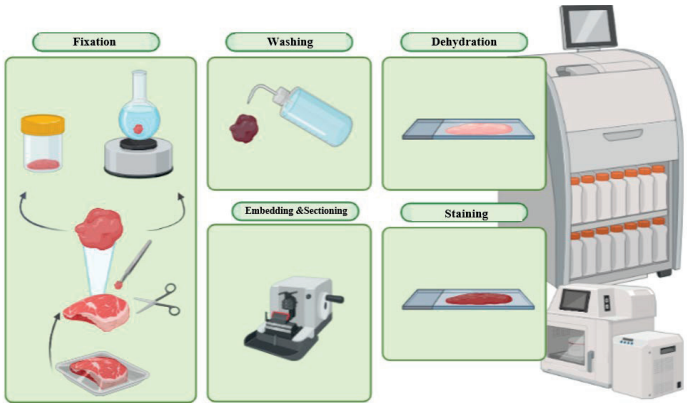

Figure 1. Histological method procedure (illustration made via www.BioRender.com)

Meat and meat products freshness evaluation by using the histological method

The main components of fresh meat are muscle fibers, connective tissue, and fat cells. Muscle fibers make up the bulk of the meat, and are organized into bundles called muscle fascicles. Each muscle fascicle is surrounded by connective tissue, which helps to hold it together and provides support. The fat cells are distributed throughout the muscle tissue, and their distribution varies depending on the cut of meat (Kalyuzhnaya, 2020).

Under a microscope, fresh meat appears as a highly organized structure of muscle fibers surrounded by connective tissue. The muscle fibers are long and cylindrical, with distinct striations that reflect their contractile properties. The connective tissue appears as a network of thin, white fibers that criss-cross the muscle tissue. The fat cells are typically small and round, and are located in between the muscle fibers (Figure 2 and Table 1) (GOST 19496-2013, 2013).

Hematoxylin stains nuclei and other acidic structures blue-purple, while eosin stains basic structures such as collagen and muscle fibers pink-red. In fresh meat samples, the histological aspect may show well-preserved muscle fibers, with a clear striated appearance and an abundance of nuclei. The fat content may also appear well-preserved, with a distinct pink staining and minimal eosin staining. The connective tissue may be visible as thin collagen fibers, which appear as pinkish strands (Petrášová et al., 2016). Moreover, fresh meat samples may show few or no signs of microbial activity, with a minimal presence of bacteria and fungi (GOST 19496-2013, 2013).

In spoiled meat samples, the histological aspect may show signs of muscle fiber degradation, with disintegration and separation of the fibers, a reduction in the number and size of nuclei, and an increase in eosin staining. The fat content may also show signs of degradation, with a loss of the normal pink staining and an increase in eosin staining. The connective tissue may appear disrupted, with a loss of elasticity and a reduction in collagen content. Additionally, microbial changes may be visible under the microscope, with an increase in the number of bacteria and fungi, which may appear as clusters or filaments. These changes can result in unpleasant flavors and odors, as well as potential health risks (GOST 19496-2013, 2013).

In ralative spoiled meat samples the general appearance has both fresh and spoiled characteristics, as it's an intermediary stage (GOST 19496-2013, 2013).

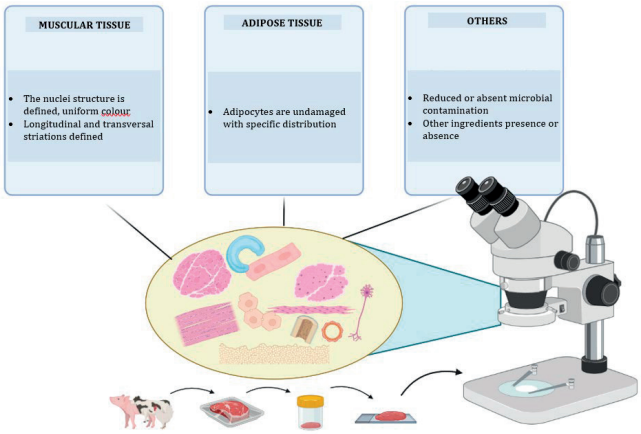

Figure 2. Main tissue characteristics in fresh meat and meat products (illustration made via www.BioRender.com)

As for cured artisan meat products, the maturation stages are determined by the muscle fibres autocatalytic destruction intensity, subsequent fragmentation into myofibrils and disintegration into sarcomeres in the form of a granular conglomerate located in the endomysium, with the preservation of the primary elements colour.

For the histological evaluation of meat products such as sausages, the muscle tissue evaluation is the first step (Khvyla et al., 2016). Fresh sausages should have a uniform and smooth appearance, with well-defined muscle fibers (Chen et al., 2019). As it changes, the muscle tissue begins to disintegrate and become more fragmented, with less distinct fibers (Khvyla et al., 2013).

Another aspect underlying the sausages freshness histological evaluation is the presence of adipocytes (Khvyla et al., 2013; Khvyla et al., 2012). Fresh sausages should have a high percentage of adipocytes, which is transposed into a moist texture (Donkova et al, 2018). Along with the spoilage progression, the adipocytes begin to disintegrate and become more fragmented, the sausage being drier and less tasty (Alshejari et al., 2017). Additionally, the presence of other types of muscle tissue (cardiac or smooth) is evaluated, depending on the nuclei location.

Table 1. Histological aspects of meat and meat products in different freshness stages (GOST 19496-2013, 2013; GOST 23392-2016, 2016; Kalyuzhnaya, 2020)

	Fresh	Relatively spoiled	Spoiled
Meat	The muscle fibre nuclei structure is clearly defined, the colour is uniform. Clearly defined and expressed muscle striations, with a uniform colour. Focal areas of cocci can be detected in fresh meats, at the section surface or in the loose connective tissue of the superficial fascia.	Nuclei in different stages of karyolysis, the colour being uneven, slightly intense and discoloured. Complete disappearance of the striations, the changes being identified up to about 15 mm deep, there are areas with a mucous appearance that have a basophilic color at the surface of the meat. Both focal areas with cocci and bacilli flora can be identified in the loose connective tissue of the superficial fascia, in the perimysium and endomysium. These aspects can be identified up to 5 mm deep.	Nuclei are almost undetectable; the colour is absent or slightly visible. Complete disappearance of the striations, the changes being identified up to more than 30 mm deep in relation to the cross-sectional surface of the sample, the color being absent or barely perceptible. Diffuse areas of bacillary flora extending up to 10 mm deep on the entire surface of the section and in the loose connective tissue of the superficial fascia, perimysium and endomysium.
Meat products – Cured meat products	Microflora is absent or up to 10 bacterial cells (cocci or bacilli) are present, without signs of muscle destruction.	Intermediate aspects between fresh and spoiled	More than 30 cocci/bacilli along with a significant degradation of muscle tissue
Meat products - Sausages	Uniform and smooth appearance, with well-defined muscle fibres. High percentage of adipocytes.	Intermediate aspects between fresh and spoiled.	Muscle tissue begins to disintegrate and become more fragmented, with less distinct fibres. Adipocytes begin to disintegrate and become more fragmented

Subsequently, the presence of integumentary epithelial structures, as well as dense connective tissue and organs, is determined. In the individual sections, the presence of starch and tissues of plant origin can also be identified.

Moreover, the presence of bacteria or other contaminants can also be evaluated (Khvyla et al., 2016).

In general, histological examination is a useful tool for determining the freshness and safety of meat and meat products (Lukášková et al., 2011; Khvyla et al., 2013).

CONCLUSIONS

In conclusion, the histological method is a powerful tool for assessing the freshness of meat and meat products. This method involves the examination of tissue samples under a microscope to detect changes in tissue structure and composition that occur as meat ages. The histological method can provide valuable information about the quality and safety of meat products, including its tenderness, juiciness, flavour, and overall palatability.

However, the use of the histological method for freshness assessment of meat and meat products is not without its challenges. These challenges include the need for specialized equipment and expertise, time and cost constraints, accuracy limitations, and a limited ability to assess other important attributes of meat quality.

Despite these challenges, the histological method remains an important tool for assessing the freshness of meat and meat products. By providing detailed information about the structure and composition of meat tissue, this method can help food producers ensure the quality and safety of their products and provide consumers with fresh, flavourful, and nutritious meat products. Further research is necessary to overcome the limitations of the histological method and to develop improved techniques for assessing meat and meat product freshness.

Additionally, the histological examination is internationally accepted for assessing the freshness of meat and meat products. The method is regulated and standardized in several countries worldwide, including EU, United States, Canada, and Australia.

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THE WORLD OF MYCOTOXINS - A SYSTEMATIC REVIEW

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Abstract

Mycotoxins are secondary toxic metabolites produced by filamentous fungi, which are predominantly found in agricultural products worldwide. Mycotoxins appear in the food chain due to fungal contamination of crops both before and after harvest. Exposure to mycotoxins can occur by consuming contaminated food (a direct factor) or by consuming feed contaminated by animals, through milk (an indirect factor). Fungal proliferation and mycotoxin production have a higher input due to environmental factors. Chemically, most mycotoxins are stable and thus survive food processing. Among the most important mycotoxins are aflatoxins, ochratoxin A, zearalenone and trichothecenes. The species that synthesize these mycotoxins belong to the genera Aspergillus, Penicillium and Fusarium and, unfortunately, they can trigger mutagenic, nephrotoxic, carcinogenic, teratogenic, cytotoxic, neurotoxic and estrogenic effects. This paper provides an overview of the world of mycotoxins, from emergence to adverse effect on contamination of agricultural products, which is of major importance as it affects food and feed safety, food security and trade.

Key words*: contamination, fungi, mycotoxins.*

INTRODUCTION

The word "mycotoxin" is derived from the greek word *mykes*, which means fungus or mold, and the latin word toxicum, poison or toxin (Singh & Mehta, 2020). Mycotoxins are toxic secondary metabolites that are produced by fungi and not just any, but filamentous fungi. Analyzing the situation, speaking more strictly, they are defined as secondary metabolites of fungal origin. They show in vivo toxicity to vertebrates after natural introduction (i.e. ingestion, inhalation, etc.) (Streit et al., 2013). Mycotoxins are toxic and harmful to humans and animals to varying degrees and can contaminate grain grains in the field as well as in storage. These result in negative effects on human and animal health. Mycotoxins are present in agricultural products such as cereals and oilseeds. If ingested in high enough concentrations, they exert severe toxic effects on humans and animals (Liang et al., 2016). While it is difficult to infer any long-term trends globally, data confirm that high mycotoxin contamination is often linked to demanding weather (Pettersson, 2012). Regarding food products, the situation is quite similar and here we are talking about the fact that low levels of contamination are frequently observed in official controls, but the maximum levels are rarely exceeded in developed countries (Ayofemi et al., 2019). Since it is very difficult to eliminate mycotoxins from contaminated commodities, it is necessary to prevent their accumulation in agricultural commodities being the most effective strategy to combat the problem (Marroquín-Cardona et al., 2014). The most important preventive measures range from crop rotation and increasing resistance to inoculation with microbial antagonists. However, unfortunately, excessive levels of mycotoxins can occur despite all preventive measures. Therefore, continuous monitoring is essential and effective decontamination strategies are needed to deal with such contaminations. The prevalence of mycotoxins in food is equal to their presence in feed, although the concentrations detected are generally lower in food (Schatzmayr & Streit, 2013). Also, depending on the potential risk of transfer of each mycotoxin, feed contamination can also pose a safety hazard to food of animal origin and contribute to the intake of mycotoxins to humans (Pinotti et al., 2016).

AFLATOXINS

Aflatoxins are carcinogenic substances produced by the species *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are mainly found in grains, peanuts, cottonseed and tree nuts. *Aspergillus flavus* can invade corn and cottonseed in the field when there is drought stress, insect or hail damage, and the presence of excess moisture (Mahato et al., 2019). *Aspergillus parasiticus* can invade peanuts in the field and during harvest if there is excess moisture, such as heavy rains when the peanuts are drying. Aflatoxin-producing fungi can also invade during storage if moisture conditions become favorable for their growth (Sineque et al., 2017). Aflatoxins are a group of structurally related compounds, the most important of which are aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2. Aflatoxins M_1 and M_2 are secondary metabolites of aflatoxin B and are eliminated through milk. Aflatoxins B_1 and B_2 are produced by strains of *A. flavus* and are most common in corn. These strains of *A. flavus* do not normally produce mycotoxins G and *Aspergillus parasiticus* produces aflatoxins B_1, B_2, G_1 and G_2. Thus, maize is most commonly contaminated with aflatoxins B_1 and B_2 and peanuts with aflatoxins B1, B2, G_1 and G_2 (Eaton & Gallagher, 2010). The best known mycotoxin found in human food and animal feed is aflatoxin B_1. In fact, aflatoxin B_1 is the most potent known hepatocarcinogen in mammals and is listed as a group I carcinogen by the IARC. The liver is the main target site of aflatoxin B_1. Acute aflatoxicosis has produced abdominal pain, vomiting, edema and even death. Aflatoxicosis outbreak was recorded four times in Kenya between 2004 and 2014, almost 600 people were affected and 211 deaths have been reported because of it (Ayofemi et al., 2019). In animals, aflatoxins can cause liver disease and are mainly associated with decreased production (milk, eggs, meat, etc.). Aflatoxin B_1 is a strong human carcinogen and high levels of exposure are known to cause liver cirrhosis and liver cancer. Aflatoxins can also cause immunotoxicity (Smith et al., 2020). FDA contamination limits for aflatoxin in grain products vary by commodity use. The contamination limit for most human foods is 20 ppb (μg/kg), but for milk and certain milk products it is 0.5 ppb (μg/kg) (Reg. (EC) No.1881/2006, Reg. (CE) No. 165/2010). Aflatoxin affects grain quality and marketability and is primarily a threat to the health of farm animals. This global scenario confirms that the contamination is highly dependent on the climatic conditions of the respective region. In general, environmental conditions such as excessive moisture, extreme temperatures, humidity, drought conditions, insect damage, cropping systems and some agronomic practices can cause stress and predispose field plants to mold and determine the severity of mycotoxin contamination (Zhang et al., 2018). The main climatic conditions leading to aflatoxin accumulation are high temperature, low rainfall and severe drought stress (Singh & Mehta, 2020).

OCHRATOXINS

Ochratoxins A (OTA), B (OTB), C (OTC) are a group of compounds developed by various species of *Penicillium* and *Aspergillus* that contaminate cereals, vegetables and compound feed. In laboratory conditions, most *Aspergillus ochraceus* strains produce OTB and OTC and in natural environmental conditions, the most predominant is OTA (Ostry et al., 2013). Ochratoxin A (OTA) is the most widespread and relevant fungal toxin in the field of mycotoxins. The results of research known over time show that in Europe at least part of the ochratoxin A in food comes from cereals and cereal products. Ochratoxin is mainly produced by *Aspergillus* and *Penicillium* species (Lhotská, 2016). Ochratoxin A is most often isolated from corn, sorghum, barley, wheat, oats and rye. It seems that barley, oats, wheat and corn, grains grown in Denmark and other Scandinavian countries, as well as in the Balkans and India, provide favorable conditions for the development of ochratoxigenic fungi, a fact that would explain such high levels of ochratoxin detected in these plant substrates (Sineque et al., 2017). In these specific climatic conditions approximately 20% of the examined samples were positive, the amount of ochratoxin A being of the order of ppm (Liang et al., 2016). Apart from cereals, ochratoxin A has been identified and isolated

from many other substrates, such as soybeans, beans and fodder peas, green and roasted coffee beans, cocoa beans, wine and grape juice, beer, white and black pepper (Ayofemi & Adeyeye, 2019). It should be mentioned that in relation to coffee beans, research in recent years shows that approximately 80% of the ochratoxin A present in the green beans is destroyed by the roasting process, so that the amount of mycotoxin remaining in the substrate represents from a medical point of view a dose without pathogenic significance (Liu et al., 2020). Ochratoxin A contamination is mainly associated with insufficient drying or improper storage and unfortunately, it is found all over the world. In temperate regions, ochratoxin A contamination is mostly due to *Penicillium verrucosum* contamination, while *Aspergillus* species such as *A. carbonarius* account for ochratoxin production in warmer regions (Visagie et al., 2014). As for feed, ochratoxin A is most commonly found in grains, but it is known to contaminate soybeans and peanuts. Because fungal development often occurs in hotspots such as a high water activity zone, in stored grain the distribution of ochratoxin A in contaminated feed tends to be very heterogeneous (Ayofemi & Adeyeye, 2019). This fact represents a challenge in terms of ochratoxin contamination testing. Ochratoxin A has been classified as possibly carcinogenic to humans (Group 2B) (Sarrocco & Vannacci, 2018). Ochratoxin A has proven over time its carcinogenic, immunotoxic and nephrotoxic virtues, manifested extremely actively and vigorously towards humans and towards various animal species (Streit et al., 2013). The primary target site is the kidney. Animal studies have shown that ochratoxin A is a potent renal carcinogen (Kovalsky et al., 2016). The International Agency for Research on Cancer (IARC) has classified ochratoxin as possibly carcinogenic to humans in group 2B carcinogen. Based on epidemiological data, there is no significant evidence for human health risks associated with exposure to ochratoxin A. The primary effect of ochratoxin in all animals is nephrotoxicity. Regarding neurotoxicity, it has been reported that ochratoxin A can be seen as a possible cause of certain lesions, including brain lesions. Also, ochratoxin A is a strong teratogen for laboratory animals, being able to cross the placenta and accumulate in the fetal tissue, causing various morphological abnormalities. (Gallo et al., 2015).

DEOXYNIVALENOL AND OTHER TRICHOTHECENES

Deoxynivalenol (DON) belongs to the trichothecene group. This is one of the least acutely toxic but is of particular interest due to its high prevalence. More precisely, deoxynivalenol is classified as B-type trichothecene and is produced by *Fusarium culmorum* and *F. graminearum* (Palumbo et al., 2020). Contamination with deoxynivalenol is observed worldwide, with cereal crops such as wheat, corn or barley being the most frequently affected (Sineque et al., 2017). Deoxynivalenol is found predominantly in cereals such as wheat, barley, corn and to a lesser extent in oats, rice, rye, sorghum. Contamination of fodder, especially silage with deoxynivalenol is regularly observed. Cold and wet weather conditions favor DON production and it was found that the timing of precipitation influences the risk of contamination more than the amount of precipitation (Oldenburg et al., 2017). In animal husbandry, deoxynivalenol, also known as vomitoxin, primarily recalls negative effects, such as: refusal of feed and vomiting in pigs (Liang et al., 2016). This mycotoxin alters the immune response and intestinal functions. Poultry are not as sensitive to deoxynivalenol and feed refusal is only observed at very high concentrations (16-20 mg/kg feed). It is known that ruminants are the animal species least sensitive to deoxynivalenol, because the rumen microflora has the ability to detoxify this mycotoxin (Rocha et al., 2017). Mycotoxin concentrations were quite wide as ranges of variation, for example between 4 and 9000 pg/kg in barley samples. Deoxynivalenol concentrations > 2 to 5 ppm induce decreased feed intake and reduced weight gain and > 20 ppm induce vomiting and feed refusal (Zhang et al., 2018). However, concentrations as low as 1 ppm have been associated with feed refusal in pigs (Schenck et al., 2019). is the main fungal species that produces trichothecenes. Toxin T-2 and HT-2 are two of the most toxic members of the trichothecene group. They belong to the A-

trichothecene type and are produced by F. sporotrichioides, *F. poae* and other *Fusarium* species. Oats and oat products were found to be particularly prone to contamination with high levels of T-2 and HT-2, followed by barley. Trichothecene contamination has been found to affect protein synthesis and exert immunotoxicity, hematotoxicity (Zhang et al., 2018). Ruminants are again protected by their microflora and have been shown to be the least sensitive to these toxins (Smith et al., 2020). Toxic effects of trichothecenes in animals (dairy cattle, pigs, broilers, and rats) include decreased plasma glucose, decreased blood cell and leukocyte counts, weight loss, food-toxic aleukia, and pathological changes in the liver and stomach (Bessaire et al., 2019). The toxins T-2 and deoxynivalenol are well-known inhibitors of protein synthesis (Khodaei et al., 2019). In general, trichothecenes exert a negative impact on the gastrointestinal tract, especially on intestinal absorption, integrity and immunity (Oldenburg et al., 2017).

FUMONISINS

Fumonisins are also counted among the mycotoxins produced by species of the genus Fusarium. In forage crops, these are most commonly produced by *F. proliferatum* and *F. verticillioides* (Jerome et al., 2018). The B-series fumonisins (FB_1, FB_2 and FB_3) are of greatest importance in terms of occurrence and toxicity, thus FB_1 is of greatest concern as it is the most widespread and toxic of the fumonisins. It was classified in class 2B, possibly carcinogenic to humans (Visagie et al., 2014). FB_1 contamination is frequently associated with corn and corn products. In a broader context, the classification of fumonisins as *Fusarium* mycotoxins is no longer 100% valid, as recently black Aspergilli, especially *A. niger*, have been found capable of producing fumonisins (Janik et al., 2021). We remind you that feed contaminated with fumonisins causes serious diseases, and here we are talking about pulmonary edema in pigs and leukoencephalomalacia in horses. In addition, fumonisins have been shown to be immunosuppressive, hepatotoxic and nephrotoxic just like other mycotoxins (Honma et al., 2004). Fumonisins, deoxynivalenol and zearalenona are considered to be the most important mycotoxins produced by Fusarium and in terms of animal health and economic losses they are the most relevant (Logrieco et al., 2018). Although FB_1 contamination is not very common in crops other than corn, mycotoxins produced by *Fusarium* in general are often found together in contaminated grains. In most cases, the resulting toxic effects are combinations of individual mycotoxin toxicity, but synergistic interactions with other mycotoxins have been observed with quite pronounced negative effects (Jouany, 2007). Unlike most mycotoxins, which are hydrophobic in nature, fumonisins are hydrophilic in nature, preventing their discovery until 1988 (Kovalsky et al., 2016). Fumonisins are frequently isolated from feed and food (sorghum, rice, corn, beer) and long-term research with FB_1 administered in feed demonstrated that this mycotoxin induced tumor formation in the kidney and liver (Marin et al., 2013). The metabolism of glycerophospholipids, as well as that of fatty acids and phospholipids, is also affected. FB_1 has the same structure as cellular sphingolipids, which are responsible for neurological and immunological diseases as well as cancer (Gallo et al., 2015). Fumonisins cause serious diseases in animals and induce esophageal cancer in humans. Human epidemiological studies in South Africa, Italy and China have shown that esophageal cancer is linked to the consumption of corn kernels containing fumonisins (Oldenburg et al., 2017). Consumption of moldy sorghum and corn contaminated with FB_1 has triggered severe poisoning with gastrointestinal disturbances and death in humans in India, and another outbreak caused neural tube disorders (birth defects of the brain, spine, or spinal cord), the last such study taking place along the Texas-Mexico border, China and South Africa, thus demonstrating the association with the consumption of corn contaminated with fumonisins (Kovalsky et al., 2016). In animals, fumonisins have been found to cause pulmonary edema and hydrothorax in pigs, leukoencephalomalacia in horses, and HCC in rats. Also, FB_1 causes reproductive damage in many animals. FB_1 negatively affects the livestock sector and food safety issues caused

by FB_1 have also received widespread attention. (Palumbo et al., 2020).

ZEARALENONE

Like the mycotoxin deoxynivalenol, zearalenone (ZEN) is produced by fungi of the genus *Fusarium*, the best known being *F. culmorum, F. graminearum* and *F. heterosporum* (the species that produce ZEN) (Rashedi M. et al., 2011). As in the case of other mycotoxins, the risk of contamination is the highest in cereal crops. Also, silage, feed and straw are prone to contamination with zearalenone (Fink-Gremmels et al., 2019). The acute toxicity of zearalenone is at a lower level and the adverse effects are caused by the interaction with the estrogen receptor. Consequently, zearalenone produces fertility problems with estrogenic symptoms such as vulva swelling and uterine enlargement (Oldenburg et al., 2017). Among animals, pigs are the most sensitive to zearalenone exposure, while poultry are tolerant, and there are few studies of adverse effects in birds, as feed is unlikely to contain zearalenone in sufficiently high concentrations. Data on dairy cows is limited, but it is known that there is a low reaction to zearalenone contamination (Anfossi et al., 2016). In animals, the main effect of zearalenone contamination is estrogenic, and pigs are clearly the most affected farm animals. It is well known that zearalenone is a non-steroidal estrogenic mycotoxin, involved in reproductive disorders of farm animals (pigs, cattle and sheep) but also causes hyperestrogenic syndromes in humans (Bueno et al., 2015). Other studies on the effects of zearalenone have revealed problems on the reproductive system, such as the enlargement of the uterus, early decline in fertility, problems of the reproductive tract, but also an abnormal level of progesterone and estradiol (Palumbo et al., 2020). In addition, another issue exemplifies that the ingestion of food contaminated with zearalenone during pregnancy resulted in a reduction in fetal weight and a reduced embryo survival rate (Jensen et al., 2019). This is because zearalenone has a structure that allows it to bind to the mammalian estrogen receptor, even if the affinity is lower compared to natural estrogens (Cheli et al., 2913). In addition, zearalenone has been shown to be hepatotoxic, hematotoxic, immunotoxic, and genotoxic. Even if the reproductive organ is the main target of this mycotoxin and has the role of inducing toxicity, adverse effects on the gastrointestinal tract have also been reported (Danezis et al., 2016). It is worth noting that the effects of zearalenone ingestion on the gastrointestinal tract are not as harmful as the other mycotoxins (Marroquín-Cardona et al., 2014). Metabolites of zearalenone (α- and β-zearalenol) were found to significantly ($P < 0.05$) decrease cell integrity, the study showed that zearalenone and its metabolites acted differently in the gut, and modulation of gene expression was responsible for the carcinogenic effects of it (Smith et al., 2020). Despite all these problems, pigs that ingested this mycotoxin did not show changes in villus height, mucosal thickness or goblet cell number (Rashedi et al., 2011). In short, zearalenone plays a negative role in the health of the intestine and beyond, even if no particular histological changes were observed (Claeys et al., 2020). Mycotoxins can cause a variety of adverse health effects and pose a serious health threat to both humans and animals (Figure 1).

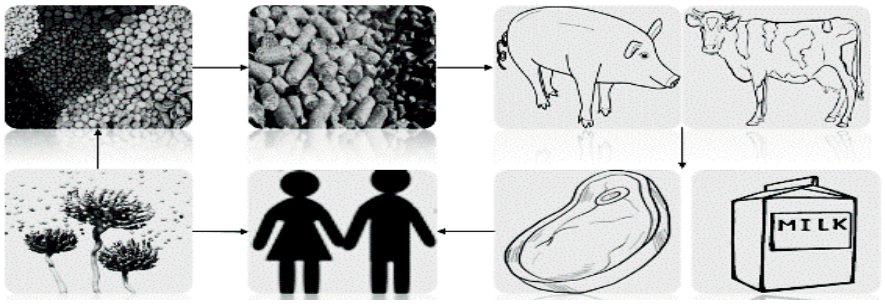

Figure 1. The chain of contamination with mycotoxins

The effects of some mycotoxins in food give signals with acute symptoms, with symptoms of severe disease that set in quickly after consumption of mycotoxin-contaminated food products (Sarrocco & Vannacci, 2018). Other mycotoxins that are found in food and not only have been linked to long-term health effects, such as even inducing cancer but also an immune deficiency. Of the several hundred mycotoxins identified to date, about a dozen have gained the most attention because of their serious effects on animal and human health and their occurrence in feed, food (Rocha et al., 2017).

CONCLUSIONS

Most grains are susceptible to mycotoxin contamination, which means that because of this, all animals are at risk of contamination. Mycotoxins trigger adverse and toxic effects in animals and affect health and productivity. Extreme weather phenomena trigger the growth and development of cultivated species, hence the presence of mycotoxins in cereals affects the agricultural and animal husbandry sectors, which represent a challenge for the future. The control along the entire traceability route contributes to ensuring the quality of the grains, in this way ensuring two important factors: quality and safety. The control of mycotoxins eliminates their entry into the agri-food chain and, in this way, the health of animals and people, as well as the environment, is protected.

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PRELIMINARY RESULTS IN INDUCING MENINGITIS IN BALB/C MICE USING A HUMAN STRAIN OF *Neisseria meningitidis*

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Abstract

Neisseria meningitidis (Nm) is the pathogen carried asymptomatically in the nasopharynx but which under certain conditions can produce meningitis or even multi-organ failure. Experimental induction of meningitis in an animal model is necessary for testing new nanopharmaceutical products. Thus, the aim of the study was to establish the concentration of Nm capable of inducing the disease. We carried out 2 studies where we tested different concentrations of Nm obtained by the nephelometric and spectrophotometric methods. In the first study we included 20 BALB/c mice, and in the second 24. Local and general clinical signs, complete with body temperature values, body weight, hematological, microbiological and histopathological examination were important indicators for the assessment of the establishment of meningitis. The clinical results have pronounced depression in the first 2 days after inoculation, then the general condition will appear. The microbiological and histopathological examination indicated the presence of bacteria at the brain level and specific meningeal lesions only in the case of the second attempt, but the early mortality, requires additional testing of the bacterial concentration that induces meningites in a stable and persistent way so that treatment against the condition can be tested later.

Key words*: intracisternal inoculation, meningitis, mouse, Neisseria meningitidis.*

INTRODUCTION

Bacterial meningitis caused by *Neisseria meningitidis* (*Nm*) is a serious inflammatory disease characterized by acute infection of the brain and cerebrospinal fluid (CSF) and is life-threatening in healthy individuals. The main injuries encountered in infected patients are those of cerebral edema, hernia, cessation of cerebral circulation (Van Deuren, 2000), mental retardation, hearing loss, hydrocephalus, cognitive disorders, sepsis and finally death, which occurs in 20% of cases , in the absence of adequate treatment (Borrow R., 2017). Serogroups A, B, C, W, X and Y are responsible for the occurrence of meningeal disease worldwide (Parikh, 2020). Bacterial strains isolated from patients with specific clinical signs of meningitis are *Staphylococcus aureus, Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes* (Sáez-Llorens, 1999) and last but not least Nm. It is carried in the upper respiratory tract by adolescents and young adults, representing the main reservoir of the bacteria. Most carried strains never cause invasive disease, but meningococci can be easily transferred through saliva droplets or direct contact from an asymptomatic carrier to an immunocompromised one (Caugant, 2009).
Nm is a Gram-negative, encapsulated, kidney shaped diplococcus that expresses pili, opacity proteins and adhesion molecules. They adhere to the non-ciliated columnar epithelial cells in

the nasopharynx or the epithelium lining the tonsils, adapt and begin to proliferate. Meningococci enter the bloodstream through capillaries and small veins in the underlying submucosal tissue. In a site with low immunity, the survival and rapid proliferation of neisseria in the blood is favored, so that within about 7 days, the disease is clinically expressed (Stephens, 1991), and death can occur in 12-24 hours (de Greeff , 2008) through septic shock or multiple organ failure that occurs in 30% of patients who contract serogroup B and C (Gedde-Dahl, 1983).

Penicillins represent one of the treatment options for meningitis because most isolated neisseria are susceptible to penicillin and ampicillin. However, there are also strains that express an intermediate resistance to these antibiotics, being isolated from patients in Europe, South Africa or the United States. In the absence of an adequate clinical response to the therapy with the mentioned antibiotics, it is recommended to change the therapy with ceftriaxone or cefotaxime (Brandtzaeg, 2012).

Vancomycin or third-generation cephalosporins are adequate for the treatment of moderate to severe meningitis (Bradley, 1997), but cephalosporins have shown little efficacy, with many treatment failures in the management of patients with pneumococcal meningitis (Shultz, 2000; Singhal, 2007). The newer fluoroquinolones seem to have stronger activity in meningitis, although the prevalence of Nm strains resistant to quinolones is increasing. (Schmitz, 2017). Therefore, for the treatment of meningitis caused by Nm and not only, it is necessary to develop antibiotic molecules with accurate drug delivery mechanisms and a broad antibiotic spectrum. In order for this to be fulfilled, researchers in the pharmacological field are testing new variants of drugs on animal models capable of expressing meningococcal disease. The murine model seems to be a suitable one, and for the induction of the disease, numerous methods have been tried through which the expression of the disease similar to humans has been induced. Thus, the pathogen was inoculated intraperitoneally (Tan, 1995), intranasally (Zwijnenburg, 2001), intravenously or intracisterna-magna (Grandgirard, 2007).

The progression of the disease, the reproducibility and the evaluation of the pathogenic characteristics are still limited and largely depends on the inoculated bacterial strain and its concentration so that the disease is reproduced as faithfully as possible. In this sense, we set out to develop a murine model of meningitis using a strain of Nm, serogroup B, on which new drugs can be later tested.

MATERIALS AND METHODS

Ethics statement

All experiments on animals took place within the "Cantacuzino" National for Medico-Military Institute of Research and Development (IC), Baneasa Animal Facility (BAF), Bucharest. The studies were approved by the competent authorities, in accordance with the provisions of Directive EU 63/2010 regarding the care, use and protection of animals used for scientific purposes. The animals included in the study come from the SPF (Specific Pathogen Free) kennel of BAF, they are housed in groups of 5, in individually ventilated cages, receiving water and food *ad libitum*, with cycles of 12 hours light, 12 hours dark. The temperature inside the cages was monitored daily, being maintained in the range of 20-22^{o} C, and the humidity was between 45-65%. The health status of the animals was monitored daily by the veterinary staff by evaluating the expression of clinical signs, measuring body temperature and weight. The conditions for excluding the animals were established *a priori* and referred to weight loss of more than 20% or their inclusion in the coma scale 1 and 2 (1 = coma, 2 = the animal does not return to the quadrupedal position after was positioned on its back, 3 = the animal returns to the quadrupedal position after 30 seconds after turning on its back, 4 = the animal stands up after 5 seconds after turning on its back showing minimal motor activity, 5 = normal behavior).

Preparation of the inoculum of Nm

The experiments were carried out in two stages, which we will call study 1 and study 2, respectively, depending on the concentration of the inoculum used to induce meningitis.

Study 1: *Nm* was grown on chocolate agar medium, 24 h, 37^{o} C, 5% CO_2. The colonies

grown on the plate were inoculated in brain-heart infusion broth (BHI) supplemented with Dextran Iron (5 mg/kg, Intrafer 200 B12, Interchemie werken, Holland), incubated in the same conditions mentioned previously. To read the turbidity of the inoculum, measured in the McFarland index, we used the nephelometric method and for the exact measurement of colony forming units per milliliter agar plating was performed. The concentrated innoculum obtained was 10^7 CFU/mL. The quantity inoculated in the animal was 10 µL, which means that the mice received bacterial concentrations 100 times lower than the bacterial inoculum, respectively 10^5, 10^4 and 10^3 CFU/mouse.

Study 2: Since the highest McFarland index that the nephelometer can express is 14iMF, the second attempt to induce meningitis was by establishing the concentration of the inoculum of *Nm* by spectrophotometry (TECAN spectrophotometer). The fresh bacteria, 24 hours old, was taken off the chocolate agar plates and inoculated in the BHI medium enriched with Dextran Iron. The vortexing process followed, then reading the density of the solution with an exponential increase (by adding additional colonies of *Nm*, until reaching the value of 0.7 optical density at 630 nm, which represents ~10^{10}CFU/mL. The same dose, of 10 µL, was inoculated to the animals, which means that they received 10^7 and 10^5 CFU/mouse.

Animals included in the study were BALB/c mice, female, 8 weeks old, which, depending on the study, were divided according to Table 1.

Table 1. Lotting of animals according to the concentration of *Nm* used

	Animal group	Number of animals/group	Concentration of *Nm* used (CFU/mL)	The method of establishing the *Nm* concentration
1	N0	5	10^5	Nephelometry
	N1	5	10^4	
	N2	5	10^3	
	Control group	5	BHI+ Dextran Iron	
2	S1	12	10^7	Spectrophotometry
	S2	12	10^5	

Inoculation of Nm in animals

Before starting the experiments, the animals were weighed (KERN 440-47N scale, Germany) and rectally thermometered (SCALA SC 28 Flex thermometer, Taiwan). On day 0, the animals were anesthetized with a mixture of Ketamine (50 mg/kg, Vetased, Farmavet, Romania) and Xylazine (3mg/kg, Bioveta, Romania). The fur from the cervical area was trimmed and disinfected with 70% ethanol and 3% betadine. The inoculation procedure took place in a laminar flow hood. Each mouse was placed on a sterile field. The operator held the animal's head in a flexed ventral position with his left hand, so that the median line of the nose was parallel to the work table. The landmark for inoculation was that of the diamond-shaped depression created at the craniocervical junction. The bacteria, regardless of the concentration, was inoculated inside the large tank, with a syringe with a 30G needle. After inoculation, the animals were placed in cages. The clinical monitoring (general clinical signs, temperature and body weight) lasted 7 days, in study 1, the animals being euthanized at the end and in study 2, the culling of 8 mice (4 from each group) at 48 h, 72 h and 7 days after inoculation.

RESULTS AND DISCUSSIONS

One-way Kruskal-Wallis ANOVA (Prism 9, GraphPad LLC, USA) was used for statistical analysis to compare the average values of weight and body temperature between groups of animals included in the study. For all analyses, a p-value < 0.05 was considered significant.

From a clinical point of view, in study 1, the animals presented a deterioration of the general state of health, characterized by weight loss (Figure 1), diarrhea, purulent ocular secretions, lethargy, hypothermia (Figure 2).

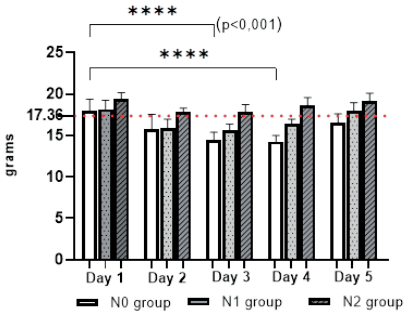

Figure 1. Body weight evolution in Study 1 (dotted red line represents the average value of the animals body weight recorded on day 0)

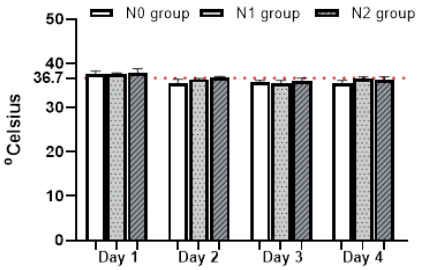

Figure 2. Evolution of body temperature in Study 1 (dotted red line represents the average value of the animals' body temperature recorded on day 0)

Regarding the bacterial concentrations used, the most brutal clinical signs were observed in the animals from the N0 group, followed successively by the other two groups, the symptoms expressed being therefore directly proportional to the aggressiveness of *Nm*. However, no animals succumbed by day 7.

In the case of study 2, the symptomatology was more pronounced, especially in the S1 group, in which mortality occurred after the first inoculation, at 24 h (2 animals). The brain was harvested from these animals to verify the viability of the inoculated bacteria and following the microbiological examination, *Nm* could be isolated. The state of health was visibly altered in the case of S1, unlike S2. Weight loss was significant after the first day of inoculation, $p < 0.001$, increasing until day 4 when $p < 0.0001$. At the end of the experiment, the surviving animals registered weight increases, compared to the previous days, indicating a value of $p < 0.05$, the values approaching those of day 0 (Figure 3).

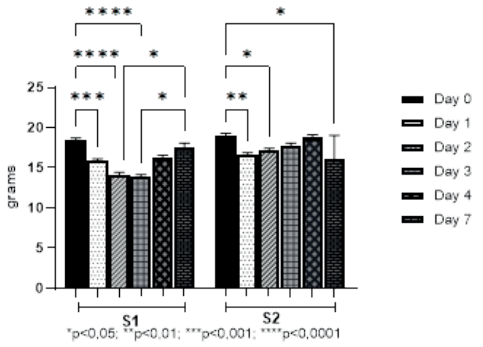

Figure 3. Evolution of body weight in Study 2

Regarding the body temperature value, a statistical significance was observed in the S1 group, on the second and third day after inoculation, when $p < 0.0001$. In the case of the S2 group, the body temperature oscillations showed no significant statistical relevance (Figure 4).

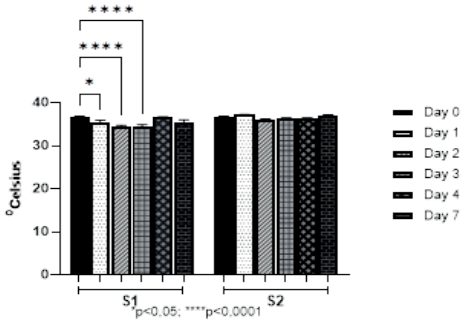

Figure 4. Evolution of body temperature in Study 2

According to the work protocol, the animals were euthanized at 48 h, 72 h and on day 7. 4 types of samples were taken: 10 µL of cerebrospinal fluid (Figure 5) inoculated on chocolate agar, pieces of brain tissue that were spread on a chocolate agar plate (Figure 6), the rest of the brain which was placed in 12 ml of BHI (Figure 7) and some brain samples for histological analysis (Figure 8). At 48h, as well as at 72 h, 2 out of 4 animals from group S1 had viable *Nm*, grown on chocolate agar plates seeded with pieces of brain tissue.

The histological samples showed in the animals of the S1 group, major foci of meningitis, blood vessels with an increased appearance in volume, discrete lesions specific to encephalitis (perivascular sheaths), and strong neutrophilic infiltrate (Figure 9).

Figure 5. Collection of 10 µL cerebrospinal fluid

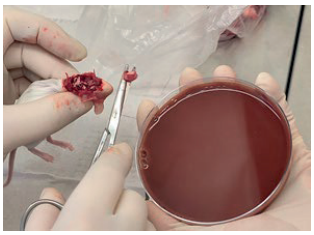

Figure 6. Collection of brain tissue and inoculation on chocolate agar medium

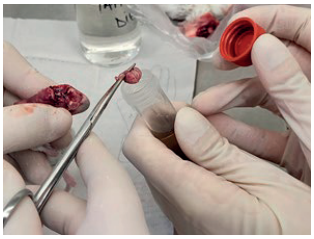

Figure 7. Harvesting brain tissue in BHI medium

Figure 8. Collection of brain tissue for histological analysis

Seven days after the inoculation, the last 6 animals, which no longer showed signs of meningococcal infection, were sacrificed, the same samples being collected, the recovery of the bacteria was no longer possible.

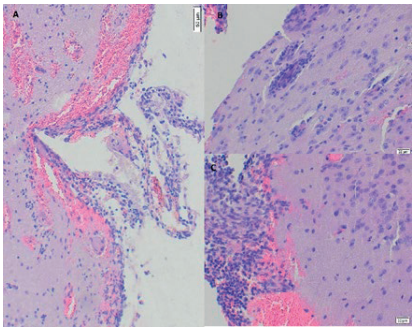

Figure 9. Histological appearance of meningeal sections: A-meningitis focus (ob. 10, Hematoxylin-Eosin staining), B, C- perivascular sheaths, neutrophilic and hemorrhagic infiltrate (ob. 20, Hematoxylin-Eosin staining)

We managed to develop a murine model of meningitis produced by *Nm* in the case of the second study, a fact demonstrated by the number of animals that responded to contact with the bacteria, thus 16.33% of them succumbed to the disease at 24 h. In 33.33% of the animals *Nm* was recovered at 48 h and in 16.6% of the animals after 72 h, the rest of the animals managing to cure the infection, an aspect explained by the fact that *Nm* is a strictly human pathogen. The symptoms expressed along with the microbiological and histopathological findings show that the infection with *Nm* in a concentration of 10^7 UFC/mouse is the closest to human meningitis, but the animals that succumbed to the disease, within the first 24 hours after inoculation, make us believe that the dose tested is not the optimal one for the animals to become stable from the point of view of the establishment of the disease, to then be used for clinical examination or testing of new drugs.

In murine models of meningitis, different ways of inducing the disease were tried, either intranasal or intraperitoneal, but complications such as sepsis or pneumonia (Zwijnenburg, 2001) make the choice of inoculation within the cisterna magna a suitable one, as the bacteria reach directly at the level of the central nervous system. The use of different concentrations of *Nm* for the induction of meningitis aimed to establish the effective level of bacteria that would satisfy the objectives of our study. Like Mook-Kanamori and colleagues (Mook-Kanamori, 2012), we compared the animals' behavior at different concentrations and could observe the aggressiveness of *Nm* at high concentrations or the fact that it is completely harmless when the concentration is low, as demonstrated by the first study tried. From a histopathological point of view, the numerous meningeal infiltrations, hemorrhages or perivascular neutrophilic sheaths, complemented by the stagnant blood that caused dilation of the vessels at the cerebral level, reflect the anatomopathological findings of human patients who died due to bacterial meningitis (Vergouwen, 2010), the same type of lesions being observed in Mtafya's studies (Mtafya, 2019). Brain infarcts or abscesses occur in human patients with meningitis, as well as in experimental rat models (Ostergaard,

2004), but in the case of our studies, this type of specific lesions was not observed and an explanation possible would be the bacterial aggressiveness, the relatively short inoculation-death/euthanasia time, the age of the animals or even the chosen animal model. However, the clinical signs expressed, the isolation of the bacterium from the brain of dead or euthanized animals and the histopathological lesions encourage us towards new attempts to create a murine model of meningitis induced by *Nm*, serogroup B, stable and reproducible. The spectrophotometric method proved to be more efficient than the nephelometric one and easier to implement. An animal model that recapitulates the pathophysiology of a pathogen similar to that in humans would be ideal, but still, hard to find. Today, several inbred and non-inbred mouse strains are available. For the study of meningitis, whether as the disease itself or testing meningococcal vaccines, inbred lines are preferred because, it seems, they better mimic the genetic diversity of humans (Tuttle, 2018). Females are chosen for such studies, they generate robust responses compared to males, the same influence of gender being observed in the case of humans (Klein, 2016). The chosen animal model is also used by other researchers, who obtained good results in the attempt to induce meningitis by inoculation of *Nm* (Pagliuca, 2019), which represents another reason that our study idea is realistic and worthy of being pursue to complete the established objectives.

CONCLUSIONS

The concentrations of *Nm* used in the first study were not effective for inducing meningitis, the animals being able to clear the bacteria. In study 2, the spectrophotometric method was useful to establish the concentration of *Nm* as accurately as possible, which determined the specific symptomatology. The concentration of 10^7CFU/mouse induced the most signs and brain lesions of meningitis and *Nm* could be recovered from the animal brains, at different time intervals. The mortality of the animals even in the first 24h after inoculation shows that 10^7CFU/mouse might be too strong for the disease to progress over longer periods of time, necessary for testing new drugs.

ACKNOWLEDGEMENTS

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EFFECT OF ISOFLURANE ANESTHESIA ON THE VITAL SIGNS MONITORING IN LABORATORY MICE

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Abstract

Inhalation anesthesia systems are used in laboratory animal experimentation due to their safety, easily adjustable dosage and rapid return to consciousness. Isoflurane is currently the most common volatile anesthetic used in mouse studies. The study aimed to investigate the influence of isoflurane on the vital functions during inhalation anesthesia in two mouse strains (inbred and outbred), suitable for blood collection and minor surgery. Heart rate, pulse distention, respiratory rate, peripheral arterial oxygen saturation and rectal temperature were measured during anesthesia and compared to the same parameters measured on awake animals. Results showed a decrease in the heart rate by 26% during 2% isoflurane anesthesia, while the breath rate decreased by 42%. Oxygen saturation remained at 95%–98% and the vascular distension caused by the pulse was relatively constant. Both groups showed a decrease in the rectal temperature by 1,6-2.2°C during anesthesia, with temperature values normalizing in 1.5-2 hours after anesthesia. The overall effects of isoflurane on mice vital signs were moderate, both induction and recovery from anesthesia proceeded quickly (1-4 minutes), with a rapid return of the animals to their normal state.

Key words*: inhalation anesthesia, isoflurane, vital signs, laboratory mouse.*

INTRODUCTION

Current attitudes toward animal research are best summarized by the "3Rs concept" (replacement, reduction and refinement). The concept of refinement aims to reduce to a minimum the pain, suffering and distress experienced by animals used for experimental purposes. Adopting best practice for commonly used procedures, such as injections, blood sampling and surgeries, can enormously improve animal welfare and can increase the reliability and validity of experimental results (Demers et al., 2006; Hubrecht & Carter, 2019).

When following the basic principles of laboratory animal welfare, the selection of an appropriate and effective anesthetic protocol for each individual animal is an essential part of laboratory animal experimentation (Aras et al., 2001; Richardson & Flecknell, 2005). General anesthesia may be used for surgical and non-surgical procedures and is also referred to as surgical anesthesia. Properly induced and maintained general anesthesia with effective monitoring is vital to maintaining animal welfare and creating reproducible studies.

Mouse anesthesia is challenging for several reasons including the animal size, metabolic rate, and the high risk of hypothermia and hypoglycemia. Moreover, anesthetic agents influence physiological parameters, further interfering with experimental results (Gargulio et al., 2012; Navaro et al., 2021). Laboratory mice are anesthetized by either inhalation of volatile anesthetics or injection of anesthetic drugs.

Inhalant anesthesia is highly demanded in rodents because the anesthetic depth can be easily controlled (Tsukamoto et al., 2015). Inhalant anesthetics available for use in laboratory animals include halothane, isoflurane, sevoflurane, and desflurane. Among them, isoflurane is most the common inhalant anesthetic used in mice (Gargulio et al., 2012; Richardson & Flecknell, 2005; Tsukamoto et al., 2015; Navaro et al., 2021). Isoflurane is

presently the animal inhalation anesthetic agent of choice for both short and lengthy procedures due to its short induction and recovery time and the reliability of its effects (Cesarovic et al., 2010). Isoflurane is also used for very short procedures because it enables mice manipulation and injection, blood collection, and minor surgical procedures (Gargulio et al., 2012).

The study aimed to investigate the influence of isoflurane on the vital functions during inhalation anesthesia in two mouse strains (inbred and outbred). Heart rate, pulse distention, respiratory rate, arterial oxygen saturation (SpO_2) and rectal temperature were measured during anesthesia and compared to the same parameters measured on awake animals. Also, a series of reflex measurements were carried out on each individual mouse to evaluate the depth of anesthesia.

MATERIALS AND METHODS

This study was carried out in compliance with the principles of ethics and in accordance with the provisions of EU Directive 63/2010 on compliance with the rules for the care, use and protection of animals used for scientific purposes.

The study was also approved by the Ethics Committee of "Cantacuzino" National Medico-Military Institute for Research and Development and approved by the competent authority.

10 NMRI female mice (outbred strain) and 10 C57BL/6 female mice (inbred strain), 12 weeks old were used in the study. The animals were provided by Băneasa SFP (Specific Pathogen Free) Animal Facility area for rats and mice of "Cantacuzino" National Medico-Military Institute for Research and Development, Bucharest.

All aspects related to animal housing and care were undertaken in accordance with the national and international regulations concerning animal testing. The animals were acclimated to the facility for 1 week before experimentation and housed under standard conditions, 10 mice per cage, 18-24℃ temperature, 35-75% humidity and light controlled conditions (12 h/12 h light and dark cycles).

For the vital signs monitoring we used MouseOx Plus Pulse Oximeter for Mice and Rats (Starr Life Sciences Corporation, USA). Heart rate, arterial oxygen saturation (SpO2), pulse distention, breath rate, and temperature were evaluated for each group (Figure 1). Animals were monitored by placing the collar sensor on the back of the neck and the rectal sensor for temperature measurement. Previously, animal hair was removed from the cervical dorsal region for a better signal detection. The awake animals were easily restrained in this position and the vital signs were recorded on the MouseOx Plus integrated software (Figure 2).

Figure 1. MouseOx Plus pulse oximeter used in mice for noninvasive physiological vital signs monitoring

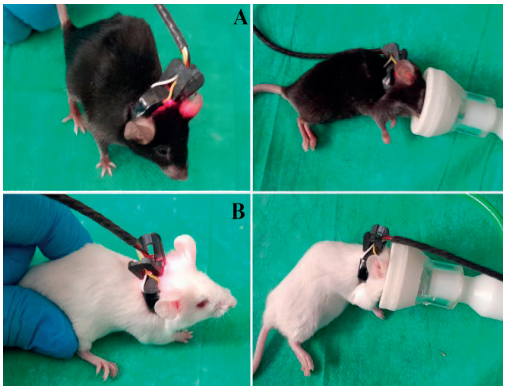

Figure 2. Vital signs monitoring on awake/ anesthetized mice: A- C57BL/6; B- NMRI

Figure 3. Ugo Basile Veterinary anesthesia workstation for small animals

Isoflurane anesthesia (Anesteran, Rompharm, Romania) was administered using an available rodent inhalant anesthesia system (Ugo Basile Veterinary anesthesia workstation 21100, Italy) which has a digital vaporizer and internal air-flow pump, an induction chamber and a nose-cone, connected to an evacuation tubing (Figure 3). The mask incorporates a latex diaphragm, which holds the rodent nose, keeping the animal in correct position and ensuring a continuous positive flow of fresh oxygen and anesthetic.

The animal was placed into the induction chamber and the O_2 flow meter was turned on to a flow rate of 1 L/min. Isoflurane exposure can be aversive so for induction gas concentration was set at 0.5% flow rate and slowly increased up to 5%. The induction time was defined as the duration between the loss of righting reflex and the commencement of surgical anesthesia.

Once loss of the postural reaction and righting reflex was confirmed, the mouse was rapidly transferred to the nose mask. Collar sensor and temperature sensor were placed on the right position and measurements were recorded for a 20 minutes period. Anesthesia was maintained with 2% isoflurane concentration at a flow rate of 1 L/min and the animal was placed on a nylon pad to maintain a constant surface temperature underneath.

A series of observations and reflex measurements were carried out on each individual mouse to evaluate the depth of anesthesia. Tail pinch, pedal withdrawal in the forelimbs/hind limbs, and abdominal skin pinch were applied at 5 min intervals by using blunt forceps containing a spacer between its arms. The reflex tests were registered as positive or negative, whether any motor response was observable or not. To reduce sources of variation in response to the stimuli, all reflex tests were carried out and assessed by the same operator. After 20 minutes aneshesia, isoflurane was turned off and each animal was monitored continuously while keeeping the nose-cone on oxygen flow running for another 2-3 minutes. To assess mice recovery after discontinuing inhalation system, the vital signs were monitored another 10 minutes and the rectal temperature was measured every 15 minutes for the next 120 minutes.

RESULTS AND DISCUSSIONS

During the study, there were no fatal events and all mice returned successfully and rapidly to their normal state (1-4 minutes). Vital signs were first measured on awake animals and then in 5 minutes from the isoflurane anesthesia being installed. Values are given as means for each group and significant differences are analyzed using an unpaired Student's T-test. P-values <0.05 were considered statistically significant.

Results showed a decreased heart rate by 24.74% in NMRI group and 28.5% in C57BL/6 group during anesthesia and a return to the initial heart rate level within 8-10 minutes after discontinuing inhalation system (Figure 4). Respiratory rate significantly decreased in the first 5 minutes after gas exposure, by 39.2% in NMRI group and 53.12% in C57BL/6 group. However, breath rate returned to the initial values in 5-7 minutes after turning off the anesthetic gas. (Figure 5). SpO_2 recorded a slight decrease during anesthesia but none of the animals showed an O_2 saturation under 95.8% (Figure 6). Under stable anesthesia, the pulse distension was relatively constant for

both groups, with a slight decrease in 5 minutes after isoflurane exposure (Figure 7).

Temperature measurement during anesthesia showed a mean decrease by 1.8°C for NMRI mice and by 2.2°C for C57BL/6 mice. After anesthesia, rectal temperature returned to the initial level within 97 minutes for NMRI mice and 123 minutes for C57BL/6 mice (Figure 8).

Finally, we evaluated the anesthetic depth. When a reflex reaction to stimulation was observed, the score was 0 and when no reaction to stimulation was observed, the score was 1. Each measurement was scored and noted, and the anesthetic depth was calculated by total score for each mouse. A score of 3 or more was defined as surgical anesthesia. Under 2% isoflurane maintenance, none of the mice showed a motor response to testing of the pedal withdrawal reflex, tail pinch or abdominal skin pinch. Based on these tests, all of the animals monitored in both groups achieved surgical anesthesia.

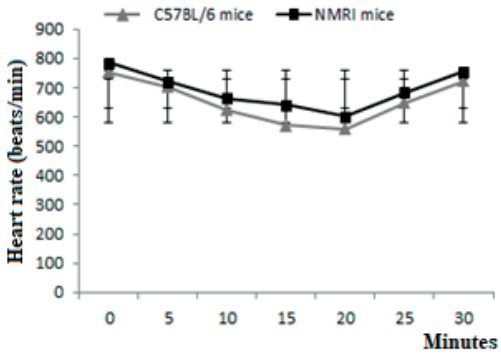

Figure 4. Heart rate measurement (beats/min) over time

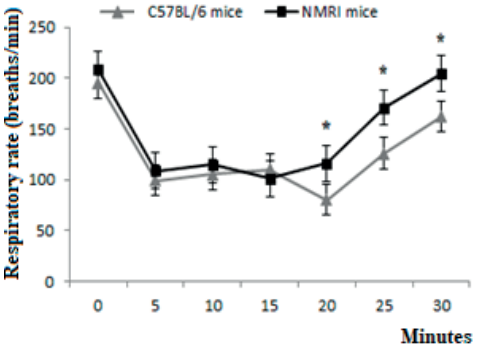

Figure 5. Respiratory rate measurement (breaths/min) over time

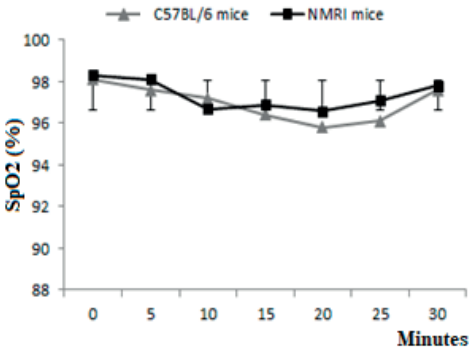

Figure 6. SPO2 (%) measurement over time

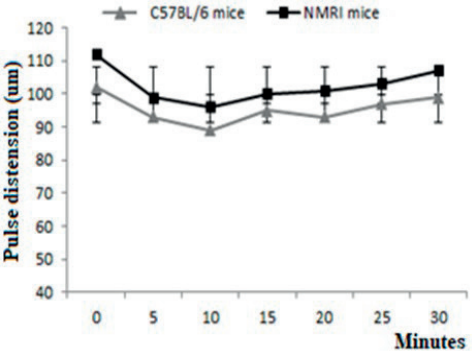

Figure 7. Pulse distention (µm) measurement over time

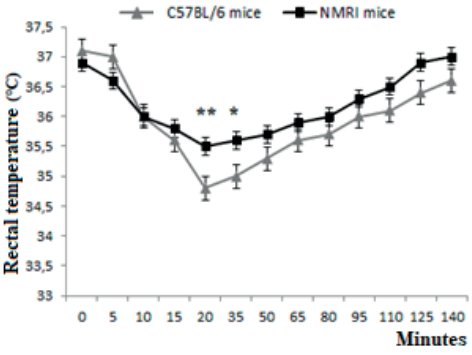

Figure 8. Temperature measurement (°C) during anesthesia and recovery

Concern regarding animal welfare and the quality of data obtained from research projects using animals is increasing.

A suitable, effective and free of complications anesthetic protocol is very important in experimental animal studies although there is no universally accepted protocol for induction, maintenance and recovery from anesthesia (Cicero et al., 2018).

The main factor in selecting an appropriate anesthesia is the required level of anesthetic depth that varies depending on the experimental procedure. Another important factor is the potential for cardiorespiratory adverse reactions. (Arunachalam & Sasidharan, 2021; Tsukamoto et al., 2015). Cardiovascular and respiratory function can be assessed by monitoring vital signs, which include the heart rate, respiratory rate, pulse distention, and arterial oxygen saturation (SPO2).

As mentioned previously, laboratory mice exhibit specific anatomic and physiologic peculiarities that influence the effects of anesthetic drugs. Due to their small body size, drug metabolism and excretion are extremely fast, reducing the half-life of injectable drugs and rendering the duration of anesthesia a more critical factor (Gargulio et al., 2012). Moreover, when compared with larger species, the higher ratio of body surface area to body volume in mice promotes heat loss and hypothermia, while their reduced glycogen reserve predisposes them to hypoglycemia. In addition, their high oxygen consumption rate reduces the survival rate for hypoxemia (Navaro et al., 2021). Small body size of mice also causes physical problems. For example, endotracheal intubation and ventilation of this species are demanding tasks, and many veterinary anesthesia monitoring devices could not be used for mice anesthesia (Ahmadi et al., 2022).

The most commonly utilized inhalant is isoflurane, although sevoflurane has also been used successfully in mice studies (Navaro et al., 2021; Richardson & Flecknell, 2005; Lipiski et al., 2017; Cesaronic et al., 2010).

For surgical intervention and short-term experimentation, isoflurane is the most frequently used anesthetic in mice studies, in part because of its moderate cardio-depressive effects in comparison to the injectable agents - pentobarbital/urethane or ketamine/xylazine mixture (Constantinides et al., 2021).

Isoflurane does not sensitize the myocardium to catecholamines, and it spares cardiac output more than other volatile agents. Indeed, isoflurane can cause a more severe respiratory depression compared with halothane and a dose-dependent hypotension (Gargulio et al.,2012). Isoflurane is poorly soluble in blood and undergo minimal metabolism. As a result, recovery after anesthetic discontinuation is rapid, even in animals with significant hepatic or renal impairment (Lee-Parritz, 2013).

Our study results showed an important respiratory depression (more pronounced for C57BL/6 group) while SPO_2 remained relatively stable under 2% isoflurane anesthesia. The heart rate recorded a moderate decrease during anesthesia with a rapid return to the initial level and slight variation of the pulse distension.

Tsukamoto et al. concluded that the heart rate decrease with isoflurane was lower than that observed in the injectable anesthetics (pentobarbital monoanesthesia; ketamine and xylazine combined; medetomidine, midazolam, and butorphanol combined), suggesting that isoflurane produces the smallest cardiac influence. However, compared to the injectable anesthetics, isoflurane administration showed a prominent decreased breath rate, respiratory depression being the major adverse effect.

Constantinides et al. compares the stability of heart rate (HR), and body temperature under isoflurane different concentrations and observed that the most stable blood pressure and heart rate accompanied an inhalant anesthetic dose level of 1.5%.

Ewald et al. showed that under stable anesthesia the vascular distension caused by the pulse is relatively constant and is of higher amplitude than the vascular distension caused by breathing while chronic exposure to excess isoflurane (2.5%) results in a decline in the pulse distension.

Picard et al. evaluated the effects of isoflurane dose on myocardial function in a murine model and concluded that light isoflurane anesthesia is preferable for hemodynamic analysis or anesthesia in a cardiac disorder model.

Various mice studies investigated the minimum alveolar concentrations, defined as the minimum concentration of inhaled anesthetic at which 50% of the animals fail to respond with purposeful movements to the testing of reflexes (Aranake et al., 2013).

The minimum alveolar concentration for isoflurane is estimated to be 1.4% to 2.0% depending on the severity of the noxious stimulus, where the stimulus is applied, and animal-specific factors such as mouse strain,

underling health conditions, age, gender, etc. (Aranake et al., 2013; Sonner et al., 2000).
Cesaronic et al. compares the effect of isoflurane and sevoflurane in laboratory mice anesthesia and established mean minimum alveolar concentration as 1.85% for isoflurane and 3.25% for sevoflurane in C57BL/6J mice. The most prominent side-effect they observed during anesthesia was respiratory depression with hypercapnia, acidosis and a marked decrease in respiration rate.
Hypothermia is one of the most common complications during anesthesia, and mice are particularly susceptible because of their high surface area to mass ratio (Navaro et al., 2021; Buitrago et al., 2008). Hypothermia has several negative consequences, including cardiac arrhythmias, hypercoagulability, increased susceptibility to infection, delayed recovery and decreased minimum alveolar concentrations for inhalant anesthetics. (Flecknell, 2016; Gargiulo et al., 2012). Baseline body temperature measured rectally for C57BL/6 mice at the start of isoflurane anesthetic exposure was previously found to be 36-37°C with a 2°C decrease in body temperature during prolonged anesthesia (Caro et al., 2013). Therefore, hypothermia must be prevented and controlled by providing heat through warming pads and infrared lamps.
An important factor that should be considered when using isoflurane is the lack of analgesic properties provided by inhalant anesthetics. During anesthesia, isoflurane provides unconsciousness so pain perception is absent but during recovery an effective analgesic protocol should be considered depending on the experimental procedure. (Navaro et al., 2021; Flecknell, 2016).

CONCLUSIONS

Safe and effective anesthetic management is a crucial aspect for the refinement of animal experimental methods.
Isoflurane concentration investigated in this study is assumed for surgical anesthesia in both mouse strains, with moderate overall effects on the vital signs and a rapid return of the animals to their normal state.
NMRI strain showed a lower decrease of the respiratory rate and rectal temperature during anesthesia compared to C57BL/6 mice. Heart rate and O_2 saturation showed similar changes during isoflurane anesthesia for both tested groups.
Additional studies are needed to determine the influence of isoflurane on vital signs across several concentrations and for long term anesthesia.

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