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Faculty of Veterinary Medicine

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Address: 105 Splaiul Independentei, District 5, Zip code 050097, Bucharest, Romania
Phone: + 40 21 318 04 69,
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SUMMARY

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ANATOMIC PARTICULARITIES OF PELVIN MUSCULARITY – AFRICAN OSTRICH (*STRUTHIO CAMELUS*)

Florina DUMITRESCU, Iulian DUMITRESCU, Cristian BELU, Diana LICSANDRU, Petronela ROȘU, Bogdan GEORGESCU, Gabriel PREDOI

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spaiul Independenței, District 5, Bucharest, Romania

Corresponding author email: dumitrescu_florina@yahoo.com

Abstract

Even though specialty literature includes a series of publications regarding the muscles of pelvic limb of the African ostrich, there are still a couple of aspects that aren't entirely understood. Therefore, a series of differences in regards to the insertions of the pectineus muscle and ambiens muscle can be described, as well as the usage of different terminology for the identification of the cranial muscles of the thigh. This study, conducted on 10 african ostriches of different ages and sexes, realized through classic methods and macroscopic investigations, aims to conduct a detailed anatomization of pelvic muscles, more precisely of the muscles whose topography is adjacent to the pelvic region. The nomenclature used was in concordance with the Nomina Anatomica Avium 1993, although this study also suggests other naming options, which from our point of view better reflect the morphological and topographical realities.

Key words: *ostrich, pelvine, muscles.*

INTRODUCTION

The ostrich is the tallest and the heaviest of all birds. While the huge ostrich is a bird, it does not fly. Instead it runs. One stride can cover up to 4.9 meters. The bird is also very fast, as it can reach speeds of up to 64 kilometers per hour on short distances, and can keep up a speed of more than 48 kilometers per hour over longer distances.

The ostrich uses its short wings for balance, holding them outstretched when it runs. Its strong legs can also be used for self-defense (7). Muscles from the lumbar and sacral regions, but most importantly from the femural and calf regions are generally regarded as high quality meat. In order to improve the quality of the meat, many scientists study the different chemical components found in the biochemical structure of the muscles, which influence taste, texture, tenderness and many other attributes that improve organoleptic features.

In already published specialty literature, the data about the musculature of the ostrich is lacklustre and sometimes disregards the notions present in the Nomica Anatomica Avium. Based on this fact, the reason for establishing this study was the desire to complete the already existing data about this species anatomy.

MATERIALS AND METHODS

The study material is represented by samples originating from 10 adult ostriches of different sexes, with a weight range between 20 – 50 kg. Several bodies originating from zoos or private breeders were brought to the Veterinary Medicine College for necropsy. Other samples were procured from butcheries. Following their dissection the samples were also used for bone preparation. The samples underwent measurements, as well as descriptions of the anatomical particularities, and werre then photographed. The identification and description of the strucutres was done according to the Nomina Anatomica Avium – 1993.

RESULTS AND DISCUSSIONS

Caudal ilio trochanteric muscle *(M. iliotrochantericus caudalis)* (Fig.1) is a small narrow muscle, flattened latero-medially. Its insertion is found on the lateral side of the ilium, dorsal from the acetabular cavity. On this level,

the muscle is marked cranially by the proximal insertion of external iliofemural muscle, and distally by the proximal insertion of the iliotibial lateral muscle. Distally, the muscle's tendon inserts on the femur at the limit between its lateral and caudal part, at the base of big trochanter.

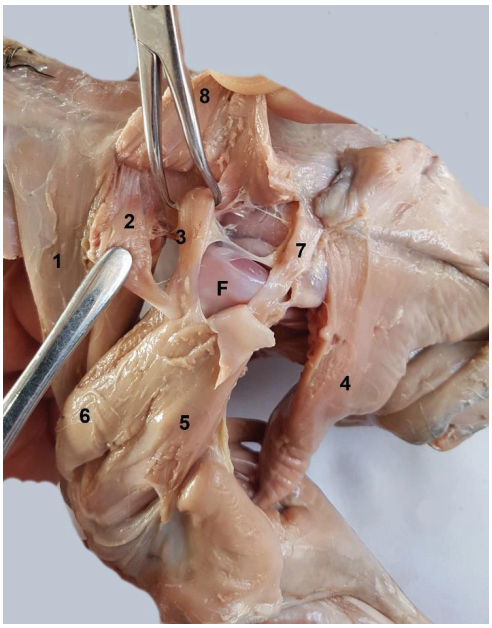

Fig. 1 Pelvis and hip muscles (lateral view) (original)
1 – iliotibial cranial muscle; 2 – iliotrochanteric cranial muscle; 3 – iliofemural intern muscle; 4 – iliofibular muscle; 5 – extern fibitibular muscle; 6 – medium femurotibial muscle; 7 – caudal iliotrochanteric muscle; 8 – extern iliofemural muscle; F- femur

The cranial iliotrochanteric muscle *(M. iliotrochantericus cranialis)* (Fig.1) is located cranio-dorsally to the coxal-femural joint, and it has rapports with the caudal-proximal edge of the cranial iliotibial muscle and the anterior edge of the medial iliofemural muscle. Its profound face provides contact with the lateral face of the proximal extremity of the ambiens muscle, while on the exterior the muscle is covered by the external iliofemural muscle. Its fixed insertion is on the lateral surface of the preacetabular part of the ilium, next to its cranial extremity. The distal tendon of this muscle inserts on the lateral surface of the femur, distally from the trochanter in the same place as the tendon of the intern iliofemural muscle.

The external iliofemural muscle *(M. iliofemoralis externus)* (Fig.1) is a muscle with a triangular shape. It is disposed in superficial plane and it fills the area between the cranial and the lateral iliotibial muscles. This muscle's proximal insertion is on the lateral surface of the preacetabular part of the ilium, near its dorsal edge, covering at this point the fixed insertion of the cranial iliotrochanteric muscle, the intern iliofemural and the medial iliofemural muscle. Distally, the flattened tendon of this muscle inserts on the lateral surface of the femur, ventrally from the trochanter.

The intern iliofemural muscle *(M. iliofemoralis internus)* (Fig.2) is totally coverd by the precedent muscle. It appears as a vertical, narrowed band which emerges to the lateral surface of the trochanter. Its proximal insertion is found on external suface of the preacetabular part of the ilium, cranio-dorsally from the acetabular cavity. The distal tendon inserts on the femur in the same place as the tendon of the cranial iliotrochanteric muscle.

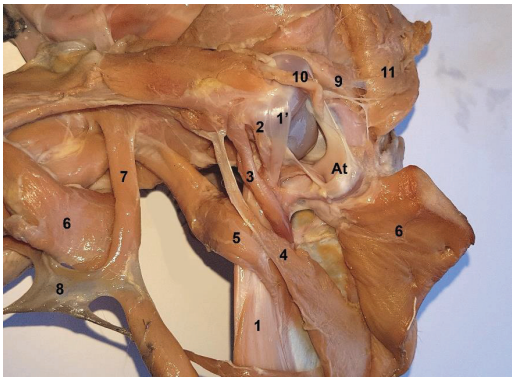

Fig 2. Profound muscles of pelvis (lateral view) (original)
1 – intern obturator muscle; 1'- intern obturator ligmanet; 2 – obturator extern muscle; 3 – ischiofemural muscle; 4 – caudofemural muscle; 5- puboischiofemural muscle; 6 – iliofibular muscle (sectioned) 7- orizontal part of crural lateral flexor muscle; 8 – quadrilater fasces; 9 – intern iliofemural muscle; 10 – caudal iliotrochanteric muscle; At-antitrochanter.

The medial iliofemural muscle *(M. iliofemoralis medius)* is placed on the profound surface of the external iliofemural muscle, in the area between the cranial iliotrochanteric muscle and the internal iliofemural muscle. This muscle has a triangular shape, with the apex oriented distally and it cranially intersects with the view the capsule of the coxal-femural joint. Its proximal

insertion is found on the external surface of the preacetabular part of the ilium in the free space between the insertion of the cranial iliotrochanteric muscle and the intern iliofemural muscle. The distal tendon of this muscle inserts on the medial surface of the femur on the line which delimits the superior and the middle third.

The caudofemural muscle *(M. caudofemuralis)* (Fig.2) is placed on the profound surface of the iliofibular muscle, being totally covered by this muscle and the iliotibial lateral muscle. Viewed fully, this muscle is oriented obliquely in a cranio-distal way, and it has a caudal muscular part and a cranially disposed, well developed tendon. The tendon inserts on the caudal surface of the femur at the delimitation between the proximal and distal third of the bone, dorsally from the insertion of the medial part of internal femuro-tibial muscle. The origin of the muscular part is found on the ventral border of caudal half from the postacetabular area of the ilium, on the first 4 coccygian vertebrae and on the superior border of the ilio-ischiatic membrane.

The ischiofemural muscle *(M. ischiofemuralis)* (Fig.2) is a relatively reduced muscle placed medio-cranio-dorsally to the caudofemural muscle, which it covers in the caudal third. Viewed as a whole, the muscle is oriented craniodistally and it can be evidenced on the profound surface of the iliofibular muscle, caudo-ventrally to the coxofemural joint. The fixed insertion of this muscle is on the lateral surface of the ischium in its cranial third, but also on the external ilio-ischiatic membrane in its cranioventral third. Cranially, the muscle has a short tendon thaat inserts on the latero-caudal surface of the trochanter, ventrally from the insertion of the tendon of the external obturator muscle.

The lateral obturator muscle (external) (Fig.2) *(M.obturatorius lateralis)* is a relatively small muscle, placed caudo-ventrally from the coxo-femural joint. The muscle presents two parts (dorsal & ventral) which are approximately merged together, which explains the difficulty met in individualizing them. The dorsal part is aligned with the tendon of the intern obturator muscle, starting from the obturator hole, and until its insertion on the femur. The origin of the lateeral obturator muscle is on the contour of he obturator hole, and its mobile insertion is found on the latero-proximal surface of the trochanter, in the same place as the tendon of the medial obturator muscle (for the dorsal surface) and on the caudal surface of the base of the trochanter, proximolaterally from the pneumatic hole (for the ventral part).

The medial obturator muscle (intern) (Fig.2) *(M. obturatorius medialis)* is placed near the ischio-pubic hole, and its appearance is flattened in a latero-medial way. Viewed laterally, the muscle presents its fibers placed horizontally in the superior half and oblique cranio-dorsally in the ventral half. The muscle's origin is on the ventral edge of the ischium, on the dorsal border and the lateral surface of the pubis as well as the lateral surface of the ischio-pubic symphysis. The muscular part continues with the tendon from the medial face of the obturator hole. It makes its way through the obturator hole medio-laterally, coming in contact with the caudal edge of it, then it goes on the superior border of the dorsal part of the lateral obturator muscle, fin order to insert on the latero-proximal surface of the trochanter.

The pubioischiofemural muscle *(M. pubi-ischio-femuralis)* (Fig.2 & Fig.3) is placed ventrally from the caudofemural muscle, craniodorsally from the crural flexor muscle, laterally and profoundly from the iliofibular muscle. From all points of view, it seems flattened latero-medially and oriented diagonally in a cranio-ventral way. The muscle is made up of two parts, and their fibers merge so the individualization between the parts is not perfect. The lateral part is placed dorsally from the medial one and it has its origins on the lateral surface of the pubis. The medial part is also divided in a dorsal part, inserted on the external surface of the ischium and on the ilio-ischiatic membrane & a ventral part, with an aponevrotic origin on the ventral edge of the ischium. Cranially, the pubio-ischio-femural muscle inserts through a tendon on the caudal surface of the femur in its distal third, proximally from the medial insertion of the gastrocnemius muscle and medially from the horizontal part of the lateral crural flexor muscle.

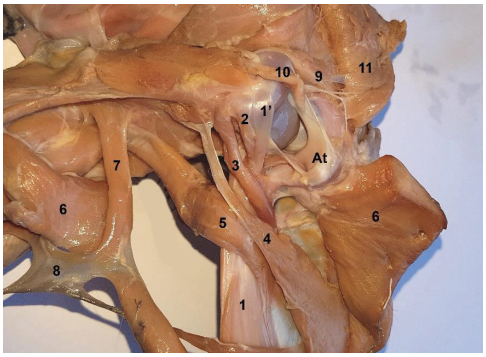

Fig. 3 Medial muscles of pelvis and hips (original)
1-Iliotibial cranial muscle; 2- ambiens muscle; 3- femurotibial accessories; 4- pectineus muscle; 5- puboischiofemural muscle; 6- obturator medial muscle; 7- crural medial flexor muscle; 8- crural lateral flexor muscle; 9- ligament of medial obturator muscle; 10- iliofibular muscle; 11- accessories part of crural lateral flexor muscle; 12- quadrilateral fascia; 13- distal bridle of medial crural flexor & distal bridle of *crus caudale* iliofibular muscle; 14- intermediary part of gastrocnemius muscle ; P- pubis; S- sinsacrum

CONCLUSIONS

Excepting the external iliofemural muscle, disposed superficially, the iliotrocantheric muscles and the other iliofemural muscles are grouped around the coxofemural joint which they help strengthen, similarly to the profound muscles of the basin in mammals.
Both obturator muscles are present; the lateral one made of two parts and reduced, while its medial counterpart is well represented.

The pubioischiofemural, ischiofemural and caudofemural muscles have their origin on the coxal bone but judging by the topography of their muscular parts they might be considered medial muscles of the pelvis.

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MACROSCOPIC ANATOMY OF PANCREAS IN RATS, GUINEA PIGS, CHINCHILLAS AND RABBITS

Florin Gheorghe STAN

University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca,
3-5 Mănăştur Str. Romania

Corresponding author email: florin.stan@usamvcluj.ro

Abstract

The aim of this paper is to provide a detailed and comparative presentation of macroscopic anatomy of the pancreas, its topography and connection elements among the experimental animal species including rats, guinea pigs, chinchillas and rabbits. Using gross dissection, the pancreas and its connection elements were studied on 10 specimens of each species presented. The triangular form of the pancreas is a common anatomical pattern in rats, guinea pigs and chinchilla with different degrees of development of the three portions. Located reroperitoneally and in relation to the duodenum, spleen and stomach, the three portions are referred as the duodenal, splenic and gastric portion or lobes with the same names. In rabbits, however, the right lobe of the pancreas has a diffuse appearance, being located largely in the mesoduoden compared to the left lobe which has a better defined shape being located in the deep wall of the greater omentum. The pancreas relations in the experimental models studied are with the right lobe of the liver, the portal vein, the right kidney, the caudal cava vein, the aorta and the emergence of the celiac and mesenteric arteries, the profound wall of the large omentum, the stomach and the transverse colon.

Key words*: pancreas, anatomy, experimental models.*

INTRODUCTION

Over the decades, the pancreas in experimental animals was intensively studied related to its physiological component. Since the insulin discovery in 1921 and its direct relationship with the glucose metabolism followed by the research involving the inflammatory, ischemic and neoplastic morbidities, it was clearly stated the need of experimental models in order to achieve a better understanding of local and systemic organic implications (Aghdassi et al., 2011; Cattley et al., 2013; Stan 2014; 2015; 2017). Moreover, pancreatic transplantation required a proper knowledge of pancreas anatomy in experimental animals in order to improve surgical techniques. With the exception of domestic animals, in which the pancreas has been extensively studied, both macroscopically and microscopically, detailed anatomical studies were performed in mouse, rats, monkeys, dogs and minipigs (Suckow et al., 2012; Pandiri, 2014; Tsuchitani et al., 2016). In this study, it was intended to present detailed description about the macroscopic anatomy of the pancreas and especially to emphasize the difference and similarities of pancreatic macroscopic anatomy in the rats, chinchillas, guinea pigs and rabbits, which may guide researchers in experimental studies.

MATERIALS AND METHODS

Ten healthy adult rats, guinea pigs, chinchillas and rabbits were used. The Institutional Bioethics Committee of University of Agricultural Science and Veterinary Medicine in accordance to Directive 2010/63 /EU of the European Parliament and of the Council on the protection of animals used for scientific purposes approved the study. Euthanasia was performed by administration of an overdose of isoflurane. The abdominal cavity was opened and the wall of it were carefully removed in order to visualize and to photograph the pancreas, its relations with the adjacent organs and its connection elements. The pancreas was divided in the following portions: duodenal segment, gastric segment and splenic segment. The duodenal segment was visualized ventrally with a minor procedure as pulling the duodenum caudally and additionally the entire pancreas were reached dorsally since the stomach and spleen were turned cranially.

Terms were used in agreement with the NAV (Nomina Anatomica Veterinaria) 2012.

RESULTS AND DISCUSSIONS

Rat

The pancreas has a lobulated pattern. On the right side it was located in the mesentery of the duodenal loop and transverse colon extending to the dorsal part of greater omentum adjacent to the stomach and spleen (Figure 1).

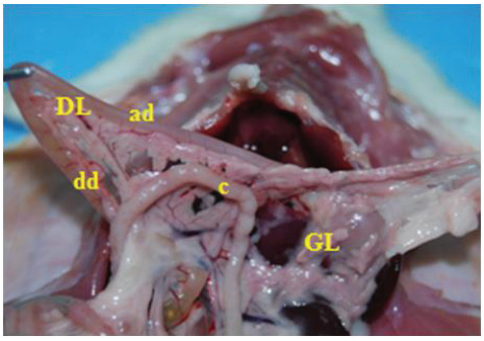

Figure 1. The duodenal lobe – DL of pancreas in rats located in the mesoduodenum. The gastric lobe – GL extends into the dorsal sheet of the greater omentum. dd – descending duodenum; ad – ascending duodenum; c – colon.

On the right side, the right lobe of pancreas (*Lobus pancreatis dexter*) or duodenal lobe was invested in the mesentery between the descending and ascending ansa of the duodenum (*Mesoduodenum*). The splenic lobe, (Figure 2) extends from the duodenal lobe toward to the spleen on the left side of the median plane, being the left lobe correspondent (*Lobus pancreatis sinister*).

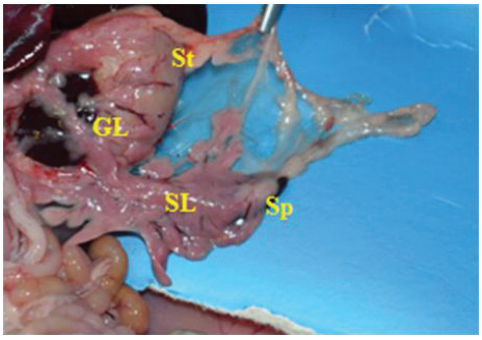

Figure 2. The splenic lobe – SL of pancreas in rats is the most developed and compact lobe. It extends between the duodenal lobe and spleen - Sp. GL – gastric lobe; St – stomach.

It was the most developed and compact lobe of the pancreas in rats. The terminal part of the splenic lobe extends into the gastrosplenic ligament (*Lig. gastrolienale*) (Figure 3).

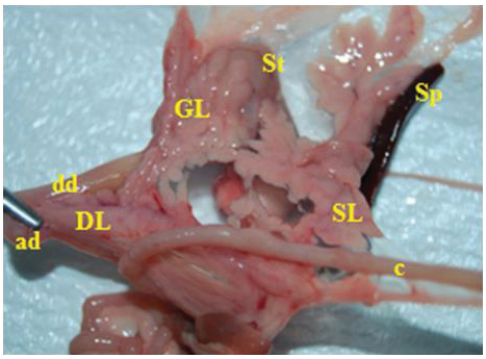

Figure 3. The three lobes compound pancreas in rat: The duodenal lobe – DL; the gastric lobe – GL; the splenic lobe – SL. The caudal part of the duodenal lobe and the dorsal part of the splenic lobe are joined together near to the colon – c.

The gastric lobe was the smallest lobe of the pancreas in rats, extending from the left portion of the duodenal lobe into the dorsal sheet of the greater omentum adjacent to the stomach.

Guinea pig

The pancreas in guinea pig consists of three lobes, each lobe being separated into a number of small lobules. The duodenal lobe lies in close contact with the descending duodenum into the mesentery between the ascending and descending duodenum (Figure 4).

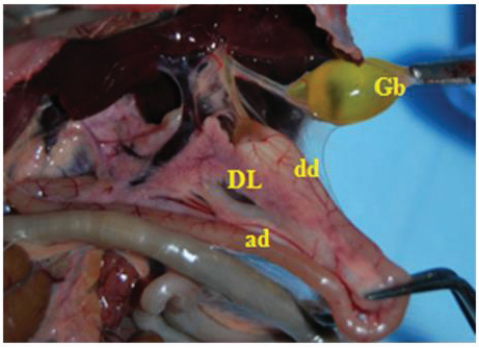

Figure 4. In guinea pig, the duodenal lobe – DL lies in close contact with descending duodenum – dd, into the mesentery between the descendant and ascendant – ad ansa of the duodenum. Gb – gallbladder.

From the proximal portion of the duodenal lobe, the splenic lobe extends to the left, in a caudal direction, near to the dorsal part of the spleen. The compact splenic lobe was the largest lobe of pancreas in guinea pig being fully attached to the gastrosplenic ligament (Figure 5).

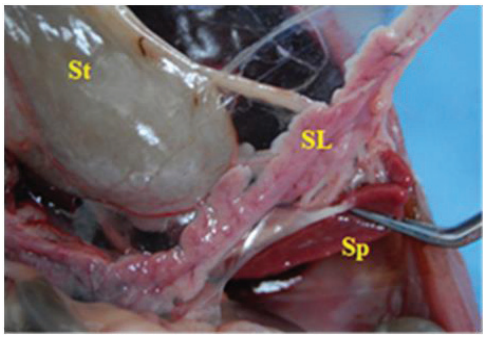

Figure 5. The splenic lobe - SL of pancreas in guinea pig extends caudally to the stomach – St, to the left on the dorsal aspect of the spleen – Sp.

Several islets of pancreatic tissue arranged in a dendritic manner caudally to the fundus of the stomach, and detached from the splenic lobe, formed the gastric lobe of pancreas in guinea pigs (Figure 6).

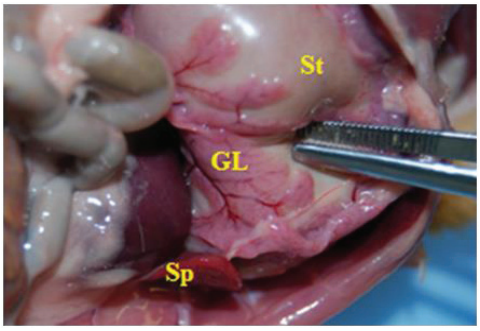

Figure 6. The gastric lobe – GL of pancreas in guinea pig arranged in a dendritic manner caudally to the stomach - St. Sp – spleen.

Chinchilla

In situ the pancreas showed the same three divisions: the duodenal lobe, the splenic lobe and the gastric lobe. The duodenal lobe, corresponding to the right lobe of pancreas (*Lobus pancreatis dexter*) was located adjacent to the duodenum, being attached to the descending loop of duodenum (Figure 7).

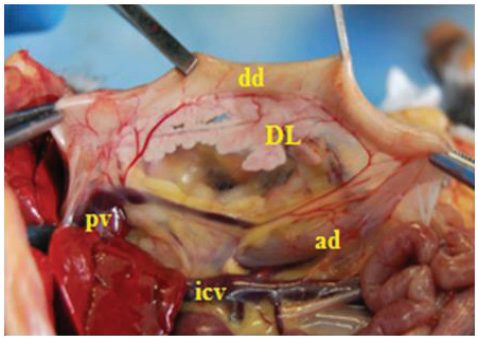

Figure 7. The well defined duodenal lobe – DL of pancreas in chinchilla lies in contact with descending duodenum – dd. The portal vein – pv, passes in close proximity of the caudal portion of the duodenal lobe. icv – inferior cava vein.

Its length does not reach the transverse portion of the duodenum and is not in contact with the ascending loop.

The portal vein was in close proximity to the caudal portion of the duodenal lobe. The left lobe (*Lobus pancreatis sinister*) or splenic lobe has a compact appearance exceeding the caudal edge of the spleen (Figure 8).

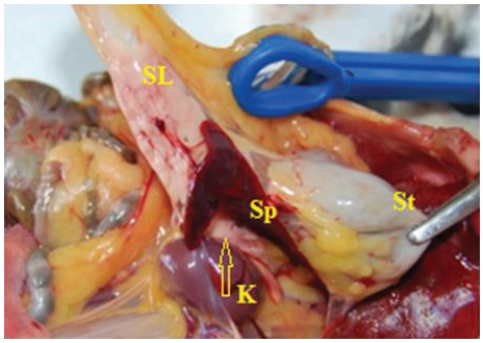

Figure 8. The left lobe of pancreas – SL in chinchilla has a compact appearance exceeding the caudal edge of the spleen - arrow. K –left kidney; St – stomach.

The gastric lobe was dispersed in multiple nodules protruding toward to the stomach (Figure 9). In chinchilla, the pancreas has a triangular shape with irregular margins after removal from the abdominal cavity (Figure 9).

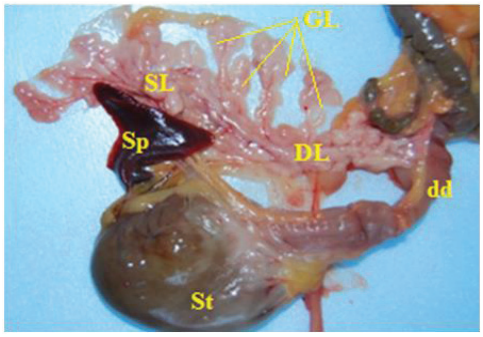

Figure 9. The triangular shape of the chinchillas pancreas. The splenic lobe – SL, was the most developed and compact lobe. The gastric lobe – GL, was dispersed in small portions protruding to the stomach – St. Sp – spleen; dd – descending duodenum.

Rabbit

The major part of the rabbit pancreas is contained into the mesoduodenum, this part being correspondent of the right lobe of pancreas or duodenal lobe (Figure 10).

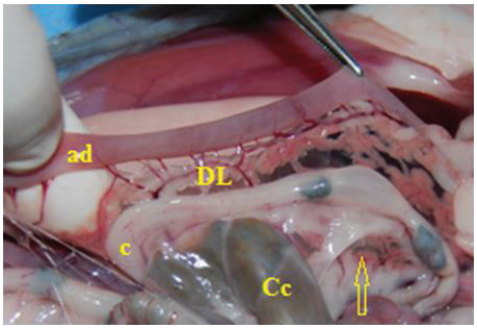

Figure 10. In rabbits, the diseminated glandular tissue of pancreas into the mesoduodenum – DL and arrow, is correspondent of the duodenal lobe. ad – ascending duodenum; c- colon; Cc – cecum.

It appears as a diffused irregular mass of glandular tissue distributed around the pancreaticoduodenal blood vessels and in more close relationship to the ascending ansa of the duodenum (Figure 10). On the lesser curvature of the stomach, and cranial part of the duodenum the gastric lobe was identified. This portion has a slightly condensed appearance (Figure 11).

The left lobe (*Lobus pancreatis sinister*) of the pancreas in rabbits located caudally from the stomach fundus into the wall of greater omentum and in close contact with spleen was assessed as the splenic lobe (Figure 12). This lobe reaches up to the ventral aspect of the left kidney.

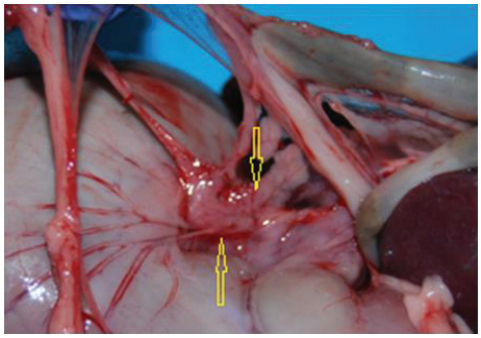

Figure 11. Slightly condensed appearance of pancreatic tissue – arrows, bounded by the lesser curvature of the stomach and cranial part of the duodenum, corresponding of the gastric lobe of pancreas in rabbit.

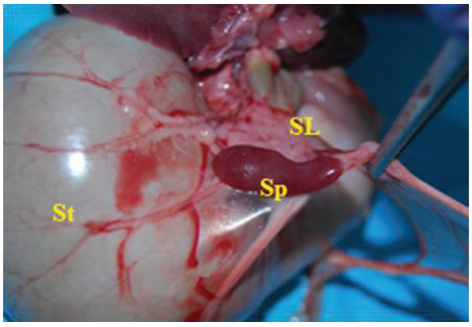

Figure 12. The condensed portion of the left pancreas in rabbits was the splenic lobe –SL. It was extended between the two extremities of the spleen – Sp and the stomach – St.

Due to the fact that pancreas is a target of a numerous diseases, from which the pancreatic cancer and diabetes mellitus are of major importance, this organ is of a great importance both of morphological and clinical interest. Several laboratory animals have been used in a numerous toxicological, pharmacological (Pandiri 2014; Stan 2015) and surgical researches in order to increase the knowledge which can be applied in humans and domestic animals. In this regard, rodents and rabbits are considered good models in clinical and anatomical studies of diverse morphological abnormalities and pancreatic disease (Aghdassi et al., 2011; Stan 2015; 2017; Tsuchitani et al., 2016).

Compared to the human pancreas, which is a compact organ, in experimental animals, the pancreas has a different appearance. Generally, two types of macroscopic anatomy is recognized: a diffuse pattern in which islets of glandular pancreatic tissue are diffusely distributed into the mesentery between the duodenal loop, found in rabbits (Barone 1997; Brewer 2006) and a more compact appearance found in domestic animals, monkeys, minipig and humans (Swindler et al,. 1973; Barone 1997; Evans and de Lahunta, 2013; Tsuchitani et al., 2016). In experimental animals there is an intermediate pattern in which the diffused distribution of the duodenal lobe alternate with a more compact pattern of the left portion (Barone 1997; Katherine Quesenberry and Carpenter 2012; Stan 2017). This is in agreement with our results which showed a compact appearance of the left portion of the pancreas in rats, guinea pigs, chinchillas and in the rabbits. Regarding the right portion assessed as right lobe or duodenal lobe, the diffused pattern of this lobe was the most pronounced in rabbits, while in guinea pigs, rats and chinchillas, the glandular tissue was compact and organized. A possible explanation of this feature is due to the large mobility of the duodenum found in rabbits.

In veterinary medicine and in accordance with the anatomical denomination (NAV) the pancreas is composed by the right and left lobe united through the body. More recent description in experimental animals uses different terms for lobe denomination like duodenal, gastric and splenic lobes (Stan 2017) or gastric lobe, duodenal head and tail (Cattley et al., 2013). In this study for a better comprehension of the macroscopic anatomy the same terms were used. Moreover, these terms have been used because the pancreas portions to which they refer are named after the organs they are in relation with. Therefore, the duodenal lobe was the correspondent of the right lobe of the pancreas or head of the pancreas. In rats, some authors have described the head of the pancreas being composed by the duodenal and parabilliary portion (Tsuchitani et al., 2016). We did not use the parabilliary terms because our results showed that the right lobe was invested into the mesentery between the ascending and descending loop of duodenum at equal distance from the two loops. The gastric lobe described in this study as in the cited literature, has no counterpart in the domestic larger species. In the human, the terms head, neck, body and tail are used to name the different regions of the pancreas. Taking into consideration the anatomical description of pancreas in humans (composed by the head, body and tail), in species presented here, the body of the pancreas extends from the head to the stomach and spleen. In this regard the gastric and proximal part of the splenic lobe is correspondent of the body of the pancreas in the mentioned species. Even in rabbit we showed the presence of a well defined gastric lobe disposed between the lesser curvature of the stomach and spleen, feature founded in other description too (Al-Saffar and Al-Hasnawy, 2014). The compact splenic lobe was found in all species of this study, being the largest lobe in rats, guinea pigs and chinchilla, as it was mentioned in other descriptions (Suckow et al., 2012; Wagner, 2014;). In rabbit this lobe was compact but regarding its length it was shorter than the duodenal lobe. In chinchilla, due to the particular triangular shape of the spleen, the splenic lobe of pancreas, especially the caudal part accounting to the tail of the pancreas, exceed the caudal part of the spleen. This pattern was mentioned by other authors too (Campbell-Ward, 2012; Ozdemir et al., 2013).

Similar with other description of pancreas in Rodents and rabbits (Katherine E. Quesenberry, and Carpenter, 2012; Al-Saffar and Al-Hasnawy, 2014; Wagner 2014), our study highlighted the pancreas partial location into the peritoneum folds between the duodenum, stomach and transverse colon, and partly location between the stomach, spleen, pancreatico-duodenal and splenic vessels. The close relation with the major vessels is of major importance in surgical experiments (Stan 2015). In this regard is worth to be mentioned the ring shape of the body of the pancreas found in a common experimental animal – the minipig. The body of the pancreas in this specie is composed of two separate portions that encompass the portal vein and make the pancreas appear to be „ring-shaped" (Ferrer et al., 2008). This patern is present in horse, pig and sometimes in cattle (Barone 1997). In the

species that were the subject of this study, we have not met this feature.

The macroscopic description of pancreas anatomy is important from the point of view of physiological, pathological and surgical studies. Experimental induction of diabetes, pancreatitis, or transplantation of pancreatic islets should take into account the segmental division of the pancreas in rodents and rabbits as it was shown in this study, the three lobe compound pancreas; the close relationship with the major vessels and the care not to injure the anatomical structure as pancreatic ducts and biliary ducts.

Due to the fact that the present study it was performed on four species, the vascular anatomy of the pancreas and the pancreatic ducts description are subjects of future reports.

CONCLUSIONS

In rats, guinea pigs, chinchillas and rabbit the pancreas presented three well-differentiated portions or lobes. These lobes have been named in relation to adjacent organs: duodenal, gastric and splenic lobe. Related to the human denomination the duodenal lobe is the head of pancreas correspondent; the gastric and the proximal part of the splenic lobe is the correspondent of the body of the pancreas and the caudal portion of the splenic lobe is the tail of pancreas correspondent.

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PERITONEAL DIALYSIS IN A CANINE PATIENT WITH KIDNEY AND LIVER INJURY CONSECUTIVE TO BABESIOSIS

Nicolae Alexandru BOCIU, Alina ȘTEFĂNESCU, Bogdan Alexandru VIȚĂLARU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spaiul Independenței, District 5, Bucharest, Romania

Corresponding author email: alexandrumv@yahoo.com

Abstract
A 4 year old male Shi-Tzu, diagnosed with babesiosis and kidney and liver failure was treated using peritoneal dialysis for six days along with hidro-electrolytic balancing and parenteral nutrition in the Faculty of Veterinary Medicine's Clinic. At presentation the patient had 6 kg, 39.9˚C, jaundice, apathy, hematuria and lack of appetite. Recommendations were complete blood count, biochemistry blood test and blood cytology. Blood test results revealed elevated levels of blood nitrogen urea (BUN = 71 mg/dl), creatinine (CREA = 4.0 mg/dl) and bilirubin (T-Bil = 11.1 mg/dl). The peritoneal dialysis was performed using Dianeal PD4 1.4%. After six days of intensive care and peritoneal dialysis blood test results were BUN = 19 mg/dl, CREA = 1.0 mg/dl and T-Bil = 0.3 mg/dl.

Key words: *peritoneal, dialysis, injury, kidney, liver.*

INTRODUCTION

Peritoneal dialysis is a modality of renal replacement therapy that is commonly used in human medicine for treatment of chronic kidney disease and end-stage kidney failure. Peritoneal dialysis employs the same principle as other forms of renal replacement therapy: the removal of uremic solutes by diffusion across a semipermeable membrane. In hemodialysis and continuous renal replacement therapy, blood is passed through straw-like semipermeable membranes, which are bathed in a dialysate. By contrast, peritoneal dialysis uses the peritoneum as a membrane across which fluids and uremic solutes are exchanged. In this process, dialysate is instilled into the peritoneal cavity and, through the process of diffusion and osmosis, water, toxins, electrolytes, and other small molecules are allowed to equilibrate. The dialysate is then removed and discarded, carrying with it uremic toxins and water. This process is repeated continuously as needed to achieve control of uremia.

Although peritoneal dialysis is used primarily for the treatment of chronic kidney disease in people, reports from as early as 1923 demonstrate its role in treating acute kidney injury. Its use has also been described for removal of dialyzable toxins and to treat pancreatitis, electrolyte and acid base abnormalities, refractory congestive heart failure, and inborn errors of metabolism. In veterinary medicine, the most common use of peritoneal dialysis is in the treatment of the acute kidney injury, though it can be used for any of the aforementioned indications as well.

One of the most common indications of dialysis in dogs and cats is acute uremia. Dialysis abating rapidly hyperkalemia and can restore the electrolyte balance, helping to stabilize the patient and providing enough time for the renal function to recover at the normal parameters.

Dialysis becomes an option when the clinical consequences of acute uremia can't be managed effectively only medical therapy after 24 - 48 hours.

Dialysis is also effective in the management of chronic kidney disease animals in terminal stages. Dialysis may improve azotemia, electrolyte and acid-base minerals disorders, but also systemic hypertension, which complicates the chronic renal disease in these animals and requiring dialysis indefinitely.

The dialysis technique is perfect for managing specific acute poisonings.

The benefits include the ability to remove toxins which are already absorbed from the intestinal lumen, the elimination of substances

that are not absorbed by the intestinal charcoal, and that both the main compound and the active toxic metabolites can be removed.
Hyperhydration consecutive to systemic arterial hypertension, ascites, limb and pulmonary edema, and congestive heart failure, are a common complication of aggressive fluid based therapy in animals with kidney failure. Circulatory overload may be life threatening and cannot treat oliguric animals. Fluid overload is a constant feature of end-stage renal disease, intravenous or subcutaneous treatments or liquid supplements administered orally. This situation becomes possible when animals have an insufficient capacity to excrete fluids from the organism. These excessive fluid attempts can be easily removed by dialysis ultrafiltration capacity.

MATERIALS AND METHODS

A 4 year old male Shi-Tzu, diagnosed in a private practice with babesiosis and kidney and liver failure was referred to the Clinic of the Faculty of Veterinary Medicine Bucharest on the 13.04.2016 with modified general status, apathy, fever, jaundice and hematuria. Clinical examination: 6 kg body weight, 39.9°C body temperature, mild icteric mucos membranes. The patient was evaluated using CBC, blood biochemistry analysis and cytology exam.
The blood biochemistry analysis on the 13.04.2016, 22:33 pm showed BUN = 22 mg/dl (8.8 - 25.9), CREA = 1.1 mg/dl (0.5 - 1.6), T-Bil = 1.1 mg/dl (0.1 - 0.6), ALP = 225 U/L (10.6 - 150), GPT = 135 U/L(8.2 - 57.3), GOT = 82 U/L (8.9 - 48.5). The CBC showed HCT = 36.3% (37.0 - 55.0), HGB 14.2 g/dl (12.0 - 18.0), RBC 5.56 10^{12}/l (5.50 - 8.50), WBC 2.8 10^{12}/l (6.0 - 17.0), PLT 99 10^{12}/l (200 - 500) on 13.04.2016.
The cytology exam revealed positive Babesia canis infestation.
The recommended treatment for the first 24 hours was hydro-electrolytic balancing and parenteral nutrition.
On the 14.04.2016, at 17:51 pm, the blood biochemistry analysis showed the next results: BUN = 71 mg/dl (8.8 - 25.9), CREA = 4.0 mg/dl (0.5 - 1.6), T-Bil = 11.1 mg/dl (0.1 - 0.6), ALP = can't measure (10.6 - 150), GPT = 143 U/L (8.2 - 57.3), GOT = 212 U/L (8.9 - 48.5). The rest of the biochemical parameters were in normal range.
Between 13 and 14.04.2016 the following treatment was administrated: fluid therapy 6 ml/kg/h with Lactate Ringer and Nephrotect (6 ml/kg/day), B_{12} s.c. 50 µg/kg, once a week and Methylprednisolone 1 mg/kg s.c. twice a day.
On the 14.04.2016 in the afternoon a peritoneal catheter was surgically placed in order to perform peritoneal dialysis.

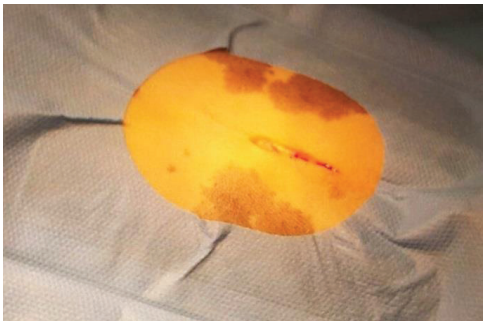

Figure. 1. The incision site, approximately 2 cm below the umbilical scar. (orig.)

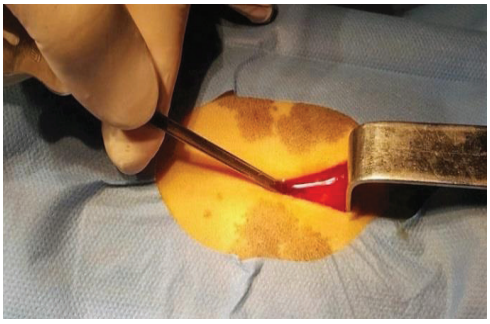

Figure. 2. Visualizing the linea alba in order to open and reach the peritoneum. (orig.)

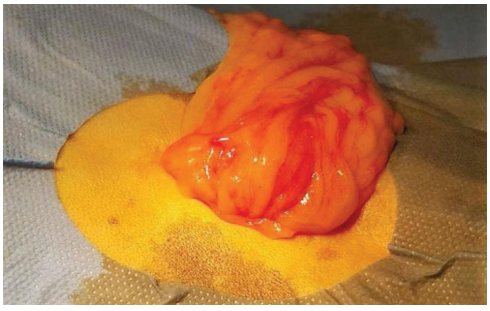

Figure. 3. Visualizing the omentum in order to perform the omentectomy. (orig.)

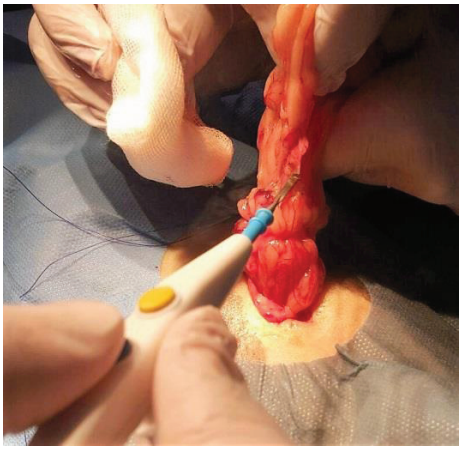

Figure. 4. The omentectomy after ligation, using an electro scalpel. (orig.)

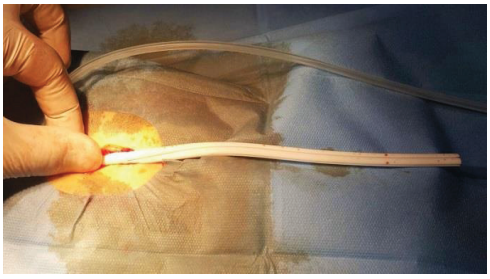

Figure. 5. A measurement of the catheter is done on spot in order to establish the catheter final length. (orig.)

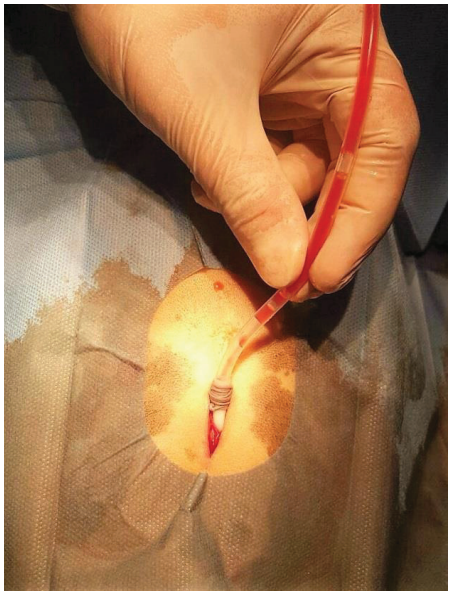

Figure. 6. The suture of the peritoneal catheter cuff, before closing the incision. (orig.)

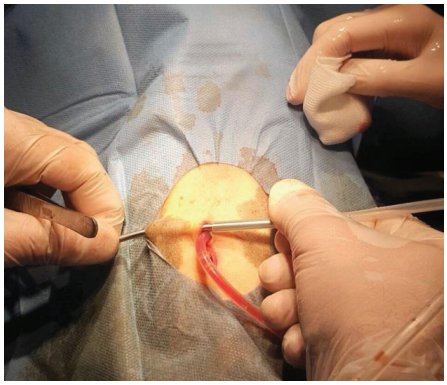

Figure. 7. The catheter passage under the skin. (orig.)

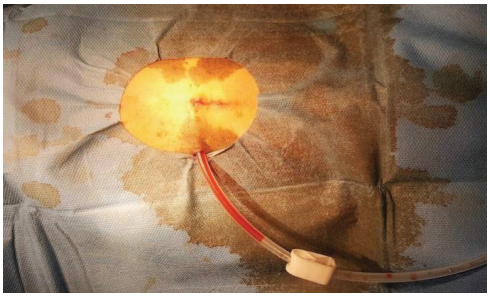

Figure. 8. Final aspect of the peritoneal catheter. (orig.)

Peritoneal dialysis was performed by the following protocol: 19^{00} - 15 ml/kg Dianeal PD4 was administered, after 30 minutes 20 ml was recovered; 20^{00} - 30 ml/kg Dianeal PD4 was administered, after one hour 80 ml was recovered; 22^{00} - 45 ml/kg Dianeal PD4 was administered, after two hours 120 ml was recovered; 00^{00} - 60 ml/kg Dianeal PD4 was administered, after four hour 300 ml was recovered. The therapy continued with 60 ml/kg every 4 hours.

RESULTS AND DISCUSIONS

On the 15.04.2016, after one day of peritoneal dialysis, the blood tests showed: BUN = 56 mg/dl (8.8 - 25.9), CREA = 2.3 mg/dl (0.5 - 1.6), T-Bil = 12.6 mg/dl (0.1 - 0.6), ALP = can't measure (10.6 - 150), GPT = 162 U/L (8.2 - 57.3), GOT = 361 U/L (8.9 - 48.5) and on the 16.04.2016 after two days of peritoneal dialysis BUN = 33 mg/dl (8.8 - 25.9), CREA = 1.4 mg/dl (0.5 - 1.6), T-Bil = 4.3 mg/dl (0.1 - 0.6), ALP = 500 U/L (10.6 - 150), GPT = 178 (8.2 - 57.3), GOT = 152 IU/L (8.9 - 48.5). On the 17.04.2016 BUN = 21 mg/dl (8.8 - 25.9),

CREA = 1.2 mg/dl (0.5 - 1.6), T-Bil = 1.7 mg/dl (0.1 - 0.6), ALP = 522 U/L (10.6 - 150), GPT = 226 U/L (8.2 - 57.3), GOT = 54 U/L (8.9 - 48.5). On the 18.04.2016 BUN = 23 mg/dl (8.8 - 25.9), CREA = 1.5 mg/dl (0.5 - 1.6), T-Bil = 1.0 mg/dl (0.1 - 0.6), ALP = 493 IU/L (10.6 - 150), GPT = 203 U/L (8.2 - 57.3), GOT = 18 U/L (8.9 - 48.5). On the 19.04.2016 BUN = 71 mg/dl (8.8 - 25.9), CREA = 1.0 mg/dl (0.5 - 1.6), T-Bil = 0.3 mg/dl (0.1 - 0.6), ALP = 119 U/L (10.6 - 150), GPT = 29 U/L (8.2 - 57.3), GOT = 12 U/L (8.9 - 48.5).

During the entire hospitalization period the hematology was performed daily and the results were in the normal physiologic ranges.

The fluid therapy was continued until the patient was discharged from the hospital.

On the 18.04.2016 the antidote for Babesiosis was administered (Imidocarb dipropionate 6.6 mg/kg i.m.).

On the 19.04.2016 we have seen an improvement of general patient status, the appetite was present, and the body temperature was 38.6˚C. On the next day, (20.04.2016), the patient was discharged from the hospital.

CONCLUSIONS

Peritoneal dialysis it is extremely laborious, but is a technically simple method and may be performed in any clinic with adequate technical assistance and supervision. Peritoneal dialysis is an effective treatment option for veterinary patients with acute kidney injury refractory to fluid therapy. It can be used as an adjunctive therapy to medical management, or can be used as a temporary means to stabilize a patient prior to a surgical procedure.

Peritoneal dialysis has a high rate of complications, but most of them are manageable with intense nursing care and careful attention to aseptic technique. Understanding the physiology of dialysis and fluid transport through the peritoneal membrane allows the clinician to make informed decisions regarding dialysate dose and treatment regimen.

Peritoneal dialysis is an important modality in the treatment of acute kidney and liver injury, consecutive to Babesia infestation.

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THE USE OF A CHROMOGENIC MEDIUM FOR THE IDENTIFICATION OF METHICILLIN-RESISTANT STAPHYLOCOCCI

N. CĂTANA, Iulia BUCUR, Corina PASCU, V. HERMAN

Faculty of Veterinary Medicine Timişoara, 300645, C. Aradului Street, No.119, Timişoara, România

Corresponding email: epirovet_tm@yahoo.com

Abstract

In the last years, an increasing attention is paid to methicillin-resistant staphylococci, isolated from animals, regardless of the species they are included in. The circulation of methicillin-resistant staphylococci strains is monitored by phenotypic laboratory techniques or with several chromogenic media. The frequency of methicillin-resistant strains was pursued on 412 strains included in S. aureus subsp. aureus and other species from the "non-S. aureus" group, based on phenotypic characters. Using the disc-diffusion method with methicillin, oxacillin and cefoxitin, 210 strains resistant to methicillin were identified and poured into a chromogenic medium named Chromatic™ MRSA. On this medium, S. aureus subsp. aureus strains resistant to at least one of the three antibiotics, formed purple to purple-orange colonies and the "non-S. aureus" strains formed white or blue colonies. All S. aureus subsp. aureus strains on this medium were methicillin-resistant, results identical to the disc-diffusion method ones. 146 "non-S. aureus" strains formed white or blue colonies, considered as methicillin-resistant, while 6 strains did not grow on this medium.

Key words: *chromogenic medium, methicillin-resistant, staphylococci.*

INTRODUCTION

Resistance to methicillin, in coagulase-positive and coagulase-negative staphylococci isolated from farm animals and pets, is very topical, which is why many researchers are studying this phenomenon in many countries. Given the large number of staphylococci species isolated from animals, which cause systemic or localized infections through pathogenicity factors (enzymes, exotoxins, biofilm), most researchers, in antibioresistance and methicillin-resistance research, use either the staphylococci divided into two groups, namely coagulase-positive and coagulase-negative staphylococci or the term "non-*S. aureus*" for all species of staphylococci except *S. aureus subsp. aureus* (Fowoyo P. T. et al., 2017; Kunz F. et al., 2011; Loeffler A. et al., 2013; Park J. et al., 2013; Saputra S. et al., 2017).

In recent years, an increasing attention has been paid to positive and negative coagulase staphylococci strains, resistant to methicillin, regardless of the species they are included in. Circulation of methicillin-resistant strains is monitored by phenotypic laboratory techniques, namely the Kirby-Bauer disk-diffusion method, the chromogenic medium method and molecular biology techniques (Dupieux C. et al., 2017; Saito E. et al., 2011).

In the screening studies conducted to monitor the circulation of methicillin-resistant strains, cromogenic media that selectively act to differentiate strains of *S. aureus subsp. aureus* versus „non-*S. aureus*" staphylococci strains are commonly used. This differentiation is based on the color of staphylococcal colonies (Saito E. et al., 2011).

The research aimed the frequency of methicillin-resistant strains, isolated from several animal species, using the Chromatic™ MRSA medium.

MATERIALS AND METHODS

The frequency of methicillin-resistant strains was followed in 412 staphylococci strains included in 22 species. Initially, the frequency of these strains was determined by the Kirby-Bauer disk diffusion method, using biodiscs with methicillin (5 μg), oxacillin (1 μg) and cefoxitin (30 μg). The methicillin-resistant strains detected by this method were the tested using the Chromatic™ MRSA medium, which is used to identify strains of *S. aureus subsp. aureus* methicillin-resistant.

This medium was provided in Petri plates as a "ready-to-use" medium by S.C. Sanimed International Impex SRL, with the following composition: peptone and yeast extract 30 g/l, sodium chloride 10 g/l, dibasic sodium phosphate 2,5 g/l, selective and matting agents 16,5 g/l, chromogenic with antibiotic mixture 0,8 g/l and agar 15 g/l.
Methicillin-resistant strains were considered strains resistant to at least one of the three antibiotics and strains which had an intermediate behavior to at least two of the three antibiotics and, in the end, 210 strains of coagulase positive and negative staphylococci were selected.

RESULTS AND DISCUSSIONS

The results regarding the behavior to the three beta-lactamases were different and showed that the resistance of the tested strains was maximum to oxacillin and minimum to cefoxitin. The presence of this phenomenon was tested, in parallel, also on the Chromatic™ MRSA medium, the results being compared with the results of the 3 beta-lactams.
On Chromatic™ MRSA, strains of *S. aureus subsp. aureus*, resistant to at least one of the three antibiotics, formed purple to orange colonies, of different nuances and the „non-*S. aureus*"strains of staphylococci, resistant to one of the three antibiotics, formed white or blue colonies.
Based on colony pigmentogenesis, this chromogenic medium allowed the differentiation of *S. aureus subsp. aureus* strains resistant to at least one of the three antibiotics or with intermediate behavior to least two of the three antibiotics. For this species, MRSA strains formed colonies pigmented in purple to purple-orange.
The results showed that all strains included in this species, based on colony pigmentogenesis, were classified as MRSA strains, these results being correlated with the results provided by the Kirby-Bauer disc-diffusion test. Also, based on the results, Chromatic™ MRSA medium can be recommended to identify the *S. aureus subsp. aureus* strains, MRSA type, in the routine diagnosis, which aimes the phenotypic characterization of strains belonging to this species (Table 1).

Table 1. Results obtained on Chromatic MRSA medium

No. crt.	Staphylococcal species	No. of strains	Chromatic MRSA	
			purple/ orange	white/ blue
1.	***S. aureus subsp. aureus***	63	63	-
2.	***S. aureus subsp. anaerobius***	3	1	2
3.	***S. hyicus***	31	-	31
4.	***S. intermedius/ S. pseudintermedius***	21	-	21
5.	***S. caprae***	5	-	5
6.	***S. epidermidis***	15	-	15
7.	***S. equorum***	5	-	5
8.	***S. lentus***	1	-	1
9.	***S. sciuri***	30	-	30
10.	***S. xylosus***	36	-	36
TOTAL		210	64	146

The strains included in 8 staphylococci species, respectively 2 positive coagulase and 6 negative coagulase, formed, on this chromogenic medium, blue or white colonies and, based on the recommendations of the producing company, could be considered strains resistant to methicillin or oxacillin. Six strains of staphylococci didn't grow on this medium, which can act selectively to some methicillin or oxacillin susceptible strains.
The results showed that staphylococci strains, included in the „non-*S. aureus*" group, resistant to methicillin and oxacillin, can be distinguished by MRSA strains with this chromogenic medium. These cultural aspects can be used as phenotypic tests in the routine diagnosis, regarding the rapid differentiation of the two staphylococci groups, namely *S. aureus subsp. aureus* and the group "non-*S. aureus*".
In the case of *S. aureus subsp. anaerobius* species, a strain formed purple-orange colonies, thus classified as MRSA type and two strains formed blue colonies, included in the „non-*S. aureus*" group.
The results obtained from these two phenotypic tests, the Kirby-Bauer disc-diffusion test with methicillin, oxacillin and cefoxitin biodiscs, correlated with chromogenic Chromatic™ MRSA medium, revealed that the phenomenon commonly referred to as methicillin resistance is present both in the strains by *S. aureus subsp. aureus*, as well as strains included in the group "non-*S. aureus*". This group included

three positive coagulase staphylococci species and all negative coagulase staphylococci species, some low pathogenic or non-pathogenic. The methicillin-resistant strains included in these species constitute a reservoir of genetic elements coding this phenomenon (SCC*mec*), which is in permanent extension.
Staphylococci, generically called methicillin-resistant, are considered zoonotic risk bacteria, because this phenomenon is associated with multiple antibiotic resistance. Circulation of methicillin-resistant strains is monitored both in human medicine and veterinary medicine and the human-animal-human circuit is a major concern for public health.
The frequency of the methicillin resistance phenomenon is studied in positive and negative coagulase staphylococci, in *S. aureus subsp. aureus* and „non-*S. aureus*" staphylococci, as well as in *S. aureus* strains referred to as "Livestock Associated-Methicillin Resistant *S. aureus*" and "Community-Associated Methicillin Resistant *S. aureus*" (Feingold B. J. et al., 2012;, Wan M. T. et al., 2012).
For this purpose the Kirby-Bauer phenotypic method, the genotypic method for SCC*mec* detection and chromogenic media are used. The use of chromogenic media is a rapid phenotypic method used in both routine diagnosis and screening research (Dupieux C. et al., 2017).
Similar results to those obtained in the own research have been reported by authors who have used different chromogenic media to detect the methicillin resistance to staphylococci isolated from farm animals for, pets and veterinary clinics (Graveland H. et al., 2009; Pletinckx L. J. et al., 2012).

CONCLUSIONS

Based on colony pigmentogenesis, the Chromatic^{TM} MRSA medium allowed the differentiation of *S. aureus subsp. aureus* strains resistant to at least one of the three antibiotics, since only the strains included in this species produce purple-orange colonies, considered as MRSA colonies.
The results obtained using the two phenotypic tests revealed the phenomenon of methicillin resistance both in *S. aureus subsp. aureus* strains and strains included in the „non-*S. aureus*" group, which confirms the animal-human epidemiological circuit of these strains in both directions.
The Chromatic^{TM} MRSA medium can be used in the routine diagnosis to monitor the frequency of the methicillin-resistant strains

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THE EFFECT OF INTRA-UTERINE TREATMENT WITH DILUTED N-ACETYLCYSTEINE ON BOVINE ENDOMETRITIS

Nicolae Tiberiu CONSTANTIN, Alexandru ȘONEA

University of Agronomic Science and Veterinary Medicine, Faculty of Veterinary Medicine Bucharest, 105, Splaiul Independenței Str., 050097, Bucharest, Romania,

Correspondig author email: constantin.ntiberiu@yahoo.com

Abstract

After the voluntary waiting period, a chronic uterine infection or a persistent inflammation in the cow may be associated with disruption of the architecture of the endometrial layer and glandular hypersecretion. The aim of our work, the first of this kind in Romania, was to evaluate the efficacy of 3% N-acetylcysteine (NAC) against the clinical endometritis. The mucolytic effect of NAC can be use to break up mucus produced by an glandular irritated layer. Besides this, NAC has a antioxidant, cytoprotection and antiinflamatory role, and at a 8% concentration it can be bactericidal. This study was carried out between 2016-2017 on 43 cows with clinical signs of endometritis after 50-60 days postpartum from a dairy farm situated in Ploiești county. The clinical endometritis was diagnosed by evalution of uterine discharge detected in the vulva. Cows were randomly splited to NAC treatment (NAC) and non-NAC treatment (nNAC) groups. All of these were clinically evaluated, and a cervical swab for microbiology laboratory was collected. The NAC group (n=21) received an intrauterine treatment of enrofloxacin+oxitetraciclin+iodoform (2.5g+5g+5g/100ml) in 20 ml dosages. After 12 hours, an infusion of 3% NAC was intrauterine administred for 3 days. The nNAC group received 20 ml of saline, for 3 days instead of 3% NAC and the same quantity of enrofloxacin+oxitetraciclin+iodoform solution. The clinical heal rate was definited as the percentage of females with no signs of clinical endometritis (clear mucus at the vaginoscopy), at the examination in first estrus following treatment. Cows were artificially inseminated following the hormonal therapy. As a main conclusion, the group of cows treated with NAC presented a pregnancy rate of 66.7% in contrast with non NAC group, where the pregnancy rate was 54.6%.

Key words: *N-acetylcysteine, cows, chronical endometritis, pregnancy rate.*

INTRODUCTION

The health condition of high-yielding dairy cows is particularly at risk in the transition period, which includes the 3 weeks before and 3 weeks after parturition, broadly correspondding to periparturient period (LeBlanc et al., 2011; Trevisi et al., 2012; Islam et al., 2014).

In this season, animals undergo pronounced physiological changes that might cause suppression of the host defence mechanisms including both the cellular and humoral response of the immune system and an increase in susceptibility to uterine and mammary gland infection (Mulligan et al., 2008; Tan et al., 2012).

Bacteria, like *Escherichia coli*, *Arcanobacterium pyogenes*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Staphylococcus aureus*, *Streptococcus uberis*, *Clostridium* spp., *Prevotella* spp. and *Fusobacterium* spp., compromises animal welfare, as well as causing subfertility and infertility because of uterine inflammation and/or infection represented by metritis, clinical and subclinical endometritis (Singh et al., 2008; Kaçar and Kaya, 2014; Zobel et al., 2014).

Clinical endometritis is basically referring to a local inflammation of the endometrium, characterized by the presence of purulent or mucopurulent (> 50% pus) material in the vagina ≥ 21 days postpartum originating from the uterus, not accompanied by systemic illness.

It affects around 20 % of dairy cows between 21 to 40 days postpartum (LeBlanc et al., 2002; Pascottini, 2016).

These diseases may prolong the days open until first service, days open until pregnancy, the intercalving period, the conception rate and the risk of cows being culled due to infertility (Đuričić et al., 2015).

Current therapies of cows suffering from endometritis aim to eliminate intrauterine (i.u.) fluid and mucus by uterine lavage and/or administration of antibiotics or other types of drugs. Recently, i.u. administration of N-

acetylcysteine (NAC) has been shown to support antibiotic therapy in cows and mares (Tras et. al., 2012; Witte et al., 2012) with endometritis. N-acetylcysteine disrupts disulfide bonds between mucin polymers and thus exhibits mucolytic properties (Matsuyama et al., 2006). Furthermore, it possesses antioxidant properties, can protect the equine colon mucosa after reperfusion injury and has protease-inhibiting capacities (Rötting et al., 2003).

MATERIALS AND METHODES

Animals

The study population consisted of 43 Holstein cows, divided into two groups (NAC treatment (NAC) *n*=21, and non-NAC treatment (nNAC) *n*=22) based on the uterine findings, from two industrial dairy farms in south of Romania. Cows were housed in ties stalls and individually fed a total mixed ration (TMR) twice daily and water ad libitum. A close-up diet was fed beginning 3 weeks prior to expected calving date, a fresh cow diet was fed beginning the day of calving through 3 weeks postpartum, and a lactation diet was fed from 3 weeks postparturition. The animals from the two groups had a physiological puerperium but the exclusion criteria included birth assistance, receiving systemic antibiotic therapy within 50-60 days prior to calving, abnormal internal genitalia (including adhesions), BCS < 2,5, systemic diseases, retention of foetal membranes, any kind of dystocia, including caesarean section, lameness, puerperal mastitis. Only cows following the second to the fourth partus were involved in the study.

Experimental protocol and clinical evaluations

All the cows were examined by rectal palpation and vaginoscopicaly examinated 50-60 days postpartum. Transrectal palpation served to assess uterine size and symmetry of the uterine horns as well as uterine fluctuation, and purulent or mucopurulent vaginal discharge. The NAC group (*n*=21) received an intrauterine treatment of enrofloxacin + oxitetraciclin + iodoform (2.5g+5g+5g/100ml) in 20 ml dosages. After 12 hours, an infusion of 3% NAC was intrauterine administred for 3 days. The nNAC group (*n*=22) received 20 ml of saline, for 3 days instead of 3% NAC and the same quantity of enrofloxacin + oxitetraciclin + iodoform solution. The clinical heal rate was definited as the percentage of females with no signs of clinical endometritis (clear mucus at the vaginoscopy), at the examination in first estrus following treatment. Healthy cows were artificially inseminated following the hormonal therapy.

Bacterial culture

A uterine and/or cervical culture is an essential tool to determine the etiology of uterine infection. Briefly, the vulva was cleaned thoroughly with a dry paper towel. A cervical swab for microbiology laboratory was collected., transferred into sterile tubes and carefully transported at 4°C to the Faculty of Veterinary Medicine, Bucharest for further bacteriology testing.

Statistical analysis

Reproductive parameters such as pregnancy rate (PR), calving to pregnancy interval (CPI), calving interval CI) and cure rate (CR) were take in consideration and calculated in Microsoft Excel 2010.

RESULTS AND DISCUSSIONS

The clinical cure rate in NAC group (77.2%) was significantly higher than nNAC group (43.4%). The group of cows treated with NAC presented a pregnancy rate of 66.7% in contrast with nNAC group, where the pregnancy rate was 54.6%. In a simmilar study, Tras and col., (2014) obtained a CR (83.3%) and PR (66.7%) significantly higher for NAC group than (55.5% and 27.8% respectively) for nNAC group. Regarding to CPI and CI reproductive parameters NAC group shows a shorter intervals in contrast to nNAC group, as we can see in tabel number 1. In the cervical swab samples of cows, *Corynebacterium* ssp., *S. aureus*, and *E. coli* were isolated.

Study parameters	NAC	nNAC
PR	66.7%	54.6%
CPI	93 days	107 days
CI	389 days	401 days
CR	77.2%	43.4%

CONCLUSION

The results suggested that NAC may be beneficial for treating genital tract infections presenting purulent discharge such as clinical endometritis due to the clinical features of NAC for cost saving, and has got no illegal residues in edible tissues of farm animals. Although it is first article in Romania of this kind, further studies are needed to clarify the efficiency of NAC intrauterine treatment in cows.

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MAGNETIC RESONANCE IMAGING FINDINGS IN 46 DOGS WITH CHIARI-LIKE MALFORMATION AND SYRINGOMYELIA

Catalina Anca CUCOȘ[1], Ateș BARUT[2], Constantinescu RADU[1], Iuliana IONAȘCU[1], Constantin VLAGIOIU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spaiul Independenței, District 5, Bucharest, Romania
[2]PetCode Animal Hospital, Ankara, Turkey

Corresponding author email: ancacucos@gmail.com

Abstract

Chiari-like malformation is a condition characterized by a mismatch in volume between the caudal cranial fossa, which is too small, and the brain, too big, leading to foramen magnum obstruction and secondary syringomyelia – fluid filled cavities within the spinal cord parenchyma. The aim of the paper was to describe and to discuss the Chiari-like malformation and syringomyelia magnetic resonance imaging characteristics, to asses the extension and severity of the neurological lesions. This research presents a retrospective study on 46 dogs, during the period 2013-2017. All dogs were subjected to magnetic resonance imaging examination; T1- and T2-weighted transverse and sagittal images of the brain, craniocervical junction and cervical spine were obtained in all cases. Each patient was assigned a grading of Chiari-like malformation and syringomyelia, according to the British Veterinary Association/Kennel Club-Chiari-like malformation and Syringomyelia Health Scheme. Chiari-like malformation and syringomyelia were shown in all cases, and although the clinical symptoms, the breed and history generates a presumptive diagnosis, the diagnosis can only be confirmed by magnetic resonance imaging examination.

Key words*: magnetic resonance imaging, Chiari like malformation, Syringomyelia, dogs, Cavalier King Charles Spaniel.*

INTRODUCTION

Chiari-like malformation (CM) in dogs is a congenital abnormality of the caudal-occipital fossa, in which the cerebellum herniates through the foramen magnum affecting the normal cerebrospinal fluid (CSF), leading to the development of fluid filled cavities in the spinal cord parenchyma, condition named syringomyelia (SM) (Rusbridge et al., 2006).

Previously, CM/SM were considered rare conditions in veterinary medicine, but nowadays are more frequent diagnosed, part to the advanced imaging tools available in veterinary medicine - magnetic resonance imaging (MRI), and also due to the increase in popularity of the miniature and toy dog breeds.

Magnetic resonance imaging is a highly sensitive method for the detection of neurological conditions. It has an important role in determining the exact location and extent of neurological lesions.

CM and SM appears to be inherited in the Cavalier King Charles Spaniel breed (CKCS) (Lewis et al., 2010). The estimated prevalence of CM in CKCS population ranges from 92% to 100% (Couturier et al., 2008; Cerda-Gonzalez et al., 2009).

Besides the Cavalier King Charles Spaniel breed, CM and SM has been also reported in Griffon Bruxellois, Chihuahua, Pomeranian, Maltese Terrier, Pug, French Bulldog and Yorkshire Terrier (Marino et al., 2012).

The risk factors in developing CM/SM appears to be miniaturisation and brachycephalism, due to the shortened skull in this dogs (Schmidt et al., 2011; Marino et al., 2012; Driver et al., 2013).

The most common clinical signs of CM/SM are neuropathic pain, yelping, vocalization on sudden posture change, scratching – with or without skin contact, scoliosis, ataxia and weakness. Not all dogs with SM presents clinical signs, this is correlated with the syrinx asymmetry, width and spinal cord dorsal horn damage (Rusbridge et al., 2007; Rusbridge, 2013).

British Veterinary Association (BVA) proposed a CM/SM classification scheme, upon which there are 3 types of CM, based on the

anatomical position of the cerebellum, which include Grade 0 CM – normal dog, without CM, Grade 1 CM - the cerebellum is indented – mild CM, and Grade 2 CM, where the cerebellum is impacted into, or herniated through the foramen magnum; and 3 types of SM, Grade 0 SM - normal (with no syrinx or pre-syrinx and normal central canal), Grade 1 SM - Central canal dilation under two millimetres, Grade 2 SM - central canal dilation of more than 2 millimetres and a pre-syrinx or syrinx.

MATERIALS AND METHODS

The study was performed over a 4-year period, 2013-2017, on 46 dogs, of various breeds, age or gender. The mean age was 49.5 months, including 28 females and 18 males, the over representative breed was Cavalier King Charles Spaniel.

In all cases the same steps were followed: physical and neurological examination, complete blood count and serum biochemistry, MRI examination of the head and cervical spine.

Every patient included in this study presented clinical signs of CM/SM, of which neuropathic pain, scratching, spontaneous vocalization and facial rubbing were the most common.

The MRI examination was performed on dogs under general anaesthesia, placed in sternal recumbency, with the craniocervical junction in an extended position. Magnetic resonance imagining scans of the brain and cervical spine were performed in every case, and in some cases the examination also included images of the thoracic spine segments. The MRI sequences included T1- and T2-weighted transverse and sagittal images, on which an evaluation of the neurological abnormalities was performed in each case. Thus, according to the CM/SM classification scheme proposed by British Veterinary Association, to each patient was assigned a certain degree of CM and SM.

The magnetic resonance images were imported into a medical image viewer (Horos™), with which we were able to perform precise measurements of the spinal cord cavities and to evaluate the CM signs - cerebellar indentation or herniation through the foramen magnum.

The spinal cord cavities measurements were achieved in sagittal and transverse planes, aiming the length and the diameter of the syrinxes (Figure 1). According to BVA, a grade between 0 and 2 was assigned to denote severity (Table 1).

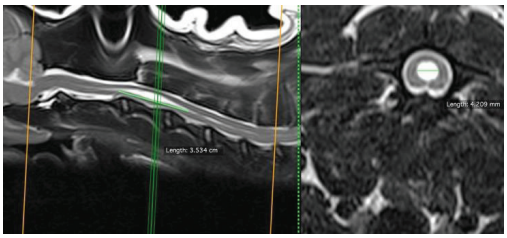

Figure 1. Magnetic resonance images from a dog with neuropathic pain. Left – mid-sagittal T2-weighted image of the cervical spinal cord. Right - transverse T2-weighted image of the spinal cord at the third cervical vertebra, illustrating a large syrinx, with the width of 4.2 mm.

Table1. Grading criteria for syringomyelia (Adapted after British Veterinary Assocication SM scheme)

Grade	Severity of syringomyelia
0	none
1	central canal dilation under two millimetres
2	central canal dilation of more than 2 millimetres, a pre-syrinx or syrinx.

The degree of cerebellar herniation was estimated according to the position of the tip of the cerebellar vermis in relation with the foramen magnum (Figure 2).

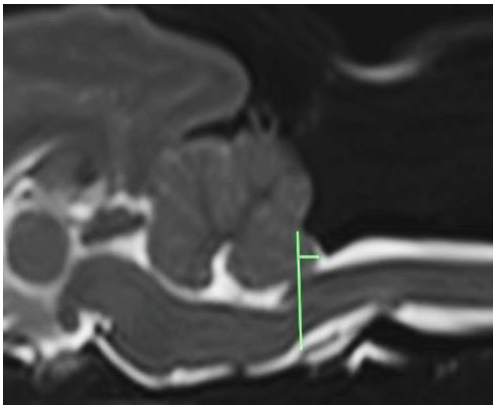

Figure 2. Mid-sagittal T2-weighted image of caudal fossa. Cerebellar herniation represents the length (horizontal line) engaged within the foramen magnum.

According to this the cerebellar herniation might be mild or marked (Figure 3).

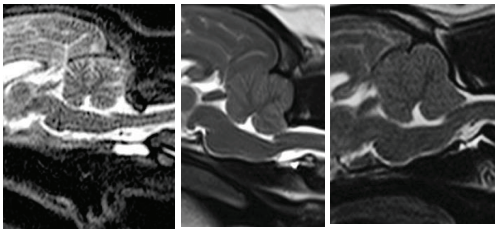

Figure 3. Mid-sagittal T2-weighted images showing a normal shaped cerebellum (left image), a mild herniation of the cerebellum (central image), and a marked deformity of the cerebellum (right image).

RESULTS AND DISCUSSIONS

The commonest magnetic resonance imaging findings were indented cerebellum, impacted or herniated cerebellum through foramen magnum, ventricular dilatation, medullary kinking, central canal dilation, cervical spinal cord syrinxes.

All of the cases presented an abnormal shaped cerebellum with overcrowding, a pointed vermis, directed caudally to the foramen magnum. In all cases the caudal part of the cerebellum presented different grades of herniation into the foramen magnum.

A study from 2014 notes that the grade of the cerebellar herniation does not predict SM, and that the cerebellar herniation progress with time (Rusbridge, 2014).

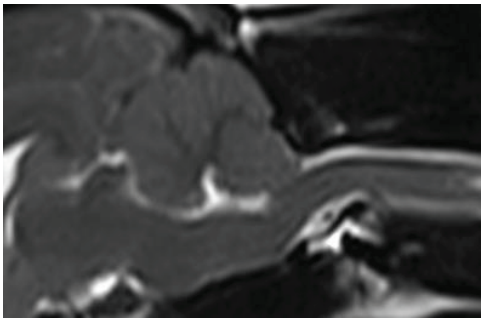

Figure 4. Mid-sagittal T2-weighted image of the caudal fossa and cranial cervical spinal cord showing medullary kinking, observed as an elevation of the medulla at the cervicomedullary junction.

Different grades of syringomyelia were observed in all 46 cases, with the transverse diameter of the syrinx ranging from under 1 mm to a maximum width of 8.3 mm. Twenty-eight dogs showed a syrinx of more than 2 mm (60,9%) compared to eighteen dogs with a syrinx less than 2 mm and/or central canal dilation (39,1%).

The syrinx length varies in each case, wide-ranging from one cervical vertebrae to almost the entire cervical spinal segment and also cranial segment of the thoracic spine (C2-T3).

Mean central canal transverse height was 3.5 mm, with a mean length of 5.6 cm.

The most common syrinx location was C2-C3 spinal segment (17.4%), followed by C3-C4 (15.2%). The MRI findings are exposed largely in Table 2.

Other MRI findings encountered were medullary kinking (Figure 4), presyrinx – spinal cord edema, that may precede syringomyelia formation (Figure 5), ventriculomegaly, bilateral otitis.

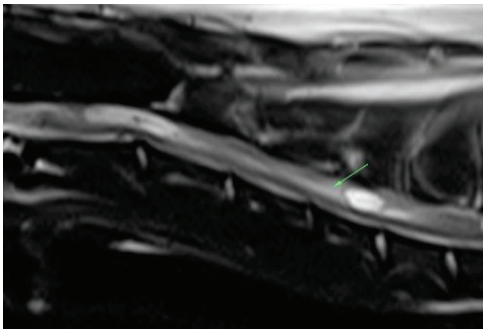

Figure 5. Mid-sagittal T2-weighted image of the cervical spinal cord segment. Note the presence of the presyrinx marked by the arrow.

Other than MRI, several diagnostic methods of CM/SM are described in the literature, these include ultrasound examination, radiography and computed tomography. However, MRI is considered the gold standard for the diagnosis of CM and SM. Ultrasonography can be used for the examination of the spinal cord and caudal fossa, but presents major limitations. Computed tomography provides important data regarding caudal cranial fossa measurements (Couturier et al., 2008).

Rusbridge in a study performed on Griffon Bruxellois dogs demonstrated that using a simple radiographic technique could be used to predict CM presence (Rusbridge et al., 2009).

Table 2. MRI abnormalities in 46 dogs with Chiari-like malformation and syringomyelia

Abnormality		Number of dogs affected
Syrinx site	C2-C3	8 cases
	C3-C4	7 cases
	C2-C4	5 cases
	C2-C5	3 cases
	Other localizations	23 cases
Syringomyelia	Grade 1 SM	18
	Grade 2 SM	28
Cerebellar herniation		46
Presyrinx		26

CONCLUSIONS

CM and SM are serious conditions that affects Cavalier King Charles Spaniel dogs and other predisposed breeds, that can be suspected based on the clinical signs and history, but it can only be confirmed by MRI.
MRI is essential for the CM/SM diagnosis, in order to fully evaluate the structural abnormalities of the central nervous system.
MRI provides accurate information that helps CM and SM staging, important for establishing the treatment, management and determining the prognosis.

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CYTOMORPHOLOGICAL ASPECTS IN GENITAL SYSTEM LESIONS OF BITCH

Georgeta DINESCU[1], Roxana PREUTU[1], Raluca Ioana RIZAC[1], Alexandru DIACONESCU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: ginadinescu2015@gmail.com

Abstract

The current paper presents some cytomorphological aspects in different lesions of the bitch genitalia system, together with their significance for diagnosis. The study was conducted between April 2016 and April 2017 (one year) on 24 bitches with genital lesions. Of the 24 studied cases, six (25%) exclusively exhibited ovarian lesions, four (16.7%) only uterine lesions, four (16.7%) vaginal lesions, while in 10 cases (41.6%) both ovarian and uterine lesions were diagnosed. The sampling for the vaginal lesions was performed both preoperative, through fine needle aspiration (FNA) and impression, and postoperative through abrasive cytology. The uterine and ovarian lesions were sampled after the ovariohysterectomy, through exfoliative and abrasive cytology. The smears were Romanowsky stained and microscopically examined with an immersion objective. The ovarian lesions were tumoral (n=3) and cystic (n=3), the uterine lesions were represented by cystic endometrial hyperplasia-pyometra complex, the vaginal lesions were tumoral – two fibromas and two transmissible venereal tumors (TVT), and the bitches that exhibited both ovarian and uterine pathologies, the uterine lesions were represented by cystic endometrial hyperplasia (CEH)-pyometra complex and the ovarian were represented by cystic ovariopathy (n=8) and tumors (n=2).The cytological examination was of maximum relevance for the tumoral lesions. For the cystic pathology it made the difference between degenerative lesions and cystic tumors. In CEH-pyometra complex, the cytological aspects were very diverse, in correlation with the evolutionary phase of the pathological process and the reactivity of the organism.

Key words*: genitalia system, bitch, cytomorphological aspects.*

INTRODUCTION

The genital system lesions are frequently involved in bitch infertility (Zubair, 2014). Pyometra is the most frequent lesion of the genital system in bitches, one in four females being affected before the age of 10 years (Baithalu, 2010; Smith, 2006).

Although cytology is a diagnosis option for pyometra, the cytology of the vaginal smear is rarely conducted, diagnosis being usually based on clinical and imaging examinations (Sfartz, 2016). In case of ovarian and vaginal lesions, the accuracy of cytology is over 90%, most often these lesions being diagnosed using cytology (Raskin, 2010; Sforna, 2003). Cytopathological diagnosis helps both diagnosis and differential diagnosis between neoplastic and non-neoplastic lesions (Beker, 2002).

MATERIALS AND METHODS

The study was conducted between April 2016 and April 2017 (one year) on 24 bitches with genital lesions. Of the 24 studied cases, six (25%) exclusively exhibited ovarian lesions, four (16.7%) only uterine lesions, four (16.7%) vaginal lesions, while in 10 cases (41.6%) both ovarian and uterine lesions were diagnosed.

The sampling for the vaginal lesions was performed both preoperative, through fine needle aspiration (FNA) and impression, and postoperative through abrasive cytology.

The uterine and ovarian lesions were sampled after the ovariohysterectomy, through exfoliative and abrasive cytology. The smears were Romanowsky stained and microscopically examined with an immersion objective.

RESULTS AND DISCUSSIONS

The following table is a synthetic presentation of the studied cases.

Although among the breeds in our study there are some prone to pyometra (Chow Chow, Golden Retriever) (Baithalu, 2010), we can not speak about breed predisposition.

Table 1. Presentation of genital lesion cases in bitch

Case No.	Breed	Age	Lesion location	Cytopathological diagnosis
1.	Maltese	14 years	Ovary	Malignant epithelial tumor – ovarian carcinoma
2.	Collie	11 years	Ovary	Malignant epithelial tumor – ovarian carcinoma
3.	Mixed breed	10 years	Ovary	Malignant epithelial tumor–carcinoma or granulosa tumor
4.	Amstaff	9 years	Ovary	Cystic ovariopathy
5.	Shar-pei	10 years	Ovary	Cystic ovariopathy
6.	Caniche	11 years	Ovary	Cystic ovariopathy
7.	Pekingese	16 years	Uterus	CEH-pyometra complex
8.	Caniche	14 years	Uterus	CEH-pyometra complex
9.	Maltese	9 years	Uterus	CEH-pyometra complex
10.	Chow-chow	7 years	Uterus	CEH-pyometra complex
11.	Mixed breed	9 years	Ovary and uterus	Ovarian carcinoma and CEH-pyometra complex
12.	Cocker	10 years	Ovary and uterus	Ovarian carcinoma and CEH-pyometra complex
13.	Mixed breed	7 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
14.	Golden Retr.	11 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
15.	Boxer	8 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
16.	Pekingese	14 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
17.	Shar-pei	10 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
18.	Mixed breed	8 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
19.	Bichon	9 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
20.	Mixed breed	10 years	Ovary and uterus	Cystic ovariopathy and CEH-pyometra complex
21.	Poodle	15 years	Vagina	Benign mesenchymal tumor - fibroma
22.	Mixed breed	11 years	Vagina	Benign mesenchymal tumor - fibroma
23.	Mixed breed	8 years	Vagina	Round cell tumor – TVT
24.	Bichon	9 years	Vagina	Round cell tumor - TVT

Typically, pyometra is a condition of middle-aged or old bitches. The average age reported in profile literature is 7-8 years (Jubb, 1993), in our case being 10.8 years.

The ovarian lesions were tumoral (n=3) and cystic (n=3), the uterine lesions were represented by cystic endometrial hyperplasia-pyometra complex, the vaginal lesions were tumoral – two fibromas and two transmissible venereal tumors (TVT), and for the bitches that exhibited both ovarian and uterine pathologies, the uterine lesions were represented by cystic endometrial hyperplasia-pyometra complex and the ovarian were represented by cystic ovariopathy (n=8) and tumors (n=2).

Macroscopy

Neoplastic ovaries appear enlarged, with a polynodular aspect, pink-grey color and high consistency (Figure 1).

Polycystic ovaries are enlarged, with uneven surface and numerous cystic structures of different sizes, thin transparent wall and aqueous or citrine liquid content (Figure 2, 3).

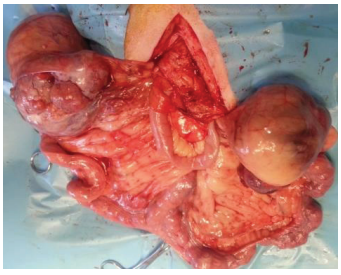

Figure 1. Bilateral ovarian tumor

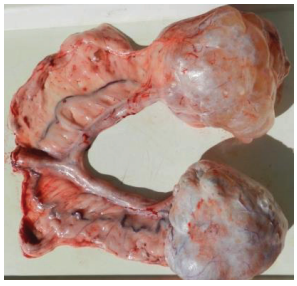

Figure 2. Cystic ovariopathy

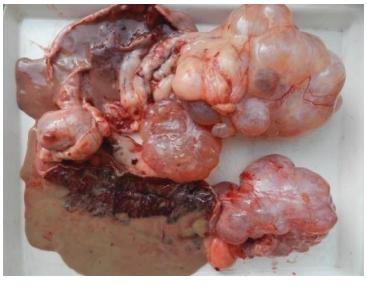

Figure 3. Cystic ovariopathy and pyometra

The uterus with pyometra is distended, and sectioning will exhibit yellow-gray-brown pus of viscous consistency (Figures 3, 4).
The vaginal masses, diagnosed as fibromas, are enlarged, with uneven surface, polynodular aspect, whitish color and high consistency (Figure 5, 6).

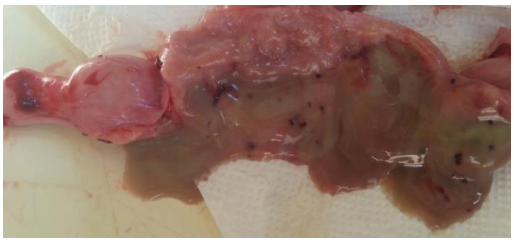

Figure 4. Uterus. Pyometra

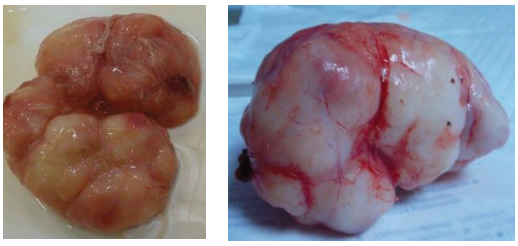

Figure 5, 6. Vaginal masses

Cytology
In case of ovarian tumors, hypercellularity and monomorphism of grouped epithelial cells were noted. Tumor cells are of medium or large size, high and variable N/C ratio, anisocytosis, anisocariosis, prominent nucleoli, variable in number and size, and unevenly distributed chromatin (Figure 7, 8)

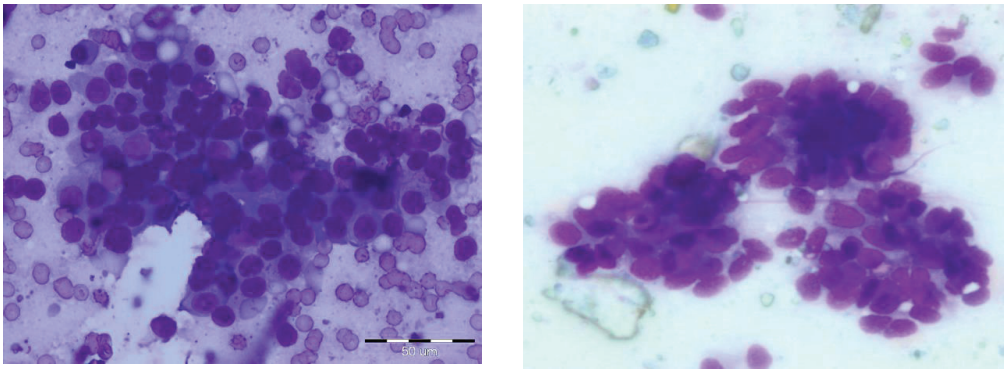

Figure 7, 8. Ovary. Monomorphic population of grouped epithelial cells, with moderate anisocytosis and anisocariosis, high and variable N/C ratio, prominent nucleoli. Malignant epithelial tumor – supposed ovarian carcinoma. M-G.G. staining, x400.

In cystic endometrial hyperplasia-pyometra complex, the aspects were very diverse, in correlation with the evolutionary phase of the pathological process and the reactivity of the organism.
Three cytomorphological patterns were generally noted:
One indicating endometrial hyperplasia. The cells are large, prismatic, with a brush border, vacuolated cytoplasm and a hyperchromic nucleus, pyknotic, basal or central. Mitoses are typical and common. In the background of the smear there are rare non-degenerated neutrophils, detritus and erythrocytes (Figure 9, 10).

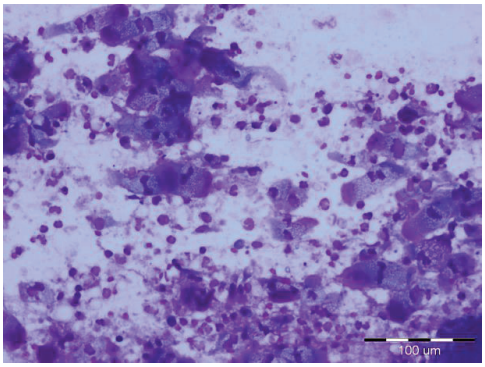

Figure 9. Uterus. Large endometrial cells, prismatic, with vacuolated cytoplasm and hyperchromic pyknotic nucleus. The background of the smear exhibits non-degenerated neutrophils, detritus and erythrocytes. Endometrial cystic glandular hyperplasia. M-G.G. staining, x200.

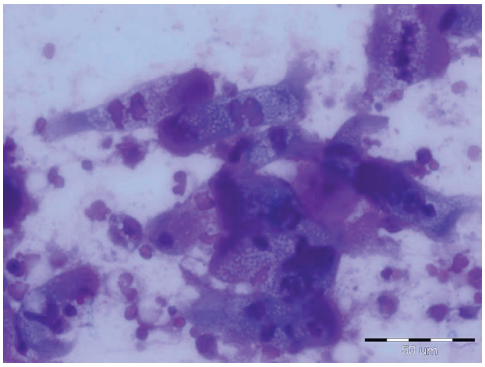

Figure 10. Uterus. Endometrial cells exhibit a vacuolated cytoplasm and a hyperchromic pyknotic nucleus. Mitoses are frequent. Endometrial cystic glandular hyperplasia. M-G.G. staining, x400.

Another pattern indicates an acute septic inflammation, in which neutrophils predominate, non-degenerated and degenerated, and bacteria are present in the smear background and in cytoplasm of the phagocytes (Figure 12).

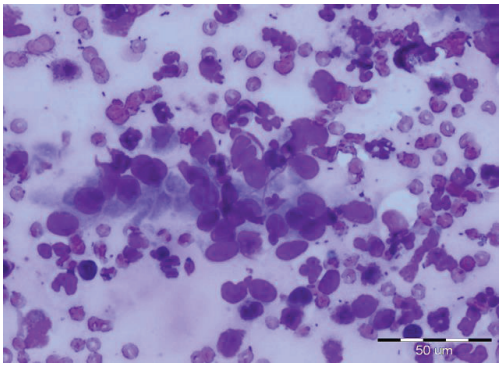

Figure 12. Uterus. Endometrial cells are rare and reactive. Bacteria are present in the background of the smear and within the cytoplasm of phagocytes. Acute septic inflammation - pyometra. M-G.G. staining, x400.

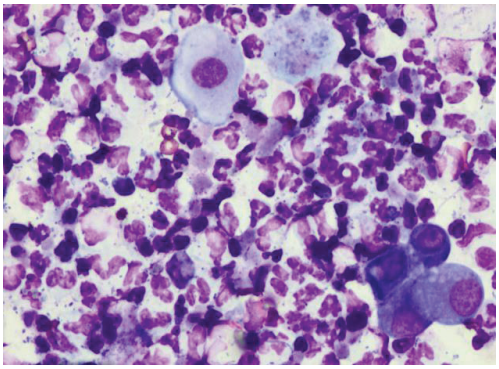

Figure 13. Vaginal smear. In the open cervix pyometra, in addition to parabasal, intermediate, superficial and anuclear local cells, a large number of degenerated neutrophils and bacteria can be noticed. M-G.G. staining, x400.

In the case of the open cervix pyometra, vaginal smears contain many degenerated neutrophils and bacteria (Figure 13).
The third pattern indicates a chronically septic inflammatory process. Local cells are rare and degenerate or absent as a result of endometrial necrosis. First, inflammatory cellularity is mononuclear, with numerous macrophages and rare plasma cells, while numerous bacteria are present in the background of the smear and within the cytoplasm of the phagocytes (Figure 14). Subsequently, the plasma cells dominate and the microbial flora is poorly represented. (Figure 15).

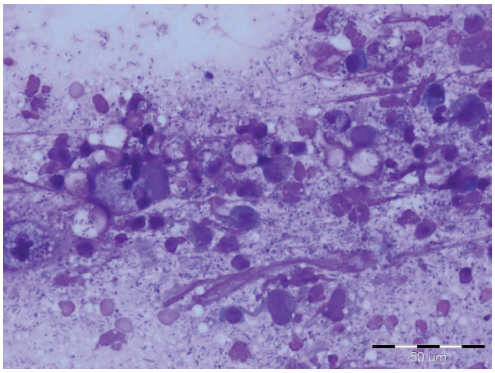

Fig. 14. Uterus. Local cells are rare and degenerate. Inflammatory cellularity is mononuclear, and in the background of the smear and within the cytoplasm of the phagocytes numerous bacteria may be found. M-G.G. staining, x400.

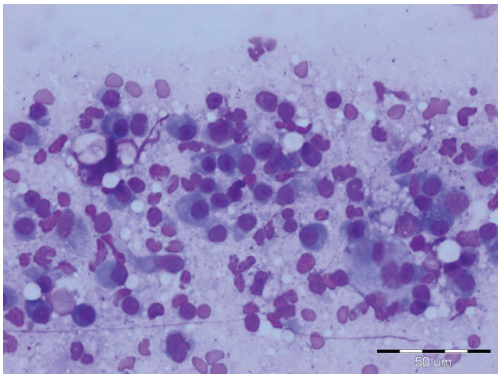

Fig. 15. Uterus. Inflammatory cellularity is mononuclear, with numerous plasma cells. M-G.G. staining, x400.

In case of vaginal masses, hypercellularity, spindle cell monomorphism with oval nuclei, uniform in size and shape, and basophilic elongated cytoplasm were found (Figure 16).
In the canine transmissible venereal tumor, the diagnosis was easily established on the basis of the monomorphic population of round cells with abundant basophilic cytoplasm, with intracytoplasmic vacuoles, round nuclei, numerous nucleoli, coarsely distributed chromatin and atypical mitosis.

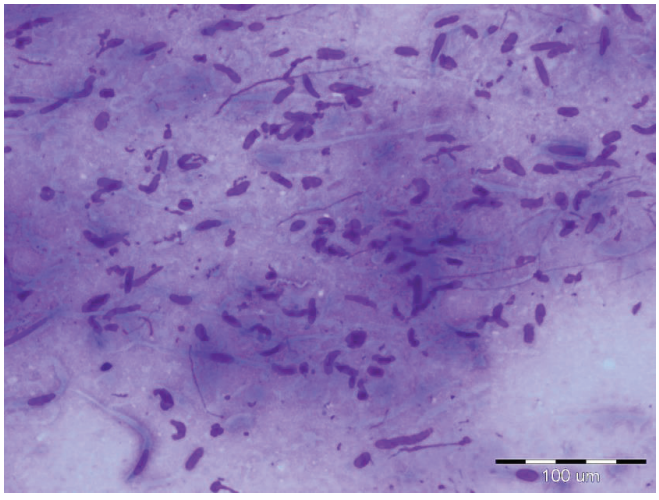

Figure 16. Vaginal mass. Hypercellularity, monomorphism with mesenchymal spindle cells, uniform in shape and size, with oval nuclei, uniform as well. Benign mesenchymal tumor – supposed vaginal fibroma. M-G.G. staining , x200.

The one year-long study of the genital system lesions in bitch aimed the epidemiological and morphological evaluation. An increased incidence of ovarian lesions (16/24) was found, most of them being accidentally identified during neutering, or in females with pyometra. Also noted was the increased incidence of the CEH- pyometra complex, in most cases

associated with ovarian lesions (n = 10), or independently (n = 4). From the analysis of these data, corroborated with the owners' history, it appears that the females had significant hormonal imbalances, mostly induced by contraceptive treatments. The incidence of vaginal lesions was relatively low (4/24), all of them being neoplasms.

The cytological examination was of maximum relevance for ovarian and vaginal tumors. In current practice, cytology testing of genital disorders is not performed through vaginal cytology, although it may capture both the vaginal lesions and the early-stage pyometra. Our study highlights the role of this simple, easy and inexpensive investigation in early detection of genital diseases in bitch and their treatment with the preservation of the reproductive capacity of the females.

CONCLUSIONS

1. Of the 24 studied cases, six (25%) exclusively exhibited ovarian lesions, four (16.7%) only uterine lesions, four (16.7%) vaginal lesions, while in 10 cases (41.6%) both ovarian and uterine lesions were diagnosed.
2. There was no breed predisposition.
3. Females aged 7 to 16 years (with an average of 10.8 years) were affected.
4. The ovarian lesions were tumoral (n = 3) and cystic (n = 3).
5. The uterine lesions were represented by CEH-pyometra complex (n = 4)
6. The vaginal lesions were tumoral (n = 2) and transmissible venereal tumors (n = 2).
7. The bitches that exhibited both ovarian and uterine pathologies, the uterine lesions were represented by CEH-pyometra complex (n=10) and the ovarian were represented by cystic ovariopathy (n=8) and tumors (n=2)
8. Three cytopathological patterns were identified in the CEH-pyometra complex: endometrial hyperplasia, acute septic inflammation, and chronic plasma cell inflammation.

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PERINEAL HERNIA MANAGEMENT OF AN INTACT MALE DOG USING DEFERENTOPEXY TECHNIQUE - CASE REPORT

Ionuţ - Cristian GÂRJOABĂ[1], Gina Teodora GÎRDAN[1]

[1]Faculty of Veterinary Medicine Bucharest, 105, Splaiul Independenţei, Bucharest, Romania

Corresponding author email: ionutvet@gmail.com

Abstract

Bilateral deferentopexy represents a surgical technique of major relevance, in preventing recurrence of perineal hernias in intact male dogs. Ductus deferens, isolated during orhidectomy, and sutured afterwards ventrally on abdominal wall, are intended to exert a cranial traction and mantainance of urinary bladder and adnexal glands of reproductive system; thereby, the exerted pressure of these viscera on perineal region is significantly diminished postoperatively, avoiding recurrence. An intact caniche male, eleven years old received a diagnosis of unilateral perineal hernia. The surgery consisted in two procedures: bilateral orchiectomy followed by deferentopexy and perineal muscle suture. Perineal hernias, encountered in males older than 7 years, occur due to prostatic hypertrophy which generates a repetitive pressure in the perineal area, the predisposing factor being the striated muscle degeneration consistent in rhabdomyolysis.

Keywords: *deferentopexy, orchiectomy, perineal hernia.*

INTRODUCTION

Bilateral deferentopexy represents a surgical technique of major relevance, in preventing recurrence of perineal hernias in intact male dogs. Ductus deferens, isolated during orchiectomy, and sutured afterwards ventrally on abdominal wall, are intended to exert a cranial traction and mantainance of urinary bladder and adnexal glands of reproductive system; thereby, the exerted pressure of these viscera on perineal region is significantly diminished postoperatively, avoiding recurrence.

MATERIALS AND METHODS

An intact caniche male, eleven years old received a diagnosis of unilateral perineal hernia acquiring a complete history followed by examination of rectal region and digital rectal palpation.

The preanesthetic patient evaluation categorized it using the American Society of Anesthesiologists (ASA) belonging to Clase II. Preoperatively, the dog received Diazepam (0.4mg/kg) intravenously [IV] and Pethidine 3.5mg/kg intramuscularly [IM]. Fifteen minutes after premedication, the patient was preoxygenated for 5 minutes before the induction agent, Propofol, was administered at 4-6 mg/kg [IV] until orotracheal intubation was achieved. Maintenance with Isoflurane (2.5-1% IV) in 100% oxygen was accomplished and analgesia was completed by Lidocaine 1%, 1.5mg/kg as a bolus followed by a Constant Rate Infusion of 40 µg/kg/min.

The surgery procedures followed two steps: bilateral orchiectomy succeeded by deferentopexy and perineal muscle suture. It has been performed an “open” technique castration, in an elliptical manner, exposing tunica vaginalis, by dissecting out the testicles and the spermatic cord **(**funiculus spermaticus) achieving a direct visualization of the cremaster, ductus deferens and spermatic vasculature (Figure 1).

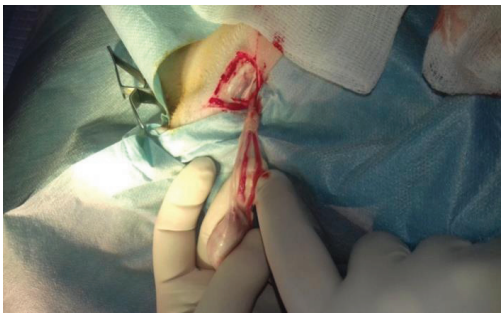

Figure 1 Exposure of spermatic cord composed of ductus deferens and adjacent vessel

Ligation of spermatic cord components has been achieved using a monofilament absorbable suture 3-0 Polydioxanone (PDS) (Figure 2).

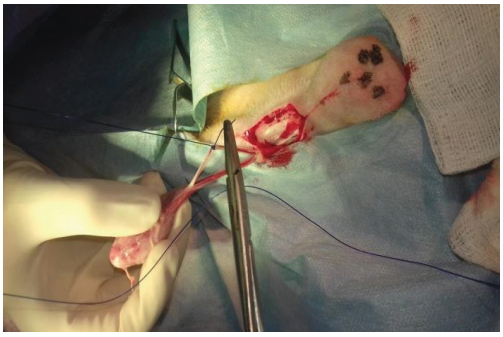

Figure 2 Spermatic cord is transected being exposed and ligated each component, respectively. ductus deferens and adjacent vessels

A parapreputial skin approach, using a ventral medial incision of linea alba was performed, deferentopexy technique, consisting in inserting each ductus deferens through inguinal canals addressing laterally on both sides of abdominal cavity wall.
A simple interrupted suture of abdominal wall using synthetic monofilament absorbable suture 0 PDS, has been completed, in order to strengthen the adhesion of ductus deferens on the abdominal wall. It is noted that clamping of ductus deferens on both sides of abdominal wall is accomplished by perforating and tunnelling it, followed by knotting both, ductus deferens, along the ventral abdominal incision (Figure 3). A single suture technique for skin closure with 2-0 PDS was accomplished.

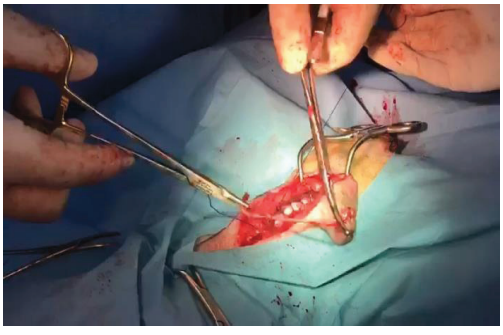

Figure 3 Clamping and knotting ductus deferens on ventral abdominal wall

Approaching the perineal hernia was made using a semicircular incision, assuming a side point between the base of the tail to the left ischial tuberosity (Figure 4).

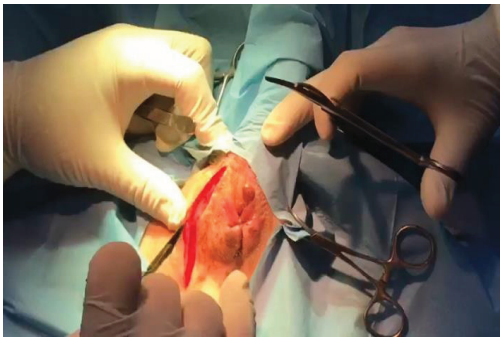

Figure 4 A semi-circular skin incision of unilateral perineal hernia

Hernial sac was isolated and various anatomical structures have been identified, including bladder, omentum and intestines.
The suture of the muscle layers with 2-0 PDS has been achieved; three sutures have been placed between coccygeus lateral muscle and external anal sphincter, between sacrotuberous ligament and external anal sphincter and three sutures between internal obturator muscle flap and external anal sphincter (Figure 5).

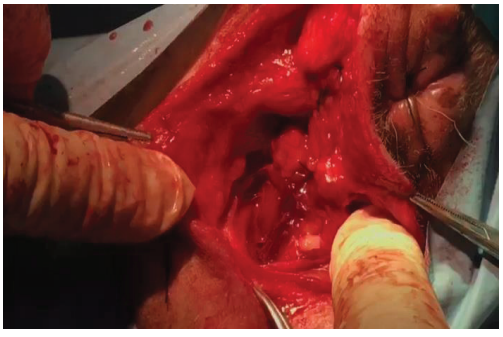

Figure 5 The appearance of herniorraphy after suture of external anal sphincter and internal obturator muscle flap

Subcuticular sutures were used for strengthen surgical wound closure with 2-0 PDS and a nonabsorbable monofilament 2-0 Polypropylene (PP) suture was used for the skin closure; this was completed by declivity to provide postoperatively drainage and efficient healing. (Commere, 2008).

RESULTS AND DISCUSSIONS

Perineal hernias found in plus seven years old dog males, it is assumed to occur due to prostatic hypertrophy which causes repetitive pressure in the perineal area, the predisposing factor being the striated muscle degeneration in this surface, a process called rhabdomyolysis. (Commere, 2008).

Anesthesia and analgesia were provided using a multimodal approach. Opioids confer optimal analgesia for different types of pain being safe when administered at clinical dosages; (Simon and Steagall, 2016). We preferred an opioid such as Pethidine, which may be more potent at κ-receptors, but having a rapid onset and a shorter duration of action, producing a short acting analgesia, which also exerts an antispasmodic activity on the smooth muscle of the large intestine. Pethidine does not cause as much constipation as Morphine and this may relate to its shorter duration of action, but it causes histamine release administered [IV]. The concentrations of Pethidine were maintained for 120 minutes after administration of a dose of 3.5mg/kg (Waterman and Kalthum, 1989). Lidocaine administered [IV] acts as a prokoinetic agent and has been used for postoperative treatment of ileus in people. It has been documented to have anti-inflammatory properties and direct stimulatory effects on smooth muscle. (Dowling, 2018).

Two hours later, the patient regained full consciouness. He was discharged 6 hours after he woke up and the owner reported two days of fecal incontinence, this status being a complication of the condition, rather than a complication of treatment (Sjollema et Sluijs, 1989). Complete healing was achieved two weeks postoperatively the dog evidencing normal urination and defecation.

A seven day Amoxicillin Clavulanate post operative therapy has been used, a broad-spectrum antibiotic prophylaxis being recommended for the immediate perioperative period, and antibiotic cover being typically continued for five to seven days after surgery (Fox, 2014). An Elizabethan collar has been used to prevent self-trauma maintaining the surgical site clean thus reducing the risk of contamination and infection (Pratschke, 2014).

CONCLUSIONS

The chance of perineal hernia occurring in intact dog males increases, these being often favoured by prostatic hypertrophy, with the involvement of prostate and bladder in herniar sac.

Elevation of the internal obturator muscle subperiostally from internal face of ischium and its suture to the perineal muscles, it's crucial for preventing future recurrence.

Orchiectomy together with deferentopexy decrease significantly the chance for recurrence of perineal hernia due to cranial orientation of the adjacent genital glands, thus reducing the pressure exerted on their perineal region (Sanspoux, 2012)

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CURRENT KNOWLEDGE ON URINE ELECTROPHORESIS IN CLINICAL MEDICINE

Ana Maria GOANȚĂ[1], Roxana IGNĂTESCU (ȚÎMPĂU)[1], Ana Maria Alexandra STĂNESCU[2], Valentina Alina SIMION[1], Lucian IONIȚĂ[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania
[2]Carol Davila University of Medicine and Pharmacy, 37 Dionisie Lupu Street, District 2, Bucharest, Romania

Corresponding author email: ana_mv@yahoo.com

Abstract

Electrophoresis defines the migration of charged particles in an electrical field in accordance to their molecular charge and size. In clinical medicine, electrophoresis is used mainly to separate and thus differentiate between and proteins in a given sample, be it serum, urine, cerebrospinal fluid or others. This paper aims to briefly describe the fundamentals and scope of electrophoresis and review the most recent knowledge on urine protein electrophoresis. Urine electrophoresis (UEP) is always evaluated in conjunction with serum electrophoresis and a measurement of total urine protein. In human medicine, proteinuria has been identified and characterised based on this criterion. UEP can also be used to differentiate between glomerular and tubular disease, based on the quantity and size of the molecules. Considering the advances in human medicine and the wealth of disorders that can present with proteinuria in animals, the authors consider that this diagnostic technique deserves more attention in veterinary medicine, in particular as a valuable aid in the detection and identification of renal lesions.

Key words*: proteinuria, electrophoresis, urine.*

INTRODUCTION

Proteinuria is defined as the presence of abnormal quantities of protein in the urine. In healthy animals, proteins that pass through a glomerulus are reabsorbed by the renal tubules or broken down by renal tubular epithelial cells (Harley L and Langston C, 2012). The characteristics of the molecule of protein (size, shape, and charge) determines its ability to pass through the glomerular filter (Latimer KS, 2011). The types of protein normally found in human urine are plasma proteins (30% albumin, 30% serum globulins) and around 40% are tissue proteins from the kidney and urinary tract (mainly Tamm–Horsfall protein, a glycosylated product from the loop of Henle, alongside protein from epithelial cells, casts or exostomes (Wein et al., 2016, Keren DF, 2003, Barrat et al, 2007; Kalantari S et al, 2013). Determining the composition of proteinuria is a non-invasive diagnostic approach that helps distinguish different disease processes. The three mechanisms that result in proteinuria are glomerular injury (with excessive filtration of protein); tubular injury (with excessive production and excretion of tubular proteins) and overfiltration of plasma protein in hyperproteinemia (Toto RD, 2004). The main causes of proteinuria in small animals are summarised in Table 1. In human medicine, clinical proteinuria (>0.5 g/24h) is recognized as the strongest risk predictor of progression to end-stage renal failure and a strong predictor of risk of cardiovascular disease (Camaré C and Caussé E, 2013; Johnson DW, 2011).

In recent years, urinary proteomics has garnered a lot of interest due to its advantages: urine can easily obtained in large quantities and without the need for qualified personnel; the protein it contains is relatively stable; its protein content is less complex and requires less processing than that of blood, which facilitates analysis and interpretation and it usually contains few cells, lipids and less soluble protein that can interfere with analysis (Mischak et al., 2010).

Determining and analysing proteinuria can be done in a multitude of ways. The most frequent is point-of-care testing using urine reagent

strips (Figure 1) in various pathologies such as diabetes, hypertension, systemic diseases, renal disease in order to monitor the effect of nephrotoxic drugs. The dipstick test is most sensitive for albumin but can detect other proteins (Harley L and Langston C, 2012) and it can be combined with the sulfosalicylic acid (SSA) precipitation test which detects all types of protein (Guedes-Marques M. et al, 2015). If the SSA test is positive but urine dipstick is negative, UEP is required to evidentiate immunoglobulin light chain excretion due to dysproteinemias (Patel VB and Preedy VR, 2016). Herbivores that produce a highly alkaline urine always obtain a false-positive reaction for proteinuria (trace or 1+) on urine dipstick tests (Constable et al., 2017). Recently, microalbuminuria assays have been recommended in both human and veterinary medicine as screening tests, as they are more sensitive than the dipstick and sulfosalicylic acid methods for albumin (Bartges J and Polzin DJ, 2011; Rangaswami J et al., 2017).

Specification

	Measurement principle
GLU	Glucose oxidase reaction
PRO	Protein-error reaction
BIL	Azo-coupling reaction
URO	Azo-coupling reaction
pH	pH indicator
S.G.	Cation extraction
BLD	Activity measurement of pseudoperoxidase in hemoglobin
BLD(10PA)	Activity measurement of pseudoperoxidase in hemoglobin
KET	Sodium nitroprusside method
NIT	Grease reaction
LEU	Activity measurement of esterase in leukocytes
CRE	Chelate competition method

	Measurement range	Visual measurement time
GLU	50-1000 mg/dl	60 sec.
PRO	15-1000 mg/dl	60 sec.
BIL	0.5-6.0 mg/dl	60 sec.
URO	2-8 mg/dl	60 sec.
pH	pH 5-9	60 sec.
S.G.	S.G. 1.000-1.030	60 sec.
BLD	hemoglobin 0.06-1.0 mg/dl	60 sec.
BLD(10PA)	hemoglobin 0.03-1.0 mg/dl	60 sec.
KET	5-150 mg/dl	60 sec.
NIT	nitrite 0.08-0.5 mg/dl	60 sec.
LEU	25-500 Leu/μl	90 sec.
CRE	10-300 mg/dl	60 sec.

Fig. 1. An example of a urine reagent test strip commonly used in veterinary medicine in Romania. GLU glucose, PRO total protein, BIL bilirubin, URO urobilinogen, S.G. specific gravity, BLD blood, KET ketones, NIT nitrite, LEU leukocytes, CRE creatinine (ARKRAY Europe, B.V.)

Due to the frequent occurence of false positive and false negative reactions, it is highly recommended to quantify and type proteinuria in a laboratory. This can be achieved through quantitative measurements with devices using various reactions (the biuret method or colorimetry) and through qualitative quantifications though various various techniques: electrophoretic methods or immunochemistry (Camaré C and Caussé E, 2013).

The urine protein to creatinine ratio (UPCR) obtained by dividing the urinary protein concentration (mg/dL) by the creatinine concentration (mg/dL) is a ratio that serves as a sensitive and reliable method to detect and quantify proteinuria (in samples that present no inactive sediment) and can replace the protein count in a 24-hour sample (Constable et al, 2017). In healthy dogs UPCR is usually <0.5 and in cats it is <0.4 (Harley L & Langston C, 2012). The normal UPCR in the horse is considered less than 1.0 (Constable et al., 2017). Values over 0.5 in dogs and cats are considered proteinuric; values > 1.0 are considered pathologic and glomerular proteinuria is usually associated with a ratio of over 2.0 (Duffy et al, 2015; Lees et al, 2004, Harley L and Langston C, 2012). In horses with proteinuria, UPCR under 13 is believed to be indicative of tubular proteinuria (Constable et al., 2017). Urine protein electrophoresis can be performed on a spot urine sample (morning preferable) or a 24-hour collection (Lee et al 2017). While serum is processed undiluted, UEP requires concentration and desalting before processing (Keren DF, 2003). For electrophoresis, urine (either from one miction or from a 24-hour urine collection) is centrifuged to eliminate the mineral and organic sediment and analysed immediately or stored at -80°C (Magdeldin et al., 2012).

Electrophoresis defines the migration of charged particles in an electrical field in accordance to their molecular charge and size and can differentiate mixed populations of protein macromolecules into large protein fractions such as albumin, alpha-1, alpha-2, beta and gamma globulins and even into subunit structures such as polypeptides that differ by a few hundred daltons or 0.1 pH units, depending on the techniques and equipment available (Westermeier R, 2016).

Table 1. Differential diagnosis for small animal proteinuria, from Gough A, Murphy K, Differential Diagnosis in Small Animal Medicine, 2nd Edition, Wiley Blackwell, 2015; modified with data from Harley L and Langston C, 2012

Proteinuria
False positives (strip test)
Contamination, e.g. benzalkonium chloride, cetrimide, chlorhexidine
Stale urine
Highly alkaline urine (pH greater than 8.0)
False positives (20% sulphosalicylic acid test)
Cephalosporins
Penicillins
Radiographic contrast media
Sulphafurazole
Thymol
Tolbutamide
Pre-renal
Fever, heat stroke
Central nervous system disease (eg. seizures)
Pancreatitis
Systemic hypertension; cardiac disease (eg. CHF)
Drug reactions
Acute pancreatitis
Hyperthyroidism (cat)
Hyperadrenocortism (dog)
Haemoglobinuria, e.g. haemolytic anaemia
Hyperproteinaemia e.g. derived from colostral proteins, monoclonal free light chain (multiple myeloma)
Myoglobinuria, e.g. muscle trauma, rhabdomyolysis
Physiological, e.g. exercise, stress
Renal
Mild to moderate
• Acute kidney injury
• Amyloidosis
• Breed-associated nephropathy (dog)
• Chronic kidney disease
• Fanconi syndrome
• Glomerulonephritis
• IgA nephropathy
• Primary renal glucosuria
• Secondary glomerular disease
• Bacterial endocarditis
• Borreliosis
• Brucellosis
• Chronic bacterial infection
• Chronic skin disease
• Diabetic glomerulosclerosis
• Dirofilariasis
• Ehrlichiosis
• Exogenous steroid use
• Feline infectious peritonitis (cat)
• Feline leukaemia virus (cat)
• Hyperthermia
• Hypothermia
• Immune-mediated hemolytic anemia
• Infectious canine hepatitis (dog)
• Inflammatory bowel disease
• Leishmaniasis
• Leptospirosis
• Mycoplasma polyarthritis
• Pancreatitis
• Polyarthritis
• Prostatitis
• Pyometra
• Pyrexia
• Rocky Mountain spotted fever (dog)
• Septicaemia
• Sulphonamide hypersensitivity
• Systemic lupus erythematosus
Severe:
• Amyloidosis
• Glomerulonephritis
Post-renal
Genital tract disease: prostatitis, vaginitis
Genital tract secretions
Lower urinary tract disease: trauma, urinary tract infection, urolithiasis
Urogenital neoplasia: bladder, ureteral, urethral, vaginal or prostatic neoplasia

Electrophoretic techniques commonly used are horizontal and vertical gel electrophoresis, agarose gel electrophoresis, polyacrylamide gels, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, native (buffer) gels, gradient gels, capillary electrophoresis, cellulose acetate electrophoresis, isoelectric focusing and two-dimensional gel electrophoresis, microchip electrophoresis. Several techniques can be used in conjunction with electrophoresis, such as immunofixation, immunonephelometry or mass spectrometry (Keren, 2003; Kurien BT and Scofield RH, 2012, Westermeier R, 2016).

In human clinical medicine, urinary protein electrophoresis is used by nephrologists and cardiologists to look for signs of glomerular or tubular proteinuria, while hematologists look for paraprotein suggestive of malignant hemopathies (Camaré C and Caussé E, 2013). UEP can help distinguish if the proteinuria is glomerular or tubular (Jenkins MA, 2009). However, urinary protein electrophoresis is a semi-quantitative method that has poor performance for the study and monitoring of proteinuria and in order to obtain more information one should investigate glomerular and tubular urinary markers such as IgG, albumin, transferrin, alpha-1-microglobulin and retinol binding protein using complementary methods such as immunonephelometry (Bastard JP et al., 2017). Nephelometry is the

measurement of scattered light used to determine the size, shape, and concentration of scattering particles; in immunoassays, these particles are the antigen-antibody complexes formed (Ackerman E, Rosevear JW, 1979). Immunoelectrophoresis includes a variety of techniques that combine electrophoresis and the precipitation reaction between antibody and antigen (Csako G in Kurien BT and Scofield RH, 2012). Immunofixation electrophoresis is an alternative to immunoelectrophoresis that helps identify specific proteins in situ (Csako G in Kurien BT and Scofield RH, 2012).

MATERIALS AND METHODS

In order to review current knowledge on urinary electrophoresis in human and veterinary medicine we searched through scientific databases using keywords such as 'urinary', 'electrophoresis', 'veterinary', 'small animal', 'large animal'. We selected those results that were relevant to the topic and presented both well established and novel uses for UEP.

RESULTS AND DISCUSSIONS

The main reason for performing UEP is to monitor and diagnose monoclonal gammopathies, which are disorders in which lymphocytes/plasma cells proliferate and produce monoclonal intact immunoglobulin (IgG, IgA or IgM), free light chain or free heavy chain protein (Tate et al, 2009; Jenkins, 2009), resulting in different electrophoretic patterns, as in chronic lymphocytic leukemia and multiple myeloma (Keren DF, 2003). In small animals, serum monoclonal gammopathies can be caused by canine ehrlichiosis, leishmaniasis, pyoderma, B‐CLL, B‐cell lymphoma, plasmacytoma and feline infectious peritonitis (FIP), and some of these protein can also be found in urine, as in myeloma or FIP (Harley and Langston, 2012; Meuten DJ, 2017). Further investigations are required to identify the type of protein involved and the cause, thus determine the severity of the lesion - benign monoclonal gammopathy, multiple myeloma or light chain amyloidosis (Tate et al, 2009; Jenkins, 2009).

Routine biochemistry in an animal may reveal a low albumin to globulin ratio, which should be followed by serum and urine electrophoresis. A decreased albumin to globulin ratio is either due to renal proteinuria and/or excessive immunoglobulin production due to antigenic stimulation or a monoclonal gammopathy (Latimer KS, 2011). B-cell chronic lymphocytic leukemia (CLL) in humans is accompanied by macroglobulinemia; in animals this is more rare and, if present, the globulin is usually IgM (identified through immunoelectrophoresis) (Meuten DJ, 2017). Electrophoresis can thus help distinguish between a T-cell or B-cell CLL.

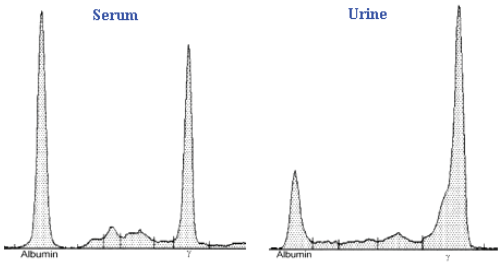

Fig. 2. An example of serum and urine electrophoretograms from a cat with multiple myeloma - a monoclonal peak in the γ region can be observed in both serum and urine electrophoretograms; the immunoglobulin was IgG (Cornell University Veterinary Diagnostic Laboratory)

In myeloma, one usually encounters a mono- or biclonal gammopathy, most frequently constituted of IgG, less so IgA and IgM, while some are nonsecretory (Meuten, 2017). These gammopathies can be identified in the serum, while in urine one can identify light chain immunoglobulins (Fig. 2) that are small enough to pass through the nephron; these globulins can determine glomerulonephritis or nephrotic syndrome (Meuten DJ, 2017). The diagnosis of B-cell or plasma cell lymphoma can be aided by UEP through the detection of large amounts of monoclonal immunoglobulin light-chain when renal damage can be excluded from the differential (Schwab M, 2011).

UEP is also useful for investigating proteinuria of renal origin and distinguishing between glomerular and tubular protein (Fig. 3). The glomerular filter is composed of three successive barries: the endothelial cells, basement membrane and podocyte foot processes that work together (Bartges J, Polzin DJ, 2011; D'Amico G, Bazzi C 2003). In

glomerular kidney disease, UEP will identify albumin as the cause of proteinuria, as larger protein molecules can be lost through urine (Lee et al, 2017; Wein et al., 2016). Tubular lesions impair the reabsorbtion of low molecular weight proteins, with increases in alpha-1 and beta-2 fractions in urine such as retinol binding protein and alpha-1 microglobulin (Jenkins, 2009). Tubular damage may result in high molecular weight protein loss in the urine, which must be differentiated though UEP immunofixation to determine its nature and origin (Lee et al., 2017; Wein et al., 2016). Yalçin A and Çetin M (2004) used UEP and imunoblotting to identify transferrin, alpha-1 microglobulin, beta-2-microglobulin and retinol binding protein (RBP) in dogs with renal disease and detected RBP in all patients with proteinuria and in two healthy dogs. Schaefer et al. (2010) also detected RBP in dogs with various pathologies diagnosed with systemic inflammatory response syndrome. Several studies identify RBP as a possible biomarker in human as well as animal kidney disease (Nabity et al., 2011). In small animal medicine, it is important to note that renal disease can be present in the absence of increased blood urea nitrogen or creatinine values and proteinuria is a negative prognostic factor in renal disease (Harley L and Langston C, 2012). On 49 dogs admitted for an increase in serum creatinine, proteinuria or both, Zini et al. (2004) performed renal biopsy and histologic examination as well as urinalysis and SDS-agarose gel electrophoresis on urine collected through cystocentesis and compared the histopathologic score with the electrophoretic pattern. They observed that dogs with glomerular disease has very similar proteinuric patterns and cannot be differentiated on this basis (Zini et al., 2004). When tubular and tubulo-interstitial lesions were detected on histology, only the most severe forms were accompanied by a tubular pattern on the urinary electrophoretogram, and the smaller the molecules detected, the more severe was the histological score (Zini et al., 2004). It is recommeded that proteinuria be treated if it persists after the inciting causes have been managed, in particular in the presence of polyuria and polydypsia (Harley L and Langston C, 2012) - see Figure 4.

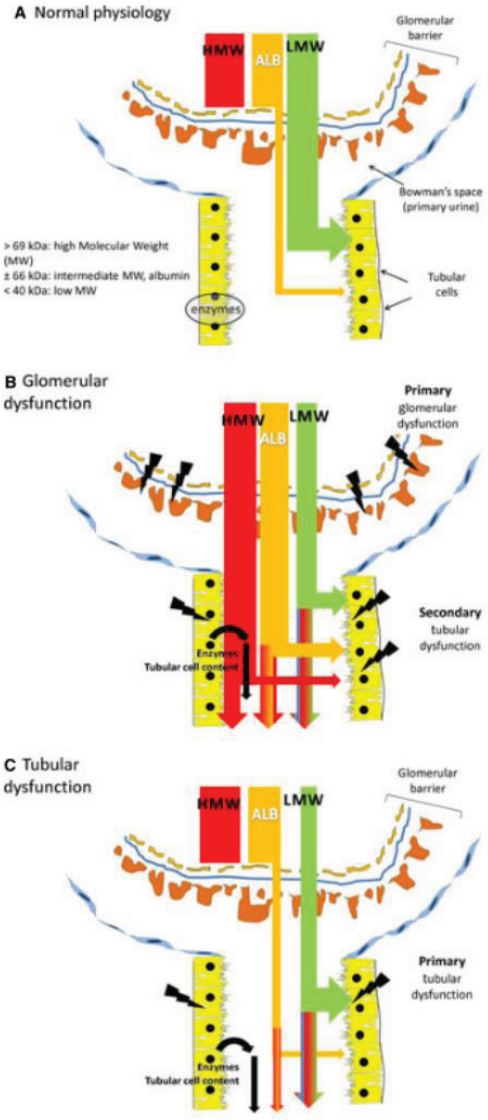

Fig. 3. Nephron filtration of plasma protein: A = physiologic; B = primary glomerular dysfunction with secondary tubular dysfunction; C = primary tubular dysfunction. In health, a small quantity of low molecular weight protein and albumin pass through the glomerular barrier to be reabsorbed by tubular cells. When different disease processes alter the permeability of the glomerular membrane and/or overcome the reabsorbtive capacity of the tubular cells, urine may contain albumin (ALB), low molecular weight protein (LMW) and high molecular weight protein (HMW) (Reproduced from de Loor et al., 2013).

Nephrotic syndrome, defined as the presence of proteinuria, hypoalbuminemia, extravascular accumulation of fluids and hyperlipidemia, is an uncommon occurence in dogs and cats with

protein-losing nephropathy (Bartges J, Polzin DJ, 2011). It presents with decreased serum albumin and γ-globulin and increased α2-albumin, while UEP demonstrates increased albumin and some increase in globulins (Keren, 2003; Longsworth LG, MacInnes DA, 1940). Nephrotic syndrome can be more readily diagnosed and its cause discovered through a complex urine examination (routine urinalysis, UEP and immunofixation), as the serum electrophoretic pattern may only become evident in severe disease (Keren 2003).

Anti-proteinuric drugs (body weight–BW)

	Feline dose	Canine dose
ACE inhibitors		
— Enalapril[a]	0.25 to 0.5 mg/kg BW, PO, q12 to 24h	0.5 mg/kg BW, PO, q12 to 24h
— Benazapril[a]	0.25 to 0.5 mg/kg BW, PO, q12 to 24h	0.25 mg/kg BW, PO, q12h
— Lisinopril[a]	0.25 to 0.5 mg/kg BW, PO, q12 to 24h	0.25 to 0.5 mg/kg BW, PO, q12 to 24h
Angiotensin II receptor antagonists		
— Losartan[b] — Telmisartan[b]	No data	0.5 to 1 mg/kg BW/d
Aldosterone receptor antagonist		
— Spironolactone[a]	0.5 to 1 mg/kg BW/d	0.5 to 1 mg/kg BW/day
Omega 3 Fish Oil[b]	Minimum of 1 g/4.55 kg BW, q24h	Minimum of 1 g/4.55 kg BW, q24h
Antihypertensive drugs (if animal persistently hypertensive despite ACE inhibitor): e.g., Amlodipine[a]	0.2 to 0.4 mg/kg BW, q12h	0.2 to 0.4 mg/kg BW, q12h

[a] Plumb DC. Veterinary Drug Handbook, 7th ed. Ames, Iowa: Wiley-Blackwell, 2011.
[b] No published dose in dogs and cats. Dose extrapolated from human data and suggested dose based on authors' experience.

Fig. 4. Antiproteinuric drugs (from Harley L and Langston C, 2012)

UEP is currently used in the search for protein biomarkers in specific diseases in order to monitor and detect diseases in earlier stages. To this end, the most common EP technique employed is capillary electrophoresis-mass spectrometry (CE-MS), which offers both high separation efficiency and molecular mass information (Shao C et al, 2011). Panels of proteins and peptides that may facilitate earlier detection of diseases such as acute kidney injury, diabetic nephropathy, glomerular diseases and distinguish between bladder, kidney, prostate cancer and other genito-urinary diseases have already been identified (Shao C et al, 2011, Siwy J et al., 2017).

Researchers are also looking at the urine proteome for early markers of non-renal disease such as acute pancreatitis, obstructive sleep apnea and non-small-cell lung cancer, among others (Barrat J, Topham P, 2007; Shao C et al 2011). Nally JE et al. (2015) devised a method to detect urinary biomarkers of chronic infection in clinically asymptomatic and serologically negative rats with leptospirosis, the most common reservoir hosts (Nally JE et al., 2015). Song et al. (2013) devised a method for the rapid detection of bacteria in urine using capillary electrophoresis with a limit of detection of 106 CFU/mL. Urine capillary electrophoresis can be used alongide complementary methods for the metabolic profiling of urine, rapid screening for drug abuse, toxic compounds and metabolites (Kohler I et al, 2013; Zhang Q et al., 2015; Wang W et al., 2010) alongside other techniques.

CONCLUSIONS

UEP is essential to determining the profile of the urinary proteome. Urine is an easily sampled biological fluid that is being searched worldwide for biomarkers of disease, both of the genito-urinary tract and of other systems. It is also useful to detect subclinical infection, toxic compounds in humans, pets and large animals or growth promoters in food-producing animals. Urine proteomics in animals and UEP in particular are not well established at the moment. We consider that serum electrophoresis and UEP should become routine investigations in animals with relevant clinical signs and for monitoring purposes and that a minimal urinalysis (dipstick and sediment) should be included in all routine examinations.

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CLINICAL EFFICACY AND SAFETY OF EXTERNAL FIXATORS USED IN ANTEBRACHIAL AND CRURAL FRACTURES IN DOGS: A REVIEW AND META-ANALYSIS

Andreea ISTRATE, Alexandra PETEOACA, Andrei TANASE, Jacqueline MOCANU, Gina GIRDAN, Emilia CIOBOTARU

University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 105 Spl. Independentei, District 5, 005097, Bucharest, Romania

Corresponding author email: andreeaistrate@gmail.com

Abstract

PURPOSE: The aim of this meta-analysis is to describe the indications, preoperative assessment, surgical technique, postoperative care, clinical results and complications of external fixators used to treat antebrachial and crural fractures in dogs.
METHODS: Databases including Pubmed, Elsevier, Science Direct, Cochrane library and other journals were searched for articles published before January 2018 for studies regarding history, clinical applications, complications, advantages and disadvantages of the external fixators technique.
With a history of over a hundred years, external skeletal fixators have been used in veterinary medicine as a common technique for stabilizing fractures over the past two decades. The external skeletal fixators use multiple percutaneous pins or wires placed distally and proximally to the fracture site or joint, coupled with an external frame, that can be linear, circular or hybrid and can be placed in various geometrical configurations. This method can be used in various situations besides fracture stabilization, including joint immobilization, limb lengthening and angular, translational and rotational limb deformities, external skeletal fixators being mechanically versatile. The minimal soft tissue and bone trauma allows for simple staged disassembly in helping promotion of bone healing by using increased loading forces on the fracture site after the beginning of healing. Also, from the reviewed sources we can easily say that some of the most common postoperative complications of these surgical approaches are pin-tract infections, bone lysis, osteomyelitis and implant failure but none of them outweigh the benefits.
CONCLUSIONS: External skeletal fixators are a biologically friendly surgical technique, used to minimize disruption of the blood supply to the soft tissue and bone being an advantageous system for management of antebrachial and crural fractures in dogs.

Key words: *dog, external fixators, orthopaedics, review, veterinary surgery.*

INTRODUCTION AND HISTORY

Every fracture has its own characteristics that make it suitable usually for a single, best, repair method. However, more than often, a number of equally valid management methods can be used and, of these, external skeletal fixation is one of the more versatile treatment options available to the surgeon (Kraus et al., 2003).
Surgery has always been dominated by the fear of pain and lethal infections. As early as the year 400 BC, Hippocrates held the opinion that immobilisation should be the aim following realignment and only if necessary and in very few cases did he use open surgery techniques for fracture repair (Adams, 1939).
After Hippocrates, in the first part of the nineteenth century, Joseph-François Malgaigne (1806-1865), a French surgeon and a medical historian, is considered to be the first to devise and to apply a practical method of external skeletal fixation, his method being used in the treatment of displaced transverse fractures of the patella, and consisted of two double hooks, which were inserted through the skin and engaged into the upper and lower borders of the patella. The hooks were connected by a screw, which drew the fragments into apposition and maintained them in position during the healing period (Malgaigne, 1847).
An adapted form of Malgaigne's screw technique was developed by Von Heine (1878), a professor of Surgery in Prague. His method involved drilling a hole through the cortical layers of the bone fragments near the fractured ends, perpendicular to the longitudinal axis,

and pushing through the holes ivory pins and fixing them in plaster of Paris by means of a Some historians consider Clayton Parkhill (1860-1902), professor of Surgery at the Gross Medical School in Denver, to be the one that first described external skeletal fixators in 1897, who designed and used a special external clamp to aid transfixation pins to be placed into long bones and to externally stabilize the fracture. (Martinez, DeCamp, 2012 -Tobias). His device consisted of four screws, two of which were inserted into each fracture fragment and the external ends of the screws were fixed together by means of a series of small plates and bolts (Parkhill, 1897; Parkhill, 1898).

In Europe, the father of external fixation devices is considered to be Albin Lambotte (1866-1955), who designs a similar apparatus as Parkhill for fractures of the femur, tibia, forearm, humerus, clavicle and metacarpals. His apparatus had the advantage over Parkhill's that it needed no additional fixation other than that provided by the 'fixateur'. His method used sharp ended pins fixed only in the cortex, inserted parallel to each other, two pins proximal and two pins distal to the fracture site, at 2 cm away, and sometimes cerclage in the case of oblique fractures (Hernigou, 2016).

The early forms of external skeletal fixation in veterinary medicine included the Stader reduction and fixation splint (1937), the Angell Memorial Animal Hospital splint (1938) and the Kirschner-Ehmer fixation splint (1940) (Petit, 1992). Despite positive reports, the use of external skeletal fixators remained limited due to relatively high complication rates, such as premature pin loosening, pin tract sepsis and associated delayed union or nonunion fractures. The development of proper transfixation pin insertion techniques and advances in pin design resulted in enhanced longevity of the pin-bone interface, which led to fewer complications. Advances in biomechanics of external skeletal fixator frame configurations led to a better knowledge of how different geometric configurations could provide mechanical support in veterinary patients with long bone fractures. Furthermore, discoveries of advanced mechanical properties using hybrid frames (e.g. type Ia/IIb or linear/circular) and tie-ins (additional connecting bars to transfixation pins or directly to intramedullary pins) have enhanced the use tube and clamps. This is considered the first form of external fixation (Hernigou, 2016). of external skeletal fixators in complex fractures (Martinez et. DeCamp, 2012, Tobias). They are now being used extensively for the treatment of long bone fractures and nonunions distal to the elbow and stifle joints, for the treatment of limb deformities and for other applications, being the standard of care in both human and veterinary orthopaedics for almost 30 years (Marcellin-Little, 2003).

COMPONETS AND TYPES OF EXTERNAL SKELETAL FIXATORS

The external skeletal fixator (ESF) system uses stainless steel pins or Kirschner wires placed percutaneously and attached to external clamps. The exposed portions of the fixation pins or wires are interconnected using connecting clamps that fasten the fixation pins to one or multiple connecting bars. Single connecting clamps are used to fasten pins to connecting bars, whereas double connecting clamps are used for connecting bars to one another (Andrerson et al., 1993).

For years, the Kirschner-Ehmer (KE) apparatus was the most commonly used system of external skeletal fixation in veterinary orthopaedics (Sherman et al., 2004). Recently, there have been developed new and improved systems, that have better biomechanics (stiffness) and allow for much simpler constructs.

The external frame of the system was at first constructed of metal or acrylic (polymethylmetacrylate) connecting bars (Willer et al., 1991), but the development of titanium and especially aluminium and carbon fibre rods have dramatically imaproved the stiffness of ESF constructs, so much that many of the cases that required 2 or more stainless steel bars now achieve superior rigidity with a single bar (Bronson et al., 2003).

Linear External Skeletal Fixation Systems

Linear ESF systems are created by using transfixation pins or Kirschner wires that can be connected to a simple bar (type Ia frame, unilateral), two connecting bars opposed 90 degrees (type Ib frame, unilateral biplanar); two connecting bars opposed 180 degrees (type IIa or IIb frame, bilateral); or three connecting

bars, two connecting bars opposed 180 degrees with one connecting bar opposed 90 degrees from the other two (type III frame, bilateral biplanar) (Fig. 1) (Johnson et al., 1999).

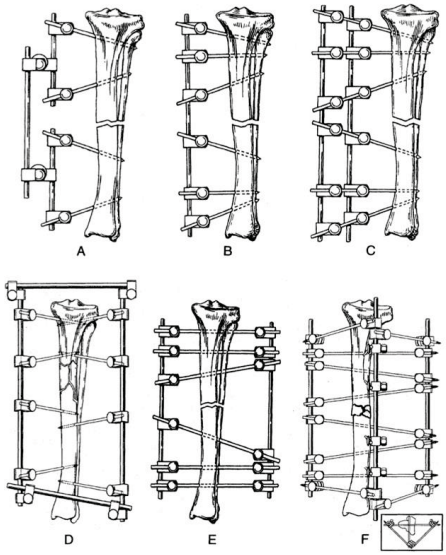

Figure 1. Common linear external skeletal fixator systems. A. type Ia, double clamp; B. type Ia, single connecting bar; C. type Ia, double connecting bar; D. type Ib; E. type II; F. type III (Adapted from Piermattei, D.L., Flo, G.L., Handbook of Small Animal Ortopedics and Fracture repair, ed. 3, Philadelphia, WB Saunders, 1997, pp. 82-85)

The most commonly used linear ESF system is the Kirschner-Ehmer (KE) splint, that comes in three sizes: small, for cats and small dogs; medium, for medium and large dogs; and large, for giant breed dogs and other species (Bouvy et al., 1993).

Another type of linear ESF is the one using acrylic pin splints, that have an acrylic column that acts as both the connecting rod and transfixation pin-gripping device. The major advantage of acrylic pin splints is that the connecting bar can be contoured to match any fracture configuration, body or joint angle and the fact that pins can be placed at any location without the difficulty of passing them through clamps, being best suited for mandibular and transarticular applications (Egger, 1992).

Circular External Skeletal Fixation Systems

Circular fixators (CEF) or ring fixators use supporting rings, connection rods, bolts and tensioned transfixation Kirschner wires (Fig. 2). The method used for placement of circular external skeletal fixation is termed the *Ilizarov method* (Marcellin-Little, 1999).

Figure 2. Full-ring circular external skeletal fixation system applied for tibia lengthening (Adapted from Zamani, A.R., Oyadij, S.O., Analytical modelling of Kirschner wired in Ilizarov circular external fixators as pretensioned slender beams, Journal of the Royal Society Interface 2009, 6:243-256)

Circular ESF systems share many of the attributes that make linear external skeletal fixation systems well suited for management of antebrachial and crural fractures in dogs. Both types of systems can be applied using closed or open fracture reduction, being useful for stabilizing highly comminuted fractures that can not be anatomically reconstructed, but circular systems have a higher bending stiffness combined with a lower axial stiffness, establishing better conditions for bone healing (Fleming et al., 1989).

Circular fixators use smaller diameter wires as fixation elements (Lewis, 2001). The fixation wires are tensioned so that construct stiffness increases, but weight bearing produces axial micromotion of the stabilized segments, which is thought to aid fracture healing (Egger et al., 1993). Given the fact that the fixation wires and pins are placed in multiple planes, soft-tissue trauma is reduced and early weight bearing in the convalescenteriod is encouraged (Ilizarov, 1992).

Fixators are constructed using three to five, but generally four rings, with two rings used to secure each of the major fracture segments. The most proximal and distal rings are usually positioned near their respective metaphyses of the fractured bone and the intermediate rings can be placed over the intact bone, adjacent to the fracture (Marcellin-Little, 1999a).
Constructs can be single-block, when the threaded bars connecting the rings cover the entire length of the limb and double-block constructs, when two separate ring blocks stabilize each fracture segment (Andreson et al., 2003).
Circular fixators can also be used for stabilizing a variety of long bone fractures, spinal fractures and luxations (Wheeler et al., 2007), to dynamically correct angular limb deformities (Lewis et al., 1999a) and to provide a rigid fixation for arthrodesis (Lewis et al., 1999b).
This type of fixation requires a high level of post-operative care and some drawbacks of this method can be the possibility of suboptimal fracture reduction, loss of limb function if transfixation wires impede muscle motion and the potentially high risk for sepsis along the wire tracts (Anderson et al., 2003).

Hybrid External Fixation Systems

Hybrid external fixation systems (HEF) are a new emerging technique for fracture stabilization in veterinary orthopaedic surgery. They combine elements from both linear and circular fixators that can be connected in a high number of combinations (Jimenez-Heras et al., 2014).
Hybrid systems have been applied for correction of growth deformities and fracture repair (Farese et al., 2002; Sereda et al., 2009).
Due to combination of both linear and circular elements, HEFs require a reduced number of rings comparatively to circular systems that usually require three or four rings, being less cumbersome and better tolerated by the patient (Kirkby et al., 2008) (Fig. 3). Hybrid fixators also share some of the positive characteristics of circular fixators like small-diameter wires that allow the fixation of small bone fragments and enable axial micro-motion, stimulating callus formation and accelerating bone healing (Goodship and Kenwright, 1985).

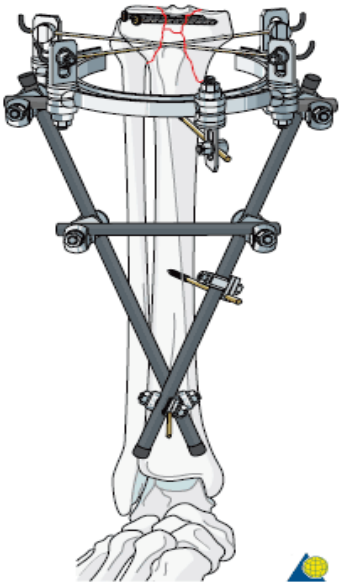

Figure 3. Hybrid fixator used for a tibial plateau fracture (Adapted from Ruedi, T.P., Buckley, R., Moran, C.G., AO Principles of Fracture Management, Ann R Coll Surg Engl, 2009, 91(5):448-449)

This type of fixation is very useful in fractures with short juxta-articular fragments, in which the circular component is used for stabilization of the small fragment while the linear component is used for the long one, but can also be useful in transverse, oblique or diaphyseal fractures (Clarke and Carmichael, 2006).
Published reports describe the use of hybrid fixation systems for fracture repair in dogs and cats and a classification of these fixators is already being reviewed (Hudson et al., 2014).
Hybrid systems can also be classified based on their ring number (Jimenez-Heras, 2014):
I. one ring included in the frame;
II. two rings included in the frame;
III. three or more rings included in the frame;
or by the number of linear elements:
A: One linear element included in the frame;
B: Two linear elements included in the frame;
C: Three linear elements included in the frame;
D: Four or more linear elements included in the frame.
Some studies have started using **Minimally Invasive Reduction Instrumentation Systems (MIRIS)** in veterinary orthopaedics, a type of minimally invasive external fixator system

developed to facilitate applications of minimally invasive plate osteosynthesis (MIPO) in human pacients (Gilbert et al., 2016).

GUIDING PRINCIPLES OF EXTERNAL SKELETAL FIXATOR APPLICATION

External skeletal fixators have been utilised for almost every bone, but for dogs and cats they are thought to be best suited in the distal limb, specifically the antebrachial region (radius/ulna) and crural region (tibia) (Harari et al., 1996, Johnston et al., 2008).

Selection of the appropriate external skeletal fixation system is always based on factors including fracture biomechanics, aetiology, location and configuration and also patient health status, age, body weight, lameness score and concurrent musculoskeletal injuries.

Due to the fact that fracture healing is completely dependent on maintaining the blood supply to the fracture site during all phases of bone healing all fixation systems used must protect, preserve and enhance vascularization at the fracture site, a method of fracture management known as *biological fixation* (Palmer, 1999).

External skeletal fixation systems meet the criteria, being a fracture stabilization method that can be applied both through a closed and open approach of the fracture site, with the surgical goal of a required open approach kept to a minimum (Martinez and DeCamp, 2012).

From the papers studied fifteen critical principles have been established for the application of external skeletal fixators for fracture management (Piermattei et al., 2006a, Brinker and Flo, 1975):

1. Aseptic techniques should be used in order to obtain a successful bone healing.
2. A proper bone surface location should be used for insertion of transfixation pins, in order to maximize construct stabilization and minimize soft-tissue damage (the proper surface for tibia fractures is medial and for radius fractures medial or craniomedial) (Marti and Miller, 1994a, 1994b).
3. The most suitable configuration of the external fixator system should be selected in order to obtain the best stabilization of the intended bone(s). Limitations of frame configurations for various regions should be taken into consideration, as well as the need for counteracting specific biomechanical forces. Frame selection will ultimately be dictated by careful consideration of both factors associated with the patient and with fracture management (Egger et al., 1986; Brinker et al., 1985; Egger, 1983).
4. Auxiliary fixation should be used when indicated. In order to achieve maximal stabilization of fracture fragments, auxiliary fixation such as intramedullary pins, lag screws, Kirschner wires and cerclage may be helpful during insertion of the external skeletal fixation pins (Aron et al., 1991, Popkov et al., 2014).
5. Fracture stabilization and reduction should be maintained during application of the external fixator frame, in order to minimize soft-tissue trauma and discomfort of the animal (Piermattei et al., 2006).
6. Insertion of pins and/or Kirschner wires through soft tissues should be made in a manner that does not distort or capture the surrounding tissues.
7. Proper pin drilling or Kirschner wire insertion technique is critical in order to maintain system integrity during bone healing and can be obtained by using a high-power drill at low speed (<150 rpm) in order to prevent thermal damage to the bone, which can result in bone resorption or pin loosening (Egger et al., 1986).
8. Proper insertion of pins or Kirschner wires through both cortices of the bone, in order to avoid weakness at the pin-bone interface or pin loosening (Dernell et al., 1993).
9. Insertion of smooth and negative-threaded-profile pins at an angle of 70 degrees to the long axis of the bone in order to obtain maximum stiffness of the fixator along with maximum pull-out resistance from the bone (Egger, 1993; Bouvy et al., 1993).
10. Insertion of pins or Kirschner wires used to create an external linear or circular skeletal fixator system in the same plane, in order to reduce pin-bone interface stress and premature resorption of bone due to unnecessary bending forces or stiff double clamps.
11. Insertion of pins or Kirschner wires at target points on the bone fragment in order to optimize the mechanical stability of the external fixation system. Studies show that maximum stability can be obtained by inserting

pins near the proximal and distal ends of the bone fragment rather than by inserting all the pins near the ends or near the fracture site (Toombs, 1994).
12. Insertion of two to four transfixation pins or Kirschner wires in each major bone fragment, in order to obtain greater stability. Studies since the 1970s have shown that three to four pins per fracture fragment increase the stiffness of the construct (Hamish et al., 2000). Using more than four pins does not increase the mechanical strength of the external skeletal fixation system (Egger, 1993; Brinker et al., 1985; Palmer et al., 1992).
13. Selection of transfixation implants, connecting bars, rings and transfixation pins of optimal size for the size of the bone involved.
14. Placement of connecting bars at an optimal distance between the pin-connecting bar clamps and the patient's skin, taking into consideration the size of the patient and the anticipated post-surgical swelling. After 10 days of usual post-surgical swelling the system might need readjustment (Piermattei et al., 2006).
15. Use of cancellous bone graft in cases of significant cortical defects, especially for architectural deficits in mature and older animals, in those with osteotomies of diaphyseal bone and in cases of nonunions (Johnson et al., 1989).

Post-operative care of ESF systems

Post-operative management of external fixators is quite controversial, the veterinary literature offering recommendations for bandaging the frames, leaving the frames uncovered, bandaging frames only in the early postoperative period and daily hydrotherapy (Harari, 1992).
In most cases gauze sponge dressings may be placed between the skin and bar-connecting clamps in order to absorb any discharge or blood from the pin-skin interface for about 5 to 7 days post-operatively. Dressings need to be changed daily until discharge stops. The frame should be bandaged in order to prevent it from getting in contact with other objects and in order to protect the patient (Lethaby et al., 2013).
If open wounds exist, they need to be treated appropriately on a daily basis. Some studies suggest allowing a dry, protective crust to form at the pin-tract drainage site, while others recommend daily cleaning with antiseptic solutions (Phillips et al., 1991).
Animals should be rechecked every few weeks and the clamps should be adjusted and tightened. Radiographs should be taken monthly in order to evaluate bone healing and decide on the best time to start disassembly of the frame. Destabilization of a type III frame to a type I 6 weeks post-operatively has been shown to enhance fracture remodelling (Egger and Histand, 1993).
Most uncomplicated fractures in adult dogs treated with ESF heal in 2 to 3 months by means of periosteal and endosteal callus formation (Harari et al., 1998).

Fixation of distal extremity fractures: radius and tibia

Radial and tibial fractures account for 30 per cent of all fractures in small animals (Ness et al., 1996) and external skeletal fixation (ESF) is a common method of stabilisation (Egger et al., 1985; Roe et al., 1985; Johnson et al., 1989; Pettit, 1992; Piermattei and Flo, 1997).
Most fractures of the radius and tibia may be treated in a closed manner and depending on the type of fracture (open or closed, comminuted or simple) with either linear, circular or hybrid external fixator frames.
External skeletal fixation systems are particularly useful in fractures of the radius and ulna due to the relative lack of surrounding soft-tissue, being the standard of care in open fractures, delayed unions, nonunions and corrective osteotomies (McCartney et al., 2010). Insertion of pins should be made on the medial or craniomedial side of the radius, the bone being more superficial on this location and the external fixator being in the position of least interference from other objects. All the linear configurations (types Ia, Ib, IIa and IIb frames) can be used, along with circular and hybrid linear-circular fixators. Type Ia frames are best suited for stabilizing simple fractures of the radius and ulna in toy breed dogs and cats, while type Ib can be usually used for comminuted fractures (Marti and Miller, 1994a, b). Circular external fixators or hybrid fixator provide an excellent alternative when a linear frame cannot be used due do severe soft-tissue trauma or in case of short fracture segments (Piermattei and Flo, 2006).

All external skeletal fixation systems can be used for tibial fractures (open or closed, simple or comminuted), more complex frames being used accordingly to the complexity of the fracture, tissue trauma and patient weight. All types of frames are applicable to the tibia, because the medial, cranial and lateral surfaces of the bone are available (Bilgili et al., 2007).
Type Ia frames are best suited for fractures of skeletally immature patients, who have a tendency of healing faster than adults. Type Ib frames are usually used for stabilizing proximal and distal tibia fractures when there is limited bone stock for pin fixation in one plane, the fixation planes being oriented at approximately 90 degrees to each other. Types IIa and IIb are indicated when no load sharing is possible in complex, nonreducible fractures. Circular and hybrid frames are usually used for treatment of epiphyseal fractures, where limited bone segments are available for fixation (Witte et al., 2014).
Studies have shown that durations of bone consolidation and external fixators were shorter for radial than tibial fractures and the hypothesis that the radius heals faster than the tibia exists (Tuhoy et al., 2014).

COMPLICATIONS OF EXTERNAL SKELETAL FIXATION SYSTEMS

While external skeletal fixation may be considered the standard of care in orthopedic surgery for management of antebrachial and crural fractures, being a versatile and useful tool, complications associated with these systems need to be taken into consideration when deciding on this surgical technique (Beever et al, 2017).
Complications associated with external skeletal fixation systems may be related to the fixation device or may be due to the character of the fracture under treatment. Complications associated with the fixation device are usually common, due to the large number of components, but have few consequences for the patient or for fracture healing (Egger, 1991).
Some of the most common minor post-operative problems are pin tract infection and premature pin or transfixation wire loosening, that can be reduced by using adequately strong and stiff frames (Egger, 1992; Jonhston et al, 1989).
Slight, serous drainage and minimal tissue inflammation around the pin-skin interface is commonly observed in all types of ESF frames and appears commonly when large muscles are transfixed (Harari, 1992). Excesive movement of the pin directly contributes to infection (Yardimci et al., 2010).
Pin tract infections occur most commonly in areas of significant penetration and disruption of adjacent soft tissues, usually being easily controlled with local treatment and antibiotic therapy. In severe cases, bacterial contamination of the pin-bone or pin-skin interface takes place, leading to superficial pin tract infection, which can progress to deep pin tract infection and associated bone lysis or osteomyelitis (Krischak et al, 2002; Dudley et al., 1997).
Implant failures include pin loosening, breakage or bending; clamp loosening and connecting bar breakage, all mechanical complications that can be avoided by adhering to known guidelines concerning frame construction and pin size, type, number and location and by constructing an external skeletal fixator frame with optimal biomechanical characteristics for the fracture treated (Anderson et al., 2003). The weakest part of any ESF system is the pin-bone interface and the junction of the threaded and non-threaded parts of the pin, thus the risk of pin breakage being higher in negative profile pins (Bennet et al., 1987; Clary et al., 1995).
Complications are considered major if they require additional surgery or substantial frame modification under general anaesthesia or if they negatively influence the expected outcome (Rovesti et al., 2007).
Issues of fracture healing may be due to the primary injury, but also due to the choices in external fixator construction. ESFs are usually chosen to treat severe fractures, which may ultimately result in issues of malunion, delayed union or nonunion, attributable to the conditions of the fracture (Anderson et al., 1996; Egger et al., 1986).
Iatrogenic bone fractures are uncommon in cats, this complication usually having contributing factors such as multiple injuries, presence of empty drill holes and inappropriate post-operative exercise restriction (Knudsen et al., 2012).

Other complications that may develop with the use of ESFs are loss of range of motion at a joint due to muscle atrophy, fibrosis, contracture or all of these conditions. Joint function is maintained only by using safe corridors for pin placement and by minimizing soft-tissue trauma. Using safe corridors for pin placement also aids in avoiding vascular injury or peripheral nerve injury (Davidson, 1997; Johnson et al., 1996).

Early ambulation hastens fracture healing by promoting axial micromotion and also prevents disuse atrophy and muscle contracture in fracture patients, especially important with bilateral fractures (Lincoln, 1992; Radke et al., 2006).

CONCLUSIONS

Although there is no perfect fixation system for each type of fracture and each clinical case should be individually evaluated, external skeletal fixators can be easily customized to accommodate almost all types of fractures of the antebrachial and crural regions being the standard of care in veterinary orthopaedic surgery.

External fixators may be applied by closed or open surgical procedures and advances in technology and surgical techniques have greatly enhanced the pin-bone interface, resulting in a significant decline of complications seen with the use of any type of external fixator frame over the last recent years, making them a safe surgical technique.

Consistent successful outcomes with limited complications and patient morbidity can be easily achieved if the guiding principles for external skeletal fixation systems application are followed.

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COMPARATIVE STUDY ON INTERLEUKIN 1, INTERLEUKIN 6 AND TUMOR NECROSIS FACTOR A IN OVARIOHYSTERECTOMIZED CATS ANESTHETIZED WITH DIFFERENT ANESTHETIC SCHEMES

Georgi MARINOV[1], Nadya ZLATEVA-PANAYOTOVA[1], Krassimira GENOVA[1]

[1]Faculty of Veterinary medicine, University of Forestry, Sofia, Bulgaria
Corresponding author email: dr.georgimarinov@abv.bg

Abstract

The use of anesthetics is an integral part of any surgery. Despite their widespread use, their mechanisms and interactions with the nervous-endocrine and immune systems are insufficiently studied.The study was conducted with healthy, adult cats subjected to anesthesia and surgery (ovariohysterectomy) in order to trace the effect of anesthesia and surgery on the secretion of proinflammatory cytokines IL1, IL6 and TNFα. The ovariohysterectomy was performed when a deep plan of anesthesia occurred. Blood samples were obtained at 0 min (prior to anesthetic administration), 30, 60, 120 min and 24 h.The chosen anesthetic schemes modulate the immune response and the response depends on the type of anesthetics used.

Key words: *cats, anesthesia, Interleukin 1, Interleukin 6 and Tumor necrosis factor α*

INTRODUCTION

The use of anesthetics is an integral part of any surgery. Despite their widespread use, their mechanisms and interactions with the nervous-endocrine and immune systems are insufficiently studied.

Possible effects of anesthesia on the immune system have been a subject of discussion since the last century. The immune system is a complex of interactions between cells, molecules and organs in order to protect the body and preserve its homeostasis. Graham E. (1911) and Gaylord H. and B. Simpson (1916) reported at the beginning of the last century about the influence of ethereal anesthesia on bacteriolisis and phagocytosis in humans and the effect of anesthetics on tumor growth in experimental animals. Recent studies have shown that anesthesia can alter the immune response by modulating the stress-response of the body and associated stress-chromosomes (Schnnemilch C., 2005).

Cytokines are key regulatory molecules for the immune response to stress, including surgical and anesthetic. They represent a heterogeneous group of proteins that act on cell-surface receptors and regulate the amplitude and duration of response by short-term secretion and self-limiting.(Sheeran P. and G. Hall, 1997).

Contemporary human studies have shown that this area of medicine has great potential for development in the direction of cytokine response control and reduction of proinflammatory activity in order to successfully recover after surgery and anesthesia (Dinarello C., 2000).

The aim of the present study was to monitor the response changes by investigating **interleukin 1, interleukin 6 and tumor necrosis factor α** in two anesthesia schemes during ovariohysterectomy in female cats.

MATERIALS AND METHODS

Animals

Fourteen mixed breed female cats at the age between 2 and 4 years, weighing 2.8-3.4 kg, were included in the study. The animals were presented from the animal protection organization. One week before the examination, the animals were kept in the University Clinic for Small Animals at the Faculty of Veterinary Medicine, University of Forestry, Sofia. They were fed commercial dry food without limitation except for the 12-hour fasting period before the anesthesia and surgery. The water was restricted two hours before surgery. Immediately prior to the operation and anesthesia, the animals were examined and determined to be clinically healthy on the basis of the physical and blood laboratory examinations. All values were within normal physiological ranges. The cats were randomly allocated in two groups (n = 7 in each group).

Anesthetic protocol

The cats were randomly allocated in two experimental groups (n=7 in each group). The

premedication in the first group (group In) was made with acepromazine maleate 0.025 mg/kg (Vetranquil®, Ceva Sante Animale) intramuscularly, and the second group (group MM) was given acepromazine maleate 0.025 mg/kg (Vetranquil®, Ceva Santé Animale), butorphanol (Butomidor®, Richter Pharma)–0.4 mg/kg, intramuscularly and meloxicam (Loxicom®, Norbrook) - 0.3 mg/kg, subcutaneously.
All animals were submitted to fluid therapy with sodium chloride 0.9 %, 10 ml/kg/h (Natrii chloridum®, Actavis) through a venous catheter 22 gauge (B.Braun) applied in v. cephalica antebrachii. Induction of anesthesia was made with propofol (Propofol®, B Braun) at 5 mg/kg body weight intravenously, fifteen minutes after the premedication.
Immediately after the application of the general anesthesia, the animals were intubated with a tube of a suitable size. The anesthesia was maintained with isoflurane (Forane®, Abbott) 2.5 vol. % in group In and 1.8 vol.% in group MM in 2.5 l/min oxygen flow by using semi-opened breathing circuit system type T/Y detail, Kuhn modification. The extubation was made 60 min later at manifestation of swallowing reflex.

Surgery protocol
Ovariohysterectomy was performed through caudal median laparotomy. The average duration of the operation was between 8 and 10 min. Surgery started 30 minutes after the initiation of anesthesia at the surgical plane of anesthesia.

Collection of blood specimens
Blood specimens were obtained from the jugular vein in sterile 2.0 ml syringes by 23 G needles at strictly determined intervals - at 0 min (before the application of the anesthetics) 30, 60, 120 min and 24 h from the beginning of the anesthesia.
Immediately after collection of the specimens, 1.5 ml of each sample was put into a sterile micro vacutainer, containing heparin and centrifuged for 15 min at room temperature for interleukin analysis.
The plasma specimens were stored at -22 °C for 27 days, prior to determination of the interleukin`s concentration.

Analytical methods of study
Interleukin 1 – (IL-1) and Interleukin 6 (IL-6) was studied using a specific Feline IL-1L и Feline IL-6L VetSet TM ELISA Development Kit, KINGFISHER BIFTECH, Inc., USA;
Interleukin studies were performed by an apparatus TRITURUS ANALYSER, USA;

Statistical analysis
All data were expressed as median and range. Differences between the two groups were analyzed using one way analysis of variance (ANOVA) and the least-significant difference (LSD) post hoc test at a level of significance 0.05. The study was approved by the Committee on Animal Ethics of the National Veterinary Service in Bulgaria.

RESULTS AND DISCUSSIONS

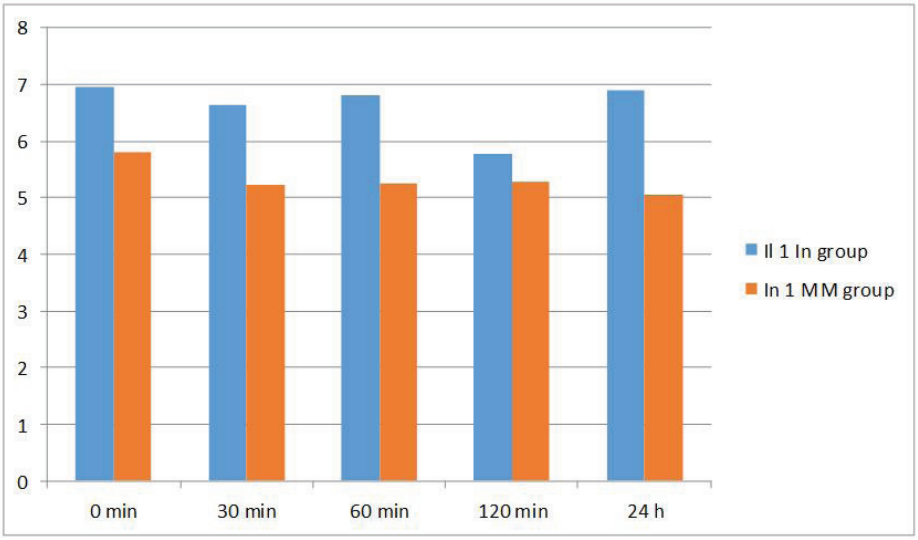
The statistical significance of the results obtained for the MM group is $p < 0.001$ and for the In group is $p < 0.05$. There were no statistically significant differences in 0 min between the two groups.
A reliable difference in In1 levels between the two groups was observed at 30 min ($p < 0.05$), at 60 min ($p < 0.01$) and at 24 h ($p < 0.001$). The IL6 concentrations were reliably elevated in the In group at 120 min ($p < 0.05$) and 24 h compared to the MM group. TNF-α is significantly lower in the MM group at 24 h compared to its established levels in the In group (Table 1).

Table 1. Changes in the levels of IL-1, IL-2 and TNF-α in both two groups

	Interleukin	0 min	30 min	60 min	120 min	24 h
In (inhalational)	**IL-1** pg/ml	6.95±1.64	6.64±1.07	6.81±0.79 *	5.78±0.37	6.91±1.36
MM (multimodal)	**IL-1** pg/ml	5.81±0.33	5.22±0.4 *** #	5.24±0.32 *** ##	5.28±0.18 ***	5.06±0.17 ***###
In (inhalational)	**IL-6** pg/ml	3.92±0.92	3.52±0.50 **	3.68±0.73 *	4.06±0.80	5.21±1.39 *
MM (multimodal)	**IL-6** pg/ml	3.82±0.47	3.95±0.41	3.39±0.16 *	3.17±0.09 ** #	3.24±0.43 ** ##
In (inhalational)	**TNF-α** pg/ml	6.41±1.45	5.59±1.20	5.81±1.93	5.29±1.12	6.62±1.33
MM (multimodal)	**TNF-α** pg/ml	5.84±0.31	5.47±0.31	5.41±0.19 *	5.81±0.19	4.94±0.55 *** ##

IL-1 in the In group was reliably reduced at 60 min (6.81 ± 0.79 pg / ml, p <0.05), but no significant changes in the concentration were observed at the end of the study period. Expressed changes in the concentrations of the studied interleukins were detected in the multimodal anesthesia group. IL-1 was reliably reduced in all study periods - 30 min (5.22 ± 0.4 pg / ml, p <0.001), 60 min (5.24 ± 0.32 pg / ml, p <0.001), 120 min (5.28 ± 0.18 pg / ml, p <0.001), 24 h (5.06 ± 0.17 pg / ml, p <0.001) compared to the initially established concentrations (5.81 ± 0.33 pg / ml) (Table 2).

Table 2. Changes in IL1 levels in In group and MM group



Significantly lower IL-6 concentrations in the In group were observed at 30 min (3.52 ± 0.50 pg / ml, p <0.01), followed by an upward trend (3.68 ± 0.73 pg / ml, p <0.05) and at 24 h were significantly higher (5.21 ± 1.39 pg / ml, p <0.05) from the original levels (3.92 ± 0.92 pg / ml).

IL-6 is unreliably increased in the MM group at 30 min, but the values found in subsequent periods are significantly lower - 60 min (3.39 ± 0.16 pg / ml, p <0.05), 120 min (3.17 ± 0.09 pg / p <0.01), 24 h (3.24 ± 0.43 pg / ml, p <0.01) from the baseline levels (3.82 ± 0.47 pg / ml) (Table 3).

Table 3. Changes in IL6 levels in In group and MM group

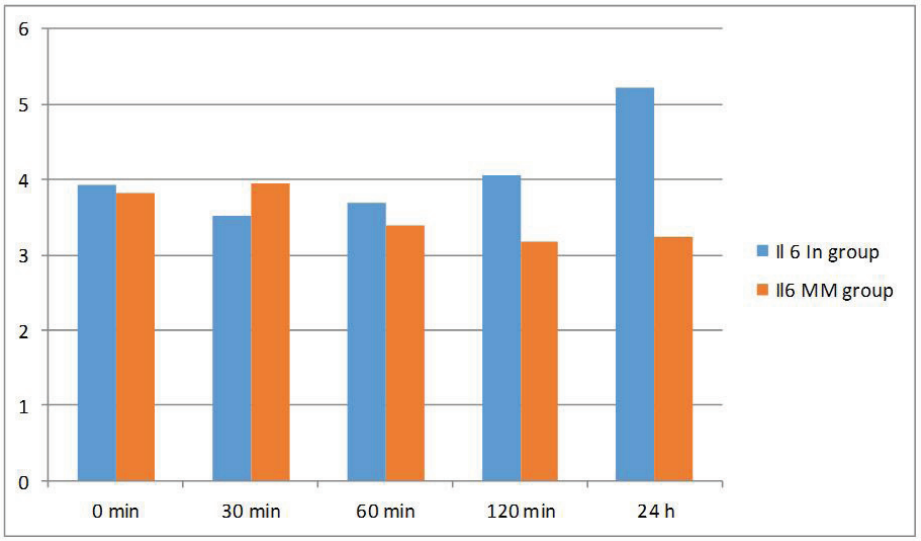
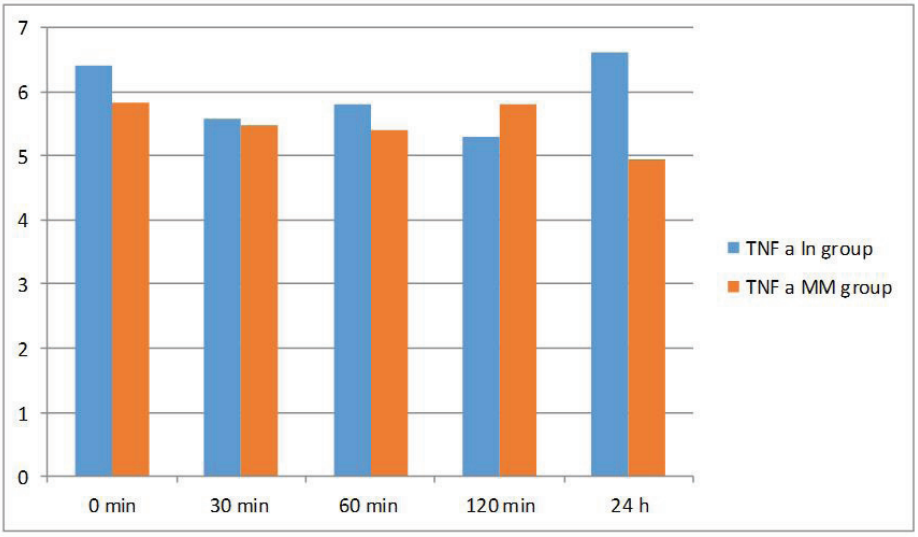


Table 4. Changes in TNFα levels in In group and MM group



An analysis of the data obtained for changes in TNF-α concentrations in group In revealed minor changes in the first three study periods, but at 24 h they were unreliably higher (6.62 ± 1.33 pg / ml) compared to baseline values (6.41 ± 1.45 pg / ml).

TNF-α concentrations in the MM group were also declined reliably to 60 minutes (5.41 ± 0.19 pg / ml, p <0.01), and the lowest reliable values compared to the baseline values (5.84 ± 0.31 pg / ml) were established to be at 24 h (4.94 ± 0.55 μg / ml, p <0.001) (Table 4).

The post-operative immune response is multifactorial and chronological. It is believed that the cytokine response changes in the first hour after anesthesia and surgery. The early pro-inflammatory response includes increased concentrations of pro-inflammatory Th1 cytokines (IL-2, IL-12, INF-γ). As a result of the surgical trauma and elevated levels of

cortisol and catecholamines in the acute phase, the release of anti-inflammatory Th2 cytokines (IL-4, IL-5, IL-6, IL-10 and IL-13) is stimulated together with suppression of cellular immunity (Ahmed W. et al., 2012). In our study, IL-6 and TNFα concentrations were reduced at the first hour in the inhalational anesthesia group, while in the MM group the two interleukins decreased reliably at 120 min. Suppression of IL-6 and TNFα in the immediate perioperative period indicates suppression of the pro-inflammatory immune response. At 24 h, the IL-6 and TNFα concentrations in MM group were significantly lower than the initial values.

Suppression of pro-inflammatory cytokines limits the possibility of infection, promotes the recovery of tissues and the organism. One of the reasons for the decrease in IL-6 and TNFα levels in our studies is probably due to the depression of adrenaline concentrations (Zlateva N. et al., 2014). The impact of multimodal anesthesia on stress-response is limited in the time of the operation and immediately after it. Increased levels of adrenaline and cortisol 24 hours after surgery are not able to suppress IL-6 and TNFα expression to such an extent. In multimodal anesthesia, interleukin concentrations are also dependent on the administered meloxicam. Blocking the synthesis of prostaglandins by influencing cyclooxygenase activity leads to a decrease in cAMP responsible for the regulation of IL-6 and IL-10. (Mahdy A. et al., 2002). On the other hand, the studies show the direct modulating action of isoflurane on cytokine secretion. According to Xu Wu et al. (2012), isoflurane anesthesia is directly responsible for the increase in IL-6, IL-1 and TNFα in rats. Stimulation of IL-6 production in isoflurane and sevoflurane anesthesia has also been proved in humans (Zhang L. et al., 2013). These results are supported by Schneemilch C. et al. (2001), which found sevoflurane anesthesia to increase IL-6 and TNF-α levels as compared to TIVA anesthesia with propofol and remifentanil.

The lack of significant change in IL-1 levels in both groups is indicative of the minor activation of the cytokine response in the direction of postoperative inflammation. High concentrations of IL-1, as a result of activation of the immune response, cause fever and mediate the response of the host to infection and inflammation. In stimulation of IL-1 production, decreased appetite, low motor activity and behavior associated with a disease state is observed (Kent S, et al., 1992), which we did not observe in the postoperative period. IL-1 concentrations at 24 h were slightly changed in the inhalational anesthesia group and were reliably reduced in the MM group.

Relatively new studies have shown that significant increase of interleukin concentrations in the post-operative period and especially of IL-1, IL-6 and TNFα, may be the cause of their crossing through the blood-brain barrier (Sanders R. and M. Maze, 2010). In response to these cytokines, microglial cells release additional cytokines that lead to inflammation of the CNS (Wan Y. et al., 2007). Pro-inflammatory cytokines play a key role in regulating immune responses in both cellular and humoral immune responses. Through their influence on T-helper lymphocytes and antigen-presenting cells, including B-lymphocytes, they induce secondary cytokine secretion and, in cooperation with them, promote the activation and proliferation of B-lymphocytes and the production of different classes of immunoglobulins.

Our study of certain factors of the immune response shows that different anesthetic agents have indirect and direct effects on the immune system. On the one hand, anesthetics alter the hormonal balance in the body that affects immune functions and, on the other hand, they directly influence the factors of non-specific and adaptive immunity. Anesthesia can both suppress and activate the immune system, which is essential for survival of patients after surgery.

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THE USE OF ENDOSCOPY IN DIAGNOSIS AND TREATMENT OF A HORSE WITH RHINOESTRUS SPP.

Cătălin MICȘA, Andrei TĂNASE, Alexandra PETEOACĂ, Andreea ISTRATE, Kassem HOUSHAIMY, Alexandru SONEA

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăşti Blvd, District 1, Bucharest, România

Corresponding author email: mcscatalin@yahoo.com

Abstract

Background: Larvae of Rhinoestruspurpureus (Diptera: Oestridae) are known to cause nasal myiasis in domestic and wild animals such as equines, giraffes and rhinos, and can also affect humans.
Case description: This paper report the case of a 4-year-old Thoroughbred breed horse that was admitted in the veterinary hospital of the Faculty of Veterinary Medicine in Perugia, Italy. The horse was presented with inflammation of the nasal cavities causing dyspnoea, sneezing, coughing and typical nasal discharge.
Methods: A flexible endoscope was used to check the nasal cavities of the horse and the presence of parasitic larvae was determined inside. After the parasitological examination, the larvae found was identified as Rhinoestruspurpureus.
Results: Clinical signs were resolved within 6 days after 3 intranasally endoscopic administrations of Clotrimazol, increasing the outcome due to local administration of the substance of choice.
Conclusion: This case report reveals the fact that flexible endoscope can be used for a better diagnosis and treatment.

Keywords: *Endoscopy, equines, Rhinoestruspurpureus*

INTRODUCTION

Larvae causing obligatory myiasis are numerous and they may affect cutaneous and subcutaneous tissues, wounds, nasopharyngeal cavities (nasal bots), internal organs and the digestive tract (bots) of domestic and wild animals and humans as well. Nasal bots belong to the Family Oestridae, Subfamily Oestrinae, which includes several important genera: Oestrus and Rhinoestrus infecting horses, Cephalopina infecting camels (Akeurin 1945). Nasal bots are widespread in Mediterranean and tropical areas and in affected animals they induce sneezing and nasal discharge which may become caked with dust making breathing very difficult. The mentioned species of larvae are host-specific but sometimes they may be deposited in human eyes inducing a painful opthalmomyiasis of short duration.

Larvae of the flies belonging to the genus Rhinoestrus (Diptera: Oestridae) cause nasal myiases of domestic and wild animals such as equids, giraffes and rhinos (Colwell et. al 2006). The myiasis caused by larvae of Rhinoestruspurpureus and Rhinoestrususbekistanicus (Diptera, Oestridae) are of importance in the horse medicine since it causes severe respiratory diseases (Di Marco et al.2001). Rhinoestruspurpureus and R. usbekistanicus cause inflammation of the nasal cavities, sinuses and pharynx, thus inducing sneezing, coughing and dyspnoea. Damage to the olfactory nerves, encephalomyelitis due to the penetration of the ethmoid bone and of the soft cerebral membrane and lesions of the upper respiratory tract and lungs may also occur (Clayton et al.2005).

Even though myiasis caused by Rhinoestrus larvaeare thought to be confined to Asiatic and Africancountries, recently it has been reported in Europe, specifically in southern Italy. The use of the endoscope in the diagnosis of equine rhinoestrosisin live animals is very useful, since, when present, larvaeare very difficult to retrieve in the pharynx without an endoscope. Serological methods for the diagnosis of equine rhinoestrosis are not available and therefore it can be achieved only by the postmortem examination (Otranto et al.2003)

This paper describes concisely the use of the flexibile endoscope for a diagnosis of nasal myiasis by *Rhinoestrus*spp. in a horse and a better treatment due to local administration of the substance.

MATERIALS AND METHODS

This paper report the case of a 4-year-old Thoroughbred breed horse that was admitted in the veterinary hospital of the Faculty of Veterinary Medicine in Perugia, Italy, with a history of anorexia and lethargy for about 2 weeks. The horse was presented with inflammation of the nasal cavities causing dyspnoea, sneezing, coughing and typical nasal discharge. The horse was in poor condition and often lethargic with weight lost. It had poor appetite, abdominal pain and suffered from lack of proper nourishment. The horse had clumps of eggs on legs, belly and mouth and the horse was seen often licking those areas. The horse was seen rubbing his face of nearby objects and biting objects to relieve irritation in mouth which had ulcers. It also presented spontaneous snorting, puffing and coughing.

Heart and respiratory rate were within normal limits. Auscultation of the heart and abdomen revealed no clinical abnormalities. Wheezes were auscultated in both sides of the lung. The mucous membranes were pink and capillary refill time was 2 sec. The mandibular lymph nodes were enlarged, moveable and lobed, but not painful. Palpation of the parotid area and larynx was unremarkable. The coughing reflex occurred spontaneously and on provocation. Neurological examination of the cranial nerves, revealed no abnormalities but the inspection and palpation of the oral cavity revealed severe irritation in gums and puss pockets.

An upper airway and guttural pouch endoscopy was performed subsequent to the physical examination. We used a flexible endoscope of 300 cm length and 10.3 mm diameter. The horse was sedated with Xylazine 1.1 mg/kg bw IV in the jugular vein.

The upper airway endoscopy revealed the following findings. The larynx was symmetrical and the epiglottis seemed to be normal. Endoscopy of the guttural pouches revealed a large number of parasitic eggs. We took a sample for parasitological examination using a Aligator biopsy forceps. We performed guttural pouch irrigation with 2% clotrimazole emulsion using a urinary catheter of 8 FG and 2.6 mm diameter. Before entering the guttural pouch, nasal passages were flushed with warm saline solution 0.9% NaCl. Subsequently the clotrimazole emulsion was instilled into the other guttural pouch and the catheter was removed.

RESULTS AND DISCUSSIONS

The results of the probe we took from the guttural pouches and gave for a parasitological examination came positive for Rhinoestrus spp. (fig. 1).

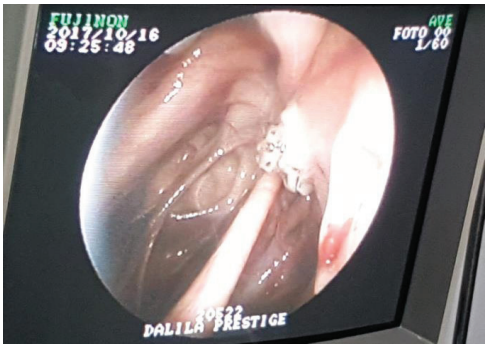

Figure 1. Evidence of larvae under endoscopic image

Therefore, we performed guttural pouch irrigations every 2 days with a 2% clotrimazole emulsion over a time period of 6 days. Under this medication, the horse's appetite improved and body condition stabilized. The severity of the dysphagia, nasal discharge, coughing, and snorting remained variable.

After 6 days and 3 intranasally endoscopic administrations of 2% clotrimazol the clinical signs were resolved and there weren't any eggs present at the endoscopic check-up of the horse's nostrals. (fig. 2).

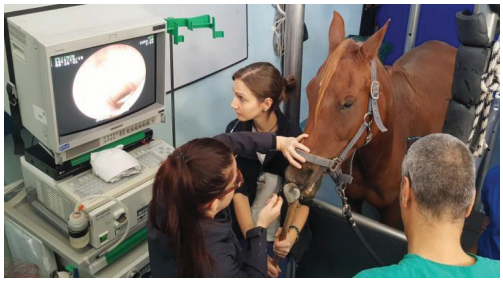

Figure 2. Endoscopic check-up after administration of 2% clotrimazole emulsion

This case report presents an uncomplicated diagnosis of a rather minimal guttural pouch mycosis with a large impact on the quality of life of a 4-year-old horse.

Guttural pouch myiasis is a rare, globally widespread, fungal disease with no predispositions to age, gender, breed, or regional origin. However, it does appear more often in stabled than in pastured horses, especially during the warm months of the year. Bad general condition, immunodeficiency, inflammation, or defects of the mucosal barrier can be initiating factors.

Complications of acute respiratory distress and aspiration pneumonia were conceivable. An indication for a thoracic radiograph was given, but due to money restrictions we refrained from this. The evaluation of a thoracic radiograph would have emphasized the clinical findings, but would not have influenced the therapy and prognosis effectively. The treatment of guttural pouch myiasis can be attempted by using topical and/or systemic antifungal medication. Various antimycotics, such as nystatin ketoconazole, miconazole, natamycin, enilconazole, clotrimazole in different pharmaceutical forms (powder, solutions), and thiobendazole, or irritant reducing solutions, such as povidone-iodine or 6% neomycin mixed with 1% gentian violet, can be used (Ragle 2003) (fig. 3).

Figure 3. Endoscopic administration of 2% clotrimazole emulsion

Unfortunately, a standard approach to the treatment of guttural pouch myiasis does not exist (Greet 1987). In this case, we choose a topical therapy with a 2% clotrimazole emulsion, which led to an excellent recovery of the mucosa lying with no signs of inflammation. The instillation of this emulsion had two advantages. Firstly Canesten® Gyn^{d} is specifically made for mucosa-associated fungal infections and secondly due to its consistency it sticks to the wall of the guttural pouch leading to a more effective, long-lasting therapy. A very likely explanation is that clotrimazole is a broad-spectrum, topical, nonsystemic, antifungal drug.

CONCLUSIONS

The clinical symptom of dyspnoea, sneezing, coughing and nasal discharge always requires a close investigation of the upper respiratory tract and both guttural pouches. Management is difficult and a standard approach to treatment of guttural pouch infestation with larvae causing myiasis does not exist. The use of flexible endoscope can be used for a better diagnosis and treatment, increasing the outcome due to local administration of the substance of choice.The use of a three-day depot clotrimazole emulsion requires further evaluation.

Using equine insect repellant and fly sheets on your horse during the summer may help to reduce the level of bot infestation - but it will be impossible to stop all flies.

Regular removal and disposal of droppings from the horse's pasture will help to prevent some of the larvae burrowing down into the soil and hatching into bot flies.

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STUDY OF MORPHOLOGICAL CHANGES IN SHEEP FLUKE

Adrian OLARIU-JURCA[1], Valentin CIULAN[1], Atila GÁL[1], Marius Stelian ILIE[1], Tijana SUICI[1], Liliana Elena TESLICI [1]

[1] Banat's University of Agricultural Sciences and Veterinary Medicine „King Michael I of Romania" from Timisoara, 119 Calea Aradului, 300645
Timişoara, Romania

Corresponding author email: olariujurca_adrian@yahoo.com

Abstract

The aim of this study is to highlight the macroscopic and especially the microscopic tissular and organic lesions of the sheep bodies in order to establish the histopathological diagnosis of fasciolosis. The study was conducted in the period February 2015-April 2017. Nineteen sheep bodies were necropsied. The sheep were aged from 7 months to 3 years and were from the breeds Turcana and Tigaie. Seven of the cases presented hepatic lesions at the necropsy exam expressed through diffuse hepatitis and interstitial fibrous hepatitis (pseudo-cirrhosis). At the necropsy exam, along with the hepatic lesions we highlighted the presence of Fasciola Hepatica in a very high number. The hepatic modification caused by Fasciola hepatica in the sheep taken into study are complex both in morphopathological shape and in extent being expressed through circulatory modifications, hypertrophy, dystrophy and lymphohistiocytic , eosinophilic and especially fibrous inflammation. The wide range of morphological modifications of the liver parenchyma, which appear in the pathological process end in liver fibrosis and pseudocirrhosis in sheep with repeated infestations. They always end up in death even though we apply repeated treatments with fasciolides. This is the reason why this disease is considered one of the most serious parasitic diseases for this animal species.

Key words*: tissular injury, necropsy, Fasciola hepatica.*

INTRODUCTION

Sheep fasciolosis is one of the most important parasitic diseases signalled in this species. It is a hepatobiliary helmintosis caused by the trematode *Fasciola hepatica.* The economic losses are translated through high morbidity and high mortality, necessity slaughter and disorders of the reproduction function expressed through irregularities of the estrus cycle and abortions. In our country the disease is caused by *Fasciola hepatica,* a foliaceous trematode, 2-3 cm in length and 0.8-1.3 cm wide. Infections with *Fasciola gigantica* and *Fasciola magna* were reported in other countries. *F.hepatica* adults can be seen free or fixed on the walls of the biliary ducts with the help of their oral sucker. The lungs, spleen, subcutaneous conjunctive, serous or intermuscular tissue are considered erratic locations. In sheep, reinfestation or superinfestations are common and this fact leads to modifications in the liver with the aspect of a mosaic. Traumatic-haemorrhagic hepatitis may be overlapped on cirrhosis or angiocholitis. There may be cases when one can see both young parasites and adults in the same liver (Cosoroabă et al., 1995; Dărăbuş et al., 2006; Paul, 1990).

The liver is the central laboratory of the organism. It is the headquarters of most fundamental pathological processes- almost every type of dystrophy, circulation disorder or inflammation is observed in this organ (Jubb et al., 1993; McGavin et al., 2001; Paul, 1990). Hepatic lesions in sheep fluke have consequences in the entire organism, ending up in lowered milk, meat and wool production but also through mortality.

MATERIALS AND METHODS

The research was based on the necropsy exams of 19 sheep bodies during the period February 2015-April 2017. The animals were aged from 7 months to 3 years and belonged to the Turcana and Tigaie breeds. Seven of the cases presented hepatic lesions expressed through diffuse and interstitial fibrous hepatitis exteriorized through the presence of white ditches on granular, nodular and lobar surface of the hepatic parenchyma. A creak is also present at sectioning. A large number of *Fasciola* parasites can be seen on seen on the

section surface. Samples of 2.5/1.5 cm fragments of lesioned liver were collected from these bodies. The samples were fixed in formaldehyde solution 10% where they were kept for 24 hours. Modelling followed and other formalin baths in the next two days for a definite fixation. The pieces were then prepared for the paraffin method. In order to accomplish the stages of this method we used the following materials and stains: alcohol 50%, 80% and 96%, absolute alcohol, amylic alcohol, paraffin, thermostat, microtome, slides, medicinal alcohol, egg white and gelatine, benzene, dropper, water, absorbent paper, Canada balm, stand. Blocks with samples (fragments) of organs with lesions were obtained through paraffining. Subsequently, the obtained blocks were cut using a microtome at 6 micrometres, after which 2-4 sections from each block were put on slides. The obtained sections were fixed on clean slides with the help of Mayer albumin. The sections were stained using the trichrome Masson method modified by V. Ciurea with methylene blue for general information and with Giemsa stain for cellular details (Olariu-Jurca, et al., 2015).

The histopathological preparations were examined using an Olympus CX41 microscope (acquired through POS CCE, DICES-MVT 2669-145), with increasing objectives. They were then interpreted and microphotographed.

RESULTS AND DISCUSSIONS

From 19 bodies necropsied in the period February 2015-April 2017, seven cases presented hepatic lesions and *Fasciola hepatica* was observed in a high number. This fact lead us to conduct histological tests in order to confirm the morphopathological diagnosis. The simple presence of *F.hepatica* does not confirm the existence of the disease, which has fatal effects on the patient.

According to the exterior exam of the bodies, two of them were in good condition and five of them were in bad condition, cachectic, yellow apparent mucosae, dry skin, dull and friable wool. Pitting edema was identified in the conjunctive tissue from the cervical and lower abdominal region (Figure 1).

After the interior examination of the abdominal cavity, the cases in good condition showed a considerable quantity (approximately 0.5 litres) of red liquid with blood clots, serohemorrhagic exudate (serohemorrhagic peritonitis). The five cases in bad condition presented 1.2 litres of citrus coloured liquid, transudate (ascites). Lower quantities of serofibrinous exudate were also present in the thoracic cavity and pericardium.

The macroscopic exam revealed physical and structural modifications in the liver (size, colour, aspect, consistency, etc.). The capsule has an irregular surface, cut by ditches, with white stripes and white nodular growths-atrophic cirrhosis (Figure 2). The lesions were also noticed in other parenchymal organs, aspects secondary to the instalment of fasciolosis.

The liver in two of the cases, was macroscopically enlarged, brown-yellowish colour, the Glisson capsule was thickened, uneven because of the ditches and nodular growths. The same colour and a relevant quantity of young fasciola is present on the section surface (Figure 3). The aspect of the lesions leads to the presumptive diagnosis of interstitial fibrous hepatitis, improperly called cirrhosis. Microscopically, we noticed the most characteristic aspects like thickened hepatic capsule, hyperplasia of precollagen and collagen fibres from the hepatic parenchyma, from the capsule and vascular walls, fibrous perihepatitis and perivasculitis (Figure 4). The instalment of this inflammation is the consequence of the liver's response to the action of the young fasciola that perforate or are implanted in the thickness of the hepatic capsule.

In some microscopic fields in the hepatic parenchyma, we found fibrosed biliary tubules and *F. hepatica* in the lumen and haemorrhagic foci - effect of the destruction of hepatic cords and sinus capillaries through the action of the parasites during migration through the hepatic parenchyma (Figure 5). The haemorrhagic and/or haemorrhagic-necrotic foci in some areas of the liver parenchyma alternate with lympho-histioplasmocytic and eosinophilic infiltrations. These are the expression of the host's first reactions to the destructive, irritating and toxic action of the parasites during migration through the liver. The histologic modifications define, in a

morphopathological plan, the superacute and acute phase of the disease expressed through haemorrhagic and/or haemorrhagic-necrotic hepatitis (Figure 6, Figure 7).

In some hepatic lobes, we could see perivascular and peritubular fibrosis and the presence of the parasite, F. hepatica. In the lumen of the biliary tubule, there was perivasculitis and fibrous angiocholitis (Figure 8). In most of the microscopic fields, the hepatocytes presented granulations and optically empty vacuoles in the cytoplasm with or without effects in the nucleus. The biliary tubules had hyperplasia, fibrosis and catarrhal exudate in the lumen while in the portal-biliary space there was perivascular fibrosis, granular hepatosis, angiocholitis and perivascular fibrosis.

In three cases, macroscopically, the liver was increased in volume, of yellow-clay colour, high consistency - fact which is perceived as a squeaking sound at sectioning, which is a consequence of fibrosis and of fibrous inflammation. Thickened biliary tubules were also noticed on section, fibrosis and the presence of a very small number of parasites. These structural modifications suggest the instalment of diffuse, fibrous, parenchymal hepatitis, improperly called cirrhosis.

Microscopically, using objective in increasing order - x10, x20, x40 - we noticed hyperplasia of the reticulin and collagen fibres around the hepatocytes throughout the entire hepatic parenchyma. There was an enhanced development of the conjunctive fibres in the Disse gaps, overlapping of hepatocytes, accompanied by atrophy caused by compression, dystrophy, necrobiosis and hepatic necrosis. These histopathological aspects certify the diagnosis of diffuse fibrous hepatitis and hypertrophic cirrhosis (Figure 8, Figure 9).

We could also notice peri hepatocytic fibroconjunctive hyperplasia in the largest part of the parenchyma, steatosis (small optically empty vacuoles) and hypertrophy of biliary ducts accompanied by pericanalicular fibrosis - hypertrophic cirrhosis (Figure 10, Figure 11, Figure 12).

Some researchers consider that hepatic fibrosis caused by Fasciola, as well as hyperplasia of the biliary ducts are the effect of the parasite's proline secretion (Jubb et al., 1993; McGavin, et al., 2001; Paul, 1990). Without denying this possibility, most researchers consider however that the hyperplasia phenomena are the expression of numerous factors induced by parasites including mechanic irritations and their antigens (Cosoroabă et al.,1995; Dărăbuş et al., 2006; Dulceanu et al., 1994; Şuteu et al., 2007).

In two cases, the liver was macroscopically decreased in volume, with an irregular surface of yellow-greenish colour of different shades and high consistency. There was a squeaky sound on section-atrophic cirrhosis. Microscopically, we noticed prominent intralobular fibrosis, which divided the lobes into pseudo-lobes, groups of cords without centrlobular veins or with veins in exocentric location (Figure 13, Figure 14). In contact with the hepatic cords, especially around the portal gap we noticed leukocytic infiltrations, predominantly eosinophilic, on a fibrosis background. We also noticed new blood vessels and biliary tubules, aspects, which suggest the instalment of the active phase of the inflammatory process-aggressive cirrhosis (Figure 15).

The microscopic and macroscopic aspects highlighted with the help of colour photographs, which closely show the morphogenesis of the patomorphic aspects in the liver, in different evolution phases in sheep fluke with reflection in the entire organism. The hepatic lesions identified along the research correspond to those described in the specialty literature (Paul, 1990; Cosoroabă et al.,1995; Dărăbuş et al., 2006; Dulceanu et al., 1994; Şuteu et al., 2007).

Figure 1. Sheep corpses with fasciolosis.

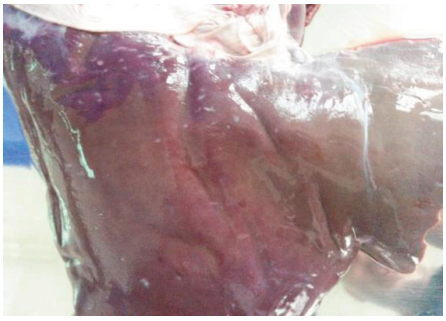

Figure 2. Sheep liver - atrophic, granular and lobar cirrhosis.

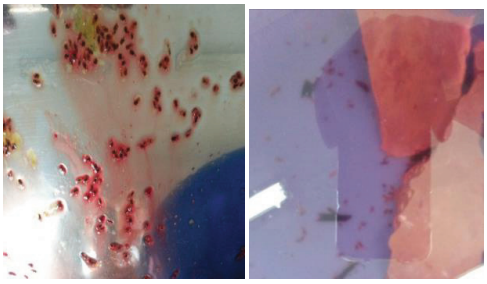

Figure 3. Sheep liver - highlighting on the section surface an appreciable amount of young fasciola.

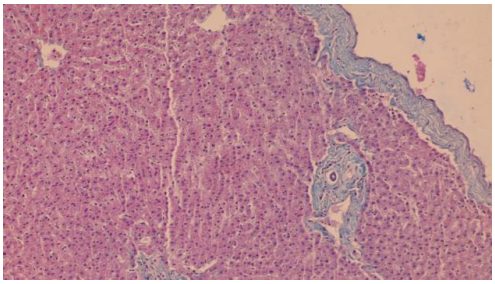

Figure 4. Fibrous perihepatitis and perivasculitis: hyperplasia of precollagen and collagen fibres from the capsule structure and vascular walls. Col. HEA x 40.

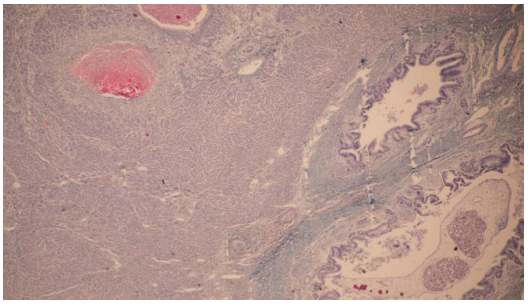

Figure 5. Hemorrhagic hepatitis in outbreaks, hemorrhagic areas, perifocal inflammatory cell infiltration and catarrhal angiocholitis with the presence of *F. hepatica* in the lumen. Col. HEA x20.

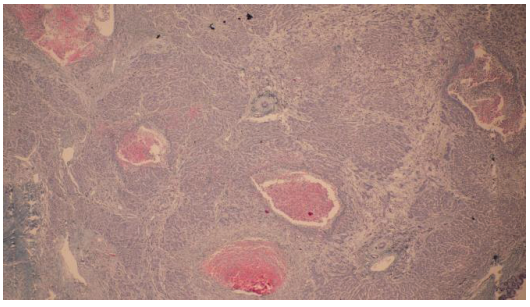

Figure 6. Hemorrhagic hepatitis in outbreaks: hemorrhagic areas and inflammatory cell infiltration.Col. HEA x 10.

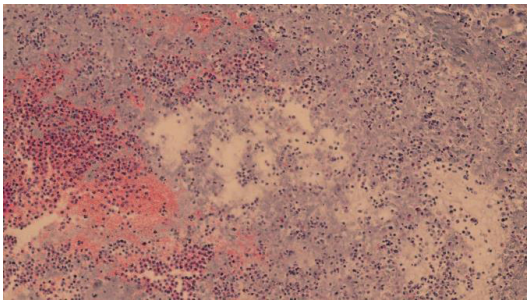

Figure 7. Hemorrhagic hepatitis in outbreaks: highlighting inflammatory cellular infiltrations: lymphocytes, histiocytes and numerous eosinophils. Col. HEA x 40.

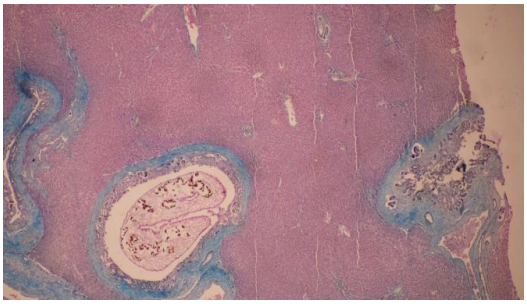

Figure 8. Perivasculitis, fibrous angiocolites and *F. hepatica* in the lumen of bile canaliculi. Col. HEA x 20.

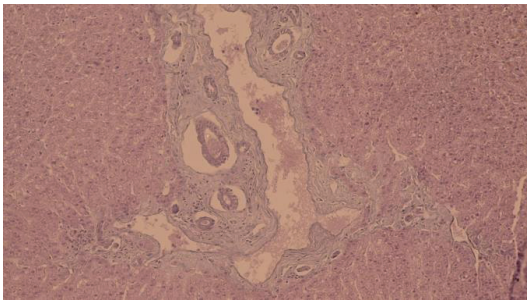

Figure 9. Granular hepatosis, angiocolitis and perivascular fibrosis. Col. HEA x10.

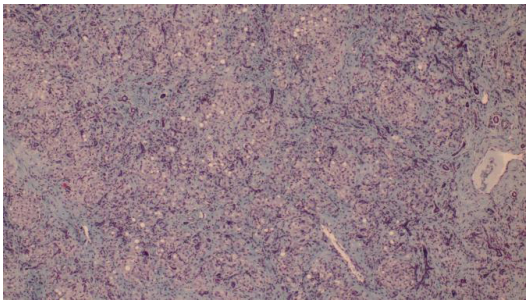

Figure 10. Hypertrophic cirrhosis: intraparenchymal fibrosis (overview). Col. HEA x10.

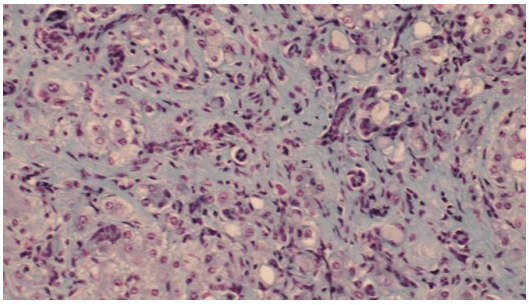

Figure 11. Hypertrophic cirrhosis: diffuse peri hepatocytic fibrosis (detail). Col. HEA x 20.

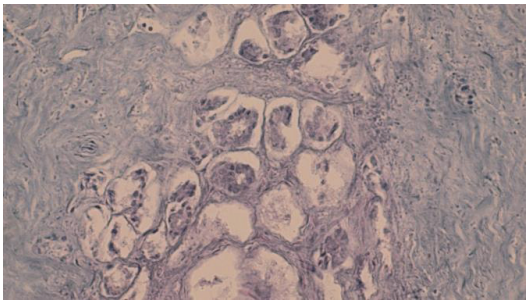

Figure 12. Hypertrophic cirrhosis: hyperplasia of the bile ducts and pericanalicular fibrosis (detail). Col. HEA x 40

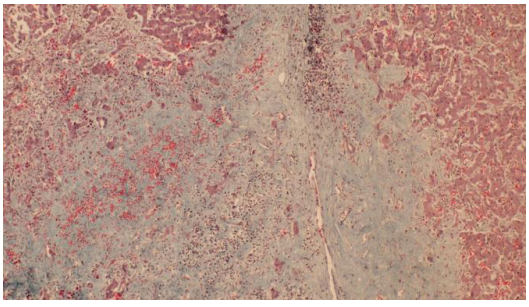

Figure 13. Atrophic cirrhosis: Perilobular massive fibrosis and leukocytic infiltration predominantly eosinophilic. Col. HEA x 20.

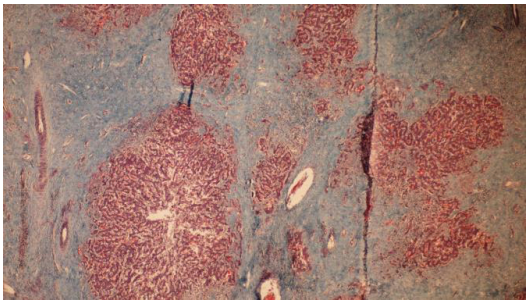

Figure 14. Atrophic cirrhosis: dividing lobules in pseudo-lobes thru fibrosis. Col. HEA x 20.

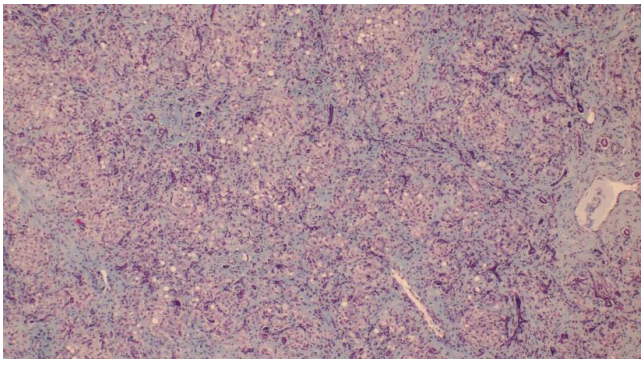

Figure 15. Aggressive cirrhosis: lympho--histo-plasmocytic infiltrations on the background of steatosis and fibrosis. Col. HEA x 10.

CONCLUSIONS

The hepatic modifications produced by Fasciola hepatica, in the sheep taken into study are complex both in form and in extent, expressed through circulatory modifications, hypertrophy, dystrophy, lymphohistiocytic inflammation and fibrous inflammation (cirrhosis).

Fibrous hepatitis in foci and diffuse-improperly called cirrhosis is the consequence of the mechanical, irritative and toxic actions of the parasite, continuous and of different intensities, produced by F.hepatica in case of periodic reinfestation.

The large range of morphological modifications in the hepatic parenchyma, which appear during the pathological process are finalised through hepatic fibrosis, pseudo-cirrhosis in repeatedly reinfested sheep that always end up with death, in spite of anti-fasciola treatments.

In the same case, in the hepatic parenchyma we noticed circulatory modifications, metabolic modifications-hypertrophy, dystrophy and fibrosis of different intensities due to periodic reinfestations which allow a succession of the evolution forms of the disease (superacute, acute, subacute and chronic) expressed in dynamics through haemorrhagic and/or necrotic-haemorrhagic hepatitis, atrophic cirrhosis and hypertrophic cirrhosis.

The identification in a singular case of a super-acute, manifestation form of fasciolosis - haemorrhagic and/or haemorrhagic-necrotic hepatitis, or of subacute or chronic-fibrous hepatitis or pseudocirrhosis is arbitrary, possible only in experimental fasciolosis where reinfestation and a diverse biocenosis for intermediary hosts are absent.

The diverse patmorphic syndrome installed in sheep fasciolosis ends in most cases with death, fact which makes this disease one of the worst parasitic diseases for this species.

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RISK FACTORS IN FELINE HYPERTHYROIDISM

Ruxandra-Anamaria OPREANU, Mario CODREANU

Faculty of Veterinary Medicine Bucharest, Splaiul Independentei 105
Sector 5 Bucharest - Romania

Corresponding author email: dr.vet.ruxandra.anamaria@gmail.com

Abstract

The aim of this article is to synthesize and systematize the risk factors in the appearance of feline hyperthyroidism presented in the literature. It is based on data and statistics extracted from numerous articles made all around the world. Data were processed and systematized based on the following factors: age, race, gender, robe color and fur length, diet, living environment, litter usage, and interaction with various chemicals. Thus, it is noteworthy that hyperthyroidism is more common in senior cats, living indoor, consuming predominantly wet food, and having contact with PBDE and PCB type substances, as well as the fact that cats from a "color point" breed are less likely to develop hyperthyroidism. Many factors have been identified that may favor the disease, but no determinant.

Key words*: hyperthyroidism, cat, risk factors, etiology.*

INTRODUCTION

Hyperthyroidism affects up to 10% of geriatric cats and is associated with increased mortality and deleterious effects on several organ systems [(1)]. Hyperthyroidism was first reported in 1979 [(2)] and is recognized today as the most common endocrinopathy in cats. Although the pathologic changes associated with hyperthyroidism (adenomatous hyperplasia, adenoma of the thyroid gland) have been well characterized the pathogenesis of these changes remains unclear. It has been postulated that immunologic, infectious, nutritional (eg, iodine), environmental (eg, toxins or goitrogens), or genetic factors may interact to cause pathologic changes. However, a substantial difficulty with any of these factors is how they could cause disease to develop in cats on different continents within a relatively short period of time. It also has been suggested that the disease may not be new, but instead is simply being diagnosed more frequently as cats live longer and as owners seek more geriatric care. [(7)]

MATERIALS AND METHODS

In order to synthesize and systematize the risk factors in the appearance of feline hyperthyroidism presented in the literature numerous researches on the risk factors and the trigger factors of feline hyperthyroidism were studied. Until now, a number of risk factors have been studied and they are systematized below. Thus, the following aspects were investigated: age, gender, breed, robe color and fur length, diet, living environment, use of litter, interaction with various chemicals.

RESULTS AND DISCUSSIONS

From an age point of view, the disease affects especially cats aged between 4 and 16 years of age predominates cats with an average age of 10 years [(5)]. The prevalence of feline hyperthyroidism observed in some studies in USA has been reported to reach 10% of all cats older than 10 years, which is equivalent to a human age of over 60 years, and the prevalence appears to increase ever further with advancing age. Similar prevalence rates have been reported in other parts of the world over the last decade, ranging from 7.4% in London to 8.9% in Japan, 11.4% in Germany, and 20.1% in Warsaw [(3)].

So far no studies have demonstrated a clear involvement of the gender in feline hyperthyroidism development. [(6)]

Regarding breed, robe color and fur length, epidemiological studies have identified Siamese, Himalayan, and Burmese breeds to be at decreased risk of developing hyperthyroidism. The characteristic colorpoint coats of

these breeds result from temperature-sensitive mutations in the tyrosinase gene which limit conversion of the amino acid tyrosine to melanin pigment except at the cooler extremities. In addition to functioning as a precursor of melanin, tyrosine is an essential precursor of thyroid hormone. It has been hypothesized that the protective effect observed in colorpoint breeds may be related to the mutation in tyrosinase that leads to relatively greater tyrosine availability for thyroid hormone production. The authors commented that the study was underpowered. Further studies, to either support or refute their hypothesis, are lacking.

Overall, the results of studies do not provide consistent evidence in support of the proposed hypothesis for an association between coat color and hyperthyroid status. However, the studies were based on the assumptions that coat color is reflective of melanin concentration and that degree of pigmentation affects relative tyrosine availability for thyroid hormone production and was subject to certain limitations. Then genetics of coat color are complex, and cats could have been misclassified because of the use of owner-reported or receptionist-recorded information. Differences in the terminology used for coat colors by breeders and geneticists and lay terminology might also have compounded this effect. Studies results indicate that certain breeds have decreased risk of hyperthyroidism and that longhaired cats are at increased risk of hyperthyroidism. Further research is necessary to determine whether pigmentation plays a role in this breed protective effect or whether this association is as a result of alternative mechanisms. (1)

The diet based on wet food was associated with increased risk of hyperthyroidism. Dietary factors that represent a risk factor in this pathology are: high feed content in iodine, goitrogenic factors, soybeans, polyphenols and resorcinol. Compared to cats that did not eat canned food, cats that ate any canned food had an approximately 2-fold increase in disease risk. Although no obvious dose–response relationship existed, cats with diets reported by the owners as being 50–74% canned and 75–100% canned food had significantly increased risk. Little evidence was found of any relationship between dry or semimoist food and disease risk. Most cats almost exclusively ate commercial cat food. Of cats receiving 80% or more of their diet in the form of commercial cat food, the risk of hyperthyroidism decreased as the proportion of commercial cat food in the diet increased. (7)

Table 1. Coat color, hair length as risk factors for hyperthyroidism (1)

Risk Factor	Category	Euthyroid n (%)	Hyperthyroid n (%)
Color/pattern	Black (reference category)	567 (76)	176 (24)
	Brown and white	12 (100)	0 (0)
	Cream	10 (91)	1 (9)
	Colorpoint	27 (87)	4 (13)
	White	74 (82)	16 (18)
	Red	203 (78)	58 (22)
	Blue	59 (78)	17 (22)
	Blue and white	48 (77)	14 (23)
	Black and white	674 (76)	208 (24)
	Tabby	550 (75)	188 (25)
	Tabby and white	148 (74)	51 (26)
	Red and white	109 (74)	38 (26)
	Tortoiseshell	222 (73)	83 (27)
	Brown	18 (67)	9 (33)
	Tortoiseshell and white	48 (67)	24 (33)
White markings	No white (reference category)	1,656 (75)	539 (25)
	Some white	1,039 (76)	332 (24)
	All white	74 (82)	16 (18)
Dilute	Not dilute (reference category)	1,583 (76)	489 (24)
	Dilute	117 (79)	32 (21)
	Unknown	1,069 (74)	366 (26)
Base pigment	Black (reference category)	1,348 (76)	415 (24)
	Brown	30 (77)	9 (23)
	Red	322 (77)	97 (23)
	Unknown (tabby)	698 (74)	239 (26)
	Unknown (tortoiseshell)	**270 (72)**	**107 (28)**
	Unknown (colorpoint)	27 (87)	4 (13)
	Unknown (white)	74 (82)	16 (18)
Hair length	Short hair (reference category)	2,459 (76)	764 (23)
	Long hair	**310 (72)**	**123 (28)**

Many goitrogenic compounds can contribute to the development of adenomatous lesions in exposed cats. These may be of particular importance, because most are metabolised by glucuronidation, a metabolic pathway that is particularly slow in the cat. Most commercial cat foods contain relatively high levels of goitrogenic compounds (e.g. phthalates). (4)

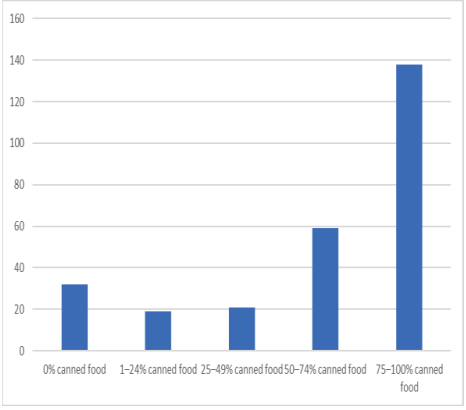

Figure 1. Relation between the percent of canned food and risk of hyperthyroidism in cats (7)

Even if some studies link the indoor living and the hyperthyroidism, in the absence of a clear explanation of the relation between litter use and hyperthyroidism, use of cat litter may simply be a marker for cats that primarily live indoors, receive better than average care, enjoy longer lives, and are more likely to reach the age at which cats develop the disease. [4]

Regarding the chemicals from the environment there where examined included those applied directly to cats to control ectoparasites, or to the environment as herbicides, insecticides, or fertilizers. Some flea-control products were associated with increased risk of developing hyperthyroidism. No obvious use–response relationship was discernable. For example, no difference in risk was found among low, moderate, and high use of flea collars. High use of flea spray and flea powder was associated with lowered risk whereas high use of flea shampoo was not associated with risk. Use of other flea products showed a monotonically increasing association with risk of hyperthyroidism, but these products were used in such a small number of cats that meaningful interpretation was not possible. Environmental use of insecticides, applied either by an exterminator or an owner, and environmental use of fertilizers and herbicides were not associated with increased risk of developing hyperthyroidism. [7]

Two classes of persistent organic pollutants, polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) are known to interfere with thyroid hormone signaling and regulation; thus, it is postulated that they contribute to the etiopathogenesis of feline hyperthyroidism and pose a risk to humans and other species. Studies demonstrate that elevated exposure to both PBDE and PCB congeners is associated with feline hyperthyroidism, supporting the hypothesis that these persistent organic pollutants may contribute to the etiopathogenesis of feline hyperthyroidism and suggests that they may have adverse impacts on thyroid health in humans and other animal species.[2]

CONCLUSIONS

Thyroid hormone function in the body is critical for proper execution of many developmental processes and the function of most organ systems throughout life across many species, thus there are many adverse implications of thyroid hormone disruption. [2]

Despite its increasing frequency, the origin and underlying pathogenesis of feline hyperthyroidism is not known, and therefore definitive recommendations in terms of prevention of the disease cannot as yet be made. [4]

Identification of a specific etiologic factor in feline hyperthyroidism will require randomized experimental trials. Such studies have limited feasibility because of the large number of cats necessary and the potential requirement for disease induction over many years. In the absence of such experiments, the need for observational studies continues. Future studies should determine detailed lifelong dietary history, micronutrient analysis of cat foods, and accurate measures of the frequency of pesticide applications on and around the cats studied. [7]

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ARTIFICIAL INSEMINATION PROGNOSIS IN CATTLE AT THE WORLD AND NATIONAL LEVEL

Marcel Theodor PARASCHIVESCU[1]

[1]Reasearch and Study Centre for Agriculture and Forestry Biodiversity, 13 September Street, no. 13, District 5, zip code 050711, Bucharest Romania, Phone: 021.318.81.06 int. 3542, Email: marceltheodor@yahoo.com

Corresponding author email: marceltheodor@yahoo.com

Abstract

There are 20 years since the title "Artificial Insemination dynamics and prognosis in cattle" was the presented thesis by me as a graduate student. A 20 years period is long enough to find out if the dynamics and the prognosis were true. That time Artificial Insemination was appreciated as a strong mean to prevent venereal diseases, and to increase bulls' fertility. It was also seen as a needed biotechnical in ET technology. From the scientifically point of view spermatozoa sexing was then preview the term of fertilization ability of sperms being fully satisfied. In breeding practice AI was seen as a very efficient mean to induce genetic progress using progeny tested sires and ET as short term way to transfer breeds of interest to new areas. It was considered AI mostly will be applied in dairy breed where all active populations will be reproduced by artificial insemination. In extensive systems of beef cattle farming AI will be few extended because of too much labor needed. For Romania AI has to be extended since the action continued to be sustained by the public finance that paid the deep frozen semen used by cow owners. They had to pay inoculation of semen only. Excepting the last prognosis all other of them fulfilled. But AI association to the other biotechnologies development has shown richer than it was then specified. In the present form progresses in biotechnologies AI assisted will be more discussed. Sperm sexing, MOET, IVF, Embryo sexing, artificial identical twins genesis, Mammal animal clone production and engineering of transgenic organisms are mentioned in relation with new organism genesis and their security food or economic importance.

Key words: *biotechnologies of reproduction, IVF, mammal animal cloning.*

INTRODUCTION

About 20 years ago I have presented "Dynamics and Prognosis concerning Artificial Insemination in Dairy Cattle" as the graduate degree in animal husbandry (Paraschivescu M. Th., 1997). The subject was interesting that time because there was a political decision of changing Artificial Insemination (AI) organization from a state one to the private ownership, based on adopted German Project paid from UE founds.

My report had to preview the dynamics of AI spreading in dairy cattle production units that were privatized, as well, and to refer about the biotechnical progress using AI in bioengineering.

From biotechnological point of view, AI had got already that time, the top of possibilities in increasing the sires' fertility, since the deep frozen semen preserves its fertilizing power for very, very, long terms and the number of spermatozoa per doses could be decreased to 20 million or less. Great success was the high selection in precision of sires by progeny testing them for milk production. Prevention of genital diseases in cows was satisfactory solved as well. Better conception rate and lower cost per calving were wanted. Practical extension of AI in dairy cattle was to be expected everywhere, up to the degree of becoming exclusive even in the commercial farms. It was a large international offer of semen apart from many countries.

The spermatozoa sexing remained a question to be solved. The target of new approaches in AI biotechnical progress had become using AI in assisting embriotransfer biotechnology both *in situ* and *in vitro* fertilization in order to answer the specialization tendency of cattle breeds. New trends in cattle breeding required disposing of cows able to biologically producing *in vivo,* in their milk, organic medicines.

LATER EVOLUTION OF AI

From the time of the mentioned report presentation had past 20 years. Having in view that I have been interested afterwards in some AI assisted reproduction biotechnologies, I thought 20 years is enough time to appreciate if prognosis was made right or wrong. Further discussions will refer to the AI biotechnics targets concerning direct effect on the dairy farm economy and on the peculiar adaptation as an adjuvant biotechnics for new approaches to using biological production of cattle.

BASIS TARGETS OF AI BIOTECHNICS

The main targets of research in AI biotechnology during this period of time have been increased conception rate and reduced costs of artificial insemination along with controlling sex of the progeny and assisting bioengineering technologies for GMO.

In order to increase the conception rate and reduce the AT service costs 3 models were followed: a) "do it yourself" insemination model addressed to the cows' owners,(Hafez B., Hafez E.S.E., 2000, Paraschivescu M.Th., 2000, Robertson E., 1999) b) dispatching the frozen semen to small territorial depots of the private veterinarian net (Paraschivescu M., 1982) or to permanent AI points financed by the AI Centers and (Paraschivescu M., 1982) c) dispatching semen every day through the itinerants AI operators employed by the AI centers.(Oţel V. et al., 1967)

a) The "do it yourself" model was promoted by the American cooperative AI Centers in order to have better approach for the optimum insemination moment and a diminished insemination cost. The possibility of "Direct insemination", that uses the straw with deep frozen semen preserved in ethilenglicol crioprotector, without open it was well received especially by farmers for "do it yourself" model of artificial insemination (Robertson E., 1999).

b) Dispatching of frozen semen to small territorial depots of the private veterinarians reduced the transport cost per dose of semen. The model was successfully implemented by Neustadt am Eisch Kunstlike Besamung Verein, greatly reducing semen dispatching cost and creating a stronger relation between the AI Center, Veterinarians and farmers. The system of AI points is preserved in Romania, but their acting rules are still confused after SEMTEST enterprises were privatized.

c) Everyday dispatching of semen was secured by many AI Centers since the model ensures good instruction of inseminators under semen producer control.

The preferred model is a question of local decision of AI Centers.

Reducing semen preservation cost by lyophilization of sperms lost interest. (Oţel V. et al., 1967)

Sperm sexing, what means separating the X chromosomes caring spermatozoa from Y chromosome caring spermatozoa, has been tried in many experiences. The experiments' goal was to obtain doses of semen certainly containing one kind of chromosomes X or Y.

In this respect 2 hypotheses were thought. The first one intended to separate the spermatozoa based on the fact that the X chromosome is the largest one and the Y chromosome is the smallest one in the gamete genome. Par consequence the caring X chromosome spermatozoa should weigh more than the y caring ones and might be separated by centrifugation.

Even there were some claims of success, the hypothesis didn't confirm. Or the centrifuges weren't sensible enough or, more probably, there are other components of sperms varying in weight and covering the difference in weight between the two mentioned chromosomes.

The second hypothesis, which looks more hopefully, is based on the complementary of genes in their pair formation in diploid genomes.

In the diploid cells genes make pairs with their copies or their mutants, only. So a small segment of Y chromosome, treated to become fluorescent, is multiplied by PCR technique and mix with the diluted semen. Thus the caring Y chromosome spermatozoa attach the fluorescent segment in pairs of genes and became fluorescent them self.

The obtained material is passed in a continuous flow through a special item able to separate the "male" spermatozoa caring Y chromosome. This is the so called FISH biotechnics of sperm sexing. (FISH makes the acronym from

"Fluorescent *In Situ* Hybridization"). (Paraschivescu M., Tibără Dana, 1989).
If FISH biotechnics of sperm sexing will get commercial application more extension seems to have using X caring sperms in commercial dairy farms avoiding the birth of male calves which are not wanted.
That will reduce the quantity of necessary milk for producing the new heifers for the farm and a higher inside herd selection intensity. Using of Y chromosome sperms is convenient for dams (mother of bulls) the only ones which are in small number in breeds.
But, par consequence, no knowledge about the genetic merit of the female sibs of the bulls will be received for the animal model BLUP. Of course there will be an interest to have only male progeny out of dams that are progeny tested.

IN SITU AI ASSISTED BIOTECHNOLOGIES

The great success of AI in increasing fertility of special progeny tested sires induced idea of increasing fertility of dams, as well. But things look differently. Meanwhile spermatogenesis and spermiogenesis are continuous processes whose product is delivered out of the organisms, oogenesis is a stopped process, within which the order I oocytes preserved in meiotic arrest in ovarian follicles of female embryos. From there they restart to maturate, one by one, periodically in pubertal female and are fertilized inside her genital tract.
More than that, the cow or heifer uterus ordinarily accepts the development of only one embryo. It is obvious that in order to put in value more than one oocyte at one ovarian cycle the intended has to be assisted by *in situ* artificial insemination and completed using foster mothers (receptors) for outside development of embryo surplus. The issue act to obtaining higher fertility of one cow is to induce polyovulation instead of single ovulation.
That is possible by stimulating follicular maturation with the gonadotropic FSH pituitary hormone. The time of ovulation should be selected in such order that the polyovulation consequence should be longer and it is recommended that the artificial insemination to be done twice at about 10 hours interval.
The last target in the field is Multiple Ovulation Embryo Transfer (MOET). The last possibility to increase the number of ova that could be fertilized from a cow would be to repeat polyovulation at shortest possible terms. That means MOET. Hypothetically the shortest term in MOET is to apply the FSH treatment immediately after uterus washing for embryo collection and inseminate the donor 10 and 11 days later.
Next washing should have place 7 days later. But nobody organized such experiment. The shortest tried term by us was applying FSH at the end of the next heat after washing and planning the artificial insemination 10 and 11 days later (Paraschivescu, M.Th. et al., 2015). Unfortunately, because of poor research resources only two heifers could be used and so erratic answers were received. Doesn't matter what term from washing of the uterus to the new follicular stimulation is engaged it is strict to know how many times uterus might be washed staying healthy (Wrathbal A., Sietmoleler P., 1998). In the mentioned experiment (Paraschivescu M.Th. et al., 2015) this problem couldn't be included. Particularly for heifers it is important to know which is the earliest age (prepuberal heifers are not excluded) which is permitting follicular stimulation and how many times could be repeated without injuring their normal development and gynecology (Wrathbal A., Sietmoleler P., 1998). Full success of such experiments in this field will be when progeny tested dams might be selected in dairy breeds improvement. Nevertheless some special MOET farms were imagined (McGuirk B., 1993, Paraschivescu M. Et al, 1989, Paraschivescu M.Th., 2010).
The last two projects, communicated but not published, refer, the first one, to a **closed MOET Farm** (fig.1) receiving embryos from AI Centers interested in ET, selling male calves for progeny testing and using heifers as receptors (foster mothers) and the second one, to a **opened MOET Farm** (fig.2) receiving embryos from the same sources but using as surrogate mothers beef cattle heifers and selling progeny of both sexes. In the last case the interest for sperm sexing is obvious.

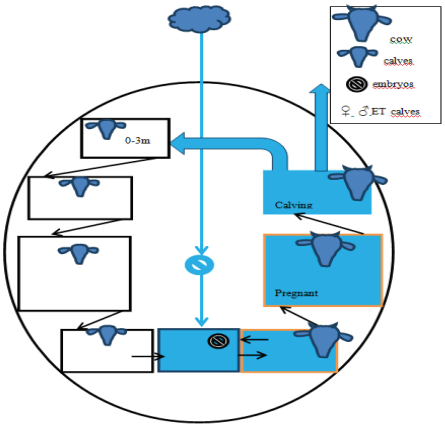

Fig.1 Closed dairy cattle MOET farm

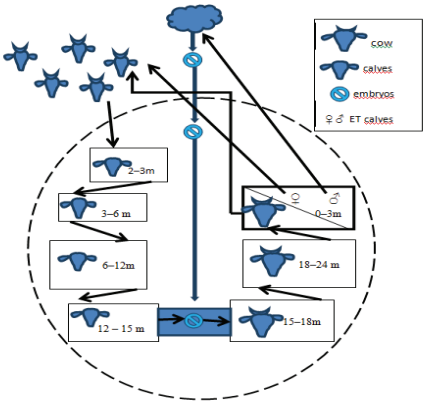

Fig. 2. Opened dairy cattle MOET farm

In the types of *in situ* AI attended biotechnologies embryo splitting and embryo cloning must be included.

IN VITRO AI ASSISTED BIOTECHNOLOGIES

Artificial insemination as the operation of fertilizing ova avoiding the mounting of females by males could be provided outside de female *in vitro* in aseptic laboratory conditions. The *In Vitro Fertilization* (IVF) gives the opportunity to access the zygote, which means that before the two genomes of the gametes are mixing (Vintilă I., 1997).

IVF difficulties are considerable. Ova have to be picked up from ovaries of living or slaughtered cows. Apart, it is necessary to have the ova as order II oocytes by inducing the maturation of ovarian follicles and keeping them. The spermatozoa have to be capacitated as the ones from the frozen semen are. IVF operation is done on clock glass under microscopic control. This way some progeny of very valuable dams could be obtained after their death.

A development of IVF is transgenesis used to obtain Genetically Modified Organisms (GMOs). Transgenesis is supposed to attach short segment of DNA to the zygote pronucleus. The foreign DNA segment attachment could be conceived by its mechanical inoculation into zygote pronucleus. Usually the male pronucleus, which is bigger, is preferred. Other means to attach the foreign DNA segment is to use compatible vectors. The vector carries the DNA segment and penetrates both pronuclei' membranes releasing the foreign matter in order to link to the respective genomes. Senday virus is the most used vector of DNA segments. Advantage of using vectors is that both pronuclei are modified and after amphimixis the chance of new genes to pair is increased. Transgenesis might be successful only when the foreign inoculated DNA segment is compatible to the host DNA. The chance to sexually reproduce an animal GMO is rare and is possible just in case both sexes genetically modified partners exist. But even in these cases the foreign genes could be eliminated during the reproduction process. Nature fights to preserve its order. The only convenient way to reproduce GMO animals is to clone them. The last procedure of artificial insemination is the mechanical monospermous fertilization of ova. It consists in preparing one sperm or head of one sperm in the wanted way, catching the piece with one inoculation needle and mechanically pushing it the cytoplasm of one mature ovocyte inside its original follicle.

In cloning superior animal organisms fertilization can be excluded if for cloning nucleus of somatic cells are used. In this case somatic diploid cells' nucleus extracted from the organism to be cloned have to be placed into the order II ovocyte cytoplasm. But the synchronization of the somatic cell nucleus formatting and of the ovocyte development has to be respected. The feasibility of such technic wonderfully was demonstrated by Wilmut

(Wilmut I and al. 1997, Wilmut I and al. 1998) when obtained Dolly, the famous creation of bioengineering.
Ignoring the great difficulties, the high costs, and the low rate of operating success, the IVF gives access to some fantastical opportunities in bioengineering. Such opportunities are using the genotypes of death female animals, practicing micro surgery on the haploid female or male genome before their amphimixis takes place, or passing over the breaks imposed by the genetic information as natural mechanisms of closed reproduction of genetic species as open ways to animal GMO bioengineering (Wiltbank M.C., 1997).
But bioengineering is costly and short time sustainable. Many times the trials to biologically reproduce animal GMO weren't successful. Species of genetic information controls the genotypes in the new generations of cloned organisms excluding the foreign genes. Cloning mammal organisms is the only hope of reproducing superior animal GMO.
The difficulties and costs of Transgenesis justify using it in pharmaceutical industry only. These biotechnics are beyond Artificial Insemination.

ARTIFICIAL INSEMINATION EXTENSION

The prognosis concerning the Artificial Insemination extension in dairy cattle breeding at the world level is confirmed as well, but not in this country. The stupid privatization of the former state SEMTEST enterprises and the clearing of the former elite dairy cattle farms with concentrated livestock, have generated a general disorder. Local AI managers weren't able to find solution and foreign specialists had no interest in repairing things.
The solution would be a simple one, namely to remake the former SEMTEST units as cooperatives of milking cows' owners. It was proposed to the EU team, but it was rejected. That was a mistake apart of Romanian leadership afraid to lose entering in Europe. Persistence of SEMTEST as local cooperatives justified maintaining elite dairy cattle farms, as the former so called "inercoop" cattle farms, for instance. The utility of the elite farms might provoke the interest of some investor in building large, concentrated, dairy cattle farms, may be at the beginning as elite and later as commercial dairy farms.
Therefore prognosis concerning AI extension in Romania didn't confirm, but for political reasons not for technical ones. Such mistakes are still going on. Promoting beef cattle husbandry, convenient for extensive agriculture, sustained by some Romanian officials, is not compatible with the Romanian agriculture specific based on hamlet location of labor neither for the European economy interested in intensive agriculture. That will decrease cattle breeders' interest for artificial insemination

CONCLUSION

Given the above information it is right to say that almost ideas of the technical prognosis thoughts 20 years ago were proven right. Additionally innovative ideas have been released globally as well.
As pure animal reproduction biotechnology, AI got the highest possible level. Much progress was wined in assisting other animal biotechnologies tending to GMO bioengineering.
Most progress concerning dairy cattle breeding refers to MOET. Unfortunately the two Romanian schemes of MOET farms were never published and no research unit lanced such projects because AI Centers acting in Romania are out of date.
Avant-garde research projects are directed to bioengineering as an AI in vitro modality to prepare medical bio products for human health care.
Concerning AI extension in dairy cattle breeding the trend was to become exclusive in most of the developed countries. In Romania things went wrong because of many political mistakes.
The future long term prognosis of AI seems to hit 3 targets: a) assisting the avant-garde biotechnics for transgenic organism enjoying pharmaceutical industry, b) assisting the MOET biotechnologies promoted to increase the female side fertility in dairy cattle breeding for breeds' improvement or higher yields of milk production in commercial dairy farms, c) extending it in current reproduction of dairy cattle to keep up the performance level of milk

production and to decrease the progeny cost up to the stage that the natural mating will be excluded.

On the contrary newly added ideas have completed the old ones worldwide.

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CLINICAL-DIAGNOSIS COORDINATES IN ETHYLENE GLYCOL INTOXICATION IN A CAT. CASE STUDY

Alexandra Mihaela POPA[1], Ana – Maria GOANȚĂ [1], Cristina FERNOAGĂ , Lucian IONIȚĂ [1], Mario CODREANU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăşti Blvd, District 1, 011464, Bucharest, Romania, Phone: +4021.318.25.64, Fax: + 4021.318.25.67, Email: codveterinary@yahoo.com

Corresponding author email: alexandrapopa613@gmail.com

Abstract

The aim of this study is to underline the significance of ethylene glycol poisoning as a differential diagnosis in young outdoor cats presented with sudden onset of lethargy in the absence of other clinical signs whose state continues to degrade over a few hours. Ethylene glycol intoxication is more common in dogs than in cats, as they are more tempted by its sweet taste. Cats are more frequently intoxicated through cutaneous absorption and grooming and have a lower minimum toxic dose. Clinical signs include polyuria, hyporeflexia and ataxia, that progress to depression, stupor and hypothermia and signs of acute kidney failure. A 9 months-old male intact cat presented in a precomatose state in our clinic. The anamnesis indicated that it was an indoor-outdoor cat with no medical history which had been been away from home for a few hours. Clinically, it presented with inappetence and lethargy. The physical examination revealed a precomatose state with hypothermia (36.8°C), dehydration, cyanotic mucous membranes, uremic halitosis, mydriasis and an absent pupillary light reflex. Blood biochemistry revealed a blood urea nitrogen of 75 mg/dL and a creatinine of 11.8 mg/dL. Repeated blood glucose measurements revealed oscilations between hypo- and hyperglycemia despite therapeutical interventions. The ultrasonographic examination showed kidney lesions suggestive of ethylene glycol toxicity. The microscopic examination of the urinary sediment revealed calcium oxalate crystals. Based on the paraclinical evidence, we suspected an intoxication with antifreeze such as ethylene glycol. The cat was treated specifically and rehydrated, but after 48 hours the its clinical state had worsened and the decision was made to put the cat to sleep. Even though the incidence is lower in cats, ethylene glycol toxicity should be added to the list of differential diagnoses in cats, in particular during the cold season when antifreeze is more commonly used and mostly when the symptomatology is suggestive of an acute intoxication.

Key words: *ethylene glycol, intoxication, cat.*

INTRODUCTION

Ethylene glycol (EG) intoxication is one of the most common causes of acute kidney injury (AKI) and the second most common intoxication encountered in pet animals. Antifreeze is the main source of exposure, but ethylene glycol is also used in a variety of household products including cleaning products, varnishes, cosmetics and aromatic extracts[4]. Ethylene glycol as such is not directly nephrotoxic; its metabolites (glycolaldehydes, glycolic acid, glycolat and oxalic acid) are the cause of renal damage [7].

Ethylene glycol intoxication is more common in dogs[10] than in cats, as they are more tempted by the sweet taste [8].

Cats, on the other hand, are more frequently intoxicated through cutaneous absorption and grooming and have a lower minimum lethal dose of just 1.5 ml/kg with a recorded mortality after ingestion of EG of 96–100% [4,5].

Ethylene glycol is rapidly absorbed from the gastrointestinal tract. The peak plasma concentration occurs about 1 hour after ingestion and approximately 50% of the ingested ethylene glycol dose is eliminated unchanged by the kidneys; however a series of oxidative reactions in the liver and kidney metabolize the rest of ethylene glycol [6].

The first step of the metabolism is its conversion to glycoaldehyde by alcohol dehydrogenase. Further glycoaldehyde is then metabolised to glycolic acid.

The metabolites of glycolic acid turn to glyoxylic acid and then oxalate. The resulting toxic metabolites cause severe metabolic acidosis and impairment of the renal tubular epithelium. One of the most toxic metabolites is

oxalate, as it cannot be metabolized further and it is cytotoxic to the renal tubular epithelium and exacerbates metabolic acidosis. Glycolic acid and oxalate are considered to be the metabolites responsible for the acute tubular necrosis associated with ingestion of ethylene glycol. The oxalate combines with calcium to form a soluble complex that is eliminated by glomerular filtration. If the concentration of the glomerular filtrate increases and the pH decreases, calcium oxalate crystals can form in the lumen of the tubes [9,12] (Fig.1)

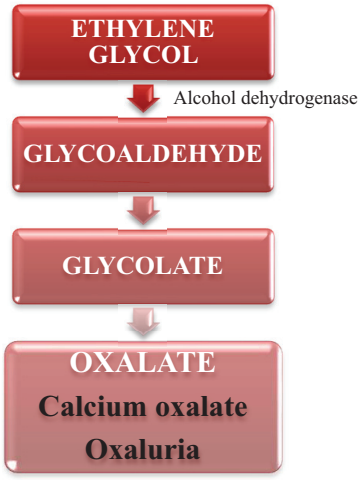

Fig. 1. Metabolic pathway of ethylene glycol

Clinical signs of EG toxicosis occur in III stages.

First stage (30 min–12 hours postingestion) is defined mainly by neurological signs such as depression, ataxia, seizures, coma, or death. As a consequence of the direct irritating effect of ethylene glycol on the mucosa, gastrointestinal signs may also appear. Research has shown that these clinical signs are due to aldehyde metabolites, hyperosmolarity, and metabolic acidosis, and resemble those of alcohol ingestion. Treatment is more likely to be successful if it is initiated in this stage [7].

The second stage occurs from 12 to 24 hours following ingestion and is determined by metabolic acidosis, CNS depression, miosis and the development of cardiopulmonary signs such as tachypnea or tachycardia [7].

The third and final stage (24–72 hours postingestion of a lethal dose) is characterized by acute renal failure and associated clinical signs (anorexia, vomiting, and other signs of uremia).

The ultrasonographic image of kidney with oxalate nephrosis points out ultrasonographic changes that varies from mild to marked increased renal cortical echogenicity with varying degrees of intensity of the corticomedullary junction ('halo' sign) and is supportive of the presumptive diagnosis of ethylene glycol intoxication [2].

MATERIALS AND METHODS

The clinical investigations, ultrasound examination and treatment methods described herein were performed in the Clinic of Medical Pathology, Faculty of Veterinary Medicine, Bucharest, on a 9 months-old intact male cat, presented in a precomatose state in our clinic. Haematological, biochemical and urine tests were conducted in the Laboratory Clinics, belonging to the Faculty of Veterinary Medicine.

The ultrasonographic examination was performed using the Esaote Pie Medical MyLab, in M and B-modes, with convex, micro-convex and linear probes with a frequency range of 5-18 MHz.

Storing and analyzing the image obtained was performed with the computer using specific morphometry software.

The anamnesis indicated that it was an indoor-outdoor cat with no medical history which had been away for a few hours.

Clinically, it presented with inappetence and lethargy. The physical examination revealed a precomatose state with hypothermia (36.8°C), capillary refill time was within 2-3 seconds, cyanotic mucous membranes, heart rate – 136 min, respiratory rate – 32 min, dehydration, uremic halitosis, mydriasis and an absent pupillary light reflex.

Blood biochemistry revealed a blood urea nitrogen of 75 mg/dL and a creatinine of 11.8 mg/dL. Repeated blood glucose measurements revealed oscilations between hypo- and hyperglycemia despite therapeutical intervention.

The ultrasonographic examination showed nephromegaly, increased renal cortical echogenicity, being markedly more echogenic than the adjacent liver with sonolucency in the

corticomedullary junction and central medullary region (the "halo sign") due to accumulation of multiple calcium oxalate crystals, particularly in the cortex and the corticomedullary junction.
The microscopic examination of the urinary sediment revealed calcium oxalate crystals, which can be detected in the urine of most animal species approximately 6–8 h after ingestion of ethylene glycol, and in cats as early as 3 h after ingestion (Fig.2)

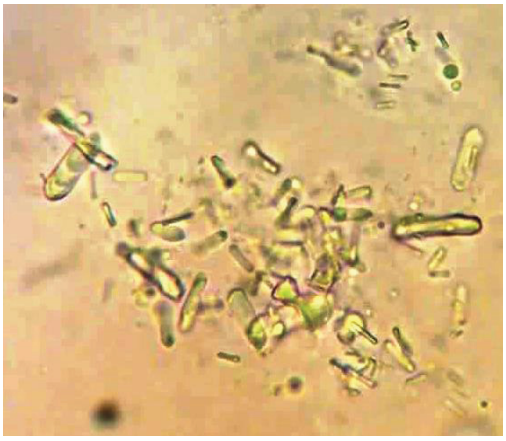

Fig. 2. Urine sediment: the presence of calcium oxalate crystal (Courtesy of the Laboratory of the Faculty of Veterinary Medicine).

The diagnosis of oxalate nephropathy associated with ethylene glycol toxicosis as the cause was supported by the history and further corroborated by the analytical findings.
Based on the paraclinical evidence, we suspected an intoxication with antifreeze such as ethylene glycol. The cat was treated specifically and rehydrated, but after 48 hours its clinical state had worsened and the decision was made to euthanise the cat.

RESULTS AND DISCUSSIONS

The clinical diagnosis of ethylene glycol poisoning can be challenging, taking into account the fact that in this case, the actual ingestion couldn't have been noticed by the owner due to the absence of the cat from home.
Clinical signs were not specific especially when considering that the simptoms could have been representative of a large number of toxic or infectious agents and are variable depending on the stage of intoxication[7].
Ethylene glycol intoxication should be suspected in cats with acute onset of signs, high values of urea, creatinine, hypocalcaemia, hyperglycaemia (50% of patients develop hyperglicemia due to inhibition of glucose metabolism, increased blood epinephrine or cortisol, uraemia[7]), azotaemia or uraemia and depression, metabolic acidosis and calcium oxalate crystalluria[13].
Ultrasound examination offered valuable information regarding renal ultrasonographic appearances, which were strongly suggestive of ethylene glycol intoxication with increased renal cortical echogenicity and the presence of a 'halo' sign (an echogenic line in the outer zone of the renal medulla, paralleling the corticomedullary junction, described also as the renal medullary rim sign).
The detection of ethylene glycol in the body can also be aided by the fact that many antifreeze liquids contain fluorescein, which is easily detectable in urine by Wood lamp examination up to 6 h after ingestion[1].
Therapy. The deciding factor in the treatment of ethylene glycol intoxication is the administration of the antidote as soon as possible. The antidote of choice is ethanol, which competes with the ethylene glycol at the active site of the enzyme alcohol dehydrogenase. Its affinity is higher than that of ethylene glycol, which leads to the excretion of ethylene glycol in an unchanged form. The second antidote, 4-methylpyrazole (fomepizole), has a similar mechanism of action – with only minor adverse effects compared to ethanol, but it is very expensive and rarely quickly accessible in common veterinary practice [6,13].

CONCLUSIONS

Ethylene glycol ingestion is a common cause of lethal intoxication particularly in cats, but prompt diagnosis and treatment with ethanol therapy can be life-saving. However, in many cases the early signs may be missed, as they can be vague and non-specific, which results in a tardy presentation of the animal to the vet.

Diagnosis of EG toxicity in the clinical setting must be made based on clinical signs as well as history and clinicopathologic findings.

The speed with which the correct diagnosis is made directly influences the prognosis as the antidote is salutary when administered within the first five hours after ingestion. Otherwise, due to the enzymatic change into metabolites, the administration of an antidote is useless.

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ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

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MEAT PRODUCTS - ARE THEY SAFE FOR GUARANTEE THE POPULATION HEALTH?

Lucian-Ionel ILIE

University of Agronomic Sciences and Veterinary Medicine Bucharest, No. 59, Blvd. Mărăşti, 011464, Bucharest 1, Romania, Phone: +4021.318.22.66, Fax: +4021.318.28.88

Corresponding author email: drlucianilie@yahoo.com

Abstract
Foodstuffs of animal origin continues to record increased demand among consumers, compared to vegetable products, because they are characterized by a high biological value which is given by the rich content of most essential amino acids (Tăpăloagă, 2014). The development of metabolic processes, where foods are involved, must be done with respect for certain values for parameters that influence consumer health. The study presents some of the ingredients involved in making meat preparations and their medical importance, the values of some parameters that characterize their quality and safety while assessing the degree of consumption of these assortments of animal food. Sometimes, food quality characteristics are omitted by consumers either knowingly (when the cost price is the one that is the prime), or because of the way the product is presented which most of the time does not reflect the true reality, but the appearance, the smell, the taste or the way of packaging make it unremitting. Although, the values for the parameters determined by us, have been within the normal limits set by current legislation, the age of consumers (especially children) is worrying and also their weight in the daily diet, the two may adversely affect the health of these consumers later.

Key words: *cold meats, food quality and safety, medical diseases, population health.*

INTRODUCTION

The cold meats (cold cuts) are meat products, usually in membranes, which is consumed without prior cooking and for the manufacture of which are used raw materials, represented by meat (cattle, swine, sheep, poultry), bacon and by-products, but also many auxiliary materials (Hubert, 2005). Among the auxiliary materials used, we are particularly interested in salt, nitrite, polyphosphates.

The increased consumption of salt, over daily requirements is associated with higher blood pressure and an increased risk of hypertension, but also with serious consequences of water retention, such as: heart failure, kidney disease and renal lithiasis, edema, stroke or osteoporosis (APC, 2017).

The sodium nitrite, used for antimicrobial properties, being bacteriostatic at a pH= 5-6 is associated with potentially carcinogenic substances, especially for the digestive tract (stomach), as a result of interaction with the proteins in the preserved food (ILSI, 2011).

Also, excessive fat consumption, especially saturated ones, is the main cause of an increased value of cholesterol at children and later for obesity and cardiovascular disease.

MATERIALS AND METHODS

A total of 30 samples, representing assortments of semi-smoked meat products, were collected and analyzed by sensorial and physical-chemical exams. Samples were collected from units with a specific profile in Romania (a selling meat and meat products unit) and were represented by pork salami, summer salami, „extra" salami, „Victoria" salami and two kinds of sausages, these assortments being the most commonly used for making sandwiches. To these samples, the values of some quality and food safety parameters have been appreciated, namely: water, salt, nitrates, fat and protein, along with sensorial properties.

RESULTS AND DISCUSSIONS

The sensorial exam has followed the appreciation of the exterior and on the section appearance, consistency, colour, smell and taste. The examination revealed normal characteristics for these samples. The pieces were whole, with clean surface, without impurities or mould islands, smooth membrane, continue, adherent to the composition, resistant to traction; under the membrane without air voids.

Table 1. Results of quality parameters assessment

No	Type of sample	Water %	NaCl %
1	pork salami	51,8	2,7
2	pork salami	52,9	1,8
3	pork salami	53,7	2,3
4	pork salami	56,3	1,8
5	pork salami	52,3	2,3
6	summer salami	51,6	2,3
7	summer salami	55,1	2,4
8	summer salami	54,2	2,2
9	summer salami	56,3	2,4
10	summer salami	49,8	2,4
11	summer salami	48,7	2,1
12	„extra" salami	52,5	2,3
13	„extra" salami	55,1	2,4
14	„extra" salami	56,9	2,1
15	„extra" salami	51,2	2,3
16	„extra" salami	54,9	2,4
17	„Victoria" salami	52,6	2,3
18	„Victoria" salami	55,5	2,3
19	„Victoria" salami	50,8	2,4
20	„Victoria" salami	54,2	2,1
21	sausages type 1	55,1	2,2
22	sausages type 1	51,7	2,2
23	sausages type 1	53,7	2,5
24	sausages type 1	55,6	2,1
25	sausages type 1	49,6	2,2
26	sausages type 2	54,2	2,5
27	sausages type 2	56,2	2,3
28	sausages type 2	46,7	2,3
29	sausages type 2	53,9	1,9
30	sausages type 2	53,6	2,2

No	Type of sample	Nitrates mg%	Fat %	Protein %
1	pork salami	5,7	28,9	16,3
2	pork salami	3,9	28,6	15,6
3	pork salami	4,5	25,4	16,3
4	pork salami	4,8	31,7	15,5
5	pork salami	2,5	28,1	15,8
6	summer salami	3,5	27,2	15,7
7	summer salami	3,3	29,4	15,4
8	summer salami	5,2	28,2	15,8
9	summer salami	6,7	30,7	15,2
10	summer salami	5,2	32,2	15,6
11	summer salami	3,2	26,8	15,7
12	„extra" salami	4,2	21,2	14,3
13	„extra" salami	3,4	24,4	16,4
14	„extra" salami	6,1	29,6	14,7
15	„extra" salami	6,2	27,2	16,2
16	„extra" salami	5,4	26,6	16,4
17	„Victoria" salami	6,4	33,1	15,4
18	„Victoria" salami	2,5	20,3	15,8
19	„Victoria" salami	4,2	27,4	16,3
20	„Victoria" salami	3,6	26,2	15,7
21	sausages type 1	5,5	26,7	15,4
22	sausages type 1	5,3	26,3	15,6
23	sausages type 1	5,2	31,8	15,8
24	sausages type 1	3,2	28,4	15,7
25	sausages type 1	2,3	26,8	16,2
26	sausages type 2	3,6	25,4	16,3
27	sausages type 2	3,5	21,2	15,1
28	sausages type 2	2,6	27,8	15,5
29	sausages type 2	6,2	27,9	15,6
30	sausages type 2	6,5	28,9	16,3

On the section, the composition it was compact, well mix, with pieces of bacon of uniform size and evenly distributed across the composition, giving mosaic look; without air voids, agglomerations of molten fat, liquid bags or albumin precipitate.

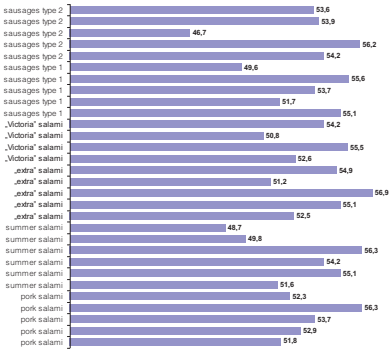

Figure 1. Water values

The consistency is firm and uniform. Smell and taste are pleasant, suitable salted and spiced.

For the physical-chemical parameters analyzed, the results were appropriate, these not exceeding the limits permitted by current legislation (Table 1). The values recorded were placed: for humidity between 46,7 and 56,9%, for salt between 1,8 and 2,7%, for nitrates between 2,3 and 6,7 mg %, for fat between 20,3 and 33,1%, for protein between 14,7 and 16,4%.

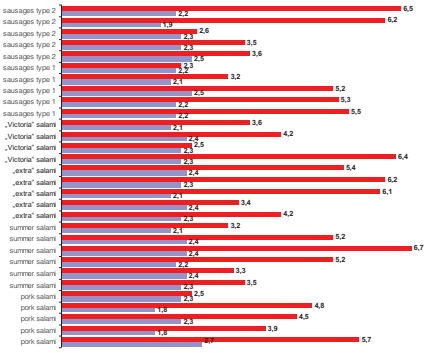

Figure 2. NaCl and Nitrates values

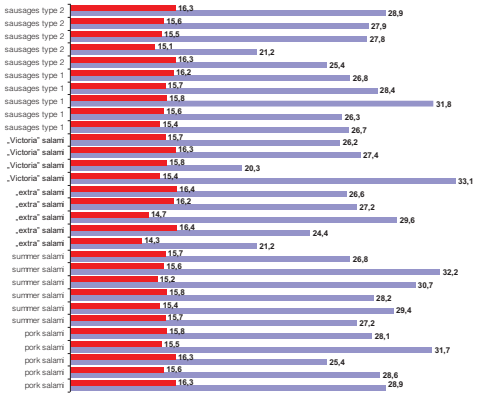

Figure 3. Fat and Protein values

CONCLUSIONS

Evaluating the main parameters that characterize the quality, safety and security of meat products must be carried out by the competent authorities on food, food additives, vitamins, mineral salts, trace elements, other food additives intended to be marketed as such, as well as materials and objects that come into contact with food, in order to verify the compliance with the legal provisions in force, relating to prevention of risks to public health, protecting the interests of the consumer and informing him correctly.

Although the analyzed samples correspond to the evaluated parameters, it is not unimportant the quantity, frequency and age at which these foods are consumed by the population.

Also, an exaggerated consumption of these products, associated with certain pre-existing medical conditions lead to these diseases worsens.

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USE OF SAİNFOİN İN RUMİNANT NUTRİTİON

Abdullah ÖZBİLGİN[1], Behiç COŞKUN[2]

[1,2]University of Selcuk Veterinary Medicine, Department of Animal Nutrition and Nutrient Disorders, in Turkey, Konya
Corresponding author email: abdullahozbilgin@gmail.com

Abstract

In this review, general characteristics of sainfoin and researches on the usability in animal feeding. Sainfoin is a perennial leguminous plant. It is a perennial feed crop with a total area of 1,914,391 hectares in Turkey. Sainfoin grows in pH 7-8 on calcareous soils on the northern slopes of valleys, plains and mountains up to 6 thousand meters in height. The content of nitrogen in the branches and leaves of the sainfoin plant is higher than in the beginning of flowering. The sainfoin contains condensed tannin. The condensed tannin removes protein degradation. Grazing ruminants may encounter many metabolic diseases related to feeding. The minimum plant condensed tannin concentration for this effect should be greater than 5 g / kg (CT) in dry matter. In the sheep study, parasitic infestation of the abomasum and small intestine causes large protein losses in sheep. İn a study reported that the presence of tannin in sheep fed had no effect on milk yield but increased the yield of tannin to 5.9%.

Key words*:Sainfoin. Abomasum, tannin, feed, sheep.*

INTRODUCTION

1. What is Sainfoin?

Sainfoin is a perennial leguminous plant. The French described the corpus with the words "Sain" and "Foin". "Cain" is healthy and "Foin" means grass. In other words, they use healthy herbs for sainfoin. This name has been passed on in English. It is called "Healthy hay", inspired from this name by a project sponsored by the European Union. In some sources it is also called "holy hay" (Carbonero 2011, Carbonero 2012). It is expressed in a word that means the favorite food of donkeys in the shade of greens (Ruprecht 2005, Smith 2011).

2. Type and varieties

There are many varieties of sainfoin (Onobrychis sativa) cultivated in Europe. The variability of these varieties is based on the number of formats in the present. O. sativa. Communis is a plant with a life span of 7 years or more without weed problems. But only its root forms in a year, its body is incarcerated and blooms once a year. In addition to being long-lived, satisfactory cultivation in arid conditions and poor soil has resulted in excessive cultivation. O. sativa Bifera (Giant Safflower) is a variant with a lifetime of one or two years. Much more than the ordinary sainfoin brings to the big body. She blooms twice in a season and she is reared twice. O. sativa. Maxima (Three Shaping Sainfoin) this plant is cut 3 times in one season. However, this feature is not fixed and can be taken in 2 forms according to the ambient conditions. For this reason, it is mixed with giant hull. O. sativa. Persica (Multi-Format that Sainfoin) of these varieties in the south west of Asia, in the Caucasus, it was reported that Turkey is an ecotype and grown in the northeast of Iran.

3. History and geographical distribution

Sainfoin spreads widely in Europe's temperate regions, Asia, Mediterranean countries and Northwest America. It was raised throughout history by locals in more temperate regions (Smoliak 1972, Clark and Malte 1913). In the 15th century the center of Europe spread to countries like Italy, England and Switzerland (Burton and Curley, 1968).

4. General features

Sainfoin is an important feature that the corpuscle grows and develops earlier than other leguminous feedstuffs (Smoliak 1972). Outside the seedling period, drought is particularly resistant to cold. It likes sainfoin, permeable, calcareous, sandy soils. It improves the weak and barren areas by putting into the planting season under all kinds of climate and soil conditions. When seeds are infected with

bacterial culture at the time of planting, nitrogen is added to the soil due to nodosity bacteria. It is included in planting seasons in arid regions, helping to narrow fallow areas. At the same time, it is an important plant which is involved in the construction of artificial lands in arid and erosion open areas. At the same time, sainfoin is a good bee.

5. Anatomic features

The plant has a thickened main stem and a large number of side spines. The root system consists of a main pile root with several large and numerous fine root roots. The pile root can be 5 cm in diameter and can go up to 1-10 m deep. Fists are most often
found on thin side roots. But there may also be some punches on the young pile roots. The body of the protector develops steeply or semi-obliquely. Plant height can be increased up to 60 cm in normal conditions and 90-100 cm in good soil. The plant gives a large number of stems from its crown. The stems are 100-120 cm long. The stalk is round. Empty the floor. In the upper parts, the inside is full and it is hairy. Leaves are reciprocal. On a leaf axis, there are mutually 7 to 15 leaflets. The long egg-shaped leaflets are covered with thin fur. The leaf axis always ends on the leaf. The flowers come out of the leaf seats and are on the handle. It is pink-colored and clustered. Each cluster contains 5-80 flowers. Fruits 5-8 mm. It is semicircular in shape. It's a single-seeded flat one. The fruit shell is veined and threaded. The seeds are kidney-shaped dirty yellow and brown. At the end of maturation, fruit bark is not opened, it is planted as fruit (Genç Ziraat 2007, Temel 2010). It is a perennial feed crop with a total area of 1,914,391 hectares (NRCS 2013) in our country. Although some sources say that the guardian may live 8-20 years (Manga 1995), the economic life in our country is approximately 5-6 years.

6. Adaptability

Sainfoin grows in pH 7-8 on calcareous soils on the northern slopes of valleys, plains and mountains up to 6 thousand meters in height. It is tolerant to cold and drought, is a bait plant resistant to bacterial infections and late spring frosts. It is a good alternate for alfalfa (Medicago sativa) in arid areas where short-term irrigation is inadequate for dry hay production (Smith 2011).

7. Nutrient content

The content of nitrogen in the branches and leaves of the sainfoin plant is higher than in the beginning of flowering. The NDF, ADF content increases with maturation of the plant, and thus the values at the end of flowering are higher than at the beginning of flowering. When the contents of condensed tannin of roots, leaves and plant are all compared with the end of flowering, flowering is higher at the beginning. The digestibility of organic matter is higher at the beginning of flowering than other phenolic periods. During the first 1.5 hours of feed consumption, the nitrogen and ammonia peak values occur in the rumen fluid and fall after 6 hours and after consumption (Theodoridou 2010). Ripe kernels contain 34 g / kg (21% crude protein) nitrogen and 20% soluble carbohydrate in the dry matter. The digestibility of dry matter is about 70% (Waghorn 1998).

Table 1. The chemical composition of the sainfoin in different regions harvested during flowering (Kaplan, 2014)

Composition	Afşin	Tekir	Pazarcık	B. konus
Dry matter	94,67	95,1	95,73	95,58
Crude protein	17,39	15,23	15,16	17,7
NDF	43,31	43,56	47,64	44,61
ADF	35,62	35,61	38,28	34,34
Ash	5,97	7,3	5,21	6,4
Condanse Tannin	4,19	5,76	9,95	6,59

8. Tannins and chemical structure

Tannins are high molecular weight compounds that bind to proteins with polyphenolic bonds. Tannins are structurally composed of 2 groups. These can be hydrolyzed and are condensed tannins (Makkar 2003). By chewing the tannin-containing feed, about 60% of the cells are torn and the resulting tannin mixes with the saliva secretion into the rumen fluid. Tannins at pH 6-7 in rumen protect the plant proteins from proteases and thus may produce an inhibitory effect on ruminal protein digestion. These tannin-protein complexes can not be digested with bacterial enzymes and pass through the rumen without digestion. Rough feed containing low levels of tannin can partially protect proteins from rumen microbial digestion. Complex proteins are secreted by

gastric enzymes present in the abomasum at pH <3.5 digestion and enhance the absorption of amino acids at pH> 7 in the small intestine (McLeod 1974, Jones 1977, Mangan 1988, Hagerman et al 1992, Mueller-Harvey 1992, Waghorn 1996, Mueller -Harvey 2006). Distribution of 4% to 10% of the tannin content of the preservative has beneficial effects on the ruminants. For example; sulfuric amino acid increases digestibility. Thus, even at low concentrations, tannins help milk production by increasing the absorption of essential amino acids from the small intestines (Min et al. 2003).

9. Condensed tannin on protein degradation

Condensed tannins are polyphenol compounds with naturally-binding capacity to proteins. It is found in leguminous foods such as Italian sainfoin, clover (Zeller-Forage 2014). Proteins in leguminous feeds can not be exploited sufficiently by ruminants due to rapid rumen dissolution (Gebrehiwot 2002, Min et al 2003, Broderick 1995). Ammonia emerges as amino acid deamination product of soluble protein in rumen 70% (McMahon 2000). However, sainfoin containing condensed tannins enhances protein utilization in ruminants by protecting microbial digestive proteins in the rumen (McMahon 2000, Waghorn 2008). Protein digestion results in a reduction in the rate of condensed tannin-fast soluble protein (Frutos 2004). In plants containing tannins the protein is reduced to rumen amoa. Tannins reduce degradation of plant proteins and thus allow proteins to pass by-pass (Aerts 1999). When given in early ripening period, when the highest content of sainfoin condensed tannin was given, the urinary nitrogen excretion reduced the ruminal degradability and digestibility of the crude protein. This is directly related to the nitrogen retention of the condensed tannin (Chung 2013).

10. Effect of sainfoin above tympany

Grazing ruminants may encounter many metabolic diseases related to feeding. It's thympany from these diseases. Tympany; chewing of pasture grasses such as alfalfa, fig, clover, destruction of the mesophyll cell wall and mixing of intracellular components into the rumen fluid. It is a gas formation shaped in the rumen. During the chewing and ruminating of cilantro in mixed climates, the tannin and soluble protein are released from the plant material (Wang 2006), and ruminally released soluble proteins cause stabilized foam to form especially in spring (Jones and Lyttleton 1971, Jones et al 1973, Howarth et al., 1978), but can reduce the production of rumen gas by the effect of condensed tannin (Chiquette 1988). At the same time, it is an important reaction that the sainfoin leaves are resistant to the mechanical condition of the epidermal layer and the chewing damage of the mesophyll cell wall. This response reduces the risk of tympany in legumes that are slower at the beginning of digestion and higher than the mechanical resistance of leaf tissues such as red alfalfa and white alfalfa (Howarth ve ark 1978, Chiquette 1988, Wang 2006).

11. Antiparasitic effect of the sainfoin

The condensed tannin with sainfoin is an important feature of the antiparasitic effect. An antiparasitic effect against Haemonchus contortus was found in a study conducted with its dry grass (Heckendorn 2007). The minimum plant condensed tannin concentration for this effect should be greater than 5 g / kg (CT) in dry matter (Li 1996). In the sheep study, parasitic infestation of the abomasum and small intestine causes large protein losses in sheep (MacRae 1993). The development of parasitic resistance to anthelmintic drugs reported in sheep, cattle and cattle in the USA and New Zeland is a major question (Waller 1994). In 55% of the sheep fed parasitic parasitic eggs. In the sheep, sainfoin can be given as coarse feed to stop the egg growth in the periparturient period (Werne et al. 2013). At the same time, condensed tannins have been proposed stratification strategies (Niezen 1998, Molan 2000). Antihelmintic pesticides grow in similar proportions when they consume Italian guacamole (Hedysarum coronarium) and alfalfa. However, unscanned icebergs grow better depending on the effect of the condensed tannin. The consumption of Italian sainfoin (Hedysarum coronarium) is 41% and 45% lower than that of the alfalfa consuming alfalfa and parasite loads (Molan 2000).

12. Effect of the sainfoin on methane production

Methane is one of the greenhouse gases such as carbon dioxide and nitrous oxide. It has been found that methane is more widely distributed in ruminant farms at 55-60% than other gases. It causes a loss of 2-12% of the crude energy (Rochfort et al, 2009). İn rumen is a product of methanogenic bacteria. Tannins cause less methane production (Mc Sweeney 2001), by inhibiting cellulolytic microorganisms or by complexing lignocellulosics to reduce fiber digestion, thus altering the amount of methaneogenesis and the type of fermentation produced in the rumen. Recently, in vitro experiments have shown that the effects of the protection on methane production can be determined (Tavendale et al 2005, Mila 2008, Bhatta 2009).

Table 2. Influence of vegetation period on wild sainfoin gas production, methane production (Kaplan 2014).

Parameters	Flowering Before	Flowering after	Seed Binding
Total gas (mL)	47.02a	44.55b	39.05b
Methane (mL)	7.41a	6.91a	6.23b
Methane (%)	15.63	15.69	15.79
ME	9.58a	9.15a	8.25b
OMS	69.47a	66.15a	59.70b

13. The Effect of Sainfoin and Tannin Resources on Milk Yield and Meat Quality

In the sheep fed with Lotus corniculatus, the condensed tannin does not affect milk secretion during the early lactation period. However, in middle and late lactation, the cigarette increases the secretion, lactose and protein ratios by about 12, 14 and 21%, respectively (Wang 1996). The addition of PEG (Polyethylene Glycol) feed additive to the condensed tannin component increases the milk yield and milk urea ratios in deciduous gardens in the gardens of the gum tree (Pistacia lentiscus) and Oak (Qercus spp.) (Decandia 2000a, Decandia M. 2000b). Petacchi et al. (2007) reported that the presence of tannin in sheep fed had no effect on milk yield but increased the yield of tannin to 5.9% (Petacchi and Buccioni 2007). Some researchers observed no difference in protein yield, protein yield, and fat yield between the two groups when they consumed alfalfa alfalfa hay in the same amount of goat and cattle (Arrigo 2009). However, Romero et al. (Romero 1997) found that cattle fed with corn silage containing less than 0.4% of tannic acid and a ration of alfalfa and 0.8% tannic acid had lower milk yield and milk fat values. Petacchi et al. (2007) reported that the presence of tannin in sheep fed had no effect on milk yield but increased the yield of tannin to 5.9% (Petacchi and Buccioni 2007).

Vasta et al. (2007) have reduced the ruminal biohydrogenation of volatile components such as skatole and indole, which are produced by the rumen microorganisms that provide tannins in their work, to conjugated linoleic acid as an antioxidant (Priolo and Vasta 2007). Tavendale et al. (2005) reported in an in vitro study that the tannins reduced skatole synthesis when sheep fed the Deli Kaplan grass (Dorycnium rectum) in their study (Tavendale et al. 2005). Priolo et al. (2007) have shown that the reduction of B12 vitamins and hemoglobin synthesis by ruminant microorganisms in the ranching rations makes the meat color pale in small ruminants. But; This effect of tannins can be eliminated by the addition of polyethylene glycol (Priolo and Vasta 2007). In the study conducted by Priolo et al. (2000), the adverse effect on the carcass quality of the condensed tannin can be eliminated by adding 40 g of PEG per kilogram of goat horn ration (Priolo et al. 2000).

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ASSESSMENT OF SEROLOGICAL TESTS OF THE INFLUENZA A INFECTION IN WILD MIGRATORY AND ZOO BIRDS DURING THE EPIZOOTIC IN BULGARIA, 2015

Georgi STOIMENOV[1], Gabriela GOUJGOULOVA[2], Georgi GEORGIEV[3], Chavdar FILIPOV[1], Roman PEPOVICH[1], Kalin HRISTOV[1], Branimir NIKOLOV[1]

[1]University of Forestry, 10 Kliment Okhridsky Blvd., 1797, Sofia, Bulgaria
[2]NDSRVMI, 15 Pencho Slaveikov Blvd., 1606, Sofia, Bulgaria
[3]Risk Assessment Center on Food Chain, 136 Tsar Boris III Blvd., Sofia, Bulgaria

Corresponding author e-mail: georgi.stoimenov.vm@gmail.com

Abstract

The aim of our study was to assess the possibility of serological tests for a detection of antibodies against Influenza A virus in wild migratory, zoo birds and alive birds, presented on markets. The samples were collected in Bulgaria during the epizootic in 2015. Totally 209 specimens have been tested, of which 179 only by ELISA and 30 both by ELISA and HI assay. Some differences during the testing of two yolk sacs of eggs, from the found death Dalmatian pelicans, have been demonstrated, where ELISA and AGID were negative, but HI was positive; the following VNR found them to be partially positive. A possible explanation for the observed contradiction could be given by the specific haemagglutinin, located on the surface of viral particle. The obtained positive serum samples of wild birds from Sofia Zoo and those from a market for alive birds have shown that, the supervision of Avian influenza should not be focused only on the migratory birds, because the disease can be introduced by an import of exotic birds and their offer through auctions and markets.

Key words*: Avian influenza A, AGID, Bulgaria, ELISA, HI.*

INTRODUCTION

Influenza A infections are among the most dangerous and significant illnesses in many species of animals and people. Their zoonotic potential has always inspired a great interest among scientists and not once has struck fear among the populations of the world. Avian Influenza is particularly crucial since waterfowl migratory birds are the main reservoir and vector of infection. They can emit the virus 30 days after infection, which is a prerequisite for its dissemination over long distances during the migration of birds and subsequent introduction into populations of domestic flocks. Poultry farming is one of the traditional breeding industries for Bulgaria. There are a lot of industrial poultry sites almost everywhere in the country. At the same time, there are many "backyard" farms where the level of biosecurity is low. In addition, Bulgaria's market share of fattened duck liver in Europe is over 20%, and it is known that a large number of low pathogenic avian influenza A viruses circulate among the ducks. The combination of these factors and the passage of large populations of wild birds throughout Bulgaria in two main migration routes, create conditions for outbreaks of avian influenza A. The strict compliance of biosecurity measures in industrial poultry farms and increased alertness of veterinary services and farmers in backyard farms are the necessary requirements for better control of the disease.

Although there are different studies on serum antibody responses in experimentally and naturally infected ducks (Suarez and Schultz-Cherry, 2000), the immune response in birds due to infection with Influenza A has not been well studied. This response is predominantly based on the virus-producing neutralizing antibodies directed against hemagglutinin (HA) and neuraminidase (NA) glycoproteins (Marinova-Petkova, 2012).

MATERIALS AND METHODS

Blood samples were derived from a farm for hunting birds (Yambol region), hunting birds

(shot around Ogosta dam), zoo birds and eggs from wild birds found dead in the Srebarna Biosphere Reserve.

Table 1. Characterization of samples, included in provided serological tests

Species	*Type of sample*	*Number of samples*	*Method*			
Pheasants	Serum	169	ELISA			
Wild birds zoo Sofia	Serum	10	ELISA	HI		
Wild birds	Serum	6	ELISA	HI		
Wild birds	Serum	10	ELISA			
Dalmatian pelicans	Yolk sac	2	ELISA	HI	AGID	VNR
Mallards	Serum	10	ELISA	HI		
Swans	Serum	2	ELISA	HI		

Antigens used in serological reactions

APMV1 (NDV), H5N1, H5N2, H5N3, H5N9, H1N1, H2N3, H3N8, H4N8, H6N2, H7N1, H7N3, H7N7, H8N4, H9N2, H10N9, H11N9, H12N5, H13N6, H14N5, H15N9-Instituto Zooprofilattico delle Venezie.

Biological systems

As biological systems in the virus neutralization reaction, we used 9-11-day old SPF embryonated chicken eggs.

Methods used for a detection of antibodies against avian influenza A virus obtained from eggs of dead wild birds

Processing of the yolk was performed according to the standard operating procedure of the Reference Laboratory in Ames, Iowa, USA:

1. Break the eggs and put the contents in a Petri dish.
2. Using a syringe, take 1 ml of yolk.
3. Mix egg yolk with 1 ml of sterile PBS in a tube.
4. Vortex the yolk-PBS mixture at maximum speed for 10-15 s.
5. Leave the mixture for 1h at room temperature, then repeat step 4.
6. Centrifuge at 1500 x g for 30 min.
7. We separated the supernatant and used it for AGID, ELISA, HI and VNR.

We have been working with several commercial ELISA kits: INGEZIM INFLUENZA A - Avian Influenza Virus Antibody ELISA Kit and Avian Influenza Virus Antibody test kit, MultiS-Screen, IDEXX. We followed the manufacturer's protocols. The standard OIE procedure was followed for HI (OIE, 2015).

Virus neutralization procedure

We made ten-fold dilutions of yolk and mixed it with an equal amount of field isolate [as we used A / Dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1)]. This was followed by each dilution inoculated into the allantoic cavity of three 10-day-old embryonated chicken eggs (ECE).

Results interpretation

In case of presence of antibodies, they should neutralize the virus and no mortality or agglutination of erythrocytes should be noted. When there is no complete neutralization of the virus, it can be read by the degree of agglutination.

RESULTS AND DISCUSSIONS

Using this serology test, of all 209 samples, 14 were found to be positive. Antibodies in two of them (mallard ducks shot around the dam Ogosta, region Montana) have been defined by us as subtype H7 by hemagglutination inhibition test (HI) (Table 2).

Table 2. Summarized positive results from ELISA testing and HI subtyping

Bird species	Methods		
	Ingenaza ELISA	IDEXX ELISA	HI for subtypes H5 and H7
Mallard 1 (Montana)	Positive	Positive	Positive for H7 Titter 1:16
Mallard 2 (Montana)	Positive	Positive	Negative for H5 and H7
Mallard 3 (Montana)	Positive	Positive	Positive for H7 Titter 1:16
2 Wild birds (Sofia zoo)	Positive	Positive	Negative
9 Mallards (Live birds market)	Positive	Positive	Negative

On March 25, 2015, together with the organ samples of the found death pelicans in the Srebarna Reserve, we received eggs found around them. We processed yolk according to the SOP (reference laboratory at Ames, Iowa, USA). The results are summarized in Table 3.

Table 3. Results of detected antibodies in yolk sac, tested in three methods

Methods	Yolk sac 1	Yolk sac 2
Ingenaza ELISA	Negative	Negative
IDEXX ELISA	Negative	Negative
Immunodiffusion test	Negative	Negative
HI	*1:512	*1:32
	**1:16	**1:8

* Used A / Dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1) antigen-field isolate

** Standard antigen H5N3, A / duck / Italy / 775/2004, IZV, Italy

Virus neutralization

The neutralization test was applied for detection of antibodies in the two yolk sacs of eggs from the found death Dalmatian pelicans. With the prepared mixture of antigen and antibody we infected 3 embryos into their allantoic cavity. After that we observed the embryos for mortality. All chicken embryos were found dead after 48 hours and their allantoic fluids were tested for hemagglutination activity via a hemagglutination assay (HA). We detected hemagglutination activity in all dilutions. The HA positive allantoic fluids were examined for hemagglutination inhibition (HI) using 4 hemagglutination units per well and hyperimmune standard serum (H5N1, H5N3) produced from Instituto Zooprofilattico delle Venezie (Comin et al., 2013; Molesti et al., 2014). The standard OIE procedure was followed for both the HA and HI assays (OIE, 2015). The titter in HI was 1:64 for the concentrated yolk and 1:256 for all other dilutions.

During the analysis of the results from the applied tests (HI, ELISA), for the presence of antibodies against Influenza A virus in wild birds, we were impressed to detect antibody response in the mallard ducks, shot around Ogosta dam, Montana. From the same ducks we received cloacal swabs and tracheal swabs and the applied subsequently rRT-PCR method for the M-gene showed negative results. In serum, tested by ELISA, the results were positive. By HI, two of the three positive samples, detected antibodies against the H7 subtype, and the third sample was negative for the H5 and H7 subtype. Possible explanation of our results may be the duration of virus-carrying and virus-excretion in ducks. Intratracheal and oral infection of ducks (*Cairina moschata*) with H3N6 subtype revealed that the virus was secreted with the high titer in faeces until the 6th day after inoculation (Webster et al., 1978). On the 7th day, only 50% of the ducks emit the virus, and on the 8th day only 1 out of 4 birds. Zarkov et al. (2011) infected intravenously with H6N2 subtype (virus isolated from mallard duck) and found that the virus excretion on day 7 after inoculation was only 50%. On the 21st day, only 29%, while on day 28 it is no longer possible to detect the virus in the cloacal swabs and oropharyngeal swabs from the infected birds (Marinova-Petkova, 2012).

Basing on this information, we can build a hypothesis that serum samples were most likely to have been taken after the period of virus-excretion and the body had enough time to produce antibodies.

The immune response in ducks due to infection with Influenza A has not been well studied, although there are various studies on serum antibody response in experimental and naturally infected ducks (Suarez and Schultz-Cherry, 2000). In an experimental infection with LPAI H7N2 virus, in white Peking ducks, Kida et al. (1980) established virus-excretion until the 7th day after infection, but the antibody response was poor. With HI low antibody titers are established (Marinova-Petkova, 2012).

The results of the study of egg yolk of found dead pelicans in the biosphere reserve Srebarna are interesting. We also found some differences during the testing with the different methods of the yolk. ELISA and AGID were negative, whereas HI showed positive results. A possible reason for the positive reactions in HI test and the negative ones of AGID and ELISA is that the haemagglutinin is located on the surface of the viral particle. They first managed to carry out antigenic irritation. In contrast, the type-specific proteins against which the body builds antibodies, are located inside the virus and are more readily available to immunocompetent cells. The contact with them occurs only after the destruction of the virions (Zarkov, 2007). Some authors' publications have found a great variety in the comparative results of HI, ELISA and AGID in terms of their sensitivity and specificity. In some cases, the applied ELISA testing is more sensitive, in other ones the HI method is more sensitive, in third case the AGID showed higher sensitivity. This fact could be explained by different ways of preparation of diagnostic tests, because there is no standard operating procedure for this. It is well seen in the results of Jin et al. (2004). They compare two types of ELISA: one developed by them and the other was a commercial product. As differences in results are 6%.

Virus neutralization reaction similar to HI proves antibodies against hemagglutinin

protein of the virus. In our case, all chicken embryos were found dead after 48 hours and their allantoic fluids were tested for hemagglutination activity via a hemagglutination assay (HA). We detected hemagglutination activity in all dilutions. The subsequent HI virus titer was 1:64 for concentrated yolk and 1:256 for subsequent dilutions. We did not have complete virus neutralization, but partially the virus was neutralized, due to the fact that there were not enough antibodies in the yolk. Many studies on the birds have shown a strong correlation between antibodies in egg yolk and/or hatchlings and circulating levels of maternal antibodies (Staszewski et al., 2007; Martyka et al., 2011).

We assume that the titer of antibodies found in yolk are low during the acute stage of infection, death has occurred relatively quickly, and the pelican organism did not have enough time to build up more antibodies.

The right approach to prevent obtaining contradictory results is to test the samples with several tests. For example, blood serums were tested with both ELISA and HI test, and the yolk samples were run through four different tests.

CONCLUSION

Studies have shown that yolk samples from wild birds can be successfully used for serological studies. The antibody titer found in the yolk sac in highly pathogenic avian influenza infection is low as the disease is in an acute form and there is not enough time to build up antibodies due to the rapid death of the birds. The positive serum samples from the zoo Sofia and those from the live bird market show that surveillance of Avian Influenza A should not only focus on migratory birds, as the disease can be introduced by imports of exotic birds (zoos birds) and by offering them in markets.

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CHARACTERISTICS OF REFRIGERATED STALLION EPIDIDYMAL SPERMATOZOA AT 24-H AND 48-H AFTER CASTRATION USING TWO COMMERCIAL EXTENDERS

Mirela TRIPON[1], Emoke PALL[1], I. MORAR[1], M. CENARIU[1], C. CRECAN[1], Denisa LUNGU[1], Lavinia SÎNTEONEAN[1], V. ILAȘ[1], A. HENEGARIU[1], Andreea. RUSU[1], I. Ș. GROZA[1];

[1]USAMV - University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăștur 3-5 street, Cluj-Napoca, Romania.

Corresponding author email: mirela.tripon@usamvcluj.ro

Abstract

Collection of spermatozoa from the cauda epididymis may be the last chance to preserve genetic material from valuable stallions in case of sudden death or emergency castration. In the present study we compared the characteristics of extended refrigerated stallion epidydimal spermatozoa at 24 and 48 hours after castration. Spermatozoa from 12 epididymides were recovered at 24 hours after the routine orhiectomy of 6 healthy stallions using the retrograde flush method and refrigerated for 24 hours. For refrigeration we used 2 commercial extenders - an egg yolk based extender (Triladyl®, Minitube) or a milk based extender (Gent®, Minitube). Concentration and motility parameters were assesed for each sample after collection and 24 hours later using computer assisted sperm anlaysis (SCA®, CASA). Viability was assessed using the eosin staining technique. Total motility, velocity, viability and percentage of progressive spermatozoa were similar among the two groups at 24-h post castration. However, at 48 hours the percentage of progressive spermatozoa was significantly higher in milk based extender. Therefore we concluded that stallion epididymal spermatozoa extended in milk protein based extenders can be successfully cryopreserved at 48 hours after routine orhiectomy.

Key words*: stallion; epididymal spermatozoa; refrigeration.*

INTRODUCTION

Collection of spermatozoa from the cauda epididymis may be the last chance to preserve genetic material from valuable stallions in case of sudden death or emergency castration. Given the fact that only a limited amount of sperm is available, efforts have been made to establish the best processing method. Extenders have a great influence on epidydimal sperm motility and milk protein based extenders seem to offer better results (Stefanie Neuhauser, 2018). Using modern protocols and new extenders pregnancy rates are increasingly higher than those reported in 2002 by Moris et. Al. (30%). Pregnancy rates in recent studies are comparable to those obtained with frozen ejaculated sperm when using pasteurised egg yolk based extenders (Stawicki, 2016). One study demonstrates 93% pregnancy rates using epididymal spermatozoa cryopreserved immediately after collection (Monteiro, 2011). Furthermore, epididymal spermatozoa stored for 24h at 5°C were used to obtain 60% one cycle pregnancy rates (Papa, 2008). Epididymal spermatozoa stored for 48h at 5°C in the epididymis seems to offer good results regarding pregnancy rates (Stawicki, 2016).
Collection of the spermatozoa from the cauda epididymis is routinely done in laboratory from refrigerated testicles at a certain amount of time post castration. Laboratories equipped for the technique might not be in range for fast shipment and often 48h are needed for transport. This has lead to the necessity to develop viable techniques to harvest and preserve epididymal stallion spermatozoa at 48h post castration.

MATERIALS AND METHODS

Animals

The testicles and epididymides were obtained from 6 healthy stallions after routine castration. Stallions were aged 4 to 8 years and all testicles were grossly normal. The breeding history of the stallions was unavailable.

Epididymal storage and collection

After routine castration the deferent duct was identified and a ligature was placed around the cut end of each deferent duct. Testicles were transported and then stored at 5°C for 24h. All epididymides were flushed using the retrograde flush technique firstly described by Monteiro et al. (2011). at 24h after castration and the first assessment was performed. From each stallion, one epididymis was flushed using 5 ml of Gent extender (Gent®, Minitube) and the second one was flushed using 5 ml of Triladyl extender (Triladyl®, Minitube). Samples were centrifuged at 1000 x g for 10 minutes at room temperature and the supernatant was removed. The sperm pellet was resuspended in 5 ml temperature-matched of the same extender. All spermatozoa were stored at 5°C for another 24 hours when the second assessment was performed.

Sperm motility and viability

Sperm motility was determined using computer assisted sperm analysis (SCA®, CASA). Viability was assessed using the eosin staining technique. Each sample was analysed at 20 minutes after collection and 24h later.

For motility assessment the samples were prewarmed at 37°C and placed on a prewarmed chamber (Leja Standard Count 4 Chamber Slide 20 micron, Leja Products B.V., Nieuw Vennep, the Netherlands) on a heated microcope stage. Using computer assisted sperm analysis we determined concentration (CONC M/mL) and motility parameters of each sample using the following parameters: total motility (TMOT %), total progressiveness (TPROG %), rapid progressiveness (RPROG %), medium progressiveness (MPROG), slow progressiveness (SPROG), velocity rapid (VELR %), velocity medium (VELM %), velocity slow (VELS %). Average values of speed determined were: curve speed (VCL), linear speed (VSL), average value (VAP), linearity index (LIN), straightness index (STR), oscillation index (WOB). Other parameters we assessed were amplitude lateral head (ALH), beat frequency (BCF), hyperactive and mucous penetration.

For viability samples were prewarmed at 37°C and equal parts semen and prewarmed eosin were mixed. A smear was prepared from each sample and analysed under the microscope at 40x. 100 spermatozoa per sample were assessed and divided into 2 groups – green viable and red non-viable and final results were expressed in percentages.

RESULTS AND DISCUSSIONS

Spermatozoa suspended in a commercial skim mik-based extender (Gent®, Minitube) had at 24h post castration a mean TMOT of 74,22% whereas at 48h TMOT was 77,59%. TPROG at 24h was 9,6% and at 48h 17,97%. Velocity was at 24h VELR 5,96%, VELM 15,35%, VELS 52,89% and at 48h VELR 14.02%, VELM 16,46%, VELS 52,89%. Viability at 24 h was 93,7% and at 48h 87,5%.

Spermatozoa suspended in a commercial egg yolk-based extender (Triladyl®, Minitube) had much lower mean parameters both at 24h and 48h post castration. At 24h TMOT was 44,93% and at 48h 23,94%. TPROG at 24h was 3.33% and at 48h 2.41%. Velocity was at 24h VELR 1,64%, VELM 5,3%, VELS 37,98% and at 48h VELR 1.27%, VELM 4,33%, VELS 18,34%. Viability was at 24h 85,3% and at 48h 78%.

The aim of this study was to evaluate motility parameters and viability of epididymal stallion spermatozoa at 24 h post castration and storage in the epididymis at 5°C and at another 24h after storage at 5°C post collection using two commercial extenders. Triladyl is an egg yolk based extender destined for use in bull but it has been successfully used for stallion ejaculated spermatozoa as well (Blottner, 2001).

The results of this study show better kinematic values of epididymal spermatozoa when using a milk-based extender. Prolonged cooled storage of epididymides prior to collection of spermatozoa (48h) do not seem to decrease the kinematic parameters (Neuhausser, 2018). In the current study, spermatozoa were stored for 24h post collection at 5°C in one of the two extenders and the kinematic parameters improved significantly in the milk base extender.

In conclusion, spermatozoa cooled stored for 24h in the epididymis and another 24h in cooled extender show adequate quality and fertilizing capacity and could successfully be used for cryopreservation.

In this study we only used young healthy stallions and two commercial extenders that were commercially available. However, new extenders should also be tested for accuracy of the method on both healthy and pharmacologically treated stallions.

CONCLUSIONS

The milk based extender used in this study improved substantially the kinematic values of epididymal spermatozoa at 48h post castration while the egg-yolk based extender showed poor results at 48h. Furthermore, parameters were much better at 24h post castration in milk based extender. Therefore, we recommend using milk based extenders for epididymal spermatozoa at 24h and 48h post castration of healthy stallions.

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HEMATOLOGICAL AND PLASMA BIOCHEMICAL VALUES IN FOUR SPECIFIC PATHOGEN-FREE MICE STRAINS PRODUCED IN THE ANIMAL FACILITY AT CANTACUZINO INSTITUTE, BUCHAREST

Cristin COMAN[1], Ene VLASE[1], Angelica DINU[1]

[1],,Cantacuzino" National Medical-Military Institute for Research and Development, Animal Facility, 103 Splaiul Independentei, District 5, 050096, Bucharest, Romania

Corresponding author email: comancristin@yahoo.com

Abstract

Laboratory mice are still the most used animal in research and animal experiments. In most experiments, knowledge of hematology and biochemistry data base is essential to achieving quality results and used the research results. Cantacuzino Institute from Bucharest is the most important provider of laboratory mice in Romania and therefore making it available to the users the hematological and biochemical physiological data of the mice strains from his animal facility was an ethical obligation. Hematologic analysis and biochemical from blood were performed in 4 strains of mice (2 inbred and 2 outbred) in both sexes and age groups, in dynamics. Hematologic analyses were made from whole blood and biochemical ones from blood plasma. The results showed differences between the lines, sexes and age categories. The results obtained can be used by the researchers on selecting the mice strains according to the purpose of the research and according to the values of the hematological and biochemical parameters. The results thereby helping to reduce stress in animals.

Key words*: mice, hematological values, biochemical values.*

INTRODUCTION

Laboratory mouse is the most common mammals for scientific purposes, whether we are talking about research, monoclonal antibody production or drug and medical devices testing. This small mammal has several qualities that make it be so used: multiplying fast, short duration of generations, the existence of many inbred lines, knowledge of the genome and extensive knowledge of its physiological and immunological characteristics (Moore et al., 2000).

The determination of the hematological and biochemical profile of mice is necessary because it is possible to characterize the animal populations used in the research, to establish research results, to anticipate pathological conditions of the animal colonies and to monitor the effectiveness of a possible treatment (Mazzacara et al., 2008).

Each institution must establish the hematological and biochemical profile of its colonies because this profile is influenced by the strain of mice, age, health, nutrition, environmental conditions, etc. Reduction of variability is a standardization condition, and if environmental conditions (air changes, temperature, relative humidity, noise and illumination) are set by regulations, other variability factors can be avoided by providing stable livestock strains with a constant profile (O'Connell et al, 2015). Reference values may be for a researcher an important research tool to initiate studies, especially if the lab does not have enough historical data on the hematological and biochemical profile.

Cantacuzino Institute from Bucharest is the largest producer and supplier of laboratory animals in Romania. With the 4 strains of mice (2 inbred and 2 outbred) we provide the same feed that we give to the animals in our facility and the same type of bedding. Our main users of mouse strains are research institutes that used mice for immunological, metabolic, oncological studies, etc., using inbred mice (BALB/c and C57Bl/6 strain) and human and veterinary medicines manufacturing plants for product safety testing using outbred lines (CD1 and NMRI). The purpose of this study was to determine hematological values (22 determined and calculated parameters) and biochemical (6 metabolic profile parameters) in 4 strains of mice, 2 inbred (BALB/c and C57BL/6) and 2

outbred (CD1 and NMRI) in both sexes at the age of 8-9 weeks and 20-21 weeks, strain breaded at specific pathogen free (SPF) animal facility of Cantacuzino Institute, Bucharest.
Establishing the hematological and biochemical profile will help researchers to choose their mice according to their intended profile, ensure the validity and reproducibility of experiments and tests, and help implement 3R by avoiding repeat analyses at the start of studies and implicitly reducing stress in animals (Schnell et al., 2002). Establishment of the basic data line is very important in the evaluation of the result of nonclinical experiments, in the confidence of test results.

MATERIALS AND METHODS

Mice
The initial colonies with which our SPF animal facility was populated were bought by the Cantacuzino Institute in 2014 from Charles River Germany. The animals used in these studies were derived from 10-generation colonies. Mice were maintained under strict barrier conditions at animals without specific pathogenic germs, confirmed status by quarterly control performed according to the recommendations of FELASA (Mahler et al., 2014). There were four groups of animals, one for each strain analysed (CD1, NMRI, BALB/c and C57BL/6). Each group was composed of 40 animals, (20 male and 20 female) that provided the statistically significant number of the study. The groups were formed at the age of 8-9 weeks of the animals, when we did the first analysis. The second was done in the same animals at the age of 20-21 weeks. The mice from these lots were the negative control groups from 4 different experiments, studies approved by the Ethics Commission of Cantacuzino Institute and authorized by Veterinary Authority from Bucharest.
Housing and husbandry
The animals were kept in polysulfones cages, 10 mice in the cage, and were individually identified by ear punch. Food from the Cantacuzino Institute was administered *ad libitum*, as well as water that is acidified and is administered in bottles. Lighting system is 12 hours light/12 hours dark, temperature 20 ± 2^0 C, relative humidity 55 +/- 10%. Mice were daily evaluated for sanitary condition and weighed monthly.
Blood collection
Sample blood collection was done after a 12-14 hour diet. The harvesting method was the retro-orbital one. After total anesthesia with an equal combination of ketamine and acepromazine, in a final volume of 0.1 ml. a total approximate amount of 200 microliters was harvested, sufficient for both types of hematological and biochemical analysis.
Hematological parameters
For the hematological determinations the blood sample were harvested on EDTA. Analyses have been made on an IDEXX Procyte 5 Diff on the same day as the harvest.
The samples were analysed hematological, being determined direct or calculated values for the following parameters: red blood cells count (RBC), hematocrit (HCT), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets count (PLT), mean platelet volume (MPV), white blood cells count (WBC), neutrophils (Ne), lymphocytes (Ly), monocytes (Mo), eosinophils (Eo), basophils (Ba), reticulocyte (RETIC), platelet distribution width (PDW), platelet hematocrit (PCT).
Biochemical parameters
For biochemical analysis, blood was collected in vacutainers with lithium-heparin. The blood was centrifuged at 12.000 rpm for 2 minutes at 40C and then plasma was extracted. The determinations were made on an IDEXX VetTest Chemistry Analyzer apparatus. The parameters analysed are: Glucose (GLU), Uric Acid (URIC), Alanine aminotransferase (ALT), Aspartate Aminotransferase (AST), Cholesterol (CHOL), Triglyceride (TRIG). The analyses were done on the same day as the harvest.
Statistics
Statistical analyses were performed with Microsoft Excel, current version. In each mouse strain, the determination of the hematological and biochemical value were expressed as mean ± standard deviation (Mean ± SD) and also minimum and maximum values.

RESULTS AND DISCUSSIONS

The results of the analyses are highlighted in Tables 1-8.

Table 1. CD1 strain – hematology value

Parameter	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
RBC 10^12/L	9.13±0.44	7.11-13.2	8.91±0.29	7.67-11.6	9.52±0.24	7.91-13.97	9.12±0.29	7.42-13.4
HCT %	51.22±0.23	35.2-68.4	48.76±0.44	37.7-72.5	56.68±0.78	39.9-74.6	53.44±0.87	41.5-76.4
HGB g/dL	13.84±0.6	11.1-17.3	14.11±0.32	11.6-18.1	14.34±0.56	11.8-17.8	15.01±0.11	12.1-18.7
MCV fL	52.83±0.12	46.5-55	54.52±0.28	44.3-60.1	56.2±0.49	46.2-61.3	57.11±0.59	46.1-64.2
MCH pg	14.95±0.37	12.9-15.5	15.22±0.27	13.2-15.9	15.36±0.76	13.6-17.1	15.85±1.33	13.9-17.4
MCHC g/dL	23.47±1.21	19.1-26.3	24.17±1.82	20.2-27.9	24.53±0.87	20.4-28.1	25.11±0.91	22.6-29.7
RDW %	15.2±0.22	14.3-17.8	16.1±1.45	15.1-19.3	16.22±0.67	14.5-18.3	16.7±0.45	15.8-20.1
RETIC %	3.75±0.31	3.2-4.2	3.47±0.21	3.1-4.3	4.72±0.21	3.6-4.91	4.47±0.67	3.7-4.9
RETIC K/µL	316.85±17.39	288.4-351	349.23±11.21	291-366	347.8± 15.3	298.1-371.3	363.2±22.11	311-384
WBC 10^9/L	9.16±0.79	3.96-13.1	8.13±2.15	4.1-13.6	10.89±1.44	5.7-14.2	11.22±0.35	5.9-15.2
NEU %	25.11±3.32	16.2-31.2	22.39±1.87	17.3-28.7	23.97±2.76	17.9-32.5	20.76±1.96	14.5-25.4
LYM %	65.23±1.65	43.1-76.1	67.44±2.34	44.8-78.3	66.07±2.82	47.8-79.2	68.2±3.12	48.3-85.4
MONO %	8.36±1.42	3.9-12.7	9.02±0.62	4.2-12.8	8.11±0.56	4.9-13.9	9.61±2.42	5.8-14.5
EOS %	0.82±0.64	0.3-3.1	0.64±0.54	0.2-3.4	1.11±0.54	0.2-2.5	0.97±0.67	0.2-2.8
BASO %	0.48±0.72	0.01-1.3	0.51±0.64	0-1.26	0.74±1.11	0-1.2	0.78±1.02	0-1.03
NEU 10^9/L	2.56±0.28	0.24-4.87	2.41±1.16	0.36-5.12	3.45±0.87	0.45-5.23	3.65±1.22	0.65-5.38
LYM 10^9/L	4.97±0.95	2.43-9.87	5.27±1.46	2.11-10.2	6.61±1.78	2.97-10.4	5.76±1.65	2.65-10.4
MONO10^9/L	0.53±0.11	0.02-0.66	0.67±0.54	0.11-0.94	0.98±0.65	0.34-1.56	1.16±0.43	0.36-1.23
EOS 10^9/L	0.09±0.06	0.01-0.43	0.12±0.46	0.01- 0.6	0.15±0.67	0.01-0.79	0.17±0.65	0.03-0.82
BASO 10^9/L	0.02±0.003	0-0.09	0.03±0.01	0-0.12	0.02±0.02	0-1.4	0.04±0.01	0-0.09
PLT K/µL	1381.7±118.36	912-1891	1411±145.46	956-1986	1572±132.12	854-1954	1480±166.32	865-2164
MPV fL	5.11±0.11	4.9-5.68	5.27±0.26	4.3-6.3	4.87±0.58	4.2-6.1	4.71±0.64	4.09-6.22
PDW fL	7.19±0.41	6-8.1	7.54±0.43	5.9-8.4	7.45±0.65	6.01-8.23	7.12±0.42	5.91-8.11
PCT %	0.65±0.33	0.53-0.87	0.51±0.43	0.42-0.76	0.61±0.76	0.48-0.88	0.54±0.54	0.41-0.91

Table 2. CD1 strain – biochemical value

Parameter	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
GLU mmol/L	8.64±0.45	8.11-9.89	8.46±0.21	8.08-9.17	9.84±0.67	8.71-10.29	9.12±0.34	8.67-9.76
URIC µmol/L	121.21±0,3	118-135	110±0.76	97-123	134±0.94	119-142	129±1.54	107-145
ALT U/L	41.32±0.76	30-61	38±1.12	28-65	57±1.39	35-78	49.24±0.98	36-72
AST U/L	82.21±0.56	69-98	86.11±0.86	71-93	95±0.82	61-102	93±0.45	76-112
CHOL mmol/L	1.49±0,85	1.32-1.65	1.87±0.31	1.23-1.96	2.19±0,32	1.76-2.21	2.21±0.81	1.56-2.36
TRIG mmol/L	0.76±0.73	0.61-0.91	0.85±0.45	0.68-1.16	0.96±0.98	0.78-1.35	1.15±0.23	1.02-1.43

Table 3. NMRI strain – hematology value

Parameter	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
RBC 10^12/L	7.89±0.44	6.93-8.66	7.94±0.53	6.97-8.64	8.72±0.24	7.11-9.12	9.23±0.19	8.12-11.01
HCT %	37.23±1.98	33.1-40.1	36.93±2.67	31.1-40.8	46.66±1.48	36.2-56.9	49.87±1.35	39.6-64.3
HGB g/dL	11.87±0.54	10.6-12.6	12±0.76	10.8-13.3	13.24±0.61	11.1-16.9	15.1±0.5	11.9-18.4
MCV fL	47.22±1.65	44.2-50.4	46.5±1.2	44.3-49	56.1±1.23	46.8-63.7	55.34±0.82	49.2-67.1
MCH pg	15.06±0.52	14.2-15.9	15.11±0.38	14.8-15.6	15.9±0.36	14.6-17.4	16.25±.034	15.21-18.1
MCHC g/dL	31.90±0.67	31-33.2	32.53±0.79	30.9-33.5	34.13±0.37	32.8-39.7	34.12±0.45	32.1-40.2
RDW %	23.49±1.94	29.9-26.4	23.48±1.44	19.9-25.2	27.87±0.92	18.7-29.9	24.6±0.87	21.8-26.8
RETIC %	5.46±1.57	2.8-7.8	4.92±1.49	2.9-8.9	5.32±1.34	2.8-8.2	5.05±0.24	3.2-5.8
RETIC K/µL	432.42±29.84	224.9-632	391.75±27.82	194-569	443.32± 28	312.-498	423.2±24.5	376-497
WBC 10^9/L	7.77±2.08	4.53-13.8	7.33±1.97	4.63-10	9.12±1.53	4.97-12.9	9.12±0.95	5.65-11.87
NEU %	27.87±6.7	17.5-46	21.03±6.45	12-33.5	27.36±4.87	19.23-35	24.22±5.76	17.23-32.3
LYM %	67.02±7.44	45.6-77.5	73.70±6.87	59.6-84.9	67.77±7.33	59.12-79.	70.55±5.98	47.23-82
MONO %	2.33±1.31	1.2-7.1	1.8±0.81	1-4.3	2.12±0.54	1.8-8.2	2.19±1.87	1.2-9.7
EOS %	2.35±0.80	1.2-4.7	3.06±0.96	1.3-4.9	2.32±0.65	0.8-2.9	2.66±0.23	1.1-3.2
BASO %	0.42±0.31	0-0.9	0.4±0.21	0-0.8	0.44±0.23	0-1	0.38±0.26	0-0.8
NEU 10^9/L	2.14±0.73	1.15-4.09	1.46±0.37	0.9-2.06	2.45±0.65	0.76-5.76	1.87±0.65	1.15-3.4
LYM 10^9/L	5.22±1.58	2.76-9.58	5.5±1.8	1.98-7.49	5.61±1.01	2.17-10.3	5.93±1.11	2.11-10.65
MONO10^9/L	0.18±0.12	0.06-0.26	0.12±0.03	0.06-0.19	0.14±0.65	0.04-0.8	0.17±0.27	0.09-0.9
EOS 10^9/L	0.18±0.09	0.1-0.46	0.22±0.08	0.1- 0.37	0.17±0.07	0.08-0.53	0.25±0.21	0.08-0.52
BASO 10^9/L	0.03±0.02	0-0.09	0.03±0.01	0-0.06	0.02±0.03	0-0.1	0.04±0.04	0-0.1
PLT K/µL	440±186.65	171-819	452±155.26	213-707	932±152	345-1176	980±152	413-1236
MPV fL	7.28±0.30	6.8-7.8	7.04±0.48	6.5-8.4	7.11±0.71	5.2-8.9	7.23±0.87	4.75-9.3
PDW fL	8.43±0.88	7.1-10.1	7.83±0.95	6.5-10.1	8.22±0.15	7.23-9.9	7.96±0.39	6.26-9.12
PCT %	0.32±0.13	0.13-0.47	0.33±0.09	0.2-0.47	0.39±0.87	0.21-0.76	0.43±0.58	0.3-0.82

Table 4. NMRI strain – biochemical value

Parameters	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
GLU mmol/L	11.24±3.34	6.84-14.06	11.88±3.6	7.75-14.66	12.24±1.32	7.13-15.7	12.68±2.6	8.12-15.2
URIC µmol/L	66.88±33.93	12-122	49.73±31.19	19-164	98.12±23.93	65-187	87.73±26.65	65-191
ALT U/L	45±13.05	23-87	44±20.75	24-91	45±3.23	32-78	44±13.75	24-89
AST U/L	108.75±4.65	66-223	87.18±3.6	60-169	123.32±4.62	82-197	107.21±2.56	80-160
CHOL mmol/L	2.80±0.47	1.99-3.61	2.44±0.23	2.1-2.82	2.91±0.65	1.72-2.18	2.57±0.29	2.21-2.76
TRIG mmol/L	0.65±0.16	0.35-0.95	0.96±0.22	0.5-1.26	0.85±0.34	0.64-1.32	0.98±0.27	0.65-1.58

Table 5. BALB/c strain – hematology value

Parameter	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
RBC 10^12/L	9.01±0.64	7.87-11.96	10.12±0.59	7.11-11.8	9.89±0.74	7.21-12.7	10.54±0.28	7.42-11.4
HCT %	54.67±0.28	31.8-69.1	52.49±0.71	30.1-71.2	55.11±0.19	33.7-70.3	54.69±1.24	34.5-73.4
HGB g/dL	14.98±0.75	11.8-17.66	15.65±0.65	10.6-17.5	15.04±0.36	10.9-17.1	14.45±0.41	10.7-17.8
MCV fL	51.67±0.22	45.3-56.1	51.43±0.42	42.4-62.3	53.9±0.56	41.9-62.8	53.47±0.67	44.7-65.8
MCH pg	14.57±0.43	12.1-16.2	14.32±0.66	13.1-16.7	15.45±0.53	12.9-17.1	14.67±0.83	12.3-17.1
MCHC g/dL	24.87±1.68	19.6-29.88	24.48±2.45	19.4-33.1	25.44±1.83	20.9-31.2	25.78±1.44	21.2-30.5
RDW %	17.8±0.77	13.8-19.1	17.9±0.66	15.1-19.3	18.12±0.33	15.2-19.5	17.7±0.52	15.2-19.6
RETIC %	2.85±0.64	2.2-4.5	2.17±0.66	1.7-3.9	3.32±0.31	2.11-4.23	3.47±0.66	2.08-4.49
RETIC K/µL	310.42±36.76	257.2-366.8	328.76±28.55	278.9-378	328.64± 25	281-381	343.8±32.56	321-396
WBC 10^9/L	7.81±0.67	3.87-12.65	6.73±0.89	3.3-11.9	8.81±0.66	4.9-12.3	7.61±0.69	4.2-12.5
NEU %	26.71±4.51	18.7-34.8	24.13±2.33	16.7-24.6	26.22±1.87	18.6-29.7	23.66±1.11	16.9-27.3
LYM %	66.12±0.8	49.7-68.7	66.23±1.34	51.7-75.3	66.11±1.74	51.2-78.3	66.5±2.05	52.8-81.4
MONO %	6.18±1.89	4.2-9.4	8.45±0.87	5.3-10.2	6.28±0.49	5.3-9.3	8.66±0.87	4.8-10.2
EOS %	0.76±0.65	0.3-2.2	0.87±0.54	0.3-2.8	1.15±0.99	0.2-3.2	0.9±0.52	0.4-2.9
BASO %	0.22±0.89	0.01-1.4	0.31±0.47	0.01-1.11	0.24±0.11	0-0.9	0.28±0.38	0-1.02
NEU 10^9/L	2.12±0.26	0.34-3.17	1.91±1.52	0.25-4.71	2.35±0.93	0.41-5.11	1.57±1.78	0.41-5.34
LYM 10^9/L	5.28±2.67	1.98-6.17	4.77±3.83	1.67-9.02	6.02±1.65	3.18-10.2	4.75±1.32	2.11-9.42
MONO10^9/L	0.44±0.31	0.02-0.87	0.35±0.18	0.05-0.63	0.78±0.25	0.31-1.2	0.56±0.54	0.23-0.98
EOS 10^9/L	0.04±0.09	0.00-0.3	0.06±0.11	0.01- 0.43	0.08±0.17	0.00-0.42	0.09±0.55	0.02-0.76
BASO 10^9/L	0.01±0.004	0-0.08	0.02±0.01	0-0.9	0.03±0.01	0-0.87	0.02±0.01	0-0.87
PLT K/µL	1118±89.65	872-1532	1265±78.94	911-1652	1342±92.34	893-1756	1143±88.82	781-1846
MPV fL	4.65±0.09	4.2-5.11	4.87±0.22	4.2-5.67	4.94±0.78	3.9-6.3	4.99±0.83	3.87-5.76
PDW fL	6.88±0.38	5.7-7.9	7.13±0.67	5.8-8.7	7.13±0.98	5.67-8.26	7.87±1.1	5.82-8.67
PCT %	0.83±0.84	0.45-0.98	0.67±0.65	0.32-0.91	0.87±0.26	0.48-1.13	0.69±0.38	0.33-0.93

Table 6. BALB/c strain – biochemical value

Parameter	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
GLU mmol/L	8.11±0.25	7.34-9.66	8.23±0.28	7.93-9.01	9.12±0.72	8.21-9.76	9.32±0.56	8.73-9.87
URIC µmol/L	111.64±0,45	93-127	115±0.96	101-129	128±0.41	104-138	131±0.39	115-141
ALT U/L	44.19±1.36	33-72	42±1.88	32-68	49±3.43	29-81	48.14±2.18	33-78
AST U/L	90±1.55	76-104	86±0.57	76-101	95±3.72	69-111	95±0.76	81-118
CHOL mmol/L	1.28±1,84	1.12-1.76	1.37±1.68	1.11-2.54	1.99±2,67	1.14-2.98	1.85±0.67	1.35-2.41
TRIG mmol/L	0.97±0.74	0.85-1.98	0.95±0.87	0.75-1.76	1.46±1.78	0.82-1.87	1.26±0.84	1.06-1.87

Table 7. C57BL/6 strain – hematology value

Parameters	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
RBC 10^12/L	8.72±0.24	8.34-9.17	8.81±0.27	8.46-9.39	9.94±0.49	9.31-10.8	10.37±0.99	8.04-11.34
HCT %	37.22±1.23	35.4-39.6	38.12±1.23	36.8-40.8	42.1±1.92	39.9-46.1	44.41±4.84	33.4-50
HGB g/dL	12.44±0.4	11.8-13.1	12.88±0.37	12.5-13.7	14.37±0.67	13.4-15.5	15.43±1.38	12.2-16.9
MCV fL	42.64±0.52	42-43.8	43.23±0.38	42.6-43.8	42.36±0.6	41.9-43.8	42.77±0.87	41.5-44
MCH pg	14.25±0.27	13.9-14.5	14.61±0.11	14.5-14.8	14.46±0.1	14.3-14.7	14.89±0.14	14.8-15.2
MCHC g/dL	33.42±0.36	32.8-34	33.8±0.23	33.5-34.3	34.12±0.39	33.6-34.6	34.82±0.84	33.7-36.5
RDW %	22.79±0.67	21.9-24.3	22.42±0.42	21.7-23.3	26.03±0.91	24.7-27	25.15±2.09	20.1-27.1
RETIC %	3.75±0.31	3.2-4.2	3.62±0.25	3-3.9	2.79±0.26	2.6-3.3	2.91±0.31	2.2-3.4
RETIC K/µL	326.85±27.19	283.4-358	318.19±22.1	261-341	250.22±82	231.2-338	301.76±44.5	213.1-340
WBC 10^9/L	3.56±0.77	1.96-4.41	3.07±0.68	1.88-4.05	6.62±2.59	2.38-9.61	5.99±1.89	3.55-9.03
NEU %	21.61±3.82	14.2-28	22.61±5.11	15.7-28.9	20±10.07	9.9-39.5	22.22±9.42	9.3-38.1
LYM %	76.23±3.71	69.9-83.1	74.87±4.97	66.9-81.7	76.66±10.5	57.6-88.4	74.71±10.23	55.4-82.8
MONO %	0.87±0.4	0.5-1.9	0.95±0.21	0.6-1.3	0.89±0.33	0.4-1.6	0.87±0.39	0.3-1.6
EOS %	0.85±0.6	0.3-2.4	1.28±0.87	0.3-3.4	2.22±1.65	0.6-3	1.96±1.1	0.6-4.5
BASO %	0.44±0.32	0-1	0.29±0.24	0-0.7	0.23±0.16	0-0.5	0.23±0.14	0-0.5
NEU 10^9/L	0.77±0.25	0.44-1.17	0.71±0.28	0.46-1.26	1.14±0.37	0.62-1.72	1.23±0.41	057-1.86
LYM 10^9/L	2.71±0.55	1.47-3.33	2.28±0.45	1.34-2.73	5.26±2.44	1.37-8.19	4.57±1.8	2.71-7.66
MONO10^9/L	0.03±0.01	0.02-0.06	0.02±0.008	0.02-0.04	0.06±0.04	0.01-0.11	0.05±0.03	0.02-0.1
EOS 10^9/L	0.03±0.02	0.01-0.1	0.04±0.02	0.01-0.09	0.14±0.11	0.03-0.39	0.19±0.06	0.02-0.27
BASO 10^9/L	0.01±0.008	0-0.03	0.009±0.00	0-0.02	0.01±0.007	0-0.03	0.01±0.01	0-0.04

PLT K/μL	1191.7±208	902-1535	895.6±102.7	717-1050	970±126	778-1129	634.55±179	336-829
MPV fL	5.89±0.12	5.7-6.1	6.02±0.11	5.8-6.1	6.08±0.25	5.8-6.5	6.48±0.26	6.1-6.8
PDW fL	6.09±0.11	6-6.3	6.19±0.12	6-6.4	6.74±0.33	6.4-7.3	7.44±0.51	6.5-7.9
PCT %	0.70±0.12	0.53-0.91	0.54±0.06	0.44-0.63	0.59±0.07	0.48-0.71	0.41±0.11	0.22-0.54

Table 8. C57BL/6 strain – biochemical value

Parameter	8 – 9 weeks				20-21 weeks			
	Male		Female		Male		Female	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
GLU mmol/L	7.04±0.71	6.62-8.57	8.46±1.83	4.59-9.89	8.93±1.49	6.49-10.5	9.78±2.4	8.04-10.24
URIC μmol/L	59.36±17.48	33-87	74.1[b]±35.1	41-140	92.2[a]±38.39	31-139	82.8±45.66	27-159
ALT U/L	40.18±3.78	27-44	47.1±4.2	37-53	48.8±2.3	36-52	52.7±3.6	38-57
AST U/L	50±9.99	32-67	44.7±3.9	37-56	55.28±11.5	35-61	49.4±4.33	41-54
CHOL mmol/L	1.19±0.19	0.91-1.46	0.86±0.27	0.36-1.31	1.78±0.25	1.51-2.37	1.67±0.12	1.47-1.85
TRIG mmol/L	1.04±0.31	0.8-1.15	0.98±0.24	0.53-1.36	1.26±0.24	0.87-1.68	1.22±0.19	0.86-1.52

In CD 1 mice strain the red blood cells values are relatively similar, having small variations with sex and age. White blood cells parameters are higher in males, regardless of age. The values are higher at 20 weeks. Values in all biochemical parameters analysed are generally higher in males than females and higher with increasing age.

In NMRI mice strain red blood cells values are approximately the same. Also, white blood cells parameters are relatively similar, regardless of age. The blood platelets are higher at 20 weeks. Except glucose and triglycerides values, in all biochemical parameters analysed are generally higher in males than females and higher with increasing age. Standard deviation and the range are large in URIC, AST and ALT, which means either that the harvesting method is not the optimal one, or that these analyses should be done by other methods at this strain.

In BALB/c mice strain red blood cells values are relatively similar, regardless of age and sex. White blood cells parameters are higher in males, but lymphocytes are higher in female. The values are little modified with age. Biochemical values are generally higher in males than females and higher with increasing age.

In C57BL/6 mice strain red blood cells values are higher in female and grow with age. White blood cells values are higher in male and increased significant in age in both sex. The other parameters of white blood cells have similar value at different age. Blood platelets range is very large in both sex and ages. At this strain biochemical values are generally higher in females than males and higher with increasing age.

In all strain blood reticulocytes and platelets have a large standard deviation and very large limits which means that there is a variability in this study, variability determined either by the blood collection method or by the analytical method.

Based on the results of this studies it was conclude that our data could be comparable with those of literature sources (Loeb et al., 1999; Mitruka et al., 1997).

CONCLUSIONS

The main goal of these studies was to provide to the users of these mice strain with the range and the average of the hematological parameters and the main biochemical metabolic parameters. The reference value presented could be used as baseline in research, testing, health evaluation etc. Because microclimate, breeding and experimentation conditions differ from unit to unit, it would be ideal for each animal facility unit that provide laboratory mice to set its own values.

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VACCINES AND VACCINATION PROGRAMS USED TO ERADICATION AND CONTROL OF RABIES IN WILDLIFE

Vlad VUTA[1*, 2], Florica BARBUCEANU[1, 2], Gabriel PREDOI[2], Constantin VLAGIOIU[2]

[1] Institute for Diagnosis and Animal Health, Bucharest, Romania
[2] University of Agronomic Sciences and Veterinary Medicine-Faculty of Veterinary Medicine, Bucharest, Romania

*Corresponding author email: vladvuta@yahoo.com

Abstract

Rabies is a fatal viral zoonosis of the central nervous system of mammals. Until recently, rabies was predominantly in domestic dogs, although outbreaks were reports in wildlife. The implementation of dog mass vaccination resulted in the disappearance of dog-mediated rabies in Europe and North America, but the disease unexpectedly re-emerged in wildlife. Oral rabies vaccination (ORV) programs in wildlife are highly effective in control and eradication the disease. After years of successful vaccination campaigns, many previously infected countries in Western, Central and Northern European have become free of rabies. All rabies vaccines used for wildlife immunization are derivatives of the original SAD strain. Currently, five vaccine strains are authorized in Europe and all are derivatives of the original SAD strain: Sad Bern, SAD B19, SAG2, GASGAS and V-RG vaccine (Vaccinia Recombinant Glycoprotein). In our paper, we described and analyzed the main characteristics of available vaccines from Europe market for oral vaccination of wild animals, taking into account the performance and the quality features. In Western Europe, rabies has been eliminated using oral vaccination with all available vaccines. Nevertheless, worries still exist related to the residual pathogenicity of attenuated live vaccines that could induce rabies in certain conditions.

Keywords: *rabies, vaccines, foxes, wildlife.*

INTRODUCTION

Rabies is a Central Nervous System zoonotic disease, with the causative agent Rabies virus, the negative-sense single stranded RNA viruses of the Lyssavirus genus within the family *Rhabdoviridae*, distributed worldwide and found in terrestrial mammals causing between 37,000 and 87,000 human deaths annually (Virus Taxonomy 9th report, 2012; WHO Expert Consultation on Rabies, second report, 2013). In Europe the major reservoir of rabies are wild animals, especially red fox (*Vulpes vulpes*) (Cliquet, 2015). Extensive oral vaccination programs (ORV) with baits for red foxes have reduced the incidence of rabies in many Western European countries (Slate et al., 2009; Zienius et al., 2011).

All rabies vaccines used in oral rabies vaccination (ORV) programs of wild animals are based on live vaccine viruses. In the EU, all rabies vaccines need to fulfill the requirements of European Pharmacopoeia monograph, Rabies vaccine (live, oral) for foxes and raccoon dogs: efficacy, safety and stability and have to be licensed or registered. Therefore, the objective of this paper was to describe and analyze the main characteristics of available vaccines from Europe market for oral vaccination of wild animals.

MATERIALS AND METHODS

Oral Rabies Vaccination Programs

The first fox rabies vaccination field trial using vaccine baits was applied in Switzerland in 1978 (Steck et al., 1982).

The strategy of ORV initially used to red fox proved to be effective in raccoon dog populations as well in several countries (Finland, Baltic countries and Poland) where this animal has a significant role in rabies epidemiology. The first field trial for elimination of rabies in wildlife in areas with a significant raccoon dog population was carried out in Finland in 1988 (EFSA, 2015).

Same time, administrative and political borders may constitute barriers to the movement of foxes, but in most cases vaccination zones need to be clearly defined and vaccination cam-

paigns synchronized across these administrative lines.
Examples of cross-border re-infections are a lot (Schaarschmidt et al., 2002). These could be prevented by synchronizing control measures on both sides of political or administrative borders and if this is not possible, by the maintenance of an immune belt or buffer zone at the border.
Vaccination programs are required to be conducted and monitored by a scientific team dedicated and deeply involved to this task. The team needs to be trained in field surveys and use validated and accreditated laboratory methods for rabies diagnosis, titration of vaccines, evaluation of bait uptake by the target species, and rabies antibody level. The entire procedure, including bait distribution in the field, needs to be very carefully processed, followed and very well documented. Based on experience in previous oral rabies vaccination campaigns, it is considered crucial that vaccination campaigns must to continue for a period of at least two years after the last reported case of fox-related rabies. The classical pattern of two vaccination campaigns per year, carried out in spring and autumn, has been shown to be successful whatever the fox population density. This two times distribution frequency has been used in all European programs of oral vaccination that resulted in the elimination of rabies (Zanoni et al., 2000; Breitenmoser et al., 2000; Bruyère and Janot, 2000; Brochier et al., 2001; Besch, 2001). Spring distribution is preferably carried out in May or June in order to increase the efficient access of fox cubs to baits. However, early spring campaign carried out in March-April was also shown to be beneficial in Belgium, Luxembourg, and several German Bundesländer (Brochier et al., 1996; Brochier et al., 2001). Where snow is abundant, its melting may degrade the vaccine baits, and in this case is preferably performed before the snow starts to melt. Autumn distribution is organized in September or October (EC, 2002).
There are used 25 baits/ campaign/km2 with a distance between flight lines of 500 meters and 150 meters altitude, by avoiding the territories of localities, water surfaces, highways (Vuta et al., 2016a; Vuta et al., 2016c).
In general, vaccination is not advised to be carried out at temperatures below 0°C, because: frozen vaccine baits do not induce a sufficient immune response and the virus titre may decrease due to freezing-thawing cycles, except for VRG which has been found to remain stable in such conditions (Pastoret et al., 1996). Vaccination using attenuated rabies virus vaccines is not recommended during hot weather. At temperatures above 30°C, melting of the bait occurs and vaccine titer decreases.

Oral animal vaccines against Rabies
In the EU, rabies vaccines used for oral vaccination need to be registered and to comply with the requirements of the European Pharmacopoeia monograph (European Pharmacopoeia, 2005) and with national regulations for veterinary biological products, with particular aspect of efficacy, safety and potency of the vaccine virus and to genetic strain stability (EFSA 2015).
Vaccine baits should contain a biomarker (usually tetracycline) to monitor the bait up-take. Vaccines for ORV currently available and authorized in the EU market are made of the following strains: SAD B19 (live attenuated); SAD Bern (live attenuated); SAG 2 (live attenuated); V-RG (live recombinant); GASGAS (live recombinant).

RESULTS AND DISCUSSIONS

At a 45 days'time following each vaccination campaign, there shall be performed the hunting of foxes in order to assess the efficiency of vaccination, for this purpose, there shall be shot 4 foxes/year/100 km2. For the monitoring of vaccination campaign, there shall be taken samples of thoracic liquids in order to determine post-vaccinal antirabies antibodies and samples of mandible in order to determine vaccinal marker (Tetracycline) (Vuta et al., 2017) (figure 1).
All vaccine strains currently used for oral vaccination programs are modified live-virus vaccines and a live recombinant vaccine.

- SAD family strains vaccines

Modified live-virus vaccines are all derived of the original SAD (Street Alabama Dufferin) attenuated virus (which was isolated from a rabid dog in the Alabama (USA) in 1935), after that it was passaged in mouse brain cells (ERA strain); then it was adapted to BHK cell line by

various passages (SAD Berne). Lysvulpen vaccine (Bioveta, Czech Republic) is an attenuated SAD Berne vaccine. SADB19 and SAD P5/88 vaccines (Impfsoffwerk Dessau-Tornau are produced by several passages on cloned BHK cell line of the SAD Berne strain (Mahl et al., 2014). Those successive and continues selections from the original strain may produce hazardous and uncontrolled results, and variants may remain pathogenic for target and non-target species.

- Vaccine strains selected by monoclonal antibodies

SAG1 and SAG2 vaccines (Street Alabama Gif) (Virbac, France) are selected from the SAD Berne strain after one and two successive mutations of the arginine 333 codon, using specific antirabies glycoprotein monoclonal antibodies, in a site of the genome whose integrity is required for pathogenicity by the oral route (Lafay et al., 1994). SAG2 vaccine is the only rabies oral vaccine registered at the European Medicine Agency, for the moment.

- Live recombinant vaccine

VRG vaccine (vaccinia recombinant glycoprotein) is a vaccinia virus (Copenhagen strain) recombinant coding for the rabies glycoprotein gene from the ERA strain. The already attenuated Copenhagen strain was even more attenuated thanks to the replacement of the thymidine kinase gene by the cDNA of the rabies glycoprotein increasing rabies immunity (Ruprecht et al., 1992).

The vaccine strain SPBN GASGAS (Rabitec, IDT, Germany) is a recombinant rabies virus

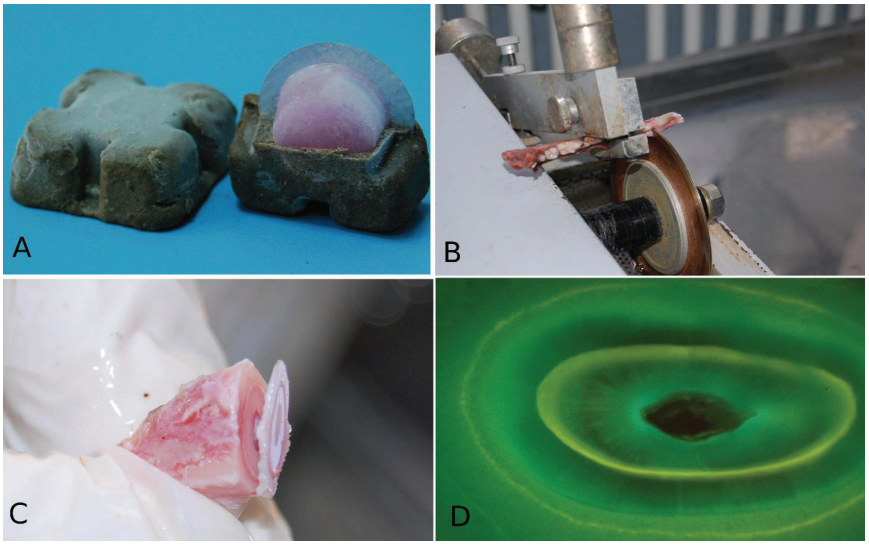

Figure 1. A-vaccine baits. B-special saw used to cut bones/teeth. C-thin bone/teeth section. D-fluorescent tetracycline biomarker deposit, detected by UV light microscopy

which has been derived by site directed mutagenesis from the most widely used oral rabies vaccine for wildlife, the passage attenuated vaccine strain SAD B19. The recombinant virus has been further attenuated compared to the SAD B19 strain by three targeted genetic modifications (EMEA, 2017).

The history of rabies vaccine-strains used in ORV programs is presented in figure 2.

Quality criteria of vaccine baits include stability testing of both vaccine (vaccine titer, genetic and thermo stability) and bait casing (appearance, melting point and temperature stability). As ORV campaigns are also to be conducted nearby human settlements, a number of minimum criteria and precaution measures have to be considered to minimize the risk of humans to come in contact with vaccine baits including mechanical stability of the vaccine bait, warning labels on blisters. Cold chain as specified by the manufacturer (usually -20°C or less) has to be maintained while vaccine baits are stored, during transportation and delivery directly to the customer. Maintenance of the cold chain

has to be documented using calibrated temperature loggers or equivalent equipment and written evidence has to be provided. Storage under any other conditions prior to distribution in the field may have high negative impact on the vaccine titer. The vaccine viral titer of all batches should therefore be verified right before the campaigns by the competent authority and approved laboratory. Such tests should be performed in qualified laboratories, preferably accredited to EN ISO/IEC 17025:2005 with documented, validated methods and standard operational procedures (EFSA, 2015).

The main characteristics of vaccines used in the EU have been summarized in Table 1 and Table 2.

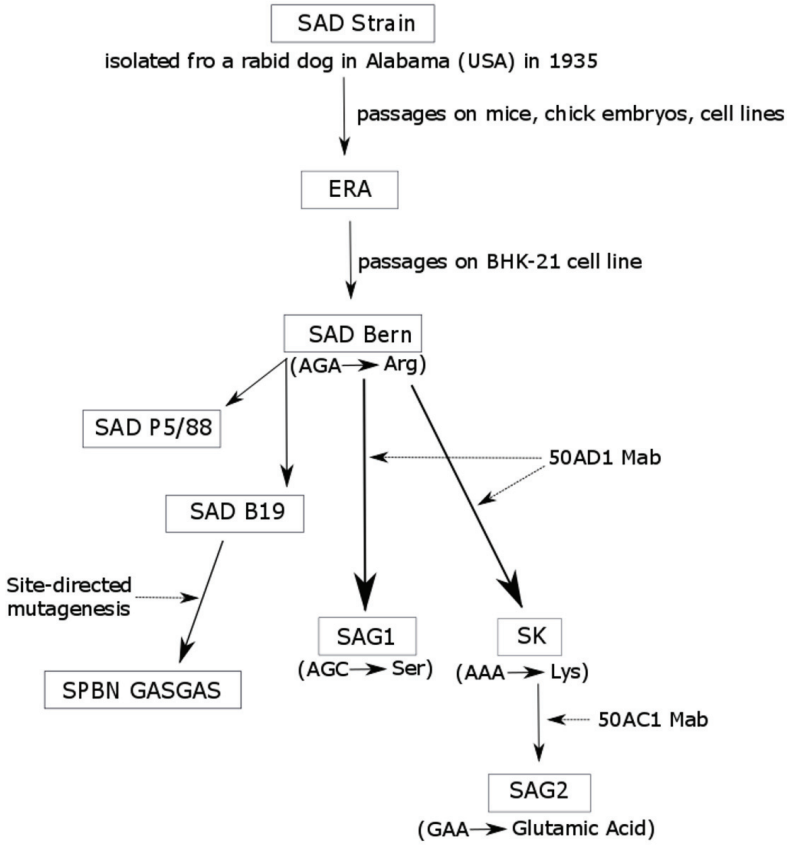

Figure 2. The history of rabies vaccine-strains used in ORV programs. ERA = Evelyn Rokitnicki Abelseth, Mab = monoclonal antibody. The rabies virus SAD strain was isolated from the salivary glands of a rabid dog in the USA during 1935, which was passaged in mice, chick embryos, and various cell lines and was re-named ERA (Evelyn Rokitnicki Abelseth). The SAD Bern strain is a cell line-adapted derivative from the ERA strain. The SAD Bern strain was cultivated using monoclonal antibodies binding specifically to one of the two major antigenic sites (antigenic site III) of the rabies virus glycoprotein, involved in pathogenicity. Under the selective pressure of these monoclonal antibodies, only variants of SAD Bern bearing an amino-acid substitution at the critical position 333 of the rabies virus glycoprotein escaped neutralisation in cell culture. An avirulent mutant, SAG1 (for SAD Avirulent Gif), in which arginine at position 333 was substituted by serine, was isolated from SAD Bern with monoclonal antibody (Mab) 50 AD1. The SAG2 strain was constructed from SAD Bern in a two-step selection procedure using neutralizing monoclonal antibodies. First, a mutant strain (SK) was selected from SAD Bern, where the arginine at position 333 was replaced by lysine. SAG2, a non pathogenic mutant resistant to neutralisation by monoclonal antibody 50 AC1 was selected from SK, where the lysine at position 333 was replaced by a glutamic acid. Therefore, SAG2 can be considered as a double avirulent mutant, since the codon GAA, which codes for glutamic acid, differs from the codon AGA from SAD Bern (coding for arginine) by two nucleotides. The vaccine strain SPBN GASGAS is a recombinant rabies virus which has been derived by site directed mutagenesis from the passage attenuated vaccine strain SAD B19. The recombinant strain has been further attenuated compared to the SAD B19 strain by three targeted genetic modifications.

Table 1. The main features of oral rabies vaccines used in the EU (Data compiled from manufacturers and EMEA)

Vaccine	**VRG**	**SAG2**	**SAD B19**	**SAD P5/88**	**SPBN GASGAS**
Proprietary name	Raboral	Rabigen	Fuchsoral	Rabifox	Rabitec
Company	Merial	Virbac	IDT	IDT	IDT
Quality					
Vaccine titer,	>8 log10 TCID50/dose	>8 log10 TCID50/dose	7 log10 FFU/ml	7 log10 FFU/ml	$10^{6.8}$ – $10^{8.1}$ FFU/1.7 ml
Thermostability, virus titre	Stable (time and temperature details not available)	0.16 log10 reduction after 2 days at 25°	0.4 log10 reduction after 7 days at approx. 25°C	0.26 log10 reduction after 7 days at approx. 25°C	The titer not decrease below $10^{6.6}$ FFU/ml after 5 days at approx. 25°C
Melting point of bait casing	> 50°C	43°C	35°C (new bait casing under development)	35°C (new bait casing under development)	Not mentioned
Safety					
Non-target species tested	**52**	approx. 30	approx. 20	approx. 15	approx. 7
Tested Horizontal transmission	None in foxes (adults and cubs), dogs, cats, cattle, ferrets	None in foxes, may be found in salivary glands of young dogs	None in foxes, rodents, skunks and dogs	None (no information on species)	May be found to the site of vaccine uptake i.e. the *tonsilla palatina*
No Reversion to virulence after	7 backpassages in mice (intracerebral and footpad), 10 backpassages in vero cell cultures, 1 backpassage in fox	5 backpassages in suckling mice	5 passages in foxes and 10 passages in suckling mice	10 passages in suckling mice	5 backpassages in NMRI mice
Efficacy					
Lowest protective dose tested	10^{7} TCID50/dose	$10^{8.1}$ TCID50/dose	$10^{6.0}$ log10 FFU/ml	$10^{6.2}$ log10 FFU/ml	$10^{6.8}$ FFU/dose (i.e. $10^{6.6}$ FFU/ml in 1.7 ml)

TCID-tissue culture infective dose; FFU-focus forming units; EMEA-European Agency for the Evaluation of Medicinal Products

Some modified-live rabies virus oral vaccines may have residual pathogenicity depending on the level of attenuation of the viral strain, as the successive selections from the original strain may produce hazardous and uncontrolled results, and variants may remain pathogenic both in target and non-target species (WHO, 2013).

Different animal species were involved in vaccine-induced rabies cases, such as foxes (Müller et al., 2009; Hostnik et al., 2014), raccoons, striped skunks and even castles (Fehlner-Gardiner et al., 2008; Vuta et al., 2016b; Vuta et al., 2017; Pfaff et al., 2018).

Table 2. The main results from safety trials carried out on target and non-target species using the VRG, SAG2, SAD B19 and GASGAS vaccine

Vaccines	**Carnivora**	**Rodents**	**Immunocompromised mice**	**Non human Primates**
VRG	No pathogenicity	No mortality	No mortality In 40 SCID mice (109 TCID50)	No pathogenicity for 11 chimpanzees (109PFU/ml) 24 Common squirrel monkeys (108PFU/ml) (Rupprecht,1992)
SAG2	No pathogenicity	No mortality	No mortality In 10 SCID mice (108 TCID50)	No pathogenicity for 10 baboons (109 PFU) (Bingham, 1997)
SAD B19	No pathogenicity in several species Pathogenic for Skunk at high doses (109 FFU) (Rupprecht, 1990; Vos, 2002)	Up to 6% mortality in several European wild species (Artois, 1992, Vos, 1999)	No mortality in 10 SCID mice (107.4FFU), mortality in 2/10 nude mice (107.3 FFU)	No pathogenicity for 12 baboons (108.3 FFU) (Vos, 1999)
GASGAS	No pathogenicity	No mortality	No mentioned	No mentioned

TCID-tissue culture infective dose; FFU-focus forming units; PFU-plaque forming units; SCID-severe combined immunodeficient mice

CONCLUSIONS

In Europe, oral vaccination by means of vaccine baits has been found to be successful in eliminating terrestrial wildlife rabies in most cases. However, the ultimate success of ORV campaigns requires a long-term strategy from competent authorities and cross-border cooperation between countries.

Rabies in wildlife was eliminated in those countries where the vaccination campaigns were planned on a national level and coordinated with neighboring countries.

The vaccines authorized in the EU for oral vaccination are Raboral (V-RG strain), Rabigen (SAG2 strain), Fuchsoral (SAD B19 strain), Lysvulpen (two dominant sub-populations of SAD Bern and SAD B19-'like' viruses) and a new vaccine, Rabitec (SPBN GASGAS) The efficacy of the existing vaccines is one of the factors that has contributed substantially to the success of rabies monitoring, control and elimination in several European countries. Very few vaccine-associated cases of rabies in the field have been reported and published so far. Nevertheless, worries still exist related to the residual pathogenicity of attenuated live vaccines that could induce rabies in certain conditions.

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