

EFFECT OF BALANCED ANAESTHESIA ON CANINE LYMPHOCYTE APOPTOSIS

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Abstract

It is well established that major surgery, anaesthesia, and sedation compromise a wide range of immune function that may predispose patients to postoperative infections, septic complications, and tumour spread.

The immunosuppressive effects of general anaesthesia are quite different and depend on the used anesthetic agents, dose and combinations. We tested the hypothesis that perioperative lymphocytopenia is due to apoptosis of these cells induced by balanced anaesthesia. The relation of lymphocyte apoptosis to the anaesthesiological stress and concentrations of the main pro- and anti-inflammatory cytokines was also investigated.

Based on the results we concluded that balanced anaesthesia used in the present study induced lymphocytopenia by activation of apoptosis of these cells which was due to the combined apoptogenic effects of halothane, fentanyl, and pancuronium, but neither to the anaesthesia-related stress-response nor to changes in the main pro- and anti-inflammatory cytokines TNF-alpha and IL-10. Total lymphocyte count was diminished on the expense of B-lymphocytes without significant changes in CD 5+ and CD 8+ cells.

Clinical implications: balanced anaesthesia disturbs normal humoral immune response by decreasing the count of B-lymphocytes for minimum 24 hours after anaesthesia.

Key words: *apoptosis, balanced anaesthesia, dog, lymphocyte.*

INTRODUCTION

It is well established that major surgery, anaesthesia, and sedation compromise a wide range of immune functions that may predispose patients to postoperative infections, septic complications, and respiratory dysfunction (Bolke et al., 2001). Many investigations reported lymphocytopenia induced by anaesthesia and surgery (Oka et al., 1996; Isitmangil et al., 2002; Yokoyama et al., 2005). Very few are studies on the immune effects of anaesthesia alone without surgery. The aim of our work was to explore the effect of balanced anaesthesia on some aspects of immunity with elimination of surgery related influences such as pain, tissue damage, blood loss and transfusion, organ dysfunction, inflammation.

The immunosuppressive effects of general anaesthesia are quite different and depend on the used anesthetic agents, dose and combinations. We investigated the hypothesis that perioperative lymphocytopenia is due to apoptosis of these cells induced by balanced

anaesthesia. Balanced anaesthesia was chosen because it has the advantages that combination of narcotic, opioid and muscle paralyzing agents enables to decrease the total dose of narcotic by 75%. Moreover, this kind of anaesthesia is very useful for cesarean section because a fetal respiratory suppression is minimal.

There are some discrepancies about induction (Matsuoka et al., 2001) or not (Ohara et al., 2005) of lymphocyte apoptosis by anaesthesia. Balance between cell survival and death is under strict genetic control. Deviations in any direction can lead to disease process. Decreased apoptosis is connected with the development of cancer (Bogler et al., 1995), autoimmunity disorders, or viral infections (Young et al., 1997). Excessive apoptosis is related to endotoxaemia, sepsis and multiorgan failure (Nielsen et al., 2005).

The relation of lymphocyte apoptosis to the anaesthesiological stress and concentrations of the main pro- and anti-inflammatory cytokines was also investigated.

MATERIALS AND METHODS

Anaesthetic protocol

The study was performed on 8 healthy mixed breed dogs, aged between 3 and 5 years, with mean body weight of 17.9 ± 3.5 kg, equalized in gender. In order to avoid an environmental stress the animals were kept under equal living conditions and fed on a commercial diet for a month. They received also antiparasitic drugs and were routinely vaccinated.

All dogs received one and the same scheme of balanced anesthesia that was achieved by a combination of halothane, pancuronium bromide, and fentanyl citrate. Atropine sulfas (Sopharma-Bulgaria, 0.02mg/kg.m, S.C.), and acepromazine maleate (Combistress, Kela-Belgium, 0.1 mg/kg.m, I.M.) were used for premedication. Twenty minutes later thiopentalum natrium (Biochemie GmbH-Austria, 10 mg/kg.m, I.V.) applied as a 2.5% solution was injected for induction of anaesthesia and endotracheal tube was inserted. Anaesthesia was maintained as follows: an inhalation agent halothane 0.5% in 100% oxygen was inhaled through a semicircuit respiratory system; a muscular paralyzing agent pancuronium bromide (Pavulon, Troyapharm-Bulgaria) was applied intravenously by 0.06 mg/kg.m initially with repetition of a half of the initial dose when a single spontaneous respiratory effort recurs; an opioide agonist fentanyl citrate (Stobium, Research Institute of Chemistry and Pharmacology-Bulgaria) was applied every 30 minutes I.V.) in dose 0.01mg/kg.m. Respiration was maintained by artificial ventilation of the lungs by oxygen flow of 20ml/kg and rate of 12 minutes⁻¹.

Depth of anaesthesia was maintained in surgical stage by tracing of some important unconditioned reflexes (eye globe in central position, on the average dilated pupils, lack of corneal, palpebral, patellar, anal, and swallowing reflexes). Venous line was created by cannulating of a cephalic vein using a venous catheter 22G, 25mm (Vygon GmbH & Co., Germany). The infusion rate of saline solution was 10ml/kg.m/h.

Total duration of anaesthesia was 120 minutes. After recurrence of four spontaneous respiratory movements 10 mg galanthamine

hydrobromide (Nivalin, Sopharma-Bulgaria) was used I.V. for recovery from neuromuscular blockade.

Extubation was performed after recurrence of the swallowing reflex. The animal was considered brought out of anaesthesia when it was able to raise his head and took sternal recumbence.

Blood sampling and processing

Venous blood samples were obtained immediately before (0 minute), during deep anaesthesia (120 minute), and on the next day (24 hour). The blood was withdrawn from v. jugularis, which was preliminarily cannulated. KF + Na₂EDTA 15mmol solution was used as an anticoagulant. Half of the collected blood was used for counting of lymphocyte numbers, measuring the proportion of apoptotic lymphocytes, and determining lymphocyte subpopulations. Plasma was separated from the second half of the collected blood after centrifuging 30 minutes by 1000 rpm and was stored at -25°C. Stored plasma was used later for quantifying of stress hormones and cytokine concentrations.

Total lymphocyte count was calculated from differential leukocyte formula on blood smear. The percentage of B-lymphocyte subset was found out by complement-zimozane indicative complexes that bind to the receptor of C3 component of the complement located on the surface of these cells (ZC-rosette test, Kajdavy-Balla & Mendes, 1976). Determination of percentage of apoptotic cells as well as T-lymphocyte subsets was performed after isolation of peripheral blood polymorphonuclear cells (PBMCs).

Collection of PBMCs

Isolation of PBMCs was performed under strict sterile conditions with the help of separation medium Histopaque with density 1.083 (Sigma Aldrich, St. Luis, MO, USA). Firstly, 2.5ml whole blood was diluted 1:1 with saline solution. After that 3ml of Histopaque was placed in tube and 4ml of diluted blood was deposited. Centrifugation in 1880 rpm for 40 minutes was accomplished and stratification was attained from the bottom to the surface as follows: red and white blood cells, separation medium, thin ring of lymphocytes, and finally plasma. The lymphocyte ring was fully drawn out with pipette, washed three times with

RPMI-1640 medium (Sigma Aldrich, St. Luis, MO, USA), and centrifuged for 10 minutes by 1500 rpm.

Annexin V-FITC-Propidium iodide staining and flow cytometry

Apoptotic lymphocytes were determined by the method of Vermes et al. (1995). This method uses the property of Annexin V to bind to the membrane phospholipid phosphatidilserine in the presence of calcium. Phosphatidilserine is expressed on the cell surface very early in apoptosis, before DNA fragmentation to occur. A percentage of the cells stained with FITC conjugated-Annexin V (Apoptest, Dako Cytomation, Denmark) from total lymphocyte count were determined by flow cytometry analysis (EPICS flowcytometric analyzer Beckman Coulter Inc., USA). The translocation of phosphatidylserine from the inner side of the cell membrane to the outer layer occurs also in necrotic cells. To distinguish cells that had lost membrane integrity, propidium iodide dye was added before analysis. Necrotic and late apoptotic cells are stained with propidium iodide (PI), a DNA-binding dye, because of the loss of the cell membrane integrity. Therefore, AV (+) /PI (-) cells are regarded as early apoptotic, AV (-) /PI (-) as vital, AV (+) /PI (+) as late apoptotic or secondary necrotic cells, and AV (-) /PI (+) as primary necrotic cells.

Lymphocyte subsets were analyzed using FITC-conjugated rat monoclonal antibodies

specific anti-canine CD-5 clone and CD8-clone (Bio Source International Inc., USA).

Determination of stress hormones and cytokines

Plasma concentrations of the cytokines tumour necrosis factor-alpha (TNF-alpha) and interleukine - 10 (IL-10) were measured by immunoenzymatic assay ELISA (Sunrise reader, Columbus washing mashine, and Magellan V3.11 software) using mice monoclonal antibodies specific for canine TNF-alpha and IL-10 (R& D Systems, USA).

Plasma adrenaline and cortisol levels were determined by radio immune assay using RIA kits (Amersham Biosciences, UK).

Statistical analysis

Statistical significant differences between three investigated periods were assessed by analysis of variance ANOVA/LSD (Statmost for Windows, DataMost Corp. 1994-1995) and probability lower than 0.05 was considered to be significant.

RESULTS AND DISCUSSIONS

Our results revealed that balanced anaesthesia with halothane, pancuronium, and fentanyl lead to decreased lymphocyte number on the expense of B-lymphocytes without changes in total T-lymphocyte number and T-suppressor cells (table 1).

Table 1. Changes in total lymphocyte count, percentage of apoptotic PBMCs, lymphocyte subpopulations, plasma concentrations of stress hormones and cytokines TNF-alpha, and IL-10 before, during and after balanced anaesthesia in dogs. Data are presented as mean ± SD.

Parameter	0 min	120 min	24 h
Lymphocytes, G/L	4.30± 0.56	1.46± 0.27*	4.42± 0.42
Apoptotic PBMC,%	1. ± 1.79	1. ± 1.52 ***	1. ± 1.70*
B-lymphocytes,%	2. ± 2	2. ± 1***	2. ± 1***
CD 5+,%	3. ± 12.4	3. ± 12.6	3. ± 6.1
CD8+,%	4. ± 7.4	4. ± 8.6	4. ± 9.7
TNF-alpha, pg mL ⁻¹	0.0± 0.0	0.84± 2.06	0.0± 0.0
IL-10, pg mL ⁻¹	36.17± 43.35	36.12± 45.90	36.44± 40.93
Adrenaline, ng mL ⁻¹	25.9 ± 4.3	51.7 ± 5.0***	31.3 ± 4.6*
Cortisol, nmol L ⁻¹	74.5± 40.7	99.1± 68.1	31.9± 25.3*

*p< 0.05, **p< 0.01, ***p< 0.001 versus initial period;

Balanced anaesthesia induced apoptosis of PBMCs that lasts at least 24 hours (figure 1).

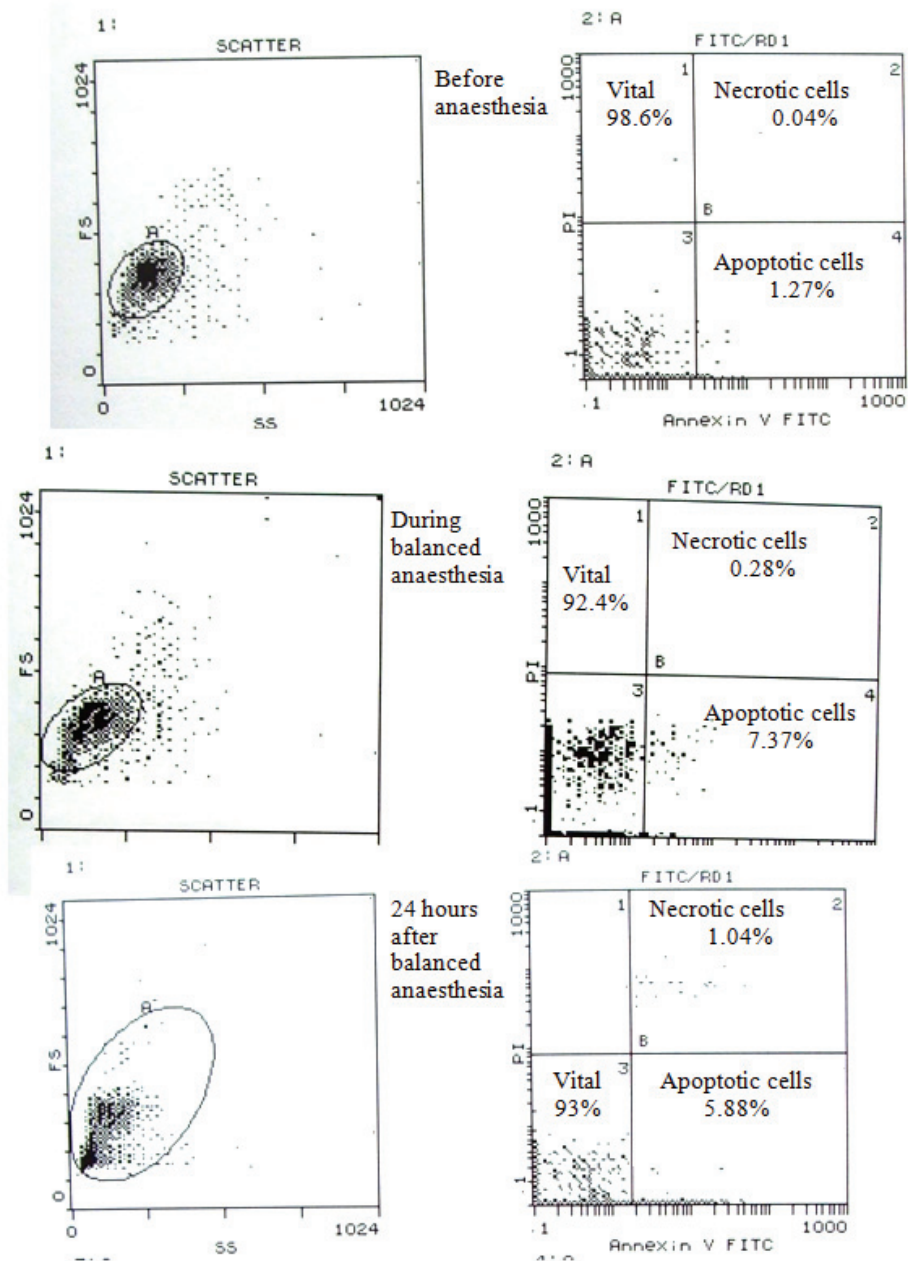


Figure 1. Flow cytometry report of discrimination between vital, early apoptotic, and secondary necrotic lymphocytes using Annexin V FITC/Propidium iodide staining in a dog immediately before, at 120 minute of balanced anaesthesia, and 24 hours after anaesthesia.

Cytokines did not show any alterations during and after anaesthesia. Plasma concentration of adrenaline increased at 120 minute of anaesthesia but with the tendency to decrease on the next day. Cortisol increased

insignificantly during anaesthesia, but 24 hours later its concentration dropped below the initial level.

Balanced anaesthesia that we performed on dogs was comprised by halothane, fentanyl and

pancuronium after induction with thiopental sodium. Barbiturates as anaesthesia-inducing agents have been reported to have a cytotoxic action on lymphocytes *in vitro* (Trowell, 1958) but at clinical dose this effect appears to be negligible.

Almost all inhalation anaesthetics induced apoptosis of normal peripheral lymphocytes *in vitro* by time and dose-related manner (Matsuoka et al., 2001; Loop, 2005). These investigations can not be extrapolated in clinical environment because they are accomplished *in vitro* on human and mice cells but not on canine cells.

Yamada et al. (2001) carried out a similar to our study on dogs anaesthetized with thiopental and halothane or isoflurane without any surgery. They concluded that both anaesthetics induced lymphocytopenia that was due to increased apoptosis of these cells.

There are several evidences that opioids modulated cell survival and death (Wang et al., 2002; Ohara et al., 2005). Immune competent cells express opioid receptors thus under the impact of the opioids they submit to apoptosis (Tegeder & Geisslinger, 2004). Fentanyl showed a strong apoptogenic effect on isolated peripheral lymphocytes *in vitro* (Delogu et al., 2004). In this research fentanyl initiated time-dependant lymphocyte apoptosis by alterations in their membrane redox metabolism. In another study Delogu et al. (2003) revealed that incubation of lymphocytes with clinically applicable concentrations of pancuronium also induced apoptosis of these cells.

Apoptosis is a genetic controlled process that could be induced by several activators such as stress, reactive oxygen species (ROS), and cytokines by three different main ways: binding of TNF-alpha to the specific receptors, mitochondrial mechanism or by apoptosis-inducing factor (AIF).

One of the most potent inducers of apoptosis is ROS. In our previous study we did not find an increased plasma malondialdehyde levels as a parameter of oxidative stress (Simeonova et al., 2004) thus we excluded the possibility of ROS-inducible lymphocyte apoptosis during balanced anaesthesia.

Direction and the strength of the immune response could be affected by cytokines. They work on an apoptosis of the cells by different

ways (Matsuda et al., 2001). IL-10 increased the viability of the cells by increasing the expression of protective molecule Bcl-2 and thus suppressing apoptosis (Cohen et al., 1997). Delogu et al. (2001b) found out that increased production of IL-10 during anaesthesia and surgery had been in correlation with the increase apoptosis of T-lymphocytes. According to our results induction of lymphocyte apoptosis by balanced anaesthesia was not in relation to IL-10 because we did not observe any statistical significant changes in its plasma concentrations. Moreover, there were not any alterations in the levels of TNF-alpha which is considered as a direct inductor of apoptosis and these results were contrary to the data of Kotani et al. (1999) who claimed that inhalation anaesthetics halothane, isoflurane, enflurane, and sevoflurane increased gene expression of proinflammatory cytokines including TNF-alpha. Obviously, the rise in pro-inflammatory cytokines has been referred to be due to the stress response to surgery but not to anaesthesia alone. The increase in concentrations of proinflammatory cytokines TNF-alpha, IL-1, and IL-6 was approximately proportional to the severity of the surgery (Kumar et al., 2002).

After examination the mechanism of stress-induced apoptosis Fumarola & Guidotti (2004) concluded that different cell types engage more than one intracellular signaling pathway for induction of apoptosis. Stress-related apoptosis is mediated by the insufficient expression of the protective molecule Bcl-2. Most of the investigations have been performed upon concomitant anesthesiological and surgical stress. We did not perform any surgery in order to avoid the influence of surgery on the immune function. In our research the anesthesiological stress response was connected with the increase plasma levels of adrenalin but not of cortisol. The endocrine parameter that has a central role in modulating cytokine synthesis and lymphocyte migration is cortisol (Wilckens and De Rijk, 1997). Glucocorticoids enhance IL-10 production which is known to inhibit T cell function. Cortisol decrease up to 2 hours in response to single fentanyl application (Hoehe et al., 1988) which explains the lack of cortisol-based stress response in our balanced anaesthesia. In

contrast, both epinephrine and norepinephrine inhibit the production of TNF-alpha while increasing the production of IL-10 (Munford and Pugin, 2001).

According to Papadima et al. (2009) combined general and epidural anesthesia failed to blunt the increase in lymphocyte commitment to apoptosis caused by surgical stress. They also concluded that decrease in total lymphocyte count and increase in percentage of the late apoptotic lymphocytes correlated positively with the increased cortisol levels. Immunosuppressive effects of surgical trauma and anaesthesia reported Yamada et al. (2002). Their results showed an increased lymphocyte apoptosis in dogs induced by general anaesthesia and surgery that correlated with the increased plasma cortisol levels. This data are not in accordance with our results because we did not find any correlation between plasma cortisol concentrations and lymphocyte apoptosis during balanced anaesthesia. Moreover, the latter authors revealed a greater increase in apoptosis of T-lymphocyte population compared with the B-lymphocyte because of a bigger susceptibility of T-cells to corticosteroids than B-lymphocytes. The reason for that difference was probably due to the influence of opioid fentanyl in our study. In vivo animal models have shown that opiate agonist suppress both humoral and cell-mediated immune response (Bayer et al., 1990; Bryant et al., 1992). In clinical conditions, however, fentanyl may suppress mostly humoral immune response.

CONCLUSIONS

In conclusion, balanced anaesthesia used in the present study induced lymphocytopenia by activation of apoptosis of these cells which was due to the combined apoptogenic effects of halothane, fentanyl, and pancuronium, but neither to the anaesthesia-related stress-response nor to changes in the main pro-and anti-inflammatory cytokines TNF-alpha and IL-10. Total lymphocyte count was diminished on the expense of B-lymphocytes without significant changes in CD 5+ and CD 8+ cells.

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