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INVESTIGATIONS ON *SORGHUM BICOLOR* SAPONINS-INDUCED CHANGES IN OSMOTIC FRAGILITY OF HUMAN AND BOVINE ERYTHROCYTES

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ABSTRACT

The in-vitro osmotic fragility of human and cow erythrocytes induced by *Sorghum bicolor* saponins was investigated. The *Sorghum bicolor* saponin caused osmotic fragilities in human and cow erythrocytes. The osmotic fragilities were found to be dose dependent and decreased with increasing concentrations. Erythrocyte osmotic fragility was higher in the sample exposed to 0.8 mg.ml^{-1} of the standard saponin than in the sample exposed to 1.6 mg.ml^{-1} of the standard saponin especially at NaCl concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.75, 0.8, and 0.85%. In the sample treated with 1.6mg.ml^{-1} of the sorghum saponin however, the erythrocytes were more resistant to osmotic lysis than those treated with either 0.8 or 1.6 mg.ml^{-1} of standard saponin especially at NaCl concentrations of 0.7, 0.75, 0.8, 0.85 and 0.9%. The bovine erythrocytes exposed to 1.6 mg.ml^{-1} of standard saponin were more stable in hypotonic solution than those treated with 0.8 mg.ml^{-1} of the standard saponin at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.75, 0.8 and 0.85% NaCl concentrations. When sorghum saponin was however introduced to the bovine erythrocytes, the erythrocyte osmotic fragility decreased considerably, in a manner that is similar to that of the human erythrocytes, especially at 0.5, 0.6, 0.7, 0.75, 0.8, 0.85 and 0.9% NaCl. Saponin increased the erythrocytes osmotic fragility of both human and bovine erythrocytes at 0.6, 0.7, 0.75, 0.8, 0.85 and 0.9% NaCl concentrations. However, bovine erythrocytes were more stable in hypotonic solution in the presence of saponin than were the human erythrocytes especially at sodium chloride concentration of 0.4, 0.5, 0.6, 0.7, 0.75 and 0.8%.

Key words: cow; erythrocytes; human; osmotic fragility; saponins; *Sorghum bicolor*

INTRODUCTION

Saponins represent a group of natural compounds, widely distributed in the plant kingdom, which display a number of biological properties (2).

Saponins (as triterpene or steroid glycosides) are naturally occurring products of high pharmaceutical importance characterized by producing foaming activity with water and haemolysed red blood cells (31). Saponins have the ability to cause lysis of erythrocytes, a property that have been employed by several authors to detect the presence of saponins in drugs. By measuring the haemolytic activity, their concentrations can be estimated (5). In general, the change in absorbance of the supernatant of an erythrocyte suspension is measured after haemolysis by a saponin or a saponin-containing drug (5).

Saponins are amphiphilic in nature. The chemical structure of saponins from different species of Medicago has especially being studied. They generally consist of a mixture of triterpene glycosides with medicagenic acid, hederagenin, Zhanic acid or soya saponenols as the main aglycones (2).

Saponins from medicago species are reported to have various biological and physiological effects like antimicrobial, fungistatic, allelopathic, cholesterol binding, cytotoxic, insecticidal, membrane depolarizing and haemolytic properties (32). Until recently, haemolytic structure-activity relationships of saponins have been based on the nature of the aglycone (35). Voutquenne *et al.* (34) reported on the influence of the sugar moiety on haemolytic activity and the synthesis of a-Hederin and related triterpene saponins. Some of the biological effects of *Sorghum bicolor* saponins have been reported by Lasisi *et al.* (15) and Soetan *et al.* (30). In continuation of

the studies on the biological effects of saponins in plants used as human foods and animal feeds, this paper examines the *in vitro* osmotic fragility of human and cow erythrocytes induced by *Sorghum bicolor* saponins.

MATERIALS AND METHODS

The *Sorghum bicolor* (Guinea corn) used for this study was obtained from the Bodija market, Ibadan in Nigeria.

Preparation of crude saponin extracts

The sorghum seeds were sun dried. The dried seeds were ground once using an electronic grinder. Nine hundred grams of sorghum seeds were exhaustively separated for 10 hours in a Soxhlet extractor using hexane (boiling range 68–69 °C). This removed the lipids and other pigments. The solvent was then changed to methanol (boiling range 64–65.5 °C) and the extraction was continued for the next 12 hours. This removed the saponins, together with low molecular weight substances or compounds such as sugars, phenolic compounds, oligosaccharides and flavonoids (10). The resulting solutions were evaporated to dryness to yield 32 g methanolic extracts. The presence of saponins in these methanolic extracts was detected by the characteristic frothing tests and thin layer chromatography.

In order to obtain partially purified saponins, the methanolic extracts were loaded on to a column of RP powder (Octadecyl slane bonded to silica gel particle size 15–25 YM JT Baker, Germany). The column was washed with water to remove the sugars and oligosaccharides while further elution with 30 % methanol (v/v) removed the flavonoid and other phenolic compounds. Subsequent elution with 100% methanol removed the saponins (10, 12). This yielded 24 g of crude saponins. The saponin extracts were dispensed into clean sterile bottles and stored in the refrigerator at -4 °C.

Confirmation of the presence of saponins in the purified fraction

The saponin extracts were subjected to thin layer chromatography (TLC) on silica gel plates (0.25 mm silica gel) using the solvent system methanol/distilled water (4 : 1). The developed plates were dried at room temperature. The visualization of saponin on the developed plates was done by spraying with 50 % (v/v) sulphuric acid. The sprayed chromatographs were allowed to dry for 15 minutes at room temperature and then heated at 105 °C for 3 minutes in an oven until the colour developed reached its maximum. The frothing test was done on the basis that the aqueous solutions of saponins form very stable foams. One ml of the concentrated methanolic solution of the extract was shaken with 5 ml of distilled water in a test tube. Formation of stable foams confirmed the presence of saponins.

Collection of blood samples

Fresh blood samples (10 mls) were obtained from the jugular vein of a one year old cow (white Fulani breed) and 6 ml blood samples were also obtained from a healthy adult human female through the ulna veins. The genotype of the human blood was haemoglobin HbAA and the blood group was O^+.

The blood samples were put into sample bottles containing 2 $mg.ml^{-1}$ ethylene diamine tetra acetic acid (EDTA) as the anticoagulant. The red blood cell (RBC) count was determined by the haemocytometer method; the packed cell volume (PCV) by the microhaematocrit method; and the haemoglobin (Hb) concentration by the cyanmethaemoglobin method. From the values obtained, the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated (28).

The erythrocyte osmotic fragility test was performed in the human and bovine blood samples as described previously (8, 23) using the following procedures. Five ml each of different concentrations of 1% phosphate buffered sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$ 0.14 $mg.ml^{-1}$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$ 0.23 $mg.ml^{-1}$), 1.6 $mg.ml^{-1}$ standard saponins and 1.6 $mg.ml^{-1}$ *Sorghum bicolor* saponins solution were prepared in 12 centrifuge tubes. The standard saponin was of analytical grade (Sigma Chemical Co, Ltd).

The pH of the buffered solution (7.4) was determined using a pH meter (Mettler Toledo MP 220). To each of the 1.6 $mg.ml^{-1}$ standard saponin and the 1.6 $mg.ml^{-1}$ *Sorghum bicolor* saponin solutions, 50 µl of a fresh blood sample was added. The contents were mixed gently and kept at 37 °C for 30 minutes and then centrifuged at 2, 500 revolutions/minute for five minutes. The supernatants were removed and the haemoglobin content determined spectrophotometrically using a spectrophotometer (Spectro sc Labomed, Inc.) at wavelength of 540 nm. The osmotic fragility curve was obtained by plotting the percentage haemolysis against the concentration of the phosphate buffered NaCl solution, using the tube with the highest fragility (0.0 % NaCl) as 100 % lysis.

RESULTS

Fig. 1 shows the osmotic fragility of human erythrocytes exposed to the standard saponin and the sorghum saponin. The erythrocyte osmotic fragility was higher in the sample exposed to 0.8 $mg.ml^{-1}$ of the standard saponin than in the sample exposed to 1.6 $mg.ml^{-1}$ of the standard saponin especially at NaCl concentrations of 0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5 %, 0.6 %, 0.7 % and 0.75 % NaCl concentration. In the sample treated with 1.6 $mg.ml^{-1}$ of the sorghum saponin however, the erythrocytes were more resistant to osmotic lysis than those treated with either 0.8 or 1.6 $mg.ml^{-1}$ of standard saponin especially at NaCl concentrations of 0.7 %, 0.75 %, 0.8 %, 0.85 % and 0.9 % NaCl concentration.

Similarly, as shown in (Fig. 2), the bovine erythrocyte exposed to 1.6 $mg.ml^{-1}$ of standard saponin were more stable in hypotonic solution than those treated with 0.8 mg. ml^{-1} of the standard at 0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5 %, 0.6 %, 0.7 %, 0.75 %, 0.8 % and 0.85 % NaCl concentrations. When sorghum saponin was however introduced to the bovine erythrocytes, the erythrocyte osmotic fragility decreased considerably, in a manner that was similar to that of the human erythrocytes, especially at 0.6 %, 0.7 %, 0.75 %, 0.8 %, 0.85 % and 0.9 % NaCl concentrations.

As shown in Fig. 3, sorghum saponin increased the erythrocyte osmotic fragility of both human and bovine erythrocytes at 0.5 %, 0.6 %, 0.7 %, 0.75 %, 0.8 %, 0.85 % and 0.9 % NaCl concentrations. However, the bovine erythrocytes

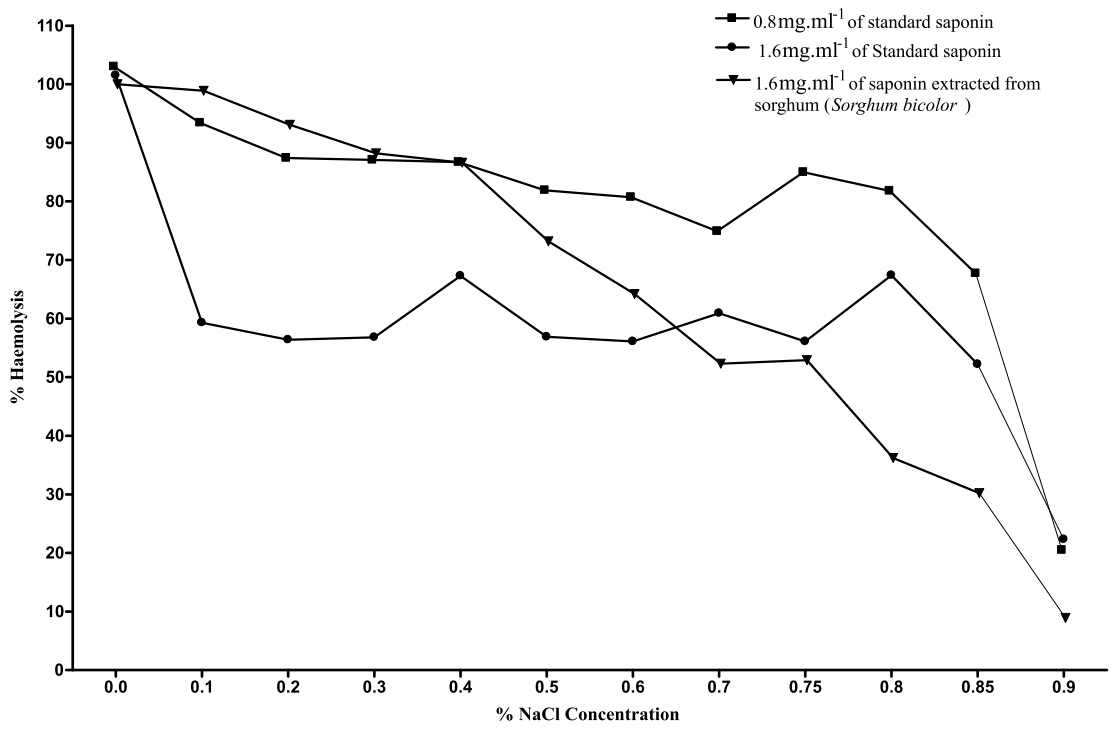

Fig. 1. Osmotic fragility of human erythrocytes exposed to 0.8 mg.ml^{-1} and 1.6 mg.ml^{-1} of standard saponin and 1.6 mg.ml^{-1} of saponin extracted from sorghum *(Sorghum bicolor)*

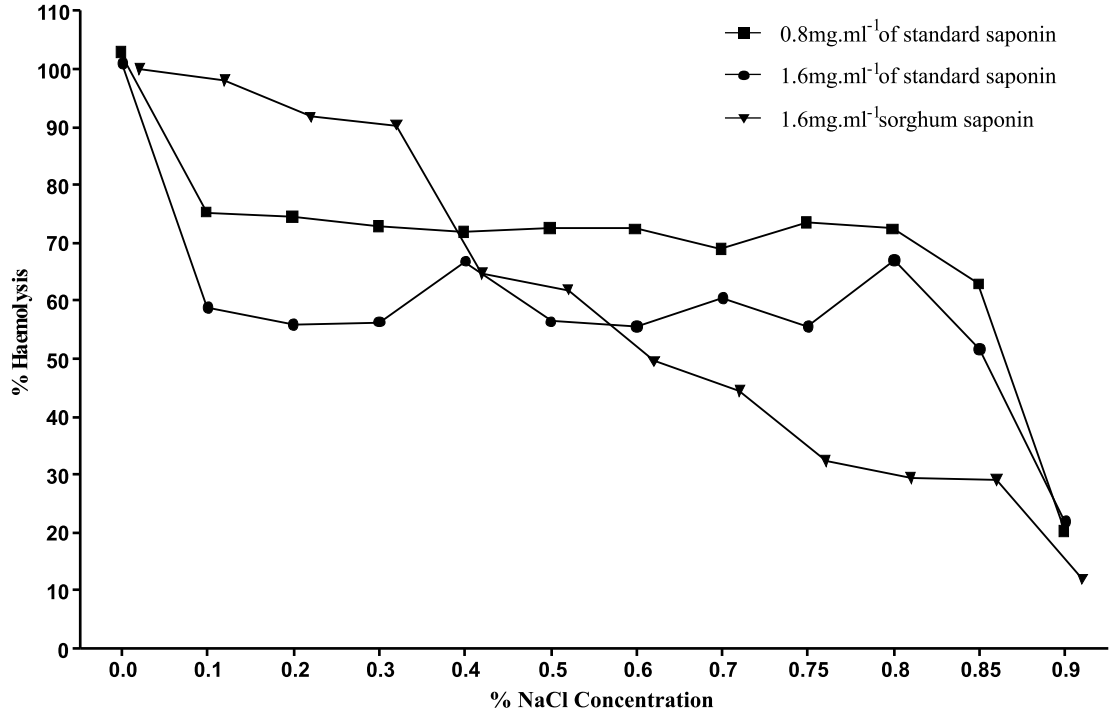

Fig. 2. Osmotic fragility of the bovine erythrocytes exposed to 0.8 mg.ml^{-1} and 1.6 mg.ml^{-1} of standard saponin and 0.8 mg.ml^{-1} of saponin extracted from sorghum *(Sorghum bicolor)*

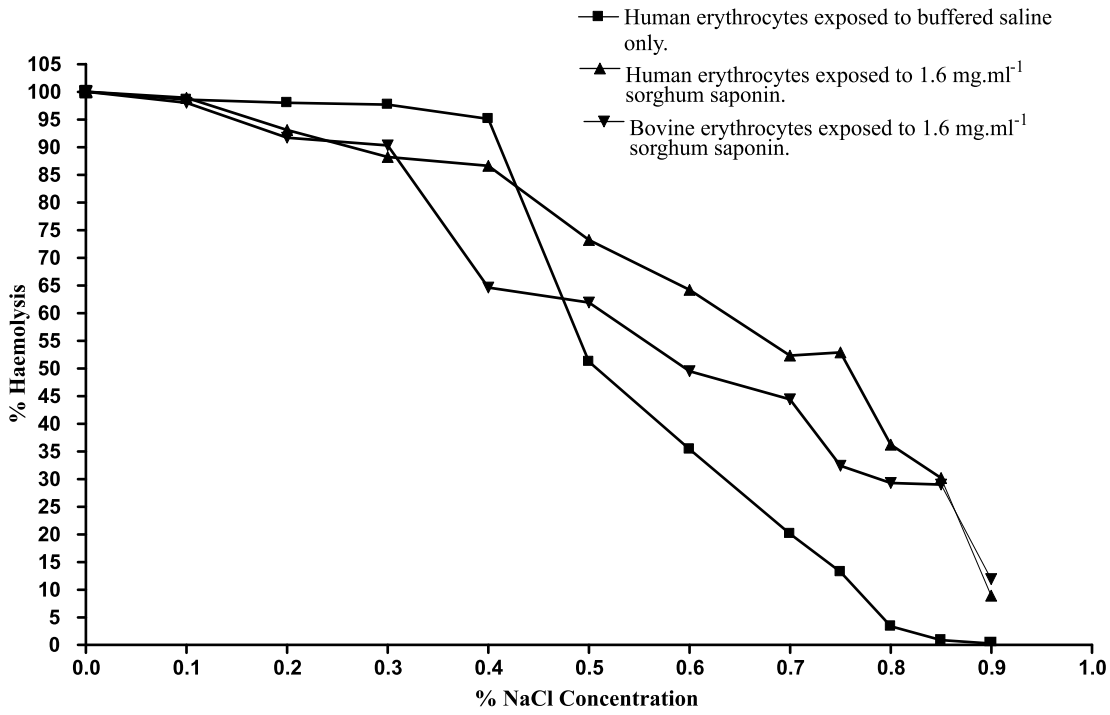

Fig. 3. Comparison of the osmotic fragility of the human and bovine erythrocytes exposed to 1.6 mg.ml^{-1} of sorghum saponin with those exposed to buffered saline only

were more stable in hypotonic solution in the presence of sorghum saponin than were the human erythrocytes especially at the sodium chloride concentrations of 0.4 %, 0.5 %, 0.6 %, 0.7 %, 0.75 % and 0.8 % NaCl concentration.

DISCUSSION

The present study has demonstrated that sorghum saponins caused the increased haemolysis of human and cattle red blood cells in hypotonic solutions. This is expected because of the previous reports of the alterations in the membrane integrity and its effects on cholesterol in the plasma membrane of erythrocytes. Lindahl ***et al.*** (18) reported that the injection of saponins in large amounts elicited various responses in different animals, some of which were erythrocyte haemolysis and growth retardation, whilst some others elicited the inhibition of smooth muscle activity (6), cholesterol depletion (29), increased enzyme activity (17) and alterations in cell membrane integrity (21). Gee and Johnson (11) have also reported pronounced haemolytic properties elicited by saponins present in some other plants.

The (osmotic fragility) haemolytic activity of the sorghum saponins was found to be dose dependent. The osmotic fragility decreased with increasing concentration of both the standard and the sorghum saponins. The osmotic resistance of erythrocytes of several mammalian species has been found to change when the temperature or pH of the surrounding hypotonic environment is altered (25).

In this study, bovine erythrocytes were observed to be more stable in hypotonic solution than were those of human erythrocytes despite exposure to similar concentration of saponin. This clearly demonstrates that species differences occur in the behavior of erythrocytes to haemolytic agents such as saponin (13, 25). Several authors have also reported different values even for the same animal species (28). This could be due to differences in the factors that are known to influence osmotic fragility such as volume, size and age of erythrocytes, temperature and pH of the medium (28). The species differences in the osmotic fragility of mammalian erythrocytes observed by Coldman *et al.* (7) were confirmed in a study by Oyewale (24). He reported that the species differences are apparently attributable to factors that vary between species such as the nature of the erythrocyte membrane or the physical and chemical constitution of the cell. It has been shown by Jain (13) that the resistance of erythrocytes to osmotic lysis may increase or decrease in some haematological disorders.

It has been reported that the osmotic fragility of human erythrocytes (1) and rat, rabbit, cattle and pig erythrocytes (23) as well as sheep and goat erythrocytes (22) decreases when the temperature or pH of the surrounding hypotonic solution is increased. Aloni *et al.* (1) reported that in contrast to the above findings, the erythrocyte osmotic fragility

increases with an increase in temperature in the camel. This effect of temperature on erythrocyte fragility is on the membrane lipids and proteins. The pH of animal blood, especially cattle (26), pig (33), dog (3) and horse (4) blood has been reported to decrease during storage for 24 hours and this is attributable to the accumulation of lactate, an end-product of glycolysis. It has also been reported that the fragility of erythrocytes in rat, rabbit, cattle and pig erythrocytes (23) as well as sheep and goat erythrocytes (22) increases as the pH or temperature of the surrounding hypotonic solution decreases. Oyewale (24) reported the effect of storage of blood on the osmotic fragility of mammalian erythrocytes. He observed significant changes in the fragility of goat, cattle, pig, mouse, rat and rabbit erythrocytes, but not in sheep erythrocytes during the storage of blood for 24 hours at 10 °C.

It has been shown that oestrogen increases the resistance of erythrocytes to osmotic haemolysis and that testosterone has no effect on erythrocyte fragility (20). The presence of oestrogens in the adult human female and the cow used in this study could affect the degree of the osmotic haemolysis of their erythrocytes by the sorghum saponins.

The curve of the osmotic fragility of human and cattle haemoglobin against the concentration of saponins is sigmoid in shape and it is similar to that of the standard osmotic fragility curve. The sigmoid shape of the osmotic fragility curve indicates that normal red cells vary in their resistance to hypotonic solutions. This resistance varies osmotically and gradually as a function of red cell age, with the youngest red cells being the most resistant and the oldest red cells the most fragile and susceptible. The old erythrocytes are more fragile than the young ones (27). The reason for this is that old red cells have higher sodium content and a decreased capacity to pump out sodium (9).

The ability of normal red blood cells to withstand low or high hypotonicity arise from its biconcave shape, which allows the cell to increase its volume by about 70 % before the surface membrane is stretched and once this limit is reached, lysis occurs.

The mechanisms by which saponins cause red cell haemolysis were also investigated by Jones and Elliot (14). They reported that a reaction of the saponin with cholesterol in the erythrocyte wall, resulting in permeability changes, might be responsible for the haemolytic activity although a number of haemolytic saponins do not form cholesyeroids. Lower (19) reported that not all saponins are haemolytically active. For example, the soy saponins have little haemolytic activity. Different saponins have different haemolytic activities and there is a wide variation among different animal species in the susceptibility of their erythrocytes to haemolysis by saponins as demonstrated by the human and bovine erythrocytes in our study. This might explain why anaemia is not observed in people consuming foods containing highly haemolytic saponins. However, it should be noted that no general correlation was found between haemolytic activity and saponin toxicity (16).

It is hoped that the data presented in this study would be useful in assessing the significance of the values that may be obtained in the tropical environment in disease conditions at least with respect to the effect of saponins on human and bovine erythrocytes.

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THE MICROMINERALS IN SERUM OF WEST AFRICAN DWARF (WAD) GOATS INFECTED WITH *TRYPANOSOMA CONGOLENSE*

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ABSTRACT

Sixteen (16) West African Dwarf (WAD) goats, ages between 12–18 months, were used for this study. Animals were grouped into two groups: group A was infected with a fresh stock of *Trypanosoma congolense* (Gboko strain) intravenously at the rate of 1.0×10^6; while group B served as an uninfected control. Samples between the infected and the uninfected controls showed very highly significant levels of sodium (Na), calcium (Ca), phosphate (PO_4), and blood urea nitrogen (BUN) ($P < 0.001$) and lower levels of potassium (K), and bicarbonate (HCO_3) ($P < 0.001$). Therefore, the alterations in the concentrations of these microminerals in the serum of goats may suggest that they may have played a possible role in the pathogenesis of caprine trypanosomosis due to *T. congolense*.

Key words: microminerals; pathogenesis; *T. congolense*; WAD goats

INTRODUCTION

African animal trypanosomosis constitutes a serious threat to the livestock production in Africa, especially in countries where *Glossina* (tsetse flies) are predominantly found (12, 17, 28 29). In Nigeria, as well as other countries in Africa, it is caused by *Trypanosoma brucei* brucei, *T. congolense*, *T. simae* and *T. vivax*, which infect cattle, sheep, goats, dogs, horses, camels and donkeys (18, 24). Sheep and goats, however, are seldom found infected with trypanosomes under natural conditions. Losos and Ikede (18) estimated that of all the diseases due to trypanosomoses in Africa, sheep and goats accounted for only 2 %. The low incidence rate may be due to their resistance to trypanosomes. However, over time, the prevalence rates have changed to 8.6 % for sheep and 8.1 % for goats (24) in Nigeria. In the Ogbomoso area of Oyo State, the prevalence rate in sheep, goats and cattle was 4.7 %, 3.5 % and 3.9 %, respectively (1).

It has been estimated that Nigeria has 22.1 million sheep and 35 million goats and the majority of the sheep and goats are found in the Northern rural areas, reared alongside cattle or with sedentary farmers. These small ruminants may therefore serve as alternatives to tsetse flies during feeding in an infested area.

Clinical manifestations of trypanosomosis in small ruminants are mainly the acute and chronic forms of the disease where anaemia is the cardinal sign (18). Numerous physiological factors can be affected from diseases like trypanosomosis and can produce variation in micromineral concentrations in the blood of previously healthy sheep and cattle (20, 30). Wellede *et al.* (32) showed that the serum iron and serum-iron binding capacity for cattle decreased when infected with *T. congolense*. Cattle infected with *T. congolense* showed an increase in the levels of serum iron (SI), total iron binding capacity (TIBC), plasma iron turnover rates (PITR) and plasma iron clearance (PIC). In treated animals, the SI and TIBC falls, with the level of SI returning to preinfection level faster than TIBC. Godwin (7) reported that, in trypanosomosis due to *T. congolense* in cattle, there was a loss of 40–45 % of the iron in circulation. However, Sarror (27) found that neither iron nor copper

deficiency played any important role in the pathogenesis of anaemia in cattle infected with *T. vivax* and found no change in the levels of these microminerals in the serum of the cattle. Copper levels may fluctuate but within the normal limits (2, 3, 14).

Kalu *et al.* (16) reported that the level of calcium in the serum of goats increased significantly during the acute stages of trypanosomosis. After chemotherapy, there were constant decrease levels of calcium, which was similar with the increase in total protein levels (15). Anosa (2) reported a decrease in phosphate levels in cattle infected with *T. congolense*. The depression of calcium observed was thought to be due to thyroid gland damage. This study was carried out to determine the serum concentration of sodium, potassium, calcium, bicarbonate, phosphate and blood urea nitrogen of goats infected with *T. congolense*.

MATERIALS AND METHODS

Experimental Design

Sixteen (16) WAD goats were used for this study. The dental eruptions were used for the estimation of their ages. The goats were housed in a fly proof pen and fed with a variety of freshly cut grass supplemented with spent grains daily. *T. congolense* was obtained from the Nigerian Institute of Trypanosomiasis Research (NITR), Vom, Jos and was screened and passed in mice as described by Losos and Ikede (18). The parasites were continually maintained in the mice until the donor animals were infected. Goats were inoculated with *T. congolense* as follows:

Group A

There were eight animals in this group and each animal was inoculated with 1.0×10^6 parasites via the external jugular vein. The animals were treated with Berenil® at the end of the 5th week post infection after the first wave of parasitaemia. The drug was given intramuscularly.

Group B

This group of eight served as uninfected controls.

Blood sampling

Parasitological examinations were carried out with 2 ml of blood collected via the external jugular vein in a Bijou bottle with EDTA as the anticoagulant. The bleeding was done twice a week.

Serum collection

Five ml of blood was collected via the external jugular vein from each animal in the different groups. Samples were collected on the same day with that for parasitological examinations. Serum was harvested in a sterilized Bijou bottle and stored at -20 °C until required.

Micromineral concentrations

Serum sodium, calcium and potassium concentrations were determined by flame photometry using Gallenkamp Flame Analyser (FGA-330). Serum phosphate and bicarbonate were estimated according to Toro and Ackermann (31). Blood urea nitrogen concentrations were measured by continuous flow analysis using an autoanalyser (Technicon, UK).

Statistical analysis

The mean values are expressed as the mean ± standard deviation for serum microminerals from the two groups of the experimental animals as represented in the figures. Data obtained from the animals in the two experimental groups were compared statistically using Student *t*-test.

RESULTS

In this study there were changes in the serum micro nutrients as a result of experimental *T. congolense* infection in the WAD goats. The mean period to detection of trypanosomes in the peripheral blood of goats following experimental infection was 7 days with initial value of $10^{2.7}$ trypanosomes per ml. Thereafter, the goats exhibited fluctuating parasitaemia with peaks of $10^{7.8}$ trypanosomes ml^{-1} at 28 and 35 days, respectively (Table 1).

The mean serum sodium concentration of infected animals increased by 10.72% (from pre-infective value of 138.00 ± 0.41 to 152.80 ± 0.52 mmol.l^{-1}) (Fig. 1), while that of potassium decreased by 32.14% (from pre-infective value of 3.64 ± 0.02 to 2.47 ± 0.01 mmol.l^{-1}) (Fig. 2).

The mean serum calcium concentrations increased by 18.11% (from pre-infective values of 9.00 ± 0.30 mmol.l^{-1} to 10.63 ± 0.03 mmol.l^{-1}) (Fig. 4). The mean serum bicarbonate concentration declined by 32.95% (from pre-infective values of 24.43 ± 0.08 to 16.38 ± 0.05 mmol.l^{-1}) (Fig. 3), while the serum phosphate concentration increased by 38.27% (from pre-infective value of 3.58 ± 0.05 to 4.95 ± 0.03 mmol.l^{-1}) (Fig. 2) and the serum blood urea nitrogen increased by 6.37% (from pre-infective values of 20.73 ± 0.14 to 22.05 ± 0.03 mg.dl^{-1}) (Fig. 3).

Table 1. Mean Parasitaemia of WAD goats infected with *T. congolense*

Days post-infection	Parasitaemia (log10 trypanosomes per ml of blood)
0	0
3	0
7	2.7
14	3.3
21	4.5
28	7.8
35	7.8

DISCUSSION

The serum microminerals have been studied by various workers in animals infected with *T. congolense*: in sheep infected with *T. congolense* by Joshua *et al.* (14) and in Yankasa sheep infected with *T. congolense* by Ogunsanmi *et al.* (22). In this study there were statistically significant increa-

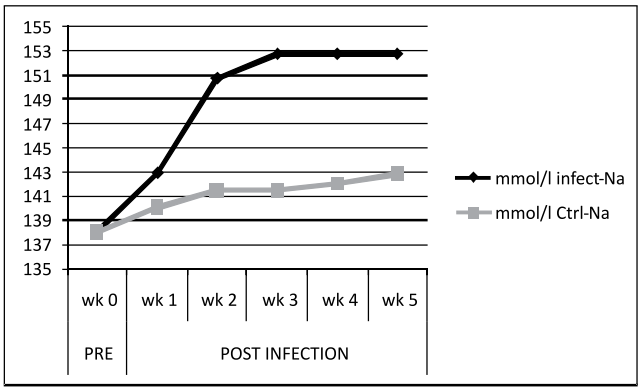

Fig. 1. Mean values of Na^+ $mmol.l^{-1}$ concentrations in WAD goats infected with *T. congolense*

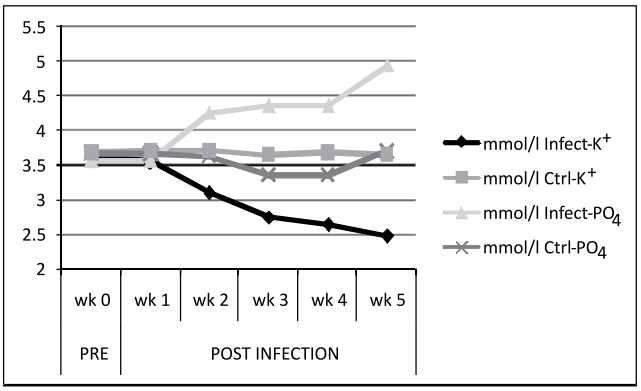

Fig. 2. Mean values of K^+ and PO_4 concentrations in WAD goats infected with *T. congolense*

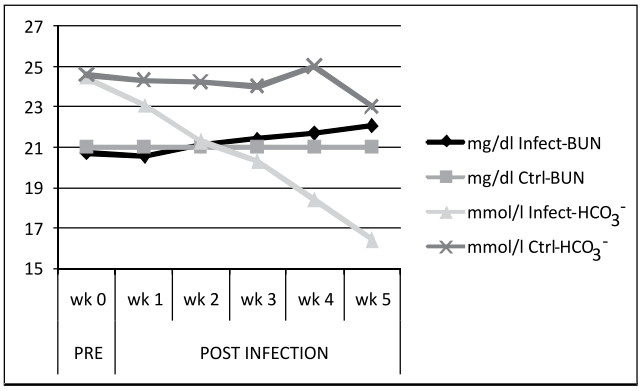

Fig. 3. Mean values of BUN $mg.dl^{-1}$ and HCO_3^- $mmol.l^{-1}$ concentrations in WAD goats infected with *T. congolense*

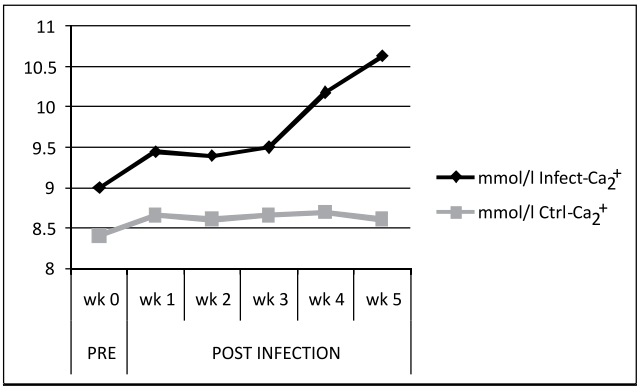

Fig. 4. Mean values of calcium concentrations ($mmol.l^{-1}$) in WAD goats infected with *T. congolense*

ses ($P < 0.001$) in serum sodium, calcium, blood urea nitrogen and phosphate concentrations up to 5th week between infected and their control groups and they correlated with the high peak of parasitaemia. This investigation has shown that goats are susceptible to experimentally induced *T. congolense* infections. The infectivity could have been enhanced by the passage in mice, as observed by Joshua (13). Finnes (8) and Losos *et al.* (18) associated trypanosomoses in cattle, sheep and goats with heavy parasitaemia, though it was not a constant finding. These studies have shown that anaemia produced from trypanosome infection in goats may be related to the level of parasitaemia. However, parasitaemia fluctuated at intervals as described by Joshua (13). The increased serum sodium concentration may therefore, be due to failure of the Zona glomerulosa of the adrenal cortex to produce aldosterone hormone that regulates sodium in the extracellular fluid (23, 33).

The concentration of calcium in the infected animals was found to increase and it correlated with the high peak of parasitaemia. This agrees with earlier observations made by Goodwin and Guy (10), Kalu *et al.* (15) and Ogunsanmi *et al.* (22). It is obvious that calcium increases in the serum but the source remains unclear; it could be that, calcium ions from the extra cellular fluid that binds cell membranes together, along with ions in the serum which are partly mobile, elevated the concentration (9). This needs further investigation.

The phosphate (PO_4) level was also found to fluctuate in a similar pattern to that of calcium, but the increase in value was significant ($P < 0.001$) between infected and the controls. Phosphate is found distributed in almost all organs of the body and plays a role in the production of ATP especially in muscles. During the course of the infection, there is a reduction in the production of ATP, thus the probable increase of phosphate in the serum. Anosa (2) suggested that calcium in conjunction with phosphorus depressed thyroid cells, but the actual roles of calcium and phosphorus during trypanosomosis are not yet known.

In this study there was an increase in the blood urea nitrogen (BUN) in all infected groups but there were no significant differences with their corresponding controls. BUN is a by-product of protein catabolism. Increased BUN levels are consistent with the results from the infection of monkeys infected with *T. b. rhodesiense* (26) and humans infected with *T. b. gambiense* (28). BUN is a product cleared from the body through the kidneys and as such, its measurement du-

ring disease are good indicators of renal function (25). The causes of the elevated BUN levels include kidney diseases such as glomerulonephritis and excessive protein catabolism and febrile conditions. Fever and glomerulonephritis are common features of trypanosomosis and presumably act together to elevate the BUN. Similar defects in renal function during trypanosomosis have been observed in man (5). Indeed, gross and histological changes affecting kidneys have been demonstrated in trypanosome-infected dogs (21) and humans (2, 3), which could explain the observed changes in kidney function in the present study.

A progressive decrease in serum potassium levels (hypokalemia) was observed in the infected animals. This may suggest a depletion of the body's potassium stores or a redistribution of potassium from the ECF into the ICF space (6). Also, it may be due to excessive renal loss of potassium which results from the action of mineralocorticoid excess and as the result of altered renal tubular function in infected animals with renal tubular acidosis or post obstructive states.

The mean serum bicarbonate levels in all infected goats were observed to have a sharp drop during the post infection period. This observation agreed with the report of Brobst (6) who reported a decreased in serum bicarbonate in *T. brucei* infected rabbits. However, our results disagree with Godwin (11) and Onyia (23) who observed an elevated bicarbonate, but then a sharp drop on day 56 in *T. brucei* infected sheep. The sharp drop in the serum bicarbonate levels might be due to acidosis associated with anaemia, renal malfunction and the release of toxic metabolites such as free acids by Trypanosomes (2, 3).

In conclusion, *T. congolense* was found to be pathogenic to goats. The increase or decrease in micronutrients concentrations fluctuated with peaks of parasitaemia. Fever and glomerulonephritis are common features of trypanosomosis and presumably act together to elevate BUN and therefore, to some extent, an increase in the levels of BUN in this study may suggest or serve as a marker in clinical diagnosis of trypanosomosis although the actual roles played by these microminerals in the pathogenesis of trypanosomosis due to *T. congolense* are not clear and therefore may need further investigation.

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THE INFLUENCE OF THREE DIFFERENT LEVELS OF DIETARY ENERGY ON THE SUSCEPTIBILITY OF WEST AFRICAN DWARF (WAD) GOATS TO EXPERIMENTAL *TRYPANOSOMA CONGOLENSE* INFECTION

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ABSTRACT

West African Dwarf (WAD) goats were placed on three different levels of dietary energy (A = low; B = medium; C = high) and subsequently infected with a virulent strain of *Trypanosoma congolense*. The influence of these three different levels of dietary energy on parasitaemia, body weight changes and haematological parameters were evaluated up to 5 weeks post infection (p. i). The respective high levels of parasitaemia and retarded growth rates were significant ($P < 0.05$) and more pronounced in group A than B or C. There were very highly significant reductions ($P < 0.001$) in the erythrocyte values (PCV, Hb and RBC) of infected goats in groups A, B and C than in the corresponding uninfected controls, while the infected groups A, B and C were significantly different ($P < 0.05$) from one another in these erythrocyte values. The declines in the erythrocyte values were worsened with the decreasing levels of dietary energy and this indicated that the dietary energy influenced the susceptibility of WAD goats to experimental trypanosomosis.

Key words: erythrocyte values; dietary energy; susceptibility; *Trypanosoma congolense;* WAD goats

INTRODUCTION

Trypanosomosis which is transmitted by the tsetse fly *(Glossina)* is an important disease of man and other animals (23). It has long been recognized as a major constraint to livestock production in tropical Africa. It is endemic in many parts of tropical Africa including Nigeria. Ruminant animals show considerable variation in their susceptibility to parasitic infections and the nutritional status of the host has been suggested as one of the possible causes of this variation (19).

Studies in cattle experimentally infected with *T. congolense* have demonstrated that the rate of the development of anaemia in cattle given ground nut cake supplementation was slower than in those that were not supplemented (8, 16). Similar observations were made by Hecker *et al.* (12) in Djallonke sheep exposed to high tsetse fly challenges. However, these results are at variance with those of Agyemang *et al.* (2) on N'Dama cattle infected with trypanosomes. The blood-stream forms of some trypanosomes scavenge blood glucose as a source of energy (5). This may partly contribute to the development of hypoglycemia observed in some trypanosome-infected animals. Indeed, Faye *et al.* (10) reported that the high energy demands of trypanosomes, infections may lead to severe energy shortage and this might be reflected in the changes in the energy and protein metabolism.

The stunting, debilitation and even death of the infected animals results in great economic losses (19). It is well documented that patho-physiological alterations occur in the cellular and plasma components of blood during infections (18). African trypanosomosis are generally characterized by haematological and serum biochemical alterations, the severity of which are often determined by the strain of the infecting trypanosomes and the overall health of

the host animals (3). Dietary energy is known to modulate the severity of trypanosomal infections in animals, (9, 21) but this has not been fully substantiated in West African Dwarf goats. The present study investigated the influence of different levels of dietary energy on the susceptibility of West African Dwarf (WAD) goats to experimental *T. congolense* infection.

MATERIALS AND METHODS

Experimental Site

The experiment was carried out at the large animal ward II, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, University of Ibadan.

Experimental Animals

A total of thirty six (36) adult male West African Dwarf (WAD) goats were used. They were acclimatized for several weeks at the experimental site. They were treated against worm infestation, haemoprotozoan diseases, such as babesiosis and trypanosomosis, as clinically indicated. Pestes des petit ruminantum (PPR) vaccine was administered to all goats.

Grouping of Animals

After the adaptation period, the goats were divided into three experimental groups (A, B and C) based on feed ration energy levels. Each group contained twelve goats. The animals were on these rations for 4 weeks before experimen tal infections were carried out. This was to create the desired differences in the nutritional status of the animals before the experimental infection. In each group, eight goats were infected with trypanosomes while the remaining four goats served as uninfected controls.

Animals in group A, B, and C were placed on low, medium, and high plane rations respectively based on different levels of dietary energy but isonitrogenous (the crude protein levels were the same). The composition of the rations and the proximate analyses of the feeds are given in Tables 1 and 2, respectively. Feed was given based on 4.0 % of their body weight (maintenance ration) and water was provided ad libitum.

Infection with Trypanosomes

The *Trypanosoma congolense* parasites used in this experiment were obtained from the National Institute for Trypanosomosis Research (NITR), Vom, Nigeria. *Trypanosoma congolense* (Binchi Bassa Strain) was obtained and subjected to six passages in albino mice, prior to use. The infected animals were inoculated intraperitoneally with equal numbers of *Trypanosoma congolense* at the rate of 1.0×10^6 ml^{-1} in sterile saline. These experiments lasted 6 weeks.

Haematology

Approximately 5 ml blood samples were collected from the jugular vein of the animals at pre and post parasite inoculations and evaluated for parasitaemia and haematological parameters. Parasitaemia was determined by haemocytometry described by Herbert and Lumsden (13) and scored by Paris *et al.* (22). Packed cell volume (PCV) was determined by the micro-haematocrit method; the haemoglobin concentration was measured by the cyanomethaemoglobin method; and the red blood cells (RBC) were determined with an electronic cell counter (Counter Coulter model ZF, Coulter Electronic, Gr. Britain) as described by Jain (15).

Determination of body weight

Each animal was put in a light bag and weighed on a hanging weighing balance (Salter suspended Model 285, Made in England). Prior to that, the weight of the light bag was determined. The weight of the animal was then obtained by the difference.

Statistical analysis and experimental design

The parasitaemia, body weight and haematological changes were observed at pre and post infection. The data were statistically compared with the respective control groups by a one way analysis of variance (ANOVA) using software SPSS Vs 10. Duncan's multiple range tests were applied to compare the significance of differences of groups ($P < 0.05$ and $P < 0.001$ were considered significant and very highly significant differences, respectively).

RESULTS

Parasitaemia

Parasitaemia was first detected at day 7 post infection (p.i.) in all the groups with log parasitaemia of 4.0 ± 0.13, 3.6 ± 0.05 and 3.2 ± 0.02 in all infected animals on low (A), medium (B) and high (C) levels of dietary energy respectively. The lowest parasitaemia level was found in the goats placed on a high (C) level of diet, while the highest level was observed in goats on the low (A) level of dietary energy as revealed by examination of the buffy coat. The parasitaemia persisted with the peak parasitaemia occurring on day 35 post infection (Table 3).

Body weight assessment

In group A, the difference in the percentage of mean weekly live weight gain of the infected animals and their corresponding controls was 48 %, while in group B it was 61 % and in group C it was 90 %. The difference of the body weight gain of the infected and their uninfected controls in A, B and C were significantly different ($P < 0.05$) (Table 4).

Haematology

After the appearance of the parasites in the peripheral blood there was a decline in the group mean PCV values of the infected goats in all of the three groups. At post infection phase (week 0–5 p.i), the percentage group mean fall in PCV values in group A was 50.65 % (from pre-infection value of 27.13 ± 0.08 to 13.39 ± 0.10 %). In group B, the percentage group mean PCV values fall was 37.79 % (from pre-infection value of 28.13 ± 0.025 to 17.50 ± 0.14 %). In group C, the percentage group mean PCV declined by 34.08 % (from pre-infection value of 27.38 ± 0.16 to 18.05 ± 0.09 %).

The percentage group mean haemoglobin concentration of infected animals dropped by 45.50 % (from pre-infection value of 9.45 ± 0.02 to 5.15 ± 1.00) and by 36.65 % (from pre-infection value of 9.55 ± 0.20 to 6.05 ± 0.03) in groups A and B, respectively, while in group C, the percentage group mean haemoglobin concentration of infected animals dropped by 32.82 % (from pre-infection value of 9.08 ± 0.02 to 6.10 ± 0.05).

There was a fall in group mean RBC counts of all infected goats in all of the three groups. In group A, the percentage group

mean RBC counts dropped by 43.46% (from pre-infective value of 12.38±0.20 to 7.00±0.20). In group B, the percentage group mean RBC counts dropped by 47.79% (from pre-infective value of 12.45±0.31 to 7.30±0.12) while in group C, the percentage group mean RBC counts dropped by 38.89% (from pre-infective value of 12.60±0.02 to 7.70±0.06).

When the group mean PCV, the group mean Hb concentration and the group mean RBC values respectively in group A, B and C were compared with their corresponding control groups, there were very highly significant differences ($P<0.001$). When the infected groups A, B and C were compared with one another in their respective group mean PCV, Hb and RBC values, there were significant difference ($P<0.05$) (Tables 5, 6 and 7).

DISCUSSION

The present study showed that dietary energy had a marked influence on parasitaemia, body weight changes and haematology during infections. The trypanosomal infections in these studies were characterised by an undulating parasitaemia and the host goats did not reveal parasites in their peripheral blood until the 7th day post infection.

Table 1. Proximate analysis of diet offered to the WAD Goats

Components	Ration		
	A	B	C
Dry matter %	72.95	75.25	77.57
Crude protein %	13.17	13.26	13.35
Aether extract %	24.64	26.92	29.20
Crude fibre %	16.82	16.82	16.81
Nitrogen free extract %	62.40	54.50	45.80
Total Ash %	3.60	11.60	15.20
Calculated gross energy ($kcal.kg^{-1}$)	2426.74	2548.57	2670.4

Table 2. Composition of rations offered to WAD Goats

Feed ingredients	Percentage of rations		
	A	B	C
Panicuum maximum	20.00	15.00	10.00
Sun dried cassava	50.00	55.00	60.00
GNC	10.00	10.00	10.00
PKC	4.00	4.00	4.00
Fish meal 65 %	4.25	4.25	4.25
Wheat offals	10.00	10.00	10.00
Oyster shells	1.00	1.00	1.00
Vitamin premix	0.50	0.50	0.50
Salt	0.25	0.25	0.25
Total	100.00	100.00	100.00

Following the establishment of infection, infected animals fed different levels of dietary energy experienced greater retardation of growth than their control groups. These findings were in agreement with those of Hecker *et al.* (12) on Djallonke sheep and Agyemang *et al.* (2) on N'Dama cattle exposed to natural fly challenges. The observation of more pronounced retardation of growth in the infected animals on a low dietary energy level in the ration cannot be attributed to a decrease in the feed intake alone, but are due to the infection, as the growth rate of these infected animals were significantly lower than that of the control animals. It is of interest to note that by the 5th week post infection, goats

Table 3. *Trypanosoma congolense* parasitaemia (mean ± standard deviation) in WAD goats kept on diets with different levels of dietary energy

	LOGIOPARASITAEMIA OF DAYS P.I.					
Planes of nutrition	**0**	**7**	**14**	**21**	**28**	**35**
Low (A)	0	4.0 ± 0.13	2.9 ± 0.02	6.8 ± 0.02	8.0 ± 0.05	8.5 ± 0.03
Medium (B)	0	3.6 ± 0.05	3.0 ± 0.04	6.2 ± 0.03	7.0 ± 0.02	8.1 ± 0.03
High (C)	0	3.2 ± 0.02	2.7 ± 0.05	6.0 ± 0.03	6.8 ± 0.03	7.6 ± 0.02

The values are expressed as the mean ± standard deviation.

All uninfected control WAD goats were aparasitaemic throughout the period of study.

Table 4. The effect of experimental *T. congolense* infection on WAD goats fed three different planes of nutrition: body weight change

Group	Status	No.	Body weight (kg)			
			Wk 1	Wk 5	Weakly mean weight gain	Overall weight gain
A	Inf.	8	8.0 ± 0.07	9.2 ± 0.07	0.24 ± 0.04a	1.2±0.07
	Non-inf.	4	7.5 ± 0.04	10.0 ±0.07	0.50 ± 0.03b	2.5±0.08
B	Inf.	8	8.0 ± 0.06	10.2 ± 0.04	0.44 ± 0.04a	2.2±0.04
	Non-inf.	4	7.5 ± 0.04	10.5 ± 0.04	0.72 ± 0.05b	3.0±0.08
C	Inf.	8	8.0 ± 0.05	12.0 ± 0.05	0.90 ± 0.06a	4.5±0.06
	Non-inf.	4	7.5 ± 0.04	12.5 ± 0.07	1.00 ± 0.06b	5.0±0.08

The values are expressed as the mean ± standard deviation.
a, b, c – the means in the same column followed by different superscripts differed significantly ($P<0.05$) in infected and control in group A, B and C

Table 5. The effect of experimental *T. congolense* infection on WAD goats at different levels of dietary energy: changes in PCV

	Wk	Infected A	Control A	Infected B	Control B	Infected C	Control C
Pre-infection	0	27.13 ± 0.08	27.45 ± 0.08	28.13 ± 0.25	27.42 ± 0.04	27.38 ± 0.16	27.87 ± 0.13
	1	25.86 ± 0.38	27.50 ± 0.13	25.78 ± 0.41	27.50 ± 0.10	26.13 ± 0.54	28.50 ± 0.72
	2	21.23 ± 0.02	27.00 ± 1.20	21.95 ± 0.05	28.50 ± 1.80	23.15 ± 0.09	28.50 ± 1.88
Post-infection	3	18.63 ± 0.24	27.00 ± 0.02	20.05 ± 0.29	28.50 ± 0.02	22.25 ± 0.14	28.50 ± 1.50
	4	16.20 ± 8.17	27.00 ± 0.04	19.13 ± 0.13	28.25 ± 0.87	19.75 ± 0.25	28.50 ± 0.30
	5	13.39 ± 0.10[b]	27.00 ± 0.04	17.50 ± 0.14[a]	29.00 ± 0.30	18.05 ± 0.09[a]	28.50 ± 1.50

The values are expressed as the mean ± standard deviation
a, b, c – the means in the same row followed by different superscript differ significantly (P<0.05) in infected A, B and C

Table 6. The effect of experimental *T. congolense* infection on WAD goats at different levels of dietary energy: changes in haemoglobin concentration

	Wk	Infected A	Control A	Infected B	Control B	Infected C	Control C
Pre-infection	0	9.45 ± 0.02	9.5 ± 0.15	9.55 ± 0.20	9.55 ± 0.20	9.08 ± 0.08	9.20 ± 0.20
	1	8.13 ± 0.02	9.45 ± 0.20	8.25 ± 0.06	9.60 ± 0.03	8.45 ± 0.06	9.20 ± 0.01
	2	7.50 ± 0.2	9.40 ± 0.01	7.40 ± 0.04	9.75 ± 0.06	7.48 ± 2.5	9.50 ± 0.05
Post-infection	3	6.25 ± 2.90	9.20 ± 0.02	7.25 ± 0.05	9.80 ± 0.10	7.15 ± 0.06	9.20 ± 0.02
	4	5.70 ± 2.50	9.25 ± 0.05	6.25 ± 0.05	9.70 ± 0.88	6.48 ± 0.05	9.20 ± 0.04
	5	5.15 ± 1.00b	9.00 ± 0.08	6.05 ± 0.03a	9.80 ± .005	6.10 ± 0.05a	9.20 ± 0.10

The values are expressed as the mean ± standard deviation
a, b, c – the means in the same row followed by different superscript differ significantly (P < 0.05) in infected A, B and C groups

on low and medium energy rations had started to lose body weight, indicating that the feed consumed was no longer sufficient to meet the maintenance requirement in the state of infection with the parasites. This observation was similar to Fagbemi *et al.* (9) in boars placed on different dietary energy and infected with *T. brucei*. Ilemobade and Balogun (14) made a similar observation on pigs infected with a chronic disease-inducing strain of *Trypanosoma simiae* and ascribed it to a combination of lowered voluntary feed intake and worsened feed conversion efficiency.

Table 7. The effect of experimental *T. congolense* infection on WAD goats at different levels of dietary: changes in the RBC counts

	Wk	Infected A	Control A	Infected B	Control B	Infected C	Control C
Pre-infection	0	12.38 ± 0.20	12.65 ± 0.15	12.45 ± 0.31	12.60 ± 0.20	12.60 ± 0.02	12.65 ± 0.25
	1	11.15 ± 0.17	12.00 ± 0.00	11.50 ± 0.10	12.90 ± 0.01	8.25 ± 2.50	12.70 ± 0.00
	2	10.68 ± 0.02	12.20 ± 0.02	10.35 ± 0.09	13.00 ± 0.00	8.25 ± 0.05	12.60 ± 0.00
Post-infection	3	9.35 ± 0.10	12.35 ± 0.05	7.35 ± 0.08	13.00 ± 0.00	8.05 ± 2.89	12.90 ± 0.00
	4	8.23 ± 0.08	12.00 ± 0.00	6.80 ± 0.15	13.00 ± 0.00	7.85 ± 2.89	12.85 ± 0.05
	5	7.00 ± 0.20	12.00 ± 0.00	6.50 ± 0.12	13.00 ± 0.00	7.70 ± 0.06	12.85 ± 0.05

There were declines in the PCV, RBC count and Hb concentration in this study. It is obvious from the results that the reduction of these erythrocytic values were more pronounced in the group of goats on a low level of dietary energy and this agrees with the observations of Fagbemi *et al.* (9) in boars placed on different dietary energy and infected with *T. brucei*. It is a well known fact that trypanosomosis causes anaemia (3, 4) and that the impairment of the re-utilisation of iron from degraded erythrocytes, as a result of blockage of reticuloendothelial iron release, results in decreased RBC values (6, 7). It is therefore possible that (adequate) dietary energy is a requirement for the re-utilisation of iron in trypanosomosis.

Another reason why the infected goats on low dietary levels resulted in a pronounced reduction in erythrocyte values was that the kinetics of erythroid cells were affected due to changes in biochemical and metabolic pathways during erythropoiesis. The low levels of dietary energy causes ineffective erythropoiesis with a prominent disorder of haeme, a pigment component of haemoglobin in the developing erythroid cells in the marrow.

Haeme is a planar molecule composed of the tetrapyrrole protoporphyrin IX, containing a central ferrous molecule. The initial rate controlling step in the heme synthesis, the delta aminolevulinic acid (ALA) synthase (5-amino levulinate synthase) reaction occurs within mitochondria. Glycine and the Krebs cycle intermediate succinyl-coA are utilized as substrates and vitamin B6 as pyridoxin phosphate is required as a cofactor. The ALA formed is transported to the cytoplasm where a series of reactions results in the formation of coproporphyrinogen III, which must enter the mitochondria for the final steps in the haeme synthesis (11).

Following synthesis, haeme must be transferred from the mitochondria to the cytoplasm for combination with the globin chains to complete the synthesis of haemoglobin. Haeme, therefore affects erythroid cell metabolism in different ways depending on the stage of the maturation process. The

higher the levels of succinyl.coA produced from the Krebs cycle, the higher the levels of haeme formed. Therefore, if the levels of dietary energy is low, the succinyl.coA produced from the Krebs cycle will be low and hence the haeme synthesis is affected and the production of haemoglobin in the erythroid cell will be affected and the levels of red blood cell production will be deminished.

The present study has shown that a high energy diet ameliorates the effects of trypanosome infection on the growth rate. Following patency, infected animals on different dietary levels developed different degrees of anaemia. This agrees with Dwinger *et al.* (8) who observed that the rate of the development of anaemia in N'Dama cattle inoculated with *T. congolense* and supplemented with extra groundnuts was slower than in unsupplemented cattle. However, their results disagree with the observation of similar degrees of anaemia in the low and high plane infected groups by the findings of Agyemang *et al.* (2) in N'Dama cattle. In agreement with these reports, the present study showed that trypanosome establishment and the rate of development of anaemia in trypanosome infected WAD goats is influenced by different dietary energy. This agrees with the observations of Fagbemi *et al.* (9) in boars placed on different dietary energy and infected with *T. brucei*. From this study it was observed that the higher the level of dietary energy, the lower the level of susceptibility in caprine experimental trypanosomosis.

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COMPARISON OF EPIDURAL ANAESTHESIA WITH BUPIVACAINE, XYLAZINE AND BUPIVACAINE-XYLAZINE MIXTURE IN CATS

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ABSTRACT

The effects of the lumbosacral epidural administration of 0.5% bupivacaine (BUP), 2% xylazine (XYL), and a mixture of 0.5% BUP and 2% XYL (BUP-XYL) were compared in five cats premedicated with an intramuscular injection of a mixture of atropine (0.04 mg.kg^{-1}) and ketamine (10 mg.kg^{-1}). Each cat was administered with each of 1 mg.kg^{-1} BUP, 3 mg.kg^{-1} XYL and 0.5 mg.kg^{-1} BUP – 1.5 mg.kg^{-1} XYL at one-week intervals. Selected anaesthetic indices were determined. The associated changes in heart rate (HR), respiratory frequency (f_R) and rectal temperature (RT) were also recorded over a 100 min period.

The time to onset of analgesia obtained with BUP (5.0 ± 1.0 min) and XYL (4.8 ± 0.4 min) were similar but significantly ($P < 0.05$) longer than with BUP-XYL (3.9 ± 0.4 min). The duration of analgesia with BUP (79.4 ± 6.3 min) was significantly longer than with BUP-XYL (72.1 ± 3.5 min) but shorter than with XYL (92.7 ± 5.9 min). The duration of recumbency with BUP-XYL (110.6 ± 4.0 min) was significantly longer than with BUP (101.8 ± 6.8 min) but shorter than with XYL (147.4 ± 15.8 min). The time to walking with BUP-XYL (12.6 ± 2.5 min), XYL (9.6 ± 2.8 min) and BUP (14.4 ± 9.3 min) were similar. The mean values of HR, f_R and RT were significantly lower with BUP-XYL and XYL than with BUP over the observation period.

It was concluded that the epidurally administered BUP appears to be better than either XYL or BUP-XYL in critically ill animals considering its long duration of analgesia, shorter duration of recumbency and rapid recovery period as well as stable rectal temperature and cardiopulmonary function.

Key words: bupivacaine; cats; epidural anaesthesia; mixture; xylazine

INTRODUCTION

The epidural nerve block is sometimes used as an alternative to general anaesthesia in well sedated or critically ill cats (14). However, the search is ongoing for an ideal epidural drug or drug combination that provide rapid onset and long lasting analgesia as well as rapid reversal of neural blockade at the end of the procedure without the development of adverse side effects in animals. The epidural administration of lignocaine (LIG) produces a rapid onset of action but it is of insufficient duration for performing major surgery in cats (1, 4, 7). Although similarly injected xylazine (XYL) produces long duration of analgesia, it is associated with a delayed onset of action and a depression of cardiorespiratory and thermoregulatory functions in the cat (1). Furthermore, the epidural administration of bupivacaine (BUP) also reportedly produces slow onset but prolonged duration of analgesia in dogs (2, 5, 10). In goats, Adetunji *et al.* (3) have reported that the epidural administration of BUP combined with XYL (BUP-XYL) produced rapid onset and prolonged duration of analgesia. To our knowledge, there are no similar reports on the use of the latter drug combination in cats.

The aim of this study was to evaluate and compare the effects of the epidural administration of BUP-XYL with BUP and XYL in cats, in terms of: time to onset; duration of analgesia; duration of recumbency; and time to walking. The associated changes in the heart rate (HR), respiratory frequency (f_R) and rectal temperature (RT) were also recorded in the absence of any surgical procedure.

MATERIALS AND METHODS

Animals

The protocol for this study was approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Uni-

versity of Ibadan. Five adult Nigerian local cats (three intact toms and two non-pregnant, non-lactating queens) weighing 1.9 ± 0.2 kg (mean ± SEM, range 1.6–2.7 kg) were used for the study. The animals were acquired from a cat market and only those with American Society of Anesthesiology (ASA) physical status 1, based on the physical examination findings, were selected for the study. The cats were housed singly in the catteries and were fed twice daily on cereal-based concentrate supplemented with fish meal *ad libitum*. Fresh drinkable water was made available free choice. The animals were allowed a period of four weeks to get them familiar with their new environment, new feeding regime and constant human handling in readiness for the trials. Just before the start of the experiments, the cats were judged to be in good general health based upon the findings at; a complete physical examination, haemogram and serum chemical profile.

Study Design

The cats were assigned to receive one of the following drug treatments at one-week intervals. The first drug treatment involved the lumbosacral epidural administration of BUP; the second treatment involved similar administration of XYL, both at recommended doses. The third treatment involved similar administration of BUP-XYL at half the recommended doses of the component drugs. Selected anaesthetic indices were calculated for each treatment group. Physiological variables were also measured immediately before the epidural administration of the drugs, and subsequently at 10-min intervals over a time period of 100 min as indicators of safety.

Experimental Protocol

Prior to each trial, food but not water was withheld from the cat for 12 h. Each cat was weighed on the day of the trial, using a weighing balance with a reading range of 0.0 kg to 20.0 kg. Premedication of the cats consisted of an intramuscular injection of 10 $mg.kg^{-1}$ of 5 percent ketamine hydrochloride (Ketamin, Laborate Pharmaceutical, India) and 0.04 $mg.kg^{-1}$ of 0.1 percent atropine sulphate (Amopin, Ningbo Chemicals International Trade Corporation, China) mixed together in the same hypodermic syringe. After the loss of the righting reflex, a treated cat was positioned in sternal recumbency with the hindlimbs directed cranially. The lumbosacral space was located and aseptically prepared for epidural puncture. The anaesthetic solution of choice was injected epidurally as previously described for cats (14). Bupivacaine 1 $mg.kg^{-1}$ (Marcain Heavy 5 $mg.kg^{-1}$; Astrazeneca, UK), XYL 3 $mg.kg^{-1}$ (Xylax 20 $mg.kg^{-1}$; Farvet Laboratories, Holland) or BUP-XYL (0.5 $mg.kg^{-1}$–1.5 $mg.kg^{-1}$) was epidurally administered to each cat at one-week intervals. The treated cat remained in sternal recumbency throughout the duration of each trial. Analgesia was assessed with the aid of haemostatic forceps clamped to the first ratchet position on the cat's hind toe-web immediately after the epidural injection and subsequently at two–min intervals until the return of the pain response.

Calculations

For each treated cat, the following anaesthetic indices were recorded:

1. Time to onset of analgesia: time interval (in min) between the epidural injection of the drug and the loss of pedal withdrawal reflex.
2. Duration of analgesia: time interval (in min) between the loss and the reappearance of the pedal withdrawal reflex.
3. Duration of recumbency: time interval (in min) between the ketamine-induced recumbency and the cat standing on its hindlimbs.
4. Time to walking: time interval (in min) between the cat's ability to stand and walk on its hindlimbs.

Measurements

In the course of each trial, HR, f_R and RT were measured immediately before the epidural injection of the drug(s) (time 0 min) and subsequently at 10-min intervals over a time period of 100 min. Heart rate in beats/min was determined with the aid of a precordial stethoscope. Respiratory frequency in breaths/min was determined by visual observation of thoracic excursions and RT was measured in degrees Celsius (^{0}C) with the aid of a digital clinical thermometer.

Statistical Analysis

The data are expressed as means ± SEM. Anaesthetic indices of the epidurally administered BUP-XYL, BUP and XYL were compared using one way ANOVA followed by the Bonferroni t procedure when a significant difference was indicated. Physiological variables were compared using the analysis of variance (ANOVA) for repeated measures in each group to assess changes from control with time. Dunnett's test was used as a post test (6). For all analyses, a value of $P < 0.05$ was considered statistically significant. The statistical package NCSS 2004 (Lange/McGraw-Hill, New York) was used (6).

RESULTS

Anaesthetic Indices

The time to onset of analgesia obtained with BUP (5.0 ± 1.0 min) and XYL (4.8 ± 0.4 min) were similar but significantly ($P < 0.05$) longer than with BUP-XYL (3.9 ± 0.4 min). Duration of analgesia with BUP (79.4 ± 6.3 min) was significantly longer than with BUP-XYL (72.1 ± 3.5 min) but shorter than with XYL (92.7 ± 5.9 min). The duration of recumbency with BUP-XYL (110.6 ± 4.0 min) was significantly longer than with BUP (101.8 ± 6.8 min) but shorter than with XYL (147.4 ± 15.8 min). The time to walking with BUP-XYL (12.6 ± 2.5 min), XYL (9.6 ± 2.8 min) and BUP (14.4 ± 9.3 min) were similar.

Physiological Variables

The mean HR, f_R and RT for each of the three treatment groups are shown in Tables 1 to 3. Mean HR with BUP-XYL ranged from 114.8 ± 11.1 to 223.2 ± 21.1 beats/min, with BUP from 203.2 ± 14.9 to 227.2 ± 6.4 beats/min and with XYL from 90.4 ± 8.5 to 201.6 ± 18.7 beats/min. The ranges of the mean f_R were from 20.0 ± 2.6 to 52.1 ± 7.4 breaths/min (BUP-XYL), from 34.8 ± 6.3 to 61.6 ± 12.2 breaths/min (BUP) and from 21.6 ± 1.9 to 30.4 ± 2.0 breaths/min (XYL). The mean RT with BUP-XYL ranged between 37.4 ± 0.3 and 38.9 ± 0.3 °C, with BUP between 38.0 ± 0.1 and 38.4 ± 0.2 °C, and with XYL between 36.3 ± 0.4 and 38.3 ± 0.2 °C.

DISCUSSION

In general, cats tend to resist handling and thus require some form of chemical restraint (9). In this trial, to ensure

Table 1. Mean ± SEM heart rate (beats/min) in five ketamine-sedated cats given epidural bupivacaine (1 mg.kg^{-1}; BUP), xylazine (3 mg.kg^{-1}; XYL) and mixture of bupivacaine and xylazine (0.5 mg.kg^{-1}and 1.5 mg.kg^{-1}; BUP-XYL)

Time interval (min)	HR (beats/min)		
	BUP–XYL	BUP	XYL
0	223.2 ± 21.1	203.2 ± 4.3	201.6 ± 18.7
10	137.6 ± 8.3*	220.8 ± 13.2*+	136.8 ± 6.3*
20	128.8 ± 11.2*	216.4 ± 12.2+	133.6 ± 10.1*
30	114.8 ± 11.1*	226.4 ± 7.5*+	117.6 ± 9.7*
40	115.2 ± 9.3*	227.2 ± 6.4*+	113.2 ± 7.7*
50	115.2 ± 8.8*	220.0 ± 10.4*+	106.4 ± 8.1*
60	116.8 ± 10.3*	203.2 ± 14.9+	101.6 ± 8.6*
70	116.8 ± 9.9*	217.6 ± 7.1*+	96.8 ± 7.3*+
80	121.6 ± 11.5*	214.8 ± 15.2+	96.8 ± 9.4*+
90	128.0 ± 11.0*	208.8 ± 10.1+	92.8 ± 7.1*+
100	127.6 ± 14.3*	207.6 ± 9.2+	90.4 ± 8.5*+

* – significantly different from 0 min in each group
+ – significantly different from LIG-BUP group ($P < 0.05$)

Table 3. Mean ± SEM rectal temperature (°C) in five ketamine-sedated cats given epidural bupivacaine (1 mg.kg^{-1}; BUP), xylazine (3 mg.kg^{-1}; XYL) and mixture of bupivacaine and xylazine (0.5 mg.kg^{-1} and 1.5 mg.kg^{-1}; BUP-XYL)

Time interval (min)	RT °C		
	BUP–XYL	BUP	XYL
0	38.9 ± 0.3	38.3 ± 0.1+	38.3 ± 0.2+
10	38.6 ± 0.3	38.1 ± 0.1+	38.0 ± 0.2+
20	38.4 ± 0.3	38.1 ± 0.1	37.8 ± 0.2*+
30	38.2 ± 0.3*	38.0 ± 0.1*	37.6 ± 0.3*
40	38.0 ± 0.3*	38.1 ± 0.2	37.4 ± 0.3*
50	37.7 ± 0.3*	38.1 ± 0.2	37.1 ± 0.3*
60	37.7 ± 0.3*	38.2 ± 0.2	36.9 ± 0.3*+
70	37.5 ± 0.2*	38.2 ± 0.3	36.8 ± 0.3*+
80	37.5 ± 0.3*	38.3 ± 0.2+	36.7 ± 0.3*+
90	37.4 ± 0.2*	38.3 ± 0.2+	36.5 ± 0.3*+
100	37.4 ± 0.3*	38.4 ± 0.2+	36.3 ± 0.4*+

* – significantly different from 0 min in each group
+ – significantly different from LIG-BUP group ($P < 0.05$)

Table 2. Mean ± SEM respiratory frequency (breaths/min) in five ketamine-sedated cats given epidural bupivacaine (1 mg.kg^{-1}; BUP), xylazine (3 mg.kg^{-1}; XYL) and mixture of bupivacaine and xylazine (0.5 mg.kg^{-1} and 1.5 mg.kg^{-1}; BUP-XYL)

Time interval (min)	f_R (breaths/min)		
	BUP–XYL	BUP	XYL
0	27.2 ± 4.4	34.8 ± 6.3	30.4 ± 2.0
10	20.0 ± 2.6*	36.4 ± 6.6+	21.6 ± 1.9*
20	26.0 ± 2.6	51.2 ± 10.8+	22.0 ± 1.1*+
30	31.4 ± 3.8	55.8 ± 12.4*+	23.6 ± 3.0*+
40	32.4 ± 4.1	61.6 ± 10.1*+	25.2 ± 2.7*+
50	31.2 ± 3.8	60.0 ± 10.9*+	27.6 ± 5.4
60	34.8 ± 7.4	56.6 ± 8.8*	25.6 ± 2.5*+
70	35.6 ± 8.2	48.4 ± 7.5*	26.4 ± 2.9+
80	40.8 ± 8.4*	52.0 ± 8.1*	27.6 ± 1.8+
90	40.4 ± 9.0	60.0 ± 12.5*+	24.8 ± 2.0*+
100	52.1 ± 7.4*	61.6 ± 12.2*	24.0 ± 1.1*+

* – significantly different from 0 min in each group
+ – significantly different from LIG-BUP group ($P < 0.05$)

complete immobility of the cats for the efficient and humane epidural puncture, ketamine was used as a chemical restraining agent in view of its efficacy, wide safety margin and ease of administration by the intramuscular route (15). Atropine (an antimuscarinic agent) solution was mixed with ketamine in the same syringe and administered together to counteract the increase salivation produced by ketamine which is liable to block the tiny airways of the cat (20).

A single epidural administrations of 3 mg.kg^{-1} of 2 percent XYL in cats (1) and 1 mg.kg^{-1} of 0.5 percent BUP in dogs have been reported (8, 10). Half of the recommended doses of the above drugs were used for the drug mixture in this trial to limit the cranial spread of large volumes of drug solutions in the cat's epidural space, thereby avoiding cardiorespiratory complications. The drug solutions were injected slowly over a period of 30 seconds to prevent spotty, incomplete analgesia, with more cranial spread and a shorter duration of block in the animals (14).

In the trial with BUP, the time to onset of analgesia, durations of analgesia and recumbency were 5.0 ± 1.0 min, 79.4 ± 6.3 min and 101.8 ± 6.8 min, respectively. Gomez de Segura *et al.* (10) reported in dogs corresponding values of 5.0 ± 0.0 min, 135.0 ± 68.0 min and 65.0 ± 26.0 min. Also, the epidural administration of XYL produced a time to onset of analgesia of 4.8 ± 0.4 min, duration of analgesia of 92.7 ± 5.9 min and duration of recumbency of 147.4 ± 15.8 min. The time to onset of analgesia and duration of recumbency obtained in this study agree with the previous similar study of XYL in cats by Adetunji and colleagues (1), who reported a time to onset of analgesia and duration of recumbency of 5.2 ± 1.4 min and 183.8 ± 21.5 min respectively. However, the duration of analgesia obtained by the latter authors (51.8 ± 6.9 min) was shorter than 92.7 ± 5.9 min obtained in this study. The reason for this discrepancy is not clear. It might be due to the use of a different brand of XYL or other factors.

In this study, the time to onset of analgesia obtained with BUP-XYL was shorter than with either BUP or XYL, so that the mixture would appear to be better than either single component drug for use during emergencies. Duration of analge-

sia with the single component drugs were higher than with the mixture and this makes the single component drugs better in animals undergoing long duration procedures. Apart from the need for extended nursing care, the prolonged recumbency obtained with BUP-XYL and XYL is generally undesirable in veterinary practices which handle day cases for reasons of limited in-patient facilities, financial constraints or inconveniences. Prolonged recumbency could also lead to hypostatic congestion in an anaesthetised cat undergoing surgery; thus BUP would be preferable to either BUP-XYL or XYL in this regard.

Both XYL and BUP-XYL produced reduced HR, f_R and RT more than with BUP (Tables 1–3). This agrees with the typical response of cats to the parenteral injection of alpha$_2$ agonists (1, 4, 19). A significant decrease in HR with XYL and BUP-XYL from 10-min to 100-min period observed in this trial (Table 1), agrees with the reports following both the intramuscular and intravenous administration of XYL. This is likely to be due to central absorption of XYL from the epidural space via the cerebrospinal fluid (14). A significant decrease in f_R obtained with XYL and BUP-XYL could also be similarly explained. It has been reported that a reduction in f_R following the administration of clinically recommended doses of XYL does not change the values of arterial pH, PaO_2 and $PaCO_2$ in dogs (16) and in cats (13). This suggests that the decreased f_R might be accompanied by an increased tidal volume to maintain alveolar ventilation (12, 17). Following the epidural administration of XYL and BUP-XYL, significant decreases in RT were observed from the 20-min and 30-min time intervals respectively till the end of the trials. This finding may relate to the depressant effect of alpha$_2$ agonist on thermoregulation as previously reported in cats (19).

The epidural administration of BUP produced increases in the mean values of HR and f_R in this trial (Tables 1 and 2). This may relate to both the sympathomimetic effect of ketamine and vagolytic effect of atropine which were left unopposed or even exacerbated by the hypotensive effect of BUP (11). This supposition is supported by the finding of a recent study that low doses of ketamine were associated with significant increases in HR, mean and systolic blood pressure, PaO_2 and temperature in cats (18).

In conclusion, BUP-XYL produced a shorter onset of analgesia than either BUP or XYL. Xylazine produced longer duration of analgesia than either BUP of BUP-XYL. However, prolonged recumbency, delayed recovery, hypothermia and cardiopulmonary depression make XYL and BUP-XYL unsuitable for use in critically ill cats. The epidural administration of BUP appears to be better than either XYL or BUP-XYL in this regard considering its long duration of analgesia, shorter duration of recumbency and rapid recovery period as well as stable rectal temperature and cardiopulmonary function.

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CONCENTRATION OF Fe, Cu, AND Zn IN BLOOD SERUM, MILK, URINE AND FAECES OF LACTATING SOW

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ABSTRACT

The aim of this experiment was to evaluate the concentration of micro-elements (Fe, Cu, and Zn) in blood serum and their excretion via milk, urine and faeces during the lactation of sows. Ten clinically healthy sows (crossbreed Large White x Landrace) were fed with the concentrated feed mixture (OŠ-09) during the entire lactation period. The blood serum, milk, urine and faeces samples were collected on the 7th, 14th, 21st, and 28th day after farrowing. During four weeks of lactation, we recorded the relatively stable concentrations of Fe, Cu and Zn in the blood serum, however the concentration of all these elements were determined to be marginally deficient. Similarly, the amounts of the studied micro-elements excreted via milk, urine and faeces (except the Zn in faeces) were also relatively stable. On the other hand, the amount of Zn in the faeces was significantly decreased in the 3rd and 4th sampling in comparison to 1st and 2nd sampling. In conclusion, the supplementation of Fe, Cu and Zn into the diet for this category of animal is recommended because the dietary levels of Fe, Cu and Zn are not sufficient to maintain the appropriate micro-element levels of sows during the lactation phase.

Key words: blood serum; faeces; lactating sows; micro-minerals; milk; urine

INTRODUCTION

The critical stages in the mineral nutrition of the sow are during the late gestation and lactation (7). The main problem is that metabolic disturbances (which often manifest no clinical symptoms), induced by the inadequate feeding of mineral nutrients, may cause the insufficient development of breeding sows and may impair their breeding performance (10).

The micro-elements (Fe, Cu, Zn) fulfil important structural, physiological, catalytic, and regulatory functions in animal bodies (14). They act particularly as components or activators of enzymes, co-enzymes, and hormonal mechanisms of animal bodies, affecting in this way the function of the cardiovascular, central nervous, immune, and reproductive systems (9).

The amount of micro-elements in the animal tissues, body fluids and excreta depends mostly on their: content in animal feed; their absorption; and the homeostatic control mechanisms of the body. Whereas, the mineral composition of sow's milk is largely under genetic control, it is also influenced by the stage of lactation and litter size as well (7).

The aim of this study was to evaluate the concentration of Fe, Cu and Zn in the blood serum, milk, urine and faeces of lactating sows during four weeks after parturition.

MATERIAL AND METHODS

We used in our experiments, ten lactating sows (crossbreed Large White x Landrace) in the age 2.5–3.0 years (average count of suckling piglets – 12) with an average body weight of 220 kg. We studied them from the 7th day to the 28th day after farrowing. During four weeks, the sows were fed with the concentrated feed mixture (OŠ-09) for the lactating pigs (Tajba a. s., SR). The diet was based on corn, soybean extracted ground meal, barley and wheat. In addition, OŠ-09 contained per 1 kg of DM: vitamin A 6000 U; vitamin D3 600 U; NC 155 g; DF 70 g; lysine 7.5 g; treonine 4.5 g; methionine and cysteine 4.0 g; Ca 7.0 g; P 5.0 g; Na 2.0 g; vitamin E

20 mg; Fe 240 mg; Zn 110 mg; Cu 20 mg. According to Šimiček *et al.* (12), the diets received covers the nutritional requirements of lactating pigs. We used a divided type of feeding (three times daily) corresponding to the count of new-born piglets (6.80 kg of concentrated feed mixture per lactating sow with 12 piglets per day). The sows were housed individualy in the standard farrowing pens with free access to water. The blood was taken from the vena cava cranialis. The blood, urine and faeces were sampled at one-week intervals: 1st, 2nd, 3rd, 4th sampling on 7th, 14th, 21st, 28th day after farrowing, respectively. Throughout the entire experimental period, milk was collected during the morning suckling of the piglets. The specimens were stored at –24 °C until their analysis. All samples were subjected to wet mineralization in a microwave oven. The concentrations of Fe, Cu, and Zn in the blood serum, milk, faeces, urine and diet were determined by flame AAS method (Perkin Elmer, AAnalyst 100).

Statistical analysis was done using one-way analysis of variance (ANOVA) with the post hoc Tukey multiple comparison test.

RESULTS AND DISCUSSION

The mineral requirements of pigs are influenced by many factors. These include the type and level of production, the breed and age of animal, the level of nutrition, as well as the quantity and chemical form of the mineral nutrients (1). For example, the concentration of Zn in the blood serum depends on the dietary level of copper, iron, calcium, phosphorus, proteins and phytates (6). Also it is important to know that during lactation a substantial amount of Zn is transferred by the mammary gland from the maternal circulation into the milk. The secretory mammary epithelial cells regulate Zn transport to ensure optimal Zn supply to the suckling neonates (5).

The concentration of Fe, Cu and Zn revealed no significant differences in the blood serum of lactating sows within the four samplings (Table 1), which is in accordance with the results obtained by Žvorc *et al.* (15). However, the concentration of all these elements could be examined as marginally deficient when compared with the physiological range of Fe, Cu and Zn which is 16.3–35.6 μmol.l^{-1}, 20.9 to 43.8 μmol.l^{-1} (4) and 15.3 to 35.2 μmol.l^{-1} (3) for pigs, respectively.

Table 1. The concentration of Fe, Cu, Zn (μmol.l^{-1}) in blood serum of lactating sows on the 7th, 14th, 21st, and 28th day after farrowing

Sampling	Fe μmol.l^{-1}	Cu μmol.l^{-1}	Zn μmol.l^{-1}
1	17.68 ± 5.29	21.44 ± 7.42	8.79 ± 1.59
2	17.23 ± 1.13	23.41 ± 6.90	10.32 ± 1.46
3	17.46 ± 1.55	22.43 ± 6.72	9.36 ± 0.73
4	17.68 ± 3.38	23.01 ± 6.31	9.36 ± 0.96

Similarly, the amount of Fe, Cu and Zn found in the milk and urine (Table 2 and 3) was relatively stable within the four samplings. However, a slight decreasing tendency in these parameters was seen between the 1st and 4th collection. The amount of Fe and Zn in milk declined as lactation progressed, and their content in milk appeared to be effected by the litter size (7). We found a very low amount of Fe and Cu in the milk of sows in comparison to the amount of Zn which was approximately 7 times higher (Table 2). That is the reason why an exogenous source of iron is routinely applied to piglets at the day of birth to prevent anaemia.

Table 2. The concentration of Fe, Cu, Zn (mg.l^{-1}) in milk of lactating sows on the 7th, 14th, 21st, and 28th day after farrowing

Sampling	Fe mg.l^{-1}	Cu mg.l^{-1}	Zn mg.l^{-1}
1	1.13 ± 0.82	1.28 ± 0.44	8.78 ± 4.14
2	1.08 ± 0.79	0.80 ± 0.23	6.24 ± 1.62
3	0.85 ± 0.51	0.84 ± 0.25	7.14 ± 3.55
4	0.88 ± 0.54	0.71 ± 0.05	7.66 ± 3.11

Table 3. The concentration of Fe, Cu, Zn (mg.l^{-1}) in the urine of lactating sows on the 7th, 14th, 21st, and 28th day after farrowing

Sampling	Fe mg.l^{-1}	Cu mg.l^{-1}	Zn mg.l^{-1}
1	0.73 ± 0.52	0.11 ± 0.13	0.50 ± 0.37
2	0.76 ± 0.54	0.10 ± 0.07	0.33 ± 0.13
3	0.76 ± 0.56	0.11 ± 0.06	0.36 ± 0.15
4	0.52 ± 0.30	0.15 ± 0.17	0.42 ± 0.33

The amount of Fe and Cu in the faeces of lactating sows was without significant differences between the four collections of the samples. Interestingly, the amount of Zn excreted via the faeces was found significantly lower on the 21st and 28th day after farrowing compared to the 7th and 14th day after farrowing (Table 4). No significant differences were found in the amount of micro-elements excreted via the faeces of prefattening pigs during entire experiment (8). Faeces play an important role in the excretion of Fe, Cu and Zn, on the basis of the homeostatic control mechanisms. When judging the amount of micro-elements found in the faeces, there is a need to consider that these concentrations are not formed only by unabsorbed, but also by endogenous and absorbed micro-minerals, that are re-excreted to the intestine by normal biological secretion. For example, the amount of Zn used during the synthesis of milk, is released mainly from the endogenous reserves (11). It is known, that sows of higher productivities have a greater loss of nutrients (which could present up to 20 %) than the sows of lower productivities (7). We presume that the depletion of these sources could lead to the increased absorption of Zn from the intestinal tract, which was observed in our experiment. Donangelo *et al.* (2) found that lactating women with lower plasma zinc

concentrations had higher efficiencies of zinc absorption. Moreover, the analyses of correlations between the blood serum, milk, urine and faeces did not reveal any significant relationships within the samplings and the combination of all these indexes should be used for the diagnostic purpose in the lactating sow.

Table 5. The concentration of Fe, Cu, Zn (mg.kg^{-1}) in faeces of lactating sows on the 7th, 14th, 21st, and 28th day after farrowing

Sampling	Fe mg.l^{-1}	Cu mg.l^{-1}	Zn mg.l^{-1}
1	441.47 ± 95.85	31.80 ± 10.26	148.57 ± 27.26a
2	377.08 ± 73.66	29.83 ± 9.27	150.75 ± 10.92a
3	362. 53 ± 99.83	20.96 ± 7.39	100.84 ± 27.24b
4	345.91 ± 116.75	21.11 ± 15.72	106.04 ± 41.09b

In conclusion, based on the findings of the marginal deficient concentration of Fe, Cu and Zn in blood serum of lactating sow, the supplementation of these micro-elements into the diet for this category of animals is recommended. The significant decreased amount of zinc excreted via faeces, which was found during the last 2 weeks of lactation, could indicate its increased absorption in the intestine during this phase of lactation.

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MERCURY AND SELENIUM CONCENTRATIONS IN MUSCLE TISSUE OF DIFFERENT SPECIES OF NONPREDATORY FRESHWATER FISH

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ABSTRACT

Concentrations of the total mercury and selenium were determined in muscle tissue in the collection of 69 individuals of five species of nonpredatory freshwater fish, common carp (*Cyprinus carpio)*, crucian carp (*Carassius carassius*), bream (*Abramis brama*), vimba (*Vimba vimba*) and barbel (*Barbus barbus*). The mean concentrations of the total mercury were as follows: barbel (0.277 mg.kg^{-1}) > crucian carp (0.063 mg.kg^{-1}) > vimba (0.038 mg.kg^{-1}) = bream (0.038 mg.kg^{-1}) > carp (0.035 mg.kg^{-1}) of the fresh weight of the muscle. The mean concentrations of the total selenium were as follows: vimba (0.335 mg.kg^{-1}) > barbel (0.254 mg.kg^{-1}) > carp (0.199 mg.kg^{-1}) > crucian carp (0.122 mg.kg^{-1}) > bream (0.119 mg.kg^{-1}) of the fresh weight of the muscle. The muscle selenium concentration did not correlate with the corresponding total mercury concentration in four of the five fish species. There was a linear correlation (R2 = 0.647) between the Se content and the total mercury content in muscle tissue of *Vimba vimba*. All of the samples of *Barbus barbus* showed the total mercury concentrations exceeding the hygienic limit of Hg for nonpredatory fish.

Key words: *Abramis brama*; *Barbus barbus*; *Carassius carassius*; *Cyprinus carpio*; fish; mercury; muscle; selenium; *Vimba vimba*

INTRODUCTION

For an assessment of the toxic effects of different pollutants in the aquatic environment, fish can be very important indicator organisms (19). A number of wide-ranging monitoring studies have been performed in order to estimate the degree of mercury contamination in freshwater ecosystems (12, 15, 31). Fish have the ability to accumulate mercury in relatively high concentrations in their tissues. The edible fish tissues are a major dietary source of this element. For most species, 60 to 90 % of the total mercury content is present as methylmercury (CH3Hg), the most toxic chemical form of this element (6). The human health hazard due to consumption of mercury contaminated fish is well known. Methylmercury is known to cause neurotoxic effects in humans and animals via the food chain. The human Reference Dose (RfD) for methylmercury is estimated to be 1×10^{-4} mg.kg^{-1} body weight.day^{-1} (27). The toxicity of mercury is known to be highly dependent on its chemical form. Organomercury is generally more toxic than inorganic mercury salts. Studies have shown that a daily methylmercury intake of 0.48 μg.kg^{-1} body weight will not result in any detectable adverse effects. Elementary mercury and insoluble HgS (Cynnabar ore) are the least toxic. There are major mercury species in the environmental and biological samples as follows: elementary mercury (Hg°), mercuric ion (Hg^{2+}), mercury ion (Hg^{+}), mercury sulphide (HgS), organic mercury species as follows: methylmercury (CH_3Hg^+), ethylmercury ($CH_3CH_2Hg^+$), phenylmercury ($C_6H_5Hg^+$) and dimethylmercury (CH_3HgCH_3). In the past organic mercury compounds were synthesized commercially and applied as biocides (5). The monitoring of Hg in the tissues of edible fish is extremely important because consumption of contaminated fish has caused serious neurological damage to newborn babies and adults (5, 27).

Selenium is an essential micronutrient for humans and other animals, but may be toxic to organisms when present in the diet at concentrations slightly higher than levels required for nutrition. Selenium is a naturally occurring element, commonly found in rocks and soil. Natural sources of selenium include Cretaceous marine sedimentary rocks, coal and other fossil fuel deposits. Selenium pollution in the environment is associated with a broad spectrum of

human activities (21). There is considerable evidence that selenium counteracts mercury toxicity in several animal species (13, 26). Fish also accumulate significant amounts of selenium and, therefore, are an important dietary source of this element. Selenium uptake by fish occurs both directly from the water across the gills and all body surfaces and across the gut from their diet (11). For all species, 15 to 35 % of the total selenium content is in the form of selenate (Se^{6+}). Occurrence of other water-soluble forms, such as selenite (Se^{4+}), selenide (Se^{2-}) or (Se°) is lower (6). The organic forms of Se, such as seleno-amino-acids, selenoproteins, enzymes, e.g. glutathione peroxidase (Gpx) are present in most of the living organisms (1, 9). Laboratory studies have shown that the organic forms of selenium, such as seleno-methionine have a higher bioaccumulation factor than the inorganic forms such as selenite and selenate (4). The data on the selenium content of freshwater fish from Eastern Slovakia rivers are not as extensive as they are for mercury.

The aim of this study was to determine the content of mercury and selenium in five species of nonpredatory freshwater fish living in the natural conditions of rivers and ponds in Eastern Slovakia and to follow up the correlation between these two elements in the muscle tissues of examined freshwater fish.

MATERIAL AND METHODS

Samples of *Cyprinus carpio*, *Carassius carassius*, *Abramis brama*, *Vimba vimba* and *Barbus barbus* were randomly obtained from various rivers (Laborec, Latorica, Hornád, Ondava, Torysa) and lakes (Domaša, Ružín, Šírava). Before analysis the samples of fish tissues (2 g of fresh tissue) were mineralized in nitric acid by microwave digestion. The concentration of the sample was 2 g per 25 ml 0.1 $mol.dm^{-3}$ nitric acid. The best quality chemicals were used for the analysis (suprapure nitric acid, Merck, Germany, and deionised water). The spectrometer was calibrated by metal standard solutions from Merck (Merck, Germany). The content of selenium was determined spectrometrically employing QZ UNICAM 939 AA spectrometer with Zeeman background correction from ATI UNICAM (Ltd. Cambridge, England) by the official methods used at the State Veterinary and Food Institute in Košice CH-1.6 (22). The certified reference materials (BCR No 60 Trace elements in an aquatic plant Lagarosiphon major, Community bureau of reference – BCR, Brussels; and GBW07603, Institute of Geophysical and Geochemical Exploration Langfang China) were used for the control of the analysis. The selenium detection limit was 0.003 $mg.kg^{-1}$. The content of total mercury was determined by Advanced Mercury Analyzer AMA 254 (ALTEC Ltd., CZ) directly in 100 mg portions of fresh fish tissues. The limit of detection for mercury was 0.002 $mg.kg^{-1}$. The results are reported in $mg.kg^{-1}$ fresh weight of fish muscle tissue.

RESULTS AND DISCUSION

The set of 69 samples of nonpredatory freshwater fish was analysed for the content of total mercury and selenium. The results are presented in Table 1.

Table 1. Total mercury and selenium content and Se/Hg ratio in nonpredatory freshwater fish muscle tissues

Species		Hg ($mg.kg^{-1}$)	Se ($mg.kg^{-1}$)	Se/Hg
Common Carp	Min	0.001	0.001	
Cyprinus carpio	Max	0.128	0.487	
	Mean	0.035	0.199	5.69
	SD	0.033	0.154	
	N	29	29	
Crucian Carp	Min	0.001	0.014	
Carassius	Max	0.239	0.354	
carassius	Mean	0.063	0.122	1.94
	SD	0.063	0.114	
	N	17	17	
Bream	Min	0.007	0.015	
Abramis brama	Max	0.117	0.242	
	Mean	0.038	0.119	3.13
	SD	0.042	0.071	
	N	12	10	
Vimba	Min	0.014	0.088	
Vimba vimba	Max	0.094	0.563	
	Mean	0.038	0.335	8.82
	SD	0.034	0.21	
	N	5	5	
Barbel	Min	0.15	0.109	
Barbus barbus	Max	0.376	0.474	
	Mean	0.277	0.254	0.92
	SD	0.107	0.124	
	N	6	6	
Hyg. Limit		0.1	ND	

The content of metals is expressed as $mg.kg^{-1}$ fresh weight
SD – standard deviation; n – number of pieces; ND – not defined

The correlations between the concentrations of Hg and Se are illustrated in Figures 1 to 5 and the relationship between the ratio Se/Hg and concentrations of Hg are illustrated in Figures 6 to 10.

The content of the total mercury and selenium in the muscle tissues of the examined freshwater fish was not constant, but varied between the levels below the limit of detection (LOD) to the maximum levels, as shown in Table 1. The wide variation in muscle tissues in mercury and selenium content, reflect different levels of environmental mercury and selenium pollution (3, 6, 17, 28). Our results confirmed that mercury and selenium concentrations in samples of fish muscle tissues were very low, which correlated with other authors (6, 7, 14, 29). Čelechovská *et al.* (7) determined the mercury in the muscular tissue of *Cyprinus carpio* L. with a mean concentra-

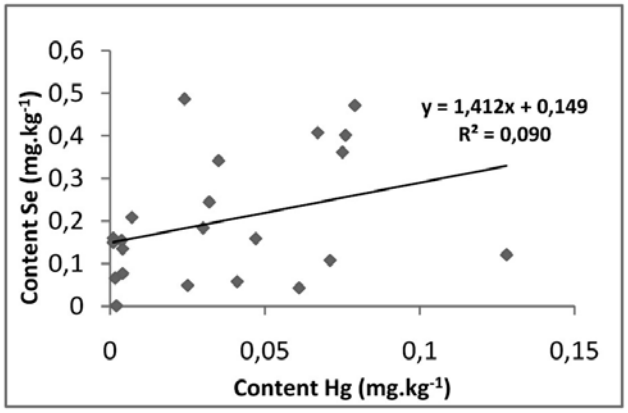

Fig. 1. Correlation between contents of total mercury and selenium in Cyprinus carpio muscle tissue

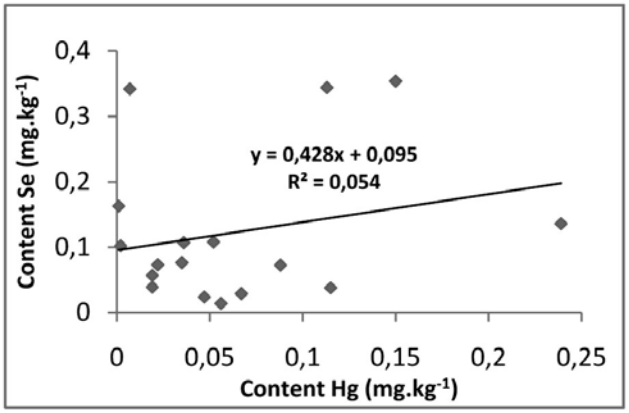

Fig. 2. Correlation between contents of total mercury and selenium in Carassius carassius muscle tissue

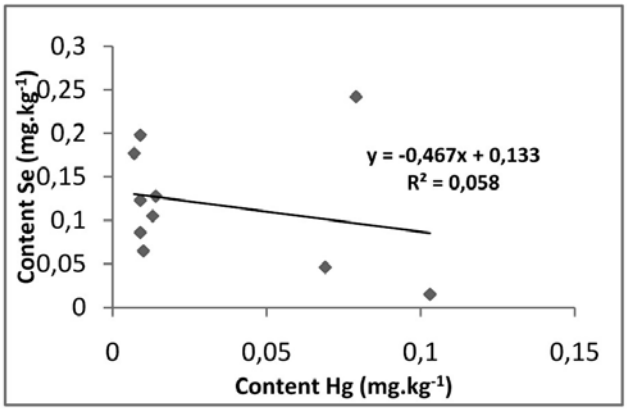

Fig. 3. Correlation between contents of total mercury and selenium in Abramis brama muscle tissue

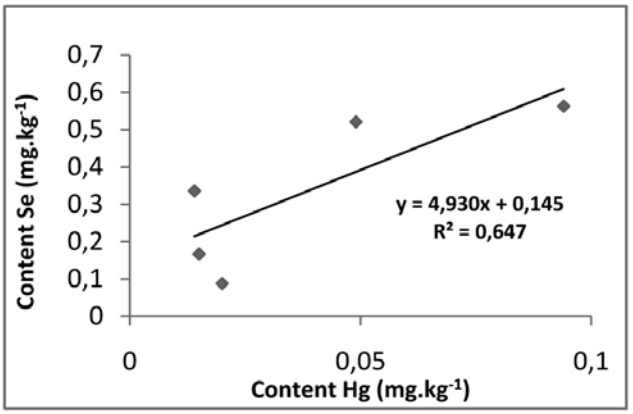

Fig. 4. Correlation between contents of total mercury and selenium in Vimba vimba muscle tissue

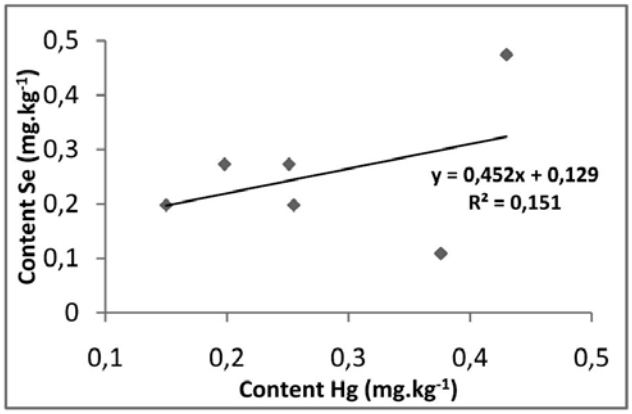

Fig. 5. Correlation between contents of total mercury and selenium in Barbus barbus muscle tissue

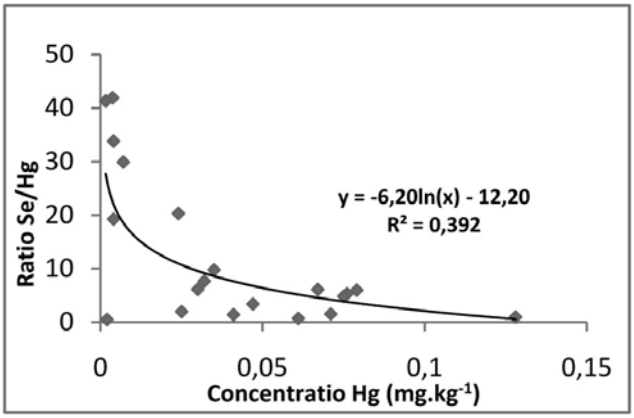

Fig. 6. Relationship between concentrations of total mercury and Se/Hg ratio in non predatory fish muscle Cyprinus carpio

tion 0.031 mg.kg^{-1} which is in good agreement with our results. Likewise, Spurný *et al.* (29) and Penaz *et al.* (24) published concentrations of mercury in muscular tissues of chub (*Leuciscus cephalus*) ranging between 0.078–0.139 mg.kg^{-1} and 0.14–0.22 mg.kg^{-1}, respectively. Jurada (14) detected a concentration of mercury ranging between 0.08 and 0.195 mg.kg^{-1} in the muscle tissues of the chub in seven localities of the Morava River. Higher concentrations of total mercury and selenium were published for marine fish (6, 18, 28). Twelve of 69 analyzed samples exceeded the hygienic

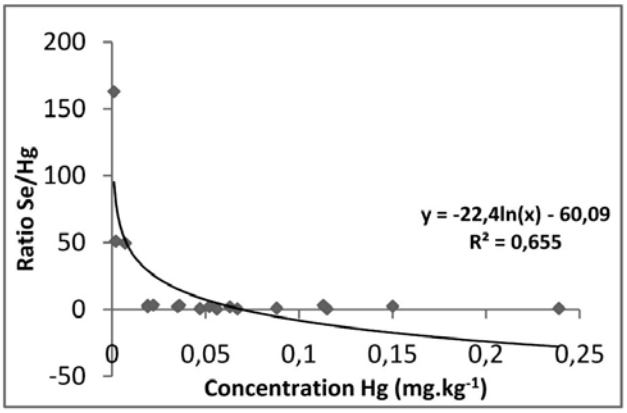

Fig. 7. Relationship between concentrations of total mercury and Se/Hg ratio in nonpredatory fish muscle Carassius carassius

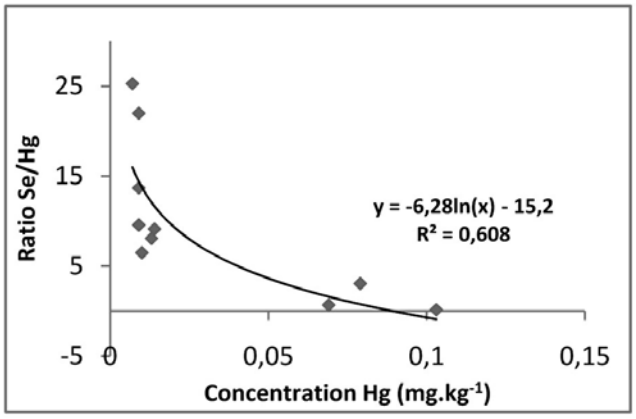

Fig. 8. Realtionship between concentrations of total mercury and Se/Hg ratio in nonpredatory freshwater fish muscle Abramis brama

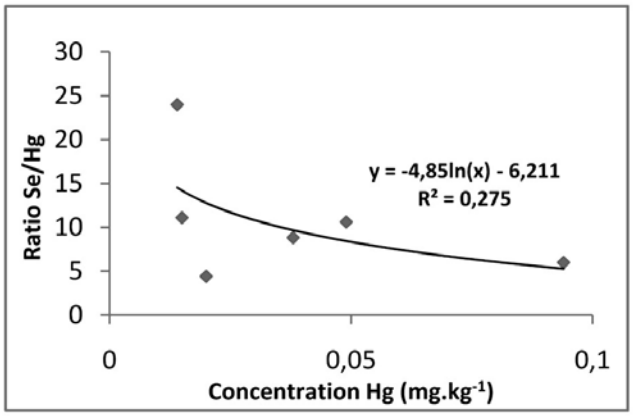

Fig. 9. Relationship between concentrations of total mercury and Se/Hg ratio in nonpredatory freshwater fish muscle Vimba vimba

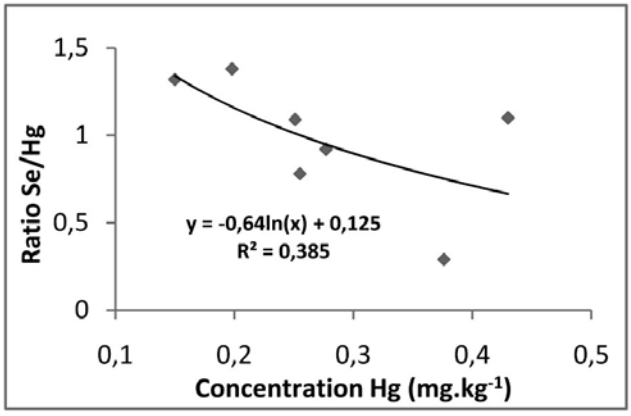

Fig. 10. Relationship between concentrations of total mercury and Se/Hg ratio in nonpredatory freshwater fish muscle Barbus barbus

limit (0.1 mg.kg^{-1}) for mercury in muscle tissue. There is no hygienic limit for selenium in fish tissues in the Codex Alimentarius of the Slovak Republic (8). The concentration of Hg was below LOD in five samples (7.25 %) and concentration of Se was below LOD only in two samples of *Cyprinus carpio*. Fifteen (22.39 %) analyzed samples exhibited higher concentrations of Hg in comparison with concentrations of Se. The ratios of Se/Hg in these samples were below level one. Similar observation for freshwater species was noted by Cappon and Smith (6). Fifteen (22.39 %) samples showed concentrations of Se higher that 0.3 mg.kg^{-1}. The mean concentration of Se was higher in samples of *Cyprinus carpio*, *Carassius carassius*, *Abramis brama* and *Vimba vimba*. The mean concentration of Hg was higher in samples of *Barbus barbus*. No correlations between the concentrations of total mercury and selenium in the examined fish species were observed (Figure 1, 2, 3 and 5) but the sample from *Vimba vimba* showed some linear correlation between these parameters (Figure 4). Robinson and Shroff (28) did not find a significant correlation between the total Hg and Se concentrations in marine fish but we observed a correlation between the Se/Hg ratio and the concentration of total mercury in muscle tissues of the examined fish species (Figure 6, 7, 8, 9 and 10). These results were in good agreement with the results of other authors (16, 32). The fish living in the rivers and ponds highly contaminated with selenium (> 10 µg Se.l^{-1}), and also marine fish, exhibited clear and strong antagonistic effect between Se and Hg in various tissues (20, 25, 28). The mercury exposure that might otherwise produce toxic effects is counteracted by selenium, particularly when the Se/Hg molar ratios approach or exceed 1 (25). The antagonistic interrelationship between selenium and mercury has been established in many studies (2, 10, 23, 30). The mechanisms of the antagonistic interactions between selenium and mercury have not been fully understood.

CONCLUSION

The mercury level in 12 of 69 examined samples exceeded the hygiene limit for Hg. The content of selenium in the muscle tissue of nonpredatory freshwater fish from rivers and lakes of Eastern Slovakia was relatively low. No correlation between the concentration of the total mercury and

selenium in the examined fish species was observed. Only the *Vimba vimba* species showed some linear correlation. There was a logarithmic relationship between the Se/Hg ratio and the total Hg concentration. Approximately 77.6 % of the analysed fish samples contained more Se that Hg (ratio Se/Hg > 1). This parameter exhibited high heterogeneity as it ranged from 0.15 in the samples of *Abramis brama* to 163 in the samples of *Carassius carassius*. Our results suggested that 77.6 % of freshwater fish were protected by selenium against Hg toxicity, but this is only an assumption.

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CHANGES OF THE CANINE ENDOMETRIUM DURING PROLIFERATIVE AND SECRETORY PHASES OF THE OESTROUS CYCLE

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ABSTRACT

The endometrium in bitches undergoes marked micro-morphological changes during individual phases of the oestrous cycle. The sections of the uterine bodies of Beagle bitches were histologically observed in the proliferative (n = 5) and the secretory (n = 5) phases of the canine uterine cycle by the image analysis system LUCIA G 4.71. The endometrium in the proliferative phase is significantly thinner than in the secretory phase (669.9 ± 62.6 µm vs. 888.8 ± 9.4 µm; P < 0.05). The endometrium is covered by a relatively low simple columnar epithelium. The individual cells of this surface epithelium have a larger centrally placed nucleus and darker cytoplasm during the proliferative phase when compared with the secretory phase. In the secretory phase, the surface columnar epithelium is significantly higher (25.0 ± 1.0 µm vs. 9.5 ± 0.7 µm; P < 0.05). Its cells are markedly larger with lighter cytoplasm and a small eccentrically situated nucleus with pycnotic bodies. These do not exfoliate into the uterine lumen. In the proliferative phase there are few straight tubular glands exhibiting patent lumens and they are situated mainly in the deeper portion of the tissue. The number of tubular glands is significantly lower than that during secretory phase (P < 0.05). The length and diameter of the tubular glands are nearly the same in both phases of the uterine cycle.

Key words: bitch; endometrium; oestrus cycle; proliferative phase; secretory phase

INTRODUCTION

The canine uterus, as in other domestic animals, undergoes marked micro-morphological changes during the individual phases of the oestrous cycle. The endometrium consists of a simple columnar epithelium, resting on a layer of connective tissue (the stroma), which varies in thickness according to hormonal influences. The simple tubular uterine glands extend from the endometrial surface to the base of the stroma, which also demonstrates a rich blood supply of spiral arteries.

In the follicular phase of the ovaries, when the follicles grow and produce estrogens, a proliferative phase of the uterine cycle occurs. The endometrium proliferates; both the number and length of the uterine glands increase (18, 2). Numerous mitoses of fibrocytes and epithelial cells of the uterine glands can be seen in the *lamina propria* mucosae (16, 15). The surface epithelial cells are of a convex shape and have irregular microvilli (6).

Immediately after ovulation of the Graafian follicles, the secretory phase of the uterine cycle begins. It is typical for the presence of a corpus luteum (CL) on the ovarian surface (luteal phase) and specific changes in the endometrium which are under the action of progesterone produced by the CL. At the beginning of this phase, a larger amount of glycogen is produced in the basal part of the epithelial cells. This results in forcing the cellular nuclei closer to the epithelial surface. Before that, the glycogen is found in the apical part of the cytoplasm in the epithelium of the uterus and the uterine glands. The endometrium enlarges markedly as a result of the hypertrophy of the uterine glands and the secretions that accumulate inside. This secretion consists mostly of glycogen, lipids, and mucus. The glands get wider and their shapes change so that they ramify in a spiral nature near their opening. In carnivores, these changes are not so marked. In addition to the uterine glands, in the mucous membrane there are also the uterine crypts (0.1–0.2 mm deep), which are of the same constitution as the uterine glands, but smaller (18, 2).

In the secretory phase, the surface epithelial cells appear more flat. At the beginning of metoestrus, the microvilli begin to abbreviate progressively and at the end of this period their numbers decrease. Microvilli are lacking completely by the end of metoestrus. The length of the metoestrous phase is influenced by the function of the CL. The production of progesterone lasts for a period of 40–90 days (3, 12, 13).

Only in carnivores there is a stromal layer (stratum subglandulare) between the glands and myometrium (which is without glands). The uterine glands are oriented obliquely to the surface of the mucous membrane (8, 18, 2).

If fertilization of the released egg does not occur, then the regressive phase begins. The preparation of the endometrium does not occur. The secretion of the uterine glands slowly subsides and glycogen disappears from the epithelial cells. The lumen of the glands gets smaller, the length shortens and the thickness of the endometrium decreases back to the level at the beginning of the proliferative phase (18, 2). In non-fertilized bitches the mucous membrane is regenerated sooner than in pregnant bitches (about day 120–150 after ovulation) (9, 10, 6, 16). Metoestrus changes over to anoestrus, i.e. the quiescent phase. The microvilli are rarely noted (6, 16).

Our work is aimed at evaluating and comparing the changes occurring in the endometrium during the proliferative and the secretory phases of the uterine cycle of Beagle bitches under normal physiological conditions.

MATERIAL AND METHODS

The excisions of the uterine bodies from 10 bitches of the beagle breed in proliferative (n = 5) and secretory phases (n = 5) of the uterine cycle were used. After fixation of the excisions in 10 % formalin, they were processed by standard histological methods. The 5–7 µm paraffin embedded sections were stained by haematoxylin-eosin (H-E). We evaluated the prepared sections under the light microscope ZEISS Axiolab connected to PC by the analogue PAL GKB CCD camera CC-8603 with image analyser LUCIA G ver. 4.71 (MuTech Corp., U.S.A). The thickness (in µm) of the endometrial bodies was determined from the surface epithelium to the myometrium. The numbers of tubular glands and their diameter were assessed by examining 1 mm^2 squares. We also measured the length of the counted tubular glands; height of the surface simple columnar epithelium and indicated its diameter in µm. We observed the changes of the cell shape of the surface simple columnar epithelium and shape of the tubular glands in the *lamina propria* mucosae. The micro-photographs were obtained by Canon Power Shot A95 fixed on the light microscopic eyepiece. The values obtained for each parameter were compared by Student's *t*-test (4, 11). All data are expressed as mean ± S.E.M (standard error of the mean). The level of significance was set at $P < 0.05$.

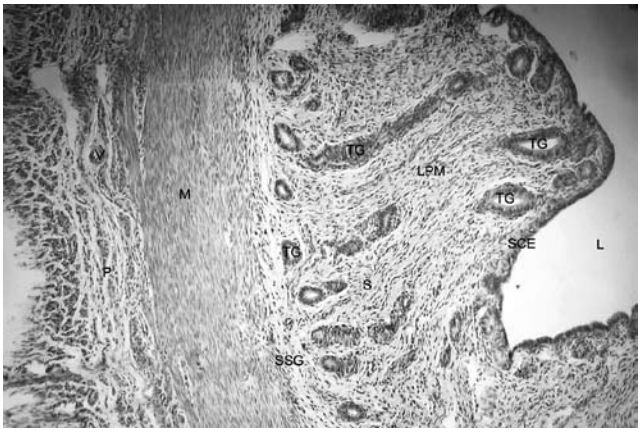

Fig. 1. Transverse section of the uterine body in the proliferative phase of the uterine cycle

In the picture there are depicted individual layers of the uterine – perimetrium (P) with blood vessels (V), myometrium (M), and endometrium with following labels: surface columnar epithelium (SCE), lamina propria mucosae (LPM) with scattered uterine tubular glands (TG) under the surface and also in the deep stroma (S). Between the lamina propria mucosae (LPM) and myometrium there is only a slender band of stratum subglandulare (SSG) where the glands are lacking. L – lumen of the uterus.
(Beagle bitch in late oestrus, H-E, × 100)

RESULTS

The endometrium of the uterine bodies in the proliferative phase (Fig. 1) illustrates the status of the ovaries without the corpus luteum. The endometrium is significantly thinner than in the secretory phase (669.9 ± 62.6 µm vs. 888.8 ± 9.4 µm; $P < 0.05$). A comparison of the thickness of the endometrium in both phases is shown in Fig. 2.

In the proliferative phase, the *lamina propria* mucosae has the cells relatively sparsely arranged (the stroma appears light) and there are only a few straight tubular glands with patent lumens (Fig. 6). These glands are situated mainly in the deeper portion and their number is significantly lower than that during the secretory phase (Fig. 7; 36.3 ± 4.8 vs. 65.5 ± 3.5; $P < 0.05$), respectively. During the secretory phase, these cells appear in copious numbers also under surface epithelium. The lumen of the tubular glands is filled with acidophilic secretions and the stroma appears dark and thick (Fig. 5). The length of tubular glands is nearly the same on average in both phases of the uterine cycle (48.9 ± 4.7 µm vs. 48.5 ± 2.5 µm; ns).

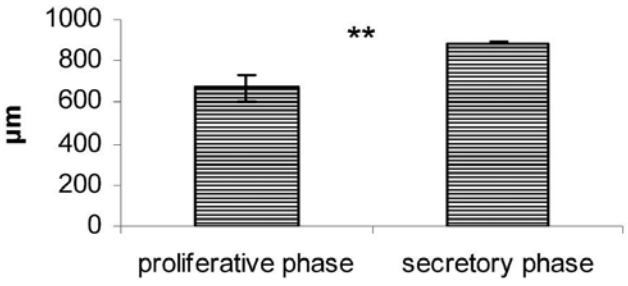

Fig. 2. Comparison of endometrium thickness in proliferative and secretory phases of the canine uterine cycle
** **– P < 0.05**

The endometrium is covered by a single layer of relatively low columnar epithelium (Fig. 1 and 3). Cells of this surface epithelium have mainly centrally placed nuclei and darker cytoplasm during the proliferative phase.

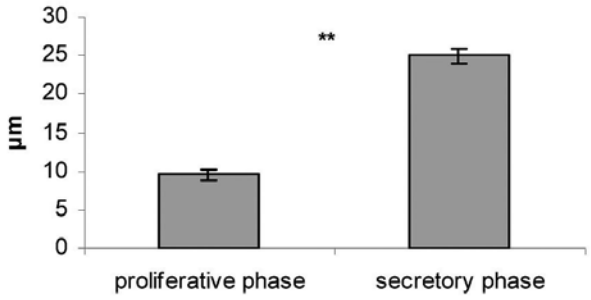

Fig. 3. Comparison of thickness of the simple columnar epithelium in proliferative and secretory phase of the canine uterine cycle
**** – P < 0.05**

In the secretory phase, the simple columnar epithelium is significantly higher than in the proliferative phase (25.0 ± 1.0 µm vs. 9.5 ± 0.7 µm; P < 0.05), respectively. Cells of this epithelium are markedly larger with light cytoplasm and a small eccentrically situated nucleus with pycnotic bodies. These do not exfoliate into the uterine lumen (Fig. 4 and 5). Histological image of the endometrium in the uterine bodies is typical for the presence of the active corpus luteum on the ovary surface during this phase (Fig. 4).

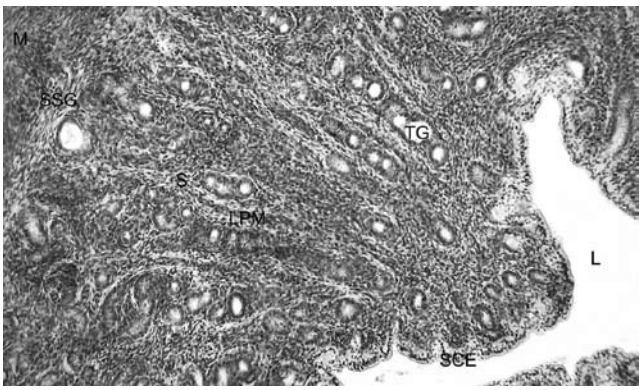

Fig. 4. Transverse section of the uterine body in the secretory phase of the uterine cycle

The endometrium of the uterine horn during the secretory phase is thicker as a result of hypertrophy of uterine tubular glands (TG) under the surface and deeper in the stroma (S). The surface simple columnar epithelium (SCE) appears lighter as a result of the enlarged cells. At the lumen of the uterus (L), these cells appear darker because of the eccentrically placed cell nuclei of the columnar epithelium. Between the lamina propria mucosae (LPM) and myometrium (M) is found very slender band of stratum subglandulare (SSG) where the glands are lacking.
(Beagle bitch in metoestrus, H-E, × 100)

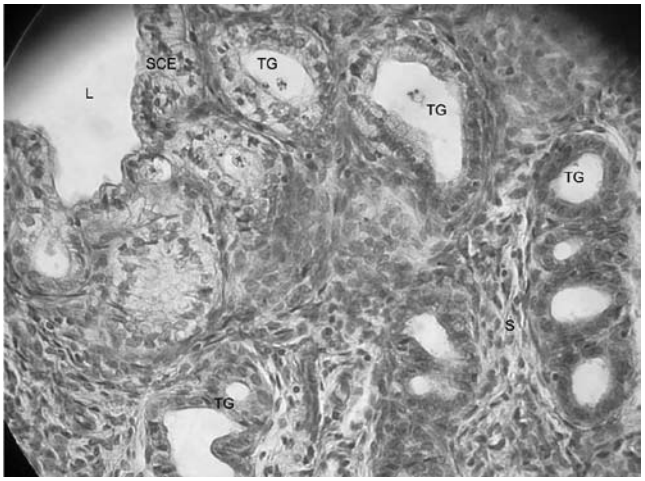

Fig. 5. Transverse section of the uterine body in the secretory phase of the uterine cycle

Uterine gland wall is formed by enlarged columnar epithelium similar to surface of endometrium (SCE). In the gland lumen the secretion is seen. Tubular glands (TG) are surrounded by stroma (S). L – lumen of the uterus.
(Beagle bitch in metoestrus, H-E, × 400)

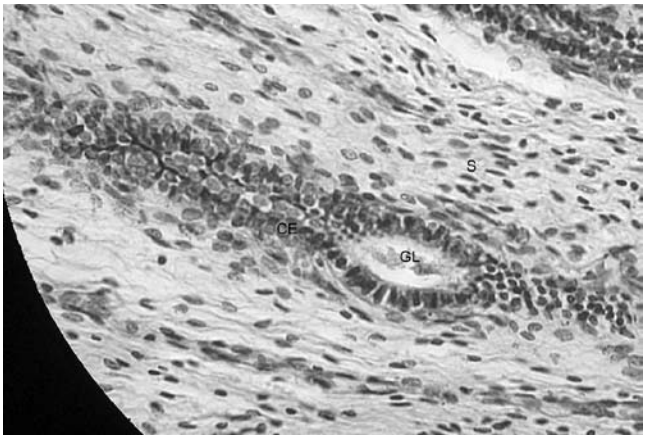

Fig. 6. Uterine tubular gland in proliferative phase of uterine cycle

The Uterine gland wall is formed by columnar epithelium (CE) similar to that of the surface epithelium. In the gland lumen (GL) secretions may be seen. The glands are surrounded by stroma (S).
(Beagle bitch in late oestrus, H-E, × 400)

DISCUSSION

The Endometrium of the uterine body, in the proliferative phase corresponds to the situation on the ovaries when the corpus luteum is present. The endometrium is enlarged several fold (roughened) in consequence of the proliferation and the increase in the number and length of the uterine glands (18, 2). We have ascertained that the endometrium in this phase is 669.9 ± 62.6 µm thick, but significantly thinner (P < 0.05) when compared to the secretory phase 888.8 ± 9.4 µm. A difference in the length of the uterine glands was not observed. The endometrium is covered by relatively low simple columnar epithelium (15). The cells of this surface epithelium have larger centrally located nuclei and darker cytoplasm during the proliferative phase.

In the secretory phase, the surface simple columnar epithelium is significantly higher (P < 0.05). The cells of this epithelium are larger with light cytoplasm and small eccentrical-

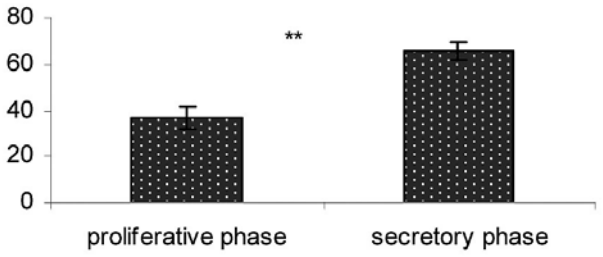

Fig. 7. Comparison of the number of endometrial glands in proliferative and secretory phases of the canine uterine cycle

** – P < 0.05

ly located nuclei, in which the pycnotic bodies can be seen. These pycnotic bodies are not extruded into the lumen. At the beginning of the secretory phase, higher amounts of glycogen is synthesized in the basal part of the epithelial cells. This has the effect of pushing the nuclei closer to the surface (18, 2). As cited in the literature (15), the light colour of the cytoplasm and the deformed nuclei are obviously caused by the accumulation of fat. Similarly, also the columnar epithelium of the uterine crypts undergoes fat degeneration (5). According to Van Cruchten *et al.* (17, 16), the cells of the surface epithelium of the endometrium in proestrus and oestrus are slightly convex and they change to a flat appearance at the end of metoestrus.

The histological picture of the endometrial body is dependent upon the presence of the active corpus luteum on the ovary during the secretory phase. The endometrium gets thick in consequence of the stromal oedema and hypertrophy of the uterine glands (18, 2). The uterus is prepared to receive the fertilized egg and at the beginning of this phase the uterine glands arise to elaborate the secretions under the influence of the elevated concentrations of progesterone (3, 12, 13). Receptors for sexual hormones have been shown to occur on most cellular types of the endometrium, especially on the cells of the endometrial glands (1). After the secretion of progesterone has finished, the surface layer is reduced and the mucous membrane renews itself, but without exfoliation (6, 7, 16, 15, 14).

We observed that in the proliferative phase the *lamina propria* mucosae have a relatively sparse number of cells in the stroma (it appears light) and few straight tubular glands with patent lumens. In contrast, during the secretory phase, the straight tubules sporadically ramify (we observed ramified glands only in one bitch). The secretion of the glands consists mainly of glycogen, lipids, and mucus. The glands get wider with the increased amount of secretion and therefore their shape changes as they spiral toward the surface of the uterine lumen. However, in carnivores their decantation is very rare (18, 2). The glands are located mainly in the deep portion and their number is significantly lower in the proliferative phase ($P<0.05$). At the same time, Van Cruchten *et al.* (16) observed that the number of uterine glands showed small variability during the oestrous cycle. During the secretory phase, the glands appear in higher numbers near the surface of the uterine lumen.

CONCLUSIONS

The status of the endometrium in the proliferative phase conforms to the situation on the ovaries where a corpus luteum is not present. The surface simple columnar epithelium is relatively low. The Cells of this epithelium have the nucleus in a central position and the cytoplasm appears darker. The *lamina propria* mucosae have relatively fewer cells and the tissue appears light. There are fewer straight tubular glandules with discernible lumens, some of which occasionally demonstrate sporadic bifurcations. These latter types of glandules are mainly localized deeper in the endometrium and their number is not very high. In the secretory phase, the surface simple columnar epithelium is higher. Its cells are evidently bigger with light cytoplasm and a small nucleus in an exocentric position where pycnotic bodies can be seen. These are not exfoliated into endometrial lumen. Tubular glandules are found in high numbers near the lumen surface of the uterus and their lumen is fulfilled with acidophilic fluid. Also under the uterine surface epithelium, tissue cells are compacted and appear dark. The histological picture of the endometrium is characteristic for the presence of an active corpus luteum on the ovary.

The results and conclusions that we came to, offer some new information about structural changes on the bitch endometrium in the proliferative and secretory phases of the Beagle bitch oestrous cycle.

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SANITARY SURVEY OF WILD RED DEER *CERVUS ELAPHUS* HUNTED IN SONDRIO PROVINCE (ITALY)

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ABSTRACT

To investigate the health conditions of the wild red deer (*Cervus elaphus*) in Sondrio Province (Central Italian Alps) 43 blood samples, 48 lung samples, 37 pulmonary lymph nodes, 34 fragments of diaphragms, 13 samples of small intestines and 30 faecal samples were collected from 61 red deer culled during the 2006 hunting season (1st September – 15th December). This study revealed the presence of respiratory syncytial virus, *Leptospira interrogans* serovar *grippotyphosa*, *Mycobacterium avium* complex and gastrointestinal parasites, and confirmed the absence of *Trichinella* sp., bovine pestivirus, bovine herpesvirus type 1, parainfluenza virus type 3, *Mycobacterium bovis* and *M. avium* subsp. *paratuberculosis*. More investigations are required to confirm the presence of *Brucella spp.*

Key words: domestic animals; Italian Alps; *L. interrogans* serovar *grippotyphosa;* sanitary conditions; wildlife diseases; zoonoses

INTRODUCTION

Monitoring disease conditions of wild animals is the first step to investigate the link between wildlife pathogens, environment, livestock and human activities. Many diseases (e. g.: tuberculosis, leptospirosis, brucellosis, ...) can infect multiple host species and these are primarily responsible for emerging (or re-emerging) infectious disease outbreaks in humans, livestock and wildlife (33, reported by 19). The data collected from wild animal disease surveys can be used to verify disease status and contribute to the maintenance of sanitary qualifications of zootechnical settlements of a territory (e.g.: repeated negative results for the presence of *Trichinella* spp. in wild carnivores, deer and wild boar, as well as in domestic species, can certify a province/region as officially clear from the disease (Reg. CE 2075/2005).

In Italy, periodic sanitary control of wildlife has been made compulsory under the DPR 607/96, art. 10, derived from the **Council Directive 92/45/EEC (11)**, in order to allow the commercialization of meat from hunted animals. The control of culled wild ungulates during the hunting season is a strategic opportunity to collect samples from carcasses in order to monitor wildlife sanitary conditions in a specific area (3).

This study was conducted to investigate the disease conditions of wild red deer (*Cervus elaphus*, Linnaeus, 1758) in Sondrio Province in order to verify the presence (or absence) of diseases that can be transmitted among wild ungulates, domestic animals and humans.

MATERIALS AND METHODS

STUDY AREA

Sondrio Province (surface area 3,212 km^2, E 9°14' – 10°37'; N 45°47' – 46°22') is situated in the Central Italian Alps and it is divided into five Hunting Management Districts, where wild ungulate species and domestic species may share the same areas during the summer period.

Wild species densities vary between districts (Table 1). Wild

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boars (*Sus scrofa*, Linnaeus, 1758) were illegally introduced to some areas in 2006, but no information is available about their density. In addition to wild ungulates, 10 975 cattle (*Bos Taurus*, Linnaeus, 1758), 17 216 sheep (*Ovis* sp., Linnaeus, 1758) and goats (*Capra* spp., Linnaeus, 1758), and 612 horses (*Equus caballus*, Linnaeus, 1758) and donkeys (*E. asinus, Linnaeus*, 1758) were present in the mountain ranges during summer.

Table 1. Pre-reproductive densities (heads.km^{-2}) of wild ungulates in Sondrio Province

Species	DISTRICT				
	CHIAVENNA	MORBEGNO	SONDRIO	TIRANO	ALTA VALLE
Red deer (*Cervus elaphus,* Linnaeus, 1758)	1.78	2.13	1.69	1.63	3.36
Roe deer (*Capreolus capreolus,* Linnaeus, 1758)	2.45	2.23	3.15	1.21	3.36
Chamois (*Rupicapra rupicapra,* Linnaeus, 1758)	2.42	5.31	5.14	1.72	1.85
Mouflon (*Ovis musimon,* Linnaeus, 1758)	3.05 (only in a Game Reserve, 22 km² of the Province surface area)				
Ibex (*Capra ibex,* Linnaeus, 1758)	3 (on 493.76 km² of the Province surface area)				

From 61 carcasses of red deer (13% of total red deer culled) shot during the hunting season in 2006 (1st September–15th December), it was possible to collect 30 faecal samples, 37 pulmonary lymph nodes, 48 tissue samples of lungs, 13 small intestines, 34 fragments of diaphragms and 43 blood samples. The blood samples were centrifuged and the sera were stored at –20 °C until analyzed. For 19 deer, only the samples were delivered to the control centre, therefore gender and age classes could not be recorded. The remaining 42 animals could be assigned to the following gender and age classes: 19 females (5 calves, 5 yearlings and 9 adults) and 23 males (6 calves, 6 yearlings and 11 adults).

The microagglutination test (MAT) (24) was used to verify the presence of antibodies against eight *Leptospira interrogans* serovars: Canicola, Pomona, Ballum, Sejroe/Hardjo, Copenagheni, Australis/Bratislava, Tarassovi, Grippotyphosa. The rose bengal agglutination test (RBT) (13, 14) was used to detect antibodies against *Brucella abortus/melitensis* since it was impossible to perform the complement fixation test (CFT; 10) because of the low quality of the sera. Sera were tested for antibodies to respiratory syncytial virus (RSV) and to parainfluenza virus type 3 (PI-3) by blocking ELISA (12). For pestivirus antibody testing, blocking ELISA was performed using anti-p80 monoclonal antibodies (9). A commercial blocking ELISA (HERDCHECK IBR GB, IDEXX laboratories, Westbrook, Maine, USA) was used to test the presence of antibodies to bovine herpevirus type 1 (BHV-1).

The lung samples were checked by immunohistochemistry (IHC), for all viruses of the RSV-family in tissue samples (D. Gelmetti, unpublished data). Diaphragms were tested by pooled sample digestion (PSD) (25) for the presence of *Trichinella* spp. larvae. Pulmonary lymph nodes were submitted for bacteriology including culture for different species of *Mycobacterium* on selective culture media and their subsequent identification by spoligotyping techniques (24). The small intestines were processed by PCR (QIAamp DNA Kit - Qiagen, for extraction of DNA; ADIAVET PARATB Kit - Adiagene, for amplification of DNA) to test for DNA of *Mycobacterium avium* subsp. *paratuberculosis.* The faecal samples were analysed by flotation (31) to look for eggs of worms, oocysts and tapeworm segments. The different parasite genera and/or helminthic groups were identified by comparison with pictures and drawings (7, 31). The frequency of presence/absence of gastrointestinal parasites between "young" (calves and yearlings) and "adult" (adults) animals was compared by Chi square test. For this test we could use data about 23 animals: 14 "young" and 9 "adult".

All analysis were performed by Diagnostic Sections of IZSLER. For each pathogen we calculated the percentage of infected hosts against the total number of hosts examined (prevalence).

RESULTS

Results are shown in Table 2.

Twenty-two positive RBT reactions to Brucella abortus/melitensis were found, but these results were omitted from the Table 2 because it was not possible to perform further analysis confirming this finding as truly positive or false reactions.

No differences were found in the presence of gastrointestinal parasites between the "young" and "adult" animals.

DISCUSSION

Leptospira interrogans serovar *grippotyphosa* has been found in red deer of Sondrio Province in previous years (2). Its reservoir host is the common vole (*Microtus arvalis*, Pallas, 1778) that is present in this area. *Leptospira interrogans* serovar *grippotyphosa* has been reported in cattle, sheep, dogs, horses and wild boar. It has also been isolated in the white-tail deer (*Odocoileus virginianus*, Zimmermann, 1780) (4, 22, 25, 28, 29, 30). In our study area, the infection in red deer appears to be occasional, possibly occurring when other infected species, such as cattle, sheep, dogs and horses, contaminate the area. *Leptospira spp.* have never been reported in roe deer or chamois living in the Sondrio area (3) and no information is available about this organism in domestic animals or in other wild species in Sondrio Province or about a possible association with deer migration.

Positive RBT reactions to *Brucella abortus/melitensis* could be due to cross-reactivity with other pathogens such as *Yersinia enterocolitica*, as is known to occur (17, 32). It was not possible to do the CFT in this study. For future studies

Table 2. Number of red deer tested and positive, and prevalence (%) for each pathogen in 2006 in Sondrio Province

Pathogen	Test	No. of animals		Prevalence (%)
		Tested	Positive	
***L. interrogans* serovars**				
Australis/Bratislava	MAT	43	0	0
Tarassovi	MAT	43	0	0
Canicola	MAT	43	0	0
Pomona	MAT	43	0	0
Sejreo/Hardjo	MAT	43	0	0
Copenhageni	MAT	43	0	0
Grippotyphosa	MAT	43	1 (titre 1:1,600)	2.3
Ballum	MAT	43	0	0
RSV	ELISA	43	1	2.3
Bovine pestiviruses	ELISA	43	0	0
BHV-1	ELISA	43	0	0
PI-3	ELISA	43	0	0
RVS	IHC	48	6	12.5
Mycobacterium spp.	Spoligotyping techniques	37	1	2.7
M. avium subsp. paratuberculosis	PCR	13	0	0
***Trichinella* sp.**	PSD	34	0	0
***Ostertagia* sp.**	Flotation	30	17	56.7
***Cooperia* sp.**	Flotation	30	7	23.3
Moniezia benedeni	Flotation	30	1	3.3
Haemonchus contortus	Flotation	30	1	3.3
Trichuris ovis	Flotation	30	1	3.3
***Bunostomun* sp.**	Flotation	30	1	3.3
***Eimeria* sp.**	Flotation	30	1	3.3

it would be essential to perform more appropriate tests in order to confirm or exclude the presence of this pathogen since it may have serious sanitary and economical implications. However, the possibility that brucellosis could be derived from local domestic animals is unlikely, as the study area has been considered clear of this infection for more than 30 years and the Public Veterinary Service carries out RBT tests on domestic animals annually to monitor the presence of this disease.

There was a positive test for RSV in one animal by ELISA. However, it turned out to be negative to the IHC test. In six other animals it was possible to detect the virus by IHC in the lung tissue, but the ELISA tests were negative. This could be due either to an early phase of the infection, during which the virus was already present in the lungs, but the antibodies had not been developed yet, or due to latency of the virus in the host. The presence of RSV in red deer does not seem to represent a sanitary risk, but it may mean that there is a circulation of the virus amongst different wild populations. For example, RSV is present in chamois and roe deer populations of the study area (3).

No positive reactions were found to Bovine Pestivirus, BHV-1 and PI-3 tests, despite the presence of positive reactions to those infections in roe deer, chamois and cattle in the study area (20).

No positive results were found to *Trichinella* spp.: this parasite has never been found in foxes or in the domestic animals of the study area (I. Bertoletti, pers. comm.).

No positive test was found for *Mycobacterium bovis*: the Province of Sondrio has been considered clear of this infection for more than 30 years, until 2006, when just a focus of the disease was found in a cattle herd in the study area (23).

With the PCR test on the small intestine, no positive animal for paratuberculosis was found, although this disease is widespread in deer populations in the neighbouring areas (Parco Nazionale dello Stelvio) (8) and in domestic animals in the study area (21). But bacteriology tests showed that one deer, culled near the boundary of the Park, had a positive reaction to *Mycobacterium avium* complex, which comprises also *M. avium subsp. paratuberculosis*.

Parasitic genera/species found in wild deer are common also in domestic animals (31). Their occurrence in deer may be a consequence of the spatial overlap with domestic ruminants during the summer grazing period. Although some parasitic species could be more frequent in young animals than in adults (e.g. *Ostertagia* spp., 31), we found no differences in the presence of parasites between "young" and "adult" animals, in agreement with the results reported by other Authors (16, 27).

Despite the small sample size, the present results provide valuable data on the health status of red deer in Sondrio Province. As the results of other studies conducted in the previous years in the localized areas of the Province show (1, 3, 5, 6), the study affirms the existence of sanitary interactions between other species, both wild and domestic.

It is important to collect samples annually to obtain more accurate information about the health status of wild animals in order to understand the real risks of transmission of those

pathogens that can have an impact on human health and/or a management and economics of livestock. Intensive collaboration between scientific institutions and hunting associations is needed in the future to increase the knowledge about wildlife diseases and to improve wildlife management strategies.

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REDUCED ALKALINE PHOSPHATASE ACTIVITY IN JEJUNAL ENTEROCYTES OF PIGLETS WITH RETARDED GROWTH

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ABSTRACT

The activity of alkaline phosphatase (AP) (EC 3.1.3.1) in the jejunum enterocytes was determined in 1 month old clinically healthy piglets with normal (12 animals, average body weight 12.5 kg) and retarded growth (12 animals, average body weight 7.5 kg). A microdenzitometric analysis was used to evaluate the differences in AP activity. When compared to normal control animals (5.3 ± 0.25), a significant decrease ($P < 0.001$) was observed in the mean density of alkaline phosphatase in the animals with retarded growth (4.4 ± 0.29). The results obtained point to a relationship between the level of the investigated enzyme in the microvillous zone of enterocytes and the functional state of enterocytes. Thus, the measurement of AP activity in the small intestine might be used as a diagnostic marker of the growth disturbances in weaned piglets

Key words: alkaline phosphatase; enterocyte; microdenzitometry; piglet

INTRODUCTION

Failure of nutrient absorption from the intestinal tract leads to the malabsorption syndrome. The clinical manifestations, which are most evident in humans and animals, may consist of: persistent gastrointestinal upset (vomiting, diarrhoea); changes in eating habits; loss in weight, and in a few cases steatorrhoea. Moreover, villous atrophy may be pronounced in the lesions located in the small intestine. Accordingly, the intestinal villi are shortened due to the loss of epithelium. In addition, the villi are broadened due to the infiltration of leukocytes into the *lamina propria*.

The brush border of the enterocytes contains many hydrolytic enzymes (disaccharidases, peptidases, and phosphatases) as well as nonenzymatic proteins, etc. (3). Among these enzymes, alkaline phosphatase is primarily found in the cell membrane where active transport takes place in the brush border of the enterocytes (12). Phosphatase's significance is indicated by its presence in the digestive tract during the embryonal development and/or during the postnatal period (5, 6). The importance in the overall metabolism of alkaline phosphatase is related to its role in the absorption of lipids (10).

MATERIAL AND METHODS

Twenty four 1 month old Slovak White x Landrace crossbred weaned piglets reared on a large-capacity farm in Košická Polianka were used in this study. The animals showed no clinical symptoms of sickness and had negative bacteriological findings. The animals were divided (dependent on their body weight) into two groups (12 animals in each). The first group (control) consisted of clinically healthy, weaned piglets with normal growth (mean body weight 12.5 ± 0.73 kg), whereas weaned piglets with retarded growth (mean body weight 7.5 ± 0.94 kg) formed the second group.

After the piglets were killed, 1 cm long samples of the small intestine were removed from the jejunum (250 cm orally from the

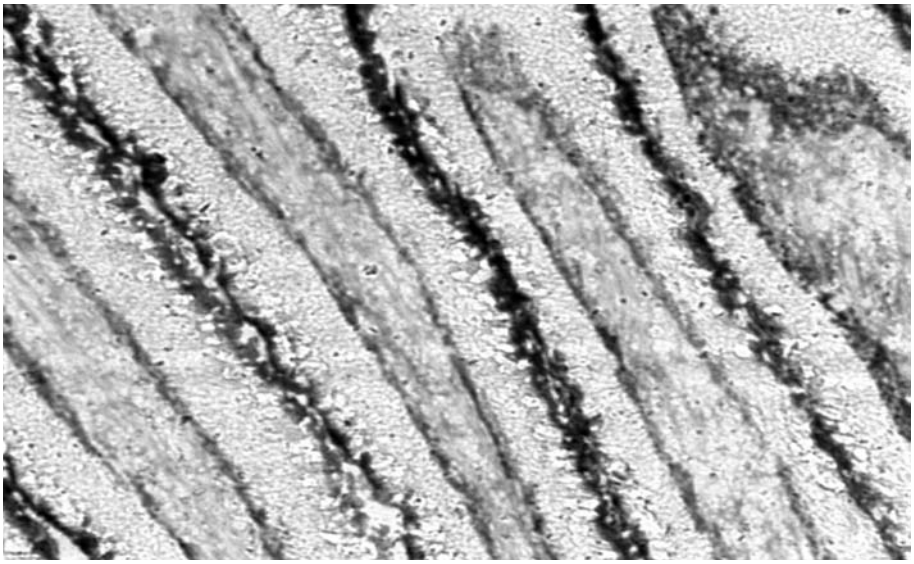

Fig. 2. Alkaline phosphatase activity along the villus axis in a 30-days-old retarded growth piglet. The incubation medium contained naphthol AS-BI phosphate and Fast blue BB. Incubation was performed at 37 °C for 10 min, using a substrate concentration of 2.0 mM and pH 9.2. The final reaction product is localized in the brush border of jejunal epithelial cells

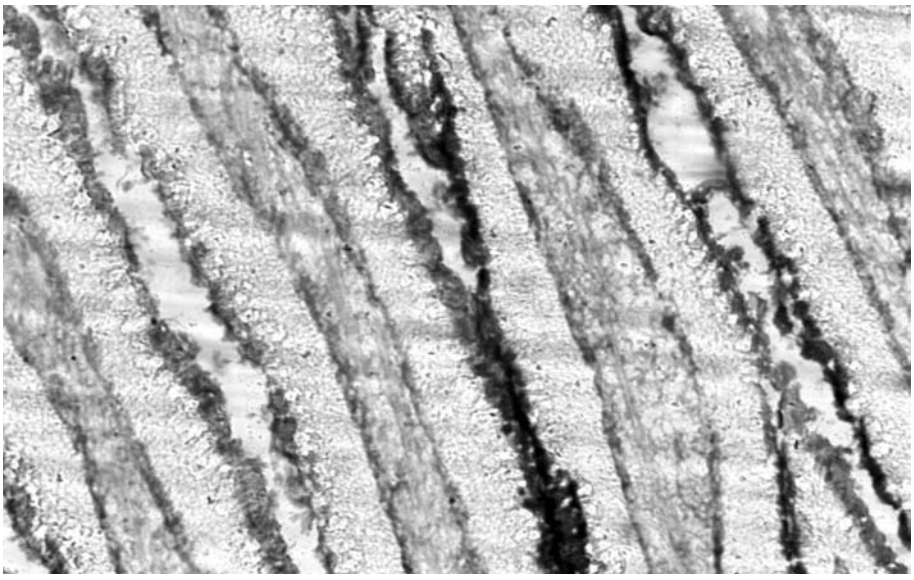

Fig. 1. Alkaline phosphatase activity along the villus axis in a 30-days-old control piglet. The incubation medium contained naphthol AS-BI phosphate and Fast blue BB. Incubation was performed at 37 °C for 10 min, using a substrate concentration of 2.0 mM and pH 9.2. The final reaction product is localized in the brush border of jejunal epithelial cells

ostium ileocoecale). Specimens were immediately frozen in cold petroleum ether at –20 °C. Frozen sections (7 μm thick) were cut on a cryostat (SHANDON CRYOTOME E, Cheshire, UK) at a cabinet temperature of –21 °C. Sections were picked up onto clean glass slides (0.96 to 1.06 mm thick) and stored in the cryostat cabinet until used. The cryostat sections were allowed to dry for 5 min at 37 °C and incubated for alkaline phosphatase (AP) activity.

The incubation for AP activity was performed as previously described by Lojda *et al.* (15) and Mozeš *et al.* (17). The medium consisted of veronal acetate buffer (pH 9.2) and naphtol – AS-BI-phosphate (Sigma-Aldrich, Deisenhofen, Germany), and stable diazonium salt Fast Blue BB (Sigma-Aldrich). The incubation lasted for less than 10 min at 37 °C. Control incubations were performed in the presence of an inhibitor (L- cystein).

After incubation, the sections were rinsed with distilled water in order to stop the reaction immediately. Post-fixation of the sections was performed in a solution of 4% (v/v) formaldehyde for 10 h at room temperature. The sections were rinsed in distilled water and mounted in glycerin jelly.

The enzyme activity was analysed cytophotometrically with a Vickers M85a microdensitometer (Biorad Instruments, York, UK). The measurements were carried out by means of an ×40 objective, an effect scanning area of 28.3 μm^2 and a scanning spot of 0.5 µm. The integrated absorbance was measured at a wavelength of 480 nm. The mask was set over at the last 30 brush border areas along the villus length (from the cryptal part to the tip) in the jejunal sections. The AP activity was calculated as the absorbance average values recorded by the instrument in $min.mm^{-3}$ brush border ± SEM.

Data between groups were compared by using the unpaired Student's *t*-test. Significance was accepted at $P < 0.05$.

RESULTS

The microdensitometric evaluation of the activity is summarized in Table 1. In both normal and retarded animals uniform distributions of alkaline phosphatase activity in the microvillus zone in terms of location and magnitude were observed. In contrast, only weak activity of this enzyme was observed in the crypts level in both groups. Nevertheless, the control animals exhibited significantly higher AP activity in the middle parts and tips of the villus (Fig. 1) in comparison with the piglets with retarded growth (Fig. 2).

Table 1. Alkaline phosphatase (AP) activity in the jejunum of control and retarded growth piglets (AP activity is given as an integrated absorbance in min. μm^{-3} brush border of enterocytes at a wavelength of 480 nm. The difference between control and retarded group is significant at the level of * – $P < 0.001$ (*t*-test))

Group	Normal	Retarded
Organ	jejunum	jejunum
AP activity	5.3 ± 0.25	4.4 ± 0.29*

DISCUSION

Short atrophic intestinal villi with flattened epithelium at the surface may be caused by many etiological factors and usually results in the development of malabsorption syndrome (19). Lojda (11) observed subtotal to total atrophy of villi at the electronmicroscopic level. In addition, he also observed defects in the microvillous zone of enterocytes. The overall absorption area was reduced, thus the functional state of the digestive tract at the level of enterocytes was adversely affected. Simultaneously with the reduction of the absorptive area, the quantity of digestive enzymes decreased which leads to malabsorption in the small intestine.

The results obtained in our experiments on weaned piglets with normal growth, point to a higher activity (when compared to piglets with retarded growth) of alkaline phosphatase which is in agreement with results obtained by Kudweis and Lojda (5, 6). These results are similar to those obtained from piglets with spontaneous and experimental viral, bacterial, and protozoal enteritis (1, 4, 6). Similarly, in the intestinal mucosa of piglets which were growth retarded, decreased AP activity was observed (7, 8). In addition, the malabsorption syndrome in broilers led to the decrease of digestive enzymes activity in the brush-border of enterocytes (9). Moreover, the AP activity in the small intestine displays circadian fluctuations closely related to food intake which was previously shown by Račekk *et al.* (18). On the other hand, it was also shown that duodenal and ileal villi were significantly higher in gnotobiotic piglets when compared to normal animals, which, however, did not lead to an overall body weight increase (2).

Disturbance of the functional state of the digestive tract, accompanied by malabsorption and body weight decrease, occurs during the reduction of alkaline phosphatase activity in the microvillous zone of enterocytes. The decrease of the alkaline phosphatase activities in the retarded group observed in our experiment, allows us to speculate that in general the AP activity reflects functional conditions of the digestive tract when normal piglets were compared to piglets with retarded growth. From this point of view these results extend our knowledge about intestinal morphology and AP activitiy in normal growing and retarded animals. Nevertheless, further detailed research is needed to: precisely clarify the (patho)physiological mechanism underlying the malabsorption syndrome; as well as to evaluate how piglets compensate for this unfavorable situation in later life.

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BENDIOCARB EMBRYOTOXICITY ON THE CHICK DEVELOPMENT FROM STAGE 20

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ABSTRACT

Carbamate pesticides generally possess low toxicity for warm-blooded vertebrates, but developmental data are scarce. We have therefore evaluated the embryotoxicity of the cholinesterase inhibitor bendiocarb in the chick embryo. The pesticide (8–1600 µg/egg) was dissolved in 10% acetone in distilled water and a volume of 200 µl from each dilution was administered over the embryo through the *membrana papyracea* at embryonic days (ED) 2 and sampling was performed on ED 9. The toxicity of bendiocarb was fairly low, although the mortality increased with advancing bendiocarb doses except for the experimental dose of 800 µg/egg. The body weight was higher at bendiocarb doses of 8–200 µg/egg and lower at doses of 400–1600 µg/egg in comparison with the control group. There was a mild but statistically significant dose-dependent reduction in the body weight; most pronounced in the treatment with a dose of 800 µg/egg (12%) in comparison with the control group. The malformations in the surviving embryos were observed rarely (<3%) and occurred in both control and experimental groups. The lethal dose of bendiocarb on ED 2 was found to be 973 µg/egg.

Key words: bendiocarb; chick embryo; toxicity; xenobiotics

INTRODUCTION

Agrochemicals, as chemicals used in agriculture, include a broad range of products used for the nutrition of plants, as well as the protection and nutrition of animals. The pesticides currently used in agriculture include a wide variety of compounds belonging to different chemical classes (7). There are pesticides, substances used as repellents or destroyers of all sorts of plant and animal pests (10). Bendiocarb (BC) is a pesticide acting upon invertebrates by irreversibly blocking the activity of the enzyme cholinesterase, which is critical in allowing muscle relaxation by removing the neuromuscular mediator acetylcholine (9). Like other carbamate insecticides, BC is a reversible inhibitor of acetylcholinesterase (AChE). The blockage of AChE caused by BC persists for approximately 24 hours and, subsequently, the situation returns to normal because the insecticide does not accumulate in mammalian tissues (16). In agriculture it is used against a variety of insects, especially those in the soil (17) and to control disease vectors (mosquitoes, flies, and other household and agricultural pests; 4).

The chick embryo is a popular model for developmental pharmacological and toxicological studies. Chicks are readily available, cost-efficient, and presents an alternative approach to the treatment of pregnant mammals. The concordance of data from CHEST (The CHick Embryotoxicity Screening Test) and mammals is excellent, and it avoids the potentially confounding effects of different maternal metabolism between species by allowing for the separate testing of human-relevant metabolites (8). Acute oral toxicity (LD_{50}) was investigated in different adult mammals: rat 34–156 $mg.kg^{-1}$, guinea pig 35 $mg.kg^{-1}$, rabbit 35–40 $mg.kg^{-1}$ (18), and also in non-mammalian species like birds: mallard duck 3.1 $mg.kg^{-1}$, bob white quail 16 $mg.kg^{-1}$, hen 137 $mg.kg^{-1}$ (18), fish 0.7–1.8 $mg.kg^{-1}$ (LC_{50}, 5), and bee 0.1 µg/bee (19). Up to this day, no detailed studies have been conducted regarding the embryotoxic effects of bendiocarb on birds which are more sensitive to the action of toxic substances. Because of that, the aim of the present study was to observe the effect of BC on chicken embryos. Chicken embryos are more sensitive than embryonic mammals to toxic substances because their action is not influenced by maternal metabolism (14).

MATERIAL AND METHODS

Fertile chicken eggs of Leghorn hybrid, variant spotted, were obtained from the animal farm Koleč, Institute of molecular genetics AS CR, Praha, Czech republic (144 eggs). They were incubated in a thermostat with forced circulation of air and temperature maintained at 37.5 ± 0.5 °C and a relative humidity of 60 %. The blunt end of the eggs was cleaned with 70 % alcohol and covered by transparent adhesive tape. The eggs were opened by the modified "window technique" (8). Embryotoxicity was observed by the application of BC on ED 2. According to Hamburger and Hamilton (1951), the ED 2 represents the 20th stage of development (5). Subsequently, using serrated scissors (FST 14071-12); an opening was cut for the application of the respective solutions. After cutting out the opening in the eggshell, doses of bendiocarb (8–1600 µg/egg) were applied to the surface (on *membrana papyracea*) of all chicken embryos.

Bendiocarb was dissolved in acetone and diluted with sterile water for tissue cultures to obtain the required concentrations. The application dose per one egg was 200 µl, with acetone concentration equal to 10 µl/200 µl of the application dose. Identical volumes of acetone solution were applied to control embryos: sterile water for tissue cultures (1 : 10), with an acetone concentration 10 µl/200 µl application dose. After application of the solutions, the eggshell openings were covered with an electrical insulation adhesive tape (15) and the chicken embryos were transferred to a thermostat and incubated under standard incubation conditions (temperature 37.5 ± 0.5 °C, 60 % relative humidity, without turning or adding CO_2). The embryos were observed during incubation and those which died were eliminated from the experiment.

The chicken embryos were dissected out of the membranes on ED 9, following bendiocarb administration on ED 2. The chick embryo's weight, malformations and mortality were recorded. A statistical comparison (Student t-test) of mortality, body weight and malformations was performed using GraphPad Prism. The LD_{50} was calculated from the regression equation, which is No. 2 in the chart, where after substituting the value of 50 (50 % mortality observed in the group) instead of an unknown y, we obtain the resulting value of the LD_{50} for ED 2.

Tested solutions with varied concentration of bendiocarb were applied directly over the embryo on the top of inner shell membrane (*membrana papyracea*), because the vascular diffusion of monitoring substance occurs and the monitoring substance is more evenly applied in comparison with the intra-amnial application. Furthermore, application on the *membrana papyracea* decreases the non-specific effects of the applied substance.

RESULTS

Bendiocarb effect on the body weight of the chick embryo

The average body weight of the control group was 1355 ± 270 mg. Up to the bendiocarb dose of 200 µg/egg, the average body weight of the chick embryo was higher than the control group. The lowest bendiocarb dose of 8 µg/egg resulted in an average body weight of the chick embryos of 1546 ± 220 mg. The body weight at this dose was the highest in comparison with both the control group (higher by 14 %) and the other experimental groups after bendiocarb administration on the ED 2. At the bendiocarb dose of 80 µg/egg, the average body weight was higher by 3 % (1399 ± 380 mg) and at the bendiocarb dose of 200 µg/egg was higher by 0.6 % (1363 ± 120 mg). The body weight at the higher bendiocarb doses (400, 800 and 1600 µg/egg) was lower in comparison with the control group and the lower doses of bendiocarb. At the dose of 400 µg/egg, the body weight was lower by 4 % in comparison with the control group (1305 ± 80 mg). The average body weight at the bendiocarb dose 800 µg/egg was lower by 12 % in comparison with the control group (1196 ± 250 mg). This is the lowest body weight in comparison with control group and other experimental groups. At the highest bendiocarb dose of 1600 µg/egg, the body weight of the chick embryos was lower by 4 % in comparison with the

Table 1. Embryotoxicity of the chick embryo after bendiocarbamate administration on 2 ED

Dose (µg)	N	Dead embryos	Dead embryos (%)	M	Average weight (g)	SD c	p-value	LD_{50} (µg/egg)
0	35	5	14	3	1.355	0.272		973
8	20	0	0	0	1.546	0.219	0.011 *	
80	22	1	5	1	1.399	0.378	0.627	
200	13	5	38	0	1.363	0.119	0.938	
400	11	5	46	0	1.305	0.081	0.659	
800	21	7	33	0	1.196	0.245	0.259 *	
1600	22	16	73	0	1.304	0.267	0.675	

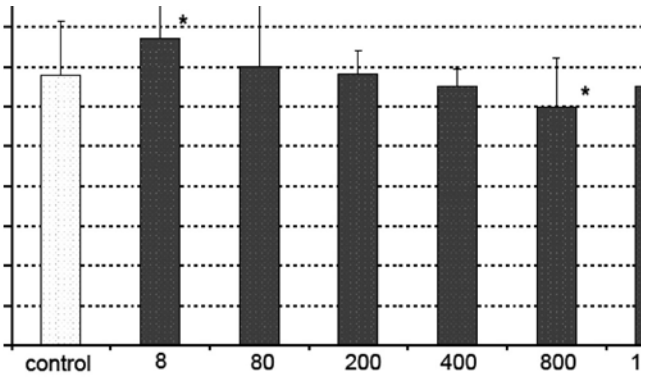

Fig. 1. Body weight of the chick embryo after bendiocarb administration

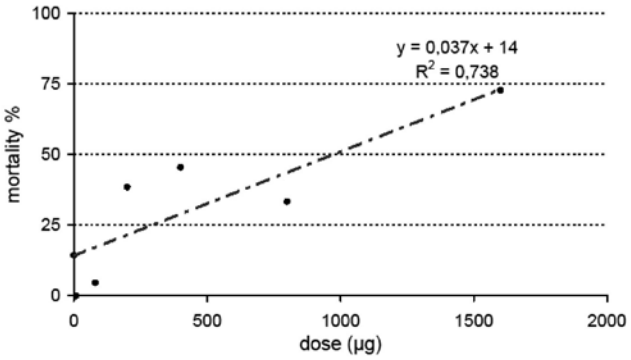

Fig. 2. Embryotoxic effect of bendiocarb on the chick embryo

control group (1304 ± 270 mg). This body weight of the chick embryos was higher than we expected (Table 1, Fig 1).

Lethal dose – LD_{50}

The chick embryos inoculated on ED 2 showed increasing mortality depending upon the bendiocarb dose; except for the dose of 800 µg/egg, which demonstrated a lower mortality than at lower doses of bendiocarb (200 and 400 µg/egg). The results of the mortality aspect (including the control group) are shown in the Table 1 and Fig. 2. The lethal dose of the chick embryos (LD_{50}) after bendiocarb administration on 2 ED was 973 µg/egg.

Malformations

We observed only 4 malformations (2.6 %) out of the total number of the chick embryos (144 individuals) who survived and were sampled on ED 9. These malformations were observed both in the control group and experimental groups. We observed monophthalmia, cleft beak, opening of the body cavity and growth retardation of the chick embryo (Table 1, Fig. 1 and 2).

DISCUSSION

Up to this time, the embryotoxic effects of bendiocarb on the chick embryo has not been studied, despite the fact that the embryotoxic effect of the carbamate pesticides (carbaryl and aldicarb) on the hatching of the chick embryos has been observed. Carbaryl was injected on ED 5 and ED 15 at 45 $mg.kg^{-1}$ egg weight and was found to be extremely toxic to the embryos on ED 5 of incubation, in comparison with treatment on ED 15. Carbaryl injected on 5 ED caused locomotion alterations and reduced hatchability of the chick embryos. Aldicarb injected on ED 5 and ED 15 at 1.5 $mg.kg^{-1}$ egg weight did not affect the hatchability of the chicks. However, the locomotion of chicks was affected in both treatment groups until 47 days after hatching (2). Reduced survival rates or lowered birth weights were observed in female rats at bendiocarb doses of 40 $mg.kg^{-1}$ from days 6–15 of gestation (6).

In our study we used white Leghorn chicken eggs. However it may not be excluded that other breeds might be just as sensitive to the bendiocarb effect. Similarly, the results of our study concluded that different commercial strains of White Leghorn chick embryos (Dekalb Delta, Hy-Vac SPF type L and Hy-Vac SPF type V) showed different susceptibilities to the induction of ventricular septal defect by 50 % ethanol (1). It is possible that the feed of hens could also play a role in the susceptibility to the effect of BC. Embryos born to vitamin-A deficient females had many congenital defects, including abnormalities of the central nervous system and the neural crest derivatives. Conversely, it was subsequently shown that too much vitamin A is equally harmful to the embryo and that a similar spectrum of defects arises in the central nervous system and neural crest derivatives (12). Deficiency or abundance of vitamin A could cause fatal consequences to the development of the chick embryo together with the bendiocarb.

In mammals, Flesarova *et al.* (4) noted fairly low toxic bendiocarb effects in rabbits after oral administration. Results of their study suggested negative effects of bendiocarb on the formation of thymus structures (4).

Our study involving administration of bendiocarb on ED 2 showed considerably lower proportion of malformations (2.6 %) compared to mortality (25 %).

The effect of carbamate pesticides on the morphogenesis and development of the chick embryo was observed after injection of carbaryl in the allantoic sac on ED 5 and ED 6. In comparison with a control group, some morphological alterations have been found in the embryos treated, at the expense of the skeleton apparatus, particularly in the most distal portions, as tibial and metatarsal angulations with their curtailment (10).

Persistent locomotion alterations were observed only at the higher doses of both carbaryl and aldicarb when the specific neurotransmitters measured returned to normal levels (3).

The average mortality in the control group was 14 % (35/5). The morality in the experimental groups was higher depending on the dose (except for the mortality at the dose

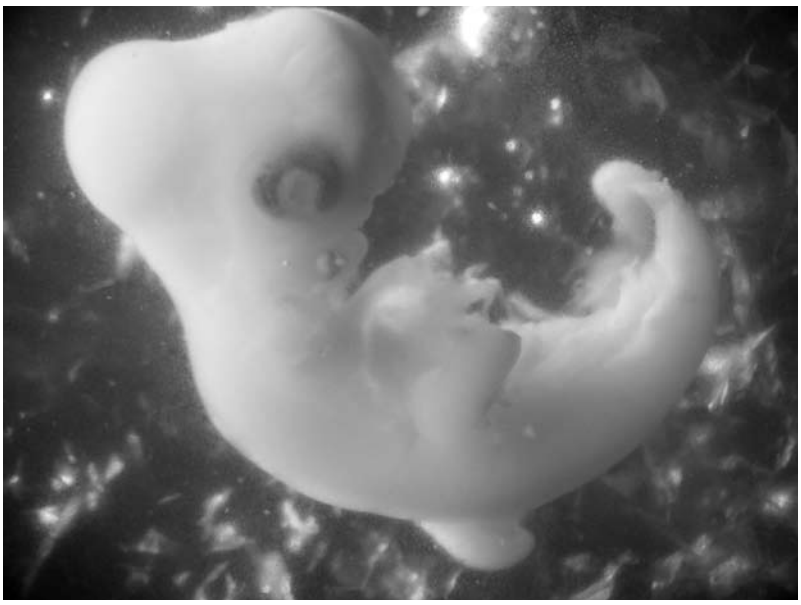

Figure 3: Growth retardation of the chick embryo

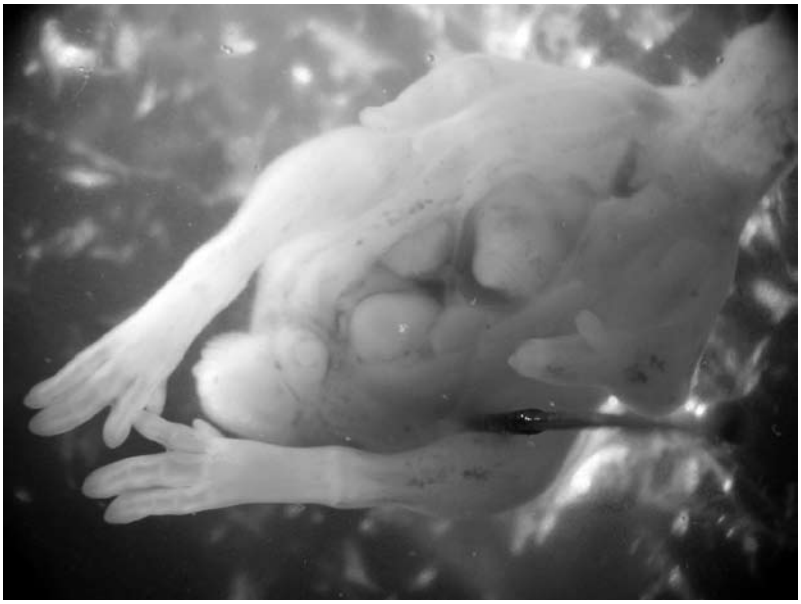

Fig. 4. Opening of the body cavity of the chick embryo

of 800 µg/egg). When an embryotoxic dose of a substance is administered into the immediate vicinity of the chick embryo, one should realize that the number of malformed embryos alone may not be an adequate indication of its teratogenic potential, especially if only a few dose levels are used (13). The first possible explanation may be that embryos suffering from a potential inborn defect, die simply because the malformation is incompatible with further life and development. This is called prenatal selection, such as a result of early spontaneous human abortions. Prenatal selection may also appear in the chicken embryos, whereas only 70 % of individuals hatch from unopened eggs. The next possible reason of progressive embryonic mortality on increasing the dose, could be the interference of some classes of substances and/or doses with basic vital functions. In mammals a third possibility must be considered – the frequent rejection of fe-

tuses as a result of intoxication, stress or some other change in the maternal organism and/or placental function (13).

CONCLUSIONS

In our study we observed fairly low embryotoxic effects to the bendiocarb dose administered on ED 2. The weight of the chicken embryos was lower depending on the dose. The chick embryos surviving the highest dose (1600 μg/egg) were only the strongest individuals (27 %). This indicated the tendency of chicken embryos to succumb to the toxic effect of bendiocarb.

ACKNOWLEDGEMENT

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3. Brown, L. W., Johnson, E. M., 1989: Enzymatic evidence of alkaline phosphatase. In **Caster, A. R.:** *Enzymology*. Plenum Press, New York, 99–101.

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ACKNOWLEDGEMENT

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