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INDUCTION OF MICRONUCLEI IN RAT BONE MARROW AFTER CHRONIC EXPOSURE TO LEAD ACETATE TRIHYDRATE

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ABSTRACT

The data concerning the mutagenic, clastogenic and carcinogenic properties of inorganic lead compounds have been conflicting. Here, we evaluated the frequency of micronuclei in bone marrow erythrocytes of male rats treated with lead acetate trihydrate. Outbred male Wistar rats were exposed daily to dose of 100 mg.l^{-1} of drinking water for 125 days. The effects of lead acetate trihydrate are both cytotoxic and genotoxic because of a decrease in the ratio of polychromatic to normochromatic erythrocytes and an increase in the frequency of micronucleated polychromatic erythrocytes, respectively.

Key words: erythrocyte bone marrow test; genotoxicity; lead acetate trihydrate; micronucleus; rat

INTRODUCTION

Pollution is one of the primary ways in which humans have caused drastic modifications of wildlife habitats. Historically we have regarded the air, water, and soil that surround us as waste receptacles and have given little consideration to the ecological consequences of our actions. As a result, wildlife populations are confronted with a bewildering array of pollutants that we release into the environment either by intent or accident. The number of chemicals that affect man and animals, e.g. synthetic chemicals, oil, acid rain and toxic metals, is increasing at alarming rates.

Heavy metals rank among the most widespread environmental contaminants. They are introduced into the environment in two main ways, by natural and anthropogenic processes. The natural processes include weathering, atmospheric deposition related to soil, processes in oceans and volcano eruptions. Heavy metals are a natural part of the Earth's surface and thus they occur in different levels throughout nature (17, 16). The anthropogenic sources are related to the burning of fossil fuels, industrial activities, agriculture, tourism and use of various means of transport.

One of the toxic metals used in industry is lead. Its absorption in organisms is affected by age, the chemical form of the lead, and minerals in the diet (e.g., iron, calcium, and zinc). Once absorbed, lead is transported to the red cells, then distributed to the blood plasma, the nervous system, and soft tissues. It is subsequently redistributed and accumulates in bone; approximately 75 % to 90 % of the lead body burden is found in bones and teeth (3, 1). Lead can induce toxic effects in several biological systems. It is known to have deleterious effects on nervous (2), renal (11), immune (27) and reproductive tissue (26). The mutagenic potential of lead is still being investigated.

The aim of this study was to evaluate lead-induced genotoxic damage in chronically exposed Wistar rats using the erythrocyte bone marrow assay.

MATERIAL AND METHODS

Animals

Eleven clinically healthy male Wistar rats SPF were obtained from the accredited breeding station Central Zodiac of the Medical Faculty, University of P. J. Šafárik in Košice, the Slovak Republic. There were six males weighing 444.2 ± 33.08

grams in the experimental group. The control group consisted of five males with mean body weight 464.0 ± 44.92 grams. The animals were housed in plastic cages (four rats per box) and acclimatized for one week before the experiment. The animals were observed prior to the study to ensure that they were healthy. Only animals found to be in a clinically acceptable condition were assigned to the study. Food and water consumption, general condition and any other clinical symptoms were monitored daily. Body weight changes were assessed weekly. During the study, food and water were offered *ad libitum*. Animal quarters were maintained at 22 °C (± 2 °C); 30–70 % relative humidity on a twelve hour light/dark cycle.

Chemical

Lead acetate trihydrate ($Pb[C_2H_3O_2]^2.3H_2O$); CAS number: 6080-56-4; purity 99.5 %) was obtained from Lachema, Brno (the Czech Republic).

Dose and exposure

Lead acetate trihydrate was applied to drinking water at a dose of 100 mg.l^{-1} for 125 days.

Animal sacrifice, bone marrow cell isolation, and slide preparation

Before the animals were killed, a 10 ml centrifuge tube was filled with 5 ml of foetal calf serum for each individual (PAN SYSTEMS GmbH, Biotechnologische Producte). All rats were killed 24 hours after the end of exposure by vertebral cervical dislocation and both femora were removed. The bones were then freed from muscle manually by the use of gauze. The proximal and distal ends of the femur were carefully shortened with scissors until a small opening to the marrow canal became visible. With a needle of about 3.0 ml serum was drawn from the tube into a plastic syringe. Then the needle was inserted a few mm into the proximal part of the marrow canal and the marrow was gently flushed in the prepared test-tube.

The tubes were then centrifuged at 800 rpm for 5 minutes. The supernatant was removed with Pasteur pipette. A small drop of the viscous sediment was put on the end of a slide and spread by pulling the material behind a cover glass held at an angle of 45 degrees. The preparations were then air dried and fixed in absolute methanol for 6 minutes. Cells were stained with May-Grünwald (3 minutes) and Giemsa-Romanowski (15 minutes).

Scoring and statistical analysis

Criteria for scoring follow those of MacGregor *et al.* (19) and OECD Guideline 474 (24). At high magnification one thousand polychromatic erythrocytes (PCEs) per animal from coded slides were screened for the presence of micronuclei (MNPCEs). Separately, the number of micronucleated mature erythrocytes (MNNCEs) was registered in a total of 1000 normochromatic erythrocytes (NCEs) per animal. Toxicity to bone marrow was estimated by the relationship between polychromatic (PCEs) and normochromatic erythrocytes (NCEs) frequency. The ratio of PCEs to NCEs was determined in the first 500 erythrocytes scored per animal.

The Student's *t*-test was used to compare MNPCEs and MNNCEs values and PCE to NCE ratios between treated and control animals for statistical significance.

RESULTS

No overt signs of toxicity were observed during of the study. Whilst, body weight was markedly affected by treatment (Table 1). The mean body weight for animals in the experimental group was 444.17 ± 33.08 g at the beggining and 429.17 ± 58.09 g at the end of treatment. There was statistically significant decrease in body weight of exposed rats at the end of treatment in comparison with the control group ($p = 0.0471$).

Table 1. Body weight changes after chronic exposure to lead acetate trihydrate (100 mg.l^{-1} of drinking water) in male Wistar rats

Animal	b.wt. (g) initial	b.wt. (g) final
K9	490	505
K10	445	465
K12	425	480
K13	430	485
K14	530	590
Mean ± SD	**464 ± 44.92**	**505 ± 49.62**
P29	425	440
P30	425	455
P33	500	315
P34	465	475
P35	410	430
P37	440	460
Mean ± SD	**444.17 ± 33.08[a]**	**429.17 ± 58.09[*]**

[a] — no significant difference; [*] — statistical significance ($P < 0.05$); K_x — control animals; P_x — exposed animals; SD — standard deviation; b.wt. — body weight in grams

Micronucleus frequencies and distributions of micronuclei observed in polychromatic and normochromatic erythrocytes as well as the ratio of polychromatic to normochromatic erythrocytes of male rats are shown in Table 2. The chronic exposure to lead acetate trihydrate resulted in a significant increase in the number of micronuclei in polychromatic erythrocytes (MNPCEs) of the male rats compared with the control group (24.17 ± 7.859/1000 PCEs *vs.* 4.00 ± 4.528/1000 PCEs; $p = 0.0007$).

No significant differences in micronucleated normochromatic erythrocytes in exposed rats were observed (22.67 ± 9.933/1000 NCEs *vs.* 16.44 ± 15.630 NCEs; $p = 0.439$).

The ratio of polychromatic to normochromatic erythrocytes of exposed rats was significantly decreased in comparison with the control (0.715 ± 0.431 *vs.* 1.343 ± 0.306; $p = 0.0232$).

Table 2. Micronucleus frequencies and distributions in bone marrow cells of male rats after chronic exposure to lead acetate trihydrate (100 mg.l^{-1} of drinking water)

Animal	PCEs	MNPCEs	MN distribution in PCEs			NCEs	MNNCEs	MN distribution in NCEs			Ratio PCEs/NCEs
			1	2	3			1	2	3	
K9	1000	6	6	0	0	1000	8	8	0	0	1.416
K10	1000	1	1	0	0	1000	7	7	0	0	1.276
K12	1000	2	2	0	0	1000	6	6	0	0	1.210
K13	1000	0	0	0	0	1000	43	33	5	0	1.819
K14	1000	11	9	1	0	1000	18	18	0	0	0.995
Mean ± SD		**4.0 ± 4.528**					**16.40 ± 15.630**				**1.343 ± 0.306**
P29	1000	29	25	2	0	1000	34	34	0	0	1.589
P30	1000	33	12	9	1	1000	34	30	2	0	0.574
P33	1000	27	21	3	0	1000	20	18	1	0	0.573
P34	1000	27	23	2	0	1000	24	20	2	0	0.503
P35	1000	16	16	0	0	1000	12	12	0	0	0.578
P37	1000	13	13	0	0	1000	12	8	2	0	0.470
Mean ± SD		**24.17 ± 7.859*****					**22.67 ± 9.933**^{a}				**0.715 ± 0.431***

^{a} — no significant difference; * — statistical significance ($P < 0.05$); *** — statistical significance ($P < 0.001$); K_x — control animals; Px — exposed animals; SD — standard deviation; PCEs — number of polychromatic erythrocytes screened; MNPCEs — number of micronucleated polychromatic erythrocytes; NCEs — number of normochromatic erythrocytes screened; MNNCEs — number of micronucleated normochromatic erythrocytes; ratio PCEs/NCEs — ratio of polychromatic to normochromatic erythrocytes; MN — micronuclei

DISCUSSION

Lead is a heavy metal whose widespread distribution in the environment has the potential to affect a large number of people and animals, thus, its genotoxicological investigation is of importance. The results of the genotoxicity and carcinogenicity studies dealing with lead compounds may be influenced by a number of factors such as cell type, duration and route of exposure. The mechanisms for the genotoxic responses to lead exposure may involve indirect damage to DNA affecting the stabilization of chromatin (14, 37) or interacting with repair processes (18, 6). Under *in vitro* conditions it affects the fidelity of DNA synthesis (7, 33). Lead is believed to covalently interact with tertiary phosphate ions in nucleic acids and proteins (9). In proteins it can also be substituted by calcium and zinc. This substitution can promote further changes in these proteins and thus can alter cellular metabolism and induce aberrant gene transcription.

Lead compounds have been classified by IARC (13) as possibly carcinogenic to humans (group 2B). It has recently been reported that diets rich in antioxidant nutrients may reduce the risk of certain types of cancer and that multivitamin dietary supplements can significantly decrease the frequency of chromosome aberrations in organisms exposed to this metal (20, 10).

Lead is highly reactive and forms numerous compounds that have very different physical-chemical properties. One important property of lead compounds in biological systems is solubility that affects bioavailability. For this study lead acetate trihydrate was chosen as one of the major soluble lead compounds.

Lead acetate trihydrate is used as a mordant in cotton dyes, as a lead coating for metals, as a drier in paints, varnishes, pigment inks, insecticides, and as a colorant in hair dyes (13).

Studies on the clastogenic effects of lead acetate trihydrate are contradictory. Lead acetate is not mutagenic in bacteria or yeast. Positive and negative results have been obtained in cultured mammalian cells *in vitro* (3). Muro and Goyer (21) detected chromosome gaps and breaks by 1% lead acetate in mouse leukocytes. An increase in the frequency of chromosomal aberrations has also been reported in human lymphocytes (5, 23). On the other hand, Bauchinger and Schmid (4) have failed to observe any significant change in the chromosomal aberrations in Chinese hamster ovary cells exposed to different concentrations of this compound. Hartwig *et al.* (12) have found that lead acetate alone did not induce DNA-strand breaks in HeLa cells or mutations at the HPRT locus, nor did it induce sister-chromatid exchange in V79 Chinese hamster cells.

When combined with UV irradiation, lead ions inhibit the removing of DNA-strand breaks and enhance the number of UV-induced mutations and sister-chromatid exchange, indicating an inhibition of DNA repair. Valverde *et al.* (34) have not detected direct induction of DNA strand breaks by lead acetate at low noncytotoxic concentrations (0.01, 0.1 and 1.0 μmol). Recent results show that low-level lead acetate exposure *in vitro* can induce significant cytogenetic damage in human melanoma cells (B-Mel) (25).

However, several *in vivo* studies confirm the clastogenic action of lead acetate in mammalian models (30, 32). Celik *et al.* (9) have reported increased frequency of micronuclei in peripheral blood erythocytes of rats after subchronic per oral exposure to lead acetate trihydrate (140, 250 and 500 $mg.kg^{-1}$ b.wt. for 10 weeks). The same result has been observed in suckling rats exposed to lead acetate either orally for nine days (daily dose 2 $mg.kg^{-1}$ b.wt.) or by a single intraperitoneal injection (5 $mg.kg^{-1}$ b.wt.) (15). Šutiaková *et al.* (31) have observed an increased frequency of micronucleated polychromatic erythrocytes in female Wistar rats after perorally exposure to 100 mg lead acetate trihydrate.l^{-1} drinking water. In BALB/c mice receiving lead acetate in doses of 50, 250, and 500 $mg.kg^{-1}$ b. wt. An increased number of micronuclei in polychromatic erythrocytes of the bone marrow has been reported by Rusov *et al.* (29). After a single intraperitoneal injection of lead acetate an increase in the relative number or size of myeloid/monocytic cells in bone marrow cells of Balb/c mice has been observed (8).

Genotoxicity induced in CD-1 mice by inhaled lead acetate aerosol (6.8 $mg.m^{-3}$) has been assessed by the comet aasay (35). A significant increase in DNA damage has been observed in kidney cells from rats perorally exposed to 107 $mg.kg^{-1}$ lead acetate (28).

A significant increase in numerical chromosome aberrations in the bone marrow cells of rats, but not the number of structural aberrations (with or without gaps) after subchronic exposure for four weeks (5 times/week) to lead acetate (10 $mg.kg^{-1}$ in distilled water *per os*) has been reported by Nehez *et al.* (22). A dose-dependant, statistically significant increase in chromosomal aberrations has also been observed in the bone marrow cells of mice exposed once daily for five days to 200 or 400 mg purified lead acetate.kg^{-1} diet (1).

The influence of lead on sperm morphology, sister chromatid exchanges or on micronuclei formation has been studied on male rabbits after exposure to doses of 0, 0.25 and 0.50 mg lead acetate.kg^{-1} b. wt. subcutaneously injected three times a week over fourteen weeks (36).

It is still a matter of speculation whether the differences in the results of genotoxic studies are due to different concentrations of lead acetate, different sensitivity of cells, or different maturity of cell systems.

In our study the effects of lead acetate trihydrate in rat males were both cytotoxic and genotoxic because of a decrease in ratio of polychromatic to normochromatic erythrocytes and an increase in frequency of micronucleated polychromatic erythrocytes, respectively.

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COMPARATIVE LIGHT AND ELECTRON MICROSCOPIC STUDIES OF UROTHELIUM OF BRACKEN FERN FED AND EBH AFFECTED COWS

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SUMMARY

A comparative ultrastructural study was undertaken in two nondescript hill cows; one fed with bracken fern (BF) @ 30 % for 60 MPF while the other was a spontaneously affected case of enzootic bovine haematuria (EBH). An earlier necropsy showed one had a thickened bladder with a few wart-like pedenculated growths, while the other showed a moderate size cauliflower-like growth on the ventral mucosal surface. Histopathologically, the BF fed cow urinary bladder showed chronic cystitis and papilloma while the EBH affected urinary bladder evinced changes of papilloma. Electron microscopic examination of urinary bladder mucosa in the BF fed cow showed widened intercellular spaces of smooth muscle cells with a greater number of dense bodies, margination of the nucleolus with clumping of heterochromatin in the nucleus, activated macrophages and numerous tonofilaments. The urothelium growth of the EBH affected cow revealed a loss of the scalloped appearance of mucosa, loosened tight junctions on the apical surface, microvilli-like structures on the urothelial cell surface, a urothelium containing numerous mitochondriae, dilated fusiform vesicles and a bizarre-shaped nucleus in smooth muscle cells. From ultrastructural studies, it was inferred that compared to the urothelium of the BF fed cow, benign changes in the EBH affected urothelium were pronounced and advanced.

Key words: bracken fern toxicity; papilloma; tumour; ultrastructure; urothelium

INTRODUCTION

Enzootic Bovine Haematuria (EBH) is a chronic affection of hill cattle, clinically characterized by intermittent haematuria, debility, chronic cystitis and/or urinary bladder neoplasia and finally death. It is an economically important disease of hill cattle in the Himalayan region of India and highlands of many other countries (5).

EBH is attributed to chronic bracken toxicity. Ptaquiloside is one of the toxins, responsible for this condition (11, 19). Experimental feeding of bracken fern for prolonged period has induced urinary bladder neoplasia in cattle in abroad (12, 13).

The target organ of EBH and bracken toxicity is the urinary bladder while the target cells of these ailments are transistional epithelial cells. Cellular and subcellular changes induced by these agents are not well known. Preliminary ultrastructural studies of the urothelium of EBH affected/bracken fern fed cattle were undertaken in our laboratory (17, 18). Ultrastructural features were described but we failed to demonstrate any virus particles in the urothelium.

Sanchez *et al.* (16) have examined the urinary bladder of two cattle affected with enzootic haematuria under high-resolution light and electron microscopy. Extensive areas of sub-epithelial haemorrhage, alternating with ulcerative lesions were observed. Epithelial cells connective tissue and endothelial cells of the *lamina propria* had large vacuole in the cytoplasm. Numerous viral elements loading large vacuoles and exocytosed mature viruses were observed on electron microscopy of the epithelial cells. These viruses had a polyhexagonal shape with an average diameter of 104 nm. The virus was identified as a cytomegalovirus. It was concluded that massive viral reproduction had caused the degeneration of endothelial cells resulting in a subsequent haemorrhagic ulcerative process causing the haematuria.

In the present investigation, a comparative study of ultrastructural changes in urothelial cells, influenced by bracken toxin and affected with neoplasia were undertaken and the results are reported in this communication.

MATERIALS AND METHODS

Two adult nondescript hill cows formed the basis of the present experimental study. Of these, Cow No. 22 was fed bracken fern powder in a concentrated ration mixture @ 30 % w/w for 60 MPF, while Cow No. 47 was affected with spontaneous EBH during the study. This animal was fed a normal ration mixture @ 1 kg.day^{-1}. In addition, both animals were served plenty of green cultivated fodder. Both animals were maintained in experimental sheds of this Division under standard management conditions. Both these animals were humanely sacrificed and detailed necropsy examination was conducted.

For histopathological studies, pieces of mucosa of urinary bladder were collected in 10 % formalin. Tissues were conventionally processed, paraffin embedded, 4—5 µm thin sections were cut and stained with H&E as per routine procedure.

For transmission electron microscopic studies in cows, samples of urinary bladder with or without tumour were collected within seconds of the slaughter of the animals. Immediately after collection, tissue pieces were transferred to a petrie dish containing a few drops of chilled 2.5 per cent glutaraldehyde. Sections in the form of 1 mm^2 were cut and fixed in 2.5 per cent gluteraldehyde in 0.2 mol phosphate buffer (pH 7.4) for 6 h at 4 °C. The tissue pieces were then washed with three changes (2 hours each) of cold 0.2 mol phosphate buffer (pH 7.4) and post fixed with one per cent osmium tetroxide for 4 hours at 4 °C. The tissues were dehydrated in ethyl alcohol, cleared and embedded in epon-araldite resin (10). Ultrathin sections (600 °A) were cut by employing ultramicrotome (Ultracut. Reichert-Jung, Austria) and mounted on copper grids and stained with uranyl acetate (20), subsequently by lead citrate (25). They were washed and allowed to dry on a filter paper in a covered Petridish at room temperature. The grids were viewed in an electron microscope (Morgagni-268 and Philips CM-10, Holland) at the Electron Microscope Facility, Department of Anatomy, All India Institute of Medical Sciences, New Delhi.

RESULTS

The Bracken fern fed cow

Necropsy findings: The urinary bladder of cow No. 22 was thickened and congested. It contained a haemorrhagic area and pea-sized nodular to pedunculated growth attached on its mucosal surface.

Light microscopy: The urinary bladder mucosa was thrown into finger-like projections or like-papilla of tongue (Fig. 1). Occasionally, focal hyperplasia of lining epithelium was also present. The lining epithelium showed cytoplasmolysis, vacuolar degeneration with a formation of cyst-like structures. Below the lining of the epithelium, numerous small blood capillaries along with fibro-cellular reaction and focal infiltration of lymphocytes were seen. These changes indicate chronic cystitis with papilloma.

Electron microscopy: The mucosa had smooth muscle cells with a widened intercellular space. One cell contained a nucleus with the presence of a few clefts, margination of nucleolus and clumping of heterochromatin (Fig. 2). Sarcoplasm showed a greater number of dense bodies and RER. Caveolae was absent. In one place, a fibrocyte was also present which had an elongated nucleus with a thin rim of cytoplasm. Vacuole and few secretory vesicles were present in the cytoplasm. It also had a thin flattened filamentous hyphae-like structure, which consisted of an organelle and certain vacuoles. Divided lymphocytes-like cells were seen. From these two cells, one cell had a round nucleus with margination of heterochromatin. Both cells contained a demarcated cytoplasmic membrane and few mitochondriae and RER (Fig. 3).

The macrophage had lysosomes with small electron dense material, dilated RER, mitochondria and coated vesicles of proteinaceous materials (Fig. 4). The cytoplasmic membrane of the macrophage was not appreciable and the nucleus was absent. In places, a large amount of tonofilaments was present (Fig. 5).

The EBH affected cow

Necropsy findings: Generalised thickening of urinary bladder wall, congested mucosa and varying sizes of haemorrhagic areas were seen. Moderate sized cauliflower-like growths were present on the mucosal surface (Fig. 6). These growths were present either on the ventral or lateral surface of mucosa in urinary bladder.

Light microscopy: The lining of the epithelium of the urinary bladder showed finger-like papillary projections (Fig. 7). From the lining of the epithelium, along with abundant loose connective tissue, stroma (*fibrovascular stroma*) were seen. Occasionally, engorged blood vessels and marked multiple focal areas of infiltration of lymphocytes were also present in the *lamina propria*. The condition was diagnosed as papilloma.

Electron microscopy: Mucosa showed the absence of the scalloped appearance of the lining of the epithelium. Intercellular space was marked. They contained an electron luscent nucleus along with other cytoplasmic organelles such as mitochondria, few lysosomes and varying sizes of vacuoles. In one place, the capillary lumen was studded with erythrocytes (Fig. 8). In other area, urothelial cells revealed the presence of an irregular shaped nucleus. Elongated villi like projections were also observed in the widened intercellular space (Fig. 9). The smooth muscle cell had a bizarrely shaped nucleus. The sarcoplasmic membrane contained dense bodies. Numerous tonofilaments and vacuolations were also seen (Fig. 10). In one place, an electron dense finger-like indentation protruding from the nucleus was also seen (Fig. 11).

DISCUSSION

Gross and light microscopic examination of urinary bladder mucosal growth of bracken fern fed cows showed changes of chronic cystitis and papilloma. Experimental feeding of bracken fern is reported to induce urinary bladder neoplasia (12, 13). In the present study prolonged

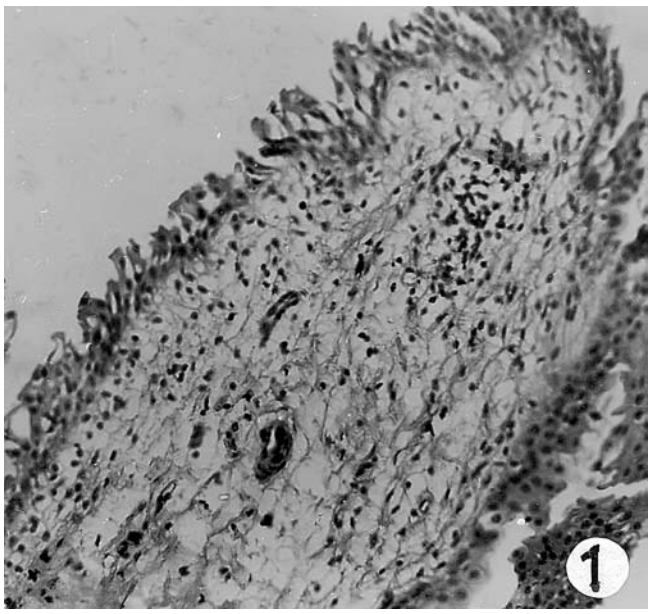

Fig. 1. Urinary bladder mucosa showing finger-like projections with infiltration of a few mononuclear cells into the *lamina propria*. Bracken fern fed cow. H&E, ×150

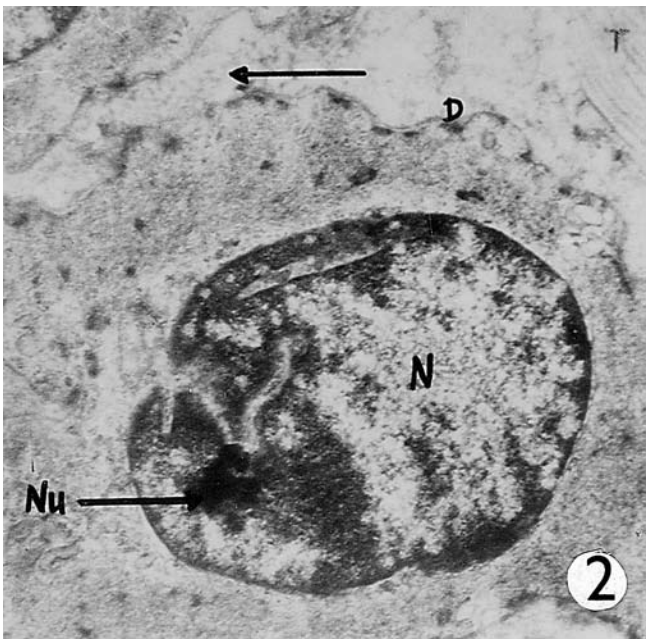

Fig. 2. Two smooth muscle cells with marked intercellular space (arrow) are seen. In one cell, the nucleus (N) shows the presence of cleft and margination of the nucleolus (Nu). The sarcoplasmic membrane has dense bodies (D). Adjacent to the muscle cells tonofilaments (T) are also seen. BF. Lead citrate & Uranyl acetate, ×2900

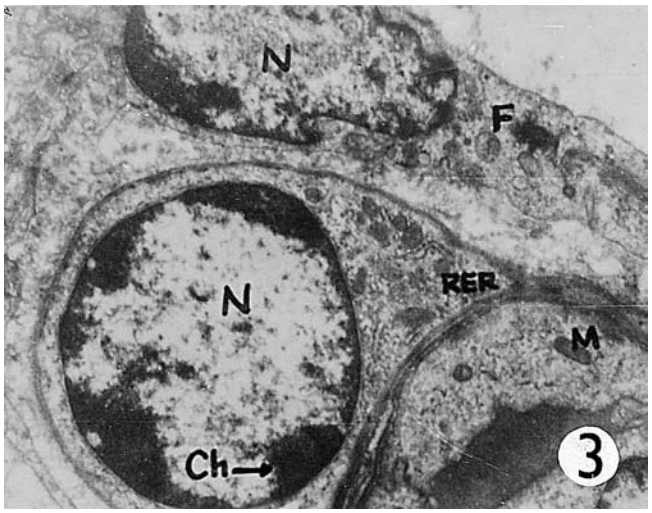

Fig. 3. Divided lymphocyte like cells are seen. Both cells have round to somewhat oval nuclei (N) with margination of the nuclear chromatin (Ch). The cytoplasm, contains mitochondriae (M) and a rough endoplasmic reticulum (RER). Adjoining these cells a fibroblast (F) is visible with a somewhat elongated nucleus. BF. Lead citrate & Uranyl acetate, ×2900

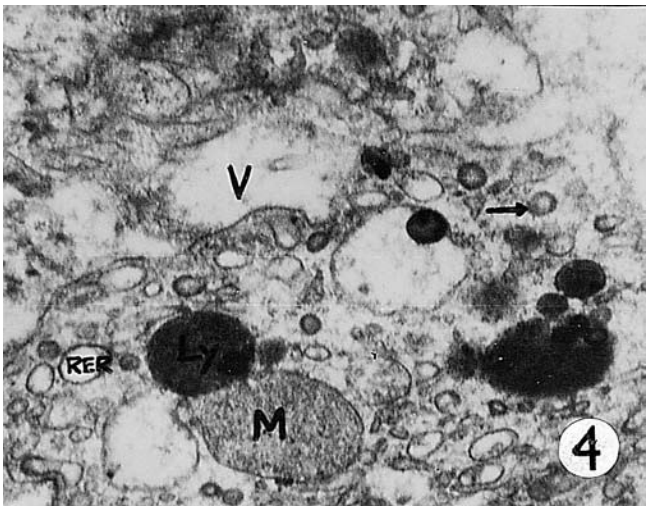

Fig. 4. Activated macrophage showing the absence of the cytoplasmic membrane. It contains lysosome (Ly), a dilated rough endoplasmic reticulum (RER), vacuoles (V), mitochondriae (M) and proteinaceous material (coated vesicles–arrow). Adjoining the macrophage, tonofilaments(T) are also visible. BF. Lead citrate & Uranyl acetate, ×5400

feeding of bracken fern for 60 MPF induced microhaematuria and a benign type of pathological change. The reason for this may be a low level of ptaquiloside (4.5 mg.kg^{-1}) present in bracken fern fed. This finding is in accordance with Rajendran *et al.* (1983) who have induced preneoplatic changes in the urinary bladder after feeding BF for 1,094 days to calves.

In the electron microscopic examination it showed the presence of smooth muscle cells with a widened intercellular space and a sarcoplasm containing a greater number of dense bodies. The nucleus showed margination of the nucleolus and clumping of heterochromatin. A few fibroblasts were also present. Occasionally, lymphocytes and macrophages were infiltrated into the muscularis layer. The macrophage contained lysosome, dilated RER and coated vesicles of proteinaceous materials. In some places,

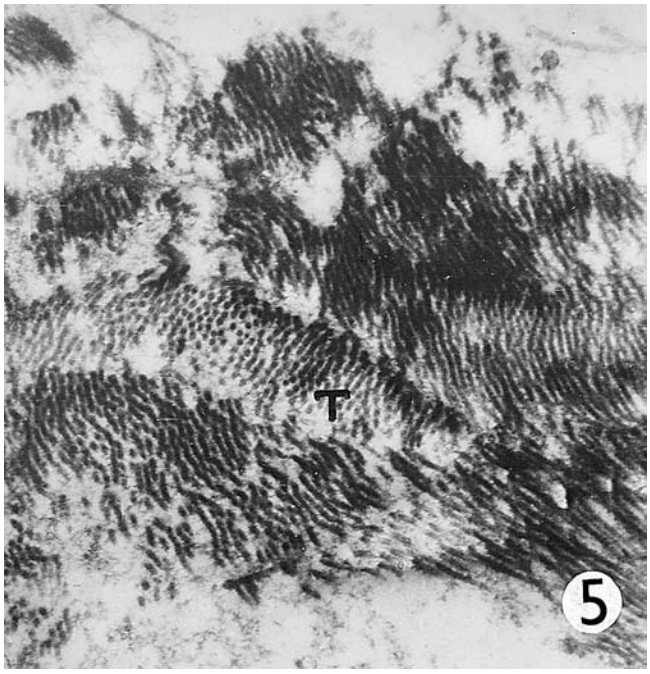

Fig. 5. Numerous tonofilaments (T) are seen in an amorphous granular ground substance. BF. Lead citrate & Uranyl acetate, ×2400

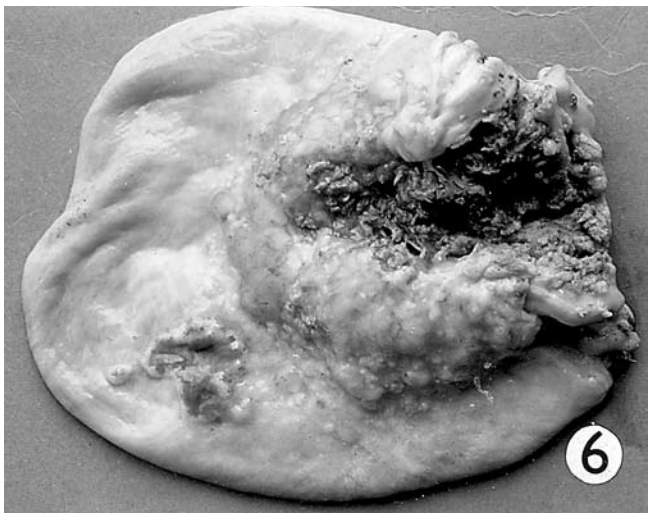

Fig. 6. Urinary bladder mucosa showing congestion and a cauliflower-like growth on its ventral surface in an EBH affected cow

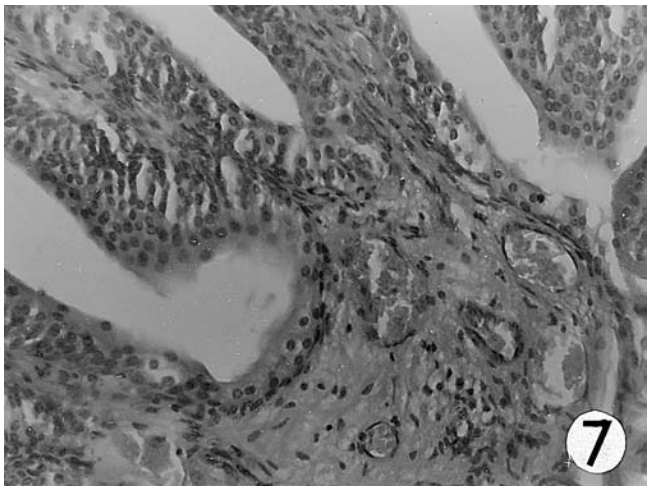

Fig. 7. Urinary bladder papilloma showing finger-like papillary projections of mucosa (longitudinal section) with fibrovascular stroma at the *lamina propria*. EBH H& E, ×150

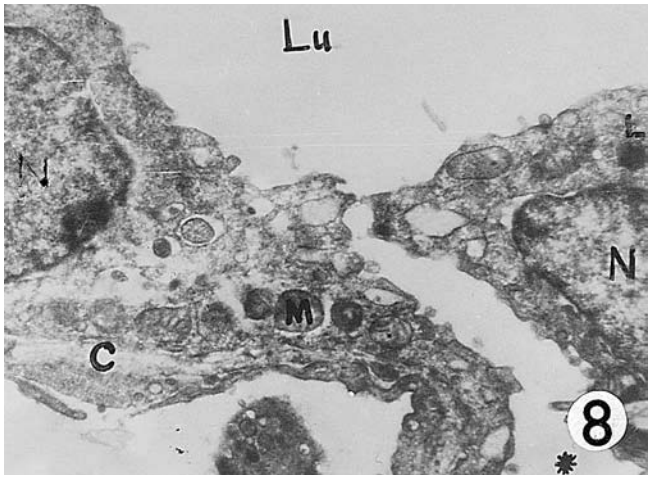

Fig. 8. Surface bordering lumen (Lu) showing part of two nuclei (N) interacellular space (*) and a cross section of a capillary along with an erythrocyte (E) in its lumina. The intercellular space is markedly widened (*). The cytoplasmic areas adjoining the intercellular junction/space show mitochondriae (M), lysosome (Ly) and vacuoles (v). EBH. Lead citrate & Uranyl acetate, ×3400

numerous tonofilaments were also present. Somvanshi (14) has observed various ultrastructural changes in a biopsy of the urinary bladder of *Polystichum* and bracken fern fed cows. This included the lining of the epithelium containing swollen and degenerated RER, pyknotic nuclei and polymorphonuclear cell and lymphocytic infiltration. In addition, the presence of the smooth muscle cell with a centrally placed nucleus and widened intercellular spaces were described.

Increased intercellular space between muscle cells may be due to cell shrinkage. Absence of caveolae in the present study may reflect poor contraction of muscle cells. This finding is supported by Cross and Mercer (4) who have described the role of caveolae in calcium transport between the cells which in turn is involved in the contraction of muscle cells. Margination of the nucleolus against the nuclear membrane in present study is in agreement with Ghadially (7) who stated that margination of nucleolus is common in cells with active protein synthesis. He has pointed out further that nucleolar margination is frequently seen in a variety of tumours. Although in the present study, a tumour was not observed, it might be due to hyperplastic changes of muscle cells due to fern feeding.

The urinary bladder was grossly thickened, congested and had a moderate size cauliflower like the growth in the EBH affected cow. Histopathologically papilloma

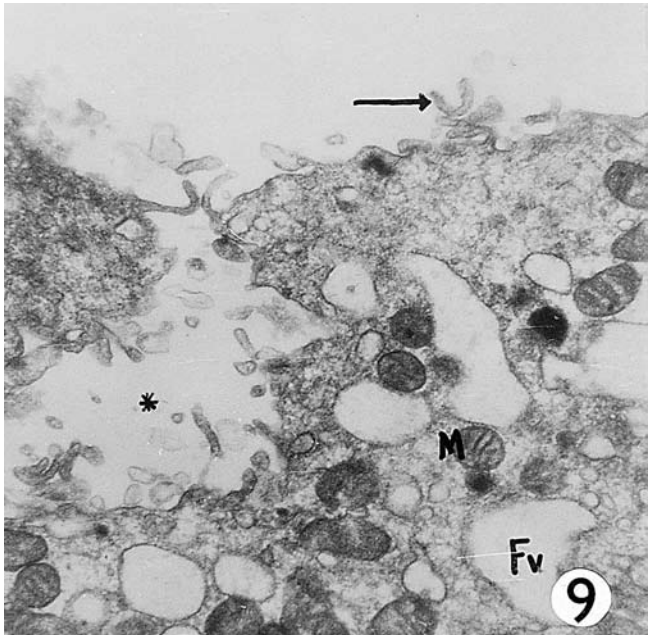

Fig. 9. Elongated villi-like projections (arrow) are seen on the cytoplasmic membranes of the urothelium and widened intercellular space (*). Part of the nucleus (N) is also visible.Numerous mitochondriae (M), dilated fusiform vesicles are present in the cytoplasm. EBH. Lead citrate & Uranyl acetate, ×4600

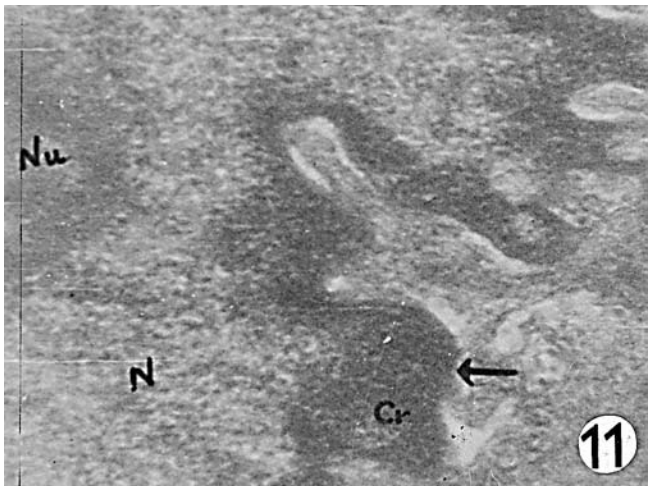

Fig. 11. Higher magnification of nuclear membrane showing electron dense finger-like projections (arrow) with margination of chromatin (Ch). The cytoplasm is electron dense. EBH. Lead citrate & Uranyl acetate, ×15500

Fig. 10. Cross section of smooth muscle cell showing bizarrely shaped nucleus (N). Nuclear membrane is thrown into finger-like indentation and the nucleolus (Nu) is present at the centre of nucleus. The sarcoplasmic membrane has dense bodies (arrow) and shows the absence of cytoplasmic organelles. Numerous tonofilaments (T) with vacuolation are also seen. EBH. Lead citrate & Uranyl acetate, ×3400

was diagnosed. Urinary papillomas are not uncommon in EBH affected cases. It is attributed due to BPV2 in some cases (3).

In the EBH affected cow, the scalloped appearance of the luminal surface of mucosa was absent. Intercellular space was markedly widened and showed the presence of micro villi-like structures over its cell surface and a tight junction at the apical portion of the luminal surface was detached The lining of the urothelium revealed the presence of a nucleus with numerous mitochondriae, dilated fusiform vesicles, RER and vacuoles in the cytoplasm. The blood capillary lumen was studded with RBCs. The later changes were similar to those observed in bracken fern fed rats in our earlier observation. The smooth muscle cell contained a bizarrely shaped nucleus with deep finger-like indentations of the nuclear membrane and electron dense nuclear heterochromatin.

Somvanshi (17) has examined ultrastructural changes of the urinary bladder in EBH affected cows. He has described the presence of bud-like structures from the surface lining of the urothelium. The urothelium showed swollen and double walled mitochondriae, vacuoles, pyknotic nuclei with electron dense heterochromatin. Infiltration of the mononuclear cells and capillary lumen containing bleb-like, structures were also present. Sanchez *et al.* (16) have ultrastructurally examined the urinary bladder of EBH affected cattle and found varying degrees of vacuolations in the cytoplasm of epithelial cell, connective tissue and endothelial cell along with numerous viral elements.

In a normal urinary bladder epithelium, the tight junctions at the apical portion of the cell membrane are a major constituent of the junctional complex, which may be responsible for the tight seal between the superficial cells (9). The degree of tight junction alteration often correlated with the tumour grade (6). Detachment of tight

junction at the apical portion of the urothelium in the present study is in agreement with Hicks (8) who has stated that the transepithelial leakiness found in bladder tumours could be explained on the basis of tight junction attenuation and its discontinuation. Further, Fulker *et al.* (6) have pointed out that the attenuation of the tight junction might resulting an influx of molecules from the urine into tissue. The presence of microvilli-like structures on urothelial surface observed in the present study has been described in inverted papilloma by Alroy *et al.* (1). This finding is further supported histopathologically as papilloma was diagnosed in the present EBH case.

The bizarrely shaped nucleus with electron dense heterochromatin in the present study is in accordance with Ghadially (7) who has described that tumour cells can assume bizarre forms of nuclei. He has further explained that hyperchromatic nuclei in tumour cells are due to the presence of numerous clumps of heterochromatin. Numerous tonofilaments were observed in urinary bladder of both bracken fern fed and EBH affected cows. In a study on the ultrastructure of canine urinary bladder carcinoma, Alroy (1) has reported that cells with squamous differentiation were rich in tonofilaments. Although ultrastructural changes in the urinary bladder described in the present study were mild the changes were comparable to those which have been explained in various tumours in different species. From ultrastructural studies, it is inferred that compared to the urothelium of the BF fed cow, benign changes in the EBH affected urothelium were pronounced and advanced.

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THE EFFECT OF SELENIUM-ENRICHED YEAST ON HAEMATOLOGICAL PARAMETERS AND SERUM CREATINE KINASE AND LACTATE DEHYDROGENASE ACTIVITIES IN LAMBS

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ABSTRACT

The objective of this study was to determine the effect of selenium (Se) from selenized yeast fed to lambs on their blood parameters, the activity of creatine kinase (CK), lactate dehydrogenase (LDH) in serum and Se in plasma. Lambs (n = 10) were randomly assigned to receive a basic diet (BD) providing a daily intake 50.6 µg of Se only or supplemented to provide 0.3 mg.kg^{-1} DM Se from the selenized yeast and giving a total daily intake 278 µg of Se per animal. Lambs which were fed additional Se had an increased (P < 0.001) concentration of Se in plasma and greater activity of creatine kinase (CK) (P < 0.05) in their serum. Oral administration of Se brought an increase in erythrocyte count and in the osmotic resistance of red blood cells (P < 0.01). The total leukocyte count (TLC) was increased in lambs given BD (P < 0.05). Selenium supplementation brought about increased cell membrane resistance and increased selenium in plasma.

Key words: blood; enzyme; haematology; selenium; sheep; yeast

INTRODUCTION

Selenium is essential for body functions such as growth, reproduction, prevention of variety of diseases, and protection of the integrity of tissues. The metabolic function of selenium is closely linked to vitamin E. Both Se and vitamin E function to protect membranes from oxidative degeneration.

Selenium is an essential constituent of the antioxidant enzyme glutathione peroxidase (GSH-Px) (20). This enzyme aids in protecting cellular and subcellular membranes from oxidative damage.

Approximately 30 to 35 selenoproteins have been identified as being expressed by tissues in mammals; however the function of 20 of them has been described (1). The biochemical reactions catalysed by mammalian selenoproteins fall into three broad categories, namely, antioxidant defense system, thyroid hormone metabolism, and redox control of cell reactions.

Selenium also has protective effect against heavy metals, including cadmium and mercury.

On the other hand, Se is among the few elements known to be capable of absorption by food and forage plants in sufficient amounts to create a toxicity hazard (12).

The minimal dietary National Research Council (NRC, 1989) (14) selenium requirements range from 0.1 to 0.3 ppm. Some animal experiments have suggested that dietary selenium may have some beneficial effects at levels above those generally accepted as adequate.

Nutritional deficiences of selenium cause white muscle disease in lambs (13), loss of glutathione peroxidase activity (20), reduced selenoproteins (28), and suppression of immunity (27).

Research has now established that the total areas of the world affected by Se deficiency are far greater and the consequences are more economically important than those afflicted with Se excess.

The purpose of this study was to determine the effect of supplementing rations with selenized yeast on selected haematological parameters and on the serum enzyme activities of lambs.

MATERIAL AND METHOD

The experiment was carried out on ten male lambs of the Valaška breed four months old divided into two groups of five animals and kept on diets that differed in the content of Se supplemented. The animals were housed in individual pens with free access to water and fed both diets used for three months before sampling. The lambs weighed from 18 to 20 kg at the end of the experiment.

The composition of the daily ration of basic diet (BD) per lamb and the daily delivery of Se by BD is presented in Tab. 1.

Table 1. The composition of the daily ration of basic diet per lamb and Se intake

Component	Amount	Dry matter (g)	Content of Se ($\mu g.kg^{-1}$ of DM)	Se intake ($\mu g.day^{-1}$)
Hay	500	440.0	61.9	27.2
Rapeseed oilmeal	40	36.3	166.5	6.1
Barley	300	259.0	66.8	17.3
Total	840	735.3		50.6

The first group was given BD with a daily Se content of 50.6 µg coming from the natural occurrence of Se in the dietary component only. The second group received BD supplemented with 0.3 mg of Se in the form of Se-yeast extract (Sel-Plex, Alltech Inc., USA) giving a daily Se intake 278.6 µg. The diet for the first group was fortified with adequate amounts of the yeast extract without Se (NUPRO, Alltech Inc., USA) to obtain the same final levels of the yeast extract as in the second group (daily intake 1.04 g of NUPRO in feed).

EU requirements related to laboratory animal welfare were met.

Sample analysis

One heparinized and one non-heparinized tube of blood were collected *via* venipuncture from each lamb at 6.00 a.m. before morning feeding. Plasma was removed after blood centrifugation at $1\,180 \times g$ for 15 minutes. Blood collected in non-heparinized tubes was centrifuged at $2\,000 \times g$ for 20 minutes and the serum was decanted and frozen.

The concentration of selenium in dietary components and sample of plasma were measured by the fluorometric method of Rodriguez *et al.* (18).

The enzymes, lactate dehydrogenase LD (LD_{50}, Bio-La-Test, CzR) and creatine kinase (CK) (CK 50, Bio-La-Test, CzR) were assayed in the serum according to procedures outlined in respective commercial kits using a spectrophotometer set at 500 and 400 nm wavelengths, respectively. Haemoglobin (Hb) was analysed using the kit, RANDOX, UK.

Th haemocytometer method was used for the total erythrocyte and total leukocyte count determination. The PCV (haematocrit) was measured by the microhaematocrit method. The erythrocyte fragility test was performed according to the method of Coles (5).

The results are expressed as mean ± S.E.M. Statistical significance was evaluated by the unpaired Student's *t*-test.

RESULTS AND DISCUSSION

The selenium concentration in plasma was significantly increased in Se-yeast treated lambs (Table 2). This agrees with the results of Neve *et al.* (15) who have reported that the selenium in plasma was increased after a 60-d selenium supplementation with DL-selenomethionine ($100\,\mu g\ Se.d^{-1}$) in a group of ten adults. These findings have been confirmed by Chen and Lin (8). The concentration of selenium in the serum of a rat was significantly increased after SeO_2 treatment compared to the control. Similarly, Rock *et al.* (17) have found that selenium supplementation to pregnant ewes either from sodium selenite or selenized yeast increased the selenium concentration in the serum of both pregnant ewes and the lambs of ewes given Se.

Table 2. The effect of supplemental Se on the plasma concentration of selenium and activities of lactate dehydrogenase and creatine kinase in the serum of lambs

Parameter	Control	Selenized yeast	
LDH ($\mu kat.l^{-1}$)	7.40 ± 0.51	7.50 ± 0.52	NS
CK ($\mu kat.l^{-1}$)	2.63 ± 0.09	1.73 ± 0.29	$P<0.05$
Se ($\mu mol.l^{-1}$)	0.39 ± 0.02	1.78 ± 0.05	$P<0.001$

Results are expressed as mean of five determinations ± S.E.M.
NS — not significant

Given the increasing number of seleno-proteins identified, it is not suprising that control mechanisms exist to regulate the priority of synthesis of different seleno-proteins. This hierarchy not only relates to the synthesis of different enzymes within the same tissue but also to the retention of Se between tissues.

For example, depletion studies have shown that Se is retained well by the brain, endocrine and reproductive organs but lost relatively rapidly from the liver and muscle corresponding well with nutritional myopathy being the most prevalent symptom of deficiency (3).

Our study shows that there was an increase in CK activity in the animals given the basic diet ($P<0.05$). However, creatine kinase activity in both control and Se-supplemented lambs belong to a physiological norm. There were no clinical cases of nutritional muscular dystrophy. Sekin *et al.* (22) have reported that CK activity is correlated with intensive degenerative changes in the muscle while Bradley *et al.* (4) have reported that the detection of CK activity is particularly useful in the diagnosis of subclinical states of dystrophy. Sobiech

and Kuleta (23) have subsequently reported significantly higher CK activity in Se-deficient lambs.

Total erythrocyte count and osmotic resistance of red blood cell were increased by Se supplementation ($P<0.01$). The white blood cell count, however, was increased in lambs given basic diet ($P<0.05$) (Tab. 3).

Table 3. The effect of supplemental Se on the haematological parameters of lambs

Parameter	Control	Selenized yeast	
RBC ($T.l^{-1}$)	5.65 ± 0.17	7.77 ± 0.45	P<0.01
WBC ($G.l^{-1}$)	7.32 ± 0.92	4.57 ± 0.33	P<0.05
PCV ($l.l^{-1}$)	0.51 ± 0.02	0.50 ± 0.03	NS
Hb ($g.l^{-1}$)	125.9 ± 10.5	133.8 ± 5.7	NS
Fragility of red blood cells:			
minimal	0.64 ± 0.02	0.61 ± 0.01	NS
maximal	0.50 ± 0.0	0.42 ± 0.02	P<0.01

Results are expressed as mean of five determinations ± S.E.M.
NS — not significant

A positive effect of selenium on the red blood cell count and osmotic resistance of erythrocytes has been observed by a number of authors but has not been confirmed by others. Doni *et al.* (6) have reported that erythrocyte fragility was decreased in selenium- supplemented animals. This agrees with the study of Chen and Lin (8) who have found that the fragility of red blood cells is significantly decreased in SeO_2-treated rats compared to controls. Similarly, Keshavarz *et al.* (9) have reported that in a trial with mice treated with different doses of sodium selenite it is shown that selenium is able to prevent erythrocyte membrane damage induced by T-2 toxin.

The protective effect of selenium may be due to its membrane stability properties, although inhibition of lipid peroxidation is likely, too. Earlier results obtained by Lumsden *et al.* (19) have indicated that the lifespan of red blood cells is increased in seleno-methionine-treated horses. Moreover, Li *et al.* (10) have found that Kaschin-Beck disease children are deficient in selenium and the fragility of erythrocytes is increased.

Rationing with 0.1 ppm of added Se as sodium selenite to sows causes significantly higher red blood cell count than the lower Se group of offsprings (16) and Thoren-Tolling (25) have indicated that piglets born from sows fed with an extremely deficient vitamin E and selenium diet showed significantly low haemoglobin values at birth and higher MCV values during the first three weeks of life than normal piglets.

In contrast, the earlier results of Hu *et al.* (7) have not indicated that dietary selenium as sodium selenite alone alters the total haemoglobin, haematocrit, erythrocyte count or osmotic fragility pattern of rat blood. This agrees with the study of Sehgal *et al.* (21) who have reported that selenium administered alone to monkeys has no therapeutic effect on anaemic monkeys but an injection of a mixture of the two antioxidants (vitamin E and Se) to seventeen anaemic monkeys resulted an increase in mean haemoglobin concentration and a decrease number of leukocytes.

In contrast to these results, Tras *et al.* (26) have found that none of the haematological parameters (red blood cell count, haemoglobin, haematocrit) are affected by a diet supplemented with vitamin E + selenium in male broiler chickens but ascorbic acid + aspirin + vitamin E + selenium supplementation significantly decreased the leukocyte count. Bednarek *et al.* (2) have reported that two i.m. injections (each of which contained 5.75 μg selenium and 75 mg α-tocopherolacetate (Ursoselevit) given to calves resulted in a higher blood leukocyte count, while the erythrocyte and haematocrit count did not show differences between control and Ursoselevit-supplemented group.

Se has diverse effects on immune cells, including increased number of lymphocytes (19).

A divergence of opinion as regards the effects of Se on haematological parameters is often presented in the literature. One of the reasons is due to the fact that the effect of selenium is mostly examined simultaneously with that of vitamin E or/and ascorbic acid which makes interpretation difficult.

Moreover, the selenium requirement is dependent on the chemical form of selenium. Most Se in feed components is present mainly as selenomethionine, and to a lesser extent seleno-cysteine; much Se added as a supplement to diets is in the form of inorganic selenite or selenate. The availability of selenite and selenate is relatively low as it is reduced in the rumen (24). The possible different effects of Se on haematology may be caused by other factors, including the duration and dosage of Se supplementation, the previous Se status of the animal, and the amounts of interfering or enhancing factors in the diet, including vitamin E, sulfur, calcium, iodine and lipids.

Further research is needed to confirm the effect of other factors affecting selenium requirements in ruminants.

CONCLUSION

Selenium-yeast supplementation to lambs increased the concentration of Se in plasma. In addition, administration of selenium brought about an increase in the erythrocyte count and decreased the fragility of erythrocytes.

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THE EFFECT OF CADMIUM ON FATTY ACID ABSORPTION ACROSS THE CAECUM EPITHELIUM IN SHEEP

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ABSTRACT

The effect of cadmium as one of the heavy metals on acetate and propionate absorption across the caecum epithelium was investigated. In the experiment caecum walls obtained from adult Merino sheep two to four years old were used. Acetate and propionate absorption was observed on an apparatus constructed in our laboratory. Determination of the acids was carried out by gas chromatography (Perkin-Elmer 8500). Absorption of separately acetate (50 mmol.l^{-1} of Thyrode's solution) and also in combination with propionate (50 + 15 mmol.l^{-1} of Thyrode's solution) across the caecum epithelium after the addition of all three cadmium concentrations (5, 20, 50 mg) was increased. Propionate absorption was not effected. Only a 50 mg addition of cadmium to combined propionate with acetate decreased propionate absorption. One of the possible explanations of this phenomenon could be that cadmium readily enters into interactions with proteins and phospholipids which are important components of the cell membranes. In the case of propionate absorption a reason might be the higher metabolization rate in the epithelium.

Key words: absorption; acetate; cadmium; caecum epithelium; propionate

INTRODUCTION

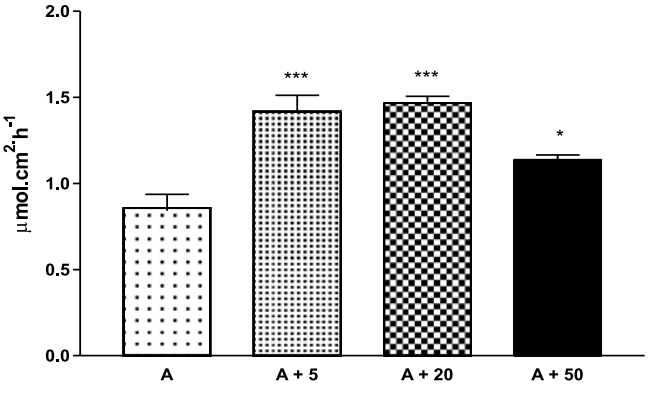
Acetic and propionic acid belong to the short-chain fatty acids (SCFA) which are a usual part of the rumen and also caecum content. They are produced during the fermentation of plant materials in the forestomach of ruminants. In the rumen and caecum epithelium they are metabolized or absorbed into the blood stream. Acetic acid is utilized as a source of energy, propionic acid is the only one short-chain fatty acid which is glucogenic. It is quantitative, the most important precursor of glucose. Practically, all propionic acid is utilized in the liver and does not reach the peripheral blood stream.

Cadmium belongs to the heavy metals group of elements and often contaminates the environment from where it enters in animal organism in its food. Although it is essential microelement for organisms it readily reacts with a number of active molecules of proteins, phospholipids, purines, nucleic acids and enzymes changing their activity which is the cause of its toxic effect (9, 12, 8). The toxic effect of cadmium is related to the amount supplied to the organism (6, 15, 10). The aim of the experiment performed was to investigate the effect of cadmium on acetate and propionate absorption across the caecum epithelium.

MATERIAL AND METHODS

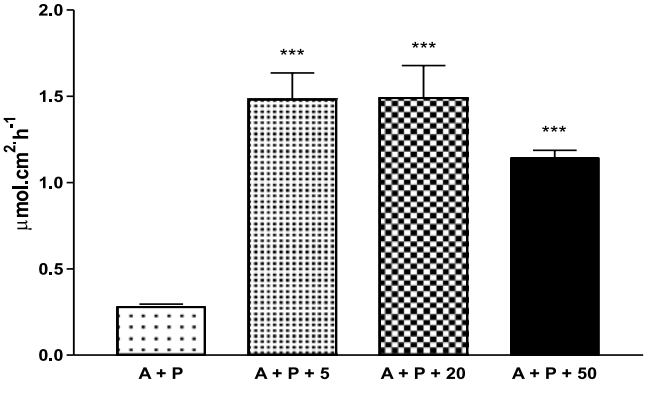
In the experiment the rumen walls of eight adult Merino sheep two to four years old were used. The animals were individually housed in sheds and given a diet consisting of meadow hay *ad libitum* and 200 grams of ground barley per animal per day. The animals had free access to water and lick salt. Immediately after slaughter and bleeding the entire digestive tract was removed from the abdominal cavity and brought to the laboratory. There the caecum was separated, its content removed and washed with lukewarm water. From the epithelium slices were cut and transfered into a glass vessel containing a saline solution.

Acetate and propionate absorption was observed on an apparatus that had been constructed in our laboratory. Furthermore, the experiment continued in accordance with (5). Each combination was tested six times (n = 6).



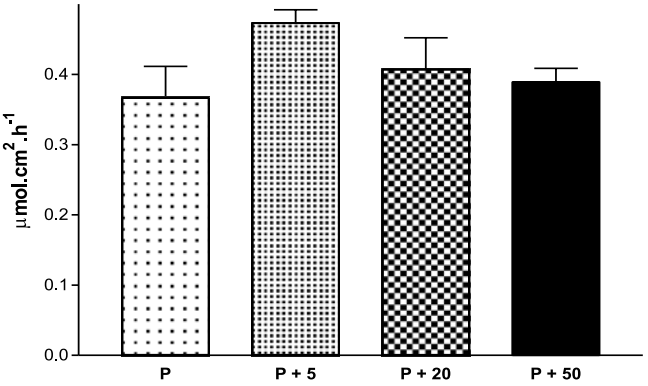
*** — P < 0.05; *** — P < 0.001; A — acetate**
5, 20, 50 — cadmium (mg)

Fig. 1. Acetate absorption across the caecum epithelium when separately administered after the addition of cadmium



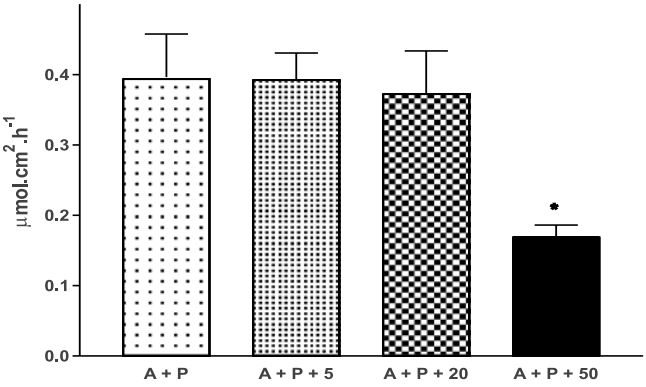
***** — P < 0.001; A — acetate; P — propionate**
5, 20, 50 — cadmium (mg)

Fig. 2. Acetate absorption across the caecum epithelium administered in combined form (A + P) after the addition of cadmium



P — propionate; 5, 20, 50 — cadmium (mg)

Fig. 3. Propionate absorption across the caecum epithelium when separately administered after the addition of cadmium



*** — P < 0.05; A — acetate; P — propionate**
5, 20, 50 — cadmium (mg)

Fig. 4. Propionate absorption across the caecum epithelium in combined form (A + P) after the addition of cadmium

Statistical analysis: The means of the individual parameters were compared using the Tukey-Kramer multiple comparison test (GraphPad Instat Software, Inc., San Diego, USA). Differences from the means marked in the graphs indicate standard error (S.E.).

RESULTS

The effect of Cd on absorption of acetate had a significant affect on both forms of acetate (separately and combined with propionate). All doses of cadmium increased acetate absorption (Figs. 1 and 2).

The different effects of cadmium on propionate absorption through the caecum epithelium was observed. Propionate absorption was not affected when separately administered (Fig. 3). Only a 50 mg addition of cadmium to combined propionate with acetate decreased propionate absorption (Fig. 4).

DISCUSSION

Cadmium belongs to the group of heavy metals the effect of which on living organisms in higher doses is unfavourable. The industrial utilization of components containing cadmium has in the recent years gained speed. Mobilization and speed of transport of this component highly exceed the possibilities of its natural recycling, which results in an increased rate of cadmium deposition in living organisms. This is also the reason that the effect of cadmium is being given great attention in the investigation of its effect on living organisms at the present time (7, 4, 13, 1, 2, 16).

Ruminants can be exposed to the effect of heavy metals by the consumption of contaminated food and water. The digestive tract of animals, and other organs are thus subjected to the direct effect of this group of xenobiotics. Since cadmium belongs to the essential microelements decreased milk production in ruminants and retarded growth of the youngs, muscular weakness, decreased motility and

death have been observed after an insufficient supply of cadmium. An increased cadmium supply up to 0.3 mg. kg^{-1} dry matter eliminated these symptoms (3). In the case of cadmium, its negative effect in greater supplies to the organism is of greater importance. The effect of cadmium on the microbial population and SCFA production in the gastrointestinal tract of ruminants has been considered in some papers (11, 14).

This led us to the idea to examine the manifestations of graded cadmium doses on the permeability rate of the mucous membrane in the rumen and caecum with the two most important short-chain fatty acids in the digestive tract of ruminants i.e. acetic and propionic acid.

The effect of Cd on the absorption of acetate had a signifficant affect on both forms of acetate (separately and combined with propionate). All doses of cadmium increased acetate absorption (Figs. 1 and 2).

The different effect of cadmium on propionate absorption through the caecum epithelium was observed. Propionate absorption was not affected when separately administered (Fig. 3). Only a 50 mg addition of cadmium to combined propionate with acetate decreased propionate absorption (Fig. 4).

One of the possible explanations of this phenomenon could be that cadmium readily enters into interactions with proteins and phospholipids, which are important components of the cell membranes. In this way destruction of the epithelial components with a subsequent increase in the transport of not only fatty acids but also other components might occur. This finding could perhaps be explained also by the different acetate and propionate metabolism in the wall of the gastrointestinal tract.

It is assumed that the luminal and basolateral epithelial surfaces differ as to the permeability of both forms of SCFA, and that the transport of SCFA can be explained by maintaining the electrochemical gradient for the hydrogenous ions between the epithelial cell content and the solutions on both sides of the membranes. SCFA transport from the caecum into the blood can therefore be explained not only by diffusion, but also that the process in question is affected by the pH of the lumen and both the cell content and the intercellular SCFA metabolism.

The assumed destruction of the caecum epithelium, which might be the reason for increased acetate absorption rate after the addition of cadmium was not demonstrateded in a similar way with propionate.

As it seems other interactions might also have occurred between the epithelium and propionate i.e. a higher metabolization rate in the epithelium and a larger molecule might have played a role instead to acetate.

The knowledge obtained is valuable for the study of fatty acid transport across digestive tract membranes in animal.

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THE COMPARATIVE ASSESSMENT OF THE HAEMATOLOGICAL VALUES IN THE AFRICAN GIANT RAT (*Cricetomys gambianus* Waterhouse) AND THE AFRICAN GRASS CUTTER (*Thyronomys swinderianus* Temminck)

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ABSTRACT

The haematological values were determined in apparently healthy young and adult wild African giant rat (*Cricetomys gambianus* Waterhouse) and also the young and adult wild African grass cutter (*Thyronomys swinderianus* Temminck). The red blood cell counts (RBC), neutrophil counts, eosinophil counts and monocyte counts, packed cell volume (PCV), haemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were similar ($P > 0.05$) in the adult African giant rat and the adult African grass cutter. The adult African giant rat however had higher white blood cell counts ($P < 0.001$) and lymphocyte counts ($P < 0.001$) than the African grass cutter. The young (12—15 weeks old) African giant rat had lower RBC counts ($P < 0.05$), PCV ($P < 0.02$) and lymphocyte counts ($P < 0.05$) but higher neutrophil counts ($P < 0.05$) than the adult (between two and three years old). However the MCV, MCH, MCHC and the total WBC, monocyte and eosinophil counts were similar in the young and adult African giant rat. All the haematological values were similar in the young (10—15 weeks old) and the adult (2—3 years old) African grass cutter

Key words: adult; African grass cutter; African giant rat; haematology; old; rodent; young

INTRODCUTION

The African grass cutter (*Thryonomys swinderianus* Temminck) is also known as the greater cane rat. They are the second largest rodents in Africa, the porcupine being the largest. They weigh an average body mass of 4.5 and 3.6 kg for males and females respectively (20). They are found in Africa south of the Sahara desert where they are semi-aquatic inhabitants of marshy areas (20). African grass cutters are important as a food source for many African cultures. They are savored because they are an excellent protein rich food (1, 3). Although efforts are in progress at domestication, they have not been successfully domesticated (10).

The African giant rat (*Cricetomys gambianus* Waterhouse) weighs 0.86 g and 1.13 kg for female and male respectively (17). They are also a ready source of dietary protein for rural dwellers. Efforts at domesticating them started about three decades ago (2) and there are some successes at domesticating then. There are also efforts in several quarters in using them as laboratory animals (11).

Although there are some reports on the haematology of the adult African giant rat (13, 14, 5, 17, 11, 12), there has not been any report on the haematology of the young African giant rat. We also observed from our search of literature that except for a report by Ogunsanmi *et al.* (10) on the effect of sex on the haematology of the the African grass cutter, there is no other report on the haematology of these rodents.

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There has not been any report on the comparative study of the haematology of these two species of rodent.

In this paper, we therefore present the influence of age and species variation on the haematology of the African grass cutter and the African giant rat.

MATERIALS AND METHODS

Two groups of wild rodents were used for the present study. The first group consisted of six adults (2—3 years old) and six young (12—15 weeks old) African giant rat (*Cricetomy gambianus* Waterhouse). The adults were captured from the wild near Ibadan, Nigeria. The young were born in captivity. They were reared in our animal house at the Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria. They were fed on a diet of commercial rat feed (21 % protein, 3.5 % fat, 6 % fiber, 0.8 % calcium and 0.8 % phosphorus, Ladokun feeds limited, Ibadan, Nigeria). They were given water *ad libitum*.

The second group of the wild rodents used for the study were the African grass cutter (*Thryonomys swinderianus* Temminck), five adult (2—3 years old) and six young (10—15 weeks old). The adults were reared in captivity at Adagro Farms at Ikotun, Lagos State, Nigeria. The young were also given birth to in captivity. They were fed with forage consisting of elephant grass (*Pennisetum purpureum*), guinea grass (*Panicum maximum*), *Leuceana leucocephala* leave, pawpaw (*Carica papaya*). Supplementary feed made up of corn offal, corn and Palm kernel cake. Water was given *ad libitum*.

Each animal in the two groups was weighed and sedated with Ketamin (Waterland Laboratories, Germany) at 30 $mg.kg^{-1}$ body weight. Blood was then collected into bottles containing ethylene diamine tetra acetic acid (EDTA) (2 $mg.ml^{-1}$ of blood). RBC and WBC were counted with haemocytometer. PCV was determined using the microhaematocrit method. Haemoglobin concentration was measured by the cyanmethaemoglobin method. From this data the MCH, MCHC and MCV were calculated (6). Blood smears were stained with Giemsa stain for differential WBC counts.

All data were analyzed statistically using Student's *t*-test.

RESULTS

Table 1 shows the erythrocyte values in the adult African giant rat and the adult African grass cutter. The mean values of RBC, PCV, Hb, MCV, MCH, MCHC were similar ($P > 0.05$) in the two species of rodents.

Table 2 presents the erythrocyte value in the young and adult African grass cutter. The mean values of RBC, PCV, Hb, MCV, MCH, MCHC were similar ($P > 0.05$) in the two age groups.

Table 3 shows the effect of age on the erythrocyte value of the African giant rat. The young had significantly lower RBC count ($P < 0.05$), PCV ($P < 0.05$) and Hb concentration ($P < 0.02$). However the values of MCH, MCHC and MCV were similar in the young and adult African giant rat.

Table 1. Erythrocyte values (mean ± SD) in the adult African giant rat and adult African grass cutter

PARAMETERS	GIANT RAT (6)	GRASS CUTTER (5)
RBC ($10^6.\mu l^{-1}$)	6.99 ± 0.84	6.27 ± 0.93
PCV (%)	43.17 ± 5.27	37.60 ± 4.51
Hb ($g.dl^{-1}$)	13.40 ± 1.85	11.92 ± 1.73
MCH (pg)	19.14 ± 0.70	18.78 ± 1.31
MCHC ($g.dl^{-1}$)	30.99 ± 0.74	31.14 ± 0.87
MCV (fl)	61.77 ± 1.97	60.25 ± 2.45

Number of animals in parentheses

Table 2. Erythrocyte values (mean ± SD) in the adult and young African grass cutter

PARAMETERS	YOUNG (6)	ADULT (5)
RBC ($10^6.\mu l^{-1}$)	6.48 ± 1.21	6.27 ± 0.93
PCV (%)	37.50 ± 5.54	37.60 ± 4.51
Hb ($g.dl^{-1}$)	11.92 ± 1.73	11.65 ± 1.16
MCH (pg)	18.57 ± 1.52	18.7 ± 1.31
MCHC ($g.dl^{-1}$)	31.81 ± 0.97	31.14 ± 0.87
MCV (fl)	58.29 ± 3.44	60.25 ± 2.45

Number of animals in parentheses

Table 3. Erythrocyte values (mean ± SD) in the adult and young African giant rat

PARAMETERS	ADULT (6)	YOUNG (6)
RBC ($10^6.\mu l^{-1}$)	6.99 ± 0.84	5.93 ± 0.59*
PCV (%)	43.17 ± 5.27	36.50 ± 2.74*
Hb ($g.dl^{-1}$)	13.40 ± 1.85	11.02 ± 0.88**
MCH (pg)	19.14 ± 0.70	18.61 ± 0.58
MCHC ($g.dl^{-1}$)	30.99 ± 0.74	30.18 ± 0.73
MCV (fl)	61.77 ± 1.97	61.72 ± 3.24

Number of animals in parentheses. Value significantly different from young giant rat at * — $P < 0.05$ and ** — $P < 0.02$

Table 4 presents the leukocyte values in the adult African giant rat and the adult African grass cutter. The neutrophil counts, eosinophil counts and monocyte counts were similar in the two rodents, however the African giant rat had higher ($P < 0.001$) WBC and lymphocyte counts.

Table 5 reveals the effect of age on the on the leukocyte values of the African grass cutter. The values of WBC counts, neutrophil counts, eosinophil counts, monocyte counts and lymphocyte counts were similar in the two age groups.

Table 6 shows that the young African giant rat had lower lymphocyte counts ($P < 0.05$) but higher ($P < 0.05$)

Table 4. Leukocyte values (mean ± SD) of the adult African giant rat and adult African grass cutter

PARAMETER	GIANT RAT(6)	GRASS CUTTER(5)
TOTAL WBC ($10^3.\mu l^{-1}$)	8.37 ± 4.01	3.23 ± 1.36*
LYMPHOCYTE ($10^3.\mu l^{-1}$)	5.60 ± 42 (67.50 ± 14.65)[a]	1.33 ± 1.09* (36.6 ± 16.60)[a**]
NEUTEROPHIL ($10^3.\mu l^{-1}$)	2.66 ± 1.58 (30.83 ± 14.63)[a]	1.83 ± 0.32 (62.20 ± 17.30)[a***]
EOSINOPHIL ($10^3.\mu l^{-1}$)	0.06 ± 0.05 (0.83 ± 0.75)[a]	0.02 ± 0.05 (0.40 ± 0.89)[a]
MONOCYTE ($10^3.\mu l^{-1}$)	0.06 ± 0.05 (0.83 ± 0.75)[a]	0.08 ± 1.10 (0.03 ± 0.05)[a]

Number of animals in parentheses
[a] – Value expressed as a percentage of total WBC count
Value significantly different from grass cutter at
* — P < 0.001, ** — P < 0.02 and P < 0.01

Table 5. Leukocyte values (mean ± SD) of the adult and young African grass cutter

PARAMETER	YOUNG (6)	ADULT (5)
TOTAL WBC ($10^3.\mu l^{-1}$)	4.23 ± 1.13	3.23 ± 1.36
LYMPHOCYTE ($10^3.\mu l^{-1}$)	1.86 ± 1.10 (41.50 ± 18.75)[a]	1.33 ± 1.09 (36.6 ± 16.60)[a]
NEUTROPHIL ($10^3.\mu l^{-1}$)	2.27 ± 1.58 (56.17 ± 18.72)[a]	1.83 ± 0.32 (62.20 ± 17.30)[a]
EOSINOPHIL ($10^3.\mu l^{-1}$)	0.04 ± 0.05 (0.83 ± 0.98)[a]	0.02 ± 0.05 (0.40 ± 0.89)[a]
MONOCYTE ($10^3.\mu l^{-1}$)	0.06 ± 0.05 (1.50 ± 1.22)[a]	0.08 ± 1.10 (0.03 ± 0.05)[a]

Number of animals in parentheses
[a] — Value expressed as a percentage of Total WBC count

neutrophil counts than the adult. The WBC counts, eosinophil counts and monocyte counts were similar in the young and adult African giant rat.

DISCUSSION

In the present study the effect of sex on the haematology of the adult African giant rat and the adult African grass cutter were not determined because Oyewale *et al.* (17) and Ogunsanmi *et al.* (10) have reported on the influence of sex on the haematology of the adult African giant rat and the adult African grass cutter respectively.

Table 6. Leukocyte values (mean ± SD) of the adult and young African giant rat

PARAMETER	ADULT (6)	YOUNG (5)
TOTAL WBC ($10^3.\mu l^{-1}$)	8.37 ± 4.01	8.50 ± 0.89
LYMPHOCYTE ($10^3.\mu l^{-1}$)	5.60 ± 1.42 (67.50 ± 14.65)[a]	4.31 ± 0.39* (50.83 ± 2.99)[a*]
NEUTROPHIL ($10^3.\mu l^{-1}$)	2.66 ± 1.58 (30.83 ± 14.63)[a]	3.94 ± 0.46* (46.33 ± 32.34)[a*]
EOSINOPHIL ($10^3.\mu l^{-1}$)	0.06 ± 0.05 (0.83 ± 0.75)[a]	0.16 ± 0.12 (1.67 ± 1.21)[a]
MONOCYTE ($10^3.\mu l^{-1}$)	0.06 ± 0.05 (0.83 ± 0.75)[a]	0.10 ± 0.06 (1.17 ± 0.75)[a]

Number of animals in parentheses
[a] — Value expressed as a percentage of Total WBC count
Value significantly different from young giant rat at * — P < 0.05

Oyewale *et al.* (17) observed that apart from WBC counts, which were higher (P<0.05) in the male than in the female adult African giant rat, the erythrocyte values and the differential WBC counts were similar in the two sexes. Ogunsanmi *et al.* (10) however reported that the erythrocyte and leukocyte values were similar in male and female African grass cutter. The haematological values obtained for the adult African giant rat and the adult African grass cutter in the present study were similar to those reported for the same species by Oyewale *et al.* (17) and Ogunsanmi *et al.* (10).

In the present study, the erythrocyte values were similar in the adult African giant rat and the adult African grass cutter (Table 1). This may be as a result of both being tropical species of rodent and they seem to share similar physiology. However when Durotoye and Oke (5) compared the haematology of the African giant rat and the Wistar rat, they observed that the MCH and MCV values were higher while the RBC counts were lower in African giant rat than the Wistar rat. The difference may be because the Wistar rat is not a tropical species of rat while the giant rat is a tropical species of rodent.

In the present study, the WBC and lymphocyte counts were higher in the African giant rat than in the adult African grass cutter, while the neutrophil counts, monocyte counts and the eosinophil counts were similar in the two species of rodents. The higher WBC and lymphocyte counts in the African giant rat may be due

to difference in the feed given to the rodents. It seems the commercial feed that was given to the African giant rat was richer in protein content than the diet given to the African grass cutter. Rekwot *et al.* (18) have reported that the White Fulani cattle placed on a high protein diet (14.45 %) had higher WBC counts than those placed on lower protein diet (8.51 %).

In the present study, it was observed that young and adult African grass cutters had similar erythrocyte values and leukocyte values. Similar observations have been made for the guinea pig (Oluwaniyi *et al.* – 16), the Nigerian local cat (Nottidge *et al.,* 7), Jack rabbit (Jain, 6) and Ayrshire cattle (Tashjain *et al.* – 19) where similar values were reported in male and the female. Olusanya (15) however has reported that MCV increased with age while WBC counts decreased with age in White Fulani cattle. Also Oduye (19) has observed that young Nigerian dogs had lower PCV than adult dogs.

The values of RBC, PCV, and Hb were higher in the adult than the young African giant rat in the present study. Two strains of rat namely the Long Evans rat and the Sprague Dawley rat were similarly reported to have higher RBC, PCV and Hb values in the adult than young (Jain – 6). Haemoglobin value has also been found to be higher in the adult than in the young Dorcas gazelle (Bush *et al.* – 4). However no age difference was reported in the erythrocyte values of White Fulani cattle (Oduye and Okunaiya, 9).

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THE REPRODUCTIVE IMPLICATIONS OF CLOMIPHENE CITRATE ON SPERM CELLS DURING THE EPIDIDYMAL TRANSIT OF SPERMATOZOA IN MALE WISTAR RATS

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ABSTRACT

The reproductive implications of Clomiphene citrate (CC) on male Wistar rats revealed the reactions of the rats to CC at different dosages (varied). Results revealed detrimental effects at a higher dosage. In the *caput* of the epididymidis, the mean percentage motility in group B Wistar rats (20.0 ± 8.66) and group C Wistar rats (23.3 ± 3.33) was significantly lower (P < 0.05) than group A Wistar rats (42.0 ± 2.00). The drug did not influence the percentage livability of the spermatozoa in the *caput* epididymidis. In the *corpus* epididymis the mean percentage motility for groups A — C were 56.00 ± 2.45, 25.56 ± 7.50, 13.00 ± 5.77, respectively. Group A value was significantly higher than those of groups B and C. The percentage livability was higher in-group A than B and C. The concentration of the spermatozoa was significantly higher in group A (78.25 ± 6.97. 106) than groups B (53.75 ± 1.89. 106), though this is not significantly different (P < 0.05). These findings were discussed in relation to the significance of the drug as a fertility enhancer in infertile animals. The reproductive implications of the drug were studied in the epididymis of Wistar rats with no adverse effects when used at the recommended dose. The drug can therefore be used to boost the reproductive potential of infertile male animals.

Key words: Clomiphene; epididymis; fertility; rat; semen

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INTRODUCTION

Clomiphene citrate (CC) is non-steroidal drug; it has a molecular formula of C_{26} H_{28} CINO. C_6 H_8 O_7. It is capable of interacting with estrogen receptor containing tissues. The first endocrine event in response to clomiphene treatment in male rats is an increase in the release of pituitary gonadotrophin (1, 2). Some scientists have reported that a significant improvement following CC treatment in terms of semen volume, density, motility and sperm concentration (3, 4). Clomiphene citrate (Clomid®) is used to trigger ovulation in women with anovulatory circles in those having secondary amenorrhoea with normal levels of follicles stimulating hormones (FSH), luitenizing hormone (LH) and prolactin (1). Women with low estradiol levels respond poorly to clomiphene citrate. When it is used as recommended, it is a safe, reliable, inexpensive therapeutic modality (5).

Epididymal transit is the movement of the sperm cells from *caput* (head) through *corpus* (body) to tail of the epididymis. Sperm cells stored in the *caudal* epididymis at the end of spermatogenesis can be influenced by age, nutrition, drug, temperature and successive ejaculations (6, 7). The normal sperm cells of Wistar rats consist of a hook shaped head (question mark shape), a thin neck, midpiece and a long tail. It is only in rats and mice that the heads of spermatozoa terminate in a distinct “hook” shape (8).

The aim of this study was to determine if clomiphene would enhance fertilization potential of the spermatozoa in terms of the motility, livability and integrity of the sperm cells. This is important in animal breeding and artificial insemination programmes.

MATERIALS AND METHODS

Sixty Wistar rats aged between 12 and 14 weeks and with a body weight of 150—180 grams were used in this study. They were divided into three groups A, B, C, of twenty rats each and were kept in a cage of about 18 by 25 inches in the Experimental Animal Unit (EAU) of the Faculty of Veterinary Medicine University of Ibadan, Nigeria. All the rats were fed on commercial rat pellets and 50 mg of Clomid® tablets were administered to group B. Each table was dissolved in 1.0 ml of distilled water and deposited into the stomach of each rat using the cannular for five consecutive days. Group C rats were treated with 0.6 mg of Clomiphene citrate that was dissolved in 1.0 ml distilled water as in earlier groups. Group A rats which is the control received 1.0 ml of distilled water. The rats from all the groups were then left for two weeks before semen samples were collected for analysis.

Spermatozoa were obtained and examined from the *caput*, *corpus* and *caudal* epididymidis. The methods of collection from these locations were generally similar to that of Akusu *et al.* (9).

The spermatozoa recovered were used to study motility, live/dead or livability (using eosin-nigrosin stains). The improved Neubaur haemacytometer method was used to determine sperm cell concentration as described by Zemjanis (10).

DATA ANALYSIS

Data obtained were subjected to Student *t*-test and chi-square test for the establishment of significance (11).

RESULTS

Results of the signs, symptoms and observable activities of the rats in groups B and C given 50 mg and 0.6 mg clomiphene citrate (CC) respectively are presented in Table 1.

Motility in the control experiments (Group A) and percentage livability were increasing as the sperm cells migrate along the *caput* through *corpus* to *caudal* epididymis. While the same increase goes for the groups B and C given 50 mg and 0.6 mg per body weight decrease ($P<0.05$) in motility when group A and the groups B and C values were compared.

Table 2 indicated that the treated group B had motility of 20.00 ± 8.66 % in the *caput* 25.56 ± 7.50 % in the *corpus* and 42.50 ± 2.50 % in the *caudal* these values are significantly lower ($P<0.05$) than the control group. The values of the concentration in group B treated with 50 mg CC is significantly lower ($P>0.05$) than that of group A treated with 1.0 ml of distilled water (Table 2). The concentration of group A control to the experiment is $78.25 \pm 6.97.10^6$ spermatozoa.ml^{-1} of the 0.6 mg. In the *caudal* (tail) epididymis the motility of group A $76.00 \pm 2.4.40$ % is significantly higher ($P<0.05$) than group C (46.67 ± 3.33 %). The percentage of the spermatozoa that were alive in the *caudal* epididymis group A was 88.00 ± 3.39 %. This is significantly higher ($P<0.05$) than 73.33 ± 6.67 % of *caudal* epididymis of group C. The percentage livability in *corpus* and *caput epididymidis* was higher in group A than in groups B and C.

Table 1. Reactions of the Wistar rats after the treatment with Clomiphene citrate

Parameter studied	Group A	Group B	Group C
Activity	Normal	Decreased	Normal
Ataxia	Absent	Present	Absent
Coma	Absent	Absent	Absent
Cyanosis	Absent	Absent	Absent
Defaecation	Normal	Increased	Normal
Forces consistency	Normal	Soft	Normal
Faecal colour	Normal	Light brown	Dark
Irritability	Absent	Decreased	Absent
Respiration	Normal	Dyspnea	Normal
Mortality	Absent	High	Absent

Table 2. Spermiogram of rats treated with Clomiphene citrate and distilled water control

Parameters	Control/Group A	50 mg CC/Group B	0.6 mg CC/Group C
A. Head of epididymis (*caput*)			
Motility (%)	42.0 ± 2.00^a	20.00 ± 8.66^b	23.33 ± 3.33^b
Percentage live	58.0 ± 2.00	52.62 ± 7.50	53.33 ± 6.67
B. Body of epididymis (*corpus*)			
Motility (%)	56.00 ± 2.45^a	25.56 ± 7.50^b	13.00 ± 5.77^b
Percentage live	66.00 ± 2.48^a	42.50 ± 2.50^b	56.67 ± 3.33
C. Tail of epididymis (*caudal*)			
Motility (%)	76.00 ± 2.40^a	42.50 ± 2.50	46.67 ± 3.38^b
Percentage live	88.00 ± 3.39^a	47.50 ± 4.79^b	73.33 ± 6.67^c
Concentration.10^6	78.25 ± 6.97	53.75 ± 1.89	62.67 ± 6.17

CC — Clomiphene citrate

$^{a, b, c}$ — numbers differently superscripted in horizontal column are significantly different ($P < 0.05$)

DISCUSSION

The decrease in the activity with some obviously abnormal symptoms and death are due to the over dosage of the rats, the signs decreased with no death when the dosage was reduced. The spermiogram in the epididymal transit revealed that more matured sperm cells exhibit highest percentage of motility increase as the maturation increase in the epididymis. This is similar with the findings of Oyeyemi *et al.* (6).

The percentage livability (percentage live) decreased significantly when compared with the value at the *caudal* epididymis $88.00 \pm 3.39\,\%$ in control group A to $47.50 \pm 4.79\,\%$ in group B when 50 mg CC was used. The findings when 0.6 mg CC was used differed with a the percentage livability value of $73.33 \pm 6.67\,\%$. This was higher than group B but lower than that of group A. This observation was similar to the report of Noseir-Wael (1) that the percentage livability of fertile animals is not superior when Clomiphene citrate was used.

In the concentration (sperm count) of the sperm cells in control group A the value $78.25 \pm 6.97.10^6$ spermatozoa.ml^{-1} was significantly higher ($P < 0.05$) than group B value of $53.75 \pm 1.89.10^6$ spermatozoa.ml^{-1}. While in group C the value of concentration is $62.67 \pm 6.17.10^6$ spermatozoa.ml^{-1}. This indicates that Clomiphene citrate reduced sperm count in a fertile male animal.

It is an established fact that matured sperm cells are in the *caudal* (tail) epididymis (6, 10). The maturation of spermatozoa usually starts in the head (*caput*) epididymis. The maturation continues at the *corpus* (body) before it gets to the *caudal*.

Clomiphene citrate in this study has shown potential to increase male fertility but the recommended dosage must be used. The semen collections form the *caudal* epididymis or from the rats must be after four weeks of treatment when spermatogenic cycle will have been completed. After this a sperm cell will migrate through the *caput* and *corpus* to *caudal* epididymis.

This study concludes that it is important that rats either for natural breeding and or artificial insemination programme should be adequately fed for maximum performance. Clomiphene citrate can be used to boost the percentage livability and concentration of sperm cells of an infertile male rat.

These two parameters are necessary for male reproductive potential on which others' motility, volume and mass activity depend (12, 13). Also the number of spermatozoa in the ejaculate is of paramount importance in fertilization (14).

The adverse effect of CC treatment on the fertile rats in the present study may be due to CC influencing effect on testosterone and or due to its antiestrogenic properties, while simultaneous use of CC in fertile male rats could have a controversial effect. Therefore Clomiphene citrate treatment that could improve citrate treatment that could improve fertility in infertile male rats (15) infertile ram(1) could also induce a deteriorative effect on sperm cells and their characteristics on sperm cells and their characteristics when used in fertile Wistar rats

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THE PHARMACOLOGICAL REACTION PROCESSES OF THE GASTROINTESTINAL SMOOTH MUSCLE OF THE NIGERIAN DUCK (*Cairina moschata*) TO ACETYLCHOLINE

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ABSTRACT

We studied the effects of acetylcholine (Ach) on the various segments of the gastrointestinal (G.I.) tract (i.e. crop, proventriculus, duodenum, ileum, caecum and rectum) of *Cairina moschata* using the modified *in vitro* muscle bath contractile method. The effect Ach on the isolated smooth muscle was evaluated in the absence and in the presence of atropine by determining the potency or the effective concentration at 50 % (EC_{50}) of a subject's response and the efficacy, which was measured by the percentage maximal response (E_{max}). The dose ratio (D.R.) was used to determine the degree of antagonism while the statistical significance of the differences of the means of control and test groups was established using Student's *t*-test. Ach produced dose-dependent contractions of muscle strips prepared from the different parts of the G.I. tract of *Cairina moschata*. Its threshold contractile response was recorded between a concentration of 1.10^{-15} and 1.10^{-9}mol, with the proventriculus having the lowest threshold value (1.10^{-15} mol). 1.10^{-7} mol atropine significantly reduced the potency (EC_{50}) of Ach in the crop, proventriculus, duodenum, ileum, caecum and rectum. The antagonism was competitive for all the G.I. sections. The D.R. values obtained confirm the effectiveness of atropine as a competitive antagonist of Ach in the various sections of the G.I. tract of *C. moschata* studied. This study demonstrates the atropine-sensitive contractile effect of Ach on the gastrointestinal smooth muscle of *Cairina moschata*, similar to reported findings in other avian species. However, *Cairina moschata* appeared to have lower threshold values for acetylcholine than has been reported in other Nigerian avian species.

Key words: acetylcholine; gastrointestinal smooth muscle; Nigerian duck; pharmacological response

INTRODUCTION

The smooth muscle of the gastrointestinal (G.I.) tract is innervated by the parasympathetic and sympathetic divisions of the autonomic system in a delicate balance that ensures effective control of the motility of the tract. The study of the autonomic control of G.I. motility is closely related to the evaluation of the pharmacodynamics of the complexes formed between the receptors and autonomic neurotransmitters regulating the motility of G.I. smooth muscle. Whereas, the control of G.I. motility is basically the same from species to species, the pattern of motility differs from species to species and even within a particular species depending on a number of factors such as genetic, dietary and climatic factors – Prendergast and Boag (23).

The clinical significance of neurotransmitter receptor research is gaining ground daily. The pathogenesis of a number of disease conditions in the body have been traced to the

Running title:
Cholinergic stimulation of gastrointestinal tract Nigerian duck

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absence of receptor type or change in the population of receptors in a tissue or dysfunction of receptors – Jackson (14), Dekeyser *et al.* (8). It is therefore imperative to identify and characterize receptors mediating neurotransmitters in as many species as possible in order to have relevant basic data for applied research purposes.

Much work has been done in characterizing the response of G.I. smooth muscle and the mediating receptor to parasympathetic stimulation in different mammalian avian species, however very little has been done in Nigerian avian species. This particular study is aimed at evaluating the pharmacological reaction processes of G.I. smooth muscle of the Nigerian duck (*Cairina moschata*) to acetylcholine as a neurotransmitter.

MATERIALS AND METHOD

General Experimental Procedure

The ducks were kept in an intensive housing system and were placed on a growers chick mash (Guinea feeds, Nigeria Limited), and allowed free access to water.

At the beginning of each experiment, a duck was killed by stunning and cutting the neck with a knife. Immediately, the bird was split open from the neck down to the vent with scissors so as to allow easy access to the gastrointestinal tract. The section to be used was cut and taken out in a Petri dish containing Tyrode solution constantly aerated by an air pump.

The section was cut into strips of about two centimetres long. The lower end of the tissue was attached to the aerating tube inside the organ bath while the upper end was attached to a simple isotonic lever counterbalanced to provide a load of two grams on the tissue. This frontal writing lever was aligned on a kymograph drum. Each strip of tissue was allowed to equilibrate for at least one hour before experimentation – Dina and Arowolo (9).

Experiment 1: Determination of Threshold and Maximum responses to Acetylcholine

After equilibrations, trial study was undertaken in order to determine the minimum (i.e. threshold value) and maximum concentration at which responses could be initiated or completed respectively. This was done by exposing the tissue to the lowest concentration of the ten-fold serially diluted acetylcholine chloride (British Drug House Chemicals Limited, England). Stronger concentrations were then applied consecutively until a threshold response was obtained. The maximum response was ascertained when increases in the concentration of acetylcholine applied did not produce a corresponding increase in the response of the tissue. This procedure was repeated several times using new tissue strips from each section of the G.I. tract.

Experiment 2: Agonist – antagonist Study

A disposable syringe (1 ml) was used to drop solution of acetylcholine into the organ bath. The quantity of the subject was increased threefold at each addition being made after the exposure to the previous addition had attained a matured level and remained steady. This was done until the maximum tissue response was attained. The volume of drug administered with each addition was adjusted to give a 200-fold final bath dilution. The subject concentration-response curves were determined from measurements made at the highest point of the contraction for each experiment after which the tissue was thoroughly rinsed several times – Dina and Arowolo (9).

The same tissue was then pre-incubated with atropine sulphate (British Drug House Chemicals Limited, England). This was done by allowing the antagonist to equilibrate in the bath medium for at least fifteen minutes. This period was sufficient to ensure adequate contact of atropine with the tissue. A single predetermined molar concentration of 10^{-7} mol atropine was used in every subject-antagonist experiment.

Without washing out atropine, agonist concentration-response curves (CRCS) were determined by adding acetylcholine to the bath at concentrations corresponding to those in the preceding subject study. This was done until the maximum subject response obtainable in the presence of the antagonist was attained. This procedure for subject-antagonist study was repeated five times using new tissue strips from each section of the G.I. tract. The values of EC_{50} and the maximal response (E_{max}) of acetylcholine alone and in the presence of 10^{-7} mol atropine were determined from the concentration-response curves.

Statistical Analysis

Student's *t*-test was employed to determine whether there was a significant difference between two mean values of the various parameters evaluated in the absence and in the presence of atropine – Bailey (3).

RESULTS

The smooth muscle of the gastrointestinal tract of *Cairina moschata* contracted in a dose dependent fashion to acetylcholine. The effect of Ach on the smooth muscle was observed as higher amplitude of contractions characterized by increased frequency of slow waves and spike formation.

The threshold and maximal responses to acetylcholine

The isolated preparations of gastrointestinal tract of *Cairina moschata* vary in their sensitive to acetylcholine. The subject being most potent in the proventriculus where the threshold response was attained at 1.10^{-15} mol and maximum response obtained at 3.10^{-6} mol. The rectum was less sensitive to acetylcholine than the proventriculus; threshold response was obtained at 1.10^{-13} mol and the maximum response at 3.10^{-4} mol. Crop responded moderately to acetylcholine with the threshold value attained at 1.10^{-12} mol and maximum response at 3.10^{-4} mol. Acetylcholine was equipotent in duodenum and caecum; threshold responses were attained at 1.10^{-11} mol, however the subject is more efficacious in the caecum with a maximal response at 3.10^{-5} mol than in the duodenum with a maximal response at 3.10^{-4} mol. The ileum appeared the least sensitive to acetylcholine with a threshold response obtained between 1 and 3.10^{-9} mol while the subject was equally effective as in other

tissues with a maximal contractile response at 3.10^{-4} mol (Table 1).

The EC_{50} values of acetylcholine in the presence and absence of atropine

Pretreatment with atropine caused a significant increase in the mean EC_{50} values of acetylcholine in all the organs; crop ($P<0.001$), proventriculus ($P<0.05$), duodenum ($P<0.05$), ileum ($P<0.001$), caecum ($P<0.01$) and rectum ($P<0.05$) (Table 1).

Percentage Maximal response (E_{max}) to acetylcholine and the dose ratio (D.R.)

Atropine caused a non-significant depression of the acetylcholine E_{max} in the crop, proventriculus, duodenum caecum and rectum. This depression was significant in the ileum ($P<0.05$) (Table 1).

The mean dose ratio (D.R.) value was above unity (1) thus suggesting a competitive inhibition of acetylcholine by atropine in the crop, proventriculus, duodenum, ileum, caecum and rectum. The effectiveness of this inhibition was highest in the ileum (Table 2).

DISCUSSION

Acetylcholine produced dose-dependent contractions of muscle strips prepared from the different parts (i.e. the crop, proventriculus, duodenum, ileum, caecum and rectum) of the gastrointestinal tract of *Cairina moschata*. The effect of Ach on the smooth muscle was observed as a higher amplitude of contractions characterized by an increased frequency of slow waves and spike formation. This pattern of contraction induced by Ach has also been observed in the G.I. tract of most mammals: canine – Furuichi *et al.* (13), Balsiger *et al.* (4); horse – Malone *et al.* (18) and rabbits – Racz *et al.* (24). A similar pattern of contractile response to Ach have been documented in other Nigerian avian species: chicken – Ameh *et al.* (1); Oke *et al.* (21) and guinea fowl – Arowolo and Olowookorun (2). The contractile effect of Ach on the smooth muscle of G.I. is no less different in non-mammalian species such as fish, amphibians or reptiles – Olsson and Holmgreen (22) and arthropods – Tuberg *et al.* (30). In all these animals, the submissions of all the workers generally agreed that the application of Ach to isolated gastrointestinal smooth muscle causes a decrease in the resting potential and an increase in the frequency of spikes, accompanied

Table 1. The molar concentrations of acetylcholine producing the threshold (T. R.) and maximal (M. R.) responses in the various sections of the G. I. tract of *C. moschata*

	Crop	Proventriculus	Duodenum	Ileum	Caecum	Rectum
T. R. (mol)	1.10^{-12} (5)	1.10^{-15} (5)	1.10^{-11} (5)	1.10^{-9} (5)	1.10^{-11} (5)	1.10^{-13} (5)
M. R. (mol)	3.10^{-4} (5)	1.10^{-6} (5)	3.10^{-4} (5)	$3.\ 10^{-4}$ (5)	3.10^{-5} (5)	1.10^{-4} (5)

( ) — Number of observations; mol — Molar concentration

Table 2. The mean ±S.E.M values of parameters obtained from acetylcholine-induced contractions of the G. I. tract of *C. moschata* in the presence and absence of atropine

		Crop	Proventriculus	Duodenum	Ileum	Caecum	Rectum
EC_{50}	TEST	$^{a}3.30\times10^{-7}\pm 3.38\times10^{-5}$ (5)	$^{c}2.60\times10^{-8}\pm 6.29\times10^{-10}$ (5)	$^{c}7.06\times10^{-9}\pm 1.35\times10^{-9}$ (5)	$^{a}1.64\times10^{-4}\pm 2.25\times10^{-5}$ (5)	$^{b}4.40\times10^{-5}\pm 1.10\times10^{-5}$ (6)	$^{c}7.20\times10^{-11}\pm 4.75\times10^{-12}$ (5)
	CONTROL	$^{a}4.18\times10^{-9}\pm 1.70\times10^{-9}$ (5)	$^{c}5.13\times10^{-9}\pm 2.97\times10^{-9}$ (5)	$^{c}3.85\times10^{-11}\pm 1.1\times10^{-11}$ (5)	$^{a}4.86\times10^{-6}\pm$) 2.37 $\times10^{-6}$ (5	$^{b}5.00\times10^{-9}\pm 1.00\times10^{-9}$ (6)	$^{c}2.24\times10^{-11}\pm 1.59\times10^{-11}$ (5)
E_{max}	TEST	$^{d}78.26\pm2.57$ (5)	$^{d}71.67\pm8.7$ 7 (5)	$^{d}83.93\pm4.49$ (5)	$^{c}47.30\pm5.98$ (5)	$^{d}71.05\pm18.62$ (6)	$^{d}81.23\pm2.55$ (5)
	CONTROL	$^{d}92.22\pm3.55$ (5)	$^{d}74.99\pm9.58$ (5)	$^{d}91.07\pm3.42$ (5)	$^{c}81.43\pm5.71$ (5)	$^{d}94.48\pm$ 4.19 (6)	$^{d}86.98\pm4.18$ (5)
D. R.		1120.26 ± 9.15 (5)	76.08 ± 64.31 (5)	280.6 ± 153.73 (5)	1354.32 ± 1235.40 (5)	137 ± 9.16 (6)	124.41 ± 13.22 (5)

Values are given in means (± S. E.); ( ) — The number of observations

[a-a, b-b] and [c-c] — Significant difference ($P < 0.001$, 0.01 and 0. 05) between test and control in each organ respectively

[d-d] — non-significant difference ($P > 0.05$) between test and control in each organ

by a rise in tension. These electrical events translate physiologically to an increase in the tone, amplitude of contractions and peristaltic activity of the stomach and intestines, as well as enhanced secretory activity of the G.I. tract – Brown and Taylor (6).

Available reports suggest that the primary action of Ach in initiating its effect is brought about by depolarization of the cell membrane, initiated by an increase in Na^+ and Ca^{2+} conductance – Feng *et al.* (12), Somylo and Somylo (27). Ach stimulates ion fluxes across the membrane and/or mobilizes intracellular Ca^{2+} to cause contractions in the G.I. smooth muscle – Sward *et al.* (29).

The Threshold and Maximum values of Ach in the G. I. of *C. moschata*

The threshold value of 1.10^{-15}—1.10^{-9} mol obtained for Ach in the G.I. of *C. moschata* was lower than the value of 1.10^{-9}—1.10^{-7} mol obtained for the Nigerian local chicken (*Gallus gallus domesticus*) – Ameh *et al.* (1), Oke *et al.* (21) and 1.10^{-9} mol for guinea fowl – Arowolo and Olowookorun (2). This indicates that the gastrointestinal smooth muscle of Nigeria ducks have comparatively higher pharmacological reaction to Ach than other Nigerian avian species already documented. In this study, the crop, proventriculus, and rectum appeared the most sensitive regions of the G.I. tract of *C. moschata* to Ach. These disparities strongly suggest regional difference in the distribution of cholinergic receptors along the wall of G.I. of *C. moschata*. Regional difference in the distribution of receptors mediating gut motility is a reflection of anatomical or physiological variations in the G.I. tract, and this phenomenon has been reported in man – Katschinski *et al.* (15) and other mammalian species – Muinuddin *et al.* (20), Randenkovic *et al.* (26).

Atropine Antagonism

1.10^{-7} moll atropine significantly reduced the potency (EC_{50}) of Ach in the crop, proventriculus, duodenum, ileum, caecum and rectum (Table 2). The antagonism was competitive for all the G.I. sections studied though the maximal response (E_{max}) was significantly reduced in the crop and caecum. The D.R. values obtained confirm the effectiveness of atropine as a competitive antagonist of Ach in the G.I. tract of *C. moschata* (Table 2). These findings are specific for atropine at the M cholinergic receptors – Ameh (1), Randekovic *et al.* (25).

Five subtypes of M receptors have been detected by molecular cloning – Dorje *et al.* (10). These variants have distinct anatomical localization and chemical specificities but atropine has high affinity for all the receptor subtypes. M1, M3, and M5 subtypes are excitatory in action and their effects are carried out through G-protein induced changes in the direction of distinct membrane bound effector molecules such as the IP3 and diacyglycerol – Barany (5), Somylo and Somylo (28). M2 and M4 receptors are reported to have inhibitory effects through interaction with a distinct group of G proteins with resultant inhibition of adenylcyclase, activation of receptor – operated K^+ channels and suppression of the activity of voltage – gated Ca^{2+} channels – Coulson *et al.* (7), Lambrecht *et al.* (16). In the gastrointestinal smooth muscle, only M2 and M3 are identified and they mediate contractions of the smooth muscle of the G.I. tract – Lecci *et al.* (17), Coulson *et al.* (7). The M2 receptor is reported to have an inhibitory effect on cAMP levels, which causes inhibition of the relaxant action of cAMP-stimulating adrenergic neurotransmitters, hence the role of M2 receptors to the motility of G.I. is considered indirect – Ehlert (11). Notwithstanding, M2 is reported to contribute 25 % of cholinergic induced contraction in rat ileum – Matsui *et al.* (19) and the population of M2 outnumbers that of M3 by a factor of about four in most instances – Ehlert (11).

This study also established the molar concentration for the threshold and maximal contractile response of the gastrointestinal smooth muscle of *C. moschata* to acetylcholine. The contraction was dose-dependent and was blocked competitively by atropine. Further study needs to be done in order to sub-classify the muscarinic receptors present in the G.I. tract of *C. moschata*.

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THE INFLUENCE OF SHORT TIME EXERCISE ON GLUCOSE AND CORTISOL CONCENTRATIONS IN THE BLOOD SERUM OF THE POLISH HORSE

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ABSTRACT

Investigations were carried out to determine the impact of short time exercise on glucose and cortisol levels in the Polish horse. The examinations were made in two stages, i.e. prior to and after short time training. Serum levels of glucose and cortisol increased after the exercise in both samplings. The level of glucose and cortisol determined in samples taken one month apart differed considerably. The data obtained are not sufficient to identify the reason for such difference.

Key words: cortisol; glucose; Polish horse; short term exercise

INTRODUCTION

Glucose is the most common sugar in a living organism. Its concentration in blood serum reflects systemic metabolism of this sugar and is regulated mainly neurohormonally. Another agent supplying energy to an organism during physical effort is cortisol. It belongs to a glyco-corticosteroid group secreted by the adrenal cortex. Cortisol is considered a stress hormone and makes up 79—90 % of total corticoids circulating in the blood. This hormone is released in "critical" situations about two to three minutes after the introduction of adrenalin and marenoradrenalin into the bloodstream which stimulates the organism and generates response to a stress situation or exercise. It induces metabolism of sugars, fat and proteins as well as of water-electrolyte. It also inhibits the activity of the immune system yet shows an anti-inflammatory action, enhances synthesis of some enzymes, influences blood circulation system (intensifies blood cells production) and cell metabolism. According to its physical activity, the energy reserve of the organism changes. Quite different is the response of all organisms to environmental changes or to various efforts (4, 5, 6, 9).

The present study was conducted to observe changes in blood glucose and cortisol in Polish horses in relation to short time training.

MATERIAL AND METHODS

Our investigations were carried out on Polish horses from the Roztoczański National Park. The animals were between two and six years of age and came from the same breeding stable. All the horses grazed together on pasture and were kept in one stable. They were calm and fit.

The herd of Polish horses included tenty horses and examinations were carried out on nine of them (five stallions and four mares). Before the collection of blood samples the horses were examined clinically (body temperature, pulse and respiration rate) and were under veterinary care throughout the experiment. All clinical examinations confirmed that they were in good health.

The load consisted of training on a training track (fifteen minutes) in the south-eastern part of Poland. Temperature during the training at both samplings ranged between 10 and 18 °C.

Two samples of blood were withdrawn in autumn with a one month interval between them. All experimental material was collected in two stages in the morning:

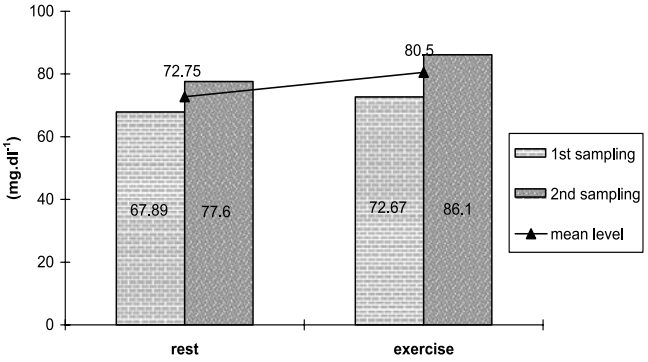

Fig. 1. Glucose level in Polish horses at rest and after short time training

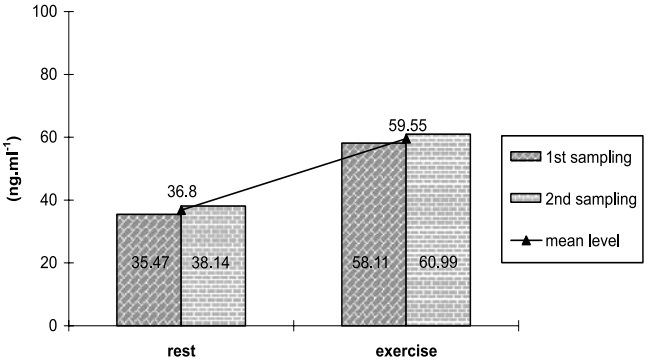

Fig. 2. Cortisol level in Polish horses at rest and after short time training

Stage one – blood samples were collected at rest and animals underwent a short-term training session; stage two – material was sampled after exercise.

Glucose and cortisol levels were determined in blood samples taken in both stages. Cortisol was determined by an immunoenzymatic method using the apparatus VIDAS and glucose by a spectrophotometric method.

Results were presented in graphic form and the significance of differences was checked by the Student *t*-test.

RESULTS AND DISCUSSION

Physical exercise requires great expenditure of energy. So to meet this demand a live organism is forced to activate available energy sources. During exercise, glucose is used as a primary energy source and in the following stage a group of mechanisms responsible for energy supply becomes important, such as cortisol and others (2, 4, 6, 9). Rose *et al.* (7) and Andrews *et al.* (1) have observed an increased concentration of cortisol and catecholamines after intensive training and subsequent increase in the level of glucose. Acute stress with short term elevation of cortisol levels may aid the re-establishment of the homeostasis of the immune system (3, 8).

The results obtained by analysis of materials obtained from the Polish horses before and after short-term exercise are presented in Figures 1 and 2.

The glucose concentration in blood may be affected by physical effort, such as training, endurance and fitness as well as the manner of riding. Various forms of activity and physical training may have a short term impact on blood glucose levels. In the case of a brief effort we have recorded an increase in glucose level (speed training) while long-term exercise results in its decrease (endurance training). During the endurance training, which lasts longer than the speed training, a fall in glucose level is reported, yet above the resting level. After the training the blood glucose level rises again and reaches normal concentration within a thirty minute rest period (9).

In horses, blood glucose is often determined in relation to training or its potential modifications to obtain information on the horse's energy reserve.

The mean glucose level in the examined horses acquired different values at individual experimental stages. In the first sampling, blood glucose reached 67.89 mg. dl^{-1} prior to the effort and increased during the training to 72.67 $mg.dl^{-1}$. In the second sampling during the rest period the mean glucose level reached 77.6 $mg.dl^{-1}$. As in the first sampling, this value was lower than that recorded after the exercise (86.1 $mg.dl^{-1}$).

In two samplings, the total post-exercise glucose level was higher (80.5 $mg.dl^{-1}$) compared to that before the physical effort (72.75 $mg.dl^{-1}$). However, the levels discussed were within the reference range reported by Winnicka (13) (Fig. 1).

A similar tendency was observed for cortisol. The mean cortisol level prior to exercise reached 35.47 $mg.dl^{-1}$ and after the exercise 58.11 $mg.dl^{-1}$. No significant differences were recorded between individual animals at rest or after the training. At both samplings after the exercise a significant ($P<0.01$) increase in the cortisol level was noted (Fig. 2). The training was conducted in the morning hours when stable cortisol levels had been observed.

Various environmental and social factors may induce a change in natural animal behaviour and thus affect the organism response in a significant way (11, 12). Also adverse environmental conditions may cause disturbances in the natural behavioral development of organism (2, 4, 5, 6). With regard to these it is difficult to explain the considerable differences in the levels of glucose and cortisol observed only one month apart with very little weather differences. Perhaps nutritional factors may also play some role. However, the results of some other authors (8, 10) do not confirm such dependence and point more to seasonal changeability.

The results obtained in Polish horses extend the body of knowledge on specific changes related to their training and exercise. More experiments are needed to identify the reasons for observed differences.

CONCLUSIONS

1. The blood glucose and cortisol levels in Polish horses were significantly lower prior to exercise compared to those after short-time training ($P < 0.01$).

2. The level of glucose and cortisol determined in samples taken one month apart differed considerably but the reason for such differences cannot be identified on the basis of results obtained in this study.

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THE EFFECT OF TIME OF THE ADMINISTRATION OF PREGNANT MARE SERUM GONADOTROPHIN ON OESTRUS SYNCHRONIZATION EFFICIENCY AND FERTILITY IN EWES

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ABSTRACT

A study was conducted on 84 Blackhead Ogaden (BHO) ewes to evaluate the necessity and appropriate time of Pregnant Mare Serum Gonadotrophin (PMSG) administration in relation to Medroxyprogesterone acetate (MAP) sponge withdrawal on oestrous response and fertility. The treatments include administration of 300 IU PMSG at: (1) twenty-four hours prior to MAP sponge withdrawal, (2) at MAP sponge withdrawal and (3) control (without PMSG administration). Regardless of the time of administration, oestrous response and pregnancy rate were higher ($P \leq 0.01$) in PMSG administered than in the control groups of ewes. Similarly, the time to onset of oestrous was earlier ($P < 0.01$) in PMSG administered than in the control groups of ewes. However, there was no significant difference ($P \geq 0.05$) in all parameters among PMSG administered groups of ewes. In conclusion, administration of 300 IU PMSG either 24 hours prior to or at MAP sponge withdrawal is important to attain better synchronization of oestrus and increased pregnancy rate from BHO sheep kept under the extensive management conditions of Eastern Ethiopia.

Key words: ewe; MAP sponges; PMSG; oestrus synchronization

INTRODUCTION

The Blackhead Ogaden (BHO) sheep is found mainly in the eastern and southeastern lowlands of Ethiopia. This sheep breed is the second largest in number next to camels in the lowland areas of Ethiopia. Meat from this breed of sheep plays a vital role in the local economy of Ethiopia and as a source of foreign currency. Apparently, little effort has been made so far to improve productive and reproductive performances of this sheep breed despite the great ambition of pastoralists to improve productivity by controlled mating (6). To this end, the owners apply traditional practices to match the lambing season with the availability of water and feed to improve the survival rate of the offspring and to match the slaughter age of animals with the season of highest market demand.

However, the progress made by pastoralists using traditional practices is far below acceptable as few of their efforts have been supported by research. Thus, the current productive and reproductive performances of the BHO sheep are far below the owners' and the country's needs (6).

Taking into consideration the importance of this specific breed of sheep on one hand and a lack of scientific information to support the traditional attempts to synchronise oestrus on the other hand, this study was initiated to assess the response of this sheep breed to the controlled-breeding techniques practised in other European sheep breeds. Thus, the synchronization efficiency in terms of inducing oestrus and fertility by using MAP sponge treatment and time of PMSG administration relative to MAP sponge withdrawal were evaluated in this experiment.

MATERIALS AND METHODS

Study site

The experiment was conducted at Alemaya University, which is situated 25 km from the town of Harar and 42 km from Dire-Dawa, Ethiopia. The site is located 90 24" N latitude, 410 5" E longitude, and at an altitude of 1,980 m above sea level. The annual total rainfall and the mean maximum and

Running Title: Effect of time of Pregnant Mare Serum Gonadotrophin

minimum temperatures of the area are 870 mm, 22.9 °C and 7.8 °C, respectively (12).

Experimental animals and their management

One hundred and twenty ewes (2—2.5 years and 15—26 kg body weight) were purchased from pastoralists. All the sheep were provided with fresh clean water throughout the experimental period and allowed to graze on natural pastures for about eight hours a day (8.00 a.m. to 12.00 a.m. in the morning and 2.00 p.m. to 6.00 p.m. in the afternoon). All animals were soaked with a broad-spectrum anti-helminthic, dipped with a standard acaricide solution for external parasites and were vaccinated against pasteurellosis and anthrax. At the end of the adaptation period, 84 non-pregnant ewes weighing between 20 and 26 kilograms were selected and assigned to their respective treatment groups.

Table 1. The effect of the time of PMSG administration in relation to Medroxyprogesterone sponge withdrawal on the oestrous response in Blackhead Ogaden ewes

Time of PMSG administration	n	Oestrous response (%)
24 hours prior to MAP sponge withdrawal	29	100.0[a]
At MAP sponge withdrawal	27	100.0[a]
Control (without PMSG administration)	28	75.0[b]
Overall mean	84	91.7

[a, b] — values in a column with different superscripts differ significantly ($P < 0.01$); n — number of ewes

PMSG treatment

At the end of the MAP sponge treatment period, ewes were randomly allotted to groups of 29, 27 and 28 animals, respectively. The first and the second groups were treated with 300 IU PMSG at MAP sponge withdrawal and 24 hours prior to sponge withdrawal, respectively, whereas the third group was kept as a control (administered with a sterile physiological solution).

Oestrous observation

The signs of oestrus were observed at eight hourly intervals following MAP sponge withdrawal for a period of 96 hours. Intact rams fitted with aprons were used for detecting ewes in oestrus. The ewes were kept in their respective groups, and each group was observed three times daily for a period of thirty minutes. Between 13 and 28 days after AI, all ewes were monitored twice daily for a return to oestrus.

AI procedures

Semen was collected from healthy rams with the aid of an artificial vagina. Following each semen collection and prior to its use for artificial insemination, the viability of the sperm was microscopically evaluated according to standard procedures (18). Fresh semen was diluted at a ratio of 1 : 2 with sterile skimmed cow milk. Cervical insemination with 0.1 ml diluted semen at 53 to 55 hours following MAP sponge withdrawal was performed.

Statistical analysis

The general linear model (GLM) procedures of SAS were used to run analysis of variance test for the effect of time of PMSG administration on the time to onset of oestrus and the duration of the induced oestrus. The categorical modeling (CATMOD) procedures of SAS were used to test the effect of duration of progestagen treatment and time of PMSG administration on pregnancy rate and oestrous response. The treatment means were compared by Duncan's multiple range test (DMRT) as described in Gomez and Gomez (7).

Table 2. The effect of the time of PMSG administration on time to onset and the duration of induced oestrus in Blackhead Ogaden ewes

Time of PMSG administration	n	Time to onset of oestrus (h)	Duration of Oestrus (h)
24 hours prior to MAP sponge withdrawal	21	32.1[b] ± 2.4	45.6[a] ± 2.7
At MAP sponge withdrawal	27	38.2[b] ± 2.5	46.6[a] ± 2.8
Control (without PMSG administration)	29	48.8[a] ± 2.9	42.8[a] ± 3.2

[a, b] — Means in a column with different superscripts differ significantly ($P < 0.01$); n — number of ewes

Table 3. Reproductive performance following oestrous synchronisation and artificial insemination in Blackhead Ogaden ewes

Time of PMSG administration	n	Pregnancy rate (%)	Non-return rate (%)
24 hours prior to MAP sponge withdrawal	29	69.0[ab]	82.8[a]
At MAP sponge withdrawal	27	74.1[a]	81.5[a]
Control (without PMSG)	28	46.4[b]	57.1[b]

[a, b] — Means in a column with different superscripts differ significantly ($P < 0.05$); n — number of ewes

RESULTS

The effects of time of PMSG administration relative to MAP sponge withdrawal on oestrous response is set out in Table 1. The response to oestrus was significantly higher ($P < 0.01$) in PMSG treated than in controlled ewes.

The duration of the induced oestrus was not significantly affected by the time of PMSG administration relative to MAP sponge withdrawal. The interval from MAP sponge withdrawal to the onset of oestrus was, however, significantly longer ($P < 0.01$) in the control ewes, compared to those treated with PMSG at 24 hours prior to MAP sponge withdrawal or at sponge withdrawal (Table 2).

The pregnancy rate was significantly lower ($P < 0.01$) in the control ewes, compared to ewes given PMSG administration regardless of the time of administration (Table 3). Furthermore, PMSG administration at the time of MAP sponge withdrawal resulted in a significantly higher ($P < 0.05$) pregnancy rate, compared to the administration of PMSG at 24 hours prior to sponge withdrawal. Similarly, the non-return rate was significantly lower ($P < 0.05$) in the control ewes, compared to ewes administered PMSG at 24 hours prior to sponge withdrawal or at MAP sponge withdrawal. There was, however, no significant difference in the non-return rate between ewes administered with PMSG at MAP sponge withdrawal and those administered at 24 hours prior to MAP sponge withdrawal. In all cases, the values obtained for the non-return rates were higher than those for pregnancy rates (63.1 % *vs.* 73.8 %).

DISCUSSION

In the present experiment, only seven out of 84 ewes failed to exhibit overt oestrus. Three of these seven animals had lost their MAP sponges at one stage of the treatment period although these were immediately replaced. The oestrous response rate might have been higher than the current value had these sponges not been lost. Even then, the oestrous response value obtained in this experiment is comparable to the values reported in the literature (4, 9, 11, 16, 19). This implies that BHO ewes maintained under traditional management conditions also respond to synchronizing agents commercialized and well adapted to other sheep breeds in other parts of the world.

Attainment of a significantly higher ($P < 0.01$) oestrous response in PMSG administered ewes, regardless of the time of application, compared to the control (Table 1) in this experiment is in agreement with previous reports (1, 2, 3, 14) which have reported a low dose of PMSG administration to result in compact and predictable oestrus in ewes treated with MAP sponges.

The time of PMSG administration relative to MAP withdrawal did not significantly influence the oestrous response. This disagrees with the previous results of Zhang and Yuan (1988) who have reported oestrous synchronization rate to be 100 % in nanny goats treated with PMSG 48 hours prior to MAP withdrawal, compared to nanny goats treated with the same amount of PMSG but at sponge removal (66.7 %). Probably, the gap between 24 hours prior to MAP withdraws and at sponge withdrawal may be too short to affect oestrous response in the present experiment.

Several factors may influence the extent of the interval between the removal of MAP sponge and the onset of induced oestrus. Generally, oestrus starts at about 36 hours after MAP withdrawal, although some ewes may be in oestrus as early as 24 hours or as late as 48 hours (8). The overall mean time interval between intravaginal progestagen sponge withdrawals to the onset of oestrus in the present trial (Table 2) is in agreement with Gordon (8) and Vancleef *et al.* (17) who have reported the time to oestrus to be 36 hours in ewes.

The current results are also fairly comparable to the findings of Greyling *et al.* (11) in MAP (60 mg) synchronized Merino ewes during the natural breeding season (30.5 hours). However, the interval observed in this study was shorter when compared to the values obtained by Greyling and Brink (9) in MAP treated Karakul ewes (62.5 ± 18.7 hours). This discrepancy in the time of oestrous onset may be due to breed, nutritional and/or seasonal differences.

The significantly shorter ($P < 0.01$) interval from MAP withdrawal to the onset of oestrus in ewes administered 300 IU PMSG, compared to control (Table 2) is in line with the available literature (1, 5, 14, 20), which has reported the use of PMSG in combination with progestagen sponges to shorten the time from sponge withdrawal to the onset of oestrus. The shortening of the onset of oestrus in PMSG treated ewes may be due to the hastening effect of PMSG on follicular maturation.

The main focus of controlled reproduction is to obtain an optimum fertility rate at the prescribed time. Although the average lambing rate recorded in this experiment (Table 3) could not be considered as optimum, it could encourage further study as controlled breeding techniques like artificial insemination and oestrous synchronization have never been tried before in BHO sheep. In fact the lambing rate recorded in the present study agreed with the values of Greyling *et al.* (10) and Hill *et al.* (13) who have reported the mean pregnancy rate following Map treatment and AI in Merino ewes to be 63.5 % and 64.6 %, respectively.

The difference between the lambing and the non-return rate was very big (63.1 % *vs.* 73.8 %). This big difference between the two may be due to the occurrence of embryonic re-absorption and/or silent heats in some of the experimental animals. Had the lambing rate been the same as the non-return rate, the lambing rate obtained in the present experiment would have been more acceptable.

In the present experiment achievement of significantly higher ($P < 0.05$) fertility rates (pregnancy and non-return rates) from ewes administered PMSG, compared to control

indicates the importance of PMSG administration for an improved lambing rate from oestrous synchronized BHO ewes. The higher fertility rate achieved from PMSG administered groups is in line with many previous works (1, 2, 3, 5, 14, 20,).

The result of the present experiment contradicts the reports of Romano *et al.* (15), who have recorded similar fertility rates between ewes administered 250 IU PMSG or no PMSG. Perhaps, 250 IU PMSG administered in their studies might have been below the threshold level to affect the fertility rate significantly.

Unlike the previous reports of Zhang and Yuan (20) who have indicated an increase in fertility when PMSG is administered 24 hours prior to pessary removal, compared to PMSG administration at sponge withdrawal, there were no significant differences in fertility rates between the two groups in the current trial. However, the results of the present trial are in agreement with Eppleston *et al.* (5), who have stated that the time of PMSG administration relative to progestagen withdrawal could not improve fertility, except shortening the time of ovulation.

In conclusion, administration of 300 IU PMSG either at 24 hours prior to, or at progestagen sponge withdrawal is required to attain better synchronisation of oestrus and an increased lambing rate from BHO sheep kept under traditional management conditions in Eastern Ethiopia.

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THE ANALYSIS OF PRION PROTEIN GENE POLYMORPHISM IN SLOVAKIAN WHITE SHORTHAIRED GOATS

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ABSTRACT

Scrapie is a fatal neurodegenerative disease of sheep and goat, grouped under the transmissible spongiform encephalopathy (TSE). Age, infectious agent, as well as host factor like the PrP gene and its allelic form influence the progress of disease. The aim of our study was to detect polymorphisms in the prion protein (PrP) gene in White shorthaired goats as well as to reveal octapeptide repeats. The amplified portion of the PrP gene (Exon 3) was subjected to polymorphism detection using Denaturing Gradient Gel Electrophoresis (DGGE) and DNA sequencing. Polymorphisms in the codons 138 (agc/agt, S → S; or agt/agt; S → S), 142 (ata/atg; I → M), 168 (cca/caa; P → Q) and 179 (gtg/gtt; V → V) were found. All animals (n = 180) in this study were homozygous having 5 octapeptide repeats. This is the first time that a dimorphism in codon 179 present in allele $A_{136}R_{154}Q_{171}/A_{136}R_{154}Q_{171}$ is published. This dimorphism is a result of the silent mutation.

Key words: DGGE; PrP genotype; scrapie; Slovakia; White shorthaired goats

INTRODUCTION

Scrapie is a fatal neurodegenerative disease associated with pathological changes in the conformation of the normal prion protein (PrP^{C}), found on the surface of neurons, resulting in abnormal PrP^{Sc} prion protein. This PrP^{Sc} protein, typical for prionoses, can be accumulated in CNS of the affected animals and humans as aggregates. Accumulation of PrP^{Sc} in the brain is considered as a diagnostic marker of prion diseases (13). The occurrence of natural scrapie in sheep is influenced by alterations in the host gene that encodes the PrP protein (10). The ovine PrP gene has three exons of 52, 98, and 4028 nucleotides in length. In exon 3, the alanine (A) to valine (V) polymorphism in codon 136 and the glutamine (Q) to arginine (R) polymorphism in codon 171 are considered to be propagating factors for the development of scrapie (4, 5, 7, 15, 17).

The association between scrapie susceptibility and polymorphisms in codon 154 is still unclear. However, there is a possibility that histidine (H) in codon 154 may offer protection from scrapie in some sheep breeds. Polymorphisms in the codons 112, 127, 137, 138, 141, 143, 151, 168, 175, 176, 180, 189, 211, and 241 are rare and have not been associated with any disease phenotype in natural and experimental scrapie (2, 6). Similarly, in goat, PrP gene polymorphisms are recorded in the codons 102 (tryptophan to glycine, W → G), 110 (threonine to proline, T → P), 127 (glycine to serine, G → S), 142 (isoleucine to methionine, I → M), 143 (histidine to arginine, H → R), 154 (arginine to histidine, R → H), 168 (proline to glutamine, P → Q), 211 (arginine to glutamine, R → Q), 220 (glutamine to histidine, Q → H) and 222 (glutamine to lysine, Q → K) (2). A disease association has also been observed with dimorphisms in the codons 142, 143 and 154 (1, 3, 8). Codon 142 (I → M) is associated with an altered disease incubation period (3).

A different kind of protein variation is found in a series of glycine-rich octa- or nonapeptide sequences in the N-terminal region of the PrP protein. A PrP allele found in cattle, sheep and goat encodes five of these sequences in the following arrangement: nonapeptide P1(1×), octapeptide P2 (3×) and

nonapeptide P3 (1×) (7, 8). In goats, a PrP allelic variant polymorphism, containing only three octapeptide repeats, is supposed to be associated with an increased scrapie incubation period in goats (9). It is possible that the different profiles of PrP polymorphisms reported for sheep and goats could lead to a differential phenotypic expression of individual scrapie strains (3).

The first case of scrapie in Slovakia was diagnosed in Merino sheep in March 2003 (11). Since then, sporadic cases of sheep scrapie have occurred; 48 cases have been reported up to 2006. However, to date, no scrapie in goats has been diagnosed in Slovakia (**www.svsr.sk**). The aim of this study was to investigate the PrP genotype in the Slovakian goat population.

MATERIALS AND METHODS

Animals

The 180 healthy White shorthaired goats (which represent the dominant bread of goat in Slovakia) in this study originate from two farms (Farm A = 102; Farm B = 78). Genomic DNA was isolated from blood leukocytes. Blood was collected in 1.5 % EDTA. DNA extraction and purification was done according to Sambrook *et al.* (14).

Analysis of octapeptide repeats

The PrP open reading frame was amplified using primers (PRNP1 and PRNP2) as shown in Table 1, which generated 349 bp amplicons in the case of 5 octapeptide repeats. The PCR cycling conditions were as follows: initial denaturation 95 °C for 5 min, followed by 35 cycles of 95 °C for 1.0 min, 65 °C for 1.0 min, 72 °C for 1.0 min with a final extension at 72 °C for 10 min. PCR products were analysed in 1.8 % ethidium-bromide-stained agarose gel.

Table 1. Primers used for PCR amplification

Primer	Sequence 5' → 3'	Product
pTK-F1	GGCCTTGGTGGCTACATGCTG	176 bp
pTK-R1	CGCCCGCCGCGCCCCGCGCCCGCCCGC CGCCCCCGCCCG TTTTTATGTTGACACA GTCATGCAC	
pTK-F2	GTGGTAGCCTCAGTCAGTGGAACA	451 bp
pTK-R2	GAGGAGGATCACAGGAGGGGAA	
PRNP 1*	ACGTGGGCCTCTGCAAGAAGCGAC	349 bp
PRNP 2*	GCACTTCCCAGCATGTAGCCACCA	

*— primers described by Walawski and Czarnik (18)

PrP gene polymorphism study

The PrP open reading frame was amplified by using primers pTK-F1 and pTK-R1 (Table 1), which generated a 176 bp amplicon, involving codons 127 to 185. Primer pTK-R1 contains a long GC part (*GC clamp*), which will remain double stranded during DGGE. The PCR cycling conditions were as follows: initial denaturation 95 °C for 5 min, followed by 30 cycles of 95 °C for 1.0 min, 59 °C for 1.0 min, 72 °C for 1.0 min with a final extension at 72 °C for 10 min. After denaturation (94 °C.10 min^{-1}) and renaturation (30 °C.10 min^{-1}) PCR fragments were separated on 6 % polyacrylamide gel (37.5 : 1; acrylamide/bis) containing a linear gradient (20—65 %) of denaturants (urea and formamide) in 0.5× TAE buffer (40 mmol Tris-acetate, 1 mmol EDTA). Electrophoresis was done at 100 V for 16 h at 58 °C in 0.5× TAE buffer in a DGGE unit (Ingeny, The Netherlands). Gel was stained using silver staining. Goats were segregated into different groups according to the DGGE profiles.

DNA sequencing

Two or three representative samples from each DGGE profile were amplified by PCR with primer set pTK-F2 and pTK-R2 (Table 1), which generated a 451 bp fragment of the coding region of the goat PrP gene. Sequencing was done on the ABI PrismTM 377 Sequencer using Big Dye Terminator Kit (Applied Biosystems). Sequences were evaluated using the Sequence Navigator Program and aligned by Multalin **(http://prodes.toulouse.inra.fr/multalin/multalin.html**). Consequently sequences were compared with known sequences of the goat PrP gene as published in Pubmed (Genebank). Sequences of novel alleles were submitted into the Genebank under the accession numbers AY897571 and AY897572.

RESULTS AND DISCUSSION

All goats were homozygous for 5/5 octapeptide repeats showing no octapeptide variability in the examined population (Fig. 1). The results are in accordance with the previous observation in goats, sheep and other related species (3). On the other hand, Goldmann *et al.* (9) reported three octapeptide repeats in goats, which are associated with an increased scrapie incubation period.

DGGE analysis segregated the samples (n = 180) into 5 groups (Table 2; Fig. 2). Further DNA sequencing revealed that all groups had either the $A_{136}R_{154}Q_{171}/A_{136}R_{154}Q_{171}$ (ARQ/ARQ) genotype, or the ARQ/ARQ allele with additional polymorphic codons (Fig. 3). DGGE profile A, showing only the ARQ/ARQ allele, without additional polymorphisms, was found in 32 (31.4 %) goats from Farm A and 16 (20.5 %) goats from Farm B.

Table 2. Occurrence of PrP alleles in Slovakian White shorthaired goats

DGGE	Genotype	Farm A		Farm B	
		n = 102	%	n = 78	%
A	ARQ/ARQ	32	31.4	16	20.5
B	ARQ/ARQ + SS_{138}	49	48.0	35	44.9
C	ARQ/ARQ + $SS_{138}PQ_{168}$	0	0	2	2.6
D	ARQ/ARQ + $SS_{138}VV_{179}$	19	18.6	21	26.9
E	ARQ/ARQ + $SS_{138}IM_{142}$	2	2.0	4	5.1

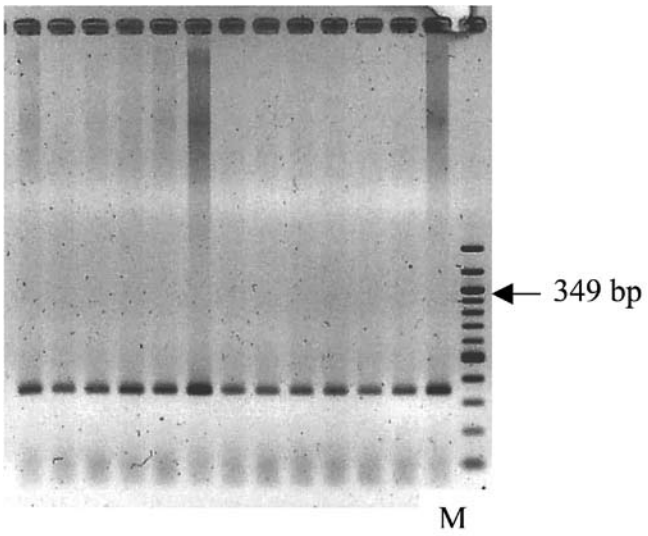

Fig. 1. Ethidium bromide stained PrP gene fragment of goat homozygous 5/5 octapeptide repeats

Legend: M: 100 bp molecular weight marker

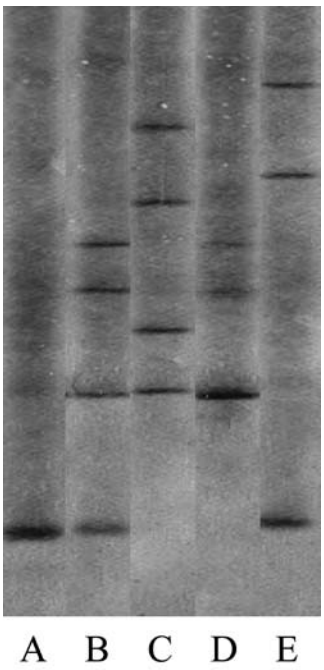

Fig. 2. Denaturing gradient gel electrophoresis of PCR-amplified DNA fragments showing different banding patterns indicating various PrP genotypes, confirmed by DNA sequencing

Legend: DGGE profile: A, ARQ/ARQ; B, ARQ/ARQ + SS_{138}; C, ARQ/ARQ + $SS_{138}PQ_{168}$; D, ARQ/ARQ + $SS_{138}VV_{179}$; E, ARQ/ARQ + $SS_{138}IM_{142}$

Among the 4 remaining clusters DGGE profile B occurred frequently, with an additional dimorphism in codon 138 (ag*c*/ag*t*). The c → t nucleotide substitution had no effect on the amino acid sequence (serine; S) in the PrP protein and is considered silent. This nucleotide substitution was also found in DGGE profiles D and E. The goats with DGGE profile C were homozygous (agt/agt) in codon 138 (Fig. 3). Earlier studies in goats (3, 8) and sheep (15, 16) have also reported a dimorphism in codon 138, however with change in the amino acid sequence (S → N, serine to asparagine). Until now this mutation has not been recorded in goats.

The codon 168 (c*c*a/c*a*a) polymorphism, detected only in 2.6 % (n = 2) of the White shorthaired goats from Farm B, consisted of a c → a nucleotide substitution (DGGE profile C, Figs. 2 and 3) causing change in the amino acid sequence of the PrP protein (P → Q; proline to glutamine). A similar mutation was observed by Billinis *et al.* (3) in goat. In this study, natural goat scrapie was strongly associated with PrP genotype $G_{49}H_{143}H_{154}P_{168}Q_{200}$ and except one, all scrapie cases were homozygous for P_{168}. The goats with DGGE profile C were also different from others because they are homozygous in codon 138 (ag*t*/ag*t*; S).

Goats with DGGE profiles D and E (Figs. 2 and 3) had a dimorphism in codon 138 as well as additional mutations in the codons 179 and 142 respectively. The mutation in codon 179 (gt*g*/gt*t*) (Fig. 3) was a silent one (valine, V). The occurrence of such a mutation has not been recorded before. In this study we found 19 (18.6 %) goats from Farm A and 21 (26.9 %) goats from Farm B with this nucleotide substitution. Amino acid change from isoleucine (I) to methionine (M) was due to a dimorphism in codon 142 (at*a*/at*g*) in goats grouped under DGGE profile E. A similar dimorphism in this codon was recorded by Goldmann *et al.* (8), where they have further described its influence on the scrapie incubation period in experimentally infected goat with BSE prion, sheep scrapie CH 1641 and ME7 strains. Studies have shown that change from isoleucine to methionine in codon 142 (genotype IM_{142}) is associated with the increased incubation period of scrapie in experimentally infected animals (8, 12). In our study we found only 2 (2.0 %) goats from Farm A and 4 (5.1 %) goats from Farm B with this genotype (DGGE profile E, Figs. 2 and 3).

The polymorphism in $A_{136}R_{154}Q_{171}$ codons is linked with the resistance or susceptibility to scrapie in sheep. The ARQ/ARQ genotype belongs to risk group R3, associated with partial susceptibility to scrapie in sheep (www.defra.gov.uk). Billinis *et al.* (3) showed that polymorphisms in the codons 143 (H/R) and 154 (R/H) can protect against natural scrapie in goats. In their study the animals with natural scrapie had genotype HH_{143} or RR_{154}, whereas, animals either heterozygotes (HR_{143}, RH_{154}) or homozygotes (RR_{143}, $HH_{15}4$) were clinically healthy as well as without any histopathological signs of scrapie. Though no polymorphism was found in codon 143 in our study, all the goats were homozygous (HH_{143} or RR_{154}) indicating their possible susceptibility to natural scrapie.

New mutations presented in the PrP gene are silent mutations, which have no effect on the primary structure

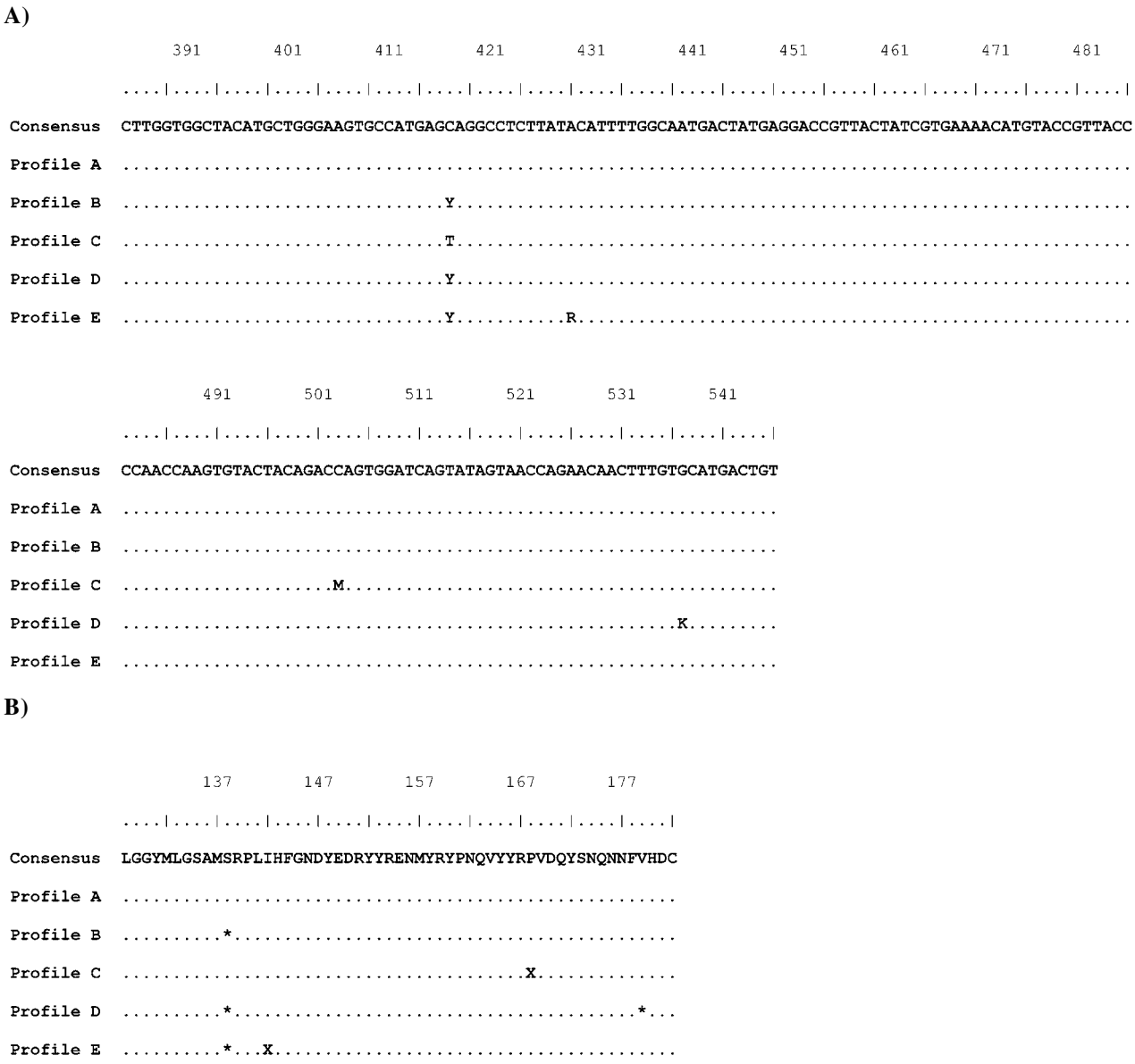

Fig. 3. Nucleotide and amino acid sequence alignment of goat PrP gene

Legend:

A) Nucleotide sequences corresponding to DGGE profiles — A, ARQ/ARQ; B, ARQ/ARQ + SS_{138}; C, ARQ/ARQ + $SS_{138}PQ_{168}$; D, ARQ/ARQ + $SS_{138}VV_{179}$; E, ARQ/ARQ + $SS_{138}IM_{142}$. The sequences are complementary to the cds part of the goat PrP gene X74758. Symbols: Y(T/C), R(G/A), M(A/C), K(G/T)

B) Amino acid sequences corresponding to DGGE profiles — A, ARQ/ARQ; B, ARQ/ARQ + SS_{138}; C, ARQ/ARQ + $SS_{138}PQ_{168}$; D, ARQ/ARQ + $SS_{138}VV_{179}$; E, ARQ/ARQ + $SS_{138}IM_{142}$. Symbols: x — mutation in amino acid; * — silent mutation

of PrP proteins, indicating no association with change in resistance or susceptibility to natural scrapie.

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CHANGES IN LIPID METABOLISM AFTER SINGLE GAMMA IRRADIATION IN CHICKS

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ABSTRACT

The present study investigated changes in the concentration of cholesterol (CHOL) and lipoperoxides (LPP) in poultry serum after single, whole-body irradiation with doses of 4.5 Gy and 15.0 Gy. The changes were observed in individual chickens at the age of 21 days and at the beginning of the experiment. The chickens irradiated with the dose of 4.5 Gy showed a significant decrease in serum cholesterol level on days 1 and 30 and an increase in the concentration of lipoperoxides in all periods investigated, significantly on days 14 and 30 after irradiation. The chickens irradiated with 15 Gy had a significantly increased lipoperoxide level one day after irradiation. A decrease in lipoperoxide level was observed in all other time periods of investigation, significantly on day 7 after irradiation. The results obtained indicate that single gamma irradiation could result in the development of free radicals.

Key words: chickens; cholesterol; gamma irradiation; lipoperoxides

INTRODUCTION

Exposure to ionizing radiation can be associated with various areas of human activities. A not negligible risk to the human population may be related to the use of artificial sources of irradiation connected particularly with diagnostic and therapeutic purposes. Also the utilisation of radioactive substances as energy sources increases the risk of contamination of the environment with radioactive isotopes. Investigation of the effect of this type of environmental pollution (6) is an important task of the present period.

The aim of the study was to observe the changes in lipid metabolism in the serum of domestic fowl at different time periods after exposure to ionising gamma radiation. Individual chickens were subjected to single, whole-body irradiation with doses of 4.5 and 15.0 Gy.

MATERIAL AND METHODS

Animals. The experiment was carried out on irradiated broiler chicks (HYBRO G breed), 21 days old at beginning of the experiment. The chickens (n = 100) were divided in three groups and were kept in previously disinfected experimental facilities from the first day after hatching (7). Water and feed was supplied to the animals *ad libitum*. They were fed a granulated mixed feed BR I and BR II intended for broiler chickens. Irradiation was carried out at the Faculty of Natural Science UPJŠ in Košice using an apparatus CHISOSTAT ^{60}Co-CHIRANA (CzR). The birds were irradiated in adjusted plexit cages.

Experimental procedure. The first experimental group of chickens (n = 20) was subjected to a single, whole-body irradiation with a dose of 4.5 Gy at the output of 0.295 Gy. min^{-1}. Blood samples were taken and analysed on days 1, 5, 14 and 30 after the irradiation. The second experimental group of chickens (n = 40) was irradiated in the same way as the first one but a single, whole-body irradiation was carried out with a dose of 15.0 Gy. Analyses were carried out on days 1, 3, 5 and 7 after irradiation. Control broilers (n = 40) were exposed to sham irradiation, i.e. were subjected to all procedures and manipulations as the irradiated groups except for gamma irradiation itself.

The chickens were killed by decapitation at 10:00 a.m. (2, 3). The mixed blood was collected in Petri dishes placed

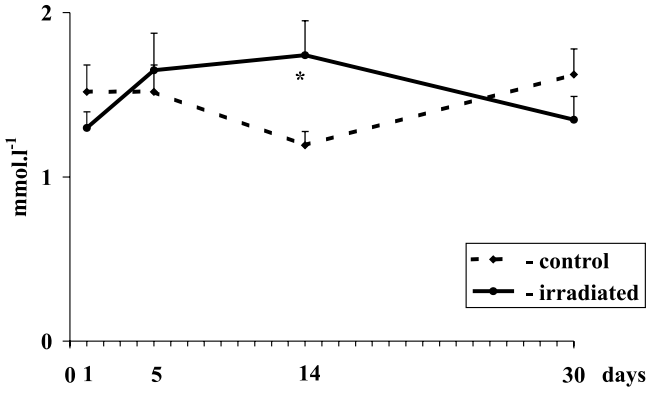

Fig. 1. Changes in the level of serum cholesterol of the first experimental group after single, whole-body irradiation of chicks, with a dose of 4.5 Gy. The values are given as means ± SEM. * — P < 0.05

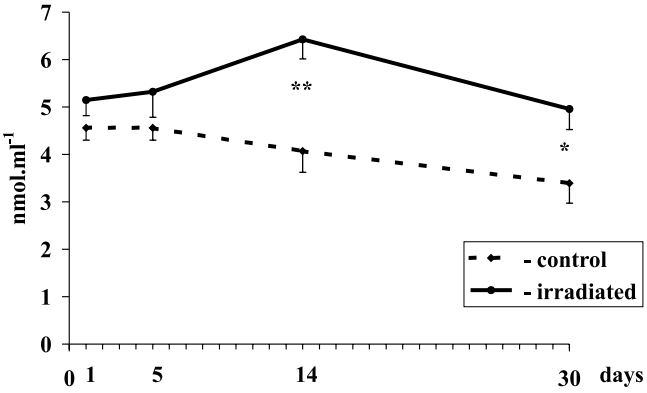

Fig. 2. Changes in the level of serum lipoperoxides of the first experimental group after single, whole-body irradiation of chicks, with a dose of 4.5 Gy. The values are given as means ± SEM. * — P < 0.05, ** — P < 0.01

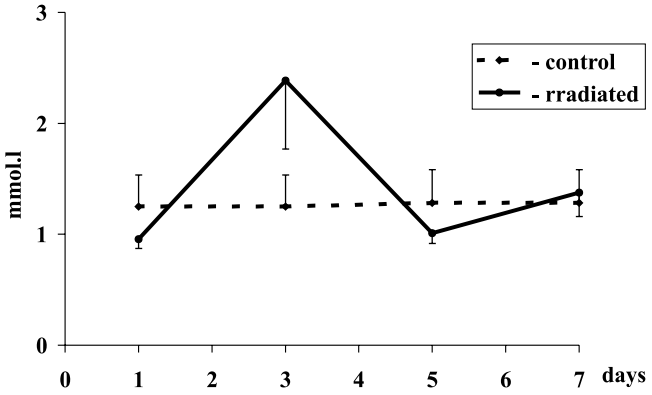

Fig. 3. Changes in the level of serum cholesterol of the second experimental group after single, whole-body irradiation of chicks, with a dose of 15.0 Gy. The non-significant values are given as means ± SEM

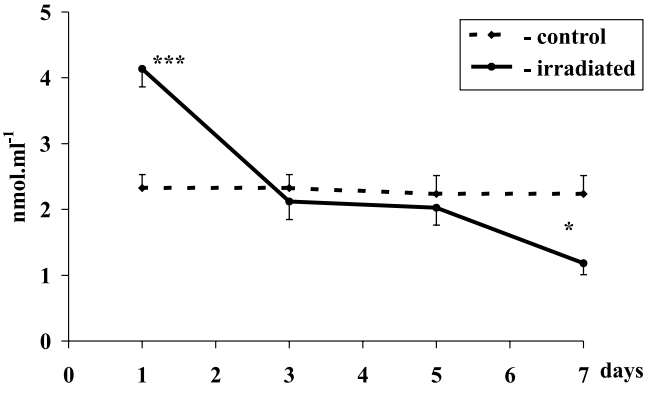

Fig. 4. Changes in the level of serum lipoperoxides of the second experimental group after single, whole-body irradiation of chicks, with the dose of 15.0 Gy. The values are given as means ± SEM. * — P < 0.05, *** P — < 0.001

on ice, centrifuged, and the separated serum was used for analyses. The experiment was carried out in summer.

Serum cholesterol levels were determined by the Bio-La test (Lachema Brno, CzR) and concentration of lipoperoxides was determined spectrophotometrically (8) using a spectrophotometer Spekol 11 (Carl Zeiss Jena, Germany).

The results obtained were processed statistically by an unpaired Student *t*-test (Prism 3.0, Software) comparing the irradiated and control groups. Individual groups consisted of between five and ten animals.

RESULTS AND DISCUSSION

In chickens irradiated with the dose of 4.5 Gy no clinical signs of irradiation disease were observed. In the second experimental group the shorter period (7 days) was used whereas $LD_{50/30}$ for chick is approximately 9—12 Gy. In our experiment the dose of 15 Gy was applied. In chickens exposed to this dose diarrhoea, joint oedema, haemorhage on legs, anaemia (10), apathy and beak discharge were observed. In necropsy 50 % reduction of Bursa Fabricii was also present in comparison with the control. During the monitored period 30% death loss was recorded in the experimental animals. Death was observed from the third day of the experiment, then no death was recorded until the seventh day of the experiment.

In animals exposed to the dose of 4.5 Gy lower cholesterol (Fig. 1) concentrations were recorded compared to controls. Later, on day 5 higher cholesterol concentrations were observed. The statistically significant increase in these concentrations was recorded on Day 14 ($P < 0.05$). In the follow-up monitored period a decrease in cholesterol concentration occurred and on day 30 cholesterol concentration was not significantly different to values measured in the control group.

Serum lipoperoxide (Fig. 2) concentration increased gradually until day 14 of the experiment when a significant increase was observed ($P < 0.01$) in comparison with the control. After this period lipoperoxide concentrations decreased, however the measured values were

still significantly higher than those in the control group ($P < 0.05$).

On day 1, in a chicken exposed to the dose of 15.0 Gy a non-significant decrease in serum cholesterol (Fig. 3) concentrations was recorded compared to the control. On day 3 a severe, non-significant increase in cholesterol concentrations was observed. On day 5, a non-significant decrease of cholesterol concentration occurred. On day 7 after irradiation cholesterol concentrations were mildly increased in comparison with the control group.

On day 1 after irradiation a significant increase ($P < 0.001$) in serum lipoperoxide (Fig. 4) concentrations was observed. Later, on day 3 and 5 the values were not different from the control. On day 7 a significant decrease in lipoperoxide concentration was recorded in comparison with the control.

The first symptoms of acute irradiation disease include inappetence, vomiting and diarrhoea, i.e. symptoms related to the gastrointestinal tract, termed intestinal syndrome, which is explained by the considerable sensitivity of the digestive tract, particularly the small intestine, to ionising radiation. It has been assumed that, besides direct damage to the gastrointestinal tract, ionizing radiation affects also nervous and humoral regulation (11). The development of post-irradiation anorexia does not necessarily require irradiation of the abdominal region but irradiation of limbs is sufficient.

With regard to the fact that in our experimental model the metabolic changes induced by irradiation could overlap with the changes related to decreased intake of feed we decided to solve this problem by overnight fasting of irradiated and control groups before taking samples for analysis.

Investigation of the effect of lower doses of ionising radiation on live organisms is an important issue of radiobiology. The effect of the levels of cholesterol have been described by Ahlers *et al.* (1). In their study rats were irradiated in an experimental field for 100 days with a daily dose of 0.024 Gy. A group of rats was analysed immediately after continuous irradiation while another group was analysed 100 days later. Long-term continuous irradiation with low daily doses caused an increase in blood glucose, serum cholesterol and phospholipides and liver cholesterol. Most of these changes receded during the period of expected recovery.

Ionizing radiation may induce intensification of lipolysis and lipomobilisation resulting in an increased supply of non-esterified fatty acids to various tissues. Concentration and production of total cholesterol and triacylglycerols in the liver increases (5). Hyperlipaemia, which predominates in the serum, can be referred to as retentive because it seems to be based on the uptake of circulating lipids by tissues due to insufficient action of lipoprotein lipase (11).

Production of free radicals is not a unique property of ionizing radiation, its unique character is related more to its ability to form these free radicals inside the cells. If the diffusion is not prevented, these free radicals react very rapidly with other radicals or molecules which harbour sensitive reactive groups. These oxido-reductive processes may result in serious structural changes in macromolecules. The resulting degree of radiation damage to cells depends on the effectiveness and rapidity of reparative processes in the body and on other biological factors (4, 9).

The chickens investigated showed an increase in cholesterol level in the later period, more intensive in birds exposed to higher doses of radiation (15 Gy). Concentration of LPP in the serum after irradiation with a dose of 15 Gy increased rapidly already on Day 1 after irradiation and then showed a rapid decrease, chickens irradiated with 4.5 Gy exhibited significant increase on Days 14 and 30.

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A COMPARISON OF TWO SUTURE MATERIALS IN THE CLOSURE OF A PYLORIC INCISION IN DOGS (A SHORT COMMUNICATION)

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INTRODUCTION

Many different types of suture materials are currently available. Suture selection has too often been guided by training, experience and preference of the surgeon rather than the knowledge of the surgeon, the physical and biological properties of the suture materials and the healing rates of wounds in various tissues (1, 3).

Surgical guts, synthetic absorbable sutures and monofilament non-absorbable sutures have been reported as ideal for the closure of hollow viscus (1, 3, 4). Although surgical guts are widely available and inexpensive, the variability in loss of tensile strength and the inflammatory reaction it induces are some of the disadvantages in its use (4, 5). Synthetic absorbable sutures which offer an advantage over surgical guts are not widely available in Nigeria and are more expensive.

Incisional dehiscence and leakage are the most common complications associated with a gastric outlet corrective procedure (6, 7). This often results from technical difficulties such as poorly placed sutures, excessive tension on the suture or inappropriate suture material (7, 9). Until now, closure of the incision into the stomach has been effected with the use of surgical gut or synthetic absorbable sutures. Since nylon monofilament is cheaper than the synthetic absorbable, and appears to have some advantage over surgical guts, it is therefore important to study if a nylon suture offers an advantage over surgical gut in the closure of a pyloric incision. This study was designed to compare the effect of either chromic catgut and nylon monofilament on the healing of a pyloroplasty incision, the nature of cellular reaction to each suture and the consequences of the cellular reaction on the animals.

Ten juvenile local dogs aged between six and nine months and comprising six intact male and four non-pregnant, non-lactating bitches with a mean body weight of 5.5 ± 1.2 kg were used. Approval for the study was obtained from the Faculty Ethical Committee on the use of animals for research. The dogs were randomly divided into two groups of five dogs each. Group A had their pyloric incision closed with a 2-0 chromic catgut (Lifetex®, Agary Pharmaceuticals), while the Group B dogs had their pyloric incision closed with 2-0 Nylon monofilament (Agary Pharmaceuticals).

The dogs were premedicated with intramuscular injections of xylazine hydrochloride (Xylaxin®, India Immunologicals) and Atropine (Non-proprietary) at the dose rate, of 1.0 mg. kg^{-1} and 0.02 mg.kg^{-1} respectively. Anaesthesia was induced and maintained using 6 % sodium pentobarbitone (Sagatal®, Kyron Laboratories) at the dose rate of 10 mg.kg^{-1} body weight. Laparatomy was carried out as described by (2). After locating the stomach and identifying the pylorus, pyloroplasty was performed using the Heineke-Mikulicz technique (6). The pyloric incision was sutured with either 2-0 chromic catgut (Group A) or 2-0 nylon monofilament (Group B). The abdominal incision was then closed routinely (6, 7).

In the course of the experiment, 5 ml of blood was obtained from the jugular vein into a sample bottle containing EDTA solution for complete blood counts. Blood samples were obtained one hour before surgery and then on days 1, 5, 7 and 14 post-surgery. Also, the dogs were observed daily for changes in appetite, faecal consistency and to see if vomiting and regurgitation occurred. In addition, the dogs were euthanized three weeks after surgery using Euthatal for necropsy changes and harvesting of pyloric tissue for histopathologic examination.

Table 1. Cellular reaction to catgut and nylon three weeks after the closure of a pyloroplasty incision in dogs

CATGUT GROUP		NYLON GROUP	
ANIMAL IDENTITY	**HISTOPATHOLOGY**	**ANIMAL IDENTITY**	**HISTOPATHOLOGY**
D1	Minimal cellular reaction	D6	Minimal cellular reaction
D2	Localized area of early granulation. *Lamina propria* showed increased cellularity, mostly lymphocytes and plasma cells. Cellular infiltration of the submucosa with neutrophils, lymphocytes and macrophages. Diffuse serosal haemorrhages	D7	Minimal cellular reaction and subserosal adipose tissue layer
D3	Minimal cellular reaction	D8	Minimal cellular reaction
D4	Minimal cellular reaction	D9	Minimal cellular reaction
D5	Minimal cellular reaction	D10	Minimal cellular reaction

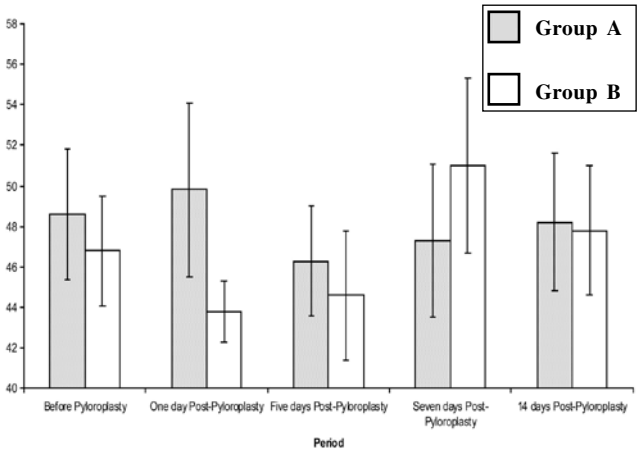

Fig. 1. Changes in Packed cell volume (PCV) following closure of pyloroplasty incision with either of chronic catgut or nylon monofilament in dogs

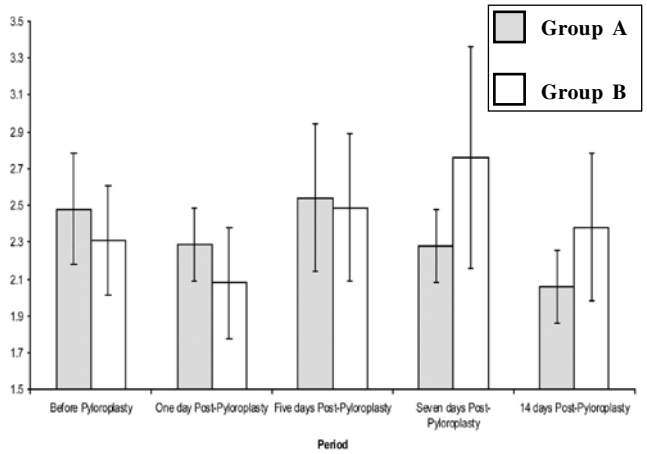

Fig. 2. Changes in Platelets count following closure of pyloroplasty incision with either of chronic catgut or nylon monofilament in dogs

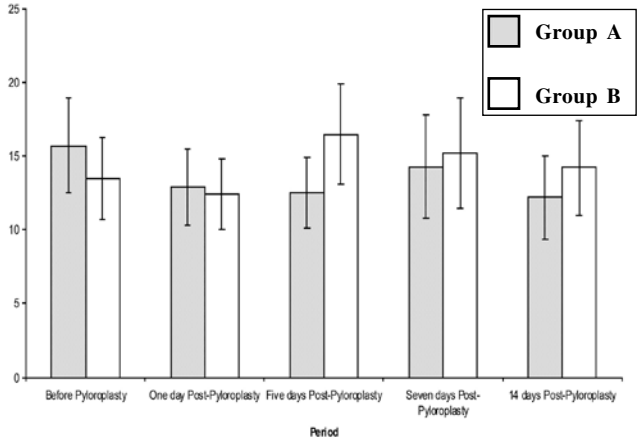

Fig. 3. Changes in Total white cell counts following closure of pyloroplasty incision with either of chronic catgut or nylon monofilament in dogs

The result of this study showed that closure of the pyloric incision in dogs with either catgut or nylon monofilament did not produce any complication associated with gastric outflow obstruction. Necropsy findings and histopathology examination revealed that all dogs had smooth mucosa and serosal surface with minimal tissue reaction except for a dog in group A that had moderate serosal adhesion of the pylorus to the caudal lobe of the liver (Table 1).

Both the packed cell volume (PCV) and platelet counts did not differ significantly ($P > 0.05$) between the two groups (Figs. 1 and 2). The total white blood cell counts (WBC) was significantly higher ($P < 0.05$) on day five post surgery in group A dogs (Fig. 3).

The result of this study showed that healing of the pyloric incision closed with either chromic catgut or nylon monofilament did not differ significantly. Also both suture materials did not significantly affect the PCV and platelet counts of the

dogs suggesting that they have no effect on the erythrocytic effects.

The rise in the WBC counts of the group B dogs on day five post surgery may suggest that nylon tends to activate immune competent cells more than chromic catgut. Soluble factors forming suture materials can interact with immune competent cells to produce an inflammatory response (8).

In general there appear to be no significant differences in the histopathology and erythrocytic value of dogs sutured with either chromic catgut or nylon monofilament. In our opinion the knot characteristics of the two suture materials are similar. Considering the high tensile strength of nylon monofilament it is thus recommended that nylon monofilament can be preferred to chromic catgut in the closure of pyloric incision.

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RECENT PROGRESS IN THE ENDOCRINE, NUTRITIONAL AND GENETIC ASPECTS OF OVINE REPRODUCTION (A REVIEW)

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SUMMARY

Recently, the updated techniques of molecular biology and endocrinology as well as visual display techniques have improved our knowledge on ovarian function in ewes. The authors summarize the physiology and genetic aspects of cyclic ovarian function, its seasonal characteristics, the influence of nutrition, and the opportunities for the induction and synchronization of oestrous cycle as well as for increasing the fertility of this species.

Key words: endocrine system; ewes; genetics; nutrition; reproduction

INTRODUCTION

Nowadays sheep breeding is a branch of animal husbandry where a lot of traditions exist with the simultaneous application of the most advanced technologies. Over the past three decades a wide range of endocrine methods, visual display techniques (ultrasonography and others) and technologies of molecular biology have caused revolutionary changes in our related knowledge.

The aim of this paper is to review recent progress in the endocrine, nutritional and genetic aspects of ovine reproduction, as well as to discuss the applicability of this knowledge in research and management of flock-level fertility in the forthcoming period.

THE OVARIAN FUNCTION

Development and endocrine function of follicles

The ewe is a seasonally polyestrous animal, following a seasonal pattern, i.e. alternating periods of anoestrous and sexual activity. According to current knowledge (18, 19, 32, 61, 64, 82) in the beginning, only intra-ovarially located growth factors (TGF, EGF, IGF-1) acting exclusively in a paracrine way, regulate the differentiation of primary follicles to small

antral follicles, which takes about 90—120 days in sheep. As soon as the follicles have reached a diameter of about two millimetres, which are histologically already tertial (antral) follicles, they become sensitive to gonadotropes: their further development – not more than five to seven days – is wavelike, basically induced by a follicle stimulating hormone (FSH), and regulated by a luteinizing hormone (LH).

On the basis of the wave-like growth of the follicles, dominant follicles (DF) develop with the ability to synthesize a gestagen-like and androgenic steroid precursor in the external (*theca interna*) cells with direct vascular connection, as well as for oestrogen (17ß-estradiol: E2) and inhibin production in the internal cell layers (granulose cells). LH-receptors appear on the granulose cells of the approximately 3.5 mm diameter follicles, respectively. The size of preovulatory follicles is about six millimetres. The subordinated follicles recruited at the same time as the DF, loose their endocrine activity and degenerate (*atresia*) without ovulation, in a similar manner to those DF-s selected during the luteal phase in cyclic individuals, or out of the breeding season during the seasonal absence of the cycle. The endocrine mechanism regulating the final maturation is similar to that observed in cattle (18, 32, 35, 37, 82).

The pulse frequency of the pulsatile-oscillating basal LH secretion is of profound importance in sheep as well: one pulse each 45—75 minutes is the predominant endocrine factor regulating the final maturation of DF. The regulatory role of LH pulsatility is completed by the intrafollicular effect of an insulin-like growth factor-1 (IGF-1) as well as by the complex enzyme-related cascade cleaving the free bioactive form of IGF-1 from its binding proteins (IGFBP) in the follicular fluid. This local mechanism plays a crucial role in the selection of DF, divergent from the development of subordinated follicles from the same follicular cohort. Free bioactive IGF-1 is the main activator of the aromatase enzyme system, which determines the ability of granulose cells to produce E2, although the insulin, 3,5,3'-triiodothyronin (T3) and leptin content of the follicular fluid may also contribute to this mechanism (32, 82, 87).

In sheep, the number of the follicular waves is generally three or four per cycle, but it has been reported to vary between five and two. As examination methods, e.g. endoscope, ultrasound etc. are fairly difficult to perform in field practice, those statements are based on limited investigations and therefore our current knowledge on the exact number of growth waves is not very reliable. In the case of four follicular waves within one cycle, the between-ovulation interval (cycle length) is approximately sixteen days; whereas, depending on the follicular waves' number, the time between two oestruses may differ between 12–13 and 19–21 days (1, 5, 7, 22, 24, 64).

Seasonal differences in ovarian function

In most of the sheep breeds we can count through ovulation and subsequent formation of *corpus luteum* (CL) secreting progesterone (P4) (*cyclic ovarian function*) at times when the day length shortens, i.e. at the end of the summer, in autumn and at the beginning of the winter (*breeding season*) (29, 80, 82, 88). Although its turnover is probably slow, in most of the modern sheep breeds the wave-like pattern of gonadotroph-dependent follicular growth and maturation exists also out of the breeding season, resulting in the regular formation of E2-producing DF-s also during the *anovulatory* (syn. *acyclic*; sometimes also called, but not fully correct: anoestrous) period. At that time, however, certain neurons of the hypothalamus (HTh) located in the ventrolateral wall of the third ventricle (*surge centre*), which are responsible for the release of large amounts of GnRH triggering the preovulatory-like LH peak, lose their E2 sensitivity. Due to this lack of hypothalamic E2 sensitivity the effect of E2 synthesized by the DF is not powerful enough to stimulate the preovulatory-like GnRH/LH release. Therefore no ovulation takes place and the DF becomes atretic.

Using other terms, in sheep the physiological prerequisite of ovulation and cyclic ovarian function is the ability of the hypothalamic surge centre to react to the E2 production of DF-s with a massive GnRH/LH release (e.g. positive feedback), and this E2 sensitivity is restricted only on the breeding season. In the last two to four weeks of the acyclic period (*transition period*) the gonadotroph-sensitive phase of the follicular development and maturation is more rapid, the follicular turnover is almost the same as within the breeding season. However, the E2-induced GnRH/LH responsiveness resulting in ovulation is still missing (2, 29, 80, 82, 88, 95). Also the E2 sensitivity of hypothalamic regions responsible for oestrous behaviour is reduced out of the breeding season. In sheep and other ruminants this E2 sensitivity is not yet been fully restored at the time of the first ovulation (29, 87): a several-day P4 rise produced by the first CL is needed for the complete return of receptivity resulting in these oestrous signs is not strongly connected to the first ovulation at the end of acyclic periods. So in sheep and goats the resumption of cyclic ovarian activity can only be demonstrated by the CL-related increase in P4 levels following the first ovulation (63, 73, 82, 87).

The ovarian function of animals not conceived by the end of the breeding season becomes acyclic again. At the end of the breeding season the thyroid hormones have a crucial role in the cessation of cyclic processes in small ruminants (93).

The change between the length of light and dark hours (photoperiod), resulting in an effect on the ovaries, follows a pathway beginning with the eyes, the optical nerve *via* the body's "biological clock" (the suprachiasmatic nuclei of the hypothalamus, HTh) and the pineal gland secreting melatonin, known as the main signalling hormone. Both melatonin production and secretion in the pineal gland and the level of melatonin pattern in the peripheral blood follows the diminution of light intensity: with the reduction of light hours the part of the day characterized with elevated melatonin levels lengthens continuously, which enhances the E2 sensitivity of GnRH-synthesizing surge centre in the HTh and also the release of gonadotroph hormones (LH and, in smaller amounts, also FSH) in the anterior pituitary gland in small ruminants.

Some details of these mechanisms mediated by dopamin, serotonin, gamma-aminobutyric acid (GABA), ß-endorfins and several other neurotransmitters (neuropeptid Y, proopiomelanocortin) are as yet still unknown (13, 47, 49, 50, 51, 52, 53, 54, 71, 81, 87, 91, 92, 98). Besides this, according to some opinions, melatonin may also act directly on the ovaries (7).

In connection with the variations in seasonality Notter and Chemineau (71) have detected differences in mela-

tonin plasma levels at night-time in certain crossbreed lines (50 % Dorset, 25 % Rambouillet, 25 % Finnish Landrace): in August the females selected for fertilization in autumn showed a significantly lower melatonin plasma levels in the evening than those which were not selected on the basis of seasonality (the above mentioned quality was significantly hereditary: $h^2 = 0.43$; $P < 0.02$). The same study showed also differences in prolactin levels during the night whereas the inheritance was not definitely shown.

The effect of melatonin is strongly connected to its specific receptors. To date, three different subtypes of melatonin receptors have now found in mammals: Mel_{1a} or MT1, Mel_{1b} or MT2 and MT3 were cloned and described. Amongst those, at this time, only the distinct significance of Mel_{1a} has been established (63). There are different sheep breeds where a polymorphism (punctual mutation) within the Mel_{1a}-gene has been found, but the exact relationship to seasonal cyclic activity is not yet clear in every detail (72, 73, 74, 97). The connection is not likely to be direct, although there are no differences in the amino acid sequences in the mentioned parts of punctual mutations within the receptors. The idea of using Mel_{1a} polymorphism as a genetic marker of ability for year-round ovarian cyclic processes was present from the beginning. But when comparing animals with different genotypes of Mel_{1a} the results were not always uniform. In a two-year French experiment with Île-de-France ewes, Hernandez *et al.* (31) failed to observe any clear connection of the Mel_{1a} genotype with the seasonal differences in daily prolactin and melatonin patterns in cyclic ovarian activity or in the growth of the animals' fleeces.

Taking into account the results of various studies, including those with contradictory findings, currently we have assumed that the Mel_{1a} receptor gene polymorphism may be a useful indicator of year-round ovarian cyclic processes in some sheep breeds. However, the real manifestation of this genetic ability requires proper environmental (mainly feeding) conditions including optimal body fat content (condition score; further details are given later). The laboratory facilities for the determination of Mel_{1a}-receptor polymorphism are available in Hungary. Within the framework of this existing cooperation the institutional background of related studies could be easily established for Slovakian researchers, too. Up to now, however, due to the lack of financial support we have not had experiences yet with diagnostic value or the practical use of this method in Slovakia.

In sheep there may be great breed-related differences in the length of the breeding seasons and the time of the first and last ovulations. Within breeds also family and/or flock-dependent variability may be obvious sometimes (18, 69, 70, 77, 80, 95). We can take into account that the length of the ovarian cyclicity is connected with the degree of domestication of various breeds as well as with geographic-climatic conditions of the area where a breed was domesticated. In breeds of temperate and cool continental climatic zones, such as the steppe-originating Merino (Rambouillet, Dorset), as well as the Finnish Landrace and the Russian Romanov breeds, the first ovulation takes place already in mid or late August, and the ovarian function of non-pregnant animals remains cyclic until the end of February, beginning of March. In some British breeds (Galway, Suffolk) the breeding season starts quiet late in mid September, but can last till the end of March or early April.

In contrast, in the Scottish Blackface ovulation is possible only from mid October to late February. In May and June even the regular waves of follicular growth cease in this breed. The Awassi represents the fat tailed breeds of the subtropical arid zone in the Middle East. The first ovulation of its breeding season is usually seen from late August to mid September in Israeli flocks but the onset of the ovarian cycle may vary within a wide range both in its home-land population and the flocks exported to the USA. The real seasonal characteristics of Awassi ewes kept under the temperate continental climate of Central Eastern Europe are still unknown. The Racka is one of the ancient breeds of the Carpathian basin existing in white, black and brownish (Gyimesi) variants.

According to our earlier experiences based on P4 determination in three blood samples of 50—50 individuals taken seven days apart almost all the well-fed ewes of these three genetic variants already had cyclic ovarian activity in late August, and those animals not conceived meanwhile remained cyclic till the end of January (6). Mouflon, one of the ancestors of domesticated sheep, shows cyclic ovarian function only between October and early to mid January, and after early March ovulation cannot be induced in them with gestagen + eCG (equine chorionic gonadotropin; syn. pregnant mare serum gonadotrophin, PMSG) administration, either (45, 46, 75).

The great variations in seasonality of cyclic ovarian function in different breeds are probably related to Mel_{1a} polymorphism. Up to now, however, this supposed connection between some Mel_{1a} gene alleles and the ability of breeds for year-round ovulation has been confirmed by studies mainly on older animals (73). The triglyceride content of subcutaneous and visceral lipid depots usually increases simultaneously with the ageing of the animals. So it may be possible that although the relationship with the age of animals is obvious the explanation may lie in the fact that body condition also improves with age (8, 12, 26). However, this hypothesis needs further improvement in studies on wider range of species and farming systems with particular attention to the role of the lipid depot localized in the hump-like fat tail of certain breeds. On the other hand, according to our current knowledge there is no confirmed connection between the Mel_{1a} polymorphism and seasonality in goats (63).

Nutritional aspects in seasonality of ovarian function

Beside the breed and genotype the nutrition of animals and in this respect their body condition is of great importance as it determines the time of the first ovulation and therefore the beginning of the breeding season, respectively the length of the anovulatory/acyclic period. To achieve that the surge centre of the HTh restores its sensitivity to E2, and so its ability to trigger the first preovulatory LH peak and ovulation, the triglyceride content of subcutaneous and visceral lipid depots (e.g. the body condition of the ewe) has to be above a certain threshold. This permissive form of regulation is supposed to interact with the endocrine consequences of changes in daylight furthermore with breed-related (and perhaps with other

unspecified genetic) factors (2, 17, 20, 32, 60, 79, 82). Breeds from the Northern and Western European areas need generally a larger depot of fat than breeds originating from the steppe or the arid subtropical zones (79).

Weak animals and especially long term deterioration of body condition causes a delay of six to eight weeks and strongly affects the time of the first ovulation within a flock. In very severe cases ovarian function may remain acyclic throughout the whole year. However, European husbandry practice and feeding does not create much likelihood of that (79). By optimising the energy intake of medium to poor conditioned animals two to three weeks before the beginning of the breeding season (*"flushing"*) first ovulation will take place earlier and the deviation within the flock will be decreased (17, 20, 32, 60, 79, 82). Compared to the significance of the energy intake on inducing ovulation the effect of the protein supply is only of secondary importance (79, 85, 86). The energy balance of an animal has a strong influence on glucose maintenance and/or glucose metabolism of the GnRH synthesizing neurons; furthermore, it works *via* the intrafollicular availability of IGF-1, insulin, leptin and thyroid hormones (2, 32, 36, 60, 68)

The *"ram effect"*

The first ovulation marking the beginning of the breeding season can be triggered if an intact or vasectomized (teaser) ram is present (*"ram effect"*). The stimulating effect of the ram results from certain species- and genus-specific odorous substances called pheromones. In some breeds (Merino, Rambouillet, Dorset, Finnish Landrace, Romanov) sometimes the pheromone effect induces ovulation out of the breeding season only through chemosensory cues (23, 41, 60, 73, 78).

Ovarian activity in the postpartum period

As seen in the bovine (33, 34, 35, 36, 38), the first postpartum (**pp**) follicular growth in sheep occurs very early after lambing and is followed by a regular wave-like pattern of follicular growth regardless of the season of lambing, the plane of nutrition and the presence or absence of suckling (82, 89, 90). However, the time of the first ovulation is determined by the season and the presence of a nursing lamb (5, 32, 55, 94) and also the nutritional influence may be of practical importance (20, 79).

The individuals conceive in the autumn's breeding period lamb in the end of the winter – beginning of spring. This lambing is followed by an approximately three-month long lactation during which the ewes usually suckle their offspring and are weaned only at the end of this period. Despite the relatively rapid onset of the wave-like follicular growth pattern in spring-lambing ewes the first pp ovulation usually occurs only in the subsequent breeding season (at the end of August, beginning of September) because of the consequences of long-lasting suckling and intensive dam-offspring bound combined with the effect of seasonality (5, 32, 55). However, depending on the breed and nutritional status of animals there are flocks where a variable number of spring-lambing ewes may ovulate 60—70 days after lambing (82). Reproductive technologies based on autumn breeding – spring lambing are robust, ewes can also easily tolerate certain degree of a temporary feed restriction in a dry summer (20, 79). However, this management system is not really cost effective when intensive dairy flocks are considered.

In those ewes, which become pregnant out of the breeding season (after oestrus induction) and lamb in the autumn period in case of nursing, the onset of the ovarian cycle is similar to that in suckling beef cows: the first ovulation may take place 35—45 days after lambing. So their re-conception is possible at the end of the same autumn period (5, 32, 55, 82, 94). Any forms of the improper energy supply of dams, however, can significantly delay the resumption of the cycle process. In a more severe case the ewes can ovulate only in the late August, early September of the next year (20, 79) which results in significant financial losses.

In intensive dairy flocks the year-round milk production provides advantages in the market and the continuous use of the expensive milking machinery is one of the major prerequisites of the financial success. These arguments justify lambing periods twice a year both in the late winter – early spring and also in the autumn season. So the common effect of milk production and seasonality at the time of the first postpartum ovulation can be of extreme importance under these conditions.

According to our recent experiences in our intensive milk producing Awassi population (25) all these principles in the pp resumption of cyclic ovarian function can serve only as guidelines. In intensive dairy flocks newborn lambs are weaned immediately or after the colostral period and fed with milk replacers thereafter. Therefore the consequences of suckling and the continuous dam-offspring bound are not present allowing the very rapid resumption of ovarian cyclic process above all in the autumn-lambing ewes. This phenomenon might provide a chance for prostaglandin F2a ($PGF_{2\alpha}$) based synchronization techniques in management of reproduction. At the same time, however, these ewes may ovulate before the completion of pp uterine involution that may also be a risk factor.

As seen in dairy cows (83) the early pp formation of the first CL and the related increase in plasma P4 may predispose the animals to uterine bacterial complications (mucopurulent-purulent forms of endometritis, perhaps pyometra) (48). On the other hand in Awassi ewes the daily milk yield as well as the fat and protein content of the milk may be very high (>2.5 kg.day^{-1}, >7.0% and >6.0%, respectively) and lactation is longer than the 180—200 days, which is completely unusual in other sheep breeds. The metabolic consequences of high milk production and the relatively stressful conditions of dairy units have been reported to depress the reproductive performance of dairy cows (12, 33, 34, 35, 36, 37, 38).

Consequently, these extremes in the milk production of dairy ewes and their interaction with other management-specific factors (two lambing seasons per year, lambing in maternity barn, early weaning, and so on) are supposed to (i) influence the onset of the cycle process and the course of uterine involution in the early weeks of lactation, (ii) interfere with the efficacy of oestrous induction/synchronization techniques administered during the last weeks of lactation, and/or (iii) depress the fertility of late-lactating animals. However, further studies are required to reveal and understand the real metabolic and reproductive characteristics of dairy ewes.

FERTILITY (OVULATION RATE, TWIN PREGNANCY, EMBRYONIC AND/OR EARLY FOETAL MORTALITY)

In ewes the twinning rate (*fertility*) is an important indicator of reproductive performance based on a breed-related genetic ability for double (or multiple) ovulation and is under the complex influence of nutritional factors, body fat content and climatic conditions (14, 20, 79, 82). It has major importance in meat-producing breeds.

The disposition towards double or multiple ovulations is an important genetically determined breed characteristic that can be easily examined with DNA-based techniques of molecular biology nowadays. In breeds belonging to the Merino group the most important genetic factor of fertility is the dominantly inherited, autosomal Booroola (*FecB*) gene, which has an additive effect on ovulation rate (OR): One copy of this gene increases the OR by about 1.5 and two copies by about 3.0 (14, 65, 66). Other genes that increase the OR are restricted to an occurrence only in certain breeds or populations, (the *FecX2* gene in Coopworth ewes in New Zealand; the Lacaune gene in the French Lacaune meat sheep population) and/or causes sterility in homozygous carrier females (the *FecX* gene in Romney sheep; the $FecG^H$ gene in Cambridge and Belclare breeds). So, compared to that of the *FecB* gene, their practical importance is limited in Central Eastern Europe (14).

In flocks the proportion of polyovulation depends also on the nutritional state and in a smaller percent on the current energy balance of animals. With the method of *"flushing"* the OR can be elevated by 20 % (17, 20, 32, 60, 79, 84, 96). Following lupin (*Lupinus albus*) seed feeding ovulation rate increases at a higher percent than with the use of other isocaloric/isonitrogen food supplement. The advantages of lupin seed feeding are evident already four to six days later. This effect is multifactorial: beside the stimulation of the glucose metabolism in the GnRH-producing neurons, it gradually activates aromatization in the gonadotroph-sensitive granulose cells of the antral follicles and increases intrafollicular IGF-1 concentration as well as its biological availability (32, 60, 67, 68). According to the facts above mentioned, flushing technology with lupin seed may be suitable to bring forward the first ovulation at the beginning of breeding season and to increase the OR.

Another possibility is to trigger multiple ovulations with pharmacological methods, e.g. administration of eCG with FSH-like activity (3, 7, 57, 59, 62, 76, 82) or as a more recent alternative, with immunisation against either *estrogen precursors* produced by the *theca interna* cells of the follicles or *inhibin* (76, 82, 96). After eCG treatment the OR depends, amongst others, on pre-treatment with gestagen (7). In Europe currently there are no commercially available preparations for active immunization against precursors of estrogens and/or inhibin. However, their introduction on the market does not depend on scientific facts but more on economic policy.

As shown in experiments with cattle (28) daily treatment with a growth hormone (GH) throughout one oestrous cycle causes an increased production of IGF-1 in the liver. Consequently, the high level of IGF-1 in the peripheral blood – and also in the follicular fluid of antral follicles – provides an ideal micro-environment for more than one member of a cohort of follicles to emerge, develop and reach the stage of dominance. So at the end more than one DF-s per follicular wave is available at the time of the pre-ovulatory LH peak and will ovulate later. The same mechanism works perfectly also in ewes (7). However, according to the actual food-safety regulations GH treatment is strictly forbidden in Europe. So we disregard its further specification.

It is important to mention that in sheep an unknown percentage (7—46 % ?!) of twin pregnancies is reduced to a single pregnancy or lost completely (60, 76, 82).

According to our current understanding, in ewes embryonic and/or early foetal mortality may occur almost exclusively in the early CL-dependent phase (in the first 50 days) of gestation mainly due to an obvious, sometimes critical P4 decrease between days 14–20 and 50. After day 50 the placenta starts to produce sufficient quantity of P4 (21, 60, 82). Nutritional and/or pharmacological methods themselves are not able to increase the chance of embryonic survival (96). Beside twins and heat stress the shortage of energy supply also and energy- and perhaps protein-overfeeding have been proven to trigger the interruption of pregnancy during the first 50 days. In dairy cows overfed with rumen-degradable protein sources the massive intraruminal production of ammonia and the subsequent urea increase may be detrimental for embryo survival (10, 11). Similar losses may occur sometimes also in alfalfa-fed dairy ewes (44).

The shortage of energy supply can reduce the secretory capacity of endometrium. The paradoxical effect of a too abundant energy intake is explained by increased P4 metabolism in the liver. Due to this latter reason it is essential to know that *"flushing"* used to induce the first ovulation of the breeding season and/or stimulate multiple ovulations should be stopped at the proper time within some days after ovulation/conception (20, 39, 40, 60, 79, 82, 84). However, in practice our currently available methods are not sufficient enough to identify the dams affected by embryonic/early foetal mortality and to reveal its current cause. So, both the real incidence of and the underlying factors leading to this form of loss remain usually undefined under flock conditions.

Other nutritional effects upon reproduction

Actual feeding may also influence the incidence of pregnancy toxicosis (ketosis), the quantity and quality of colostrum, the viability of the newborn lambs and sperm production in rams (17, 20, 39, 40, 42, 60, 79). However, this complex issue cannot be discussed entirely within the framework of this paper.

PHARMACOLOGICAL METHODS USED TO MANIPULATE OVARIAN FUNCTION

In management of ovine reproduction there are two main forms of pharmacological methods used to manipulate the ovarian function (76, 82): (a) the induction of cycles in acyclic animals, furthermore (b) the synchronisation of oestrus and ovulation in animals with cyclic ovarian activity.

Induction of ovarian cyclic process in seasonally acyclic ewes

Ovulation with subsequent cyclic ovarian activity can be induced out of the breeding season in the spring to the early summer period (pregnancies for autumn lambing) or before the beginning of the breeding season in mid to late August (shortening the transition period). The methods that are used are more or less independently of season whereas the results are better in August than in April to June (76, 82). Good body condition is an important prerequisite of the success (79). The ovarian response to these treatment procedures can be enhanced by a preceding three to three weeks energy supplementation (*"flushing"*) and/or the exposure of males (*"ram effect"* related to pheromone exposure) (23, 41, 60, 73, 76, 82).

The ten to fourteen-day **gestagen** treatment combined with the administration of 400—600 IU eCG at the time of gestagen removal is the most common method in a practice, which has been used successfully on thousands of animals for many years. As an active ingredient both the natural P4 and its synthetic analogues (medroxyprogesterone acetate, MAP; cronolone syn. flourgeston acetate, FGA and others) can be administered as an intravaginal pessary, sponge or as a subcutaneous implant (3, 9, 57, 58, 59, 62, 76). During the ten to fourteen-day gestagen treatment the pulse frequency of the oscillating basal LH release is suppressed. So the final maturation of DF-s is blocked, the most of them become atretic. Injection of FSH-like eCG at the time of gestagen removal induces a simultaneous recruitment of a new cohort of follicles. Soon after gestagen removal LH pulsatility accelerates, which meanwhile increases the E2 production and maturation of one or two selected DF-s. Additionally, the P4 (-like) priming enhances the E2 sensitivity of the hypothalamic surge centre resulting in ovulation at about the 48th to 60th hour (82).

During the FGA treatment a slight decrease in ACTH and cortisol levels, an increasing tendency of plasma leptin and weaker ACTH-induced cortisol-responses were observed. After removal these clinically negligible alterations disappeared completely (43). Beside their low cost and general acceptance the main advantage of these gestagen-based methods is that the animals can be mated or inseminated at a fixed time, e.g. 48 and 60 hours (2×) or about 55 hours (1×) after removal. The only disadvantageous aspect of their use is that each animal must be caught and handled twice i.e. at the time of administration and removal of the gestagen source.

Melatonin treatment is a recent alternative of gestagen-based methods. It can be administered as a subcutaneous implant releasing the ingredient from the vehicle continuously for approximately eight weeks. By the end of this eight week period while elevated melatonin levels (additional to the circadian changes of this hormone) are seen, the pulsatile pattern of GnRH and LH secretion has been modified: both the pulse frequency and concentration range of the basal LH secretion increases. First ovulations are expected to occur about five to six weeks after the melatonin insertion and the ovarian function becomes cyclic thereafter. Due to the biodegradable feature of its vehicle there is no need of implant removal. However, ovulations and oestruses are only induced but not synchronized within the flock: so an additional synchronization procedure is needed for a fixed-time insemination (7, 15, 54, 76, 80, 82, 87).

Melatonin implants are mostly administered around the time of the summer solstice (mid June) to trigger the first ovulation at the beginning of the breeding season (15, 76, 82). In Mediterranean breeds, however, good ovarian response has also been observed after a late winter to early spring implant insertion (15). Further studies are needed to reveal (i) the real efficacy of melatonin treatment in breeds of steppe origin kept under Central Eastern European conditions, (ii) its interaction with lactation and Mel_{1a} genotype as well as (iii) its supposed direct effect on ovarian structures and embryonic cells.

Although in some studies about half of the treated ewes ovulated and became pregnant (30) due to the lack of P4 priming the GnRH treatment is usually considered not powerful enough to induce ovarian cyclic process out of the breeding season in sheep (76, 82).

Synchronisation of oestrous and ovulation in cyclic animals

In cyclic animals the oestrus and ovulation can be synchronized with the same **gestagen**-based methods as used for induction of ovulation and cyclic processes although the dose of certain gestagens (FGA) may be higher in than out of the breeding season. The luteolytic dose of $\mathbf{PGF_{2\alpha}}$ might be an alternative of gestagens that seems to be very attractive from food safety considerations. Its disadvantage is, however, that in sheep the luteolytic dose of $PGF_{2\alpha}$ is relatively high and therefore expensive (56, 76, 82).

The administration of $PGF_{2\alpha}$ may damage the wave-like dynamics of the follicular growth and the CL development causing great differences in the time of oestrus and ovulation (4). Furthermore, its administration is completely ineffective in acyclic individuals (56, 76, 82). The latter argument is of importance because nowadays there is no available diagnostic method applicable in the everyday practice of the ovine reproduction that can provide reliable information concerning the presence or absence of ovarian cyclic rocesses and/or the current stage of the cycle.

In dairy cows the combined administration of GnRH-$PGF_{2\alpha}$-GnRH (**OvSynch** or **GPG** protocol) is more and more popular. During the nine to twelve weeks of lactation this treatment is expected to induce ovulation and cyclic processes in acyclic individuals and synchronize ovarian activity in cyclic cows. Although its effect is more reliable in the latter (already cyclic) animals fixed time insemination is possible in both cases (27, 76, 82). The food safety consideration of this procedure is excellent. So its administration seems to be promising firstly in dairy flocks.

The reason for not having much experience in sheep is the high price of products furthermore the fact that in ewes there might be remarkable individual variations in the dynamics and per-cycle number of waves of follicle growth. Greek experiments have tested the success of this method on 2.5—4.0 year old ewes with an average body condition (n = 28) in the middle of the biological breeding season (16). According to the species-specific dynamics of the follicular growth the first treatment with GnRH (day 0; resulting in ovulation and development of

CL or intrafollicular luteinization) was followed by induction of luteolysis by $PGF_{2\alpha}$ on day 5 and 48 hours later a second dose of GnRH was administered. The ewes were inseminated with fresh diluted semen (intrauterine deposition, through laparoscopy) 16—20 hours after the second GnRH. Half of the animals became pregnant.

Despite these relatively promising preliminary results further studies are required to demonstrate the reliability of this method in sheep. The authors of this review of literature are sceptical concerning its applicability as a single technique out of the breeding season. However, the species- (and breed) specific version of OvSynch may represent some value in the reproductive management of dairy flocks after a preceding melatonin administration.

CONCLUSION

The experiences of these cited studies confirm that the practical implementation of all this knowledge gives a opportunity for an immense development of sheep husbandry in all areas of Central Eastern Europe. There are good conditions and also a great demand for the practical application of these methods and procedures in both our countries.

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