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The 55th Anniversary of The Foundation of The Veterinary College The Present University of Veterinary Medicine in Košice

Dr. h. c. Prof. Rudolf Cabadaj, DVM, PhD, rector of UVM

Every individual, society and organisation has recourse sometimes to the collection of facts, data and events that took place or occurred recently and also long ago. This year is a good opportunity and even a duty to do this at our University of Veterinary Medicine in Košice which celebrates the 55th anniversary of its foundation.

During this period at its head seven rectors and deans have come and gone. Each one, together with other members of the administration, departments, clinics, institutes and sections, and in fact together with the entire personnel, has achieved a considerab-le piece of work. Even so, on these occasions, one should mention especially the academician Ján Hovorka – the first rector of our UVM.

The first attempt to establish a Veterinary College in Slovakia dates back to the end of 1939. The impulse for this was the closing of the Czech universities, which lasted until the end of World War II. This raised a critical situation for Slovak students who had studied at the Veterinary College in Brno.

The efforts of the chairman of Veterinary chamber of that time, Pavel Opluštil, DVM, as well as the head of veterinary services Milan Kukliš, DVM, to establish a place for veterinary studies for students and candidates from Slovak territory failed to find the understanding of governing authorities. The competent organs recommended that Slovak students of the Veterinary College in Brno complete their studies abroad (Vienna, Zagreb, Budapest).

In 1945 the Veterinary College in Brno (the only veterinary education institution in former ČSR) faced serious problems related to the optimisation of studies in individual classes with regard to both the reconstruction of buildings and other facilities and the personal appointment of teachers to teaching posts, particularly with regard to the extremely high numbers of students. Founding an additional Veterinary College in the restored CSR became an extremely topical issue.

In 1949 Prof. Ján Hovorka, DVM, took the initiative and together with the head of the Veterinary section of the Commissionariate for agriculture and land reform in Slovakia, Samuel Adamaťˇ, DVM, they took organised steps towards founding a veterinary school in Slovakia. The task was not simple because other cities in Czech territory were also suitable candidates. Finally, despite additional complications associated with the selection of the seat of the future veterinary school in Slovakia (Bratislava, Žilina, Martin), due particularly to the help of Laco Novomestský and JUDr. Vladimír Clementis, the final decision was in favour of Košice.

On the 12th October, 1949, Prof. Ján Hovorka, DVM (a teacher at the Agricultural and Forestry College in Košice) was appointed the rector of a newly established school, which was opened on the 3rd of October, 1949, with 105 students enrolled in the first year of study.

The years that followed were very difficult. Many facilities and buildings inherited from the former so-called Komensky institute had to be reconstructed before they could be suitable for the education of young veterinary specialists, who were awaited eagerly and urgently by animal production in Slovakia. Reconstruction, the completion of equipment and improvement of the working environment continued for decades and has not stopped even to this day. The teaching staff is complemented and harmonically renewed. Despite demanding conditions the teachers increase their qualification harmonically to which witness is borne by results obtained in the field of scientific research (see FOLIA VETERINARIA, 48/1 - Supplement, 2004). The accession of the Slovak Republic to the European Union that took place this year also opened new prospects for our activities. Our progress can be supported by grants from EU developmental funds. I believe that we will make the most of these opportunities.

In this year 2004 our University will Implement significant changes in the teaching of clinical subjects concerning animal species in accordance with the requirements of European legislative and advanced veterinary education.

After the success with the study branch Food Hygiene we will launch in the academic year 2004/2005, in agreement with the Act No. 131/2002 of the Civil Code about Universities in the wording of its recent provisions, a first-stage university study "Cynology", believing that cynology, as an important part of veterinary studies, requires education according to an individual curriculum. We should not underrate the operation and prosperity of independent institutes and specific purpose establishments despite many complications related to their financial and economical status.

In my capacity as the rector of UVM in Košice I wish to thank all, who in any possible way have contributed to the positive and progressive efforts and activities of our Alma Mater, who avow loyalty to it and enjoy collaboration with its personnel or at least remember it in the best sense and contribute to its good name.

I use this opportunity to greet all members of the Veterinary chamber of SR and look forward to our further collaboration. The Rector's insignia – the gold chain - a gift from Veterinary chamber to the Veterinary College in Košice on the occasion of its founding in 1949, reminds me of this pleasant duty.

Vivat, crescat et floreat Alma Mater nostra!

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REVIEW ARTICLE: VITELLOGENIN AS A BIOMARKER FOR THE EXPOSURE OF FISH TO ESTROGENIC CHEMICALS

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ABSTRACT

The assessment of fish reproductive performance is increasingly used to evaluate the impact of environmental disturbances. Many natural and man-made chemicals are able to mimic the action of natural sex steroids. Exposure to environmental steroids can disrupt normal reproductive development. Vitellogenin (Vtg), a precursor of egg-yolk in fish and other oviparous animals, may be used as a bio-marker for the estrogenic contamination of water environment.

Key words: aquatic environment; reproduction; vitellogenesis; xenoestrogens

INTRODUCTION

As a consequence of twentieth century industrial development the amount of chemicals in the environment has increased dramatically. About 80,000 man-made chemicals are today in common use, and another 1,000 are added annually (33). It is obvious that effluents are highly complex mixtures of chemicals. Furthermore, they also contain many natural chemicals (sources include excretion by people, and decomposition of plant material). A number of these natural and man-made substances have the ability to interfere with the endocrine system of animals (39).

Fish have a major advantage over mammals in studies of endocrine disrupters in that they produce large numbers of both eggs and sperm and release them to the external environment, so that the effect of potential endocrine disrupters can be readily examined in both sexes. An assessment of pollution induced reproductive dysfunction, however, requires a simple biomarker. Vitellogenin (Vtg), an egg yolk precursor protein, is used as a suitable and valuable biomarker of reproductive disruption in fish (19).

VITELLOGENESIS UNDER PHYSIOLOGICAL CONDITIONS

Vitellogenesis is defined as an estradiol-induced hepatic synthesis of egg yolk protein precursor, vitellogenin, its secretion and transport in the blood to the ovary and its uptake into maturing oocytes (24). Vitellogenin is defined as a female-specific serum protein that contains phosphorus, lipids, carbohydrates, calcium and iron (25). In oviparous animals, the accumulation of yolk materials into oocytes during oogenesis and their mobilization during embryogenesis are key processes for successful reproduction (2).

Most reproductive functions, including vitellogenesis, are dependent upon the regulatory activity of the brain-pituitary–gonadal axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which stimulates the release of gonadotropins (GtHs) from the pituitary. Two forms of gonadotropin have been isolated in fish: GtH I and GtH II, which are analogous to mammalian follicle-stimulating hormone (FSH) and luteinising hormone (LH), respectively. In females GtH I is involved in vitellogenesis, while GtH II plays a role in final oocyte maturation and ovulation (30). In males, GtH I

is involved in spermatogenesis and GtH II is involved in final sperm maturation and spawning (31). GtH I also stimulates oocytes to synthesize estrogens in females and Leydig cells to synthesize androgens in males. The major estrogen in female fish is 17 β–estradiol (E_2) and the major androgen in male fish is 11-ketotestosteron (30).

E_2 produced by the ovarian follicular cells in response to GtH I is transported in plasma attached to sex steroid-binding protein (SBP) (36). In the liver, E_2 is retained in target cells by high affinity binding to a specific steroid-receptor, the E_2-receptor (ER) (17). The hormone-receptor complex binds tightly in the nucleus to estrogen responsive elements (ERE) located in DNA. The receptor-DNA interaction results in a transcription of specific mRNA and its translation into the protein, vitellogenin (21). Thereafter, the vitellogenin molecule is lipidated, glycosilated and phosphorylated and then secreted into the serum for transport to the ovary (2). The uptake of vitellogenin by growing oocytes is rapid, specific and saturable, and occurs by receptor mediated endocytosis (27, 28). The vitellogenin molecule is cleaved proteolytically into main yolk components in the course of its translocation from the oocyte surface to the yolk deposition sites. Finally, components such as lipovitellins, phosphitins and phosvettes are deposited within membrane-bound spherical yolk bodies (24).

ESTROGEN MIMICS IN AQUATIC ENVIRONMENT

Environmental estrogens present in aquatic environment are chemical pollutants that can disrupt the endocrine system of animals by binding to and activating the estrogen receptor. They include both natural and synthetic steroid estrogens, as well as a variety of estrogen-mimicking chemicals (so called xenoestrogens). Natural steroid estrogens, 17 β-estradiol, estron and estriol, are produced by all vertebrates, including humans. They pass through the body and are metabolized to inactive conjugates (glucuronides and sulfates) prior to their excretion (39).

A synthetic estrogen that has been identified in surface waters is 17 α-ethynylestradiol (EE_2). Ethynylestradiol is the active component in the oral contraceptive pill. The metabolic processing of ethynylestradiol in the body is similar to that for natural estrogens, in that it is excreted primarily in conjugated forms.

The presence of 'free' estrogens in the aquatic environment probably results from their enzymatic deconjugation by bacteria in the environment. There are enzymes in sewage treatment works (STWs), such as β-glucuronidase, that are capable of hydrolyzing steroid glucuronides. β–glucuronidase is found in many species of bacteria, including *E. coli*, which are probably the most common bacteria in sewage (39).

The effluent from STWs discharging into British rivers contain 17 β–estradiol and estron at concentrations ranging from 1 $ng.l^{-1}$ up to almost 80 $ng.l^{-1}$ and 17 α-ethynylestradiol at concentration 0.2–7 $ng.l^{-1}$ (5). Larsson *et al.* (23) found 17 α-ethynylestradiol (4.5 $ng.l^{-1}$), estron (5.8 $ng.l^{-1}$) and 17 β-estradiol (1.1 $ng.l^{-1}$) in effluent water from a Swedish sewage treatment works receiving mainly domestic waste water. Belfroid *et al.* (3) have detected estron (up to 47 $ng.l^{-1}$) and 17 β-estradiol (1-12 $ng.l^{-1}$) in waste water and estrogenic hormones (below 1–5 $ng.l^{-1}$) in surface water in the Netherlands. Vader *et al.* (40) have described the biodegradation of 17 α-ethynylestradiol by nitrifying bacteria in an activated sludge system. Natural estrogens present in the aquatic environment also include mycoestrogens (produced by fungi) and phytoestrogens (synthesized by plants). Based on structural similarities, these phytoestrogens and mycoestrogens can be divided into several groups: isoflavonoids (e.g. genistein, equol, daidzein, Biochanin A), coumestans (coumestrol), and ligands (enterolactone) (39).

For aquatic animals, the routes of exposure to phytoestrogens include uptake from diet and also from water. Ishibashi *et al.* (13) have detected genistein, daidzein, coumestrol and equol in commercial fish diets and determined the estrogenic activity of the fish diets using male goldfish by measuring plasma vitellogenin (VTG) levels as a biomarker of estrogen exposure. Kiparissis *et al.* (20) have detected genistein in wood pulp (30 $mg.kg^{-1}$) and mill effluent (13.1 $mg.kg^{-1}$) collected from a bleached kraft mill in Ontario, Canada. Trembly *et al.* (37) have observed elevated plasma vitellogenin levels in sexually immature rainbow trout after 21-d exposure to β-sitosterol and Canadian bleached mill effluents (BME) establishing the presence of estrogenic compounds in BME and confirming the estrogenicity of β-sitosterol.

A wide range of man-made chemicals is estrogenic. Many of these chemicals are widely used in major industries such as agriculture, the petrochemical industry, the plastics industry, and the soap and detergent industry. Very high volumes of some of these estrogenic chemicals are used, which leads to the appearance of significant amounts in the aquatic environment (34). These estrogenic chemicals enter rivers in effluent from sewage treatment works (STWs), which usually receive both domestic and industrial inputs (33). Man-made chemicals that have been reported acting as estrogen mimics include: organochlorine pesticides (6), polycyclic aromatic hydrocarbons (26), polychlorinated biphenyls, dioxins (39), alkylphenolic chemicals (15), phthalates (14), bisphenol A (34).

Most of these xenoestrogens are only weakly active, with potencies three or more orders of magnitude less than that of natural estrogens, such as 17 β-estradiol (39). Most of them are lipophilic and hydrophobic and, hence, have a strong tendency to bioconcentrate and bioaccumulate in aquatic animals, both plants and animals. They are much more likely to be preferentially concentrated in a few tissues, such as fat. They may be physiologically inactive while stored in adipose tissue, but when this fat is mobilized (which often occurs during reproduction), the compounds may be freed to act elsewhere or they may be metabolized into other compounds that may or may not be active as estrogens (34, 39).

It is unlikely that fish are exposed to just one estrogenic chemical, but instead are likely to live in water that contains many different estrogenic chemicals. Thus, it is possible that fish living in an estrogenic environment might show a more pronounced 'feminizing' response when exposed to the mixture of chemicals than they would if they were exposed to a single estrogenic chemical at the same concentration (34).

VITELLOGENIN AS BIOMARKER OF EXPOSURE TO ESTROGENIC SUBSTANCES

As mentioned above, vitellogenin synthesis is estrogen dependent and, hence, is normally limited to the reproductive female fish. Male and juvenile fish possess the vitellogenin gene, which remains inactive under normal conditions, but can be induced by exposure to (xeno-) estrogens. Production of vitellogenin by male and juvenile fish, or non-vitellogenic females can therefore provide a bioindicator of exposure to environmental estrogens (4, 19).

The vitellogenin content of the plasma of female fish is dependent on the age and the stage of the reproductive cycle (32). An increase of plasma vitellogenin concentration under normal levels can indicate increased or decreased egg numbers and size, or spawning failure. High vitellogenin in females is therefore a warning of possible disruption to the normal reproductive function (19).

Vitellogenesis can also act as a biomarker in the reverse sense, that is abnormally low vitellogenin production. Clearly, this is not relevant in males and juveniles in which vitellogenin concentration is not normally detectable. In females, however, a low plasma vitellogenin concentration is indicative of malfunction at other points in the reproductive endocrine system, possibly resulting in inhibited ovarian growth.

For vitellogenin quantification in fish plasma, whole body homogenate or liver, various methods have been developed. These include e.g. radioimmuno-assays (38), enzyme-linked immuno-sorbent assays (4), immuno-histochemistry (42, 43), gel electrophoresis and Western blot (8). Vitellogenin is a species-specific protein. Vitellogenin assays based on polyclonal antibodies are generally restricted for use in homologous species, but some antibodies do cross-react with vitellogenin in other species (38).

In the Czech Republic the vitellogenin content of fish blood plasma from contaminated and control sites on the Tichá Orlice river (*Salmo truta*) (22, 35), Jihlava river (*Barbus barbus*) (29, 35) and Labe river (*Leuciscus cephalus*) (35) was studied during the monitoring of surface waters. In all cases, the plasma vitellogenin content in male fish from contaminated river sites was higher than in male fish from the control river sites.

CONSEQUENCES TO FISH OF ESTROGENIC CONTAMINATION OF WATER

Reproductive development is a continuous process throughout ontogeny. Consequently, it is susceptible to the effects of (xeno-) estrogens at all stages of the life-cycle, including fertilization, embryonic development, sex differentiation, oogenesis or spermatogenesis, final maturation, ovulation or spermiation, and spawning.

Given the energy cost in reproduction and the long decision time, it seems most likely that (xeno-) estrogen induced Vtg synthesis may cause an imbalance in the reproductive strategy of a given fish population. Protein synthesis is energy expensive so (xeno-) estrogen induced Vtg synthesis may result in wasteful use of stored energy resources. Furthermore, maturation has developmental priority over somatic growth. The ecological implication of this might be failure in the reproduction of affected individual fish and, in the long-term, affecting recruitment of the entire population (2).

Jobling *et al.* (15) have observed elevated plasma Vtg concentration accompanied by a decreased rate in testicular growth of rainbow trout after exposure to alkylphenolic chemicals. Jobling *et al.* (16) have detected disruption in the gonadal development of wild roach (*Rutilus rutilus*) living in UK rivers receiving large volumes of treated sewage effluent, ranging from malformation of the germ cells and/or reproductive ducts to altered gamete production. They also found intersex fish that had an altered endocrine status and an elevated concentration of plasma vitellogenin. Hecker *et al.* (11) have shown the reciprocal relationship between inhibitory effects on gonadal growth, maturation, and sex plasma steroids of wild bream (*Abramis brama*) in the Elbe River, Germany, and exposure to pollutants, such as organotins, pesticides, or metals. Folmar *et al.* (8) have observed vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio)* captured near a major metropolitan sewage treatment plant in Minnesota, USA.

In addition to effects on gonadal development or function, organ toxicity, particularly in the liver and kidney, may be toxicologically relevant in fish exposed to estrogenic chemicals. A few studies have reported kidney impairment based on the presence of amorphous eosinophilic material within the kidney or glomerulus, glomerular degeneration, tubule necrosis and interstitial cell hyperplasia upon estrogenic chemical exposure in fish (43, 12, 9). Also, the livers of estrogen-exposed fish have been reported to be enlarged, have 'hole-like' lesions, accumulate amorphous eosinophilic material, exhibit changes in hepatocyte vacuolization, have less basophilic hepatocytes, or simply resemble the liver of a vitellogenic female with increased basophilia and enlarged nuclei (43, 12, 9, 10, 7). Finally, the accumulation of acellular, amorphous eosiniphilic material in the abdominal cavity around major organs leading to ascites and/or abdominal distension has been frequently reported in fish exposed to estrogenic chemicals (43, 12, 41).

CONCLUSIONS

Of all the environments, the aquatic ecosystem is at greatest risk from pollutants since all chemicals, whether initially released on land, into the atmosphere or directly into rivers will eventually find themselves in the rivers and oceans as the final repository (18). A rapidly increasing number of chemicals, or their degradation products, are being recognized as estrogenic, albeit usually weakly. These chemicals enter water-ways *via* the effluent from STWs (and possibly from other sources of effluent) and are absorbed and bio-accumulated in sufficient concentrations to induce physiological responses in fish, which are indicative of exposure to estrogens (34). The presence of vitellogenin in male and juvenile fish, and also increased or decreased vitellogenin in females, can be used as a biomarker for estrogenic contamination.

Nevertheless, an assessment of the effects of estrogenic

chemicals on the fish organism can be useful complete with, for example, histological examination of the gonads, determination of the sex plasma steroid concentrations (17 β-estradiol, 11-ketotestosteron, testosteron), plasma thyroid hormones concentration, plasma cortisol concentration, sperm morphology and motility, egg number and size, fertilizing ability, etc (1, 18).

Fish provide a very useful model for determining the effects of environmental pollutants on the vertebrate reproductive system. Although the endocrine system of fish differs in the details of its structure and hormones, reflecting the enormous variety of reproductive strategies, the effects found in fish could be expected to have parallels in mammals (18). Fish is globally a very important food resource. Bio-accumulation of the toxicant in the fish and further concentration up in the food chain, may eventually become a threat to human health.

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CATECHOLAMINE LEVELS AND ACTIVITY OF MONOAMINE OXIDASE IN THE PINEAL GLAND OF SHEEP AFTER ADMINISTRATION OF FSH

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ABSTRACT

The influence of hormonal preparations of FSH in a dose of 480 IU on levels of catecholamine (dopamine, norepinephrine and epinephrine) and the activity of their degradation enzyme monoamine oxidase (MAO) in the pineal gland of sheep was investigated in the oestrous period employing radiochemical methods. The pineal gland reacted to the hormonal preparation FSH with decreased levels of norepinephrine and dopamine ($p < 0.001$) and with an increase in MAO activity ($p < 0.01$). We suggest that FSH administration affects catecholamine levels and the activity of monoamine oxidase in the studied areas of the brain of sheep by means of a feedback mechanism.

Key words: FSH superovulation; monoamine oxidase; pineal gland; sheep

INTRODUCTION

Hormonal preparations generally used for the induction of superovulation in farm animals affect steroidogenesis in ovaries and influence hypothalamic nuclei and their gonadotropic receptors through a feedback mechanism (18, 2). High concentrations of circulating oestrogens act specifically upon adrenegic receptors and affect the levels and metabolism of catecholamines in the central and peripheral adrenergic system (5, 11, 12).

Simultaneously with changes in the metabolism of catecholamines, some changes in activities of monoamine metabolism enzymes were recorded after hormonal treatment (1). Monoamine oxidase (MAO) is an enzyme which plays an important role in the degradation of catecholamines by oxidative deamination and participates in the regulation of the functionally active pool of monoaminergic neurotransmitters in the nervous tissue. Some authors (7, 9, 15) have discovered that oestrogens also modify the enzymatic activity of degradation enzymes of catecholamines – such as MAO and catechol-o-methyl-transferase (COMT) – in the hypothalamus and striate region. Our studies were aimed at the investigation of changes in catecholamines and their degradation enzyme MAO in the pineal gland of sheep after administration of FSH.

MATERIAL AND METHODS

In our study we used twenty sheep of Slovak merino breed age three to four years, of mean body weight 42 ± 3.1 kg in their oestric period (September – October). The sheep were fed a standard melasse feed with vitamine additives twice daily. The oestrus of all sheep was synchronized with intravaginal polyurethane sponges (Agelin Spofa, Prague) twenty milligrams of chlorsuperlutin which was instilled for a period of thirteen days. The first group (ten animals) served as controls. After completed synchronization of the oestrus, the sheep of second group ($n = 10$) were hormonally stimulated by the administration FSH *ad usum vet.* (Spofa, Prague). FSH was administered to the sheep three-times daily for two days in overall doses of 480 IU. The animals were slaughtered 104 hours after the first dose of FSH.

We quickly removed the brain. Samples were obtained from the pineal gland. The tissues were immersed in liquid

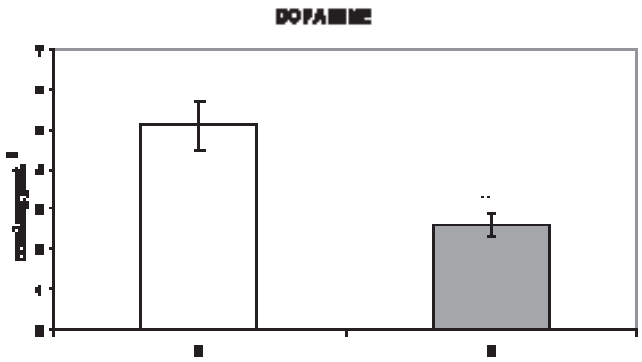

Fig. 1. The effects of oestrus synchronization and hormonal stimulation (480 IU FSH) on the levels of dopamine in the pineal gland. The results are expressed in nmol.mg protein^{-1} (means ± S.E.M.): C — the control group with synchronized oestrus, E — synchronized oestrus and stimulated by administrated 480 IU FSH. Significant difference from controls: ** — $p < 0.01$

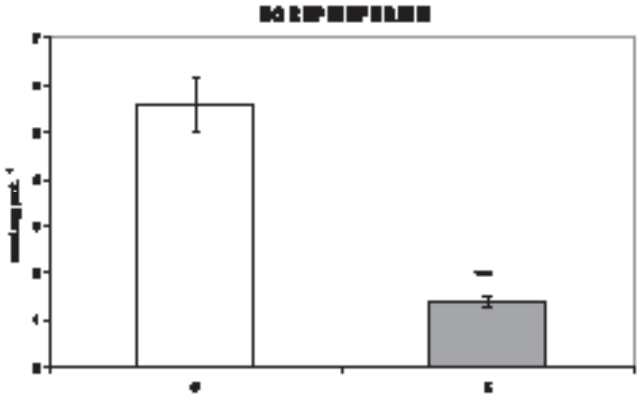

Fig. 2. The effects of oestrus synchronization and hormonal stimulation (480 IU FSH) on the norepinephrine levels in the pineal gland. For other details see Fig. 1.; * — $p < 0.001$**

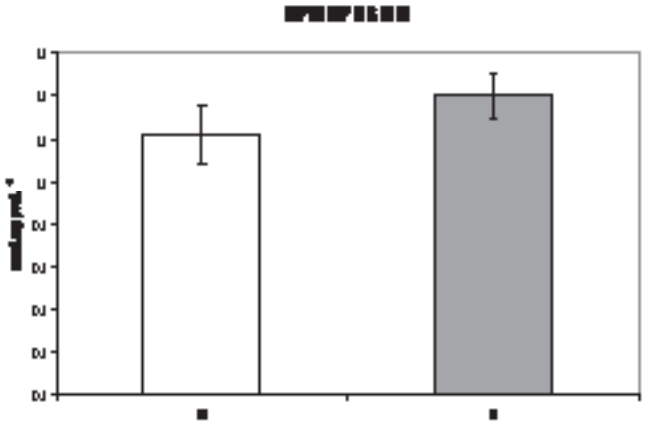

Fig. 3. The effects of oestrus synchronization and hormonal stimulation (480 IU FSH) on the epinephrine levels in the pineal gland of sheep. For other details see Fig. 1

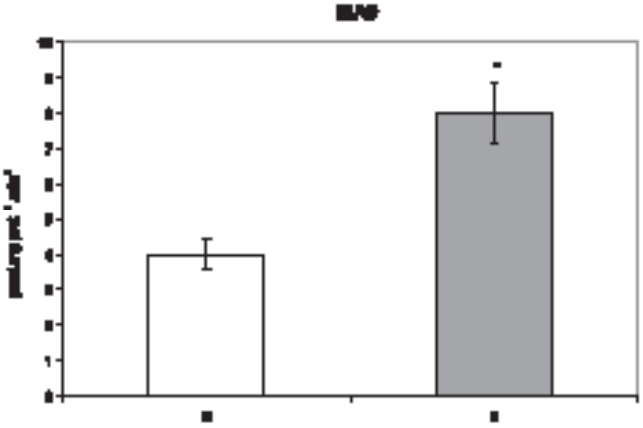

Fig. 4. The effects of oestrus synchronization and hormonal stimulation (480 IU FSH) on the activity of monoamine oxidase in pineal gland of sheep. The results are expressed in nmol.mg.protein^{-1}.min^{-1}. For other details see Fig. 1; * — $p < 0.05$

nitrogen where they were stored in the frozen state until further processing. Samples for radioenzymatic determination of catecholamines were homogenized in microhomogenizers in cooled $HClO_4$ (0.4 mol.l^{-1}) with the addition of reduced glutathione (0.05 ml.l^{-1}) at 1 µl per 1 ml tissue, and they were centrifuged at 15 000 × *g* at 0 °C for 30 min.

Catecholamines were determined by the radioenzymatic method according to Johnson *et al.* (6) in 50 µl plasma (in parallel samples). The radioactivity of catecholamine derivates was measured on a scintillating spectrometer Packard Tri Carb in a ^{3}H channel. The results are expressed in catecholamine nmol.mg proteins^{-1}. Proteins were determined in identical homogenate tissues (8).

Due to higher concentrations of catecholamines in the brain, the pituitary gland was diluted with redistilled water in the ratio 1 : 20. The coefficient of the methodical variation, calculated from ten repetitions, of one sample was 4.2 % for norepinephrine (NE) and 4.1 % for dopamine (DA).

For the determination of MAO, the tissue was homogenized in saccharose (0.25 ml.l^{-1}) and the radiochemical method according to Wurtman and Axelrod (20) was used. ^{14}C-5-(hydroxy)-tryptamine (Amersham, England) with a specific activity 15.5×10^{-7} Bq.nmol^{-1} in a dose of 6.25 nmol per sample was used as a substrate. The substrate is specific for the determination of MAO A and MAO B forms. The activity of MAO was measured using a Packard Tri Carb scintillating spectrometer in the ^{14}C channel. Proteins were determined in the same tissue homogenates. The results were statistically processed by the non-paired *t*-test and are given as means ± S.E.M. in pmol mg prot.$^{-1}$.min.$^{-1}$.

RESULTS

After hormonal stimulation of sheep ovaries with 480 IU FSH, a significant decrease of dopamine levels ($p < 0.01$) in pineal gland of sheep (Fig. 1) was observed in comparison with the control group. The most marked changes in the concentrations of norepinephrine ($p < 0.001$) after FSH application (from 5.450 ± 0.6 to 1.350

± 0.09 nmol.mg.prot.$^{-1}$) were recorded in the pineal gland (Fig. 2). The levels of epinephrine were unchanged after hormone administration (Fig. 3). FUS (Fig. 4) increased activity of the degradating enzyme of catecholamines (MAO) in the pituitary gland ($p < 0.05$). In the pineal gland (Fig. 4) a significant increase of MAO activity and decrease dopamine and norepinephrine were found as shown in the correlation.

DISCUSSION

The pituitary FSH hormone in redundant amounts induced luteolysis 48 hours after its administration, after which polyovulatory oestrus followed (10, 16). Most authors (3, 4) prefer FSH preparations to serum gonadotropins (PMSG) in biotechnically directed reproduction because FSH is a better as PMSG regulator of the superovulatory process. This ability is due to its short half-life in the organism, and the more stable gonadotropic effect of FSH (10), although it has to be given several times daily. Fernandez-Pardal *et al.* (5) have found changes in the activity of monoamine oxidase and catecholamine levels in the ovaries and uterus in ovariectomized HCG- and LH-treated rats, and an increase in the levels of cAMP, which they correlated with an increase in steroidogenesis after hormonal stimulation.

The hormonal preparations used for inducing superovulation of farm animals influence the catecholaminergic system of the hypothalamus and its controling centres of reproduction (18, 11, 12, 13, 14). Schewe *et al.* (16) have found an eight- to ten-fold increase in 17α-oestradiol with a peak at 24–36 hours after FSH administration. The high levels of oestrogens act on adrenergic receptors and influence both the function and levels of catecholamines in tissues and plasma (11, 9). Tobias *et al.* (19) have found a reduced norepinephrine turnover and subsequent LH decrease in blood plasma after administration of oestrogens to ovariectomized rats, although the activity of tyrosine hydroxylase and turnover of dopamine in the hypothalamus increased.

Monoamine oxidase is responsible for intraneuronal metabolism of monoamines and regulation of their active pool in the nervous system. It follows from our previous results (12, 14) that hormonal preparations (PMSG, HCG and FSH) influence MAO activity in the hypothalamus of sheep.

In the pineal gland, catecholamines participate in the regulation or modulation of melatonin secretion. The adrenergic neurones increase their activity in darkness, when the synthesis of NE is elevated. Norepinephrine acts on alpha- and beta-receptors of pinealocytes and stimulates the activity of N-acetyltransferase (NAT) – the enzyme synthetizing melatonin (17). Our experiments have detected increased activity of monoamine oxidase in the pineal gland ($p < 0.05$) after administration of FSH which is consistent with the decrease of norepinephrine and dopamine.

From our previous works (12, 14) it may be suggested that FSH markedly influences the activity of MAO in the uterus and in the centers controlling reproductive functions of sheep. We propose that the given changes in MAO activity in the pineal gland of sheep are associated with the observed changes in the levels of catecholamines related to steroid alterations after administration of the gonadotropic hormone (1).

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ABATTOIR STUDIES ON PARAMPHISTOMES RECOVERED FROM CATTLE IN MASVINGO AND MANICALAND PROVINCES OF ZIMBABWE

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ABSTRACT

Parasites were collected from 3225 cattle from various localities in Masvingo and Manicaland Provinces. On slaughter their rumens and reticulum were cut open and their inner walls were examined for parasites. Identifications were based on the morphological structures of flattened specimens and measurements of diagnostic features from median sagittal section. Analysis of the structures of the acetabulum, pharynx and genital atrium revealed the following parasites in the area: *Calicophoron microbothrium, Calicophoron clavula, Calicophoron phillerouxi, Calicophoron sukari, Calicophoron calicophorum and Calicophoron raja*. The visible damage by adult parasites on the hosts' tissue were as a result of their sucking the reticulum and rumen mucosa into the acetabulum, which eventually nipped off, leading to slightly hardened areas devoid of rugae. The average number of cattle infected annually was 25.41 %, but from some areas sampled up to 80 % of the cattle were infected.

Key words: *Calicophoron*; cattle; paramphistomes; reticulum; rumen; Zimbabwe

INTRODUCTION

Masvingo and Manicaland Province lie approximately between 17 ° and 22 ° parallels south and between 30 ° and 33 ° longitude East. The altitude is 400 m in the south and 1 200 m in the north. The annual average temperature is 26 °C. The rainfall that comes between mid-November and late March averages 500 mm *per annum*.

Dams, among them Lake Mutirikwi, the second largest dam in the country, are the main sources of drinking water for livestock and wildlife in this province. The numerous dams form suitable habitats for aquatic snails among which are *Bulinus* spp. and *Lymnaea* spp. and *Biomphalaria* spp. some which are intermediate hosts for paramphistomes (1, 3, 4, 6, 8, 9). The Gonarezhou National Park which has many wild ruminants among them those habouring paramphistomes in Zimbabwe, occupies a sizable portion of the area of these provinces, bringing livestock into contact with wildlife and thus facilitating cross infections between livestock and game.

Although no study has been done on paramphistomes from cattle in these provinces *Cotylophoron cotylophorum, Carmyerius spotiosus* and *Carmyerius bubalis* were recovered from the stomachs of *Alcelaphus* spp., *Tragelaphus spekei* and *Tragelaphus stresiceros* in Zimbabwe (23). *Calicophoron raja* was recovered from *Connochaeters taurinus* while *Calico-*

phoron microbothrium (syn. *Paramphistomum microbothrium*) was recovered from *Aepyceros melampus*, *Kobus leche* and *Taurotragus oryx* in Zimbabwe (10, 12).

Paramphistomes have been reported in most parts of the world among ruminants (7, 10, 11, 12, 20, 24, 31). Adult parasites are not normally associated with clinical disease, however, occasional outbreaks of parasite gastroenteritis in susceptible livestock is associated with migration of immature parasites through the upper small intestine (2, 5, 16, 30). Generally cattle not previously exposed are susceptible (2, 5, 16, 21). In areas under study there is a lot of movement of cattle due to the current resettlement programmes. These movements of cattle create the suitable conditions for outbreaks of paramphistomiasis.

The aim of this study was to determine the identity, incidents and distribution of paramphistomes that occur in cattle from Masvingo and Manicaland provinces. Not all paramphistome species are responsible for disease in livestock, it is therefore important to have accurate information about which species occur so that if pathogenic ones are found preventive control measures before outbreaks occur (28). To date there is no literature or information on the identity, incidents and distribution of paramphistomes in cattle from in Masvingo and Manicaland provinces. Such information when obtained can be used eventually to construct predictive models of outbreaks of disease and such models are important in implementation of control measures that are effective and economic (21).

MATERIALS AND METHODS

Examination for parasites was done on the inner walls rumen and reticulum of 3225 cattle slaughtered in Bulawayo Cold Storage Commission Abattoir and Tenda Meats Masvingo. The cattle under study originated from different parts of Masvingo province, namely: Chartsworth, Chiredizi, Chivi, Gutu, Masvingo, Mwenezi, Nuanetzi and Triangle Estates and Manicaland Province, namely: Buhera, Chipinge, Mutare and Nyanga. The rumen and reticulum were cut open with a sharp knife and the inner walls examined for parasites. The parasites were hand picked into normal saline solution. In the laboratory the uteri of some parasites were teased for recovery and measurements of egg size. Some specimen were flattened dorsoventrally between microscope slides held by rubber bands to facilitate examining diagnostic features. Some were preserved in formol saline or 70 % ethanol for median sagittal sectioning in order to determine species using the keys of Näsmark (20), Yamaguti (31) and Eduardo (12).

The incidents and degree of infection with mature paramphistomes was recorded. The number of cattle infected was expressed as a percentage of the total number of cattle examined in each area.

RESULTS

On average 25.41 % of the 3225 cattle examined in the province are infected with paramphistomes. The percentage of cattle infected is shown in Table 2. Six species were identified using the keys of Näsmark, (20), Yamaguti (31) and Eduardo (12). These were *Calicophoron microbothrium, C. clavula, C. phillerouxi, C. sukari, C. calicophorum* and *C. raja*. Measurements and features used for identification and confirmation of species are given in Table 1 and Figs. 1A-H. Mixed infections were noted in some cases, but this could not be quantitatively determined as the parasites can only be identified to species level only after both the sagittal sections and flattened specimens had been examined.

There were no externally observable symptoms distinguishing the infected cattle from those not infected. There were no preferential infections based on breed of cattle or their age. As the parasites attain their maximum size their grip on the walls of the stomach, reticulum and rumen was noticeably less tight judging by the fact that they could easily be detached by hand from the host with minimum force. Nests of parasites numbering between 20 and 2 500 were found in the folds of the rumen and between papillae in the reticulum, where they adhered to knobbed parts of the mucosa. Occasionally the worms nipped off the mucosa sucked into the acetabulum leading to slightly hardened areas devoid of rugae and papillae. The geographical locations where the parasites were recovered are shown in Table 2.

The species recovered are here after described:

Calicophoron microbothrium Fischoeder, 1901 (synonym *Paramphistomum microbothrium*)

Description:

The body is conical. When fresh, the acetabulum and

Table 2. Prevalence of paramphistome species found in Masvingo-Manicaland Provinces

Location	No. of cattle examined	% Cattle infected	Range of parasite per animal	Species recovered
Buhera	70	50	25–2000	A; E; F
Chartsworth	32	13	10–2000	A; B
Chipinge	97	21	10–500	A; B; D; E
Chiredzi	545	19	40–1000	A; B; C; D; E; F
Chivi	20	15	20–300	A; E
Gutu	156	24	30–2500	A; B
Mashingwe	25	80	20–2000	A; B;
Masvingo	861	25	10–2000	A; B; C; D; E; F
Mutare	99	10	20–1000	A; B
Mwenezi	840	30	10–1500	A; B; C; D; E; F
Nuanetzi	349	30	50–2500	A; B; E; F
Nyanga	15	80	20–1500	A; B; E; F
Triangle Estates	116	4	20–200	A; E; F
Total	3225	25.41		

Key: A = *C. microbothrium*; B = *C. clavula;* C = *C. sukari;* D = *C. phillerouxi;* E = *C. calicophorum*; F = *C. raja*

Table 1. Morphometric data in µm for different species of paramphistomes found in Masvingo-Manicaland Provinces

Parameters	*C. microbothrium*	*C. clavula*	*C. phillerouxi*	*C. sukari*	*C. calicophorum*	*C. raja*
Body length	10000 ± 1682	8000 ± 250	7500 ± 1250	6635 ± 432	11000 ± 150	8700 ± 1150
Body breadth	2460 ± 45	3300 ± 225	2775 ± 13	2550 ± 112	4875 ± 780	3300 ± 600
Acetabulum diameter	2100 ± 291	2161 ± 270	1905 ± 98	1688 ± 108	3225 ± 710	1800 ± 600
Pharynx length	600 ± 56	750 ± 150	750 ± 45	713 ± 19	900 ± 135	900 ± 135
Anterior testis breadth	750 ± 75	1050 ± 75	1350 ± 150	900 ± 225	1275 ± 62	1800 ± 0
Anterior testis length	1200 ± 285	2250 ± 75	1800 ± 150	1750 ± 25	2175 ± 113	1950 ± 375
Posterior testis breadth	750 ± 75	1050 ± 75	1350 ± 150	825 ± 262	1275 ± 262	1800 ± 0
Posterior testis length	1200 ± 85	2250 ± 75	1800 ± 150	1538 ± 131	2175 ± 113	1950 ± 375
Pars prostatica breadth	210 ± 45	300 ± 60	360 ± 45	300 ± 30	300 ± 0	300 ± 15
Pars prostatica length	450 ± 15	450 ± 75	450 ± 15	420 ± 15	600 ± 150	300 ± 0
Eggs breadth	80 ± 3	60 ± 5	90 ± 5	85 ± 5	90 ± 10	90 ± 15
Eggs length	150 ± 4	120 ± 15	150 ± 5	158 ± 6	165 ± 8	180 ± 15

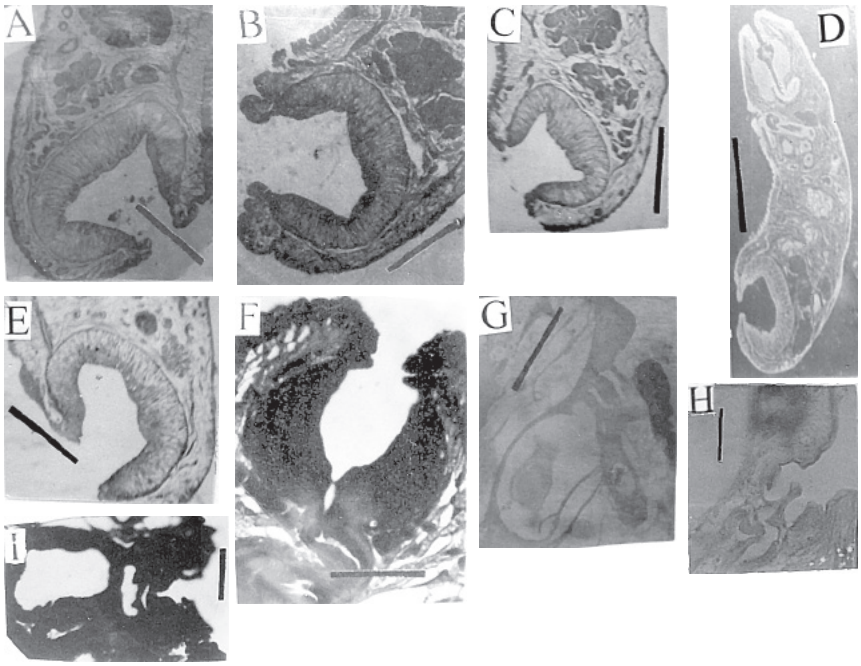

Fig. 1. A) ***Calicophoron microbothrium*** **posterior region showing testis lobulation (scale bar = 1,000 µm). B)** ***Calicophoron calicophorumposterior*** **region showing flaps around the acetabulum (scale bar = 1,000 µm). C)** ***Calicophoron sukari*** **posterior region (scale bar = 1,000 µm). D)** ***Calicophoron clavula*** **whole mount (scale bar = 2,000 µm). E)** ***Calicophoron phillerouxi*** **posterior region (scale bar = 1,000 µm). F)** ***Calicophoron microbothrium*** **pharynx region (scale bar = 500 µm). G)** ***Calicophoron phillerouxi*** **genital atrium (scale bar = 300 µm). H)** ***Calicophoron microbothrium*** **genital atrium (scale bar = 200 µm). I)** ***Calicophoron clavula*** **genital atrium (scale bar = 300 µm)**

the pharyngeal region are red while the rest of the body is yellowish-white. The tegument is marked by well-defined transverse wrinkles. The genital pore is clearly visible, lying encircled by an oval swelling. It lies about one fifth of the body length from the oral end. Measurements of diagnostic structures are shown in Table 1 for sectioned specimens and for flattened specimens.

The acetabulum is of the *Paramphistomum* (*sensu* Näsmark, 1937) (Fig. 1A). The dorsal exterior muscles are divided into two parts, the dorsal exterior one (de_1) and the dorsal exterior two (de_2). The (de_1) is larger and strongly developed than the (de_2) is smaller, sparse and more in numbers. The dorsal interior muscle units (di) are well developed and correspond to the ventral interior muscle layer (vi) in development. All the muscle units are of the same size in the (di) layer. In the (vi) the last ten muscles are smaller than the rest. In the ventral muscle layer (ve) the units are developed to the same extent as the (de_1). The muscle units occupying the central position are larger than the rest and give the impression of two muscle units joined together.

The pharynx is of the *Paramphistomum* type (*sensu* Näsmark, 1937) (Fig. 1F). The anterior sphincter is absent and interior circular layer consists of small wall defined units arranged in a single row along the whole length of the pharynx. These units are of the same size throughout. The interior longitudinal layer occupies from one quarter to one third of the thickness of the pharynx. The posterior sphincter and lip sphincter are absent. The exterior circular layer is well developed and clearly seen in groups of loosely packed units arranged along the whole length of the pharynx, about one fifth of the width of the pharynx from the exterior margin. The external longitudinal layer is very narrow abut one tenth of the width of the pharynx. The middle circular layer is absent. The basal layer is present and is made

of two layers the inner layer is less developed than the out layer.

The oesophagus has no bulbous expansion and its wall has two thin muscle layers, the inner layer is longitudinal, while the outer layer is circular. A thick tegument like layer lines its lumen. The gut caeca makes six identical bends on either side of the body. After the last bend that is on the ventral side, the terminal part of the caeca turns dorsally. Their blind ends lie on each side of the acetabulum. The testes are in tandem and they are deeply lobed.

The excretory bladder consists of gland-like tissue of irregular thickness. It opens through the excretory pore about one third of the body length from the posterior end. In flattened specimens the ovary lies between the posterior testis and the inner margin of the acetabulum. The Mehlis' gland lies ventral to the ovary in the median sections. The Mehlis' gland lies besides the ovary in flattened specimens. The Laurer's canal crosses the excretory canal and it opens dorsally close to it. Clusters of vitelline glands are conspicuous in flattened specimens. They extend from the posterior margins of the pharynx to the acetabulum. They occupy the space between the lateral margins of the body and the caeca. The genital atrium is of the microbothrium type (*sensu* Näsmark, 1937) (Fig. 1H). It lies close and posterior to the gut bifurcation.

The genital papilla lies either behind the ventral atrium or protrudes. The sphincter papillae is present and is made of loosely packed tissue. The ventral sphincter is absent. The radial musculature of the genital fold is easily seen and well developed. The *pars prostatica* is small in relation to the body size. It is barrel shaped. It opens to the genital papilla through the *ductus ejaculatorius*. Dorsally the *pars prostatica* connects the *pars musculosa* that is long and makes a few loops and then connects to the *vesicula seminalis* that makes many closely packed irregular loops. In the median sections the *vesicula seminalis* appears as a solid mass with an indistinct lumen. In flattened specimens it branches into two *vasa deferentia* that lead to the anterior and posterior tests. The uterus is wavy and runs dorsal to the testes close to the middle of the body. It opens into the genital papilla through the *metatherm. The eggs are filled with evenly scattered granules and are light blue-green.*

***Calicophoron clavula* Näsmark, 1937**
(synonym *Paramphistomum clavula*)

Description:

The body is conical (Fig. 1D). The tegument has transverse wrinkles. The colour of the body is pink when fresh. The genital pore is clearly visible lying flat with the body there is no swelling on this region as in *C. microbothrium*. It leis about one fifth of the body length from the oral end. Measurements of diagnostic features are shown in Table 1 for median sagittal sections.

The acetabulum is of the *Paramphistomum* type (*sensu* Näsmark, 1937). It follows the same description outlined for *C. microbothrium*. The pharynx is of the *Param-phis-tomum* type (*sensu* Näsmark, 1937), and therefore corresponds to the description for *C. microbothrium*. The oesophagus resembles that of *C. microbothrium.* The caeca makes six bends on either sides of the body after the last band that is towards the ventral side; the blind ends of the caeca turn dorsally. The caeca terminate on the lateral sides of the acetabulum. The excretory bladder opens three tenths of the body length from the posterior end. The testes are situated diagonally one behind the other in the mid-third of the body. They are deeply lobed. The ovary lies between the posterior testis and the margin of the acetabulum. The Mehlis' gland lies close to the ovary. The Laurer's canal runs directly to the dorsal side of the body and opens close to the excretory pore. Clusters of vitelline glands extend from the pharynx to the acetabulum between the lateral margins of the body and the caeca. The genital atrium lies close and posterior to the gut bifurcation. It is of the *Clavula* type (*sensu* Näsmark, 1937) (Fig. 1I).

The genital papilla has a large sphincter papilla. The genital sphincter is loosely packed and occupies a wide area. The radial muscles are easily seen. The *pars prostatica* is large and barrel-shaped. It connects to an inflated *pars musculosa* that makes a few coils before connecting the *vesicula seminalis* that makes many coils and then branches into two *vasa deferentia*, which lead to the anterior and posterior testis. In the median sagittal sections the *vesicula seminalis* appears as a solid mass with an indistinct lumen. The uterus is wavy and runs in the midline dorsal to the testes. The eggs are operculate and light green with granules evenly scattered in the yolk.

***Calicophoron phillerouxi* Dinnik, 1961**
(synonym *Paramphistomum phillerouxi*)

Description:

The body is conical, the surface with papi1lae around oral opening. The acetabulum is subterminal and of the paramphistomum type *(sensu* Näsmark, 1937). The pharynx is *Paramphistomum* type (*sensu* Näsmark, 1937) with weakly developed middle circular muscle units and the internal surface with small dome-shaped papillae. The oesophagus wall musculature is moderate and uniform in thickness with no bulb or posterior sphincter: hyaline layer throughout lines its lumen. Caeca are on lateral sides of the body, forming dorso-ventral bends, reaching the level of the acetabulum with the blind ends directed dorsally. Testes are deeply lobed and directly tandem in the posterior two thirds of body. The seminal vesicles are deeply coiled and thin-walled. The *pars musculosa* is well developed, thick-walled and convoluted and similarly the *pars prostatica* is also well developed (Fig. 1G).

The ovary is round to subspherical, post-testicular and anteriorly dorsal to acetabulum with the Mehlis' gland close to it. The Laurer's canal crosses the excretory vesicle and opens on dorsal surface. The uterus winds forward dorsal to testes and ventral to male ducts. The

vitellaria are in lateral fields, extending from level of pharynx to acetabulum. The genital pore opening is on ventral surface at level of oesophageal bifurcation or posterior to it, en-circled by a round or oval swelling. The terminal genital atrium is of the *Microbothrium* type (*sensu* Näsmark, 1937) (Fig. 1G). The excretory vesicle is dorsal to acetabulum.The excretory pore opens on the dorsal surface at level of posterior testis and anterior to opening of Laurer's canal.

***Calicophoron sukari* Dinnik, 1962**
(synonym *Paramphistomum sukari*)

Description:

The body is conical with papillae on the surface around oral opening, ventrally around genital pore region and on the acetabular opening. The measurements of diagnostic features for this species are shown on Table 1. The acetabulum is subterminal of the *Paramphistomum* type (*sensu* Näsmark, 1937) (Fig. 1C). There are no (de_2) muscle units. The pharynx is of the *Paramphistomum* type (*sensu* Näsmark, 1937) its internal surface has small dome shaped papillae. The oesophagus wall musculature is moderate and uniform in thickness, no bulb or posterior sphincter with the lumen having two types of lining. The anterior third is lined by a hyaline layer and the posterior two-thirds by ciliated epithelium. The caeca are on lateral sides of the body, forming dorsoventral bends, reaching level of acetabulum with blind ends directed ventrally.

The testes are deeply lobed and directly tandem in posterior two thirds of body. The seminal vesicle is thin walled and coiled. The *pars musculosa* well developed, thick and convoluted. The *pars prostatica* also well developed. The ovary is spherical, post-testicular and anterior to acetabulum. The Mehlis' gland lies close to ovary while the uterus winds forward dorsal to testes. The Laurer's canal crosses the excretory vesicle and opens on dorsal surface. *Vitellaria* lie in lateral fields, extending from level of oesophagus to acetabulum. The genital pore opens at the level of the oesophageal bifurcation. The terminal genital atrium is of the *Microbothrium* type (*sensu* Näsmark, 1937). The excretory vesicle is dorsal to the acetabulum. The excretory pore opens on the dorsal surface at level of posterior testis and anterior to the opening of Laurer's canal.

***Calicophoron calicophorum* Fischoeder, 1901**

Description:

Body pea-shaped, may bend slightly ventrally, distinctly broader in acetabulum region. The surface has papillae densely arranged around oral opening and extending ventrally to about midbody, except for an oval area surrounded by a ridge around the genital pore region, which is free of papillae. There are much smaller papillae also present randomly arranged around acetabulum opening.

The acetabulum is subterminal, of the *Calicophoron* type (*sensu* Näsmark, 1937) in median sagittal section (Fig. 1B). The pharynx is of the *Calicophoron* type (*sensu* Näsmark, 1937) with the internal surface lined by small papillae. The oesophagus may be straight or bend dorsally depending on state of relaxation of the parasite. The musculature of its wall is uniform in thickness and has no bulb or posterior sphincter, hyaline layer throughout its length lines its lumen. Caeca are in the lateral sides of body, forming irregular dorso-ventral bends reaching posterior level of acetabulum with the blind ends directed posteriorly.

The testes are deeply lobed, obliquely tandem or sometimes nearly side-by-side in middle of body. The seminal vesicles are thin-walled and coiled. The *pars musculosa* is long, well developed, thick-walled and convoluted. The *pars prostatica i*s well developed and almost straight. The ovary subspherical unlobed, post-testicular, dorsal to acetabulum and may be displaced on either side of median line by the excretory vesicle when this is full. The Mehlis' gland lies close to ovary.

The Laurer's canal crosses the excretory vesicle and opens to the dorsal surface. The uterus forms coils running forwards dorsal to testes, then ventral to male ducts. The vitellaria are in the lateral fields, extending from level of pharynx to acetabulum.

The genital pore is at the level of the posterior to oesophageal bifurcation and is surrounded by an oval area bounded by a ridge free of tegument papilla. The terminal genital atrium is of the *Calicophoron* type (*sensu* Näsmark, 1937), when everted it forms a genital pillar with genital pore opening at tip. The excretory vesicles dorsal to acetabulum and the excretory pore opens on the dorsal surface at level of posterior testis and anteriorly to opening of Laurer's canal.

***Calicophoron raja* Näsmark, 1937**

Description:

The body is conical, bending slightly ventrally and distinctly broader at acetabular region surface with papillae densely on area immediately around genital pore including genital pillar.

Acetabulum is subterminal, Pisum type (*sensu* Näsmark, 1937) with the (de_2) units fewer and more irregularly spaced. The pharynx is *Calicophoron* type (*sensu* Näsmark, 1937) with its internal surface lined by small papillae. The oesophagus may bend dorsally and has a musculature of its wall moderate and uniform in thickness. There is no bulb or posterior sphincter and the lumen lined by hyaline layer throughout. Caeca are on the lateral sides of body, forming dorsoventral bends, reaching level of acetabulum with blind ends directed ventrally.

The testes are lobed and obliquely tandem in posterior half of the body. The seminal vesicle is thin-walled and coiled. The *pars musculosa* is well developed, long, thick-walled and convoluted. The *pars prostatica* is well developed

The ovary is subspherical, unlobed, post-testicular and dorsal to acetabulum. The Mehlis' gland lies close to ovary with the Laurer's canal crossing excretory vesicle and opening on dorsal surface posterior to excretory pore.

The uterus winds forward dorsal to testes then ventral to male ducts. The *vitellaria* lie in lateral fields, extending from level of pharynx to acetabulum. The genital pore is at the level of the oesophageal bifurcation. The terminal genital atrium is of the *Raja* type (*sensu* Näsmark, 1937) characterized by presence of a genital pillar or column, which is covered by densely, arranged small tegumental papillae. The excretory vesicle is dorsal to the acetabulum. The excretory pore opens on the dorsal surface at level of posterior testis and anteriorly to the opening of Laurer's canal.

DISCUSSION

Although the percentage of cattle infected is high the number of parasites per herd was relatively low. It should be noted that the percentage take of paramphistomes is less than 20% (16, 21). These seemingly low figures of less than 2,000 in the stomach will have preceded higher numbers in the duodenum where the pH and oxygen tension is low and the parasites are normally buried in the submucosa away from the harsh conditions in the gut lumen.

The fluctuation of numbers among individual animals could arise as a result of some cattle drinking from drying water bodies where the levels of cercaria always tend to increase per unit volume of water (21). Development of immunity in cattle is low thus unlikely to affect parasite loads significantly (15). The areas sampled are geographical located such that they reflect the true distribution of the paramphistomes in the provinces. No area showed low levels of parasites.

For effective diagnosis and control of parasitic diseases, it is essential that parasite isolates be accurately identified by method that is simple and reproducible (19). In our study we have positively identified six species using previously constructed identification keys proposed by Näsmark (20), Yamaguti (31) and Eduardo (12).

C. microbothrium was identified based on the histology of the structures of the acetabulum pharynx and the genital atrium, which are in agreement with previous descriptions (16, 25, 31). It seems that *C. microbothrium* is the most frequent ruminant paramphstome in Africa in view of the fact that it has been reported in all places where studies on ruminant paramphistomes have been made (6, 13, 20, 21, 23, 25, 26, 27, 30). The present study shows it is the most frequently encountered paramphistome in Masvingo and Manicaland Province.

C. clavula, which resembles *C. microbothrium* in many respects, was identified based on the histological structures of the genital atrium, which are peculiar to this species, which is in agreement with the descriptions by other workers (10, 12, 20).

C. philerouxi, which resembles *C. microbothrium* in many respects, was identified based on the histological structures of the *pars musculosa* and genital atrium, which are reduced and was further confirmed by the deeply lobed tandem testes, which is in agreement with the descriptions of other workers (10, 12, 20).

C. sukari which resembles *C. microbothrium* in many respects was identified based on the histological structures of acetabulum which lack (de_2) and the tegument papillae which are more pronounced in the oral pore than in than in the genital pore, a features which are peculiar to this species within this genus, which is in agreement with earlier findings (12, 20).

C. calicophorum was identified on the basis of the genital atrium, which protrudes as a long shaft, well-developed *pars prostatica*, and deeply lobed testes, which correspond to similar diagnosis for this species in previous literature (12, 20, 31).

C. raja, which resembles *C. calicophorum* in many respects was identified based on the histological structures tandem testes and of the genital atrium which has tegument papillae on its protruding shaft both which are in agreement with the descriptions in previous literature (12).

C. microbothrium was found in all areas sampled and is known to cause paramphistomiasis even when it occurs in small numbers (15, 22). Chronic infections with paramphistomes can result in loss of, weight, milk production and plasma proteins (16, 17, 18). The results of our study point to the fact paramphistome infections should not be ignored as untold loses due to them could be occurring unnoticed.

C. calicophorum was previously reported in wild life in Zimbabwe (23). Our study shows it is present in cattle in Manicaland and Masvingo provinces. Very low numbers of this species can also causes serious disease conditions therefore there is need to establish its epidemiology (16, 21)

While the parasites described for the first time in the Manicaland and Maswingo provinces have not been assayed for their involvement in disease it should be noted that these parasites were previously reported in Kenya (20, 23). Their occurrence in Manicaland and Masvingo has the following implications: firstly that these parasites have always been present here but no one had come across them in their study, and secondly the parasites were introduced into Manicaland and Masvingo province by the movement of cattle across borders and have eventually now been established. There is a probability that they could be infecting the same snails as other described in which case they could affect its development in the snail synergistically or antagonistically (1, 3, 29).

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THE INFLUENCE OF DIFFERENT TREATMENT LENGTH ON THE INDUCTION OF MICRONUCLEI IN BOVINE LYMPHOCYTES AFTER EXPOSURE TO GLYPHOSATE

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ABSTRACT

Glyphosate is a broad-spectrum herbicide widely used to kill unwanted plants both in agricultural and in non-agricultural landscapes. The herbicide has been expanded in use particularly in applications involving plant varieties that are genetically modified to tolerate glyphosate treatment. To our knowledge, there is a minimum of reports available describing the cytotoxicity or genotoxicity effects of glyphosate on domestic animal cells even though many of the environmental mutagens are associated with a reduced productive and reproductive efficiency of livestock. The purpose of this study is to provide evidence of the genotoxic potential of glyphosate and different treatment length on *in vitro* cultures of bovine lymphocytes using CBMN assay. Cells were cultured as whole blood and treated with glyphosate at doses between 28 and 560 µmol.l^{-1} for twenty-four hours and forty-eight hours. Significant elevations in the induction of micronuclei were found after the application of glyphosate at a dose of 280 µmol.l^{-1} ($P < 0.05$, donor A) and at a dose of 560 µmol.l^{-1} ($P < 0.05$, donor B) only for forty-eight hours. The treatments with glyphosate for forty-eight hours induced simultaneously decreases in the percentages of mononucleated cells and increased percentages of bi-, tri- and tetranucleated cells in comparison with treatments lasting twenty-four hours.

Key words: bovine peripheral lymphocytes; glyphosate; micronucleus; treatment length

INTRODUCTION

Glyphosate was first reported as a herbicide in 1971. Since then it has been sold around the world and is formulated into dozens of products by many pesticide companies.

Glyphosate belongs among nonselective herbicides that inhibit plant growth through interference with the production of essential aromatic amino acids by the inhibition of the enzyme enolpyruvylshikimate phosphate synthase (21). It is expanded in use particularly in applications involving plant varieties that are genetically modified to tolerate glyphosate treatment.

In these cases weeds are killed but the crop is unaffected.

While glyphosate itself may be relatively harmless (6, 17) some of the products with which it is formulated, namely surfactants, have a less benign reputation. Adam *et al.* (1) have found that preparations that contained a surfactant POEA (polyoxy-ethyleneamine) were more toxic than glyphosate alone.

According to the World Health Organisation, the oral LD_{50} in rats of pure glyphosate is 4.320 mg.kg^{-1}. In spite of low toxicity, some laboratory studies have reported adverse effects in each standard category of testing: eye and skin irritation (20), cardiac depression (19), vomiting (9), pulmonary oedema (9, 14), reproductive problems (16, 22), non-Hodgkin's lymphoma (5).

A part evaluation of glyphosate has focused on the genotoxic effects of glyphosate but some *in vivo* and *in vitro* studies have produced equivocal results (11, 12, 3, 15). These conflicting genotoxicity results may be attributable to different methodologies and treatment protocols. For example, glyphosate used in commercial products differs from pure glyphosate or

a different treatment length was used. The treatment protocol of seventy-two hours (glyphosate was added immediately after mitogen stimulation) used by Lioi *et al.* (12) is unusual because chemicals that reliably produce chromosomal aberrations in stimulated lymphocytes can do so after a four hour exposure and often twenty hours of exposure (21). As an alternative to classical metaphase analysis, the frequency of micronuclei in treated cells provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosomal loss (4).

In the cytokinesis-block method (CBMN), enumeration of micronuclei is restricted to cells that are blocked from undergoing cytokinesis and which are consequently easily recognisable as large binucleate cells. This method is feasible because it is possible to accumulate CB cells by pulsing cultured lymphocytes with cytochalasin B (Cyt-B), an inhibitor of cytokinesis. The level of CB cells accumulated will depend on the proportion of cells in culture that respond to mitogen. The number of CB cells varied from individual to individual with frequencies generally between 5 % and 50 %.

Here, we present our *in vitro* findings on the genotoxic and cytogenetic effects of glyphosate in bovine lymphocyte cultures using biomarkers of chromosomal damage (micronuclei) and cell cycle kinetics (CBPI) comparing *twenty-four hour and forty-eight hour treatment protocols.*

MATERIALS AND METHODS

Lymphocyte cultures

Adding 0.5 ml blood to set up whole blood cultures to 5 ml culture medium consisting of RPMI 1640 supplemented with 15 % foetal bovine serum, L-glutamine, 15 µM HEPES (Sigma, St. Louis, MO, USA), 250 U.ml^{-1} penicillin, 250 µg.ml^{-1}, streptomycin and phytohaemagglutinin (PHA, 180 µg.ml^{-1}, Welcome, Dartford, UK).

Chemicals

Isopropylamine salt of glyphosate, Monsanto, Antwerp, Belgium

Components	CAS No.	EINECS/ ELINCS No.	% by weight (approximate)
Isopropylamine salt of glyphosate	38641-94-0	254-056-8	62
Inert ingredients			38

Glyphosate was diluted in sterile water immediately prior to treatment and added to the cultures at concentrations of 28, 56, 140, 280, and 560 µmol.l^{-1}. The test chemical was added to the cultures twenty-four hours and forty-eight hours after they were initiated (treatment time was forty-eight hours and twenty-four hours, respectively). The herbicide was present in lymphocyte cultures until the end of cultivation at seventy-two hours. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 µM) was used as positive control agent.

Cytochalasin B (Sigma, St. Louis, MO, 6 µg.ml^{-1}) was added to the cultures at forty-four hours post-initiation as described by Fenech and Morley (4).

MN analysis

For the micronucleus test, at least 1000 binucleated lymphocytes (those that have undergone one mitotic division) were scored for the number of micronuclei.

The cytokinesis block proliferation index (CBPI) was calculated by scoring at least 500 cells per dose or sample (18). This index is based on the formula:

CBPI = [MI + 2MII + 3(MIII + MIV)]/total, where MI-MIV represent the number of cells with one to four nuclei. As a result of the addition of cytochalasin B, nuclear division was not affected, but cell division was arrested. Cells that underwent one division would have two nuclei and cells that underwent two divisions would have three or four nuclei. Cells that responded to PHA stimulation but did not complete one division had only one nucleus.

The statistical evaluation of the results was carried out using Fisher's exact test for micronucleated cells and χ^2 test for CBPI.

RESULTS

Genotoxic effects of glyphosate in whole bovine lymphocyte cultures

Tables 1 and 2 show the frequencies of MNi obtained after treatment with the herbicide glyphosate. In each table, the data obtained from the different experimental conditions are shown: treatments lasting for twenty-four hours and forty-eight hours. Whole blood lymphocyte cultures were established from venous blood of two donors and treated with a broad range of concentration of glyphosate (28—560 µmol.l^{-1}). The slight increases in the MNi frequency ($P < 0.05$) were found in both donors only at the higher glyphosate doses (280 µmol.l^{-1} and 560 µmol.l^{-1}, respectively) at the treatment lasting for forty-eight hours. The dose-responses were not observed in any of donors. The results from the experiments for twenty-four hours showed no statistically significant increases in the frequency of micronuclei.

In summary, the genotoxic effect of glyphosate by the CBMN assay was minimal.

Proliferative effects of glyphosate

Table 1 and 2 also indicate the percentages of cells with different numbers of nuclei (1N—4N). The comparisons between treatment times were done for each concentration of glyphosate. The treatments with glyphosate for forty-eight hours induced decreases in the percentages of mononucleated cells but increases percentages of bi-, tri- and tetranucleated cells in comparison with treatments lasting for twenty-four hours.

Cell cycle progression can be delayed as a consequence of cellular toxicity due to *in vitro* treatments (18). In order to control lymphocyte proliferation, the cytokinesis

Table 1. Induction of micronuclei (MN) in bovine lymphocyte cultures exposed to glyphosate and percentages of cells with different numbers (N) of nuclei after 24 hour and 48 hour treatment times: Donor A

Concentration µmol.l^{-1}	Treatment time	% cells with indicated No. of nuclei 1 N	2 N	3 N	4 N	CBPI	Total MN
Control	24 h	74.6	22.0	1.2	2.2	1.29	14
	48 h	51.2	34.2	6.0	8.6	1.63	20
28	24 h	67.0	29.0	3.0	1.0	1.37	9
	48 h	51.0	32.0	8.0	9.0	1.66	19
56	24 h	77.0	22.0	0.6	0.4	1.24	12
	48 h	49.0	32.8	10.0	8.2	1.55	26
140	24 h	81.0	18.2	0.4	0.4	1.20	14
	48 h	43.8	44.2	6.8	5.2	1.68	22
280	24 h	82.0	17.4	0.6	0.0	1.19	20
	48 h	49.0	32.8	8.2	10.0	1.55	40*
560	24 h	80.0	19.2	0.4	0.4	1.20	14
	48 h	48.4	37.0	8.0	6.6	1.66	29
MMC (0.4 µM)	24 h	44.0	48.0	2.8	5.2	1.69	35***
	48 h	63.8	30.4	4.0	1.8	1.42	55***

*—Statistical significance at $P<0.05$; ***—Statistical significance at $P<0.001$

Table 2. Induction of micronuclei (MN) in bovine lymphocyte cultures exposed to glyphosate and percentages of cells with different numbers (N) of nuclei after 24 hour and 48 hour treatment times: Donor B

Concentration µmol.l^{-1}	Treatment time	% cells with indicated No. of nuclei 1 N	2 N	3 N	4 N	CBPI	Total MN
Control	24 h	80.2	18.4	1.0	0.4	1.21	15
	48 h	59.6	30.2	2.8	7.4	1.51	13
28	24 h	83.8	14.2	0.6	1.4	1.18	12
	48 h	67.8	23.8	5.0	3.4	1.41	18
56	24 h	78.4	18.6	1.4	1.6	1.25	10
	48 h	66.0	25.6	4.0	4.4	1.43	13
140	24 h	80.8	17.2	1.2	0.8	1.21	16
	48 h	65.0	25.0	5.2	4.8	1.45	20
280	24 h	78.8	19.2	0.8	1.2	1.24	11
	48 h	61.8	27.4	5.0	5.8	1.49	23
560	24 h	79.2	18.2	1.4	1.2	1.23	15
	48 h	64.4	24.6	5.6	5.4	1.47	27*
MMC (0.4 µM)	24 h	57.0	38.6	2.0	2.4	1.50	39***
	48 h	40.0	49.6	5.4	4.8	1.75	36***

*—Statistical significance at $P<0.05$; ***—Statistical significance at $P<0.001$

block proliferation index (CBPI) has been used in which both tri- and tetranucleated cells are supposed to be in their third cell cycle. Surrallés *et al*. (18) assume that these cells are derived from binucleated cells undergoing one further mitosis. However, a statistically significant inhibition of CBPI was not observed with increased doses of glyphosate or treatment length in both donors.

DISCUSSION

Several studies have previously reported an association between glyphosate exposure and adverse effects in human (5) but there is a lack of reports available describing the cytotoxicity or genotoxicity effects of glyphosate on domestic animal cells even though many of environmental mutagens are associated with a reduced productive and reproductive efficiency of livestock.

We studied the genotoxicity and proliferation as a result of glyphosate exposure of bovine lymphocytes *in vitro*. Our study of the genotoxic effects of herbicide in cell cultures showed micronuclei increases only at higher glyphosate doses for forty-eight hours. The results from the experiments for the observation of maximum frequencies of micronucleated fibroblastic cells after seventy-two and ninety-six hours of treatment suggest

that long treatment times favour an accumulative effect. It would be advantageous for cells undergoing more than one cell cycle for the optimization of the results (13).

Lioi *et al.* (12) have reported that glyphosate produced an increased frequency of chromosomal aberrations in cultured bovine lymphocytes at concentrations ranging from 17 to 170 μM for herbicide and used lymphocytes separated by Ficoll-Hypaque gradient density that were cultured for seventy-two hours. From the results reported by Li and Long (10) it is evident that administration of glyphosate to rats did not produce an increase in frequency of chromosomal aberrations. Similarly, De Marco *et al.* (3) and Rank *et al.* (15) have found that glyphosate alone was not active in chromosomal damage.

Bolognesi *et al.* (2) have obtained in Swiss/CD-1 mice, a weakly positive glyphosate-induced increase in the bone marrow micronucleus assay. Their results were in contrast with those of Kier *et al.* (7) who reported no increased micronucleus formation.

Cell cycle related parameters are also evaluated to determine their association with micronuclei induction. CBPI is a most comprehensive test because it covers all stages of cell cycles. *Lymphocyte proliferation* is an extremely complex system influenced by many factors. Micronuclei can only be expressed if cells divide. Lymphocytes from different individuals respond quite differently to PHA so that the proportion of dividing cells in culture differs from individual to individual.

Kirch-Volders et al. (8) have reported that there are not enough data to define the most appropriate treatment/harvest times for CBMN test. They suppose that treatment should ideally cover cells in different cell cycle stages. We have carried out comparisons between twenty-four hours and forty-eight hours treatment protocols. There were observed differences in response between the treatment times in lymphocyte cultures. The frequencies of binucleated and multinucleated cells increased in dependence on treatment length in all the concentrations tested.

In conclusion, glyphosate in a treatment lasting forty-eight hours only weakly increased the frequency of micronuclei in bovine lymphocyte cultures but more complete covered cells in different cell cycle stages in comparison with treatment lasting twenty-four hours. Further studies are needed in this area, as genetically modified plant varieties are likely to be used more extensively throughout the food chain in humans and animals.

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THE SEROPREVALENCE OF *Ehrlichia canis* IN DOGS IN EAST SLOVAKIA

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SUMMARY

The etiologic agent of canine monocytic ehrlichiosis (CME) is the rickettsia *Ehrlichia canis*. They are small Gram-negative coccoid bacteria that are parasitic on circulating monocytes in clusters of organisms, called *morulae*. *E. canis* is transmitted by the brown dog-tick *Rhipicephalus sanguineus*. The distribution of CME is related to the distribution of the vector and has been reported occurring in Asia, Africa, Europe and America. To date no case reports of CME have been documented in Slovakia. This study is to find out the seroprevalence of *E. canis* in dogs in Slovakia. Serum samples from 78 dogs were collected from different parts in Eastern Slovakia, and were tested by an indirect immunofluorescence technique (IFA) and by ELISA Immunocomb analysis (Biogal Kibbutz Galed Israel). On the IFA analysis 29 dogs (37.2 %) reacted to *E. canis* antigens (with a titer 1:64). According ELISA Immunocomb analysis (Biogal Kibbutz Galed Israel) positive results are expressed in scales units 1–6; degree > 3 is considered as positive. Sixteen dogs (55.2 %) had antibody titers of 'S' units < 3, eleven dogs (37.9 %) had antibody titers of 'S' units = 3, one dog (3.4 %) had antibody titer of 'S' units = 4, and 1 dog (3.4 %) had an antibody titer of greater than 5 'S' units.

Inspite of the fact that this is the first finding of *E. canis* antibodies in dogs in Slovakia, it is recommended positive sera with WI and/or PCR are investigated, to characterise and confirm the *Ehrlichia* species.

Key words: ***Anaplasma phagocytophila;* antibodies; dogs; *Ehrlichia canis;* rickettsia; seroprevalence**

INTRODUCTION

Ehrlichiosis is a tick-transmitted rickettsial disease in which, depending on the *Ehrlichia* spp. involved, mononuclear cells, granulocytes or platelets are subject to parasites. In dogs raised in endemic areas, infection is common but this rarely develops into clinical disease. However, the disease is very common in dogs imported into endemic areas.

Canine ehrlichiosis was first recognized as a disease entity in dogs in Algeria in 1935, and has since been reported world wide (8, 4).

Ehrlichia canis is a small, Gram-negative, coccoid bacterium that lacks lipopolysacharide endotoxin and is parasitic on circulating monocytes intracytoplasmically in clusters of organisms called *morulae (11).*

The distribution of Ehrlichia canis is determined by the distribution of the vector ticks and this includes most of the tropics, subtropics, warm temperate climates and Finland (4, 27, 14). *E. canis,* is transmitted by *Rhipicephalus sanguineus* (10) and experimentally by the tick *Dermacentor variabilis* (12). Infection occurs *via* salivary secretions when an infected tick ingests a blood meal. The incubation period of *E. canis* infection varies between eight and twenty days followed by acute, subclinical and in some cases, chronic phases. It is an obligatory intracellular parasite multiplying inside cytoplasmatic vacuoles in mononuclear phagocytic cells. Spread haematogenously, the parasite causes splenomegaly, hepatomegaly and lymphadenopathy. Vasculitis can develop affecting various organs including the lung, kidneys and possibly the meninges.

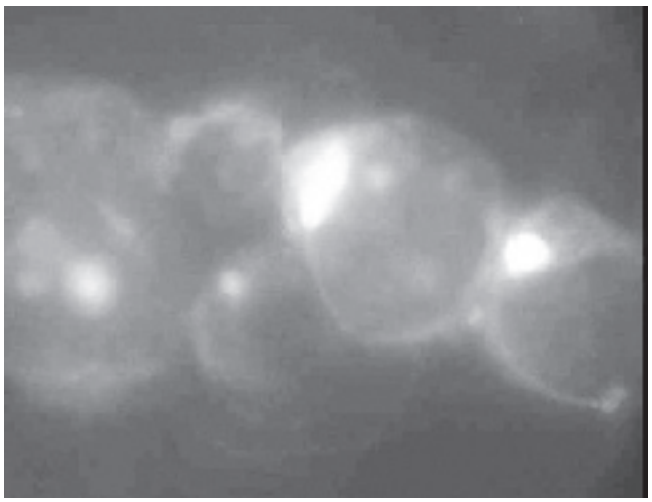

Fig. 1. IFA

Fig. 2. ELISA Immunocomb

The immune mechanism plays a major role in the pathogenesis of the disease. Thrombocytopenia, anaemia and leukopenia are often seen during the acute phase due to bone marrow suppression and may persist during the subsequent subclinical phase. The disruption of platelets can result in haemorrhages, especially epistaxis. Severely affected animals are susceptible to secondary infection (14).

E. canis specific immunoglobin G (IgG) antibodies can be detected by IFA 21 days after infection. Titres persist in untreated dogs, and in most instances gradually decline after effective treatment over a period of three to nine months (25, 1). Increased *E. canis* titres may persist in dogs that have clinically recovered from ehrlichiosis for several years (16). A positive IFA titre is considered indicative of infection or past exposure. But antibody generated to one species of *Ehrlichia may cross-react with another species that falls within the same genogroup.*

It is recommended to investigate positive sera with WI and/or PCR to characterize and specify the Ehrlichia species.

We present here the first report of the detection of E. canis specific antibodies in Slovakia.

MATERIAL AND METHODS

Seventy-eight sera from dogs seropositive for Lyme disease were used. Most of the animals were hunting dogs from eastern Slovakia and, they had suffered exposure to ticks. This sample was classified based on age, sex and breed of the dogs (36 males, 42 females; 16 dogs were less that one year old, 51 adult and 11 more than 8 years old). The sera were stored at –20 °C until assayed for antibodies titres.

Antibody detection by IFA: The IFA test was performed with *E. canis* (Israel strain No. 611, Dr. Trevor Waner, Israel Institute for Biological Research, Israel) as described by Ris- tic *et al.* (19) and Keysary *et al.* (13). Briefly, *E. canis*-infected DH82 cells (> 80 % cells infected) were pelleted and then resuspended in growth medium, 5 µl of the twofold serum dilutions were placed on glass slides prepared with acetone-fixed infected DH82 cells. The slides were incubated in a humidified chamber at 37 °C for thirty minutes, washed gently with water, air-dried and subsequently 5 µl of anti-dog IgG:FITC conjugate solution (Sigma chemical Co., USA) was added to each well. After similar incubation the slides were washed, dried and examined under fluorescent microscope.

Sera reactive at the dilution of 1:64 were considered positive.

***E. canis* antibody ELISA assay:** *E. canis* IgG antibody titres were determined by using a commercial ELISA test kit containing combs sensitised with *E. canis* antigen derived from mouse J774.A1 infected cells (ImmunoComb, Biogal Kibbutz Galed, Israel).

The sera were diluted 1:36 in buffer and incubated with the antigen spots for five minutes. After washing to displace bound antibodies, the combs were allowed to react for five minutes with whole molecule goat anti-dog IgG alkaline phosphatase conjugate (Jackson Immunosearch Laboratories Inc., Baltimore, PA). After two successive washing steps, bound antibodies were detected with a precipitating chromogen, 5-bromo-4-chloro-3-indolyl phosphatase and nitro-blue tetrazolium (BCIP/NBT) (Biosynth International, Skokie, IL). The concentration of *E. canis* antibodies for each sample was reported employing a scanning designed for automatic reading of the colour

Table 1. The seroprevalence of *E. canis* among dogs in eastern Slovakia

Variable	Number of dogs	Positive	Prevalence %
Sex			
Male	36	13	36.1
Female	42	16	38.1
Total	78	29	37.2
Age			
Young (1 year)	16	7	43.8
Adult (1 to < 8 years)	11	19	37.3
Old (> 8 years)	11	3	27.3
Breed			
Pure bred	74	28	37.8
Mixed breed	4	1	25.0

intensity of the reaction spots on the comb (Immunoscan, Organics, Yavneh, Israel).

RESULTS

Seventy-four dogs were apparently healthy and four dogs had symptoms of weakness, gastrointestinal problems and movements' difficulties. 37.2 % (29/78) of the sera tested were reactive to *E. canis antigens* (titer 1: 64) by using IFA (Fig. 1).

The results of the ELISA ImmunoComb analysis showed that 16 dogs (55.2 %) had antibody titers of 'S' units = 2, eleven dogs (37.9 %) had antibody of 'S' units = 3, one dog (3.4 %) had antibody titer of 'S' units = 4, and one dog (3.4 %) had an antibody titer of greater than 5 'S' units (Fig. 2). Seroprevalence was similar for male and female with 38.1 % and 36.1 % respectively. There were no differences in the prevalence of antibodies for young dogs (43.8 %), adult dogs (37.3 %), or old dogs (37.3 %). Most of the dogs were pure bred (94.9 %), out of them 28 dogs (37.8 %) were positive (Table 1).

DISCUSSION

To date no case reports of CME (canine monocytic ehrlichiosis) have been documented in Slovakia. No publications have been presented establishing whether the disease exists in this part of the world, although the disease is considered to be distributed worldwide. This study sets about, with the aid of the Immunocomb and IFA, to establish the prevalence of the exposure of dogs to *E. canis.*

To date, the only country in the region, which has shown the presence of E. canis antibodies, is Poland (17), no such evidence has been noted in other countries in the region, such as Hungary, Ukraine, Czech Republic and Romania. Trotz-Williams and Trees (24) have shown in their study that among European countries, where *E. canis* occurs with the highest prevalent rate, are Italy, Spain, Portugal, as well as France and Germany.

The data obtained in this study have shown that E. canis antibodies were found in 37.2 % of the dogs tested, and the seroprevalence of the female and male was similar with 38.1 % and 36.1 %, respectively.

The most acceptable serological test for suspected ehrlichial infection is the IFA test (19, 4, 11, 15, 27); a positive IFA titer is considered indicative of infection or past exposure. It does not detect the actual ehrlichial organism, but rather ehrlichial-reactive antibody in the serum.

Dot-ELISA (ImmunoComb, Biogal) tests have recently been developed for use in the clinic. These tests require the minimum of equipment and will make serologic diagnosis of CME available on a wider basis.

When assessing IFA antibody titers to *E. canis* in dogs it is essential that the diagnostician take into account the range of cross-reactivities that may confound the diagnosis (27). In areas endemic to other *Ehrlichia* species, cross reactivity between *E. canis* and *E. ewingii*, *Neorickettsia risticii* and *Anaplasma phagocytophila* should be taken into consideration.

The *Anaplasma phagocytophila* genogroup is chiefly reported as a febrile disease of goats, sheep, cattle, horses, dogs, and humans in the UK, Netherlands, Scandinavia, Spain, France, Germany, Switzerland, Italy, Greece, Slovenia, Poland, Slovakia, and Southeast Europe including Bulgaria, Albania, and Turkey (18, 9, 3, 6, 23, 7, 5, 21). Evidence of natural *A. phagocytophila* infection has also been detected in foxes, free-ranging jackals and deer in Switzerland, Israel, and southern Germany, respectively (18, 4, 27).

Pusterla et al. (18) have shown that the prevalence of *E. canis* and *A. phagocytophila* among dogs in Switzerland was 2.2 % and 7.5 %, respectively. However, most of the dogs that were found positive to *E. canis* antigen had a history of travelling to countries, where *E. canis* is endemic. Špitálska and Kocianová (22) and Derdáková *et al.* (7) have shown evidence for *A. phagocytophila* in *Ixodes ricinus* ticks from southwestern and eastern Slovakia.

The vector *Rhipicephalus sanguineus* is believed to be the major vector of *E. canis* worldwide (10, 28, 15). The distribution of *R. sanguineus* is established in the tropics, subtropics, and warm temperate climates, respectively. Yet *R. sanguineus* can also occur in colder areas of Europe such as London (UK) and Denmark (20). Ploneczka and Smielewska-Los (17)) have reported also one case of *R. sanguineus* occurrence in Poland. Nevertheless, we should not exclude *E. canis*, since positive results in both serological tests have been confirmed.

Due to the confusion caused by cross-reacting infections, it may be desirable under optimal conditions to test the sera against a number of agents. Microscopic demonstration of typical intracytoplasmic *E. canis morulae* in monocytes is seen only during acute stage of the disease. Therefore, other methods as PCR, Western immunoblotting should be carefully evaluated for final clarification of the agent involved.

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INDUCING HETEROPHILIA IN THE CHICKEN INTESTINE BY *Eimeria colchici*

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ABSTRACT

The aim of this study was to create a concept of "preventive activation" of heterophils as a means of promoting the defence of poultry against pathogens by the introduction of a pheasant *Eimeria colchici* into Leghorn chicks as a non-specific host and to study the activation and migration of heterophils to the site of infection. In the Leghorn chicks, the total number of heterophils counted in the different sites of intestine was smaller than in the natural host. The number of counted heterophils showed, as in natural host, a gradual increase along with the development. In the Leghorn chicks, we saw only a first change schizont development. The low number of heterophils with Leghorn chicks as a part of host inflammatory response is due to an incompletely developed parasite along with minimal tissue damage. These changes can be thought as a model for the preventive activation of heterophils as a means of promoting the defence of poultry against pathogens and finally as an "immune-potentiality" creation in immune responses to any infectious organisms.

Key words: *Eimeria colchici;* heterophils; specific and non-specific host

INTRODUCTION

Heterophils, the avian counterpart to mammalian neutrophils are important mediators of natural resistance against bacterial infections in poultry These phagocytic cells act early during an infection to restrict bacterial growth to a level that can be eliminated by the acquired host defences generated later. Heterophils are especially important in young chickens that have not yet developed an acquired immunity to pathogens (10).

Their importance in host defences invading pathogens appears to be due to their ability to ingest and kill a wide variety of microbial pathogens. Nevertheless, the question remains whether these cells can be readily activated so that their functions are significantly enhanced. The term "activation", as used here, is described as the rapid and selective up or down regulation of the physiologic and biochemical properties and functions of phagocytes (1).

In mammals activation is triggered by a number of different chemical signals, of both host and pathogen origin, produced locally at the site of infection of inflammation that regulate the functions of neutrophils (11). Macrophages activation has been carefully investigated in poultry (3), whereas, few reports have been documented the activation of its heterophils. Natural activation of heterophils in the intestine is followed by infection of extracellular parasites that are too large to be consumed by phagocytes (17).

The majority of the named species of *COCCIDIA* within the family Eimeriidae are intestinal parasites belonging to the genus *Eimeria (12).*

There is a marked host and characteristic site specificity of *Eimeria* spp. in the digestive tract. It is rare for this protozoan to occur naturally in more then one host species (2). Doran (4), however, was able to produce an experimental, patent infection in Leghorn chickens, chuckar partridge, ring-necked

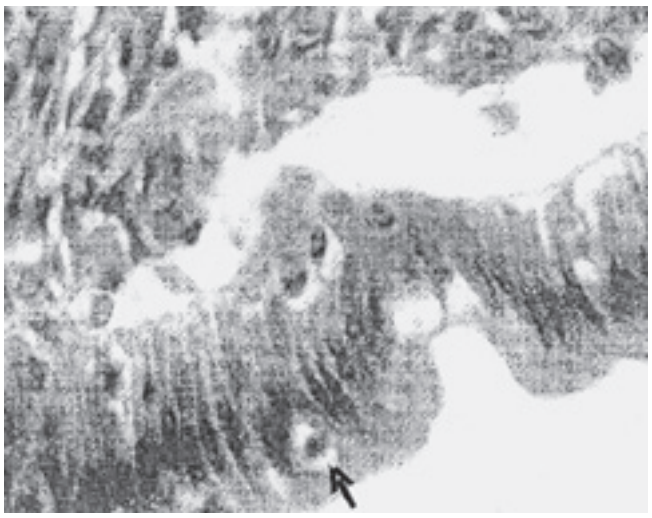

Fig. 1. The first generation of schizonts (arrow) of *Eimeria colchici* in the duodenal villi of a chicken intestine 12 h p. i. Haematoxylin-eosin, × 800

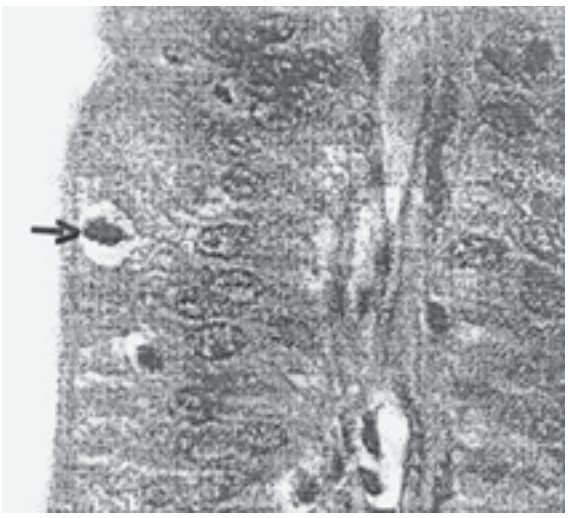

Fig. 2. The first generation of schizonts (arrows) of *E. colchici* in the epithelial cells of the *caecum* of a chicken 12 h p. i. Haematoxylin-eosin, × 800

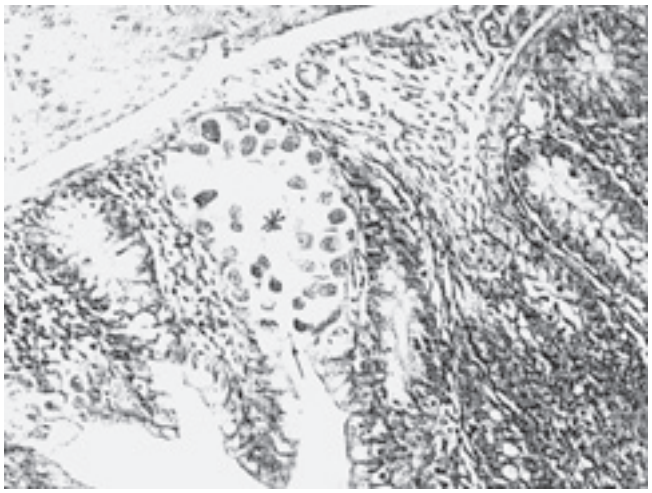

Fig. 3. Numerous first generations of schizonts (asterix) of *E. colchici* in the caecal crypts of pheasant chicks 24 h p. i. Haematoxylin-eosin, × 400

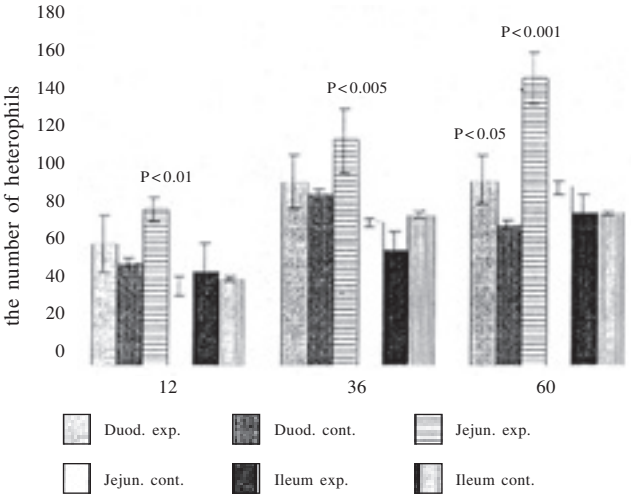

Fig. 4. The number of heterophils in the small intestine of Leghorn chicks after *E. colchici* infection (1520 μm²)

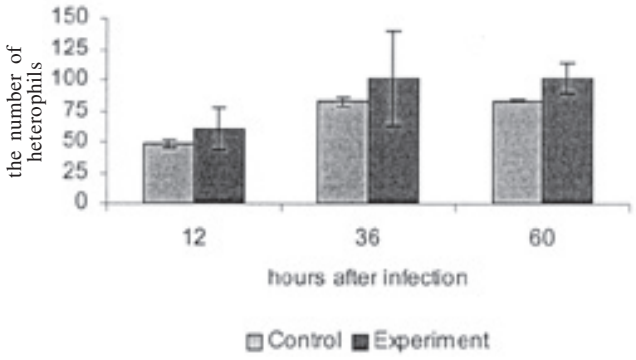

Fig. 5. The number of heterophils in the *caecum* of chicken after infection by *E. colchici* (1520 mm²)

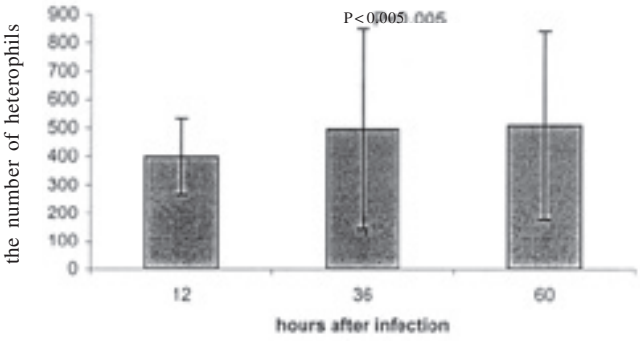

Fig. 6. The average number of heterophils in the *caecum* of pheasants after infection by *E. colchici*

pheasant and bobwhite quail using a turkey coccidium, *Eimeria dispersa*. It has been shown previously that infection with *Eimeria colchici* from a pheasant produces an infection in turkeys (15) and in chickens where only the first generation of schizonts was found in the epithelial cells of the intestine. There was an infiltration of heterophils and mononuclear cells in the intestine and a marked increase in CD3 positive cells from 36 hours post infection onwards (14).

The aim of this work is to create a concept of "preventive activation" of heterophils as a means of promoting the defence of poultry against pathogens. Preventive activation involves the introduction of a pheasant Eimeria colchici into chickens as a non-specific host and to study the activation and migration of heterophils to the site of infection.

MATERIAL AND METHODS

The pathogen, experimental animals and description of experiment

A pure culture of *Eimeria colchici* was obtained by means of single oocysts isolation agar (9). Twenty-five coccidia free two-week old pheasant chicks were infected intraproventriculary with single oocysts of the pathogen. The chicks were killed at day 6 post infection. Oocysts were collected from faeces and after sporulation in 2 % potassium bichromate solution they were used for the experimental infection.

Thirty coccidia free 14-day-old Leghorn chicks and thirty pheasant chicks were divided into groups as follows:

a) The experimental groups. Each of the chicks was *per os* inoculated with 1.10^5 sporulated oocysts and pheasant chicks with a suspension of pure strains of *E. colchici* at the dose of 5000 (only 0.5 % of the parasite, used in non-specific host) oocysts per chick.

b) The control groups

There were fifteen animals in each group.

All animals were raised under specific pathogen free conditions. Non-medicated commercially available food (BR I) and water were given *ad libitum*. Parts of the intestine (*duodenum, jejunum, ileum* and *caecum*) were taken from the infected and control animals for histological examination 12, 36 and 60 hours post infection.

Histological examination

The intestinal samples were fixed in 10 % neutral formalin and subjected to routine processing. Eight mm thick histological sections were cut and stained with haematoxylin-eosin.

The parasite localization, tissue inflammatory response and its destruction were observed.

The size and number of schizonts in the epithelial cells of the small intestine were measured in fifty microscope fields after the calibration of the eyepiece micrometer.

The number of heterophils was counted by light microscope (1000 × magnification). The pathogen and heterophils were counted in 100 fields (1520 mm^2).

Statistical analysis

Results were expressed as the mean ± SD and evaluated by two-tailed paired Student *t*-test. A confidence level of $P<0.05$ was considered significant.

RESULTS

Histological examination of Leghorn chick intestines showed, that the pheasant coccidium *E. colchici* invades the epithelial mucous cells of caecal mucosa and all parts of the small intestine.

By sixty hours post infection, the number of *E. colchici* schizonts in the Leghorn chick's caeca (1.4 ± 1.2) was significantly lower ($P<0.005$) when compared to the pheasant chick's caeca infested with *E. colchici* (1653 ± 663.5) evaluated at a similar time p. i. Measurement and comparison of the schizonts size revealed further differences between the non-specific and specific host, i.e. Leghorn chick's *versus* pheasant chick's. In the non-specific host, schizonts found in the *duodenum* (Fig. 1), *jejunum*, *ileum* and *caeca* (Fig. 2) measured 5.7×3.8 µm at 12 h p. i. and 9.5×7.6 µm at 36 h p.i. A much lower number of schizonts were found at 60 h p. i. These were identical in size (5.7×3.8 µm) to those found in the 12 h p. i. and were localized mainly in the *duodenum*. In contrast the examination of the pheasant host, infected with its host-specific parasite species, *E. colchici*, showed a number of marked differences. The first generation of schizonts in pheasant chicks were fully developed at 24 hours p. i., filled caecal crypts in huge numbers, and measured 18.7×15.1 µm (Fig. 3).

The number of oocysts needed for effective infection in the chickens was 200 × higher then needed for pheasants (5.10^3 compare to 1.10^6 in chickens).

The invasion of the parasite into the tissue in the pheasant was much more aggressive than in the chickens. There was no invasion of the parasite to the submucosa and *lamina propria* in the chickens and mucosal destruction was less prominent.

Comparison of the number of heterophils in the *duodenum, jejunum, ileum* and *caeca* in non-specific host Leghorn chicks and in specific host pheasant chicks at 12, 36 and 60 hour intervals p. i. showed significant differences. In the *duodenum* of the experimental Leghorn chicks at 60 hours p. i. there was a significant increase ($P<0.05$) of heterophil infiltration in comparison with the control chicks. In the *jejunum* infiltration by heterophils at 12, 36, 60 hours p. i. significantly rose from 83 ($P<0.01$), 120 ($P<0.005$) to 153 ($P<0.001$), compared to the control group (42, 76 and 95, respectively) (Fig. 4). The average number of heterophils in the *caeca* of the experimental Leghorn chicks (Fig. 5) showed a significant increase at 60 hours p. i. ($P<0.05$) as compared with the control group but in much smaller numbers than in the specific host – pheasant chicks (Fig. 6) (101 and 496, respectively).

DISCUSSION

The experimental infection of pheasant *Eimeria colchic*i caused a significant increase of heterophil numbers in both pheasant and chicken intestines as a part of the host inflammatory response. The number of counted heterophils showed, similarly as in the natural host, a gradual increase along with the development of the infection. In the chickens as in the non-specific host, the total number of heterophils counted in the different sites of the intestine, was smaller.

Each stage of the *Eimeria* biological cycle is a source of antigenic material and can target the host protective response. *Eimeria* has variety of locations in the intestine during its life cycle: i.e.lumen, extra/intracellular spaces of the mucosa and the intracellular environment of the enterocytes and other epithelial cells. Therefore in the extracellular and luminal phases the parasite is susceptible to immune response, while inside the cells, the parasite is inaccessible to many of these factors (13). In our study, we found less number of heterophils in the chickens compared with the pheasants. The smaller tissue destruction in the chickens along with non extra-cellular phases of the parasite reduced the inflammation response in the intestine and therefore a smaller number of immune cells was observed.

It is well known that recruitment and activation of circulating heterophils with rapid migration to the site of bacterial invasion is an effective host defence against bacterial invasion such as salmonellosis (7).

In our previous work one-day-old White Plymouth Rock chicks were experimentally infected with *Salmonella enteritidis* PT4. It was accompanied by marked heterophilic infiltration but without intracellulary located bacteria and with a decrease in the size of these cells. These observations indicate heterophils degranulation and kill the bacteria by the release of antimicrobial peptides (16). This assumption correlates with the study where the evaluation of heterophils for *in vitro* microbicidal activity against selected avian pathogens was done. The ability of these peptides at 16 $mg.ml^{-1}$ concentration was confirmed in the reduction of *Bordetella avium, Escherichia coli,* and *Salmonella enteritidis* survival (5).

Five bactericidal peptides (chicken heterophil peptides CHP1 and CHP2; turkey heterophil peptides THP1, 2 and 3) were purified from avian heterophil granules. All peptides were cationic and rich in cysteine, arginine and lysine. Both chicken peptides and THP1 shared a sequence homology at 22 residues and a cystein motif, which was similar to that of bovine beta-defensins. The beta-defensins found in heterophil granules can kill a wide variety of bacterial pathogens and are a major component of the heterophil antimicrobial arsenal (6).

In this study observed increase number of heterophils has shown the positive result of pheasant *Eimeria colchici* infection in chickens to evoke their antimicrobial activity against following antibacterial infection.

In the pheasant, the *Eimeria colchici* parasite is highly pathogenic and is the causative agent of acute coccidiosis. The infection is characterised by high morbidity and mortality rates producing a large number of oocysts. In our work most of the first generation schizonts were found in the intestinal crypts of the *caeca* and *posterior part* of intestine 24 hours post infection.

In the chicken chicks, only first generation schizonts were found in the epithelial cells of the intestine. The parasite did not develop further than first stage of schizont. Their number was fewer than in the pheasant. The parasite was much smaller and not fully developed. The infection was much more prominent after twelve hours and there was a significant decrease of the amount of parasite seen after sixty hours. Some of the chickens were contaminated while in the others no schizonts were found. This confirms that the pheasant coccidia possess characteristics that make them more suited to invade the natural host than a foreign host. The number of oocysts needed for effective infection in the chickens was 200 × higher than needed for pheasants.

The invasion of the parasite to the tissue in the pheasant was much more aggressive than in the chickens. It caused severe damage to the epithelial cells. If the epithelial cells are killed, the epithelium cannot be replaced except by encroachment from undamaged areas. Some stages of development in the coccidia avoid the problem (of massive destruction and non-regenerate epithelium) by migrating from the epithelium to the *lamina propria* (8). In our experiment, the massive damage to the epithelium in pheasant came together with observation of the parasite in deeper layers of the intestine. That was not observed in the chickens.

After the pheasant *Eimeria colchici* infection of chicken as a non-specific host heterophilia, an incomplete parasitic infection and mild inflammatory response were observed. These changes can be thought as a model for the preventive activation of heterophils as a means of promoting the defence of poultry against pathogens and finally as an "immune-potentiality" creation of immune responses to any infectious organisms.

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THE STRUCTURAL COMPARTMENTS OF A CELL DEMONSTRATED ON A SERTOLI CELL WITH A MINIREVIEW OF ITS STRUCTURE AND FUNCTIONS

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SUMMARY

We present a new functional compartmentalization of the structures of the cell. According to Cheville (10), six mutually co-operating systems can be found in the cell: cell surface, proteosynthetic system, vacuolar system for intracellular transfer of substances and intracellular digestion, smooth endoplasmic reticulum as the site of lipid biosynthesis and detoxication compartment, energy-transforming mitochondria and cytoskeleton. We have used the Sertoli cell as a model for functional classification. It is a unique cell having many functions and due to this the structure of Sertoli cells is extremely various and variable. This has enabled us to demonstrate all intracellular systems on a single type of cell. The paper also briefly reviews current knowledge on the structure and function of the Sertoli cell in mammals.

Key words: classification of cell structures; Sertoli cell

INTRODUCTION

For almost half a century textbooks, monographs and papers have described the structure/ultra-structure of a cell using the same traditional pattern of the general architecture of a cell. According to this each cell consists of amorphous cytoplasm or hyaloplasm (cytosol), which contains organelles and inclusions (paraplasm). The organelles are considered the internal organs of the cell. Inclusions (paraplasm) are lifeless accumulations of metabolites (lipid globules, glycogen, secretory droplets, protein crystals, and pigments) which are not needed for maintaining cell life (not necessary for the life of the cell). Such a classification is man-made and artificial.

Cheville (10) has proposed a classification of the cell based on a functional approach. The aim of this paper is to demonstrate an updated classification of all intracellular structures from the functional point of view using the Sertoli cell as a model. We demonstrate intracellular systems using our original electron micrographs, knowledge and experience more than 25 years study of Sertoli cell in 12 mammalian species. The structures of the Sertoli cell in relation to their functions are described successively within individual cellular systems according to the suggested functional classification. We also briefly review current knowledge on the structure and function of the Sertoli cell in mammals.

MATERAL AND METHODS

We studied the Sertoli cells of the bull, ram, billy-goat, stallion, boar, dog, tomcat, fox, roe-buck, fallow-deer, mouse and rat, intraabdominal testes of unilateral and bilateral cryptorchid boars and the testes of bulls after a 16 month long experimental cryptorchidism. The experiments were carried out in accordance with conditions specified by the *Ethics committee* and the *Act on experiments on animals* (7, 8). The testes were obtained by castration, from animals slaughtered in an abattoir, and from wild animals, which had been shot. The samples for transmission electron microscopy (TEM) were

fixed immediately in 3 % glutaraldehyde or in a mixture of 2.5 % paraformaldehyde and 2 % glutaraldehyde pH 7.2—7.4 and post-fixed in 1 % OsO_4. Samples were further processed routinely and embedded in Durcupan ACM. Ultra-thin sections were double stained by uranyl acetate and lead citrate and photographed under electron microscopes Tesla BS 500, JEM 1200 EX and JEM-100 CX II (Jeol, Japan).

SYSTEMS OF THE CELL – CLASSIFICATION ACCORDING TO THE FUNCTION

According to the functional approach the cell has six different, mutually co-operating systems:

1. The surface of the cell. The cell is covered by plasma membrane which serves as a barrier that ensures transport of substances into and out of the cell and reception of physical and particularly of chemical signals. Various specialized structures are found on the cell surface including microvilli, cilia, intercellular junctions and some other structures, such as folds of plasma membrane located in the basal part of the cell constituting, for example, the sodium pump.

2. The nucleus – nucleolus – ribosomes – granular endoplasmic reticulum and Golgi complex is the system which includes cell organelles that decode the genome and produce proteins. The pale, eukaryotic nucleus, large nucleolus, and abundant granular endoplasmic reticulum are found in the cells which produce large amount of polypeptides/proteins.

3. The vacuolar system transports substances within the cell between the organelles, and it performs the discharge of substances out of the cell for secretion of the cell. The system participates also in intracellular digestion by lysosomes.

4. The smooth endoplasmic reticulum is a site of lipid synthesis and also the detoxification system of a cell. It may have specialized functions, e.g. sarcoplasmic reticulum (smooth endoplasmic reticulum in skeletal and cardiac muscles), which stores, releases and takes up the calcium cations necessary for muscle contraction.

5. The mitochondria are organelles responsible for transformation of energy. They produce molecules which can be used by other systems of a cell as a source of energy.

6. The cytoskeleton is composed of microtubules, microfilaments and intermediate filaments; it also includes a pair of centrioles. The system acts as a structural support (skeleton) of the cell, it participates in a movement of the cell, intracellular transport and movement of organelles and cell inclusions inside the cell.

These individual systems are developed differently in various types of cells according to the functions fulfilled by the respective cell. For example, immunoglobulin-producing plasma cell is specialized solely to this specific function. Because of this the plasma cell, apart from the cell surface and mitochondria, which transform energy, has practically only one system for synthesis of a specific protein.

THE STRUCTURE AND FUNCTION OF THE SERTOLI CELL

Sertoli cells are the only somatic cells of seminiferous tubules, they are essential for spermatogenesis. Sertoli cells are exceptional cells as they perform a wide range of functions. The first among them is the supportive and trophic function for the developing spermatogenic cells of the seminiferous epithelium for which they have been named sustentacular or supporting cells and nurse cells providing special environment and numerous factors necessary for the normal development of spermatozoa. Sertoli cells have a phagocytic ability engulfing and disintegrating degenerating and dead cells of the seminiferous epithelium and residual corpuscles. They release sperm cells from the seminiferous epithelium into sperm passages and form the blood-testis barrier. Sertoli cells are both endocrine and exocrine cells; the evidence of paracrine secretion of Sertoli cells has increased. Foetal Sertoli cells produce a glycoprotein AMH (anti-Müllerian hormone) which inhibits the development of Müllerian duct. The Sertoli cells of adults produce the hormone, inhibin. The exocrine function of Sertoli cells is associated particularly with the secretion of the androgen binding protein (ABP), many polypeptides and other proteins and testicular fluid. Sertoli cells in tubuli recti produce a plug which serves as a one way valve and prevents the retrograde flow of testicular fluid carrying immotile testicular sperms into the rete testis and following excurrent sperm ducts.

Not even one of the almost two hundred different types of cells of the body of animals and humans (1) fulfils so many functions as the Sertoli cell. To perform all these functions, Sertoli cells are equipped with wide rank of different structures as seen by their extreme structural variety when observed either under transmission or scanning electron microscope (for a review see 17, 12, 38, 46, 13, 19, 21).

1. The surface of Sertoli cells is articulated. The cell membrane produces all known types of intercellular junctions: impermeable tight junctions, adherent junctions including desmosomes and communicative gap junctions of the nexus type. Adherent junctions ensure cohesion of cells and hold spermatogenic cells and maturing spermatids in seminiferous epithelium (29, 6). They play an important role in sperm release and turnover of basal junctional complexes (27). Important are also the tight junctions (Fig. 1), owing to which Sertoli cells form a blood-testis barrier (42, 14, 18, 6). Developing spermatogonial cells as they mature produce their own surface antigens which are recognized as foreign by immune system. Thus the blood-testis barrier formed by the Sertoli cells protects developing spermatogonial cells, which is crucial for the maintenance of spermatogenesis. For details on intercellular junctions of Sertoli cells see reviews by Pelle-tier and Byers (34); Parreira *et al.* (33). Microvilli and kinocilia we did not observe in Sertoli cells.

2. The proteosynthetic system is well developed already in foetal Sertoli cells which produce a glycoprotein AMH, the antimüllerian hormone (35, 53). Sertoli cells of sexually mature animals produce approximately 100 various proteins and polypeptides, among them the hormone inhibin (50, 25, 40), an androgen-binding protein – ABP (37, 52, 20), and **m**any

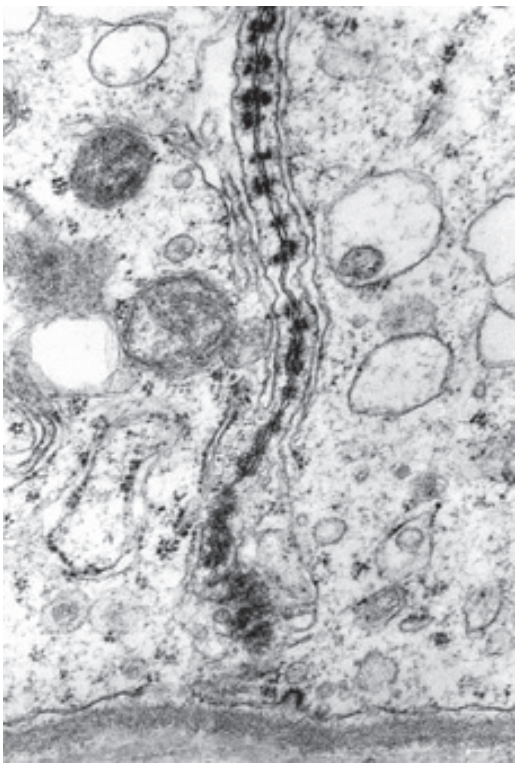

Fig. 1. Cell surface: impermeable tight junction *(zonula occludens)* between the two neighboring Sertoli cells. Dog. Magn. 40,000 ×

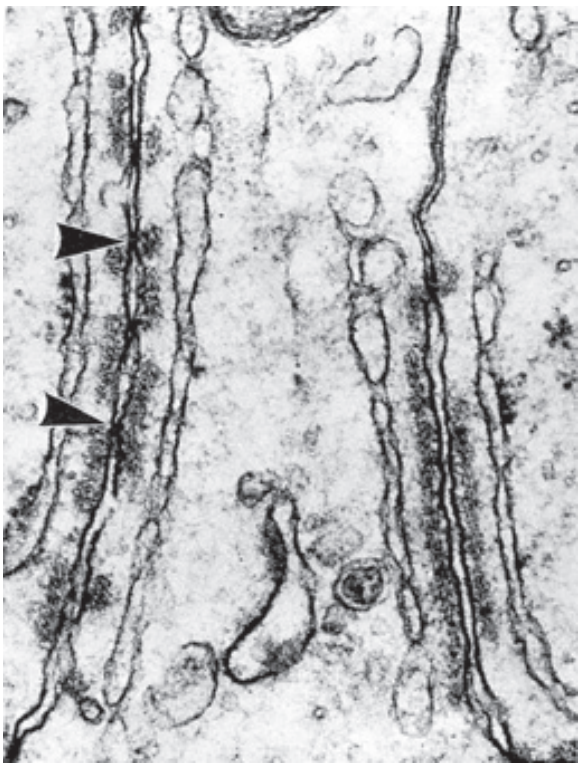

Fig. 2. Two tight junctions at high magnification. Fusion of neighboring plasma membranes *(arrowheads)* seals intercellular space. Dog. Magn. 100,000 ×

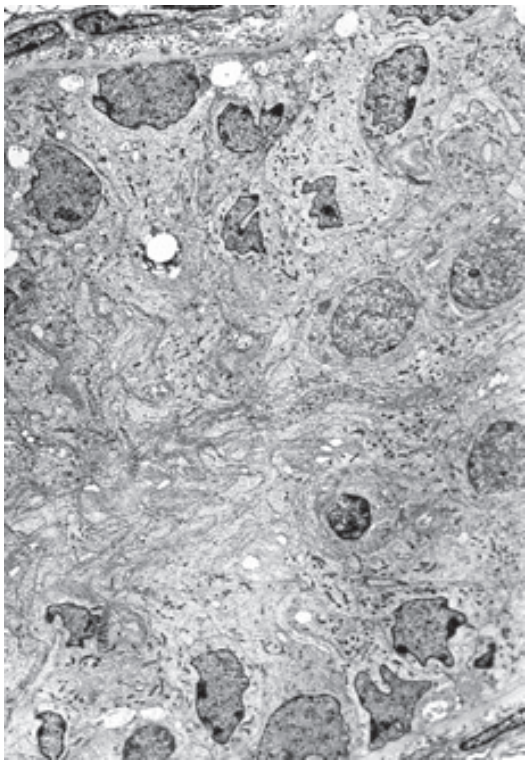

Fig. 3. Sertoli cells of foetuses and prepubertal animals have an oval nucleus with shallow invaginations, rough endoplasmic reticulum, ribosomes and mitochondria. Seminiferous tubule of a prepuberal fallow deer 9 months old. Magn. 2,500 ×

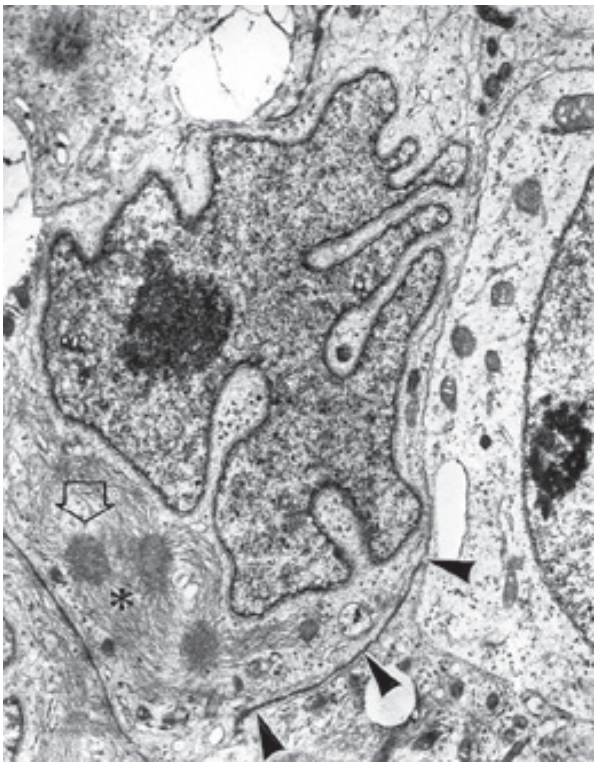

Fig. 4. Sertoli cell nucleus in adults has many invaginations of nuclear envelope containing ribosomes. Nodulous corpuscle (*arrow*) of smooth endoplasmic reticulum (*asterisk*) and tight junction (*arrowheads*). Fallow deer, October. Magn. 10,000 ×

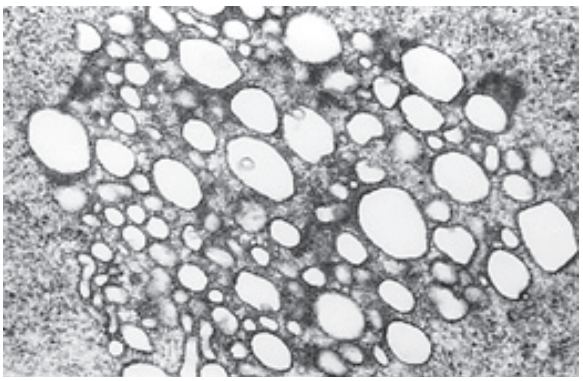

Fig. 5. Vesicular nucleolus – species specific structure of Sertoli cells in ruminants; it is the site of origin of ribosome precursors. Bull. Magn. 20,000 ×

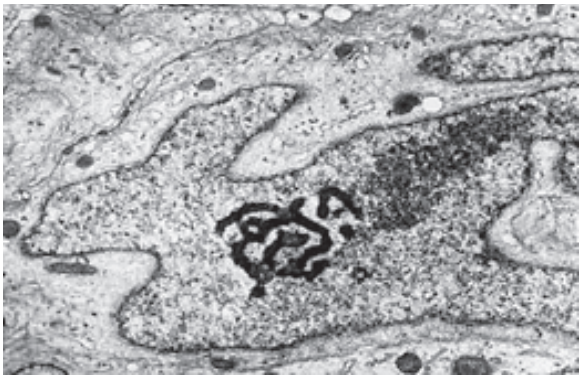

Fig. 6. Sertoli cell nucleus with reticular nucleolus; deep invaginations of nuclear envelope contain ribosomes. Fallow deer, November. Magn. 10,000 ×

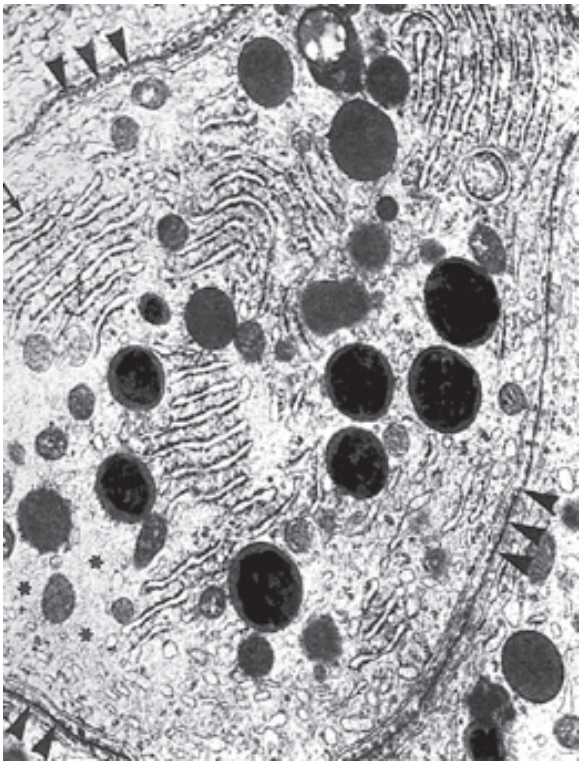

**Fig. 8. Basal part of Sertoli cell. There are many lipid droplets (black), abundant rough endoplasmic reticulum (*arrow*), some smooth endoplasmic reticulum, intermediate filaments (*asterisks*) and tight junctions (*arrowheads*).
Rat, Magn. 26,000 ×**

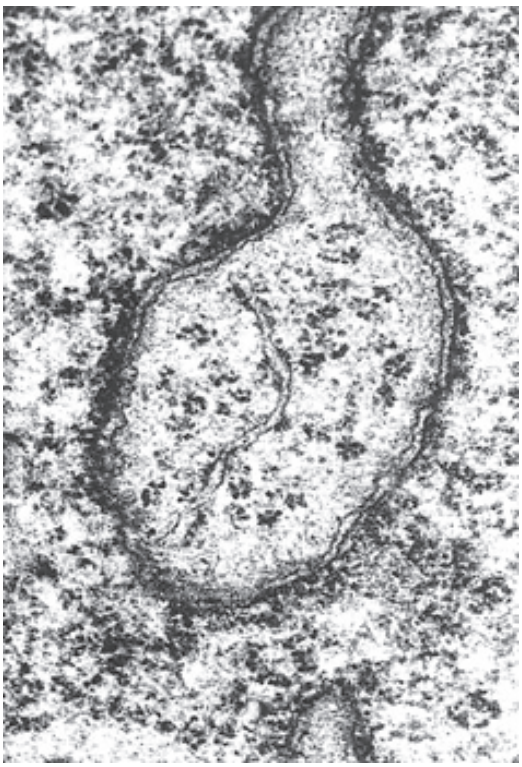

Fig. 7. Free and membrane bound ribosomes in invagination of Sertoli cell nuclear envelope. See also perinuclear web of intermediate filaments. Bull. Magn. 60,000 ×

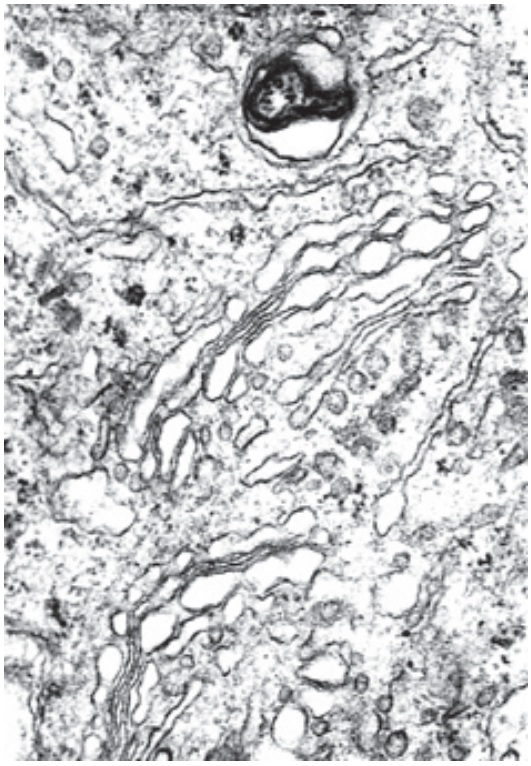

Fig. 9. Dictyosome, a part of well developed Golgi complex of a Sertoli cell at the onset of spermatogenesis. Roe-buck, April. Magn. 40,000 ×

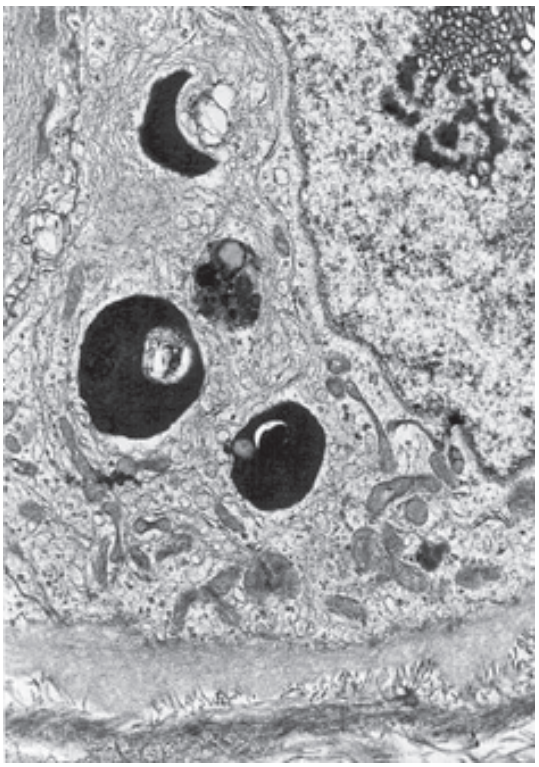

Fig. 10. Basal part of a Sertoli cell at the end of spermatogenesis contains lipid droplets and smooth endoplasmic reticulum. Electron-dense bodies are digested spermatogenic cells removed from seminiferous epithelium by Sertoli cell. Roe-buck, August 31. Magn. 15,000 ×

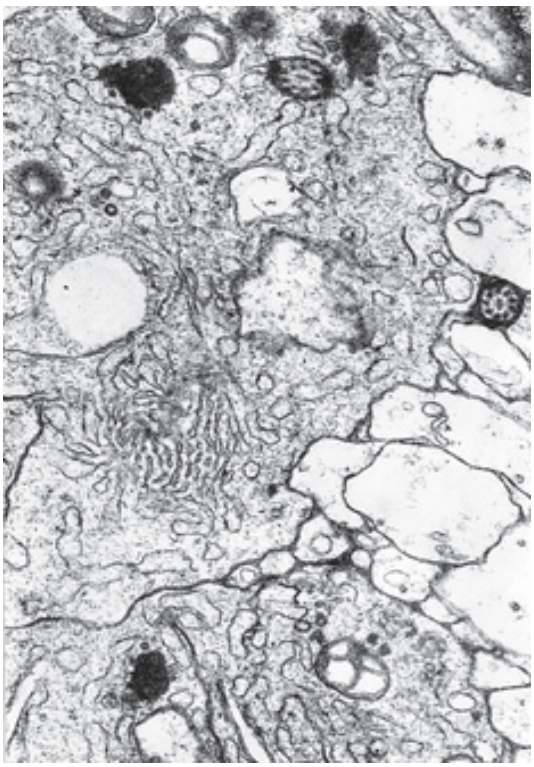

Fig. 11. The upper part of cytoplasm of Sertoli cells at the time of sperm release is highly vacuolated. Fallow deer, October. Magn. 24,000 ×

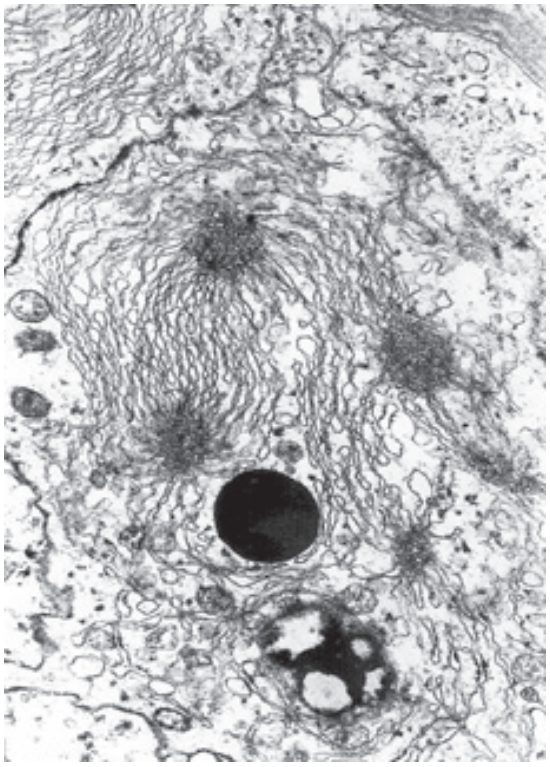

Fig. 12. The smooth endoplasmic reticulum occupies large volume of Sertoli cells in some stages of spermatogenesis. It forms nodulous corpuscles – specific structures of in Sertoli cells of some hybrids. Fallow deer, November. Magn. 20,000 ×

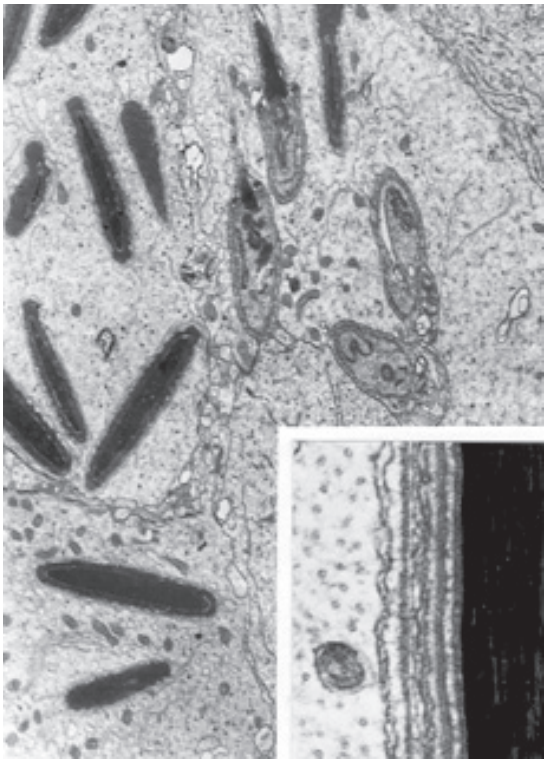

Fig. 13. Sperm heads (nuclei black) in the cytoplasm of Sertoli cell rich in cytoskeleton. Fallow deer, October. Magn. 5,000 x. Inset. Many cross cut microtubules can be seen at higher magnification. Fallow deer, October. Magn. 50,000 ×

other regulatory factors (28, 41). Due to considerable genome expressivity, Sertoli cells have an extremely euchromatic nucleus with very fine chromatin. Sertoli cells of foetuses and immature animals (9, 44, 49) have an oval and smooth surface nucleus (Fig. 2). The nucleus of Sertoli cells of adults has a highly articulated surface with numerous invaginations of the nuclear envelope (Figs. 3, 4) which usually contain ribosomes. Besides the euchromatic nucleus, the proteosynthetic function is also associated with a large nucleolus (Figs. 5 – 7) and abundant granular endoplasmic reticulum with a large number of ribosomes (Fig. 8). Ribosomes originate in the nucleolus (51).

Sertoli cells have a prominent nucleolus; its structure varies and is species specific. The nucleolus of Sertoli cells of rodents has two dark heterochromatic, DNA containing satellites (32, 17). The Sertoli cell of the bull has a vesicular nucleolus (60, 17, 30, 6, 16, 45, 56). A vesicular nucleolus was also found in Sertoli cells of other ruminants (17, 61, 30, 36, 4, 3, 24, 57, 23, 2) except fallow-deer (62, 58). In camels (Tylopoda), which were previously included among ruminants in terms of zoological taxonomy, Sertoli cells have a reticular nucleolus instead of a vesicular nucleolus (31). The secretory function of Sertoli cells is also related to a well-developed Golgi apparatus (Fig. 9).

3. The vacuolar system of Sertoli cells is well-developed in certain stages of the seminiferous epithelium cycle related to the secretion of testicular fluid (39). This system is also related to intracellular digestion. Sertoli cells have well-developed lysosomes; they occur in great numbers particularly in wild animals at the end and immediately after the reproduction season (Fig. 10). Multiplication of lysosomes is associated with cessation of spermatogenesis and elimination of sperms and their developmental stages of germ cells from the seminiferous epithelium (Fig. 11).

4. The smooth endoplasmic reticulum is extensive (Figs. 10 and 12). In some stages of the seminiferous epithelium cycle it is one of the best developed organelles of Sertoli cells (43, 5). The membranes of smooth endoplasmic reticulum in the basal part of Sertoli cells are often arranged in concentric whirls mainly in ruminants. The smooth endoplasmic reticulum is the site of steroid synthesis, its volume in steroid-secreting cells is directly related to hormonal activity of these cells (11, 26, 12, 13, 63). However, the function of the smooth endoplasmic reticulum in Sertoli cells has not yet been fully clarified. Part of the smooth endoplasmic reticulum in the Sertoli cells of the hybrids of deer (22) and fallow-deer (Fig. 12) is arranged in nodulous corpuscles. Neither the origin nor function of nodulous corpuscles is known.

5. The mitochondria are numerous but small, mostly of elongated shape, and round in cross sections. A rather large number of mitochondria containing many transversal parallel cristae mitochondriales is associated with rather high energy demands on the Sertoli cells. Mitochondrial tubules typical for the mitochondria of steroids producing cells are not common in the Sertoli cells of most mammalian species. Mitochondria with tubular and tubulo-vesicular cristae were found in rat and mouse Sertoli cells, respectively (38).

6. The cytoskeleton of Sertoli cells is very well-developed (Fig. 13) which is related particularly to their supportive function for spermatogenic cells (15, 17), intracellular transport and transport of cells across the seminiferous epithelium (55). There are microtubules (17), actin (54) and intermediate vimentin filaments (47, 48, 59).

CONCLUSION

A new principle of compartmentalization of the cell has been proposed recently according to which the classification of the cell constituents is based on a functional approach. The cell consists of six functional mutually co-operating compartments. We have described them using the Sertoli cell as a model. The Sertoli cell is a unique cell having many functions, more than any other cell of the body, which enables us to demonstrate all six compartments (functional systems within a cell) in a single cell.

The systems are (1) The cell surface which serves as a membrane barrier, receives chemical signals and transports molecules into and out of the cell. (2) The nucleus – the nucleolus – ribosomes – rough endoplasmic reticulum – Golgi system, encompassing organelles which encode, decode, synthesize and secrete proteins. (3) The vacuolar system responsible for intracellular digestion and transport related to secretion of the cell. (4) The smooth endoplasmic reticulum as a site of lipid synthesis and detoxification system of the cell. (5) The mitochondria are organelles providing energy for the cell. (6) The cytoskeleton represents a structural framework of the cell and for the movement of organelles and inclusions within a cell.

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THE RELATIONSHIPS BETWEEN CONCENTRATIONS OF SELENIUM AND GLUTATHIONE PEROXIDASE ACTIVITY IN THE WHOLE BLOOD OF DAIRY COWS

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ABSTRACT

In this work we studied the relationships between concentrations of selenium and GSH-Px activity in the whole blood of 57 healthy dairy cows. Between the recorded values we calculated the regression function ($y = 0.0031 \times - 0.8749$) and degree of linear dependence ($r = 0.892$; $p < 0.001$). As we found a high range and variability in the results, we divided them into three groups according to the activities of GSH-Px (1st group: $< 500\ \mu kat.l^{-1}$; 2nd group: 500–800 $\mu kat.l^{-1}$; 3rd group: $> 800\ \mu kat.l^{-1}$). Statistical evaluation showed markedly lower standard deviation and variation coefficient in all the groups. A close relationship with high significance was found for enzyme activities over 500 $\mu kat.l^{-1}$ (2nd group: $r = 0.633$, $p < 0.001$; 3rd group: $r = 0.597$, $p < 0.01$). These results confirmed the possible use of GSH-Px activities for the indirect assessment of selenium status in the whole blood of dairy cows.

Key words: dairy cowss; glutathione; peroxidase; selenium

INTRODUCTION

In farm animals, an optimum supply with trace elements depends on soil composition, fertilisation, and exploitation. Requirements of these elements are influenced by species, rearing technology, type of diet, production level, stage of pregnancy, health status, and, not least, genetic predispositions.

As in other elements, sufficient selenium status in the body depends on its soil content (1). The first studies of this element dealt mainly with its toxic effects on animals and humans.

Because selenium acts through various selenoproteins, it has many biological functions. Together with vitamin E, as a biological antioxidant, it is involved in the biosynthesis of coenzyme A. Selenium is also catalytic in enzymatic systems and it is a structural element of gluthatione peroxidase (GSH-Px, E.C.1.11.1.9.) (12). In its active centre, GSH-Px has a special amino acid, selenocysteine, which is directly involved in the double electron reduction of peroxide substrate. To eliminate hydrogen peroxide by this enzyme, there is a need to regenerate gluthatione in a reduced form. This is done by the enzyme gluthatione reductase (GR, E.C.1.6. 4.2.) and pyridine coenzyme NADPH. With a lack of NADPH, the function of this enzyme, and consequently functions of the whole enzymatic antioxidative system are disturbed.

Selenium deficiency leads to various diseases, which have an endemic character. From the pathological, morphological, and clinical points of view, these diseases resemble avitaminosis E. Among them, the most frequent are muscle dystrophy in calves, young cattle – heifers after turning to pasture (6), lambs, foals, dietetic hepatosis in pigs, muscle dystrophy in poultry, exsudative diathesis, and encephalomalacia in poultry. Moreover, various reproduction disorders occur (infertility, degeneration of seminiferous tubules), reduced *libido* in sheep, disorders of ovarian cycle, and increased occurrence of infertility (3).

High selenium concentrations are accompanied by severe disorders of the central nervous system, loss of vision, paralysis, increased salivation, swallowing disorders, hair losses, and abdominal pain. Selenium intoxication occurs also in incorrect therapy of its deficiency (16).

Because clinically manifested diseases are diagnosed only in a severe selenium deficiency, there is a need to detect sub-

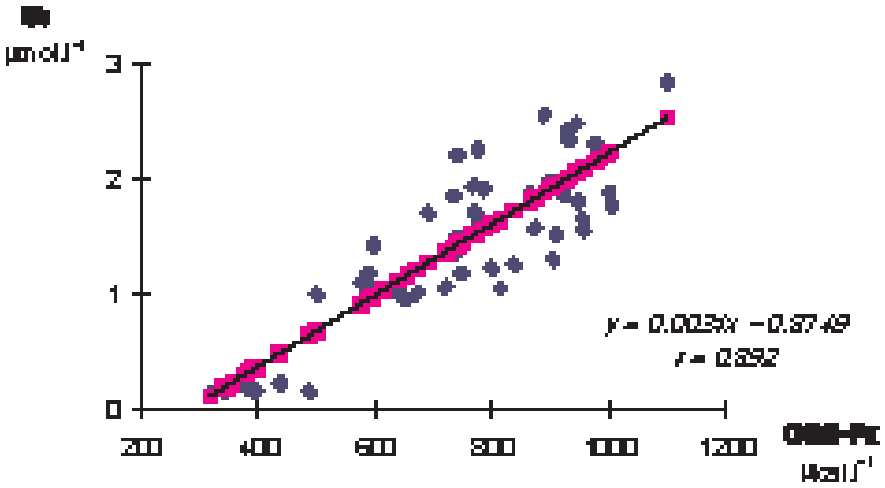

Fig. 1. Relationship between selenium concentration and GSH-Px activity in the whole blood of dairy cows

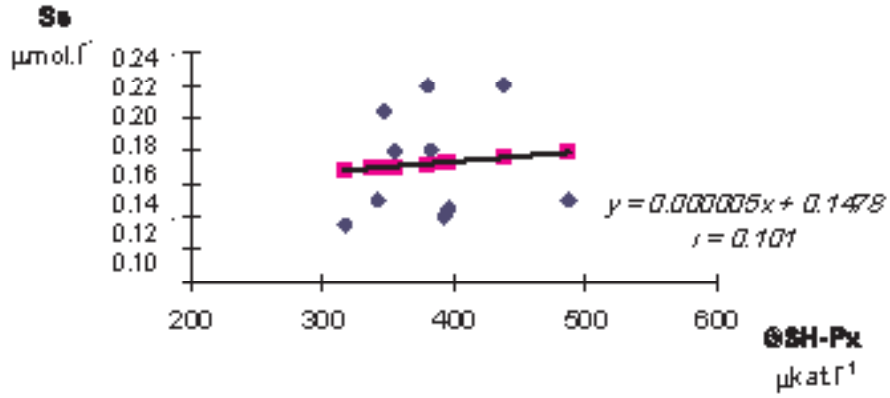

Fig. 2. Relationship between selenium concentration and GSH-Px activity in the whole blood of dairy cows (1st group)

clinical metabolic disorders with the use of the most precise available methods. Direct analysis of selenium and estimation of glutathione peroxidase activity in the whole blood are the principal methods estimating selenium status in the body of dairy cows (9).

Our study was aimed at evaluating relationships between GSH-Px activities and selenium concentrations in the whole blood of dairy cows and to judge the possible use of GSH-Px activities in indirect determination of selenium because of better availability and fewer costs of this method.

MATERIAL AND METHODS

The study was carried out in 57 healthy dairy cows from four large-scale Slovakian farms. Blood samples were collected from the jugular vein into flasks with addition of heparin stored at –20 °C. In the whole heparinised blood the enzymatic activity of glutathione peroxidase (E.C.1.11.1.9.) and selenium concentrations were analysed.

The activity of GSH-Px was estimated by an automatic biochemical analyser Alizé (Lisabio, France) with the use of diagnostic kits Ransel (Randox, UK) according the method of Paglia and Valentine (10). The GSH-Px catalyses glutathione oxidation at the presence of cumenhydroperoxide substrate (pH = 7.2; 37 °C). Glutathione reductase and NADPH transform oxidized glutathione to the reduced form. Oxidation of NADPH to $NADP^+$ was recorded as a decrease in absorbency at 340 nm, which is directly related to the sample GSH-Px activity.

Selenium concentrations in the whole heparinised blood samples was estimated by atomic absorption spectrophotometric (AAS) method (Perkin – Elmer 4100 ZL, USA), hydride technique FIAS 100, after a preceding wet mineralisation in a microwave oven MLS 1200 (Milestone, Italy).

Statistical analyses of the results were done in MS Excel. Between results of these two methods linear correlation (correlation coefficient r), regression function (y – anticipated Se values, x – estimated GSH-Px activities) were calculated.

RESULTS

The main statistical values of estimated enzymatic activities of GSH-Px and selenium concentrations in the whole blood of dairy cows are presented in the Table 1. Correlation between the obtained results is presented in Figure 1.

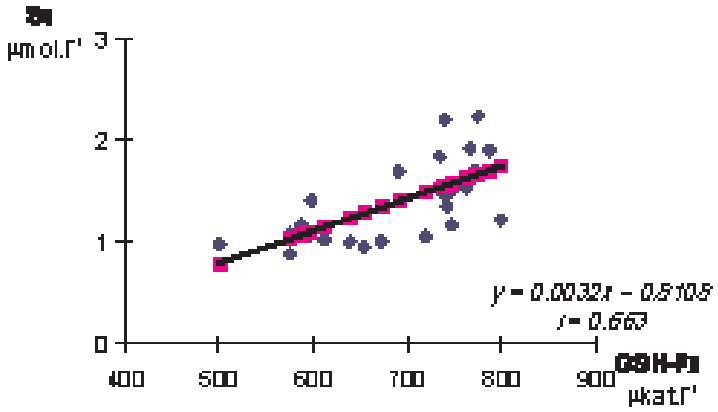

Fig. 3. Relationship between selenium concentration and GSH-Px activity in the whole blood of dairy cows (2nd group)

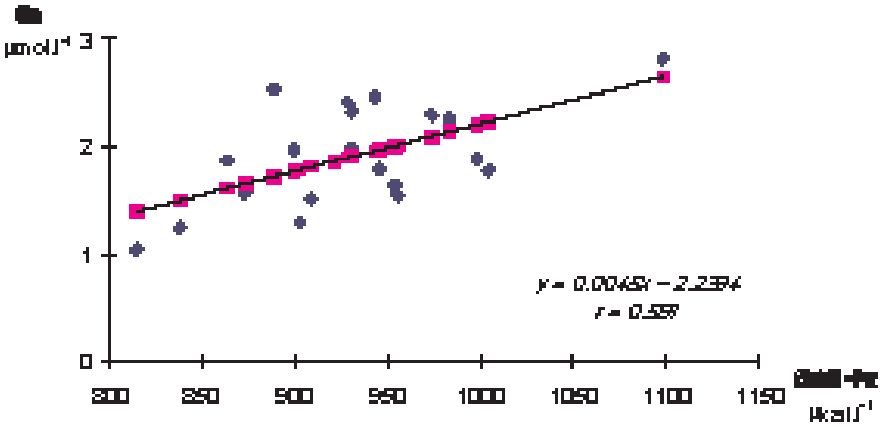

Fig. 4. Relationship between selenium concentration and GSH-Px activity in the whole blood of dairy cows (3rd group

Table 1. The main statistical values of the estimated enzymatic activities of GSH-Px and selenium concentrations in the whole blood of dairy cows (n = 57)

	GSH-Px µkat.l^{-1}		Se µmol.l^{-1}
average	719.80		1.35
min.	317.07		0.14
max.	1098.80		2.82
SD	211.56		0.73
CV%	29.39	54.07	
r – coefficient of linear correlation		0.892	
linear regression		y = 0.0031x – 0.8749	
p		< 0.001	

Enzymatic activities of GSH-Px were arranged in ascending order into three groups (1st group < 500 µkat.l^{-1}, 2nd group 500–800 µkat.l^{-1}, 3rd group > 800 µkat.l^{-1}) together with corresponding concentrations of selenium. Results within each group were also statistically evaluated including correlation and regression analyses (Tab. 2, Figs. 2–4).

DISCUSSION

Glutathione peroxidase plays an important role in the body, especially in the antioxidation system. Selenium, as a structural part of this enzyme, protects, together with vitamin E, the cells against oxidative damage. Glutathione peroxidase protects the cells by its action in the cytosol and vitamin E in the cell membrane (14). Estimating this enzyme in the whole blood samples is assumed as a simple and fast method of body selenium state assessment. The advantage of this method is the relatively adequate stability of GSH-Px in erythrocytes (11) and 98 % of GSH-Px activity bound to the erythrocytes (13).

Because selenium is incorporated into erythrocytar GSH-Px during erythropoesis, enzymatic activity depends on average selenium availability during development of the whole erythrocyte population. Therefore, estimating the whole blood GSH-Px indicates the long-term and plasma GSH-Px short-term selenium status of the body (2). These data were confirmed also by Kováč and Sankari (7), who have found the highest correlation between whole blood selenium concentrations and erythrocytar GSH-Px activities (r = 0.992), and between selenium concentrations GSH-Px activities in the whole blood (r = 0.981).

Table 2. The main statistical values of the estimated enzymatic activities of GSH-Px and selenium concentrations in the whole blood of dairy cows divided by three GSH-Px levels

	1st group		2nd group		3rd group	
	GSH-Px μkat.l^{-1}	Se μmol.l^{-1}	GSH-Px μkat.l^{-1}	Se μmol.l^{-1}	GSH-Px μkat.l^{-1}	Se μmol.l^{-1}
GSH-Px μkat.l^{-1}	< 500		500–800		> 800	
n	11		25		21	
average	379.31	0.17	691.97	1.40	931.28	1.91
min.	317.07	0.14	500.88	0.88	814.53	1.04
max.	487.22	0.22	798.82	2.24	1098.80	2.82
SD	49.21	0.03	84.18	0.41	62.62	0.47
CV %	12.97	17.65	12.17	29.29	6.72	24.61
r – coefficient of linear correlation	0.101		0.663		0.597	
linear regression	$y = 6.10^{-5} x + 0.1478$		$y = 0.0032x - 0.8108$		$y = 0.0045x - 2.2394$	
p	> 0.05		< 0.001		< 0.01	

In our work, whole blood selenium concentrations with an average value 1.35 μmol.l^{-1} ranged from 0.14 to 2.82 μmol.l^{-1} and GSH-Px activities (average 719.80 μkat.l^{-1}) from 317.07 to 1098.80 μkat.l^{-1} (Tab. 1). Correlation analysis showed the degree of dependence between the measured indices (r = 0.892; Fig. 1). The observed close and highly significant relationships of the indices (p < 0. 001) have been confirmed by many authors.

Koller *et al.* (5) have reported correlation coefficient r = 0.870, Khan *et al.* (4) r = 0.920 and Mass *et al.* (8) r = 0.970. By regression analysis we estimated the following regression equation:

$y = 0.0031x - 0.8749$

x – measured activities of GSH-Px in μkat.l^{-1}

y – anticipated values of Se in μmol.l^{-1}

Because of the different relationships between selenium concentrations and GSH-Px activities published in the literature, in the evaluation of GSH-Px activities and their use in selenium status assessment, there is a need to use reference values estimated by the corresponding laboratory. While Tasker *et al.* (15) have reported calculated activity 300 μkat.l^{-1} with selenium concentration 0.96 μmol.l^{-1}, Ortman and Person (9) have calculated GSH-Px in the range 1400–1600 μkat.l^{-1} for 1.14–1.30 μmol.l^{-1} of selenium. In our laboratory conditions, with the use of the most common reference selenium value (0.89–1.27 μmol.l^{-1} in the whole blood of dairy cows) in our regression equation, we obtained the corresponding GSH-Px activity in a range of 569.32–691.90 μkat.l^{-1}.

As we have found a wide range of the indices and high variability in the results (GSH-Px – 29.39 %; Se – 54.07 %), we have divided the data into three groups according the GSH-Px values. Statistical analysis has shown markedly lower values of standard deviations and variation coefficient in all the three groups (Tabs. 1–2).

The values in the 1st group (GSH-Px < 500 —kat.l^{-1}) do not correlate and their relationships are insignificant (r = 0.101; p > 0.05; Fig. 2). A close and significant relationship (r = 0.663; p < 0.001) has been found in the 2nd group (500–800 μkat.l^{-1} GSH-Px; Fig. 3). Enzymatic activities over 800 μkat.l^{-1} correlated with selenium concentrations with r = 0.597 (p < 0.01; Fig. 4). A higher correlation in the 2nd and 3rd group compared with the 1st group, indicates that indirect assessment of selenium status by GSH-Px analysis is useful mostly for GSH-Px activities over 500 μkat.l^{-1} (2nd group $y = 0.0032x - 0.8108$; 3rd group $y = 0.0045x - 2.2394$).

CONCLUSION

Following these findings, we can conclude that there is a relationship between enzymatic GSH-Px activity and selenium concentration in the whole blood of dairy cows. Therefore, it is possible to use the enzyme estimate for the indirect evaluation of the body selenium status in veterinary diagnosis, most of all because of the better availability and lower cost of this method.

ACKNOWLEDGEMENT

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THE EFFECT OF DIETARY SELENIUM AND VITAMIN E ON NON-SPECIFIC IMMUNE RESPONSE IN HEALTHY PUPPIES AFTER VACCINATION

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ABSTRACT

We studied the effect of feeding of dietary selenium and vitamin E on immune functions of healthy puppies after vaccination. Fourteen dogs of various breeds and sexes were divided into two groups; an experimental and a control group. The control group received a standard diet and the experimental group was fed with an antioxidant-supplemented diet including an inorganic form of selenium (5 mg per day) and vitamin E (50 mg per day) for four weeks. All dogs were vaccinated against canine distemper and parvovirosis. Blood samples were collected and the level of the phagocytic activity of leukocytes, the phagocytic index of leukocytes, the total leukocyte count and blastogenic activity of lymphocytes to mitogens were investigated. This study showed that antioxidant-supplemented dogs have significantly higher immunological values than dogs fed a standard diet. The results of this study indicate that antioxidants are effective as immunostimulators and can be used to increase the immune response in young growing dogs.

Key words: antioxidants; immune system; dogs; vaccination

INTRODUCTION

All animals are particularly vulnerable to infectious diseases at certain life stages. During the first year of life puppies encounter a series of stressful events, which may compromise the activity of their immune system and increase their susceptibility to infection. Potentially stressful situations include: weaning, growth, worming, and the series of vaccinations and so on (4). These stressful events lead to the increased production of free radicals. However, free radicals are useful only when they are produced in the right amount, at the right place and at the right time. Alteration in any of these parameters leading to free radical imbalance can result in oxidative damage and destruction of cell membranes, cell DNA and other cellular components (6). These events lead also to alteration of the immune cell functions (7).

In the body free radicals are kept under control with the anti-oxidant defence system. Antioxidants are often referred to as “free radical scavengers”, they protect the cell membrane and the cytosolic components against free radical damage (5). The antioxidant system consists of two groups: antioxidant enzymes which are mostly synthesised by the body and antioxidant nutrients which are taken into the body with the food (vitamins – E, C, A, betacarotene, bioflavanoids; minerals – selenium, zinc, copper, manganese). Some antioxidants are an integral part of the immune cell membranes that protect against free radical damage (7).

The aim of the present study was to observe the effect of vitamin E and selenium on the total leukocyte count, phagocytic activity and phagocytic index of leukocytes and blastogenic activity of lymphocytes to mitogens in vaccinated puppies.

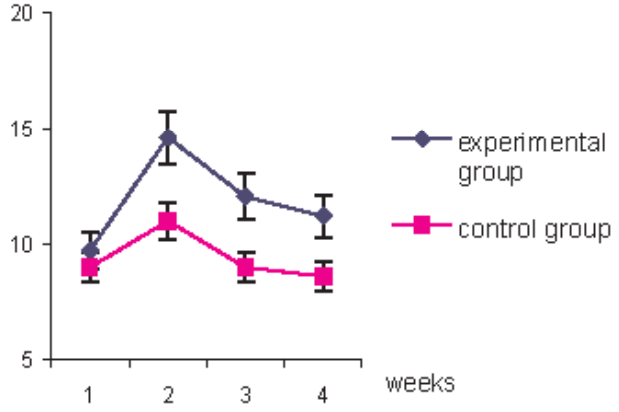

Fig. 1. Total leukocyte count

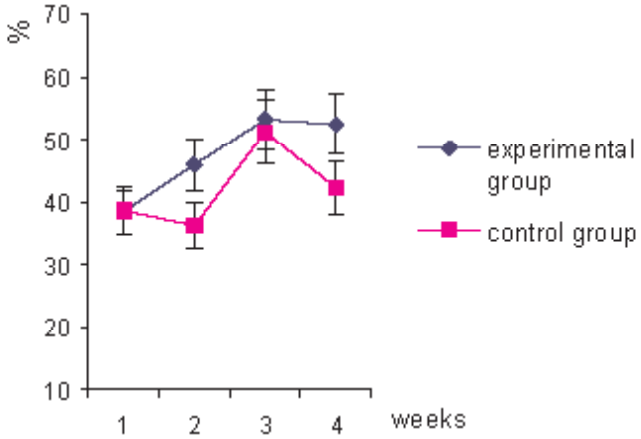

Fig. 2. Phagocytic activity of leukocytes

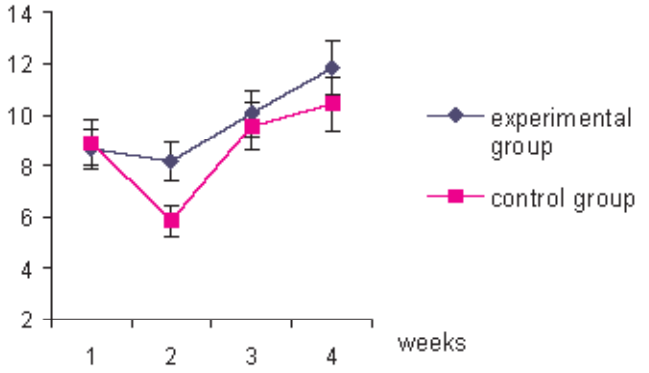

Fig. 3. Phagocytic index of leukocytes

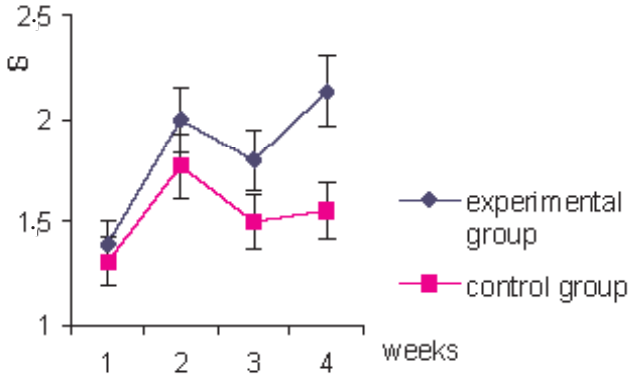

Fig. 4. Blastogenic activity of lymphocytes to ConA

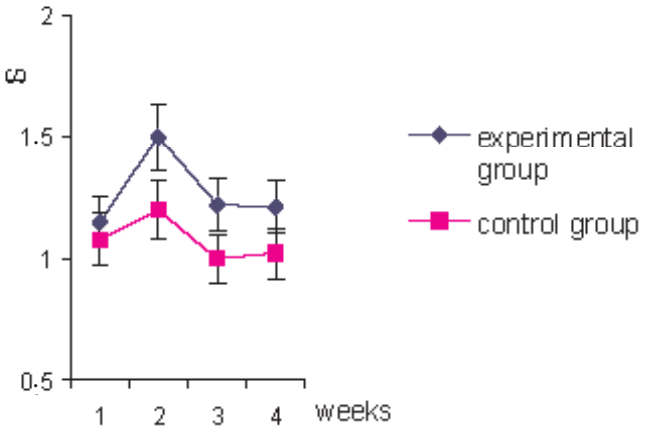

Fig. 5. Blastogenic activity of lymphocytes to PHA-A

MATERIAL AND METHODS

Animals: In this study fourteen dogs of various breeds and sexes with an average age of six months were used. The animals were divided into two groups – an experimental group (seven dogs) which was fed with an antioxidant-supplemented diet (inorganic form of selenium: five mg per day and vitamin E: fifty mg per day) for four weeks and a control group (seven dogs) which was fed with a standard diet. All dogs were vaccinated and revaccinated (fourteen days after vaccination) against canine distemper and parvovirosis. Seven days before vaccination supplementation with selenium and vitamin E in the experimental group was started.

Blood collection

Peripheral blood samples were obtained by *v. cephalica* puncture and placed into a tube containing heparin (5 IU of heparin.1 ml^{-1} of blood). Blood collection was carried out one day before and eight days after the supplementation of antioxidants three times during this experiment.

The evaluation of the parameters of cellular immunity

The total leukocyte count was determined using common haematological methods.

The phagocytic activity and phagocytic index of blood leukocytes was examined as described by Větvička *et al.* (17). 0.1 ml of fresh heparinized blood (5 IU of heparin.1 ml^{-1} of blood) was mixed with 0.05 ml of 2-hydroxyethylmetacrylate particles (MSHP, diameter 1.2 mm; ARTIM Prague) and incubated for one hour at 37 °C with occasional shaking. The phagocytic activity (PA) of neutrophils (Ne) was expressed as the percentage of the cells phagocytizing three or more MSHP, and as the index of phagocytic activity (IPA) representing the ingestion ability of neutrophils (the ratio of the number of phagocytized MSHP and the number of all potentially phagocytizing Ne).

Blastogenic activity of lymphocytes to mitogenes. Lymphocytes were separated from blood on the verografin density

gradient. Concanavalin A (Con A, Sigma Chemical Co., USA) and phytohaemagglutinin (PHA-A, Sigma Chemical Co., USA) were used for stimulation in the concentration 25 $mg.ml^{-1}$ and 20 $mg.ml^{-1}$, respectively. The level of blastogenic response of the lymphocytes was estimated using the EB fluorescence test (12) and expressed as the fluorescence intensity of stimulated and unstimulated cells, and the stimulation index (SI). The FI was measured by a spectrofluorometer (Jasco FP-550, Japan).

Statistical analyses. The immunological parameters of puppies supplemented with antioxidants were compared with puppies fed with a standard diet. The significance of differences was checked by the Anova-Manova test.

RESULTS

When comparing the results of the immunological parameters of puppies supplemented with antioxidants after vaccination with puppies fed with a standard diet significant differences were found. The total leukocyte count (Fig. 1), the phagocytic activity of leukocytes (Fig. 2) and phagocytic index of the leukocytes (Fig. 3) were significantly higher in the experimental group of puppies, which were supplemented with selenium and vitamin E. Also the blastogenic activity of lymphocytes after stimulation with Con-A (Fig. 4) and PHA-A (Fig. 5) had increased.

DISCUSSION

Recent studies have found that vitamin E and selenium are important nutrients and higher intake of these antioxidants is needed to maintain normal immune functions. Vitamin E, tocopherol, acts as an antioxidant in cellular membranes and as a free radical scavenger by blocking the peroxidation of polyunsaturated fatty acid (13, 17). In immune cells, the tocopherol content is known to be higher than in other cells, because the cellular membrane plays an important role in the immune response (3).

The mechanism of the enhancement of the immune response by vitamin E may be related to the reduction of an immunosuppressive effect of prostaglandins. It has been reported that a high vitamin E diet decreases the production of PGE_2 (prostaglandin E_2) from macrophages and enhances cellular immune functions (10, 11, and 16). PGE_2 inhibits lymphocytes proliferation; production of IL-2 (interleukin-2) and activity of NK-cells (natural killers cells). However, the precise mechanism by which vitamin E stimulates immune functions remains unclear (11).

Selenium intake, too, is inevitable for the normal functioning of the immune system; it is connected with a higher concentration of selenium in the spleen, lymphatic glands, liver and also with a particularly high activity of glutathione peroxidase in leukocytes (15). Selenium is able to enhance the expression of the a and b subunits of the interleukin-2-receptor (IL-2R) on the surface of activated cells; this, in turn, results in enhancement of immunologic functions. IL-2 affects the function of a number of cell types, among which T- and B-lymphocytes, NK-cells, macrophages, and neutrophils are primary targets (8).

The positive effect of antioxidants has been described in many studies. For example, a study in rats indicated that the ability of T-cells to proliferate in response to the mitogens, Concanavalin A, phytohaemagglutinin and lipopolysacharide related positively to plasma tocopherol and selenium concentrations (2). Moriguchi *et al.* (10) also found that the addition of macrophages pre-treated with both vitamin E and Con-A to a lymphocyte culture could induce even higher proliferation of lymphocytes compared to macrophages pre-treated with Con-A alone. This suggests that vitamin E has the ability to stimulate macrophages directly. In addition, NK-cell activity and the phagocytic function of polymorphonuclear cells were also increased in antioxidant supplemented animals (1, 9). Related studies has been carried out on puppies, which were fed with an antioxidant enriched diet and received their routine vaccinations. An increase of the phagocytic activity of leukocytes was detected (4).

The result of our study indicates the positive influence of antioxidants on the immune system of puppies. We have found that a diet enriched with antioxidants enhances the immune response of puppies. Total leukocyte count, phagocytic activity and the functional activity of lymphocytes after vaccination were significantly higher in the experimental group of puppies, which had been supplemented with selenium and vitamin E. Accordingly we can conclude that antioxidants functioning as the immunostimulans can be used to increase the immune response in young growing dogs.

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RUMINAL ENZYME ACTIVITIES IN SHEEP FROM AN INDUSTRIALLY EXPOSED AREA

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ABSTRACT

The aim of the study was to evaluate the effects of some pollutants on alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransfe-rase (GGT) and glutamate dehydrogenase (GDH) enzyme activities of rumen fluid. The experiments were carried out on six ewes from an industrially exposed area and six ewes from a control area.The activity of individual enzymes of the rumen fluid (ALT, AST, GGT, and GDH) of sheep from the industrially exposed area were significantly higher. The concentrations of copper and zinc were significantly higher in the rumen fluid of sheep from the industrially exposed area with no significant differences for lead and cadmium. These results indicate that copper and zinc affect the activity of several rumen enzymes, playing an important role in the metabolism of nitrogenous compounds.

Key words: enzyme; metal ion; rumen; ruminant; sheep

INTRODUCTION

The nutritional requirements of ruminants are different from those of monogastric animals. Rumen microbes can synthesize enough amino acids and peptides from the inorganic nitrogen in ammonia or other nitrogen source and carbon skeletons and sulphur precursors.

Ammonia assimilation by rumen microbes depends on rumen pH (24), rumen ammonia concentration (14) and ruminal ammonia-assimilating enzyme activity.

A number of ammonia-assimilation reactions by rumen bacteria are known.

In the recent past, factories producing copper and mercury have altered the agricultural environment. Jenčík *et al.* (10) has reported that soil and plant biomass sample analyses from localities situated maximally ten kilometres from copper and formerly mercury-producing works showed significant soil and biomass contamination by mercury, lead, cadmium, copper, and zinc ions.

Many papers have dealt with the study of the effectiveness of various compounds added separately to the ruminal activity of several enzymes (18, 21,23). Little is known about the effects of pollutants on the ruminal enzyme activity of animals in an industrially exposed area when the resultant enzymatic activity reflects mutual interactions of individual pollutants.

The objective of the present study was to determine the effect of ions causing air, soil and biomass contamination in an industrially exposed area on some enzyme activities of rumen fluid.

MATERIALS AND METHODS

The experiment were carried out on twelve one-year old female sheep ofmixed breed, weighing from thirty to thirty-five kilograms. The animals were divided into two groups. Six sheep in the first group were from a laboratory farm and were fed with hay *ad libitum* and 300 grams of barley for one day and they had free access to water. The second group was grazed on natural pasture in a territory near a copper production works.

Throughout the experiment, the animals were maintained according to the principles governing the care of laboratory

animals. Rumen fluid was collected from sheep using a stomach tube.

The ruminal contents were then strained through eight layers of cheesecloth. The enzymes, alanine aminotransferase, ALT (ALT 360, BIO-LACHEMA-TEST, CzR), aspartate aminotransferase, AST (AST 360, BIO-LACHEMA-TEST), gamma-glutamyltransferase, GGT (GGT 100, BIO-LACHEMA-TEST), and glutamate dehydrogenase, GDH (GL 442, RANDOX, UK) were assayed in rumen fluid according to procedures outlined in respective commercial kits using a spectrophotometer set at 510, 510, 430 and 340 nm wavelength, respectively.

Rumen copper and zinc concentrations were determined by flame atomic absorption spectrophotometry (Perkin–Elmer, Model A Analyst 100). Rumen cadmium and lead concentrations were determined by non-flame atomic absorption spectrophotometry (Perkin–Elmer, Model ZL 4 100).

The results are given as a mean ± SEM. Statistical significance of the differences between values was determined by Student's *t*-test.

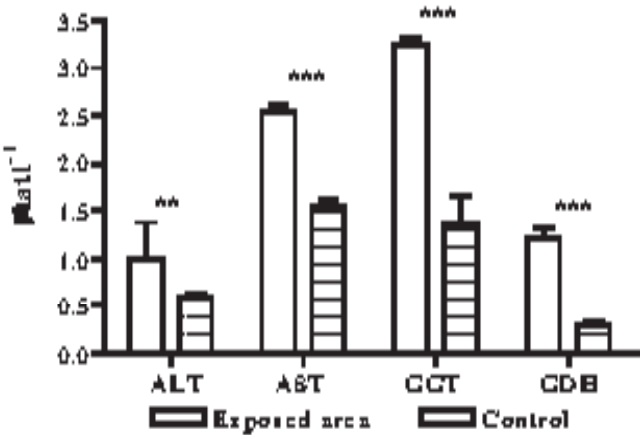

Fig. 1. Ruminal enzyme activities in sheep. Each value represents the mean ± S.E.M. n = 6 (P < 0.01, ***P < 0.001)**

RESULTS

Fig. 1 shows the activity of several enzymes of the rumen fluid of sheep from an industrially exposed area (experimental) and a non-industrially exposed area (control).

Comparison of the activity of individual enzymes of the rumen fluid of sheep from the industrially exposed area and non-industrially exposed area showed that ALT, AST, GGT and GDH activities were significantly higher in the rumen fluid of sheep from the industrially exposed area (0.998 ± 0.383 μkat.l^{-1} *vs* 0.585 ± 0.049 μkat.l^{-1}, $P < 0.01$; 2.549 ± 0.069 μkat.l^{-1} *vs* 1.562 ± 0.049 μkat.l^{-1}, $P < 0.001$; 3.250 ± 0.052 μkat.l^{-1} *vs* 1.365 ± 0.307 μkat.l^{-1}, $P < 0.001$ and 1.216 ± 0.118 μkat.l^{-1} *vs* 0.299 ± 0.027 μkat.l^{-1}, $P < 0.001$, resp.).

Table 1 shows the concentrations of copper, zinc, cadmium and lead of the rumen fluid of sheep from the industrially exposed area (experimental) and non-industrially exposed area (control).

Comparison of the concentrations of individual ions of the rumen fluid of sheep from the industrially exposed area and non-industrially exposed area showed that the concentrations of copper and zinc were significantly higher in the rumen fluid of sheep from the industrially exposed area.

DISCUSSION

Our results show that metal ions have affected the activity of the rumen enzymes tested. Data from our study indicate that ALT, AST, GGT and GDH activities were significantly higher in the rumen fluid of sheep from the industrially exposed area. Concentrations of copper and zinc were significantly higher in the rumen fluid of sheep from the industrially exposed area.

This is in contrast to previous research from our laboratory in which copper was found to inhibit both the urease and glutamate dehydrogenase activities of the rumen fluid (6). The discrepancy between the two studies may relate to the duration of copper supplementation. In the present study, rumen fluid was collected from sheep from industrially exposed areas, whereas Faixová and Faix (6) incubated rumen fluid with copper ion for thirty minutes. In addition, in the present experiment rumen fluid also contained other ions.

Copper is an essential element required for a number of biochemical functions. Engle and Spears (4) have reported that copper stimulates growth in swine and alters lipid metabolism in steers but does not effect ruminal fermentation, whereas Forsberg (7) has observed that

Table 1. The concentration of copper, zinc, cadmium and lead in the rumen fluid of sheep. Each value represents the mean ± S.E.M. (n = 6)

	Copper (mg.l^{-1})	Zinc (mg.l^{-1})	Cadmium (mg.l^{-1})	Lead (mg.l^{-1})
Control	0.133 ± 0.016	1.533 ± 0.235	0.013 ± 0.001	0.01 ± 0.001
Exposed area	0.383 ± 0.016	5.967 ± 0.159	0.008 ± 0.004	0.01 ± 0.0005
P <	**0.001**	**0.001**	**NS**	**NS**

NS — not-significant

Cu concentrations of 21 $\mu g.ml^{-1}$ can decrease fermentative activity and growth of certain populations of bacteria *in vitro* studies. Furthermore, Odenkir-chen *et al.* (16) have observed that supplementing 2 *g* $CuSO_4$ per animal per day is recommended as the maximum dose in cattle to overcome copper deficiency.

Ingestion of quantities of Cu slightly higher than required may cause accumulation in the tissues and haemolysis. Sheep are more sensitive to high copper supplementation than other farm animals (22).

Metal ions like copper, cadmium, zinc and lead are well known to bind to the -SH groups in the proteins or enzymes, thereby interfering with enzyme activity.

Wallace and McKain (25) have reported that copper, chromium, and mercury inhibited *Prevotella ruminicola* dipeptidase activity to 15, 15 and 5% of control activity. Similar results have been found by Fah- my *et al.* (5) who has described the effectiveness of heavy metals as inhibitors of camel rumen urease at a concentration of 0.005 $mmol.l^{-1}$ in decreasing order $Hg^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+}$ with 97, 94, 90, 61 and 7% inhibition, respectively. Ozcan (17) has observed a decrease in plasma aspartate aminotransferase in rat fed elevated levels of both zinc and copper.

On the other hand Zaki *et al. (26) have reported an increase in the levels of alanine aminotransferase and aspartate aminotransferase in Japanese quails fed elevated levels of Cu. These results are also consistent with findings in sheep when chronic copper poisoning was characterized by increased serum AST level. Thus, AST has been considered a good marker of copper status (15).*

The results of our study showed that zinc concentration and enzyme activities tested were significantly higher in rumen fluid of sheep from an industrially exposed area.

This is in agreement with the findings of Wallace and McKain (25) who have reported that cobalt, manganese and zinc stimulated P. ruminicola dipeptidase activity by 189, 30 and 26%, respectively. On the other hand, Martinez *et al.* (13) have reported that salts of divalent ions such as Sn^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+} produced 90, 82, 65 and 44% inhibition of amylase activity, respectively when assayed at a relative concentration of 5×10^{-3} $mol.l^{-1}$.

Zinc is an essential element in nutrition. Zinc is integral component of metalloenzymes, including glutamate dehydrogenase, lactate dehydrogenase, alkaline phosphatase as well as hormones, such as testosterone and prolactine. Thus, certain enzymes such as alkaline phosphatase have been considered a good indicator of zinc status (9, 2, 19). Many enzymes may become non-functional in the absence of zinc (8). The integrity of cell membrane depends upon loosely bound ionic zinc (20).

Ruminal fluid lead concentration was similar between treatments (Table 1). Bersenyi *et al.* (1) have reported an increase in levels of the aspartate aminotransferase activity in rabbits supplemented with 4.1 mg of $Pb.kg^{-1}$ DM from $Pb(NO_3)_2$ and 30 mg of $Hg.kg^{-1}$ DM from $HgCl_2$. Exposure to Pb significantly decreased red blood cell count, haemoglobin concentration and packed cell volume. The concurrent administration of lead and selenium to broiler chickens produced more deleterious effects characterized by adverse changes in haematological parameters and severe alterations in serum total protein, aspartate aminotransferase, and alkaline phosphatase levels (11).

Ruminal fluid cadmium concentration was similar between treatments. Cadmium is an essential microelement. Cadmium deficiency in ruminants leads to depression of milk production, growth retardation in young animals, muscle weakness, reluctance to move or even death. Increased concentration of cadmium in the body is toxic. The toxic effect of cadmum arises from its ability to bind to protein by thiol groups and may mimic or displace other metals such as zinc. Its toxicity depends on dosage, the method of administration, form, length of exposure and animal species (12).

El-Sebai *et al.* (3) have observed an increase in serum aspartate aminotransferase and gamma-glutamyltransferase and a decrease in alkaline phosphatase and glutathione peroxidase in chicken fed elevated levels of Cd. Bersenyi *et al.* (1) have reported that rabbits supplemented with 2.3 mg of Cd. kg^{-1} DM from $CdSO_4$ had increased alanine aminotransferase activity.

Informations on zinc toxicosis is largely anecdotal. Most cases are due to ingestion of zinc pennies, hardware or zinc oxide by dogs. Birds can be exposed to zinc toxicity if they are housed in flights or cages made of galvanized wire. Lead paint, industrial discharges and automobile emission (in past) are the major exogenous sources of lead exposure. The principal source of air, soil and water contamination by copper is caused by copper and iron manufacture. Cadmium gets into the environment from iron foundries. Heavy metals released into the atmosphere, finally accumulate in the soil and water and enter the food chain.

These results indicate that chronic exposure to pollutants in industrially exposed areas can alter the ruminal enzyme activities of sheep. Further research is needed to determine the role of other pollutants in the metabolism of ruminants.

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THE IMPACT OF VITAMIN E AND ROSEMARY ON THE OXIDATIVE STABILITY OF POULTRY MEAT AND GROUND MEAT PRODUCTS

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ABSTRACT

In our project, the protective effect of the natural antioxidants, rosemary (500 mg.kg^{-1}) and vitamin E (40 mg/*per capita*/day) applied in feeding mixtures from the 21st to 42th day to broiler chickens was observed. Afterwards, the oxidative stability of ground meat and poultry sausages was measured. After slaughter and processing the meat samples were frozen and stored for fourteen days in a freezer at –18 °C. Frozen breast muscles of individual groups of chickens were ground and stored another fourteen days in a refrigerator at 1 °C. Poultry sausages were produced from the frozen thigh muscles of individual groups of chickens. The synergy effect of vitamin E applied in feeding mixtures and rosemary added to ground meat was evaluated. Before the meat was ground, the meat samples of broiler chickens saturated by vitamin E were enriched with 50 mg. kg^{-1} of rosemary powder FlavorGuard P (Chr. Hansen, Denmark). The application of vitamin E and rosemary powder FlavorGuard P into feeding mixtures of broiler chickens increased the oxidative stability fats in meat. The extra addition of rosemary to meat enriched with vitamin E suppressed the oxidative processes more significantly and increased the oxidative stability of poultry meat and poultry sausages. The use of frozen meat and low temperatures (1 °C) during the storage of ground meat also significantly suppressed the oxidative processes.

Key words: broiler meat; lipid oxidation; malondialdehyde; rosemary; vitamin E

INTRODUCTION

Lipids are an important component of food and they have insufficient status in human nutrition. Lipids influence nutrition, energy and the sensory value of food. Foods with a higher fat content are preferred, because fat improves taste and aroma and makes them more attractive for consumers. Lipids are also a source of the lipofilic vitamins (A, D, E, K) and polyunsaturated fatty acids. Dominantly ω3-polyunsaturated fatty acids (PUFA) are important. Nutrition enriched with ω3-PUFA reduces risk of formation of cardiovascular diseases, hypertension and arthritis (4).

Apart from these positive factors, fat consumption in food is accompanied with some negative factors with direct or indirect impact on human health. Among these negative factors are primarily the receipt of energy additives, which are added to fats to correct their properties (stabilisators, emulgators, antioxidants, colours, etc.) and also the consumption of adverse metabolites present in fats or formed from fats (2).

Lipids and PUFA mainly are sensitive to various changes such as hydrolysis and oxidation. Lipid oxidation is an autocatalytic process occurring in food and biological membranes, which leads to significant damage in food quality (5). This process is supported by various factors, the most important of which is the content of PUFA. They can be used as a substrate for the initiation of oxidative process. The main catalysers of oxidation are the highly reactive free radicals such as superoxide anion (O_2^-), hydroxyl radical (OH°), proxy radicals (ROO°), which contain one or more free electrons. Other factors, which support lipid oxidation, are substances

such as myoglobin, haemoglobin, cytochroms and transport metals (Fe). They are produced during the process of meat ripening. The level of oxidation is also increased, because of damage to muscle membranes in processes such as the boning of meat, grinding or cooking (12).

Products formed in the process of fat degradation must be considered very carefully, because of their negative impact on human health. They are included in the mechanisms of such diseases as arteriosclerosis, cancer, suppression of immunity, Parkinson's disease, Alzheimer's disease and numerous cardiovascular diseases (14).

At the present time, there is a greater concentration focused on lipid protection against oxidation, and there are many ways of achieving this aim: the decreasing of temperature in space, prevention of air access, elimination of factors supporting oxidation and addition of antioxidants (2). Nowadays, the most effective inhibition of oxidative processes is the use of antioxidants (18).

Primary antioxidants are, for instance, ascorbic acid and its derivatives, tocopherols, phenol substances and propyl gallate. During recent years, all over the world antioxidative preparation based on rosemary extracts has become very popular in the field of meat processing and meat product production. The antioxidative effect in selected types of preparations ensures natural phenol substances present in spices (18).

The purpose of our project was the evaluation of the influence of natural antioxidants, applied in feeding mixtures, on oxidative changes in fats in ground poultry meat and poultry sausages during storage in chilled conditions. The synergy effect of vitamin E applied in feeding mixtures and rosemary powder added to meat before grinding and their influence on the oxidative stability of fats was also measured.

MATERIAL AND METHODS

In our experiment, the meat of broiler chickens, fed from 21st day of experiment with feeding mixtures saturated with natural antioxidants, was used. In group R rosemary powder FlavorGuard P (Chr. Hansen, Denmark) was applied mixed with the feeding mixture in a dose of 500 mg.kg^{-1}. In group E vitamin E (Hydrovit E forte, PHARMAGAL, Slovak Republic) was applied in a daily dose of 40 ml *per capita* into drinking water. The control group (K) was fed with feeding mixtures without the addition of antioxidants. Immediately after slaughter and processing of the carcasses, the poultry meat was boned and the skin was removed.

The breast muscles were packed into PE sacks (1 kg), frozen and stored for fourteen days at –18 °C. The frozen samples were cut on the cutter and ground for five minutes. For evaluation of the synergy effect of vitamin E applied in the feeding mixture and rosemary powder FlavorGuard P added before grinding, rosemary powder in a dose of 50 mg.kg^{-1} was mixed into meat saturated with vitamin E. The ground meat was stored in PE sacks for fourteen days in a refrigerator at 1 °C.

The thigh muscles of individual broiler chickens were frozen at –18 °C, stored for fourteen days and used for the production of poultry sausages. Four groups of samples were produced: K – control group; E – meat of broiler chickens fed with feeding mixtures saturated with vitamin E; R – meat of broiler chickens fed with feeding mixtures saturated with rosemary powder and E + R – meat of broiler chickens fed with feeding mixtures saturated with vitamin E and with the addition of rosemary powder in a dose of 50 mg.kg^{-1} before grinding.

The meat was cut on the cutter of frozen meat. After the addition of a salting mixture and in group E + R rosemary powder also, the meat was ground for two minutes. The addition of ingredients according to recipe (Table 1) followed and the meat was ground for another three minutes. After mixing the meat with the ingredients filling into pork intestines followed. Thermal treatment followed at 70 °C for ten minutes in the centre of sausages. The total time of thermal treatment was three and a half hours.

Table 1. Poultry sausage – recipe

Ingredients (g)	R	K	E	E + R
Meat	2000	2000	2000	2000
Flour	90	90	90	90
Salting mixture	55	55	55	55
Water	300	300	300	300
Black spice	5	5	5	5
Red spice	4	4	4	4
FlavorGuard P (mg)	–	–	–	100

K — control; E — meat of broiler chickens fed with vitamin E (40 mg/*per capita***/day);** R — meat of broiler chickens fed with rosemary powder (500 mg.kg^{-1}); E + R — meat of broiler chickens fed with vitamin E (40 mg/*per capita*/day) and with addition of rosemary powder (50 mg.kg^{-1}) before grinding

The decomposition of fats was evaluated by the method of measurement of the thiobarbituric acid value (TBA), the expressed amount of malondialdehyde calculated for one kilogram of the sample. The evaluation of TBA was carried out according to Grau *et al.* (6) and measured spectrophotometrically at 532 nm (Helios γ, v. 4.6, Thermo spectronic, Great Britain). The examination of the samples was carried out at 0, 7, 9 and 14 days of storage in chilled conditions. The values of selected parameters, recorded in tables, are average values derived from the calculation from six samples of meat and meat products.

RESULTS AND DISCUSSION

The lipids in poultry exhibit a higher degree of unsaturation than red meats due to the relatively high content of phospholipids. The degree of unsaturation of the phospholipids of the subcellular membrane is an important factor in determining the oxidative stability of meats, with the oxidative potential increasing as the degree of unsaturation of the lipids in the meat increases (3). In relation to the character of the process of auto

Table 2. Determination of TBA, presented as a malondialdehyde, in grinding poultry meat (breast) stored at 1 °C, after feeding of antioxidants

Time of storage (day)	Malondialdehyde (mg.kg^{-1})			
	K	R	E	R + E
0	0.031 ± 0.005	0.029 ± 0.004	0.030 ± 0.004	0.025 ± 0.003
3	0.039 ± 0.008	0.030 ± 0.005	0.031 ± 0.007	0.027 ± 0.003
7	0.068 ± 0.019	0.056 ± 0.007	0.052 ± 0.002	0.031 ± 0.007
9	0.079 ± 0.007	0.043 ± 0.013	0.042 ± 0.003	0.032 ± 0.004
14	0.227 ± 0.020	0.177 ± 0.008	0.151 ± 0.009	0.081 ± 0.010

K — control; E — meat of broiler chickens fed with vitamin E (40 mg/*per capita***/day);** R — meat of broiler chickens fed with rosemary powder (500 mg.kg^{-1}); E + R — meat of broiler chickens fed with vitamin E (40 mg/*per capita*/day) and with addition of rosemary powder (50 mg.kg^{-1}) before grinding

oxidation, the effect of antioxidants is more significant, the sooner they are applied. The ideal situation is for the fats to be protected immediately after the slaughter of the animals (15, 17). This protection can be achieved through feeding antioxidants to live animals (3).

The oxidation of lipids is influenced by the addition of antioxidant substances. The practical application of antioxidants can be difficult from the hygiene and technology point of view. It is more advantageous, if natural antioxidants are included in feeding mixtures (11). Feeding poultry a higher level of natural dietary antioxidants provides the poultry industry with a simple method for improving the oxidative stability, sensory quality, shelf life and acceptability of poultry meats.

Vitamin E and rosemary belong to substances with a significant antioxidative effect. They are important for the stabilisation of the optimal healthy status of poultry and as prevention against diseases. Higher concentrations of vitamin E in feeding mixtures have a positive impact also on feed conversion, the increase of daily gains and average weight of broilers, and the decrease of feed consumption (3).

The results of the examination of malondialdehyde in frozen breast muscles, ground for five minutes and stored at 1 °C are shown in Table 2. Immediately after grinding, the MDA amount in all samples was low. This status is probably due to the freezing of meat, when temperature of meat after grinding was –3.5 °C and as the period of storage in the refrigerator did not exceed 1 °C. During the first nine days of storage in all samples only a slight increase in MDA concentrations was recorded. The highest amount of MDA on day nine of storage was in the control group (0.079 mg.kg^{-1}). The lowest amount of MDA in the samples and the best antioxidative effect were measured in meat samples with the combination of antioxidants, vitamin E and rosemary powder.

Subsequent storage of the samples caused an increase in MDA concentrations. The application of antioxidants to feeding mixtures increased the oxidative stability of lipids in meat in comparison with the control group. Better oxidative stability was also evident in meat samples of broilers fed with vitamin E and rosemary alone. The most significant effect was achieved by a combination of vitamin E saturated in feeding mixtures with rosemary powder added into the meat before grinding. A significant antioxidative effect of added antioxidants was confirmed.

The method of meat processing, temperature of storage has a significant influence on oxidative stability of lipids (1, 12). Decreasing the temperature in space makes the process of all chemical reactions slower, included lipid oxidation. The combination of freezing and addition of antioxidants, primarily the combination of vitamin E and rosemary (9, 13), significantly suppresses the process of lipid oxidation.

The process of production of meat products (cutting, grinding, and mixing) causes degradation of the muscle membrane system and has a strong influence on the oxidation of intracellular fat, primarily phospholipids

Table 3. Determination of malondialdehyde in poultry sausages after addition of antioxidants

Time of storage (day)	Malondialdehyde (mg.kg^{-1})			
	K	R	E	R + E
1	0.444 ± 0.003	0.378 ± 0.011	0.397 ± 0.007	0.326 ± 0.009
7	0.430 ± 0.012	0.391 ± 0.008	0.342 ± 0.008	0.319 ± 0.011
14	0.525 ± 0.008	0.399 ± 0.015	0.359 ± 0.012	0.304 ± 0.005

K — control; E — meat of broiler chickens fed with vitamin E (40 mg/*per capita*/day); R — meat of broiler chickens fed with rosemary powder (500 mg.kg^{-1}); E + R — meat of broiler chickens fed with vitamin E (40 mg/*per capita*/day) and with addition of rosemary powder (50 mg.kg^{-1}) before grinding

(2). Thermal treatment makes the oxidative processes faster, which significantly changes the amount in thiobarbituric acid value.

The level of oxidative damage of lipids in the production and storage of poultry sausages is recorded in Table 3. Immediately after the production of sausages a higher level of oxidative damage of lipids was recorded. This situation is related to the thermal treatment of poultry products. The highest amounts of MDA were measured in products produced from the meat of the control group (0.444 mg.kg^{-1}). The lowest oxidative damage of lipids was recorded in sausages produced from meat of broiler chickens fed with vitamin E and with the addition of rosemary powder before grinding. The storage of products at 4 °C influenced only MDA concentrations in the control group, when a significant increase in the amount was measured (0.525 mg.kg^{-1}). On the other hand, in other groups the opposite effect was recorded (E, E + R) or the stabilisation (R) of MDA amounts.

The antioxidative effect of rosemary is based on the ability to deactivate free radicals, which are produced in the process of auto oxidation (16). Rosemary extracts, as antioxidants, have been successfully used for the stabilisation of smoked products (10), cans (7), pork meat (13), poultry meat and products (8). Knowledge in current literature corresponds with the results, which have been recorded in our experiment. Antioxidative activity is significantly obvious in systems containing tocopherols (16).

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