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EARLY IMMUNOLOGICAL EVENTS IN GERM-FREE PIGLETS MONOASSOCIATED WITH NONPATHOGENIC OR VIRULENT STRAIN OF *SALMONELLA TYPHIMURIUM*[*]

ČASNÉ IMUNITNÍ REAKCE U BEZMIKROBNÍCH SELAT MONOASOCIOVANÝCH S NEPATOGENNÍM NEBO VIRULENTNÍM KMENEM *SALMONELLA TYPHIMURIUM*

I. Trebichavský

Institute of Microbiology, Nový Hrádek, Czech Republic

ABSTRACT: An early response (6 and 24 hrs) of germ-free miniature piglets to oral treatment with virulent LT2 strain or avirulent SF1591 rough mutant of *S. typhimurium* was studied. Six hours postinfection, bacteria colonized the gut except for the rectum and penetrated into the mesenteric lymph nodes. In addition, LT2 strain was found in the spleen, blood and lungs. The virulent strain damaged ileal enterocytes and caused immigration of myeloid cells into the *villus lamina propria*. Virulent but not nonpathogenic bacteria in lymphatic organs (but not in the gut) were stained with mAb recognizing heat shock protein HSP65. No significant change in the distribution of lymphocyte subpopulations could be found six hours postinfection.

swine; gnotobiotic piglets; *Salmonella typhimurium*

ABSTRAKT: Byla studována časná odpověď (6 a 24 hodin) bezmikrobních miniaturních selat na perorální aplikaci virulentního kmene LT2 nebo nepatogenního SF1591 mutantu *S. typhimurium*. Šest hodin po infekci bakterie kolonizovaly celé střevo kromě rekta a pronikly do mesenteriálních lymfatických uzlin. Virulentní kmen byl nalezen také ve slezině, krvi a plicích. Virulentní kmen poškodil ileální enterocyty a vyvolal vcestování myeloidních buněk do *lamina propria* klků. Virulentní, ale ne nepatogenní bakterie v lymfatických orgánech (ale ne ve střevě) byly pozitivní s protilátkou namířenou proti stresovému proteinu HSP65. Šest hodin po infekci nebyla zjištěna žádná významná změna v distribuci subpopulací lymfocytů.

prase; bezmikrobní selata; *Salmonella typhimurium*

INTRODUCTION

In swine, salmonellosis is most often caused by *Salmonella choleraesuis* and *S. typhimurium* (official designation, *S. enterica* serotype Typhimurium). The pathogenesis of *Salmonella* infections is complex and many virulence factors have recently been identified (Bäumler, 1997; Groisman and Ochman, 1997). Upon penetration of the intestinal mucosae, *Salmonella* spp. are confronted by macrophages that line the lymphatic sinuses of regional lymph nodes and survive within their phagocytic vacuoles (Kaufmann 1996). Salmonella infections of the pig were studied predominantly in conventional animals. The germ-free pig, however, represents an excellent model of salmonellosis and provides interesting results (reviewed by Trebichavský et al., 1998). In this study, germ-free piglets were orally infected with two strains of *S. typhimurium* differing in pathogenicity and their early response was described.

MATERIAL AND METHODS

Animals

Seventeen miniature germ-free (GF) piglets of Minnesota-derived breed were delivered by hysterectomy, held in sterile isolators and fed a milk diet supplemented with vitamins (Mandel, 1997). 1-week-old GF piglets were divided into three groups:

- five control GF piglets
- six piglets monoassociated with virulent streptomycin-resistant smooth LT2 strain of *S. typhimurium*
- six piglets monoassociated with nonpathogenic SF1591 rough mutant of *S. typhimurium*

Salmonella was described previously (Dlabač et al., 1997). The SF1591 is a stable chemotype Ra mutant of *S. typhimurium* M206 with a deletion in the His locus, the LPS of which has a complete polysaccharide. Twelve piglets were associated by feeding 10^8 bacteria

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in milk diet. Seven infected piglets were sacrificed six hours postchallenge, five piglets were sacrificed twenty four hours postchallenge. Three piglets in each of these groups were associated with nonpathogenic rough mutant. Experiments were approved by the Ethical Committee of the Institute.

Enumeration of *Salmonella*

The intestinal contents and samples of mesenteric lymph nodes (MLN), liver, spleen and lungs were aseptically dissected and homogenized in deionized water. Suspensions were serially ten- fold diluted in saline and 0.1 ml of appropriate dilutions (or 0.1 ml of blood obtained by cardial puncture) were inoculated onto Endo plates. After 24 hrs of culture at 37 °C, colonies were counted.

Immunofluorescence on frozen sections

Samples of terminal ilea, spleen and MLN were snap-frozen in isopentane and liquid nitrogen. Cryostat sections were air dried, fixed for 5 min in cold acetone, washed and kept in a deep freezer. Before use, they were pre-incubated with 10% normal pig serum in 37 °C and incubated with optimally diluted mouse monoclonal antibodies recognizing SLA-DR (porcine MHC II molecule) or HSP 65 (heat shock protein) which were detected by fluoresceinated swine anti-mouse Ig purchased from Medicamenta (Vysoké Mýto, Czech Republic).

APAAP on frozen sections

Cryostat sections of ilea were washed in TRIS-buffered saline and treated with monoclonal antibodies directed against pig CD2, CD4, CD8, TCR γδ, IgM, porcine differentiation antigens SWC3 and SWC6 and MIL-4 mAb (see later). Positive cells were labelled by the DAKO alkaline phosphatase-anti phosphatase kit (DAKO, Glostrup, Denmark). Anti-mouse Ig was diluted 1 : 25 and APAAP (alkaline phosphatase- antiphosphatase) was diluted 1 : 50. A fast red system with levamisole inhibition was used according to the manufacturer's instruction for the detection of enzymatic marker.

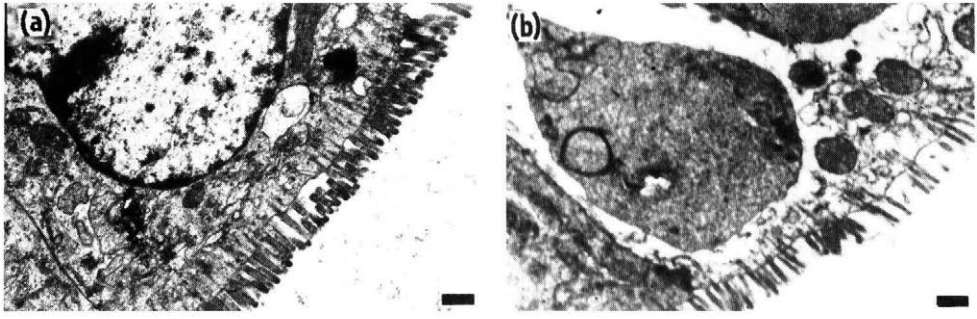
Antibodies

Nomenclature for swine leucocyte differentiation antigens was recently reviewed by Saalmüller (1996). The following mouse anti-pig monoclonal antibodies (mAb) were used as primary immunoreagents: anti-CD2 (MSA4, IgG2a), anti-CD4 (10.2H2, IgG2b), anti-CD8 (76-2-11, IgG2a), anti-δ (PGBL 22A, purchased from VMRD, Pullman, WA), anti-sIgM (LIG4, IgG1), anti-SLA-DR (MSA-3, IgG2a), anti-SWC3 (74-22-15 mAb recognizing pig macrophages and granulocytes, IgG1), MIL-4 (*lamina propria* mast cells, granulocytes and other cells, IgG1 Haverson et al. 1994). The ML30 mAb recognized a conservative epitope of the *Mycobacterium leprae* heat shock protein (HSP) 65 shared with mammalian HSP (Ivanyi et al., 1983).

The anti-SWC6 rat mAb (MAC320, IgG2a) recognizing pig null lymphocytes (mostly γδ T cells) was also used and detected by APAAP system.

Electron microscopy

Samples of ilea were immediately fixed in 1% glutaraldehyde in 0.2M sodium cacodylate buffer pH 7.2 and postfixed in 1% buffered osmium tetroxide. Dehydrated samples were embedded in Vestopal W polyester resin (Serva, Heidelberg, Germany). Ultrathin sections stained in uranylacetate and lead citrate were observed in a Tesla BS500 transmission electron microscope (Brno, Czech Republic).



1. Electron micrographs showing pig ileal enterocytes six hours after peroral infection with *Salmonella typhimurium* (bars = 0.5 μm).
a) nonpathogenic SF1591 rough mutant did not cause any ultrastructural changes.
b) virulent LT2 strain damaged epithelial brush border and cytoplasm.

RESULTS

Gnotobiotic piglets associated for six hours with *Salmonella typhimurium*

Ileal villi of germ-free piglets lacked intraepithelial and stromal lymphocytes and their enterocytes contained large cytoplasmic vacuoles. In piglets associated with nonpathogenic Salmonella rough mutant, enterocytes were intact and their brush border and cytoplasm expressed normal ultrastructural features (Fig. 1a). Bacteria were cultivated from the entire gut, except the rectum, and from mesenteric lymph nodes (two in three piglets). They were not stained by ML30 antibody (anti-HSP65).

In contrast, microvilli and apical cytoplasm of numerous ileal enterocytes were heavily damaged in all piglets associated with the virulent LT2 strain (Fig. 1b). The MIL-4$^+$ cells (large myeloid cells according to their morphology) occurred in the stroma of ileal villi. The bacteria were present in the entire gut, except the rectum, and in all MLN. Furthermore, they were found also in the spleen, lungs (one piglet examined) and peripheral blood (three piglets in four). The LT2 bacteria which penetrated into the spleen and MLN (but not intestinal bacteria) were stained by ML30 mAb. Host's cells were not stained by this antibody in this stage.

High immunofluorescent positivity for SLA-DR (porcine MHC II) molecules was observed in cryostat sections of mesenteric lymph nodes of all infected piglets in comparison with MLN of germ-free piglets. No significant immigration of cells with lymphocyte markers (sIgM, CD2, CD4, CD8 and $\gamma\delta$ TCR) and SWC3$^+$ cells into ileal villi could be detected by APAAP immunohistochemistry six hours after oral association with Salmonella.

Gnotobiotic piglets associated for twenty-four hours with *Salmonella typhimurium*

Both bacterial strains were cultivated from the whole gut, MLN, spleen, liver, lungs and blood (rough mutant in two piglets in three). Piglets associated with virulent LT2 strain had devastated ileal epithelium and were in septic state. Some cells in the *villus lamina propria* and lymphatic organs of piglets challenged with the virulent strain expressed positivity with the ML30 mAb. In contrast, no clinical abnormalities were detected following challenge by SF1591 bacteria and all piglets associated with this mutant thrived. High immunofluorescent positivity for SLA-DR was observed in cryostat sections of the ileum and lymphatic organs of all piglets associated with Salmonella.

DISCUSSION

Early effects of *Salmonella typhimurium* were studied in gnotobiotic piglets fed milk supplemented with bacteria. The milk was found in the ileum of young piglets three hours after suckling (Gaskins and Kelley, 1995). Thus, six hours postinfection, bacteria could be in contact with ileal epithelium for about three hours (pig terminal ileum with a continual Peyer's patch is the major site of Salmonella penetration in oral route of infection). Interestingly, this short period was sufficient for the virulent strain to cause bacteremia. Ileal mucosa of 1-week-old germ-free piglets (control piglets in this study) has recently been described (Trebichavský et al., 1997).

T cells and NK cells are important sources of protective type-1 cytokines, particularly tumour necrosis factor-α and gamma interferon (Nauciel et al., 1992) induced by interleukin-18 (Mastroeni et al., 1998). Cytokines derived from T cells of *Salmonella enteritidis* immunized pigs successfully protected weaned piglets from *S. choleraesuis* infection (Genovese et al., 1998).

The aim of this study was to determinate possible early redistribution of $\gamma\delta$ T and NK lymphocytes. Like myeloid cells, these cells are engaged in the mechanisms of innate immunity (Timonen, 1997; Boismenu and Havran, 1998) and it would be therefore interesting to find that they are capable of early migration. No such a study has been done, to our knowledge, in germ-free animals a few hours postinfection. Lymphocyte migration into intestinal villi is easily detected in gnotobiotic piglets because these structures lack them. The *lamina propria* of newborn and germ-free piglets is devoid of T cells (Haverson et al., 1999).

We have recently described an increase in MAC320$^+$ $\gamma\delta$ T cells 24 hours after Salmonella infection (Trebichavský et al., 1999). In this study, we concentrated on the early period. Using APAAP immunohistochemistry and a panel of monoclonal antibodies, no significant difference between germ-free and infected piglets could be found in the distribution of ileal lymphocytes. These results were confirmed also in peripheral blood and lymphatic organs by quantitative flow cytometry (unpublished results) of eleven lymphocyte subpopulations (Šinkora et al., 1998) including one NK phenotype (CD3$\bar{\varepsilon}$ CD4$^-$ CD8^{low} CD2$^+$) and three subpopulations of $\gamma\delta$ T cells.

Both bacterial and host heat shock proteins (HSP) 60/70 are expressed in Salmonella gastrointestinal infections and can play an important role in the development of autoimmunity (Res et al., 1991). In this study, we have observed binding of the ML30 mAb (anti-HSP65 of GroEL family) to virulent bacteria present in lymphatic organs of infected gnotobiotic piglets. The Salmonella GroEL protein, an analog of the mycobacterial 65 kDa heat shock protein, was shown to be upregulated when *Salmonellae* were growing intracellularly within macrophages (Buchmeier and Heffron, 1990). Avirulent *S. typhimurium* cells do not survive within macrophages (Fields et al., 1986). This was probably the reason why SF1591 bacteria penetrating into lymphatic organs of infected piglets were not stained by the ML30 antibody. Stress proteins can be expressed in

mammalian cells also constitutively. Low immunostaining of enterocyte basal part by the same ML30 antibody was previously found in older conventional but not newborn piglets (Trebichavský et al., 1993). Control samples of ilea from GF piglets did not show such a labeling.

The present study has described an early cellular effect of oral challenge with *S. typhimurium*. Inflammatory cytokines induced in gnotobiotic piglets by defined strains of Salmonella will be the aim of future studies.

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Contact Address:

RNDr. Ilja Trebichavský, CSc., Mikrobiologický ústav AV ČR, 549 22 Nový Hrádek, Česká republika
Tel. +420 441 47 82 16, fax +420 441 47 82 64, e-mail: treb@biomed.cas.cz

GOITROGENIC EFFECTS OF EXTRACTED RAPESEED MEAL AND NITRATES IN SHEEP AND THEIR PROGENY*

STRUMIGENNÍ ÚČINEK ŘEPKOVÉHO EXTRAHOVANÉHO ŠROTU A DUSIČNANŮ U OVCÍ A JEJICH POTOMSTVA

J. Kursa[1], J. Trávníček[1], W. A. Rambeck[2], V. Kroupová[1], J. Vítovec[1]

[1] *University of South Bohemia, Faculty of Agriculture, České Budějovice, Czech Republic*
[2] *University Munich, Institute of Physiology, Physiological Chemistry and Animal Nutrition, Munich, Germany*

ABSTRACT: Three groups of the breed Šumava sheep were fed meadow hay completed with extracted rapeseed meal, containing 4.2 mmol of glucosinolates, and 4 g of sodium nitrate per animal per day for 382 days. Moreover, two of the groups received parenterally 0.25 mg iodine and 0.15 mg sodium selenite per animal and per day at weekly intervals. The fourth group, also supplemented with iodine and selenium, was fed a diet without the addition of nitrate in which extracted rapeseed meal was replaced with oats meal. Biochemical analyses included determination of iodine concentrations in colostrum and milk of ewes and iodine, thyroxine and triiodothyronine concentrations in blood plasma of lambs from birth up to the age of 60 days. Thyroids of five necropsied lambs were weighed and examined by light and electron microscopy. Hypothyreosis with sporadic occurrence of congenital goitre and low concentrations of iodine and thyroxine in blood plasma were observed in the progeny of the ewes receiving in their diet extracted rapeseed meal and nitrate without iodine and selenium supplementation. The histological structure of the enlarged thyroid indicated 3rd stage of goitre. Electron microscopy revealed multilayer arrangement of follicles, cystic extension of cisterns of the endoplasmatic reticulum, numerous activated mitochondria, and pyknotic nuclei displaced towards the cytoplasmatic membranes of thyreocytes. Biochemical analyses demonstrated low concentrations of iodine in colostrum and milk (57 ± 36 and 27 ± 15 μg/l, respectively) and of iodine and thyroxine in blood plasma of lambs (69 ± 24 μg and 101 ± 27 nmol/l, respectively). On the other hand iodine concentrations in colostrum and milk were significantly ($P < 0.01$) higher in the groups receiving the same doses of goitrogens, but supplemented with iodine alone (colostrum 334 ± 330 μg/l; milk 101 ± 41 μg/l), or iodine and selenium (colostrum 430 ± 520 μg/l; milk 70 ± 16 μg/l). Also higher were mean iodine and thyroxine concentrations in blood plasma of lambs born in these groups. The highest concentrations of iodine in milk and colostrum (2 083 ± 1 423 and 198 ± 8 μg/l, respectively) were found in the group fed the diet free of extracted rapeseed meal and nitrates and supplemented with iodine and selenium. The differences between this and any other group were highly significant ($P < 0.01$). Mean concentrations of thyroxine and triiodothyronine in blood plasma of lambs of the individual groups correlated with iodine concentrations in colostrum and milk of the dams. The differences among groups were nonsignificant. The experiment demonstrated goitrogenic effects of glucosinolates presented in extracted rapeseed meal and nitrate, which can be compensated by supplementation of iodine or iodine + selenium.

sheep; glucosinolates; supplementation; iodine; selenium; thyroid gland; goitre; thyroxine; triiodothyronine; blood; colostrum; milk

ABSTRAKT: V pokusu se 24 ovcemi, plemene šumavská ovce, ve věku 12 až 18 měsíců byl 382 dnů sledován účinek dlouhodobého příjmu řepkového extrahovaného šrotu a dusičnanu v dietě s cílem určit jejich vliv na štítnou žlázu bahnic a potomstva a postihnout kompenzační efekt jodu a selenu. Ovce byly umístěny v experimentální stáji fakulty. Byly rozděleny na čtyři skupiny po šesti kusech (tab. I). Základ celoroční krmné dávky tvořilo luční seno, napájecí voda pocházela z ústředního zdroje. Ovce tří pokusných skupin (E1, E2, C1) přijímaly v doplňkové krmné směsi s řepkovým extrahovaným šrotem v průměru 4,2 mmol glukosinolátů a 4 g NO_3^- na kus a den. V krmné směsi pro čtvrtou skupinu (C2) byl řepkový extrahovaný šrot nahrazen ovesným šrotem bez přídavku $NaNO_3$ (tab. II). Suplementárním zdrojem jodu pro ovce ve skupinách E1, E2 a C2 byl roztok jodidu draselného u selenu pak přípravek Selevit inj. ad us. vet s účinnou látkou bezvodým seleničitanem sodným. Injekční aplikace se uskutečňovala v týdenních intervalech v dávkách odpovídajících příjmu 0,25 mg jodu a 0,15 mg selenu pro kus a den. Byl sledován zdravotní stav ovcí a jejich potomstva, modifikovanou metodou dle Sandell-Kolthoffa stanovena koncentrace jodu v kolostru a v mléce bahnic, zjišťována koncentrace jodu a hormonů tyroxinu a trijodtyroninu

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v krevní plazmě jehňat od narození do 60. dne věku a provedena pitva, histologické a elektronmikroskopické vyšetření štítné žlázy pěti jehňat. K připouštění, uskutečněném přirozenou plemenitbou harémovým způsobem, byli použiti dva berani stejného plemene. Krmná dávka s řepkovým extrahovaným šrotem neovlivnila negativně reprodukční ukazatele (tab. III). Ve skupině C1, přijímající řepkový extrahovaný šrot a dusičnany bez suplementace, se tyreostatický účinek glukosinolátů projevil hypotyreozou vyjádřenou vznikem kongenitální strumy a změnami funkčních parametrů štítné žlázy (tab. IV). Pitva jehněte s klinickými příznaky strumy určila 2,5 až pětinásobné zvýšení hmotnosti štítné žlázy, histologická struktura parenchymu odpovídala třetímu stadiu strumy. Elektronmikroskopické vyšetření (TEM) strumy prokázalo ve vrstevnatě uspořádaných folikulech cysticky rozšířené cisterny hrubého endoplazmatického retikula, hojné aktivované mitochondrie a pyknotická jádra odtlačená k cytoplazmatickým membránám tyreocytů (obr. 2). S histologickým a elektronmikroskopickým nálezem na štítné žláze korelovaly velmi nízké hodnoty tyroxinu (46 až 37 nmol/l), trijodtyroninu (2,6 až 1,9 nmol/l) a nízká hladina jodu krevní plazmy (45 až 26 μg I/l) v prvém až třetím dnu života (tab. V). U jehňat z ostatních pokusných skupin od matek se suplementací (E1, E2, C2, č. 2, 13A, 21), která byla klinicky zdravá, byla sekčně vyšetřena a měla normální histologickou stavbu štítné žlázy se individuální koncentrace tyroxinu pohybovaly mezi 73 až 252 nmol/l, trijodtyroninu mezi 4,6 až 8,3 nmol/l, a obsah jodu v krevní plazmě mezi 80 až 236 μg/l. Tyto individuální biochemické profily jehňat korespondují s rozdíly průměrů funkčních parametrů štítné žlázy mezi jednotlivými skupinami. Příjem glukosinolátů a dusičnanů bez suplementace vyvolal u ovcí nízké vylučování jodu mlezivem (57 ± 36 μg I/l) a mlékem (27 ± 15 μg I/l; tab. VI) s následnými nízkými koncentracemi tyroxinu (101 ± 27 nmol/l) a jodu (69 ± 24 μg I/l) v krevní plazmě jehňat. Statisticky významně vyšší ($P < 0,01$) obsah jodu v kolostru a v mléce bahnic se stejnou strumigenní zátěží, ale s adicí jodu (skupina E2) nebo jodu a selenu (skupina E1), dosáhl hodnot 334 ± 330 μg I/l, resp. 430 ± 520 μg I/l v mlezivu a 101 ± 41 resp. 70 ± 16 μg I/l v mléce. Dostatečné saturaci jodem u zvířat v těchto skupinách též odpovídají u sajících jehňat skupinové průměry hladin jodu v krevní plazmě 127 ± 45 μg I/l (skupina E2) a 139 ± 51 μg I/l (skupina E1) a tyroxinu 115 ± 30 nmol/l (skupina E2) a 127 ± 39 nmol/l (skupina E1). Nejvyšší obsah jodu v mlezivu (2083 ± 1423 μg I/l) a v mléce ovcí (198 ± 8 g I/l) byl zaznamenán ve skupině C2 s krmnou dávkou bez řepkového extrahovaného šrotu a s přídavkem jodu a selenu. Rozdíly mezi touto a ostatními skupinami byly statisticky vysoce významné ($P < 0,01$). Skupinové průměry koncentrací tyroxinu a trijodtyroninu v krevní plazmě jehňat korespondovaly s obsahem jodu v mlezivu a v mléce jejich matek, rozdíly mezi skupinami nebyly statisticky významné. Z výsledků pokusů je patrné, že štítná žláza ovcí reaguje citlivě na působení glukosinolátů v řepkovém extrahovaném šrotu s přídavkem dusičnanu, které lze eliminovat adicí jodu, příp. jodu a selenu.

ovce; glukosinoláty; přídavek jodu a selenu; štítná žláza; struma; tyroxin; trijodtyronin; krev; mlezivo; mléko

INTRODUCTION

Along with the medical research of iodine deficiency in the human population, which has been included among the cardinal preventive projects of the World Health Organisation (Delange et al., 1997), agricultural scientists pay increased attention to sufficient supply of iodine to animals. In the Czech Republic, the significance of the relation between iodine deficiency and performance of farm animals is increasing due to frequent occurrence of goitre in young farm animals (Čada, 1988) data on further adverse effects of iodine deficiency published abroad (Groppel, 1991), and reports of iodopoenic disturbances occurring even after 40 years of iodine supplementation of table salt (Zamrazil et al., 1997). Among other suggestions, the benefits of inclusion of iodine into the natural food chains is emphasized. The most effective physiological way is to increase iodine content in natural foods including meat, milk, milk products and eggs.

Epizootiological analyses revealed several factors participating in the occurrence of clinical and subclinical hypothyreosis in animals (Herzig et al., 1999). The primary deficiency induced by insufficient iodine intake is due to feeding of unsupplemented roughage and grain meals of exclusively local origin (Kursa et al., 1997). This fact is associated with historically known iodine deficiency in the environment of the Czech Republic, therefore there was occurrence of goitre in humans and cretinism in newborns (Pohůnková and Němec, 1988; Hníková, 1995; Stárka, 1995).

The data presented in this paper were obtained within a research project encouraged by results of epidemiological studies and preventive actions aimed at the control of iodopoenic situations in animal herds in South, West, and Central Bohemia and in Moravia. Previous screenings have showed that, in terms of the current knowledge, the supply of iodine to animals was insufficient and thus conditions enhancing the effect of goitrogens were prepared (Čada, 1988; Herzig et al., 1999; Písaříková et al., 1996).

The increasing interest in goitrogenic effects of rapeseed is justified by the current position of this species among other oil-producing crops and growing use of rape products (extracted rapeseed meal and rapeseed cake) as components of concentrates (Emanuelson, 1989; Šimek et al., 1999). Currently, data on the relation between the content of glucosinolates in rape and the activity of the thyroid gland gained in experiments in laboratory animals and results of experiments in swine (Schöne et al., 1997) are available. Ehlers et al. (1994) recommend to consider the variety of rape, to observe rules for dosage, and to ensure excessive saturation with iodine when ERM is fed to cattle.

Besides biochemical tests, the application of morphological and histological methods is inevitable for

the investigation of physiological events and pathological conditions of the thyroid gland (Pícha et al., 1970). Their relevance to the diagnostics of thyreopathies was confirmed by Čada (1988) who disposed of a rich necropsy material and described the development of lesions characterising goitre due to maternal iodine deficiency.

Weighing and partial histological studies of the thyroid gland were extensively applied by Groppel (1991) and other members of his team (Schöne et al., 1997). An increase in the weight of the thyroid gland in pigs fed a concentrate containing 15% (Corino et al., 1991) of ERM was also demonstrated.

The objectives of the experiment were to find effects of long-term feeding of ERM and sodium nitrate on the thyroid gland of ewes and their progeny and to assess the compensatory effect of iodine and selenium supplementation. This model experiment was based on the recent occurrence of spontaneous goitre in ruminants in several areas of the Czech Republic.

MATERIAL AND METHODS

Tests of effects of goitrogenic agents were done in 24 ewes of the breed Sumava sheep aged 12 to 18 months. The sheep were purchased from a breeding herd in the district of Prachatice which is an area with increased occurrence of goitre. The animals were divided into four groups of six (E1, E2, C1, C2). The design of the experiment, composition of feed ration, and data on iodine and selenium supplementation are given in Tab. I. Composition of concentrate is given in Tab. II.

The sheep were housed in groups in a deep-litter barn, fed twice a day, and had free access to crockery troughs with potable water supplied from a public source.

In all the groups, the major component of the ration was meadow hay harvested in the area of origin of the sheep. Hay was completed with the concentrates A and B prepared in the experimental feed mill of the Central Agricultural Control and Testing Institute, Lysá nad Labem, Czech Republic. Vitamin additives Combinal A forte, Combinal D forte, and Combinal E forte were administered in drinking water. Moreover, feeding carrot was offered and iodine-free lick salt was available.

The sheep of the groups E1, E2, and C1 received daily the concentrate B containing extracted rapeseed meal (ERM – 82.5%) and sodium nitrate (NO_3 – 1.9%). The dose was 287 g per animal and day and corresponded to a daily intake of 4 g of sodium nitrate and 4.2 mmol of glucosinolates per animal and day. The content of glucosinolates was determined by gas chromatography after derivatisation to silyl compounds (Zukalová and Vašák, 1978). The group C2 received the concentrate A in which ERM was replaced with oats meal and urea. The experiment was started by a 7-day adaptation period during which the animals received one half of the concentrates. Additional sources of iodine and selenium for the sheep of the groups E1, E2, and C2, administered from 1st to 264th day of the experiment, were a potassium iodide solution and Selevit inj. ad us. vet. containing anhydrous sodium selenite as the active substance. The supplements were administered subcutaneously at weekly intervals in doses corresponding to 0.25 mg of iodine and 0.15 mg of selenium per animal.

The ewes were bred naturally from day 81 to day 187 using two breeder rams tested for fertility. The rams were kept alternatively with the individual groups always for a two-week period. Highly pregnant ewes were separated into individual boxes and kept there with their lambs 3 to 5 days after parturition. This period was prolonged in ewes which gave birth to twins.

The ewes were weighed before the experiment, on experiment day 220, and after the experiment. Considering the differences in the initial live weight, the relative growth rate was calculated using the formula (Karakoz, 1968):

$$Q = \frac{Yt - Yo}{Yo}$$

The lambs were weighed immediately after birth and at the age of 10 days. Blood samples were collected from *v. jugularis* immediately after birth, 24 and 48 hours thereafter and on post-partum days 10, 30 and 60, always between 07.00 and 10.00 a.m.

I. Goitrogenic effect of extracted rapeseed meal and nitrates in ewes and their progeny – experimental design

Group	Feed ration (animal/day)	Goitrogen	Supplementation
E1 $n = 6$ Sheep Nos. 1–6	hay 1.4 kg concentrate B 287 g lick	ERM $NaNO_3$	iodine 0.25 mg as a KI solution once per week s.c. selenium 0.15 mg as Selevit once per week s.c.
E2 $n = 6$ Sheep Nos. 13–18	hay 1.4 kg concentrate B 287 g lick	ERM $NaNO_3$	iodine 0.25 mg as a KI solution once per week s.c.
C1 $n = 6$ Sheep Nos. 7–12	hay 1.4 kg concentrate B 287 g lick	ERM $NaNO_3$	0
C2 $n = 6$ Sheep Nos. 19–24	hay 1.4 kg concentrate A 287 g lick	0	iodine 0.25 mg as a KI solution once per week s.c. selenium 0.15 mg as Selevit once per week s.c.

Iodine concentration was determined in the first colostrum sample collected immediately after parturition and in milk samples collected at the end of the experiment (day 382), i.e. 61 to 140 days after parturition.

Iodine in blood plasma of lambs and in colostrum and milk was determined after alkaline digestion using the Sandell-Kolthoff spectrophotometric method as modified by Bednář et al. (1964). T_4 and T_3 were determined by radioimmunoassay using the commercial kit supplied by Immunotech (Prague).

One lamb of each group and another female lamb showing clinical goitre (8B) were killed on day 13 and necropsied. Samples of the thyroid gland, collected immediately after the killing, were examined using light and transmission electron microscopy. The weight of the glands was determined using laboratory balance. The samples for light microscopy were fixed in 10% formaldehyde and processed by the conventional paraffin technique. Approximately 5-μm-thick sections were stained with haematoxylin-eosin. Samples for transmission electron microscopy were collected from the lambs Nos. 7, 8B, 13A and 21. The samples were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer for 12 h and then washed in cacodylate buffer and post-fixed with 1% osmium tetraoxide. After treatment in ascending ethanol series, the samples were embedded in Durcupan. Ultrathin sections were viewed at 80 kV in the electron microscope Philips 420.

The significance of among-group differences in mean concentrations of iodine in colostrum and milk, and in mean concentrations of thyroxine, triiodothyronine, and iodine in blood plasma were analysed by the *t*-test using the STAT-plus software (Matoušková et al., 1992).

II. Composition of concentrates

Component	A (kg)	B (kg)
Calcium carbonate $CaCO_3$	4.8000	4.8000
Sodium chloride NaCl	1.8000	1.8000
Zinc oxide ZnO	0.0087	0.0087
Copper sulphate $CuSO_4.5H_2O$	0.0018	0.0018
Flour	9.0000	9.0000
Sodium nitrate $NaNO_3$	0.0000	1.9000
Urea	1.5000	0.0000
Oats meal	82.9000	0.0000
Extracted rapeseed meal	0.0000	82.5000

RESULTS

The results of the three weightings (days 1, 220, and 380) are shown in Tab. III. It is evident that the growth of the ewes receiving ERM + nitrate (groups E1, E2, and C1) was slightly more rapid during the first phase of the experiment. Towards the end of the experiment, the development was influenced by individual differences in metabolic demands associated with lactation. The most marked decrease in live weight (group E2) was observed in ewes sucked twins.

In terms of the basic fertility parameters, the reproductive performance in the groups E1, E2, and C1 with the pregnancy rate 100% was good. Pregnancy was uncomplicated in all the ewes. The first lamb was born on experiment day 249, i.e. 168 days after the beginning of the mating period. The last lamb was born in the group C1 on experiment day 321, i.e. 140 days after the termination of the mating period.

III. Live weight (kg) and reproductive performance of ewes

Parameter	Group			
	E1	E2	C1	C2
	ERM + NO_3 suppl. I + Se $n = 6$	ERM + NO_3 suppl. I $n = 6$	ERM + NO_3 no supplements $n = 6$	no goitrogens suppl. I + Se $n = 6$
Initial mean weight	41.0	43.8	44.5	43.3
Relative growth rate on experiment day 220 (%)	32.9	34.2	32.4	28.4
Mean weight on experiment day 220 (kg)	54.5	58.5	58.9	55.6
Relative growth rate on experiment day 380 (%)	35.1	24.4	27.2	31.2
Mean weight on experiment day 380 (kg)	55.4	54.5	56.6	56.8
Number and % of pregnant ewes	6/100	6/100	6/100	5/83.3
Days from the beginning of the breeding period	194.6 ± 25.3	193.2 ± 21.1	211.2 ± 21.7	208.5 ± 12.3
Gross natality	1.15	1.5	1.15	1.15
Net natality	1.15	1.5	1	0.9
Number of twin births	1	3	1	2
Lamb sex – males	4	3	3	6
– females	3	6	3	1
Mean birth weight of lambs	4.6 ± 0.5	3.7 ± 1.1	4.5 ± 0.8	4.4 ± 0.4

IV. Absolute and relative weight and histological pattern of the thyroid gland, and postnatal thyroxine (nmol/l T_4), triiodothyronine (nmol/l T_3), and iodine (μg I/l) concentrations in blood plasma of lambs of the groups receiving goitrogens and differing in iodine and selenium supplementation

Group	Lamb No.	Sex	Birth weight (kg)	Weight on day 11 (kg)	Clinical finding	Thyroid gland weight (g)	Thyroid gland relative weight (%)	Histological pattern	T_4	T_2	I
E1	2	male	4.9	9.0	normal	0.9	0.010	normal hist. structure	159	7.8	115
E2	13A	male	3.5	6.2	normal	1.7	0.027	normal hist. structure	134	4.6	89
C1	7	male	4.8	8.7	normal	1.9	0.022	normal hist. structure	195	10.3	121
C1	8B	female	3.2	*3.3	goitre	4.4	0.133	various stages of parenchyma maturation, collapsed follicles, most of the colloid vacuolised, abundant interstitium = 3rd stage of goitre	46	2.1	45
C2	21	male	4.1	8.2	normal	1.0	0.012	differentiated pattern	252	7.3	111

* on postpartum day 3

V. Individual concentrations of thyroxine (nmol/l T_4), triiodothyronine (nmol/l T_3), and iodine (μg I/l) in littermates with different manifestations of hypothyreosis (group C1, No. 8B, 8A), in necropsied and clinically health lambs with normal histological structure (group E1, E2, C2, No. 2, 13A, 21), and their group means in lambs from birth to day 60

Group	Lamb. No.	Clinical finding	Iodine								T_4								T_3							
			Age (days)						Group mean		Age (days)						Group mean		Age (days)						Group mean	
			0	1	2	10	30	60	$\bar{x}$	$s_{\bar{x}}$	0	1	2	10	30	60	$\bar{x}$	$s_{\bar{x}}$	0	1	2	10	30	60	$\bar{x}$	$s_{\bar{x}}$
C1	8B	goitre	45	26	57	35*					46	40	37						2.1	1.9	2.6					
	8A	indifferent	52	37	53	32	38	33			53	34	39	40	91	46			6.5	2.6	6.4	6.4	5.3	3.8		
E1	2	normal	115	111	122	–					159	111	75	78					7.8	7.2	8.1	8.4				
E2	13A	normal	89	80	54	144					134	132	116	101					4.6	5.6	5.6	5.2				
C2	21	normal	111	136	206	236					252	176	156	73					7.3	7.8	8.3	7.3				
C1									69	24							101	27							6.2	1.1
E1									139	51							127	39							7.2	1.1
E2									127	45							115	30							6.1	1.1
C2									186	60							134	50							6.2	1.3

* postpartum day 3

Iodine concentrations C1 vs. E1 ($P < 0.05$) Differences in mean concentrations between T_4 and T_3 were insignificant

C1 vs. E2 ($P < 0.05$)

C1 vs. C2 ($P < 0.01$)

VI. Effect of extracted rapeseed meal and nitrates on mean concentrations of iodine in colostrum and milk of groups differing in iodine and selenium supplementation

		Iodine concentration (μg I/l) Group			
		E1 ERM ± NO_3 suppl. I + Se $n = 6$	E2 ERM ± NO_3 suppl. I $n = 6$	C1 ERM ± NO_3 no suppl. $n = 6$	C2 no goitrogens suppl. I ± Se $n = 4$
Colostrum up to 6 h after parturition	$\bar{x}$	430	334	57	2 083
	$s_{\bar{x}}$	520	330	36	1 423
Milk within day 61 and 140 of the lactation period	$\bar{x}$	70	101	27	198
	$s_{\bar{x}}$	16	41	15	8

Significance of differences among group means:
Colostrum: C1 vs. E1, E2, C2 ($P < 0.01$) Milk: C1 vs. E1, E2, C2 ($P < 0.01$)
C2 vs. E1 ($P < 0.05$) C2 vs. E1 ($P < 0.01$)
C2 vs. E2 ($P < 0.01$) C2 vs. E2 ($P < 0.01$)

A substantial difference in net birth rate was found between the groups receiving the goitrogenic agents and additional iodine and selenium (E1, E2) and the control group C1 (goitrogenic agents without additional iodine and selenium) on one side and the group fed the diet without gitrogenic agents (C2) on the other side. The highest number of live lambs was born in the group E2 (goitrogenic agents + iodine). Pregnancy rate in the group fed the conventional ration (C2) was lower than the breeding objective of the breed Sumava sheep. It should be noted that one sheep of this group was infertile in spite of being in a very good condition and did not show any apparent deviation from the normal state of health including gonadal organs.

Clinical findings in the thyroid gland

No clinical abnormalities were detected by adspection and palpation of the thyroid glands except for the ewes 7, 8, and 10 of the group C1. The latter three ewes developed a slight enlargement making the thyroid gland palpable on experiment day 56. The enlargement showed rather a decreasing than an increasing tendency.

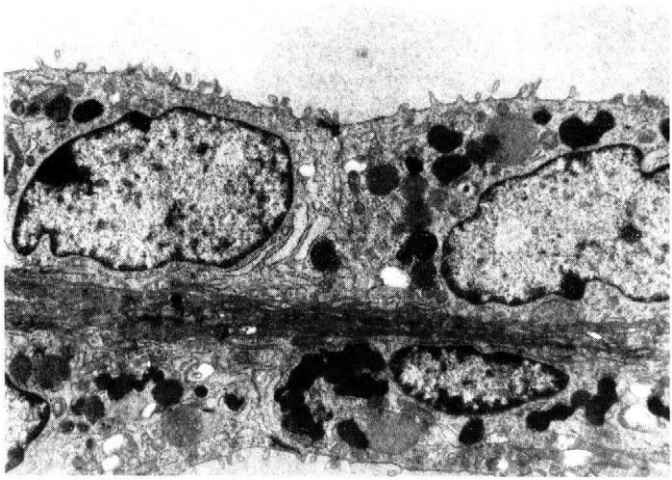
Typical goitre developed in the lambs. One female lamb (No. 8B) of the group C1, born with a low viability and live weight, was lethargic and developed thyroid enlargement palpable as two distinct painless, oval, rigid, and elastic structures with sizes of 3–4 x 2 cm. Since the lamb did not respond to supportive therapy and the clinical state rather deteriorated, it was decided to kill it at the age of 3 days. Indefinite findings on thyroid glands persisting up to the end of the experiment were further recorded in the lamb No. 8A and the well developed lamb No. 12 of the group C1. A change of the ration led to extinction of the enlargement. Slight enlargements in another two well-doing lambs (Nos. 9 and 10) disappeared spontaneously without any change in the ration.

Thyroid gland functions

Individual concentrations of T_3 and T_4 in the lambs for which clinical and necropsy data, including thyroid gland weights and microscopic findings, were available for comparison (Tabs. IV and V) indicated an insufficient supply of iodine to the thyroid gland in the lambs of the group C1. Such deficiency was evident from the thyroid hyperplasia in the lamb No 8B, absolutely low concentrations of T_4 (46 to 37 nmol/l) and T_3 (2.6–1.9 nmol/l), and a very low concentration of iodine in blood plasma on the 1st and 3rd day after birth (45 to 26 μg/l). All the values differed considerably from those found in lambs No. 2, 13A, and 21 of the other groups (E1, E2, C2) in which the concentrations of T_4, T_3, and plasmatic iodine ranged from 73 to 252 nmol/l, from 4.6 to 8.3 nmol/l, and from 80 to 236 μg/l, respectively (Tab. V).

Relevant to etiopathological considerations is the fact that, in the lamb No. 8A – a littermate of the lamb 8B mentioned in the preceding paragraph – the concentrations of plasmatic iodine and T_4 were very low, while the concentration of T_3 did not differ from those found in the other groups and the palpatory finding was indefinite. The poor stability of the thyroid gland of the lamb 8A is documented also by fluctuations of functional parameters up to day 60 after birth when low concentrations of plasmatic iodine and T_4 were found, concentrations of T_3 were variable. Individual findings corresponded to the results of biochemical function tests of the thyroid gland carried out in the groups during the postnatal development (Tab. V).

Mean concentrations of iodine in colostrum and milk in the individual groups of ewes are given in Tab. VI from which the effects of differences in feed composition and iodine supplementation on the metabolism of iodine are apparent. Feeding of the goitrogenic ration (without addition of iodine and selenium) in the group C1 resulted in extremely low excretion of iodine by the mammary gland both at the beginning and towards the end of the lactation period. The iodine concentrations



1. Normal thyroid gland of a lamb. Thyreocytes lining the follicles are arranged in a single layer, their cytoplasm contains numerous mitochondria and lysosomes, and numerous cytoplasmatic papillary projections protrude into lumina of the follicles; TEM, 500x

in colostrum (57 ± 36 μg/l) and milk (27 ± 15 μg/l) were far below the physiological values. In this respect, the differences between C1 and any other group were highly significant ($P < 0.01$).

Iodine concentrations in colostrum and milk of ewes of the experimental groups fed the ration containing goitrogens and supplemented with iodine and selenium or iodine alone, i.e. 430 ± 520 μg/l for colostrum and 70.0 ± 16.0 μg/l for milk in the group E1, and 334 ± 330 μg/l for colostrum and 101 + 41 μg/l for milk in the group E2, reached the levels indicating a satisfactory supply of iodine. The highest concentrations of iodine in colostrum (2 083 ± 1 423 μg/l) and in milk (198 ± 8 μg/l) in the late lactation phase were found in the group C2 fed the conventional ration and supplemented with iodine and selenium. In spite of a high variation, the concentrations differed highly significantly ($P < 0.01$) from those found in the other groups.

Necropsy, histological and TEM findings

As shown in Tab. IV, no gross lesions were found upon necropsy of four clinically normal lambs (Nos. 2, 13A, 7, 21). All the lambs were in a good nutritional state and no enlargement or asymmetry of thyroid lobes were seen.

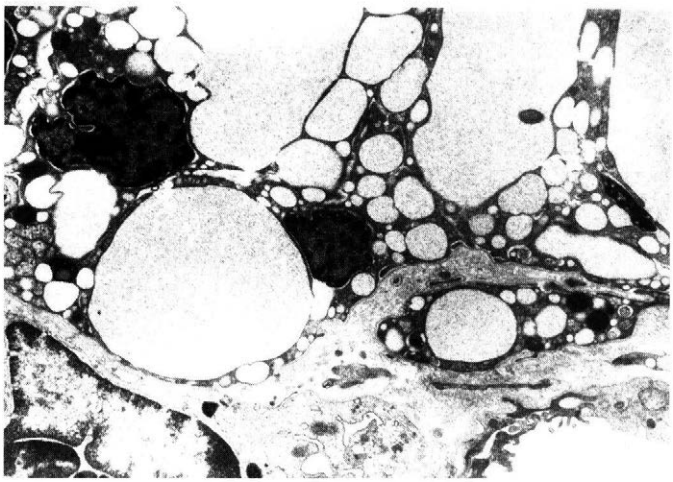
Asymmetry of the thyroid gland due to enlargement of the left lobe, moderate oedema of the surrounding tissues and dilatation of jugular veins was found in the lamb No. 8B. Further findings included disseminated atelectatic lung lesions, moderate liver swelling, greenish-brown discoloration of kidneys with enlarged pelvises, infiltration of fibrous and lipid tissues surrounding the kidneys, and brownish-grey thin pasty ingesta with large milk coagula in the abomassum.

Absolute weight of the thyroid gland of the lamb 8B corresponded to 2.5-fold to 5-fold of the absolute weight of a normal gland.

Normal histological structure of thyroid glands of lambs of the groups E1 and E2, fed the ration containing the goitrogens and supplemented with iodine + selenium and iodine alone, respectively, indicated a sufficient supply of iodine to the ewes. The structure of the thyroid parenchyma of the lamb 8B (group C1 fed the ration with ERM and nitrate) with clinically apparent goitre corresponded to 3rd stage of goitre. Thyreocytes of the morphologically normal glands of the lambs 7, 13A, and 21 were arranged usually in a single layer and their cytoplasm contained activated mitochondriae, lysosomes and abundant rough endoplasmatic reticulum with moderately enlarged colloid-containing cisterns (Fig. 1). In the enlarged thyroid gland of the lamb 8B, thyreocytes formed a multilayer structure, colloid-containing cisterns of the endoplasmatic reticulum were cystically enlarged, numerous activated mitochondriae and pyknotic nuclei were displaced to the cell periphery, and cytoplasmatic and nuclear membranes disintegrated here and there (Fig. 2). Such lesions along with the low T_4 and T_3 concentrations indicated thyroid hyperplasia and hypofunction as a result of iodine deficiency in the dam.

Dominant in the histological pattern (90%) of the thyroid gland of the clinically normal lamb No. 21 of the group C2 fed a conventional ration and supplemented with iodine and selenium was a well developed structure with regular follicles and homogeneous dark stained colloid-containing sporadic macrovacuoles.

Two intercurrent extrathyroidal diseases were recorded during the experiment. One ewe (No. 4 in the group E1) developed mastitis with subsequent hypogalactia which affected the growth and development of her twin lambs. The ewe No. 20 of the group C2 with an symptomless history died suddenly on experiment day 281, i.e. 194 days after the beginning of the mating period. At necropsy, the death cause was identified as acute heart failure associated with the approaching parturi-



2. Goitre. Cystically extended cisterns of the crude endoplasmatic reticulum and pynknotic nuclei of thyreocytes; TEM, 5x

tion, and dilatation of forestomachs overfilled with ingesta corresponding in composition to the ration. The weight of her two dead foetuses was extremely high (4 120 and 3 950 g) for twins of the breed Sumava sheep.

DISCUSSION

The ovine thyroid gland has become the subject of many studies owing to its relative sensitivity to the supply of iodine and effects of goitrogenic agents. Clinical manifestations of iodine deficiency in sheep are similar to those seen in other farm animal (Barberan and Valderrábano, 1987). Sheep as a suitable animal for experimental studies of deficiency diseases, in particular of their effects on the development of foetuses, were recommended also by Potter et al. (1982), Nasseri et al. (1986), Alexander et al. (1990), Wu et al. (1992).

The concentration of 21.51 µmol of glucosinolates per 1 g of ERM, which made up 82.5% of the concentrate B, indicates that the rape cultivars grown currently in the Czech Republic still contain considerable amounts of progoitrogens, in particular the progoitrin alkylglucosinolate (17.74 µmol per 1 g) as a precursor of goitrin with a strong goitrogenic effect described by Zech et al. (1993), Schöne (1999) and other authors. Goitrin is known to inhibit the production of T_4 and transfer of iodine in the thyroid gland. This thyreostatic effect was demonstrated experimentally in dairy cows (Ehlers et al., 1994) and also in our experiments in sheep. The length of the Ehlers' and our experimental periods was approximately the same (14 vs. 13 months) and the similarity of results pertained to the low content of iodine in the milk of the experimental groups in which the competitive effect of glucosinolates in the thyroid gland was not compensated by increased supply of iodine, and to the development of a metabolic situation with a hypothyreotic tendency. The more dramatic course of hypothyreosis manifested by the development of goitre in the progeny of the ewes receiving goitrogens in the feed was apparently due to a higher proportion of ERM in the ration, higher content of glucosinolates in ERM, cumulative effects of sodium nitrate, and a lower content of iodine in the basic ration. Parameters of performance were also similar. While both Emanuelson (1994) and Ehlers et al. (1994) reported a tendency towards higher milk yield and higher content of milk proteins, the results of our experiments indicated a favourable physical condition of ewes and birth rate and birth weight of lambs.

Both the results of laboratory examinations and clinical and post-mortem findings indicate the severity of iodopoenia developing in the group C1, as well as the effect of the supplementation in the groups E1 and E2. In this respect, valuable information was obtained from the monitoring of iodine content in milk, which is generally regarded as a reliable indicator of iodine supply (Kaufmann et al. 1998; Herzig et al., 1999).

A fact to be regarded in among-species comparative considerations is that the concentrations of iodine in sheep and goat colostrum and milk are higher than in cow's milk. Therefore, concentrations below 79 µg/l for ovine colostrum and 62 µg/l for ovine milk should be interpreted as signs of deficiency (Groppel, 1991). A similar experience was reported by Azuolas and Caple (1984) who observed goitre in lambs fed ovine milk containing 45 to 98 µg iodine per litre. Our data are consistent with the results of Mason (1976) who observed goitre in 80% of lambs born to ewes receiving 30 µg of iodine per animal per day whose milk contained 45 µg iodine per litre. On the other hand, all lambs born to ewes receiving 80 or 120 µg of iodine per animal per day whose milk contained 95 and 131 µg of iodine per litre, respectively, were free from goitre.

In our experiments, the mean concentration of iodine in milk of the iodine-supplemented ewes was above the lower margin of the physiological range, was higher in the groups E2 and C2, and markedly reflected the state of health of the ewes and lambs, which were free from goitre, and the corresponding concentrations of T_4 and T_3 in blood plasma of lambs. On the other hand, the significantly lower concentration of milk iodine in the ewes of the group C1 indicated pathophysiological consequences of the absence of iodine supplementation allowing glucosinolates and nitrate ions to inhibit the transport of iodine into the thyroid gland. Such effect of glucosinolates resulting in low iodine concentration in milk and also lower concentration of T4 in blood plasma of cows fed rapeseed cake was described by Papas et al. (1979).

The high content of iodine in colostrum and its individual variations are well-known facts. Nevertheless, the increased amount of iodine excreted in milk of ewes fed a ration free of goitrogens (group C2) cannot be overlooked and is indicative of normal iodine metabolism if the intake is sufficient. The same applies also to milk secreted in the late lactation phase. The more conspicuous is the highly significant difference between the groups C2 and E1. Although the supplementation in both the groups was the same, ewes of the group E1 excreted in colostrum and milk 2.8 to 4.8 times less iodine due to the long-term intake of ERM and nitrate ions. It is important, however, that, thanks to the compensatory effects of supplementation, the mean iodine concentrations of 70 µg/l (E1) and 101 µg/l (E2) were high enough to meet physiological requirements of sucking lambs. The sufficient supply was apparent from the concentrations of iodine, T_4, and T_3 in blood plasma of lambs expressed in terms of group means of six analyses done from birth to the age of 60 days. The highest values of the three analytes were found in the first hours after birth.

The interest in the role of selenium in the pathogenesis of thyroid disorders is encouraged on one side by the fact that selenium deficiency is associated with an excess peroxide which can damage thyreocytes (Corvilain et al., 1993), and on the other side by the presence of selenium in molecules of enzymes controlling the production of thyroid hormones, their transformation and eventual destruction in target tissues. They include selenoprotein 5'-deiodinase consisting of three enzymes of which Types I and III contain selenium in their molecules. The three enzymes represent an interconnected regulatory system which is responsible for deiodination of thyroxine and prompt provision of a sufficient amount of the active hormone triiodothyronine (Anonym, 1996; Köhrle, 1996).

In our experiment, the selenium supplementation was motivated by papers recommending such treatment for areas where naturally low concentrations of selenium in blood of the human population are recorded (Contempre et al., 1991). Such treatment was recommended also in veterinary literature (Todorova et al., 1997), particularly for animals receiving goitrogens in their rations (Donald et al., 1993). Relations between metabolism of selenium and goitrogenic effect of cyanogenetic glycosides and functional changes in the thyroid gland reported (Gutzwiller, 1993). Bíreš et al. (1996) found low concentrations of selenium in blood serum and goitre in goats in Slovakia.

Further information from areas with notorious occurrence of myopathies due to selenium deficiency (George et al., 1966; Langlands et al., 1981; McCoy et al., 1997) is consistent with our experience indicating that nutritional myodystrophy in lambs occured in areas which, to a certain extent, overlapped areas of occurrence of goitre (Kursa et al., 1997). The assumption that endemic selenium deficiency diseases of animals resulted from low selenium content in soil, crops and animal tissues (Anke et al., 1983; Kursa and Kroupová, 1975) is supported by the recently demonstrated correlation between low levels of selenium in the human population of South Bohemia and metabolism of iodine and thyroid hormones (Kvíčala et al., 1997).

In our experiment, the effect of selenium was apparent from a comparison of iodine, T_4 and T_3 concentrations in blood plasma of the progeny of the groups E1 and E2. Both were fed the ration containing the goitrogens (ERM and nitrate) and were supplemented with iodine, but the group E1 received additional selenium in a dose used in sheep also by Todorova et al. (1997). All the parameters of the thyroid function were higher in lambs of the group E1. A similarly conceived experiment in sheep grazing in an area affected by the occurrence of selenium deficiency diseases and supplemented with iodine + selenium or iodine alone was carried out by Donald et al. (1993) who used potassium thiocyanate as the goitrogenic agent. The difference in the supplementation had no apparent effect on the thyroid function parameters in lambs born by the ewes receiving the goitrogenic agent.

Worth mentioning is also the finding of an insignificant decrease of T4 and increase of T3 in sheep fed a diet with a low content of selenium and supplemented with 0.3 ppm sodium selenite (Bik and Kondracki, 1997).

The most apparent manifestation of iodine deficiency is the enlargement of the thyroid gland resulting from hyperplasia due to the effect of thyreotrophin activating the thyroid gland by a feedback mechanism in order to compensate hypothyreosis (Podoba and Langer, 1993). Nevertheless, the decisive criterion for assessing the severity of the insufficiency is the histopathological pattern (Seffner and Groppel, 1986; Čada, 1988).

The results of histological and, as the case may be, histometrical examination are valuable particularly in cases when a limited number of samples is available or if clinically complicated cases are to be explained. Although only five thyroid glands were weighed and examined histologically within our study, we consider the results useful, because they confirmed the clinical findings and the clarification causes of differences in the

biochemical parameters as described and interpreted above. From results of extensive epidemiological studies in the human population of the United States, Gaitan et al. (1989) concluded that the polyfactorial character of pathogenesis of goitre and complex interactions of intrinsic (immunological and genetic) factors and specific extrinsic factors (environment) must be considered in the analyses and assessment of the effects of goitrogenic agents. Such conclusion may also explain the fact that advanced goitre with a histopathological finding typical of 3rd stage developed in our experiments in only one lamb (group C1 receiving ERM + nitrate without supplementation). Individual differences in responses of the thyroid gland to thyreostatic effects of sodium hypochlorite were reported also by Bekeová et al. (1998) who assume that such differences are due to both intrinsic factors and current environmental conditions.

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Contact Address:

Prof. MVDr. Jaroslav K u r s a , DrSc., Jihočeská univerzita, Zemědělská fakulta, Studentská 13, 370 05 České Budějovice, Česká republika
Tel. +420 38 777 26 20, fax +420 38 777 26 21, e-mail: travnic@zf.jcu.cz

MILK COMPOSITION IN RESTRICTEDLY FED RATS*

ZLOŽENIE MLIEKA U POTKANOV S OBMEDZENÝM PRÍSTUPOM K POTRAVE

Š. Mozeš[1], Ľ. Lenhardt[2], Ľ. Raček[1]

[1]*Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic*
[2]*University of Veterinary Medicine, Košice, Slovak Republic*

ABSTRACT: The milk fat and protein concentrations in Wistar rats fed *ad libitum* or restrictedly (with free access to food for two hours daily, from 8.00 to 10.00) were measured on day 2, 5, 8, 11, 14, 20 and 25 of lactation. The results showed that restrictedly fed dams, in comparison with those fed *ad libitum*, had a significantly increased milk fat content during the whole lactation period; the protein content was significantly increased from the 5th day of lactation. The significantly decreased weight gains and body weight of the pups coming from the nests of restrictedly fed mothers, as compared to those fed *ad libitum*, show that these animals were considerably undernourished.

lactation; feeding regime; milk fat; milk protein

ABSTRAKT: V práci boli stanovené koncentrácie tuku a proteínu v mlieku potkanov kmeňa Wistar kŕmených *ad libitum*, alebo s obmedzeným prístupom k potrave (s voľným prístupom k potrave na dve hodiny denne, od 8.00 do 10.00) na 2., 5., 8., 11., 14., 20. a 25. deň laktácie. Výsledky ukazujú, že matky s obmedzeným prístupom k potrave, v porovnaní s matkami kŕmenými *ad libitum*, mali signifikantne zvýšený obsah tuku v mlieku počas celej laktačnej periódy, zatiaľ čo obsah proteínov bol signifikantne zvýšený až od 5. dňa laktácie. Signifikantné zníženie prírastkov i telesnej hmotnosti u mláďat pochádzajúcich od matiek reštriktívne kŕmených, v porovnaní s mláďatami matiek kŕmenými *ad libitum*, ukazujú, že tieto rozdiely sú dôsledkom výraznej podvyživy.

laktácia; potravinový režim; mliečny tuk; mliečny protein

INTRODUCTION

Growth and development of the rat pups during the suckling period depends mainly on the supply and composition of mother's milk. Approximately 15–16 days after birth milk is the only source of food and water for the rat pups (Babický et al., 1972). Milk production of *ad libitum* fed dams culminates about day 14 of lactation (Knight et al., 1984), and is mainly related to litter size. Evidence has been provided that the quantity of milk produced by the rat dams increases proportionally with the number of nursed pups, and is sufficient for the optimal nutrition of about 12 pups in the litter (Wurtman and Miller, 1976; Kumersan et al., 1967; Hausberger and Volz, 1984). According to data rat milk is rich in fat and protein but relatively poor in carbohydrates (Cox and Mueller, 1937; Luckey et al., 1954). It has been reported that milk protein and carbohydrate concentrations do not change, only milk fat concentration varies considerably during lactation (Luckey et al., 1954; Godbole et al., 1981). Other results revealed that the developmental patterns for protein, carbohydrate and fat were not very pronounced, although protein and carbohydrate increased to some extent in early lactation and later decreased (Keen et al., 1981). Our previous results showed only slight differences in the mean milk fat values and no changes in the milk mean protein concentration in normally fed rat dams during lactation (Mozeš et al., 1993). In relation to the feeding conditions it has been reported that the low protein diet resulted in a lower nitrogen content and an elevated total fat content of milk (Crnic and Chase, 1978). In the dietary obese rats, as compared with the lean controls, about twice higher content of milk fat and only small differences in the milk protein content were found (Rolls et al., 1981).

This study was undertaken to follow the milk fat and protein concentrations during the lactation period in dams fed restrictedly.

MATERIAL AND METHODS

Two groups, by fifteen (a total of 30), of first-time-lactating Wistar rat dams mated at the age of 4 months were used in an experiment. The first group of dams

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was fed *ad libitum* (standard laboratory diet – Velaz/Altromin 1520 DOS 2b, Prague) all the time. The food restricted group was allowed free access to food for only two hours daily (from 8.00 to 10.00 a.m.). Dietary treatment began from weaning and continued throughout the experiment (i.e. before and during pregnancy until day 25 of lactation). Both groups of dams had free access to tap water.

On the first day after birth the litters were adjusted to eight pups per nest. The litters were individually housed in Plexiglas cages in a temperature-controlled environment of 22 °C with a 12L : 12D regime. The dams were milked and the pups weighed on the 2nd, 5th, 8th, 11th, 14th, 20th and 25th day of lactation. Food intake of the mothers was controlled daily from day 2 to day 14 of lactation. Data are presented for ten dams in both groups (a total of 20); the data for the remaining rats were excluded because of the reduced number of pups in the litters.

Two hours before milking (at 8.00 a.m.) the dams were separated from their pups which were kept in a constant temperature chamber at 34 ± 1 °C during that time. 10 min before milking the dams received an inj. of 2 IU of oxytocin intraperitoneally. During the milking procedure (about 2 min in each rat) they were anaesthetized with diethylether. Milk was obtained by gentle pressure with the thumb and the finger on the nipple after preceding massage of the mammary gland. The droplets of milk were collected in standard glass disposable micropipettes (20 µl content, 64 mm length, CMS Houston, Tx) – 3 tubes in each rat (2 tubes for fat and 1 tube for protein estimation).

Milk fat was determined by the crematocrit method of Lucas et al. (1978). About 18 µl of milk (56–58 mm column) was drawn by capillarity into glass tubes which were then sealed in a flame at the empty end and centrifuged at 12 000 rpm for 10 min. The whole column and the crematocrit were measured to the nearest of 0.05 mm. Fat concentration was expressed in g/100 ml milk by the formula given by Nagasawa et al. (1989): milk fat = 0.1 x (crematocrit in % – 0.59)/0.146.

Milk protein concentration was analyzed by the method of Lowry et al. (1951). The values were expressed in g/100 ml milk. Collected milk samples (glass tubes filled end to end to contain 20 µl) were stored frozen (–20 °C) until analysis.

Statistical analysis of the acquired data (milk fat and protein, weight gains and body weight of the pups, food intake of dams) was one-way analysis of variance (ANOVA).

RESULTS

A restrictive feeding regime of rat dams influenced their milk fat and protein concentration to a remarkable degree. In comparison with the dams fed *ad libitum*, they showed a significant increase of milk fat concentration during the whole lactation period; protein concentration significantly increased from the 5th day of lactation (Tab. I). The ratio of these two components (fat/protein) in milk of restrictedly fed dams, despite of increased milk protein, showed a more pronounced increase of milk fat: 1/0.53–0.68 vs 1/0.75–0.84 in dams fed *ad libitum* (Tab. I).

I. Milk fat and protein concentrations (in g/100 ml milk)

Dams fed *ad libitum*			
Day	fat	protein	ratio
2nd	14.5 ± 0.98	10.9 ± 0.43	1/0.75
5th	14.8 ± 0.75	11.2 ± 0.34	1/0.76
8th	15.0 ± 0.67	11.5 ± 0.44	1/0.77
11th	14.3 ± 0.62	11.1 ± 0.37	1/0.78
14th	13.2 ± 0.40	9.6 ± 0.30	1/0.72
20th	12.8 ± 0.62	10.8 ± 0.37	1/0.84
25th	12.9 ± 0.62	10.9 ± 0.45	1/0.84
Dams fed restrictedly			
Day	fat	protein	ratio
2nd	21.4 ± 0.84***	11.3 ± 0.28	1/0.53
5th	22.0 ± 1.00***	13.6 ± 0.55*	1/0.62
8th	22.8 ± 1.33***	15.3 ± 0.68**	1/0.67
11th	22.9 ± 1.02***	15.1 ± 0.50***	1/0.66
14th	23.0 ± 1.01***	15.3 ± 0.61***	1/0.67
20th	22.7 ± 0.85***	15.4 ± 0.48***	1/0.68
25th	21.9 ± 0.74***	14.3 ± 0.53**	1/0.65

Values are means ± SEM of 10 determinations
* $p < 0.002$; ** = $p < 0.001$; *** = $p < 0.0001$

II. Weight gains and mean body weight of pups (in g/pup)

Body weight		
Day	AL	R
2nd	6.28 ± 0.24	6.55 ± 0.19
5th	9.48 ± 0.35	9.07 ± 0.36
8th	13.86 ± 0.52	11.31 ± 0.46**
11th	19.11 ± 0.70	13.27 ± 0.50***
14th	26.08 ± 1.07	14.62 ± 0.51***
20th	39.99 ± 1.33	17.36 ± 0.58***
25th	62.75 ± 1.36	22.14 ± 1.13***
Weight gains		
Lactation days	AL	R
3–5	1.07 ± 0.07	0.81 ± 0.10*
6–8	1.45 ± 0.08	0.75 ± 0.10***
9–11	1.75 ± 0.17	0.63 ± 0.09***
12–14	2.32 ± 0.25	0.45 ± 0.05***
15–20	2.32 ± 0.09	0.46 ± 0.10***
21–25	4.55 ± 0.13	0.96 ± 0.17***

Values are means ± SEM by eighty pups of AL and R groups
AL = dams fed *ad libitum*, R = dams fed restrictedly
Weight gains are expressed in g/pup/day
* $p < 0.05$; ** $p < 0.002$; *** $p < 0.0001$

III. Mean food intake of lactating rats (in g of food/rat/day)

Days	AL	R
3–5	26.9 ± 1.78	16.7 ± 0.66*
6–8	34.3 ± 1.45	18.9 ± 0.71**
9–11	44.0 ± 1.89	19.9 ± 0.92**
12–14	50.0 ± 1.65	21.5 ± 0.82**

Values are means ± SEM of 10 determinations
AL = dams fed *ad libitum*, R = dams fed restrictedly
* $p < 0.001$; ** $p < 0.0001$

Different feeding regimes of the dams significantly influenced weight gains and body weight of the pups. The pups coming from litters of restrictedly fed dams, as compared to the pups of the dams fed *ad libitum*, showed significantly decreased weight gains from day 2; body weight of these pups significantly decreased from day 8 (Tab. II).

The mean food intake (measured from the 2nd to the 14th day of lactation) was significantly different and showed a gradual increase in both groups of the dams. Nevertheless, the increase of food intake in *ad libitum* fed dams was more pronounced than in those fed restrictedly (Tab. III).

DISCUSSION

The results described above indicate that under certain feeding conditions not only fat, but also protein content in milk may be significantly changed. The mean values of milk fat and protein concentration recorded in our experiment in *ad libitum* fed dams are in agreement with those reported by others (Cox and Mueller, 1937; Luckey et al., 1954; Godbole et al., 1981). The mean values of milk fat concentration recorded in restrictedly fed dams are comparable to those found in rats submitted to different dietary manipulations. It was reported that rat dams fed *ad libitum* a low protein diet produced a significantly higher fat content (24.5 vs 17.7 g/100 ml) and a decreased nitrogen content in the milk (Crnic and Chase, 1978). In addition to an increase in milk lipid concentration and a decrease in milk protein concentration dietary protein restriction following parturition also reduced the milk lactose concentration in rat dams (Pine et al., 1994). In comparison with their lean controls, an about twice higher concentration of milk fat (24.4 vs 12.5% w/v) and only small differences in the milk protein content were found in dietary obese rats (Rolls et al., 1981). On the other hand, not only increased milk lipid but also high milk protein and lactose values were observed in rats fed high lipid diet during pregnancy and lactation (Del Prado et al., 1997). In our experiment simultaneously with the increased fat content a significantly increased milk protein content was also recorded in restrictedly fed rats. Nevertheless, the fat/protein ratio revealed a more pronounced increase of the fat content in the milk of restrictedly fed rats. The remarkable increase of fat and protein contents in the milk of restrictedly fed rats suggests a proportionally decreased water content in their milk. Since milk is the only source of water and food for the rat pups for 15–16 days after birth (Babický et al., 1972) it is apparent that it can induce serious early and late effects in these animals. We recorded in this connection that the pups of restrictedly fed mothers revealed a characteristic feature – a thin looking skin and a generally dehydrated appearance from about the 2nd or 3rd day.

The gradual increase of food intake observed in lactating *ad libitum* fed dams is in agreement with earlier studies (Babický et al., 1970, 1973). In dams fed restrictedly a slight increase of food intake during lactation was also observed; nevertheless, this increase was insufficient since greater differences in the amount of food eaten by *ad libitum* and restrictedly fed dams were observed until weaning. The mean food consumption of the latter represents about 60% or 40% of the *ad libitum* food from day 3 to day 14 of lactation, respectively.

Body weight gains and differences in body weight observed in the present experiment show that the pups of restrictedly fed mothers were considerably undernourished; which became more pronounced with duration of lactation. It is noteworthy that the body weight at birth in the pups of differently fed mothers was similar although there was a different number of newborns. While the mean number of newborns per one mother in *ad libitum* fed dams was about 11–12 pups, this number reached about 6–7 pups in restrictedly fed dams.

It is well known that the milk production in rat dams is altered by chronic dietary restriction which in turn may affect the growth of their pups. In rats receiving food restricted to 70% or 40% of the *ad libitum* intake prior to breeding and throughout lactation a reduced milk yield was observed. While the birth weight of the pups did not differ between these groups, the litter number and litter weights were proportional to the maternal dietary intake (Young and Rasmussen, 1985). Similarly, the pups nursed by long-term food restricted dams were smaller at 14 days of age and utilized milk energy less efficiently than those nursed by dams fed *ad libitum* (Rasmussen and Warman, 1983; Sadurskis et al., 1991). Likewise long-term dietary undernutrition, food consumption at 80% or 40% of the voluntary intake from day 7 to day 14 of lactation resulted in lowered mammary gland weight, body weight and body fat in lactating rats and led to decreased milk production (estimated from litter weight gain and litter weight) as compared to dams receiving the control diet (Taylor et al., 1986; Grigor et al., 1987). On the other hand, the concentration of total lipids, total proteins and lactose in milk were not affected by such short-term food restrictions (Grigor et al., 1987).

To conclude, the significant increase of milk fat and protein concentrations found in restrictedly fed rat dams shows that milk composition depends on the ac-

tual feeding conditions. The changes observed in the milk composition of restrictedly fed dams combined with apparent undernutrition of the pups may result in various metabolic disorders in these animals.

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Contact Address:

MVDr. Štefan Mozeš, CSc., Ústav fyziológie hospodárskych zvierat SAV, Šoltésovej 4, 040 01 Košice, Slovenská republika
Tel. +421, fax +421 95 76 21 62, e-mail: mozes@saske.cz

PREVALENCE OF ANTIBODIES TO *BORRELIA BURGDORFERI* IN GAME ANIMALS IN SOUTH MORAVIA, CZECH REPUBLIC*

PROTILÁTKY PROTI *BORRELIA BURGDORFERI* U LOVNÉ ZVĚŘE NA JIŽNÍ MORAVĚ, ČESKÁ REPUBLIKA

Z. Juřicová, J. Halouzka, Z. Hubálek

Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Department of Medical Zoology, Valtice, Czech Republic

ABSTRACT: In a game animal survey, sera of 673 animals were examined for *Borrelia burgdorferi* s. l. antibodies: 55 roe deer (*Capreolus capreolus*), 209 fallow deer (*Dama dama*), 55 red deer (*Cervus elaphus*), 80 mouflons (*Ovis musimon*), 150 wild boars (*Sus scrofa*) and 124 pheasants (*Phasianus colchicus*). The study was carried out with inhibition haemagglutination (IHA) and indirect immunofluorescence (IFA) assays. Using IHA, *B. burgdorferi* antibodies (titres from 1 : 80 to 1 : 320) were found in 30.9% of roe deer, 50.7% of fallow deer, 34.5% of red deer and 37.5% of mouflons. The percentage of reactors in IFA was 25.3% in wild boars and 13.7% in pheasants (titres from 1 : 64 to 1 : 256).

Borrelia burgdorferi; seroprevalence; indirect haemagglutination assay; indirect immunofluorescence assay; hunting game

ABSTRAKT: Séra celkem 673 kusů zvěře: 55 srnců (*Capreolus capreolus*), 209 daňků (*Dama dama*), 55 jelenů (*Cervus elaphus*), 80 muflonů (*Ovis musimon*), 150 divokých prasat (*Sus scrofa*) a 124 bažantů (*Phasianus colchicus*) byla vyšetřena na protilátky proti *Borrelia burgdorferi* s.l. Vzorky byly vyšetřeny testem nepřímé hemaglutinace (IHA) a nepřímé imunofluorescence (IFA). Za použití IHA byly zjištěny protilátky proti *B. burgdorferi s. l.* (titr od 1 : 80 do 1 : 320) u 30,9 % srnců, 50,7 % daňků, 35,4 % jelenů a 37,5 % muflonů. Pomocí IFA byly protilátky (titr od 1 : 64 do 1 : 256) prokázány u 25,3 % divokých prasat a 13,7 % bažantů.

Borrelia burgdorferi; séroprevalence; test nepřímé hemaglutinace; test nepřímé imunofluorescence; lovná zvěř

INTRODUCTION

Ticks of the genus *Ixodes* are the most important vector of *Borrelia burgdorferi* sensu lato (Bb). In the Czech Republic, the first borreliae were observed in ticks by Kmety et al. (1986). The isolation of *B. burgdorferi* from *I. ricinus* in the Czech Republic was reported by Hubálek et al. (1990). Borreliae were detected in larval (Halouzka et al., 1995), nymphal and adult stage of ticks (Hubálek et al., 1991; Pospíšil et al., 1992), and in mosquitoes (Halouzka et al., 1998) in southern Moravia. Infected ticks have been found in a number of forest habitats. Small mammals (rodents and insectivores), birds and lizards are parasitised by larvae and nymphs while large mammals (mainly artiodactyls, carnivores, leporids) are parasitised by adult ticks. We may presume that the game animals can be included as occasional hosts of Bb. Antibodies to Bb were detected in various species of game animals in Europe: deer, wild boar and fox in France (Doby et al., 1991a, b; Perez-Eid, 1995), deer in the United Kingdom (Muhlemann and Wright, 1987), deer in Denmark (Webster and Frandsen, 1994), deer in Germany (Matuschka et al., 1993), roe deer in the Netherlands (Dorrestein et al., 1996), deer and wild boars in Bulgaria (Angelov et al., 1995) and hares, deer, wild boars and pheasants in the Czech Republic (Sýkora et al., 1990; Treml et al., 1994; Juřicová et al., 1996; Zeman and Januška, 1999). Several studies have reported antibodies to Bb in white-tailed deer (*Odocoileus virginianus*) from the eastern United States (Magnarelli et al., 1984a, b; 1986, 1991; Gill et al., 1993; Mahnke et al., 1993).

In the present paper, we report the results of a serological survey for antibodies to Bb in game animals from the southern part of the Czech Republic (South Moravia). Our aim was to determine if antibodies to Bb were widespread in this area.

MATERIAL AND METHODS

Serum samples were collected from 55 red deer (*Capreolus capreolus*), 209 fallow deer (*Dama dama*), 55 roe deer (*Cervus elaphus*), 80 mouflons (*Ovis musimon*), 150 wild boars (*Sus scrofa*), and 124 pheasants

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I. Prevalence of antibody titres to *Borrelia burgdorferi* determined by indirect haemagglutination assay, 1993 to 1997

Host	*n*	Titre			Total positive	%
		1 : 80	1 : 160	1 : 320		
Capreolus capreolus	55	10	4	3	17	30.9
Dama dama	209	65	25	16	106	50.7
Capreolus elaphus	55	16	3	0	19	34.5
Ovis musimon	80	17	10	3	30	37.5
Total	399	108	42	22	172	43.1

II. Prevalence of antibody titres to *Borrelia burgdorferi* determined by indirect fluorescent assay

Host	Year	*n*	Titre			Total positive	%
			1 : 64	1 : 128	1 : 256		
Sus scrofa	1993–1997	150	23	13	2	38	25.3
Phasianus colchicus	1995	124	14	3	0	17	13.7
Total		274	37	16	2	55	20.1

(*Phasianus colchicus*) captured in three districts of South Moravia (Brno suburb, Břeclav, Hodonín) in 1993–1997. Blood samples were collected by cardiac puncture (ruminants, wild boars) or by *vena ulnaris cutanea* puncture (pheasants) during the fall hunting seasons. Sera were stored at –20 °C until use.

An indirect immunofluorescence assay (IFA) was performed according to Webster and Frandsen (1994) to detect IgG immunoglobulins to Bb, using *Borrelia garinii* BR-14 (local strain) as the antigen. For antigen preparation, borreliae were grown at 33 °C in BSK-H (Sigma) medium. Ten to 12-day-old cultures were centrifuged at 8 500 x g for 15 min at 5 °C and washed three times in phosphate buffered saline solution (Dulbecco A). The concentration of the final preparation was approximately 100 organisms per optical field. Conjugates were commercial (ÚSOL, Praha) fluoresceinlabelled rabbit anti-swine (RASw/FITC IgG; wild boars) and rabbit anti-chicken (RACH/FITC IgG; pheasants). Results were read by Olympus fluorescence microscope (Japan) at a magnification 400x. Positive serum samples were defined as any full staining, though weak, of most bacteria. Titres of 1 : 64 and higher were taken as positive. IFA method was used when specific conjugates were available.

An indirect haemagglutination assay (IHA) with BAG-Borrelia-HA-Test was performed according to directions of the manufacturer (BAG-Biologische Analysensystem GmbH). *B. burgdorferi* sensu lato was used as the antigen source. Titres 1 : 80 and higher were taken as positive.

The χ^2 was used for statistical evaluation of 2 x 2 contingency tables.

RESULTS

Serological results confirmed an exposure of different game animals to Bb in the study. Serum samples collected from all species contained antibodies. The overall seropositivity observed in ruminants by IHA was 43.1% (Tabs. I, II). The prevalence of seroreactive specimens was 30.9% in roe deer, 50.7% in fallow deer, 34.5% in red deer and 37.5% in mouflons. Prevalence of Bb antibodies in fallow deer was significantly higher than in the other species: roe deer ($\chi^2 = 6.87$; $P < 0.01$), red deer ($\chi^2 = 4.57$; $P = 0.04$), mouflon ($\chi^2 = 4.06$; $P < 0.05$). Differences of all other pair-wise comparisons were insignificant ($P > 0.40$). Antibody titre ranged between 1 : 80 to 1 : 320. Ruminants had the highest prevalence at an end-point of 1 : 80. The overall seropositivity found in IFA in the other vertebrates was 20.1%. Antibodies to spirochetes were found in 25.3% of wild boars and 13.7% in pheasants. Pheasants had a significantly lower frequency of Bb antibodies than wild boars ($\chi^2 = 5.72$; $P < 0.02$). Antibody titre ranged between 1 : 64 to 1 : 256. Most seropositive animals, however, were positive at the 1 : 64 dilution. The highest titres (1 : 256) were detected in wild boars.

III. Number of IHA positive sera of deer (*C. capreolus*, *D. dama*, *C. elaphus*) by the age groups

Age	Positive IHA		Total
	n	%	
Fawns	35	42.7	82
Yearlings	8	30.8	26
Young adults	13	50.0	26
Adults	86	46.5	185

Deer were grouped into four age classes (Tab. III) according to Bosler et al. (1984): fawns (less than 12 months), yearlings (12–23 months), young adults (24–35 months) and adults (36 months and older). The majority of seropositive animals were older than 2-years but there was no significant difference ($P > 0.10$) in the prevalence rate of Bb antibodies between the age groups of deer.

IV. Number of IHA-positive deer sera by the sex

Species	Male			Female			Sex effect	
	number	positive	% positive	number	positive	% positive	χ^2	P
Roe deer	19	8	42.1	18	8	44.4	0.021	0.886
Fallow deer	35	17	48.6	106	55	51.4	0.116	0.734
Red deer	29	9	31.0	22	10	45.5	1.113	0.292

89 young game animals of unrecorded sex (18 roe deer, 67 fallow deer and 4 red deer) are not included

Antibodies to spirochetes were found in both males and females of all three species at approximately the same frequency (Tab. IV) and no significant sex-associated differences were observed.

DISCUSSION

Antibodies to Bb were found in all species of examined game animals. The results of both IHA and IFA show the decreasing frequency with increasing antibody titres which is similar to the pattern reported by other authors (Doby et al., 1991b; Magnarelli et al., 1984a, b, 1986; Mahnke et al., 1993). Based on serologic results, Bb is present in South Moravia, and wild ruminants, boars and pheasants are being exposed to infection agents frequently. Our previous studies demonstrated the presence of Bb in ticks, mosquitoes and rodents collected in the same region (Hubálek et al., 1998).

The proportion of seropositive fallow deer was markedly higher (50.7%) than that of other animals. Similarly, high seroprevalence in fallow deer was recorded in the United Kingdom – 86.0% (Muhlemann and Wright, 1987). Differences between fallow deer and other animals are unexplained at present. On the other hand, we found an approximately equal frequency of seropositive roe deer (30.1%) and red deer (34.5%). Incidence of antibodies to Bb was higher in the central part of Bohemia: 61.9% in roe deer and 60.3% in red deer (Zeman and Januška, 1999). Relatively low seroprevalence rates were observed in France: 18.0% in roe deer, 20.0% in red deer (Doby et al., 1991b) and 17.4% in roe deer and 23.9% in red deer (Perez-Eid et al., 1995). In Bulgaria, 82.8% of roe deer and red deer had antibodies to Bb (Angelov et al., 1995) and in the Netherlands, 13.0% of roe deer were positive (Dorrestein et al., 1996). In Germany, positive findings were recorded in 45.0% of red deer but only in 22.0% of roe deer (Matuschka et al., 1993). In total, 37.5% of mouflons were seropositive, whereas in the central part of Bohemia antibodies to Bb were detected in 76.5% of them (Zeman and Januška, 1999).

Antibodies to Bb were found in 25.3% of wild boars. The records of antibodies corresponded to observations of Doby et al. (1991b) and Perez-Eid (1995), who detected antibodies in 19.7% and 19.0% of wild boars in France, of Angelov et al. (1995) in 24.7% in Bulgaria and with our previous observations – 18.2% (Juřicová et al., 1996). On the contrary, incidence of antibodies in boars was higher in the central part of Bohemia – 40.0% (Zeman and Januška, 1999).

Relatively low seroprevalence has been observed in pheasants (13.7%), whereas in previous years the percentage of reactors was 24.3% (Juřicová et al., 1996). The lower prevalence of Bb antibodies in pheasants was caused by low age of examined birds (58 of 124 pheasants were young). Antibodies to Bb in pheasants have often been found in England (P. A. Nuttall, pers. comm.).

There is no significant difference in the prevalence rate of Bb antibodies between the age groups of deer ($P > 0.10$). We also found no sex effect in prevalence rates, which agrees with findings of Bosler et al. (1983), Doby et al. (1991b), Webster and Frandsen (1994) but disagrees with the results of Lane and Burgdorfer (1986) indicating that infection is independent of sex only for certain species of deer.

Serologic testing of deer sera is suitable for determining the presence or absence of Lyme borreliosis agent in forested areas. Detection of antibodies in sera from these and other mammals indicates the past exposure to Bb. Seropositivity does not mean that these mammals are spirochetemic and infectious. Jaenson and Tälleklint (1992) found that roe deer serve as a principal blood source for all stages of *I. ricinus*, although they do not appear to serve as a major reservoir of Bb. Similarly, white-tailed deer appear to be an incompetent reservoir of Bb in the USA (Telford et al., 1988).

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Contact Address:

RNDr. Zina Juřicová, Csc., Ústav biologie obratlovců AV ČR, Klášterní 2, 691 42 Valtice, Česká republika
Tel. +420 627 35 29 61, fax +420 627 35 23 87, e-mail: juricova@brno.cas.cz

PRESENCE OF ANTIBODIES TO *CHLAMYDIA PSITTACI* IN FARM-MANAGED PHEASANTS *PHASIANUS COLCHICUS* AND PIGEONS *COLUMBA LIVIA* F. *DOMESTICA*

VÝSKYT PROTILÁTOK PROTI *CHLAMYDIA PSITTACI* U BAŽANTOV *PHASIANUS COLCHICUS* Z FARMOVÉHO CHOVU A HOLUBOV *COLUMBA LIVIA* F. *DOMESTICA*

M. Trávniček[1], L. Čisláková[2], J. Miško[1]

[1]*Univerzity of veterinary Medicine, Košice, Czech Republic*
[2]*Faculty of Medicine of P. J. Šafárik University, Košice, Slovak Republic*

ABSTRACT: Results are presented of serological examinations of 194 pheasants (*Phasianus colchicus*) and 114 pigeons (*Columba livia* f. *domestica*) for the presence of antibodies to *Chlamydia psittaci*. Farm management of pheasants is carried out in a pheasantry at Rozhanovce run by the University of Veterinary Medicine at Košice. Pigeons were entrapped in farm buildings of Rozhanovce village. Seropositivity was detected in 40.7% of pheasants (28.5–45.9% in dependence on sampling date) by a micro-method of complement fixation reaction when a genus specific antigen *Ch. psittaci* (Bioveta, Ivanovice in Haná, CR) was used. The unapparent form of chlamydiosis was demonstrated by the antibody levels (1 : 128). 64.0% of pigeons showed seropositivity while differences in the number of positive birds and antibody levels were large in relation to sampling dates. 35.7% of birds showed seropositivity with maximum antibody titer 1 : 128 in a group of 42 pigeons entrapped in January 1999 while a group of 72 pigeons entrapped in April 1999 comprised 80.5% of birds with seropositivity and antibody titer 1 : 1 024. These results document an acute form of chlamydiosis and suggest that in the spring season pigeons are a very important source of chlamydiosis for the human and animal populations.

Chlamydia psittaci; antibodies; *Phasianus colchicus*; *Columba livia* f. *domestica*

ABSTRAKT: V práci autori prezentujú výsledky sérologického vyšetrenia u 194 bažantov (*Phaisanus colchicus*) a 114 holubov (*Columba livia* f. *domestica*) na prítomnosť protilátok voči *Chlamydia psittaci*. Bažanty pochádzajú z farmového chovu, ktorý sa praktizuje na účelovom zariadení Rozhanovce, patriaceho Univerzite veterinárskeho lekárstva v Košiciach. Holuby boli odchytené v hospodárskych budovách obce Rozhanovce. Mikrometódou komplement viažúcej reakcie (KVR) za použitia rodovošpecifického antigénu *Ch. psittaci* (Bioveta, Ivanovice na Hané, ČR) sme zistili séropozitivitu u bažantov u 40,7 % (28,5 až 45,9 % podľa dátumu odberu). Zistené hladiny protilátok (1 : 128) svedčia o inaparentnej forme chlamydiózy. U holubov sme zistili séropozitivitu u 64,0 % s výrazným rozdielom v počtoch pozitívnych vtákov a hladinách protilátok v závislosti od dátumu odberu. V skupine 42 odchytených holubov v januári 1999 bola pozitivita u 35,7 % s maximálnym titrom protilátok 1 : 128, zatiaľ čo v skupine 72 holubov odchytených v apríli 1999 bola pozitivita zistená u 80,5 % a titer protilátok dosiahol 1 : 1 024. Tieto výsledky svedčia pre akútnu formu chlamydiózy a naznačujú, že holuby v jarnom období sú zvlášť významným zdrojom chlamydiózy pre ľudskú a zvieracú populáciu.

Chlamydia psittaci; protilátky; *Phasianus colchicus*; *Columba livia* f. *domestica*

ÚVOD

Chlamydióza vtákov bola potvrdená u viac ako 130 druhov zahrňujúcich voľne žijúcich vtákov a tiež hrabavú a vodnú hydinu. Vtáky predstavujú významný zdroj infekcie pre ľudskú populáciu a tiež pre iné druhy zvierat. Úlohu, ktorú zohrávajú v problematike chlamydiózy vtákov bažanty, ešte nie je dostatočne zdokumentovaná. Výskyt ornitózy u bažantov po kontakte s hydinou publikoval Crosse (1990) a izoláciu *Chlamydia psittaci*

z pečene a ďalších orgánov bažantov a hrabavej hydiny popisujú Bejleri a Berxholi (1987). Uvedené literárne zdroje nepotvrdzujú prenos chlamydií na ľudí. V našich podmienkach najvýznamnejší zdroj chlamýdiovej infekcie pre iné druhy zvierat a pre človeka predstavujú voľne žijúce holuby, čo potvrdili Řeháček a Brezina (1976), Řeháček a i. (1984), Pospíšil a i. (1988), Kociánová a i. (1993), Čisláková a i. (1998). V ČR je situácia podobná, o čom svedčia viaceré práce autorov Pospíšil a i. (1996), Věžník a i. (1996), Věžník a Pospíšil (1997).

Chlamýdie sú do vonkajšieho prostredia vylučované trusom a sekrétmi z očí a horných dýchacích ciest vtákov. K prenosu na iné zvieratá dochádza predovšetkým inhaláciou infekčného prachu, resp. aerosolu, a priamym kontaktom. Kontaminácie krmiva a vody a následné infikované zvieratá je považované za zriedkavejšiu formu prenosu nákazy. Prezentovaná štúdia chce prispieť k rozšíreniu informácií o výskyte protilátok proti *Ch. psittaci* u bažantov a holubov.

MATERIÁL A METÓDY

Ako materiál boli použité krvné séra od bažantov (194 vzoriek), ktoré pochádzajú z farmového chovu Rozhanovce, ktorý je Účelovým zariadením Univerzity veterinárskeho lekárstva v Košiciach. Vzorky boli získané v júli 1998 a následne v apríli a júli 1999. Odchyt holubov (114 ks) bol urobený v hospodárskych budovách v obci Rozhanovce v januári a apríli 1999. Obec Rozhanovce sa nachádza vzdušnou čiarou asi 4 až 5 km od Košíc, kde koncentrácia voľne žijúcich holubov je vysoká.

Krvné séra boli vyšetrované mikrometódou KVR (Sever, 1962; Lenette a Schmid, 1974). Za pozitívne sme považovali séra s titrom protilátok 1 : 16 a vyššie. V KVR bol použitý rodovo špecifický antigén *Ch. psittaci* pre KVR (Bioveta, Ivanovice na Hané, Česká republika). Ako doplnkové údaje uvádzame počty vzoriek reagujúce v riedení 1 : 8 a počty antikomplementárnych sér.

VÝSLEDKY

Séropozitivita u bažantov sa pohybuje od 28,5 do 45,9 %. Najvyšší počet bažantov (135 ks) a súčasne najvyššiu séropozitivitu (45,9 %) sme zaznamenali v apríli 1999. Maximálne výšky titrov protilátok (1 : 128) sme zaznamenali v júli 1998 a apríli 1999 (Tab. I).

Výsledky vyšetrení u holubov potvrdili v januári 1998 séropozitivitu u 35,7 % (42 ks) a v apríli 1999 u 80,5 % (72 ks). Výška titrov u prvého vyšetrenia dosiahla riedenie 1 : 128 a pri druhom vyšetrení 1 : 1 024 (Tab. II).

DISKUSIA

Kontakt medzi voľne žijúcimi holubmi a bažantmi odchovávanými farmovým spôsobom je nepriamy, keď holuby, resp. iné vlne žijúce vtáky zalietajúce za potravu, môžu kontaminovať krmivo, resp. prostredie voliér.

Pozitívne sérologické nálezy u 194 vyšetrovaných bažantov (Tab. I) potvrdzujú prítomnosť *Ch. psittaci* v ich populácii. Najvyššiu séropozitivitu sme zistili v apríli 1999, čo pravdepodobne súvisí s jarnou selekciou bažantov (výberom najvhodnejších jedincov do znáškových voliér a likvidáciou vyradených kusov). Výška titrov naznačuje, že sa jedná i inaparentnú formu chlamydiózy, ktorá je u vtákov veľmi častá. Význam bažantov ako rezervoárových zvierat nie je ešte dosta-

I. Protilátky proti *Chlamydia psittaci* u bažantov z farmy Rozhanovce – Antibodies to *Chlamydia psittaci* in pheasants from Rozhanovce farm

Odchyt[1]	Počet vyšetrených[2]	Pozitívne[3]		Výška titrov[5]								Antikomplementárne[6]
		počet[4]	%	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1 024	
Júl[7] 1998	35	10	28,5	–	4	5	–	1	–	–	–	–
Apríl[8] 1999	135	62	45,9	13	22	17	8	2	–	–	–	8
Júl[9] 1999	17	7	41,2	–	1	4	2	–	–	–	–	7
Spolu[10]	194	79	40,7	13	27	26	10	3	–	–	–	15

[1]trapping, [2]number of examined birds, [3]positive, [4]number, [5]titer level, [6]anti-complementarily, [7]July, [8]April, [9]July, [10]total

II. Protilátky proti *Chlamydia psittaci* u holubov odchytených v obci Rozhanovce – Antibodies to *Chlamydia psittaci* in pigeons entrapped in Rozhanovce village

Odchyt[1]	Počet vyšetrených[2]	Pozitívne[3]		Výška titrov[5]								Antikomplementárne[6]
		počet[4]	%	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1 024	
Január[7] 1999	42	15	35,7	–	7	3	3	2	–	–	–	–
Apríl[8] 1999	72	58	80,5	–	10	14	6	9	11	6	2	–
Spolu[9]	114	73	64,0	–	17	17	9	11	11	6	2	–

For 1–6 see Tab. I; [7]January, [8]April, [9]total

točne objasnený (Salish a i., 1996). Výskyt ornitózy u bažantov po kontakte s hydinou popisuje Crosse (1990), o izolácii *Ch. psittaci* z pečene a ďalších orgánov bažantov informujú Bejleri a Berxholi (1987). Tieto literárne zdroje však nepotvrdzujú prenos chlamýdií z bažantov na ľudí.

Výsledky vyšetrení u holubov (Tab. II) odchytených v januári 1999 (42 ks) so séropozitivitou 35,7 % a maximálnou výškou titra protilátok voči *Ch. psittaci* 1 : 128 sú prakticky zhodné s výsledkami zistenými u bažantov a svedčia o inaparentnej forme chlamydiózy. Výsledky sérologického vyšetrenia u skupiny holubov odchytených v apríli 1999 sú však výrazne odlišné a svedčia o akútnom priebehu chlamydiózy minimálne u 19 ks (titre 1 : 256 až 1 : 1 024). Celková výška séropozitivity, ktorá dosahuje 80,5 %, svedčí o vysokej premorenosti holubov, čo potvrdili aj iní autori (Pospíšil a i., 1996; Čisláková a i., 1998). Predpokladáme, že vysoká pozitivita a detekcia hladín protilátok až v riedení 1 : 1 024 súvisí s nasledujúcimi skutočnosťami: holuby prežívajú zimné obdobie v priestoroch, kde je ich koncentrácia a kontakt mimoriadne vysoký; vtáky sú po zimnom období v dôsledku nedostatočnej výživy oslabené, čo môže viesť k aktivácii infekčného procesu. Veková skladba odchytených holubov je rôzna. Vo februári a v marci sa liahnu prvé holúbäta, u ktorých po infikovaní chlamýdiami sa môže vyvinúť akútna forma ochorenia s príslušnou sérologickou odozvou.

Význam holubov, ako zdroja chlamydiózy pre ľudí, potvrdilo u nás viacero autorov (Hrúzik a i., 1970; Řeháček a i., 1984; Pospíšil a i., 1988). Dolet holubov na veľké vzdialenosti a vysoká premorenosť tohoto druhu vtákov je dôvodom prečo ich považujeme za významný zdroj chlamydióz aj pre zvieratá. V prípade bažantov odchovaných farmových spôsobom, holuby môžu svojimi exkrementami infikovať krmivo, vodu a prostredie voliér, zatiaľ čo priamy kontakt je vylúčený. Určitú úlohu pri šírení chlamydiózy môžu zohrať aj drobné cicavce, ktoré podľa zistení Číslákovej a i. (1999) majú protilátky voči *Ch. psittaci* a kontaminujú prostredie voliér a hál na farme bažantov.

ZÁVER

Výsledky sérologických vyšetrení potvrdili prítomnosť chlamýdiovej infekcie u bažantov, pričom hladiny protilátok svedčia o inaparentnom priebehu ochorenia. Vysoké hladiny protilátok u holubov odchytených a vyšetrených v apríli 1999 však svedčia o akútnom priebehu chlamydiózy. Holuby v tomto období môžu byť zdrojom infekcie nielen pre ľudí, ale aj bažantov, resp. iné druhy vtákov a cicavcov.

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Kontaktná adresa:

Doc. MVDr. Milan Trávniček, PhD., Univerzita veterinárskeho lekárstva, Komenského 73, 041 81 Košice, Slovenská republika

Tel. +421 95 633 21 11–15, e-mail: travnicek@uvm.sk

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