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Review Paper

ENDOCRINE DISRUPTING CHEMICALS AND DOMESTIC ANIMALS

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Summary: Endocrine disrupting chemical is a term for a wide range of chemicals that could affect development and/or function of endocrine system in animals. First reports about potential endocrine disrupting effects of certain chemicals in the environment were published almost twenty years ago, based on the reports about problems with sexual development/differentiation of certain wild animals living in heavily contaminated environments like certain lakes in Florida, US. Subsequent studies have shown potential endocrine disrupting effects for many different chemical substances, ranging from pesticides, plastics, pharmaceuticals and others, and currently there is no known structure/function relationship that would suggest whether certain chemical might have endocrine disrupting properties. However, almost all studies showing endocrine disrupting properties have been performed *in vitro* or with laboratory animals so there is no conclusive answer whether such chemicals might pose a threat for human and animal health in everyday life. Interesting, though, are observations that male reproductive health has detoriated in the last decades. There are several reports that incidence of hypospadias, cryptorchidism and testicular cancer has increased, although these reports with the exception of testicular cancer, are often disputed. Similarly, several reports suggest fall in semen quality in human males in the last several decades, although again, this reports are often disputed and since there are no large prospective studies, we do not have a conclusive answer whether there really are any adverse effects occurring with human reproductive health. If such reports are reflecting real situation and if endocrine disrupting chemicals are involved in the etiology of such problems, one would expect similar trends to be observed also in domestic animals, which are usually exposed to similar environment as humans. However, there are very few studies systematically and chronologically examining reproductive health of domestic animals. Two published studies examined bull semen quality and could not detect detoriating quality. However, bulls in breeding centers are selected for their reproductive performance and therefore do not represent a situation in the normal population.

Key words: endocrine disruptors; reproduction; domestic animals

Introduction

It has been almost twenty years since term "endocrine disrupting chemical" was formed and first suggestions that certain chemicals in our environment could affect development and/or function of reproductive system appeared, although deleterious effects of chemicals like DDT and PCBs on wild populations of birds and some other animals was noted already in the sixties (1-3). First suggestions of direct effects of certain chemicals on development of sexual organs arose from observations about unusually high incidence of sexual development disorders in some wild animals in certain heavily polluted areas (1). At about the same time, first reports about increase in problems with male reproductive function appeared. Namely, several reports suggested that incidence of cryptorchidism and hypospadias increased during last 50 years (4-6) and there are several reports that semen quality in humans had detoriated during the same period (7). Although these findings are certainly not conclusive and are often disputed (8-13), there is one condition that is widely accepted as being on the rise and this is testicular cancer which is now the most common cancer

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in young males (14-18). Sharpe and Skkakebaek (19) suggested that all these problems could have a common origin in increased exposure to estrogens or chemicals with estrogen activity during male fetal development. This gave rise to an endocrine disruption hypothesis, proposing that different pollutants present in our environment could have deleterious effects on development and/or function of endocrine system. From initial observations that certain chemicals could affect development of male sexual organs, it is now thought that endocrine disrupting chemicals could influence other parts of endocrine system such as thyroid (20), and have perhaps even role in the obesity epidemics (21-23). Since many reports suggested that male reproductive health had detoriated in the last decades, it would be expected that similar trends would be also observed in domestic animals, since they are usually exposed to similar environment as humans. In the present article, evidence for effect of endocrine disrupting chemicals on domestic, in particularly farm, animals, will therefore be reviewed.

Endocrine disrupting chemicals

Initial suggestions that synthetic estrogens cold have deleterious role on development of sexual organs came from unfortunate episode with the use of strong synthetic estrogen Diethylstilboestrol (DES) in 60's and 70's as a treatment for risky pregnancies to prevent spontaneous abortions. Unfortunately, follow up studies have shown that DES had deleterious effect on the development of sexual organs in both male and female fetuses which was evident by increase in the vaginal clear cell carcinoma in female offspring and increased incidence of hypospadias and cryptorchidism in male offspring of treated women (24, 25). These reports were somewhat similar to the reports about increased incidence of hypospadias and cryptorchidism in general population, suggesting that common cause might connect both problems. This gave rise to the endocrine disrupting chemicals theory (19). Since first reports about endocrine disrupting chemicals, many man-made chemicals have been identified as being able to affect endocrine system in animals *in vivo* or *in vitro*. Endocrine disrupting chemicals are today considered as a varied group of chemicals that include certain pesticides (DDT, organophosphorous pesticides, possibly atrazine; 26-33), polychlorinated and polybromated biphenyls (PCBs and PBBs; 34-37), plasticizers (octylphenol, nonylphenol; 38-40), plastic components (phthalates, bisphenol A; 26, 41-43) and also some naturally occurring estrogenic compounds, so called phytoestrogens, such as coumestrol and genistin (26, 44). Although phytoestrogens have usually very low affinity for steroid hormone receptors, their high concentrations in many baby formulas and infant foods does present a cause for concern (44, 45).

Endocrine disrupting chemicals could affect different biochemical pathways. From initial observations that endocrine disrupting chemicals might act as estrogen agonists or androgen antagonists by binding to estrogen or androgen receptors, respectively, it became clear from *in vitro* studies that these chemicals could affect other receptors such as certain membrane receptors (for example serotonin and dopamine receptors), other nuclear receptors (thyroid receptors, peroxisome proliferator activated gamma receptors), and enzymatic pathways such as steroid hormone production (reviewed in 2). Endocrine disrupting chemicals are a diverse group of compounds with very diverse mode of action and at present, there is no known relationship between structure and function of such compounds that would allow predictions whether certain chemicals might have endocrine disrupting properties.

Many studies in laboratory animals have convincingly shown that endocrine disrupting compounds could affect endocrine development in laboratory animals. It is much less clear, however, if and how such chemicals affect human health. Although some reports from wild animals and humans from certain heavily polluted areas due to industrial spills convincingly demonstrated that some chemicals could indeed affect development and/or function of endocrine system in wild animals or humans, such cases are not really relevant for general human population, which is exposed to much lower doses of such chemicals (3, 28, 46). Therefore, at present we do not have an answer whether human reproductive health has really detoriated during the last several decades, and it is even less clear if endocrine disrupting compounds could have contributed to increased incidence of human reproductive problems and perhaps obesity.

Endocrine disrupting chemicals and animal health

If claims about detoriating reproductive health in humans are true and increase in reproductive problems in men is indeed caused at least parti-

ally by endocrine disrupting chemicals, one would expect similar trends to be observed also in domestic animals, which are usually exposed to similar environment as humans. Although there are no systematic records about reproductive health of pet/companion animals, reproduction in farm animals is usually closely monitored for the purpose of successful breeding and good records exist for reproductive performance of such animals. Interestingly, though, only few studies addressed the issue of farm animal reproductive health from the endocrine disrupting chemicals perspective.

Endocrine disrupting chemicals and female reproductive health in farm animals

Reproductive success is one of the most important factors affecting economic success of dairy farms and reproduction of dairy cows is therefore closely monitored. Interestingly, there are several reports suggesting that reproductive success of dairy cows has indeed detoriated during recent decades (47-50), although it would be overwhelming simplification to connect this observation with endocrine disrupting chemicals. Reproduction in dairy cows is influenced by many different factors such as genetics, age, hormonal status, ovarian reserves as well as exogenous factors such as well being of animals including physical activity and sun light exposure. Therefore, it would be very challenging to connect any single factor with reproductive decline, especially considering that dairy production has intensified together with strong genetic selection of certain traits that might also affect successful reproduction. However, there are some field studies suggesting that certain pollutants could affect reproductive health of dairy cows (51) and endocrine disrupting chemicals do accumulate in liver and fat of animals grazing on pastures fertilized with sewage sludge (52). A number of laboratory studies, mostly in sheep, have shown that endocrine disrupting chemicals could indeed affect reproductive health of animals acting directly in the gonads or through the hypothalamic-pituitary system. Octylphenol has been shown to advance puberty in sheep and sows (40, 53). Furthermore, DES and some other endocrine disrupting chemicals could reduce FSH levels in sheep fetuses at midgestation, and this is thought to influence early follicular development in female lambs, causing premature puberty and some ovulatory problems in adult ewes (54, 55). Several endocrine disrupting chemicals have been also shown to affect steroid production in pig ovarian cells (56-58). These studies therefore suggest that female reproductive axis could be sensitive to some endocrine disrupting chemicals, although these were all laboratory studies and it is difficult to extrapolate such results directly to the general populations of farm animals.

Endocrine disrupting chemicals and male reproductive health in farm animals

Male mammals are thought to be especially vulnerable to the effects of endocrine disrupting chemicals due to the nature of development of their reproductive system. In male mammals, Sry gene present on the Y chromosome induces development of testis and testis with its hormonal secretions governs further development of male phenotype (59, 60). Since male fetal development is completely dependent on the proper secretion/exposure to sex steroid hormones, endocrine disrupting chemicals could interfere with these processes. Many studies in laboratory mice and rats have shown that different endocrine disrupting chemicals could indeed influence development of male sexual organs including testes, epididymes and prostates (reviewed in 2, 3). Studies in farm animals are scarce; nevertheless, there are some reports that endocrine disrupting chemicals could indeed affect development of male reproductive system also in sheep. Several studies have shown reduced sperm counts and increase in sperm abnormalities in rams exposed either pre- or postnatally to different estrogenic compounds such as DES; octylphenol and organochlorine pesticides (40, 54, 61) and one study reported that prepubertal exposure of pigs to estradiol or di-ethlyhexyil-phthalate affected testis size and testosterone secretion in adult boars. However, in this study animals were treated intramuscularly so it is difficult to extrapolate these results to usual, oral exposure to endocrine disrupting chemicals (62).

Bull breeding centers usually keep careful records of bulls' reproductive performance that would allow retrospective studies of bulls' semen characteristics. Surprisingly, though, there are only two such studies published , together with a meta-analysis study of published data (63-65). None of these three studies have reported any decline in any semen characteristic monitored during several decades, suggesting that no similar decline as reported in humans, has occurred in cattle. However, one has to keep in mind that such data is heavily confounded by the

fact that bulls are selected also for their reproductive performance and therefore, such data could not be a true reflection of what might be happening in the normal population.

Conclusion

Although a number of studies in laboratory animals (and some in farm animals) have convincingly shown that endocrine disrupting chemicals could influence development and function of different cells in the testis and ovary such as Sertoli cells, Leydig cells, granulosa cells and oocytes and perhaps even adipocytes, there are no conclusive data that could link everyday exposure of humans or domestic animals to endocrine disrupting chemicals with their reproductive health. Therefore, carefully designed prospective studies will be needed to establish firstly, whether reproductive health in animals or humans has indeed detoriated in recent decades and, secondly, whether endocrine disrupting chemicals might have been involved in this problems.

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HORMONSKI MOTILCI PRI DOMAČIH ŽIVALIH

G. Majdič

Povzetek: Izraz hormonski ali endokrini motilci se uporablja za različne kemične snovi, ki lahko vplivajo na razvoj in/ali delovanje endokrinega sistema. Prva poročila o mogočem hormonskem delovanju posameznih kemičnih snovi v okolju so bila objavljena pred malo manj kot 20 leti kot poročila o povečanem pojavljanju nepravilno razvitih spolnih organov pri nekaterih divjih živalih, ki živijo v zelo onesnaženih območjih, kot je npr. okolica nekaterih jezer na Floridi v ZDA. Nadaljnje raziskave so pokazale, da lahko na delovanje endokrinega sistema vplivajo številne kemične snovi, kot so različni pesticidi, plastične, farmacevtske ter številne druge snovi. Zaenkrat ni znana povezava med strukturo kake molekule in njenim delovanjem, kar bi lahko nakazovalo, da bo določena kemična snov delovala kot hormonski motilec. Velika večina raziskav o hormonskih motilcih je bila narejena *in vitro* ali pri laboratorijskih živalih, tako da zaenkrat nimamo odgovora na vprašanje, ali hormonski motilci v nizkih koncentracijah lahko vplivajo na zdravje ljudi in živali tudi v naravnem okolju ob relativno nizki izpostavljenosti le-tem.

Približno istočasno kot prva poročila o morebitnem endokrinem delovanju nekaterih kemičnih snovi so se pojavila tudi prva poročila o naraščanju pojavljanja nepravilnosti na moških spolnih organih. Več raziskav je pokazalo, da se je povečalo število nepravilno razvitih sečnic (hiposadija) in nespuščenih mod in da narašča pogostnost raka na modih pri ljudeh v zadnjih desetletjih, vendar pa so ta poročila, razen tistih o naraščanju pogostnosti raka na modih, še vedno neenotna. V preteklih letih so se pojavila tudi poročila o zmanjševanju števila semenčic pri moških, vendar jih je izjemno težko potrditi ali ovreči, saj nimamo velikih načrtovanih raziskav, temveč smo odvisni le od retrospektivnih študij. Če podatki o povečevanju napak pri moških spolnih organih držijo in če so pri povečevanju težav udeleženi hormonski motilci iz okolja, bi pričakovali pojavljanje podobnih težav tudi pri domačih živalih, ki so običajno izpostavljene vplivu podobnega okolja kot ljudje. Vendar je izjemno malo raziskav, ki bi se ukvarjale z načrtnim in sistematičnim kronološkimo proučevanjem razmnoževanja pri domačih živalih. Dve retrospektivni raziskavi o kakovosti semena bikov iz osemenjevalnih centrov nista ugotovili zmanjševanja števila semenčic ali povečevanja napak na semenčicah, vendar pa je potrebno vedeti, da so biki v osemenjevalnih centrih selekcionirani tudi glede na njihove razmnoževalne sposobnosti, zaradi česar ta populacija ne odraža nujno dejanskega stanja v naravnem okolju.

Ključne besede: hormonski motilci; razmnoževanje; domače živali

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Original Research Paper

LEVAMISOLE INCREASES LEUKOCYTE COUNT AND IMMUNOGLOBULIN LEVELS IN YOUNG BOARS

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Summary: Levamisole has been used as an immunomodulator in the treatment of some diseases and conditions include parasitic invasions and immunocompromised conditions in domestic animals. The aim of the study is usage of levamisole to modulate the immune responsiveness in young boars in condition of intensive exploitation like handling using restraint. Therefore, hematological and immunological variables were assessed in Swedish Landrace boars treated with levamisole for three consecutive days. Levamisole treatment effected a significant increase in total leukocyte count immediately upon the last dosage (day 3, $p<0.01$), which persisted to days 4, 5 and 6 ($p<0.01$ to $p<0.001$). Also, the percentage of neutrophilic granulocytes increased significantly on days 5, 6 and 7 ($p<0.001$ and $p<0.0001$), whereas the percentage of eosinophil cells decreased significantly on days 7 and 11 after levamisole administration ($p<0.01$ and $p<0.001$). Lymphocyte, basophil and monocyte counts were unaffected by levamisole treatment. Serum IgG concentration was significantly elevated on days 5, 6, 7, 11 and 14 ($p<0.01$ to $p<0.001$, respectively), whereas IgA concentration showed a significant increase only on day 14 ($p<0.01$) after levamisole administration. Levamisole treatment had no effect on serum levels of IgM. Cortisol levels remain unaffected throughout the period of observation. Levamisole treatment increased nonspecific cellular and humoral immunity in boars.

Key words: levamisole; leukocytes; immunoglobulins; boars

Introduction

Both veterinary and human medicine need a drug that would influence immune reactivity in conditions such as inflammation, infections, malignant diseases, and even in the prevention of stress effect on the immune function. Many studies have shown that immunomodulatory substances have a limited mode of action (2, 9).

Levamisole (*levo* isomer of 2,3,5,6-tetrahydro-6-phenylimidazo (2,1-*b*) thiazole) is primarily used as a broad-spectrum anthelmintic. However, a number of clinical and experimental trials have shown that levamisole may be beneficial as an immunomodulator in the treatment of some diseases (8, 21, 25), yet only partially meeting the expectations, as its therapeutic efficacy depends on several factors such as the dose administered, timing, and immune status of animal or human patients, which is of utmost importance. The drug exerts stimulatory action on the immune and inflammatory functions of leukocytes through stimulation of lymphocyte proliferation, cytotoxicity, chemotaxis, spontaneous motility and phagocytosis (25). Previous investigations have suggested that levamisole changes balance between the cAMP and cGMP cyclic nucleotides by increasing intracellular levels of cGMP in leukocytes (12, 21).

The aim of the study is usage of levamisole to assess the immunomodulatory effect in boars in condition of intensive exploitation like handling using restraint. Therefore, levamisole is administered in a recommended immunostimulatory dose of 2-3 mg/kg body weight to young boars for three days. Changes in total and differential leukocyte count and humoral nonspecific (immunoglobulins) immunity were monitored for more than two weeks

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after levamisole treatment. Cortisol concentrations were simultaneously observed because the handling procedure used in blood sampling may stress boars.

Materials and methods

Boars

Fourteen boars (Swedish Landrace) aged about 7 months and weighing between 105 and 125 kg were used. The boars were housed in individual pens in a stable at a temperature of 12-18 °C. Boars were fed individually 3 kg of standard corn-soy ration per day (components: starch 37.96%, sugar 3.02%, crude protein 16.99%, crude fiber 5.26%, crude fat 5.39%), with free access to water.

On each of the three experimental days, levamisole and saline were administered before 10.00 a.m. Both groups of boars were handled using restraint with a snare in the procedures of levamisole and saline administration and blood collection. The experimental group of seven boars were intramuscularly primed with levamisole (Nilverm®, Pliva, Zagreb, Croatia; the preparation contains 75 mg levamisole hydrochloride/mL) at the immunostimulatory dose of 2.5 mg/kg body weight on three consecutive days. The control group of boars (n=7) were intramuscularly administered 1 mL of sterile 0.9% saline on three consecutive days as a placebo.

Blood sampling

Blood samples from the treated and control boars were obtained between 8.00 and 10.00 a.m. on day 1 before the first levamisole injection, on day 3 after the third levamisole injection, then on days 4, 5, 6, 7, 11, 14, 18 and 22 after the last drug dosage. Briefly, the boars were restrained with a snare and blood was collected from jugular vein by sterile syringe (Becton Dickinson S.A., Fraga Huesca, Spain). Blood samples were immediately transferred to glass tubes containing EDTA solution for plasma samples (K3E 15% DB Vacutainer®, Preanalytical Solutions Belliver Industrial Estate, Plymouth, UK) and tubes for serum samples (SST, DB Vacutainer®, Preanalytical Solutions Belliver Industrial Estate, Plymouth, UK). Blood samples were centrifuged at 750 x g for 10 min, and then serum was separated and stored at -20 °C until analysis.

Hormone assays

Cortisol concentrations were determined by radioimmunoassay using commercially available RIA Coat-A-Count Kit (Diagnostics Products Corp., Los Angeles, USA) according to the manufacturer's instructions. Samples were quantified with average intra- and inter-assay coefficients of variation (n=5) of 7.5% and 12.0%, respectively. The assay sensitivity was 0.1 nmol/L.

Total and differential leukocyte counts

Total leukocyte counts were determined on a Baker System 9120 CP cell counter (Serono-Baker Diagnostics Inc., Allentown, PA, USA). For leukocyte differentiation, blood smears were stained with May-Grünwald-Giemsa. The percentage of neutrophils, lymphocytes, eosinophils, basophils and monocytes was counted by use of a microscope (Carl Zeiss, GF-Planchomat, Jena, Germany) at x100 magnification with oil immersion lens. A total of 100 leukocytes were counted from each slide, classified as different cell types, and expressed in percentage.

Immunologic parameters

Immunoglobulins were tested by radial immunodiffusion method (20). Test plates for determination of IgG were prepared by dilution of 2 g of agarose in 100 mL of barbiturate buffered saline (0.1 M) with the addition of anti-pig IgG antiserum (1:10) and 0.1% sodium azide. Five microlitres of reference standard solutions of IgG and diluted serum samples (1:20) were pipetted to a separately identified well of test plates. The plate was securely covered and incubated for 48 to 72 hours at room temperature. After incubation, the plates were removed and placed over a source of illumination to clearly see precipitation rings. The external diameters of the rings were measured to the nearest 0.1 mm by using an ocular scale. A reference curve was plotted using the diameters measured from standard solutions. From the reference curve, the IgG concentration of each diluted test sample was calculated by multiplying the concentration read from the curve by the dilution factor to obtain the actual concentration. Intra- and inter-assay coefficients of variation were 3.5% and 5.4%, respectively .

The concentrations of immunoglobulins A and M were determined by use of commercially available pig IgA and IgM VET-RID kits (Bethyl Laboratories,

Inc., Montgomery, Texas, USA). Intra- and inter-assay coefficients of variation (n=5) were 1.8% and 2.8% for IgA, and 0.5% and 1.2% for IgM, respectively.

Statistical analysis

Data were analyzed by Statistica® software ('99 Edition, Copyright 1984-1999, StatSoft®, Inc., Tulsa, USA). Results were expressed as mean ± SEM. Differences in total and differential leukocyte counts and humoral immune parameters in the treated and control group were examined using analysis of variance. To evaluate differences in the means between control and treated groups of animals at specific time points we used *t*-test for independent samples. The differences between values were considered significant at $p \leq 0.05$.

Results

Cortisol concentrations

The mean serum cortisol response in the groups of boars is shown in Figure 1. The administration of saline and levamisole had no effect on cortisol concentrations throughout the period of observation.

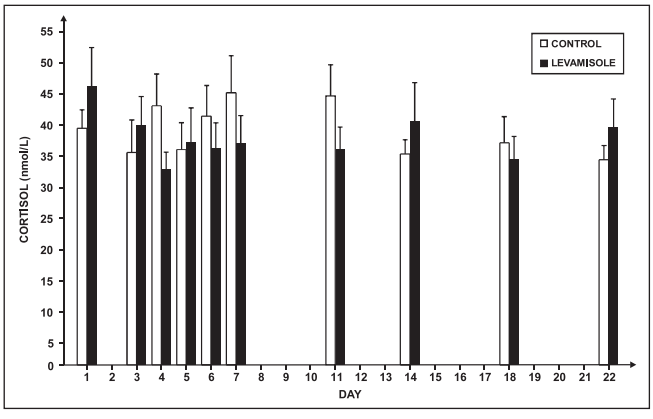

Figure 1: Serum cortisol concentration (mean ± SEM; nmol/L) in boars treated with levamisole (n=7) and saline (n=7)

Alterations in total and differential leukocyte counts

Changes in total and differential leukocyte counts in the experimental and control groups of animals after repeated levamisole and saline challenge are shown in Figures 2 to 4. The levamisole treated animals showed a significant increase in total leukocyte count immediately after the last levamisole dosage on day 3 ($p<0.01$), which persisted on days 4, 5 and 6 ($p<0.01$ to $p<0.001$, respectively) (Fig. 2). There were no significant differences in lymphocyte count between levamisole treated (49.7 ± 1.02 to 55.3 ± 4.85 %) and control (49.8 ± 3.02 to 53.8 ± 1.98 %) group of boars throughout the study period. Basophile and monocyte counts were not influenced by levamisole application either, so there were no differences between levamisole treated (basophiles: 0.57 ± 0.297 to 1.14 ± 0.594 %; monocytes: 0.58 ± 0.202 to 1.2 ± 0.632 %) and control animals (basophiles: 0.50 ± 0.341 to 1.20 ± 0.508 %; monocytes: 0.75 ± 0.387 to 1.0 ± 0.516 %). However, levamisole significantly increased the percentage of neutrophilic granulocytes on days 5, 6 and 7 after treatment ($p<0.001$ and $p<0.001$) (Fig. 3). The levamisole treated animals showed a significantly lower percentage of eosinophil cells on days 7 and 11 after the last dosage ($p<0.01$ and $p<0.001$, respectively) (Fig. 4).

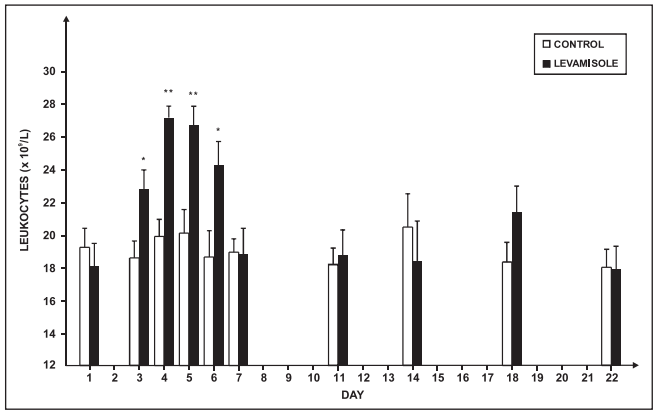

Figure 2: Total leukocyte count (mean ± SEM; $x10^9$/L) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$; ** $p<0.001$

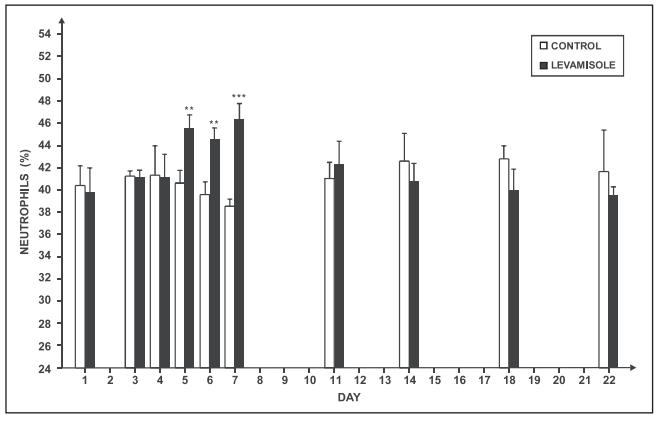

Figure 3: Neutrophil percentage (mean ± SEM; %) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: ** $p<0.001$; **** $p<0.0001$

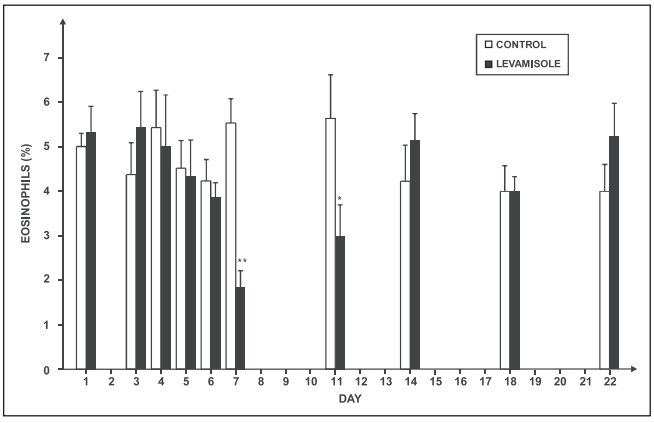

Figure 4: Eosinophil percentage (mean ± SEM; %) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$; ** $p<0.001$

Immunologic measures

The mean serum concentrations of immunoglobulin IgG, IgA and IgM levels after injections of levamisole and saline are shown in Figures 5, 6 and 7. Serum IgG concentration was significantly elevated on days 5, 6, 7, 11 and 14 ($p<0.01$ to $p<0.001$, respectively) (Fig. 5), whereas IgA concentration was significantly increased on day 14 ($p<0.01$) after levamisole administration (Fig. 6). Levamisole treatment had no effect on serum levels of IgM (Fig. 7).

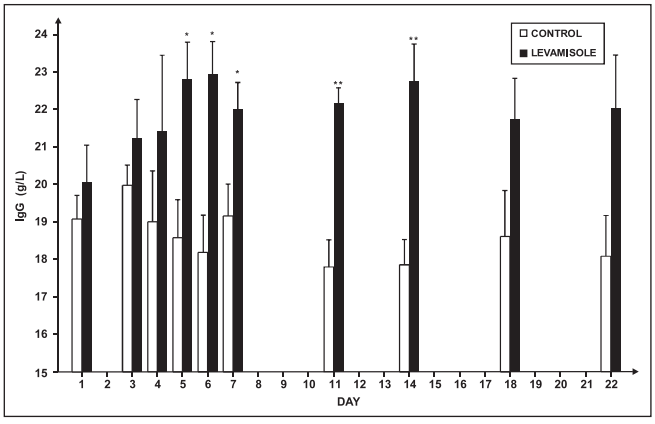

Figure 5: Serum IgG concentration (mean ± SEM; g/L) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$; ** $p<0.001$

Discussion

Different situations in modern farming are considered stressful to pigs, e.g., physical restraint and type of housing (10, 16). In this study, handling procedure by snare restraint on saline and levamisole

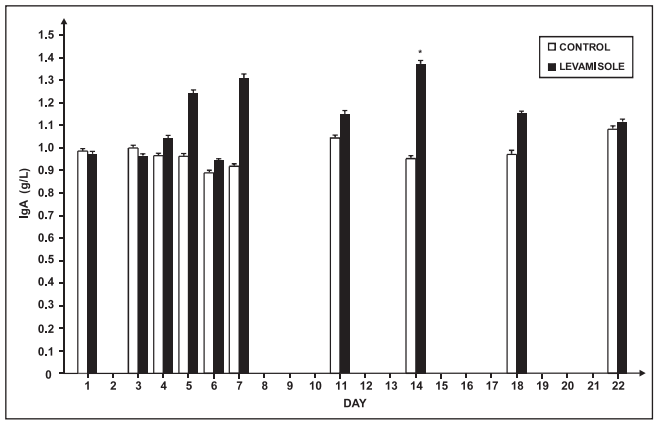

Figure 6: Serum IgA concentration (mean ± SEM; g/L) in boars treated with either levamisole (n=7) or saline (n=7). Significant differences between groups: * $p<0.01$

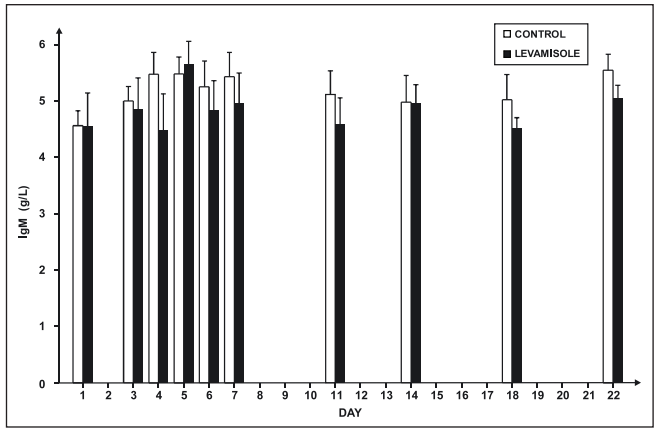

Figure 7: Serum IgM concentration (mean ± SEM; g/L) in boars treated with either levamisole (n=7) or saline (n=7)

administration as well as on blood sampling did not produce a stress condition in boars. Cortisol response remained stable throughout the experimental period in both groups of animals.

In contrast to higher anthelmintic dosage, levamisole was demonstrated to restore immune function in a dose of 2-3 mg/kg body weight, and has been recommended to be administered for three consecutive days or once a week as a single dose. Previous studies of levamisole as an immunomodulatory drug have suggested an immunity improvement to occur in cells with impaired function (8, 25). Therefore, the results and suggestions of different experiments have been rather contradictory and inconclusive (2). However, in the present study repeat levamisole injection changed total and differential leukocyte count in young healthy boars. Levamisole induced a significant increase in total leukocyte count immediately after the last dosage

(day 3), which persisted on days 4, 5 and 6 after treatment. Also, levamisole significantly elevated the percentage of neutrophilic granulocytes on days 5, 6 and 7, whereas the percentage of eosinophil cells significantly decreased on days 7 and 11 after application. On the other hand, lymphocyte, basophile and monocyte counts remained unaffected by levamisole treatment throughout the study period. In previous study is determined that levamisole treatment of boars with 2.5 mg/kg for three days increase leukocytes and monocytes 7 weeks after test termination (23).

The use of levamisole in the immunostimulatory dose of 2.5 mg/kg body weight in weaned piglets infected with enterotoxigenic *Escherichia coli* strain before vaccination suggested that levamisole restored leukocyte count to normal rather than stimulating it above the normal level (3). Also, the phagocytic function in vaccinated pigs was impaired by their pretreatment with levamisole. The administration of levamisole in the same dose (2.5 mg/kg body weight) enhanced lymphocyte proliferation in artificially reared pigs (13). It has also been reported that levamisole can enhance immune responses to viral antigens and reduce the incidence of disease (1, 11). Recent studies suggested that levamisole has a tendency for selective induction of protective mucosal cell-mediated immune response in pigs vaccinated against *Escherichia coli* (3, 5, 6).

The effect of levamisole on immunoglobulin synthesis in animals or humans is not completely understood, and it seems to primarily depend on the dosage used and duration of treatment (2, 22, 25). In our study, levamisole treatment of boars for three consecutive days in a dose of 2.5 mg/kg body weight led to a significant increase in serum IgG concentration on days 5, 6, 7, 11 and 14 after treatment. At the same time, serum IgA concentration was significantly increased only on day 14, whereas serum IgM levels were unaffected by levamisole treatment. Levamisole was demonstrated to increase serum IgG levels in foals from mares treated with levamisole (18). Therefore, the authors assumed that levamisole could protect foals against infectious disease during the postnatal period. Also, the colostrum of mares stimulated with levamisole was characterized by an increase in IgG, however, IgA and IgM classes remained unaffected.

Levamisole has a broad, well-defined activity on T lymphocytes. It has also been postulated that any effect on B lymphocyte function may probably occur indirectly through stimulation of T lymphocytes and macrophages rather than B lymphocyte cells themselves (8, 25). A recent study has shown that levamisole has a highly selective activity on immune response by resetting the immune balance of T lymphocyte subsets towards Th1 response (24, 26). On the other hand it has been shown that levamisole enhance both Th1 and Th2 type response (17). Levamisole has been shown to increase serum antibody titters after immunization, the number of leucocytes, phagocyte activities, the expression of cytokines by monocyte/macrophages, lymphocyte proliferation and antitumor responses (4, 7, 14, 15, 19, 23, 27).

In conclusion, the present study showed that administration of levamisole in recommended immunostimulatory dose for three consecutive days stimulated elevation of total leukocyte count and percentage of neutrophil cells in healthy boars. Also, levamisole induced an increase in serum IgG concentration that persisted for ten days after levamisole application. These observations suggest that levamisole also enhances immune response in cells with normal function and may be effective as a protective drug against diseases or stressful situations.

Acknowledgements

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LEVAMISOLE ZVIŠUJE ŠTEVILO LEVKOCITOV IN RAVEN IMUNOGLOBULINOV PRI MLADIH PRAŠIČIH

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Povzetek: Levamisole je v uporabi kot imunomodulator za zdravljenje nekaterih bolezni in stanj, kot so vdor zajedavcev in stanje imunske zavrtosti pri domačih živalih. Cilj študije je z uporabo levamisola uravnati imunsko odzivnost pri mladih prašičih v stanju intenzivnega izkoriščanja, kot je ravnanje s prašiči z uporabo prisile. V ta namen so bili ocenjeni hematološki in imunološki parametri pri prašičih švedske deželne pasme, ki so jih tri zaporedne dni zdravili z levamisolom. Zdravljenje z levamisolom je značilno vplivalo na povišanje skupnega števila levkocitov takoj po zadnjem odmerku (3. dan, $p<0.01$) in je trajalo do 4., 5. in 6. dneva ($p<0.01$ do $p<0.001$). Prav tako se je značilno povišal odstotek nevtrofilnih granulocitov 5., 6. in 7. dan ($p<0.001$ in $p<0.0001$), medtem ko se je odstotek eozinofilnih granulocitov značilno znižal 7. in 11. dan po uporabi levamisola ($p<0.01$ in $p<0.001$). Število limfocitov, bazofilnih granulocitov in monocitov je ostalo po zdravljenju z levamisolom nespremenjeno. Serumska koncentracija IgG je bila značilno povišana 5., 6., 7., 11. in 14. dan ($p<0.01$ do $p<0.001$), medtem ko je bila koncentracija IgA značilno povišana le 14. dan ($p<0.01$) po uporabi levamisola. Zdravljenje z levamisolom ni vplivalo na raven IgM v serumu. Raven kortizola je ostala v času opazovanja nespremenjena. Zdravljenje z levamisolom je pri prašičih povišalo nespecifično celično in humoralno imunost.

Ključne besede: levamisole; levkociti; imunoglobulini; prašiči

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Original Research Paper

MOLECULAR CHARACTERIZATION OF *MYCOBACTERIUM AVIUM* SUBSP. *AVIUM* FROM ANIMALS IN CROATIA USING IS901 RFLP AND MIRU-VNTR TYPING

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Summary: *Mycobacterium (M.) avium* subsp. *avium*, the causative agent of avian tuberculosis, primarily affects the birds but may often be isolated from granulomatous lesions in pigs and occasionally from cattle and other animals. In this study, a total of nine *M. avium* subsp. *avium* isolates collected between 2001 and 2006 from poultry (n=4), wild boars (n=2), pigs (n=2) and cattle (n=1) were investigated by IS*901* restriction fragment length polymorphism (RFLP) analysis using two restriction endonucleases (*Pvu*II and *Pst*I) and by mycobacterial interspersed repetitive units – variable-number tandem repeat (MIRU-VNTR) typing. Digestion with the restriction endonuclease *Pvu*II resulted in three RFLP types F, Q and M. Digestion with *Pst*I was successfully accomplished in eight isolates demonstrating four RFLP types A29, A31, A32 and A33, of which the last three have not been described before. Combination of *Pvu*II and *Pst*I restriction patterns revealed four RFLP types F-A29, F-A31, F-A32 and M-A33, respectively. No epizootiological connection was found among the isolates expressing the predominant RFLP type F-A29, which was discovered in pig, wild boar and poultry. MIRU-VNTR typing resulted in four MIRU-VNTR types; among them, two were regarded as new. The most frequent type 34131127 was detected in four isolates from wild boars, pig and poultry. The combination of both typing methods revealed seven distinct RFLP/MIRU-VNTR genotypes; among them, six were unique.
This work represents the first genotyping research of *M. avium* subsp. *avium* strains isolated from different animal species in Croatia. Notwithstanding the small number of investigated isolates, the results indicate a relatively high genetic diversity of *M. avium* subsp. *avium* in animals and suggest a combination of RFLP and MIRU-VNTR typing as a suitable approach to genotyping of *M. avium* subsp. *avium* isolates.

Key words: IS*901* RFLP; MIRU-VNTR typing; avian tuberculosis; pigs; poultry; cattle; wild boars

Introduction

Mycobacterium (*M.) avium*, comprising organisms that range from ubiquitous mycobacteria causing opportunistic infections in a variety of hosts to obligate pathogens of birds and ruminants, is currently divided into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *hominissuis* (1-3).

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M. avium subsp. *avium* is the causative agent of avian tuberculosis; it may infect many animal species but birds are particularly susceptible to infection which often leads to fatal outcome. In farm animals, particularly in pigs and cattle, it causes mycobacteriosis with tuberculous lesions mostly localized in the lymph nodes of the head and intestine (4). *M. avium* subsp. *avium* genome contains mobile elements, e.g. insertion sequences IS*901* and IS*1245*, which are used as markers for identification and typing.

Molecular techniques with a high discriminatory power, e.g. restriction fragment length polymor-

phism (RFLP) and pulsed-field gel electrophoresis (PFGE) are considered as a useful tool for the epidemiological studies of *M. avium* infections. IS*901* RFLP typing is used for the differentiation of *M. avium* subsp. *avium* isolates despite its rather limited polymorphism (5, 6). Lately, other typing methods that target different structures in the genome have been developed with the aim to facilitate and accelerate strain typing. Recent studies identified loci containing variable-number tandem repeats (VNTRs) of specific mycobacterial interspersed repetitive units (MIRUs) in *M. avium* isolates. This PCR-based typing method has been investigated as an alternative and rapid tool for genotyping of *M. avium* isolates in the past few years (7-10).

The aim of this study was to characterize *M. avium* subsp. *avium* isolates from poultry, pigs, cattle and wild boars by using IS*901* RFLP analysis and MIRU-VNTR typing based on some of the recently described markers (9).

Materials and methods

Mycobacterial isolates

A total of nine *M. avium* subsp. *avium* isolates, obtained between 2001 and 2006 from six regions in Croatia, were studied. The selection of isolates was based on their animal origin, namely they were isolated from different animal species with distinct biological and ecological traits. One isolate originated from cattle and two from pigs from two farms located in distinct regions; these animals showed positive reaction to avian tuberculin and were slaughtered. Two wild boar isolates from different regions were obtained from the laboratory strain collection. A total of four poultry isolates originated from animals that died of avian tuberculosis on small farms in two different regions (Table 1).

Identification of the isolates

Isolates were identified as *M. avium* with molecular identification kit GenoType Mycobacterium CM (Hain Lifescience, Germany) and as *M. avium* subsp. *avium* by IS*901* PCR using primers described previously (11). Amplification products were run on 2% agarose gels and stained with ethidium bromide.

RFLP analysis

RFLP typing was performed according to previously published instructions (12, 13) with slight modifications described by Pate et al. (14). RFLP types were analysed with BioNumerics software (v. 4.0, Applied Maths, Belgium), using *M. avium* subsp. *avium* strain R13 as a reference for band normalization and UPGMA (Dice coefficient) algorithm to

Table 1: Animal isolates of *M. avium* subsp. *avium* investigated in this study: origin, IS*901* RFLP types and MIRU-VNTR types

Isolate code	Region	Host	Sample	Year of isolation	*Pvu*II *Pst*I IS*901* RFLP type[a]	MIRU-VNTR type[b]
S44	VP	pig	SLN	2004	F-A29	34131127
S49	KK	pig	SLN	2002	F-A31	34131137
DS126	VV	wild boar	MesLN	2004	F-A29	34131127
DS125	SM	wild boar	MesLN	2003	F-A32	34131127
P127	Z	poultry	L	2001	F-A29	22131127
P128	S	poultry	L, I, S	2004	Q-ns	35131127
P129	Z	poultry	L	2005	F-A29	34131127
P130	Z	poultry	L	2006	F-A29	ns
G83	Z	cattle	MedLN	2004	M-A33	35131127

Legend: SLN – submandibular lymph node, MesLN – mesenteric lymph node, L – liver, I – intestine, S – spleen, MedLN – mediastinal lymph node, ns – typing not successful

[a] RFLP types are designated according to Dvorska et al. (2003) – the nomenclature established and used at Veterinary research Institute, Brno, Czech Republic

[b] MIRU-VNTR types are designated by the number of tandem repeats in the following sequence: TR 292-X3-25-47-3-7-10-32 (Thibault et al., 2007)

generate dendrograms with 1.2% position tolerance. The nomenclature of RFLP types described herein is in concordance with the nomenclature established and employed at the OIE Reference Laboratory for Avian Tuberculosis in Brno, Czech Republic (13).

MIRU-VNTR typing

PCR amplification of the eight loci described by Thibault et al. (9) was applied with slight modifications, as described by Pate et al. (15). PCR products were analysed by agarose gel electrophoresis and detected by ethidium bromide staining. Reference strain *M. avium* subsp. *avium* R13 was used as positive control. MIRU-VNTR types described herein were designated by the number of tandem repeats in the following sequence: TR 292-X3-25-47-3-7-10-32.

Results

RFLP analysis

Digestion with *Pvu*II resulted in three RFLP types F, Q and M (Figure 1) of an average similarity of 93.5% (data not shown). The majority of isolates (7/9) were of RFLP type F, which was found in pigs, wild boars and poultry. One poultry isolate showed RFLP type Q, while a single isolate from cattle demonstrated RFLP type M (Table 1).

In eight isolates, four RFLP types were detected by *Pst*I digestion: A29, A31, A32 and A33 (Figure 1), exhibiting an average similarity of 93% (data not shown); digestion failed in one poultry isolate. RFLP type A29 was observed in five isolates from poultry, pig and wild boar. Unique RFLP types A31, A32 and A33 were detected in pig, wild boar and cattle, respectively (Table 1).

Parallel digestion with both restriction endonucleases resulted in four combined *Pvu*II *Pst*I RFLP types F-A29, F-A31, F-A32 and M-A33. The predominant RFLP type F-A29 was detected in different time periods and regions in five isolates from poultry, pig and wild boar. Unique RFLP types F-A31, F-A32 and M-A33 were found in pig, wild boar and cattle, respectively (Table 1).

MIRU-VNTR typing

Tested isolates demonstrated four MIRU-VNTR types, including one type which could not be fully determined due to repeated absence of locus TR32 amplification product. The types differed either in

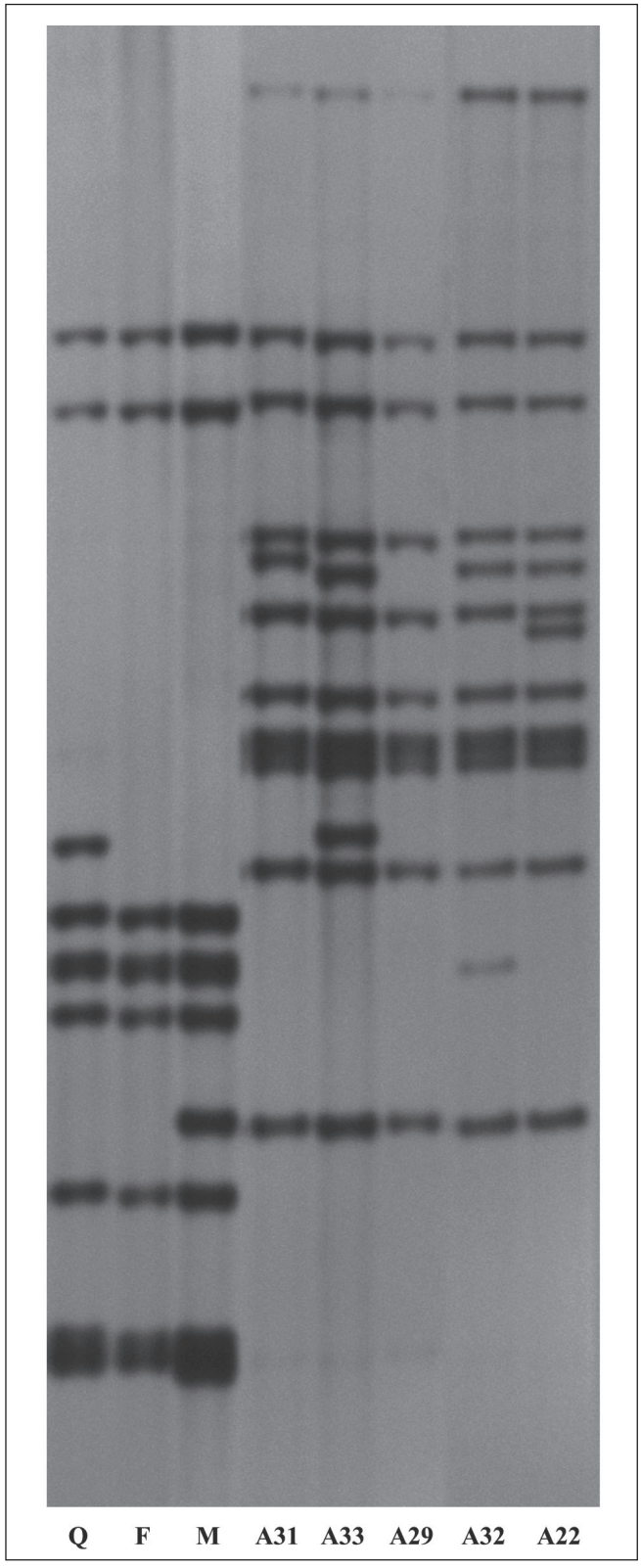
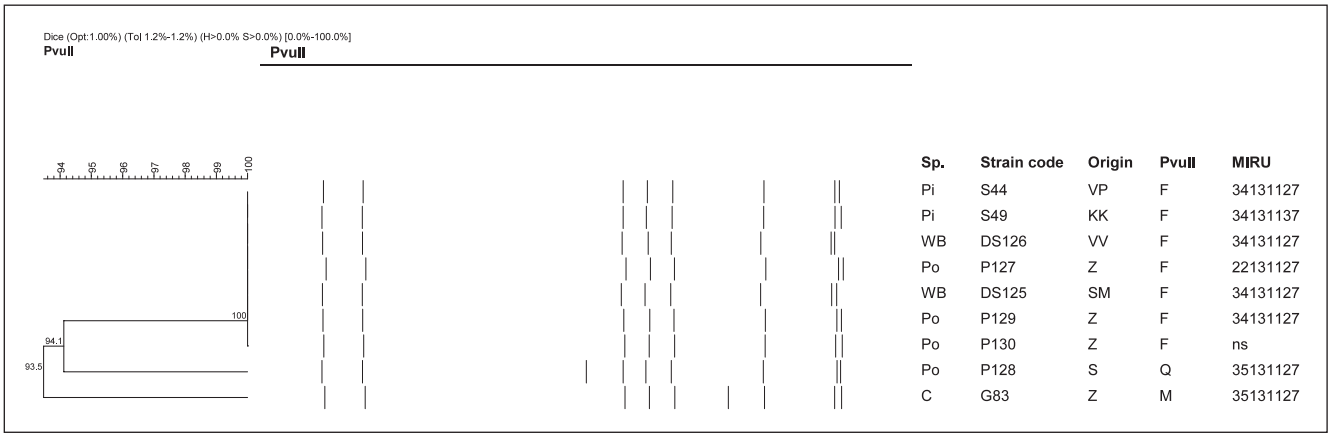


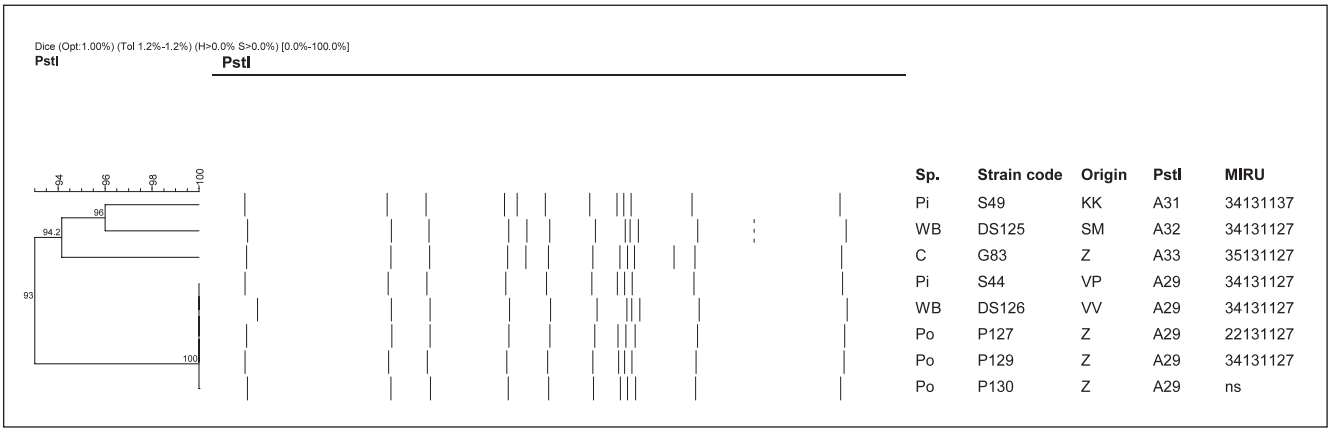
Figure 1: IS*901* RFLP types discovered in nine *Mycobacterium avium* subsp. *avium* isolates in this study: *Pvu*II RFLP types Q to M and *Pst*I RFLP types A31 to A22. RFLP types are designated according to Dvorska et al. (2003). Reference *Mycobacterium avium* subsp. *avium* strain R13 showed the *Pvu*II *Pst*I RFLP type F-A22



Sp.	Strain code	Origin	PvuII	MIRU
Pi	S44	VP	F	34131127
Pi	S49	KK	F	34131137
WB	DS126	VV	F	34131127
Po	P127	Z	F	22131127
WB	DS125	SM	F	34131127
Po	P129	Z	F	34131127
Po	P130	Z	F	ns
Po	P128	S	Q	35131127
C	G83	Z	M	35131127

Figure 2: Comparison of *Pvu*II IS901 RFLP types and MIRU-VNTR types of nine *Mycobacterium avium* subsp. *avium* isolates from animals (UPGMA dendrogram, Dice coefficient, 1.2% position tolerance)

Legend:
Sp. – host species; Pi – pig; WB – wild boar; Po – poultry; VP, KK, VV, Z, SM, S – codes of regions; *Pvu*II – *Pvu*II RFLP type; MIRU – MIRU-VNTR type; ns – typing not successful



Sp.	Strain code	Origin	PstI	MIRU
Pi	S49	KK	A31	34131137
WB	DS125	SM	A32	34131127
C	G83	Z	A33	35131127
Pi	S44	VP	A29	34131127
WB	DS126	VV	A29	34131127
Po	P127	Z	A29	22131127
Po	P129	Z	A29	34131127
Po	P130	Z	A29	ns

Figure 3: Comparison of *Pst*I IS*901* RFLP types and MIRU-VNTR types of nine *Mycobacterium avium* subsp. *avium* isolates from animals (UPGMA dendrogram, Dice coefficient, 1.2% position tolerance)

Legend: See Figure 2

single locus (X3 or TR10) or in two loci (TR292 and X3). The most frequent was type 34131127, detected in four isolates: two from wild boars, one from pig and one from poultry. These isolates were obtained from different regions in different time periods. Three other isolates, originating from the same time period but from different animal species, shared a common type (35131127). The remaining two types were unique. The results are summarized in Table 1.

RFLP and MIRU-VNTR typing

The combination of both typing methods revealed seven distinct RFLP/MIRU-VNTR genotypes, i.e. R1-M1, R2-M2, R3-M1, R1-M3, R4-M4, R1-M4 and R5-M4 (Table 1). Among these, only genotype R1-M1 was found in several isolates, namely one from pig, one form wild boar and one from poultry, originating from different regions. The remaining genotypes were unique.

Discussion

The prevalence of *M. avium* subsp. *avium* in pigs varies and depends on the presence of this subspecies in the environment. Some early studies failed to isolate *M. avium* subsp. *avium* from pigs (16, 17), while other reports described different proportions

of *M. avium* subsp. *avium* in pigs, rising up to more than 50% (18-22). A recent study in Croatia reported 21.1% of *M. avium* subsp. *avium* in pigs (23), which was about 45% less compared to the data from one of the past reports (24).

Development of genotyping methods enabled differentiation within *M. avium* subsp. *avium* isolates. In spite of rather limited polymorphism reported in the first IS*901* RFLP studies (5, 6), the method has been used for typing of *M. avium* subsp. *avium* isolates in several other studies (13, 14, 25-27). Its discriminatory power was improved by using a combination of different restriction endonucleases in parallel to increase the number of discernable RFLP types. The first extensive IS*901* RFLP study (13) revealed 25 *Pvu*II RFLP types and 25 *Pst*I RFLP types which gave a total of 52 combined *Pvu*II *Pst*I RFLP types. Several other RFLP types have been identified (14, 25, 26). In order to compare RFLP types detected in our study with the previously identified, they were submitted to the database of IS*901* RFLP types reported by several central European countries, managed by the OIE Reference Laboratory for Avian Tuberculosis in Brno, Czech Republic.

Using the restriction endonuclease *Pvu*II, three RFLP types were detected. The predominant type F was found in isolates from different animal species from different regions, which was in congruence with the results of Dvorska et al. (13, 25, 27), Moravkova et al. (26) and Pate et al. (14). RFLP type M that was detected in cattle in our study, was found also in a pig in Slovenia (26). RFLP type Q has also been detected previously (13, 14, 26).

Digestion with restriction endonuclease *Pst*I revealed four RFLP types. Among them, types A31, A32 and A33 have not been found in the database and were regarded as new. The most prevalent type A29, found in poultry, wild boar and pig, was detected also in poultry in Slovenia (14). In our study, digestion with *Pst*I failed in one isolate, however similar cases were observed before (14).

Parallel digestion with both restriction endonucleases resulted in four *Pvu*II *Pst*I RFLP types. The predominant type F-A29 was detected in five isolates from poultry, wild boar and pig. This RFLP type was first described by Pate et al. (14) in one isolate from poultry. The remaining unique *Pvu*II *Pst*I RFLP types described herein (F-A31 from pig, F-A32 from wild boar and M-A33 from cattle) were detected for the first time.

In the reports published up to date, MIRU-VNTR typing of *M. avium* was used for differentiation of *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* strains (7-10), but not for differentiation within *M. avium* subsp. *avium* which was aimed for in the present study. Our results show that the method provided less discrimination among *M. avium* subsp. *avium* isolates compared to RFLP. Among nine isolates of different origin, four isolates exhibiting two different RFLP types shared a common MIRU-VNTR type. The same applied for three other isolates of different origin that exerted different RFLP types but shared the MIRU-VNTR type. These results are in congruence with the commonly reported lower discriminatory power of MIRU-VNTR compared to RFLP genotyping of *M. avium* (9, 10, 15, 28). Nevertheless, discrimination of RFLP typing could be improved by MIRU-VNTR analysis, since two of the five isolates from our study exhibiting F-A29 RFLP type demonstrated a different MIRU-VNTR type. However, one of these two types could not be fully determined due to the absence of TR32 amplification product, but was deducted from the calculations of the allelic diversity (*h*) for this locus (*h*=0.00) performed in a study by Pate et al. (15) on 41 *M. avium* subsp. *avium* isolates; this type most probably represented MIRU-VNTR type 35131127 and was regarded as such. In the case of one isolate, both RFLP and MIRU-VNTR typing generated unique profiles. In general, the combination of both methods in our study subdivided the nine isolates into seven RFLP/MIRU-VNTR types, which was more discriminative than applying RFLP or MIRU-VNTR typing alone (obtaining five or four types, respectively). The complementarity of both typing methods was published before when the increased number of discernable types obtained from the combined approach was reported (9, 10, 15, 28).

The loci tested in this study for MIRU-VNTR typing exhibited a relatively low allelic diversity, namely a limited polymorphism was documented only for loci TR292, X3 and TR10. The reason for the observed phenomenon might lie in the selection of the markers, which was done on the basis of complete genome sequences of *M. avium* subsp. *hominissuis* strain 104 and of *M. avium* subsp. *paratuberculosis* strain K10, respectively, since it has been reported(29) that the IS*901*-positive strains contain certain genomic regions that vary between *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*. Nevertheless, the MIRU-VNTR diversity of *M. avium* subsp. *avium* isolates observed in this study is considerably higher compared to diversity established among Slovenian *M. avium* sub-

sp. *avium* isolates (15): herein, four types were detected among nine isolates while in Slovenia, a total of five MIRU-VNTR types were identified among 41 isolates. Types 34131127 and 35131127 were found in both countries with the former being the most prevalent one, which was detected in poultry, pig, wild boar and cattle. The remaining types described in this study seem to be unique among the types discovered in previous publications, although the comparison with the previously described types is hampered due to diverse typing schemes used. However, the main reason for the incongruence among the types most probably lies in the fact that previous studies (9, 28) regarded *M. avium* subsp. *hominissuis* isolates instead of *M. avium* subsp. *avium* isolates. This indicates that *M. avium* subsp. *avium* harbors unique genomic elements not found in other *M. avium* subspecies.

This is the first genotyping study of *M. avium* subsp. *avium* isolates from different animal species in Croatia. Considering the small number of investigated isolates, a relatively high genetic diversity of *M. avium* subsp. *avium* was observed. The combination of RFLP and MIRU-VNTR typing seems to be a suitable approach to genotyping of *M. avium* subsp. *avium* isolates. However, it should be remarked that MIRU-VNTR typing needs improvement in terms of identifying suitable markers for this subspecies. In order to get a better perspective on the genetic diversity of *M. avium* subsp. *avium* strains in Croatia, the research should undoubtedly be expanded by testing a larger collection of strains.

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MOLEKULARNA OPREDELITEV MIKOBAKTERIJ PODVRSTE *MYCOBACTERIUM AVIUM* SUBSP. *AVIUM* PRI ŽIVALIH NA HRVAŠKEM Z METODAMA IS*901* RFLP IN MIRU-VNTR

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Povzetek: Mikobakterije podvrste *Mycobacterium (M.) avium* subsp. *avium*, povzročiteljice aviarne tuberkuloze, prizadenejo predvsem ptice. Pogosto jih lahko izoliramo tudi iz granulomatoznih sprememb pri prašičih, redkeje pa pri govedu in drugih živalih. V okviru te raziskave smo z analizo polimorfizmov dolžin restrikcijskih fragmentov (RFLP) na podlagi cepitve DNK z restrikcijskima endonukleazama *Pvu*II in *Pst*I in s tipizacijo na podlagi razpršenih ponavljajočih se enot v genomu mikobakterij (spremenljivega števila tandemskih ponovitev MIRU-VNTR) opredelili devet sevov podvrste *M. avium* subsp. *avium*, izoliranih v obdobju 2001–2006 pri perutnini (n=4), divjih prašičih (n=2), domačih prašičih (n=2) in govedu (n=1). Po cepitvi DNK z restrikcijsko endonukleazo *Pvu*II smo ugotovili tri tipe RFLP (F, Q in M), medtem ko je bila cepitev s *Pst*I uspešna le pri osmih izolatih, pri katerih smo ugotovili štiri tipe RFLP: A29, A31, A32 in A33. Zadnji trije tipi v literaturi še niso bili opisani. S kombinacijo rezultatov obeh cepitev za posamezni izolat smo določili štiri kombinirane tipe RFLP: F-A29, F-A31, F-A32 in M-A33. Med izolati prevladujočega tipa F-A29, ki smo ga odkrili pri domačem prašiču, divjem prašiču in perutnini, nismo ugotovili nobene epizootiološke povezave.

S tipizacijo smo ugotovili štiri tipe MIRU-VNTR, med njimi dva nova. Najpogostejši tip 34131127 smo odkrili pri štirih izolatih iz divjih prašičev, domačega prašiča in perutnine. Kombinacija obeh tipizacijskih metod je razkrila sedem različnih genotipov RFLP/MIRU-VNTR, šest izmed njih je bilo unikatnih.

To je prva raziskava na področju genotipizacije mikobakterij podvrste *M. avium* subsp. *avium* pri različnih živalskih vrstah na Hrvaškem. Rezultati kljub majhnemu številu v raziskavo zajetih izolatov nakazujejo precejšnjo genetsko pestrost mikobakterij te podvrste, kombinacijo metod RFLP in MIRU-VNTR pa kot uporaben pristop h genotipizaciji izolatov mikobakterij podvrste *M. avium* subsp. *avium*.

Ključne besede: IS*901* RFLP; tipizacija MIRU-VNTR; aviarna tuberkuloza; domači prašiči; perutnina; govedo; divji prašiči

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Original Research Paper

PATHOPHYSIOLOGY OF DIABETES MELLITUS AND ITS RELATIONSHIP WITH OBESITY IN CATS

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Summary: Diabetes mellitus is one of the most common endocrinopathies in cats, and its incidence has increased in recent years associated with a significant increase in the percentage of obese cats. The main factors in the pathophysiology of this disease are the development of insulin resistance, dysfunction and / or loss of β cells, deficient secretion of insulin and islet amyloidosis (IA). Insulin sensitivity is significantly reduced in obese patients, therefore, several authors have tried to find linking factors between obesity and FDM. Among these, alterations in glucose transport, increase in triglycerides and fatty acids, deposits of amyloid in the islets and effect of hormones such as leptin and proinflammatory cytokines have been considered.

Key words: feline obesity; diabetes mellitus, feline adiponectin, feline leptin

Introduction

Diabetes mellitus is a disease of the endocrine pancreas characterized by a relative or absolute deficiency in insulin secretion. In the cat, unlike dogs, antibodies against β cells or insulin have not been detected yet; therefore it has been concluded that the autoimmune destruction, characteristic of diabetes mellitus type 1, does not seem to be a factor in the etiology of Feline Diabetes Mellitus (FDM) (1). Pathophisiology of FDM is similar to that seen in diabetes mellitus type 2 (DMT2) in humans. In both of them it is characterized by insulin resistance, deficient insulin secretion, deposit of amyloid in the islets and dysfunction and loss of β cells.

The incidence of DMT2 in the human population has increased in recent years, primarily associated with an increase in obesity and physical inactivity related to the sedentary lifestyle that man has taken. Metabolic diseases associated with obesity affect over 50% of the adult population (1, 2). This is a phenomenon that also affects felines who share the same environment as humans. Cats have evolved from animals that lived in open environments hunting foods high in protein, to a closed environment where they eat commercial foods high in carbohydrates and spend long periods of time sleeping (3). Approximately 25-40% of domestic cats are considered overweight and it has been found that obesity increases the risk of developing feline diabetes mellitus (FDM) from 3 to 5 times (4).

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Pancreatic Physiology

Pancreatic β cells, which represent approximately 70% of pancreatic endocrine cell population, secrete insulin in response to a influx of glucose through glucose transporters (5).

Insulin binds to its receptors, leading to the phosphorilation of tyrosine residues, thus initiating the signal pathways to perform its action. These receptors are glycoprotein molecules that can be found in various tissues, but in greater proportion in adipose tissue, muscle, heart and liver. Since the lipid bilayer of the cell membrane is impermeable

to glucose,it needs a system of active transport of carbohydrates to get through it. The GLUT4 is the major glucose transporter which responds to insulin. It is mainly located in muscle and adipocytes, and has a great significance in maintaining glucose homeostasis (6). In the absence of insulin or other stimuli, 90% of GLUT4 is retained in intracellular vesicles of deposit; on the contrary, in the presence of stimuli, these vesicles translocate and merge with the cell membrane. This process results in the incorporation of the GLUT4 to the cell membrane and the consequent passive entry of glucose. The cells containing the GLUT4 transporter are also stimulated by physical activity, independently of insulin action (5). In the liver, glucose diffuses freely even in the absence of insulin. The main effects of insulin on the liver are getting glucose trapped inside the liver cells, increasing the enzymatic activity that promotes lypogenesis and glycogenesis, and inhibiting the enzymatic activity that contributes to the processes of gluconeogenesis and glucogenolysis. Other effects of insulin are promoting the entry of amino acids, potassium, magnesium and phosphorus into cells. It also promotes fat storage by stimulating the activity of lipoprotein lipase and the entry of free fatty acids into the adipocyte.

Pathophysiology of Feline Diabetes Mellitus (FDM):

The FDM can be immune mediated (rarely diagnosed) (type 1), or be associated with obesity (type 2), or diseases and drugs that increase insulin resistance such as acromegaly, hyperadrenocorticism, pancreatitis and treatment with corticosteroids or progestagens (type 3). Its development also depends on factors such as age, body weight, sex, genetic predisposition and many others (7, 8). Comparing the clinical behavior and histopathology of the islets, it can be assumed that between 85 and 95% of cats with FDM have DMT2 (9, 10).

Insulin resistance and dysfunction and loss of β cells, is critical for the development of the FDM. Insulin resistance is a pathological condition where the biological response to insulin is diminished, affecting the entry and utilization of glucose by the peripheral tissues, thus leading to compensatory hyperinsulinemia. Insulin resistance also promotes a shift from glycolysis to gluconeogenesis in the liver cells, increasing this way plasmatic glucose levels. Pancreatic β cells eventually fail to compensate the insulin-resistant status, leading to relative insulin deficiency and consequent hyperglycaemia, glucose intolerance and at last, diabetes (7). When glycaemia exceeds the capacity of the renal tubules to reabsorb glucose, the consequent glucosuria determines osmotic diuresis and polyuria. Then compensatory polydipsia prevents dehydration. Hence the presentation of the classic signs: polydipsia and polyuria. The effects of hyperglycaemia can be divided into three phases: insulin resistance, depletion of β cells and glucose toxicity. Initially, exposure to high glucose levels leads to a potentially reversible decrease in insulin production. More prolonged exposure causes depletion of β cells, so that insulin stores are depleted. However, it is a reversible process since there are no alterations in insulin synthesis. Glucose toxicity is an irreversible status, since the cellular defects impair insulin production. The severity of glucose toxicity depends on the degree of hyperglycaemia; insulin secretion can be supressed after two days of persistent hyperglycaemia. The histologic abnormalities associated with glucose toxicity include glycogen deposits and cell death (5,7). A significant number of diabetic cats require insulin therapy to regulate glucose levels. However, it has been shown that between 30 and 85% of diabetic cats do not become insulin-dependent after an initial period of insulin therapy and adequate diet (5). The difference between cats that require or do not require insulin is not clear, although it seems that the degree of loss of β cells and insulin, play an important role.

The presence of islet amyloidosis (IA) and partial loss of β cells are important factors in the pathogenesis of DMT2 and FDM (7, 11). Islet amyloidosis is the result of a deposit of amyloid polypeptide derived from the islets (IAPP), which is co-secreted with insulin by pancreatic β cells. IAPP and insulin are co-regulated, and the production and secretion of both of them are upregulated by insulin resistance (7). IA in the FDM is associated with a loss of approximately 50% of β cells, whereas non-diabetic cats presenting IA show a lower degree of cell loss (7,10,12). It has been proposed that fibrillar forms of IAPP are cytotoxic and can trigger apoptosis, creating a potential link between the IA and the progressive loss of β cells in FDM and DMT2 (7,11).

Obesity and feline Diabetes Mellitus

The regulation of glucose metabolism in specific tissues such as muscle and adipose tissue has been identified as an important factor in insulin sensitiv-

ity. It is also known that the entry of glucose into the tissues is impaired by obesity causing insulin resistance (13,14). Obesity is associated with reversible insulin resistance. It produces changes in insulin secretion and also affects its action, either by alterations in insulin receptor or by post-receptor defects (15,16). The pattern of fat accumulation in obese individuals also affects the severity of insulin resistance. In humans, abdominal obesity is more associated with insulin resistance and risk of developing diabetes than peripheral obesity. Interestingly, the Burmese cat develops an accumulation of abdominal fat, unlike the domestic cat which presents accumulation of fat in the subcutaneous inguinal area (10). Despite these findings, in a study performed by nuclear magnetic resonance imaging it was found that obese cats presented abdominal fat equally distributed subcutaneously and intra-abdominally, suggesting that both may be involved in determining insulin sensitivity (17). In obese cats, the first phase of insulin secretion is significantly reduced or absent, while the second phase is increased compared to animals in their optimal weight (5,11,15).

A study in 34 obese and 14 cats with optimal weight showed that on average all obese cats presented glucose intolerance and abnormal insulin secretion after a high dose of glucose (1g/Kg of body weight). Furthermore, the obese group showed higher basal glucose levels compared to lean animals, although they remained within the normal range of plasmatic glucose (8).

It has been found that cats with diabetes are 6 times less sensitive to insulin than healthy cats (5,7,12). In a study where normal weight cats were allowed to reach obesity, it was observed a 52% decrease in peripheral insulin sensitivity. After losing weight the cats improved glucose tolerance. Other studies showed that cats with normal weight and normal glucose tolerance but with lower insulin sensitivity than the average population, increased 3 times the risk of developing glucose intolerance when gaining weight (3,5,18). This might suggest that there is a genetic predisposition to low insulin sensitivity, as demonstrated in the Burmese breed that in addition to other environmental factors (in this case obesity) leads to glucose intolerance. Other authors found that an increase in 1kg of body weight is associated with approximately a 30% of loss in insulin sensitivity and glucose effectiveness (17), concluding that body weight is an important factor in changes in insulin sensitivity. There are different theories that try to explain why obesity affects insulin secretion and which would be the factors that lead obese cats to reach the diabetic status. One hypothesis is that the hyperstimulation of β cells in the insulin-resistant status caused by obesity, promotes the development of IA, which would replace the functional β cells (19, 20). This way cats would loose the ability to control insulin resistance with compensatory hyperinsulinemia. In human it has been proposed that free fatty acids (FFA) play an important role in the impairment of β cell function (21,22); thus several studies have been performed trying to find the mechanisms by wich lipotoxicity could be involved (23,24). However, it has been concluded that the effects of FFA are influenced by concomitant glucose concentration and therefore, elevated FFA associated with normal glucose concentrations should not harm β cell (25, 26).

Cats that have been obese for a long period of time present dyslipidemia characterized by hypertriglyceridaemia associated with an increase in the very low density lipoprotein fraction (VLDL) and increases in plasmatic non-esterified fatty acids (27,28). Hoening (2002) (8) has proposed that non esterified fatty acids might be involved in defective insulin secretion in cats. Despite this study, it remains uncertain if lipotoxicity can harm β cell function in cats or if this effect is concomitant with hyperglycaemia, like in human. In a recent study the expression of glucose transporters GLUT1 and GLUT4 in muscle and adipose tissue in obese and normal weight cats was evaluated. Each animal was examined at the begining of the study (when they were lean) and after a period of 6 months, being fed ad libitum. The authors found that after weight gain GLUT1 expression was not affected, whereas GLUT4 expression decreased significantly in both muscle and adipose tissue (Figure 1) (6). This confirms that obesity affects insulin action by alterations in GLUT4 and supports the hypothesis that insulin resistance in obese cats is, at least in part, determined by a significant decrease in glucose transporters. The authors also noticed that this defect occurs even before the patients present glucose intolerance. Obesity and insulin resistance also have been related to alterations in adipokines and hormones, including leptin and adiponectin. Leptin is an important regulator of body fat. It decreases food intake, increases energy expenditure, stimulates lipolysis and inhibits lipogenesis (29,30). High leptin levels are most commonly associated with insensitivity of the leptin receptor and peripheral resistance (30). Deficiency and / or

leptin resistance in mice leads to polyphagia and decreased energy expenditure, therefore promoting obesity and insulin resistance (30). It has been postulated that there is an increase of 3 times in plasmatic leptin concentration as result of weight gain in cats (3). Furthermore, it has been found that leptin concentrations are significantly higher in obese cats than in lean ones; and that it decreases with weight loss (17).However, one study showed that in both obese cats and those with normal weight, high leptin levels were related to insulin resistance, independently of the degree of adiposity (3,31).

Adiponectin is synthesized exclusively by adipocytes both in human and cats (32,33). This citokyne enhances insulin sensitivity through different mechanisms, such as activation of AMP-activated protein kinase (AMPK) (34) and inhibition of essential gluconeogenesis enzymes (35). In addition, it increases fatty acid oxidation in skeletal muscle and liver (33, 35). Adiponectin is decreased in obese human and is associated with reduced ability of insulin to induce phosphorylation of tyrosine residues of its receptor, leading to insulin resistance (35,36,37). Recently, it has been found that plasmatic adiponectin concentration is significantly decreased in obese cats compared to normal weight cats (Figure 2) (32). This suggests that hipoadiponectinemia might be related to pathophysiology of insulin resistance and diabetes mellitus in obese cats, just like human. Hiperglucagonemia has been well studied in obesity and DBT2 and it is thought to be secondary to a reduction in insulin action in α cells. Glucagon concentrations are significantly increased in obese cats, and might be important in the progression from obesity to diabetes, since glucagon increases insulin resistance and can exacerbate the depletion of β cells (8). Finally, another factor to consider is the intolerance to carbohydrates in felines. High carbohydrate diets decrease insulin sensitivity and cause hyperinsulinemia compared to high protein diets (8). It has been suggested that cats, being strict carnivores adapted to diets high in protein and low in fat and carbohydrates, are inherently less sensitive to insulin and less able to handle high doses of carbohydrates than omnivorous species. It has been found that cats fed with commercial diets high in carbohydrates develop chronic hyperinsulinemia, increased demand of insulin and progressive destruction of the islets (15). On the other hand, it appears that diets high in protein decrease the required dose of insulin in diabetic cats (5,15). As it can be seen, there are several factors that have proved to be important in the development of diabetes mellitus in obese cats. However it remains to investigate in more depht the pathogenesis of insulin resistance and FDM in order to carry out new treatments and preventive options.

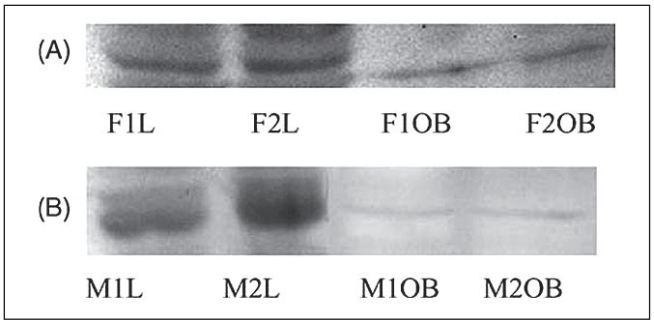

Figure 1: Representative Western blot picture of GLUT4 protein expression in fat (F; part A) and in muscle (M; part B) in a cat before (L) and after (Ob) a 6-month period of ad libitum food intake. (Taken from Brennan et al, 2004. Permission obtained from Dom Anim Endocr, Elsevier)

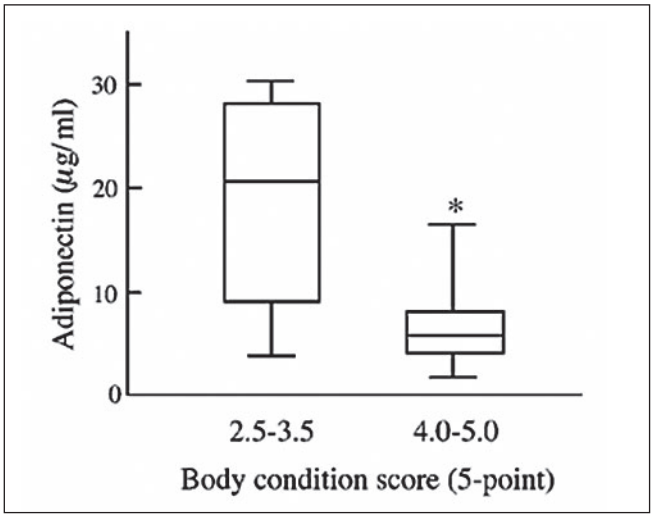

Figure 2: Plasma adiponectin concentrations in cats visiting the veterinary practice. Twenty-two cats were divided to 2 groups depending on their five-point scale body condition scores (BCSs). Normal, BCS=2.5–3.5 (n=11), obese, BCS=4.0–5.0 (n=11). The values are presented as boxplot chart. *, p=0.006 vs normal. (Taken from Ishioka et al, 2009. Permission obtained from Japanese Society of Veterinary Science)

Conclusions

The pathophysiology of FDM is multifactorial, as a consequence of both genetic and environmental factors .The clinical and physiopathological similarities between FDM and DMT2 makes the cat an important animal model to study this disease in hu-

mans; even more considering that obesity is a global problem that affects both the human population and their pets. Studies about insulin resistance and β cell dysfunction associated with obesity and adipokines have opened a great area of investigation. Taking into account this relationship, and until new therapeutic options are carried out, promoting physical activity and giving adequate diets to feline patients are key factors in the preventive treatment of FDM.

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PATOFIZIOLOGIJA DIABETESA MELLITUSA IN NJENA POVEZAVA Z DEBELOSTJO PRI MAČKAH

N.B. Rotlewicz, M.F. Gallelli, M.F.Cabrera Blatter, D.D. Miceli, V.A. Castillo

Povzetek: Diabetes mellitus je ena izmed najpogostejših endokrinopatij pri mačkah. Njena razširjenost v zadnjem času narašča in je povezana s značilnim povečanjem deleža predebelih mačk. Glavni dejavniki v patofiziologiji te bolezni so razvoj odpornosti proti inzulinu, slabo delovanje ali izguba celic β, nezadostno izločanje inzulina in amiloidoza (AI) otočkov. Občutljivost na inzulin je značilno zmanjšana pri predebelih pacientih, zato veliko avtorjev želi najti pri mačkah povezavo med debelostjo in diabetesem mellitusom (FDM). Proučevali so spremembe v prenosu glukoze, povečanje trigliceridov in maščobnih kislin, odlaganje amiloida v otočke in vpliv hormonov, kot so leptin in predvnetni citokini.

Ključne besede: mačja debelost; diabetes mellitus; mačji adiponectin; mačji leptin

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Article in proceedings of a meeting or symposium: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

NAVODILA AVTORJEM

Slovenski veterinarski zbornik (Slovenian Veterinary Research) objavlja izvirne prispevke, ki še niso bili objavljeni oz. poslani v objavo drugam. Za vse navedbe v prispevkih so odgovorni avtorji. Uredniška politika obsega publiciranje znanstvenih člankov, preglednih znanstvenih člankov, strokovnih člankov, povzetkov disertacij in drugih prispevkov, kot so kritične presoje o vsebini razprav, objavljenih v zborniku, kratke znanstvene prispevke, pisma uredniku in drugo. Avtorji pošljejo prispevke na naslov uredništva. Glavni urednik pregleda vse prispevke. Za vse članke je obvezna strokovna recenzija, za katero poskrbi uredništvo.

Prispevki naj bodo napisani v angleškem jeziku, z naslovom, povzetkom in ključnimi besedami tudi v slovenščini. Obsegajo naj največ 12 strani, kar pomeni 27 vrstic na stran s približno 75 znaki v vrstici. Prispevki naj bodo poslani v elektronski obliki v katerem koli urejevalniku besedil za okensko okolje. Zaželjena je uporaba elektronske pošte (slovetres@vf.uni-lj.si) in avtorji naj predlagajo tri možne recenzente. Besedilo naj ima dvojni razmik med vrsticami, pri čemer naj bodo vrstice na levi strani oštevilčene. Besedilo naj bo na levi strani od roba oddaljeno 4 cm.

Naslovna stran prispevkov se začne z naslovom, sledi ime in priimek avtorja. Kadar je avtorjev več, jih ločimo z vejicami. V naslednjih vrsticah je v rubriki Addresses of authors: za dvopičjem treba navesti polno ime in priimek ter naslov(e) avtorja(ev), tj. ustanovo, ulico s hišno številko, pošto in kraj. Vse navedene podatke ločujejo vejice. Sledi vrstica, kjer je treba navesti ime ter elektronski (E-mail:) in poštni naslov ter telefonsko številko (Phone:) odgovornega avtorja.

Sledi besedilo povzetka Summary v obsegu 200 do 300 besed. V naslednji rubriki Key words: se za dvopičjem navedejo ključne besede. Posamezne besede ali sklopi besed morajo biti ločeni s podpičjem.

Znanstveni članki in tisti, ki so prikaz lastnih raziskav in dognanj, morajo vsebovati še naslednje obvezne rubrike, s katerimi avtor sam naslovi ustrezne dele besedila v prispevku: Introduction, Material and methods, Results, Discussion in References. Pregledni članki naj vsebujejo uvod, poglavja, ki so glede na vsebino smiselno naslovljena, in literaturo. Podatke o financerjih ali drugih zadevah, pomembnih za prispevek, npr. o tehnični pomoči, avtorji navedejo v rubriki Acknowledgements, ki se uvrsti pred rubriko References. Za rubriko References sledijo spremna besedila k slikam.

Priloge, kot so tabele, grafikoni in diagrami naj bodo smiselno vključene v besedilo. Slikovni material naj bo poslan posebej v obliki bmp, jpg, ali tif.

Priloge in slike morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. Vsi navedki (reference), citirani v besedilu, se morajo nanašati na seznam literature. V besedilu jih je treba oštevilčiti po vrstnem redu, po katerem se pojavljajo, z arabskimi številkami v oklepaju. Prvi navedek v besedilu opredeli številko oziroma vrstni red ustreznega vira v seznamu literature. Če se avtor v besedilu ponovno sklicuje na že uporabljeni vir, navede tisto številko, ki jo je vir dobil pri prvem navedku. Citirana so lahko le dela, ki so tiskana ali kako drugače razmnožena in dostopna javnosti. Neobjavljeni podatki, neobjavljena predavanja, osebna sporočila in podobno naj bodo omenjeni v navedkih ali opombah na koncu tiste strani, kjer so navedeni. V seznamu literature so viri urejeni po vrstnem redu. Če je citirani vir napisalo šest ali manj avtorjev, je treba navesti vse; pri sedmih ali več avtorjih se navedejo prvi trije in doda et al.

Da bi se morebitni popravki lahko objavili v naslednji številki, jih morajo avtorji pravočasno sporočiti glavnemu uredniku.

Načini citiranja

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