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Morphological and immunohistochemical characterization of alterations induced by doxorubicin and the chronological course of recovering of various tissues in rabbits

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Abstract

Alterations caused by doxorubicin (DOX) in rabbits and their course of recovering was characterized morphologically and immunohistologically. After the 2nd DOX dose lymphatic organs were markedly hypocellular, testes and skin were atrophic, and myocardial degeneration occurred. Two weeks after the last DOX dose hematopoetic organs appeared to be hyperplastic. After six weeks hematopoetic tissues and testes were normal, whereas the skin was still atrophic. Myocardial fibrosis, as well as renal cysts and fibrosis developed. In conclusion, using doxorubicin the different reaction patterns of the various organs should consider forensic aspects (i.e. potentia generandi) and careful interpretation of experimental results.

Key words: doxorubicin, toxicity, histopathology, immunohistochemistry, rabbit

Introduction

Doxorubicin (adriamycin), an anthracycline antibiotic, is used in chemotherapy of various neoplasia. The side-effect cardiotoxicity (Suzuki et al. 1997) is used as an experimental model in different species. However, other organs (i.e. hematopoetic tissues, skin, kidneys, intestine) are affected by anthracyclines, too (Van Vleet et al. 1979, Maral and Jouanne 1981). The aim of this study was the morphological and immunohistological characterization of the alterations caused by doxorubicin (DOX) in rabbits and the chronological course of recovering (regeneration or repair respectively) after treatment in various organs.

Materials and Methods

Thirty four 3 months old, male white New Zealand rabbits were treated intravenously with 3 mg/kg doxorubicin hydrochloride (Ribodoxo®-L 50) once a week for six weeks. All procedures were done in accordance with the “Principles of laboratory animal care” (1985). Nine rabbits died during the treatment period after the 1st (n=1), 2nd (n=1), 3rd (n=3) or 2 days after the 6th (n=4) DOX dose. Seven animals died two weeks after the last (6th) DOX dose. Twelve rabbits were anesthetized (isoflurane) and euthanized by explantation of the heart six weeks after the last DOX administration (day 84). Six rabbits served as untreated controls.

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Specimens of the heart, bone marrow, spleen, intestinal lymph nodes, skin, testes and all major organs were fixed in 4% formalin, embedded in paraplast and stained with hematoxylin and eosin, and Picro-Sirius-Red stained slides were additionally examined by polarized light (Grüninger 1996). Immunohistochemistry was performed to detect PCNA (proliferating cellular nuclear antigen), CD79 (B-lymphocytes), MAC 387 (macrophages). Results were evaluated semiquantitatively.

**Results**

**Skin.** After two weeks most of the rabbits developed alopecia at the neck and in two cases perinasal and periocular, too. The skin at the neck showed histopathologically moderate orthokeratotic hyperkeratosis and severe epidermal and adnexal atrophy. The proliferation marker PCNA was detected only in single epidermal and adnexal cells. After the end of treatment increasing numbers of adnexal cells but only few epidermal cells expressed PCNA. Six weeks after the last DOX dose only in 5 of 12 animals the epidermis appeared to be normal compared to the controls. Seven rabbits still showed skin atrophy with mild adnexal PCNA expression.

**Testes.** After 2 applications of DOX the testes showed signs of increasing atrophy resulting in tubuli seminiferi mainly consisting of mildly vacuolized Sertoli cells and only single remaining PCNA expressing spermatogonia. Testes were still atrophic 2 weeks after the last DOX dose, but after four more weeks in 8 of 12 rabbits normal active spermatogenesis was observed. Three rabbits showed signs of beginning testicular regeneration, while the testes of one animal were still completely inactive and atrophic.

**Spleen, intestinal lymph nodes.** After 2nd DOX administration spleen and lymph nodes showed marked depletion of lymphoid cells. B-lymphocyte follicles (CD79 +) were small and the number of MAC 387 positive cells (macrophages) and the proliferation activity (PCNA) were diminished. Two weeks after the last DOX dose lymphatic follicles were moderately hyperplastic and the number of MAC 387 positive cells increased mildly. Four weeks later spleen and lymph nodes appeared to be normal in all treated animals compared to the controls.

**Bone marrow.** Already in the rabbit which died after the first DOX dose severe hypoplasia of the bone marrow had developed. Three rabbits showed signs of hemorrhagic diathesis (bleedings in the skin, gastrointestinal tract, retrobulbar hematoma) after the 2nd DOX dose. Two weeks after the last DOX dose hematopoiesis was active again and bone marrow appeared to be mildly hypercellular compared to the findings 4 weeks later. The number of MAC 387 positive cells increased markedly after the end of treatment.

**Heart.** In rabbits, which died during treatment hydrothorax (n=7), hydropericard (n=6) as well as ascites (n=6) were observed. The cardiac ventricles of DOX treated animals were markedly dilated. After the 2nd DOX dose histopathologically signs of a diffuse mild eosinophilic degeneration, loss of cross striation and/or vacuolar degeneration occurred. Later, metabolic active fibroblasts building collagen fibres appeared and few lymphocytes and macrophages were scattered in the interstitium. Six weeks after the last application mild diffuse myocardial fibrosis but only single inflammatory cells were observed. Examination of Picro-Sirius-Red stained slides in polarized light revealed mainly yellow lightening collagen fibres which can be interpreted as collagen I according to Grüninger (1996).

**Intestine.** Moderate diarrhea accompanied by a moderate mucosal atrophy and mild lympho-plasmacellular enteritis were the main findings in four animals which died during the treatment period.

**Kidneys.** No gross lesions of the kidneys were seen. However, after the 3rd DOX dose multiple mildly dilated tubules appeared in the inner cortex. Six weeks after the last treatment numerous tubular cysts, lined by atrophic epithelial cells and surrounded by mild (n=4) to severe (n=8) interstitial fibrosis had developed. In polarized light Picro-Sirius-Red stained slides showed that fibrotic areas mainly consisted of collagen I fibres.

**Lungs, liver, brain, skeletal muscles, bones** were normal in all treated and control animals.

**Discussion**

Cardiomyopathy induced by anthracyclines is a well described side effect which is often used in experimental studies. However, the effects on other organs are described sparsely (Van Vleet and Ferrans 1980, Suzuki et al. 1997) but may be responsible for the death of numerous animals during the experiments because of dermatitis, diarrhea, hemorrhagic diathesis and opportunistic infections. In our study bone marrow, spleen and lymph nodes showed marked hypocellularity after the 2nd DOX dose corresponding to Van Vleet et al. (1979), but already two weeks after the last DOX dose the immune system appeared to be very active again, especially B-lymphocytes and macrophages were proliferating. Testicular atrophy resulted from DOX by the induction of apoptosis of germinal cells (Shinoda et al. 1999) and a decreased proliferation activity (PCNA expression). We showed that regeneration of the testes was possible, but it took about 6 weeks. In contrast, corresponding to the findings of Maral und Jouanne
(1981), the skin was still atrophic six weeks after the last DOX dose in most cases. The proliferation activity (PCNA expression) reappeared first in the adnexal epithelia but was retarded in the basal layer of the epidermis. Alterations in the heart and kidneys were in accordance to those described in the literature (Fajardo et al. 1980, Suzuki et al. 1997). In contrast to regenerative tissue the reparative processes leading to fibrosis were finished in full extent after six weeks. In both organs predominantly collagen fibres type I were produced.

In conclusion, using doxorubicin the markedly different reaction patterns and regeneration time of the various organs should be considered. These results may be important in the view of forensic aspects (i.e. potentia generandi), in further therapy consequences (i.e. cardiac or renal insufficiency) and in the interpretation of experimental results. It seems likely, that DOX may influence pathogenetic pathways by the modified expression of growth factors and inflammatory reactions during immune suppression and following reactivation.

References

Historopathological pattern of alimentary tract of pigs fed with fish and rape oil supplemented feed

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Abstract

The effect of 3 and 6% of fish oil and 3% of rape oil addition to feed on histopathological pattern of the porcine alimentary tract was studied. The lesions were observed in all the animals. The most desirable alimentary tract pattern was observed in the pigs nourished with feed containing 3% of fish oil, and those fed with addition of 6% fish oil up to the slaughter revealed the greatest number of undesirable lesions.

Key words: pig, histopathological examination, alimentary tract, fish oil, rape oil

Introduction

Because of the fatty acids profile, especially Essential Fatty Acids (EFA), including DHA and EPA, some plant and fish oils are popular in swine feeding. A wide-scale study on fat in a human diet showed that the consumption of fats rich in saturated fatty acids should be limited to 30 – 35% of daily energy intake (Grys 1998, Ziemlański 1998). Profitable influence of diet rich in fish oil on human and animal health was simultaneously observed. Fish fat differs from animal fat and vegetable oils in the composition of fatty acids and the concentration of EPA and DHA n-3 family (Kolakowska and Kolakowski 2001).

It has been repeatedly shown that a diet deficient in essential unsaturated acids or undesirable n-3 to n-6 acid ratio leads to disturbances in metabolism which, in consequence, may be related to diseases of blood circulation, nervous system, skin neoplasma, immune system disturbances or vision problems (Cichon 1998, Pike 1999, Kolanowski 2000).

The significance and activity of eicosanoids in prophylaxis and therapy of many diseases were the subjects of many studies but there is not much information about the influence of PUFA on alimentary tract condition.

The aim of this study was to determine the effect of a 3 and 6% supplement of fish oil and a 3% supplement of rape oil with or without the carency on the histopathological pattern of alimentary tract in pig.

Materials and Methods

The experiment was carried out with the use of fish and rape oil. Fish oil originated from the drip formed
during fish heat treatment prior to tinning and from the fish meal production, specifically from fish waste and non-consumable fish. The fish oil was preserved with liquid Rendox anti-oxidant (1 kg/t). Refined consumable rape oil under the brand name “Uniwersalny” was purchased in retail. The oil samples were analysed for odour, consistency, acidity number, peroxide number, water content, petroleum benzine insoluble compounds and protein content.

The qualitative and quantitative analyses of the fatty acids were carried out using the Peisker method (1964) in a gas chromatograph – PYE Unicam – series 104 with a flame-ionizing detector (FLD).

The experiment was carried out on 24 crossbred fattened pigs (F1: WBP and PBZ sows with Duroc and Pietrain boars) that weighted approximately 60 kg. The animals were kept in individual non bedding grating pens. They were divided into four groups: fattening pigs from control group (K) – were fed without oil supplements, pigs from the first experimental group (D1) – with 3% supplement of refined rape oil (“Uniwersalny”), in the second experimental group (D2) – with 3% supplement of fish oil and in the third experimental group (D3) – with 6% supplement of fish oil. The animals were fed for 30 days with balanced feed produced in the industrial feed factory. Oils applied were mechanically mixed with feed directly before animal feeding in order to obtain homogenous consistency. Ten days prior to slaughter, the D1, D2 and D3 pig groups were divided into two sub-groups. The pigs from groups D1a, D2a and D3a were fed oil-free feed, whereas the diet of the rest of the animals remained unchanged until the slaughter. After the slaughter, the segments of the stomach, duodenum, jejunum and colon were sampled for histopathological analyses. They were preserved in 10% neutralised formalin and embedded into the paraffin blocks. Microtome sections were stained with haematoxylin and eosin (HE) and with the PAS method according to McManus.

### Results and Discussion

The studied fish and rape oils, in spite of their different composition, fully met the qualitative requirements for the first choice feed fats (Table 1). Fish oil contained a wider composition of fatty acids, which makes it more biologically attractive in comparison with rape oil (Table 2). See fish oil are more biologically attractive since besides n-6 linoleic acid (LA) (C18:2) and n-3 α-linolenic acid (α-LNA) (C18:3), it also contains other specific metabolites such as n-3 eicosapentaenoic acid (EPA) (C20:5) and n-3 docosahexaenoic (DHA) (C22:6) which do not occur in rape oil (Cichon 1998, Ziemlański 1998, Kołakowska and Kołakowski 2001).

The results of the histological analysis of parts of the alimentary tract are given in Table 3. Stomach hyperaemia was found in one of the animals that were fed with rape oil supplemented feed (group D1a) and in all the animals in group D1. Hyperaemia of the mucose membrane was observed in the duodenum in groups K and D1. Jejunum hyperaemia occurred in groups K, D1 and D1a (rape oil supplement) as well as in groups D3a and D3 (fed with the 6% fish oil supplemented feed). Histiocytal infiltrations occurred in the tunica mucosa of the duodenum and jejunum only in a few pigs of group K. Also in group K, eosinophil cell infiltration was found in mucose membrane of the stomach, duodenum and jejunum. Similar infiltrations in jejunum were observed in all the animal groups, however, in groups D1a and D1 they were only present in some animals. Lymphocytal infiltration occurred in all the examined parts of the alimentary tract in animals representing the control group and groups D3a and D3. Enlargement of the lymphatic follicle was observed mainly in the wall of the stomach in group K. The excess of mucous and exfoliation of epithelium were found in almost all groups, especially in the jejunum and stomach, except from the groups D2 end K and also in the duodenum and colon, except from the group K.

The histiocytal and lymphocytal infiltrations in the wall of the duodenum and jejunum correspond to the deformation and the atrophy of the villi, especially in the control group and in the animal group fed with 6% fish oil supplemented feed. Necrosis of the peak part of the duodenum villi occurred in a few animals from group K and D1. This lesion was also observed in the jejunum in one case from group D2. Microscopic examination also showed different organ lesions, which were found mainly in the control group and in the group fed 6% fish oil supplemented feed. The results obtained from animals fed with 3% fish or rape oil supplement could suggest its beneficial effect.

Table 1. Chemical analysis of the oils used in the experiment (according to the PN-R-64806:1997 norm).

<table>
<thead>
<tr>
<th>Specification</th>
<th>Fish oil</th>
<th>Rape oil</th>
<th>Permissible value for feed fats of 1st choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content and volatile compounds</td>
<td>0.55</td>
<td>0.04</td>
<td>up to 1.5</td>
</tr>
<tr>
<td>Naphthyl ether insoluble substances</td>
<td>0.05</td>
<td>0.02</td>
<td>up to 5</td>
</tr>
<tr>
<td>Acid number as KOH/g</td>
<td>4.70</td>
<td>0.12</td>
<td>up to 50</td>
</tr>
<tr>
<td>Peroxide number meq O₂/kg</td>
<td>4.14</td>
<td>1.03</td>
<td>up to 10</td>
</tr>
<tr>
<td>Protein content</td>
<td>0</td>
<td>0</td>
<td>up to 2</td>
</tr>
<tr>
<td>Energy MJ/kg</td>
<td>43.33</td>
<td>41.73</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 2. The composition of the fatty acids of the oils used for the experiment.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>SFA</th>
<th>MUFA</th>
<th>PUFA</th>
<th>PUFA n-6</th>
<th>EFA n-3</th>
<th>EPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish oil</td>
<td>23.59</td>
<td>44.35</td>
<td>32.06</td>
<td>8.72</td>
<td>16.78</td>
<td>6.60</td>
<td>12.34</td>
</tr>
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<td>Rape oil</td>
<td>34.16</td>
<td>32.98</td>
<td>32.00</td>
<td>29.77</td>
<td>3.02</td>
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Table 3. Microscopic lesions in the pigs alimentary tract.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>K n=4</th>
<th>D1 n=3</th>
<th>D1a n=3</th>
<th>D2 n=3</th>
<th>D2a n=3</th>
<th>D3 n=4</th>
<th>D3a n=4</th>
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<td>3</td>
<td>1</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>duodenum</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>jejunum</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>1</td>
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<tr>
<td>colon</td>
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<td>2</td>
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<tr>
<td>deformation and atrophy of villi</td>
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Explanation:

a) K – control group, D1a, D2a, D3a– the experimental animal groups with 10 day waiting period, D1, D2, D3 – animal groups fed with oil supplemented feed until the day of slaughter, D1 and D1a – 3% rape oil supplement, D2 and D2a – 3% fish oil supplement, D3 and D3a – 6% fish oil supplement

b) the number of animals with lesions is given in columns
on the condition of the alimentary tract of the animals examined.

The most desirable therapeutic results (the lowest number of histopathological lesions in the examined parts of the alimentary tract) were obtained in the animal group fed with 3% fish oil supplemented feed.

Acknowledgments

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References


Nitric oxide synthase (NOS) in dorsal root ganglia neurons associated with the porcine IMG: occurrence and reaction to mechanical injury

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Abstract

The present study was aimed at disclosing the distribution, morphological features and nerve lesion-induced plasticity of NOS-immunoreactive sensory neurons associated with the porcine IMG by means of combined retrograde tract-tracing using Fast Blue (FB) and double-immunofluorescence. The vast majority (up to 90%) of FB-labelled, IMG-projecting afferent neurons were located in L2 and L3 DRGs with the rest dispersed in Th14 to L5 DRGs. NOS-immunoreactivity was found in a small subset of DRG cells (approximately 2.5%), being predominantly confined to the medium- and small-sized perikarya. No changes in the number of FB-labelled neurons expressing NOS after nerve lesion were observed.

Key words: sensory neurons, inferior mesenteric ganglion, dorsal root ganglia, nitric oxide synthase (NOS), neuronal plasticity, pig

Introduction

It has been suggested that DRG neurons may influence the activity of postganglionic sympathetic neurons including those found in IMG, in both direct (through their dendritic arborizations within the ganglia), as well as indirect way (i.e. through axonal collaterals forming synaptic contacts with either preganglionic sympathetic neurons or interneurons within the dorsal horn; for review, see Majewski 2000). Thus, as NO, a short-living, gaseous messenger molecule has recently been shown to be widely used by mammalian sensory and autonomic neurons (for references, see Lukáčová et al. 2003), in the present study, we decided to elucidate the distribution pattern of NOS-IR DRG neurons associated with IMG in the pig. Moreover, as nerve lesion has in the recent past been shown to induce a strong upregulation of nNOS in DRG neurons in rats (for references, see Bergman et al. 1999) and monkey (Zhang et al. 1993), thought to represent adaptive responses serving to reduce the deleterious effects of the peripheral nerve damage and to promote survival and regeneration of the lesioned sensory pathways, we have also investigated putative changes in the expression pattern of NOS in DRG neurons supplying the porcine IMG after nerve injury.
Materials and Methods

Twelve female piglets (8 weeks old), divided into two groups (n=6 each): control animals (CA) and animals undergoing right IMG ganglionectomy (GA group) three weeks after FB application, were injected with 5% aqueous solution of FB into the right IMG. Seven days after the ganglionectomy (performed in GA group), animals of both groups were sacrificed by an overdose of sodium pentobarbital (Vetbutal®, Biowet, PL). Ten µm thick cryostat serial sections of Th₆ – S₂ DRGs were subjected to routine single-labeling immunofluorescence using serum raised in mouse and directed to NOS (1:1500, Sigma, PL). Sections were then incubated with goat anti-mouse IgG conjugated to biotin (DAKO, Dk) and afterwards, visualized by a streptavidin-CY3 complex (Jackson, USA). Retrogradely labeled/single-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each fifth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Moreover, the size of the neurons studied was measured. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Fig. 1. DRG L₁, control pig. Retrogradely labeled NOS-IR neurons. x 200.
Fig. 2. DRG L₁, axotomized pig. The number of FB+/NOS-IR neurons appears unchanged. x 200.

Results and Discussion

FB+ afferent neurons were found in Th₁₅ to L₅ DRGs. As the vast majority of these perikarya (approximately 90% of all retrogradely labeled cells) were located in L₂ and L₃ DRGs, the present data are based on the results obtained from sections of L₂ and L₃ DRGs considering all counted FB+ neurons as 100% population. NOS was present in 2.5 ± 1.2% of all FB-positive sensory neurons. The vast majority (63.5 ± 10.3%) of NOS-IR, IMG-projecting neurons belonged to the class of medium-sized cells. Neurons
with a small diameter constituted 34.9 ± 9.3%, while large NOS-IR, FB+ sensory neurons were found only occasionally (1.5 ± 1.5%). Changes in the number of NOS-IR cells (4.0 ± 0.2%) induced by mechanical injury of the peripheral processes of FB-positive neurons were statistically not significant when compared to CA group. Previously, the presence of NOS has been reported in DRG neurons of humans (Terenghi et al. 1993) and many others mammalian species (for references, see Lukáčová et al. 2003), including the pig (Majewski et al. 1995). The present results are in agreement with those mentioned above. It was shown that sciatic nerve ligature induced an increase in the number of NOS-IR DRG neurons in rats (Cizková et al. 2002) and rabbits (Lukáčová et al. 2003), but the same injury induced a distinct decrease in the number of NOS-IR cells in monkey DRGs (Zhang et al. 1993). Quite recently, Renganathan et al. (2000) demonstrated that NO, implicated to be an autocrine regulator of neuronal survival, has been up-regulated predominantly in small-sized c-type neurons. As the vast majority of NOS-IR afferent neurons supplying porcine IMG were of medium size, the lack of injury-induced changes in the number of studied cells implicates that NO is not involved in the survival mechanisms of this subset of porcine afferent neurons.

Acknowledgements

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References


Proliferative enteropathy (PE)-induced changes in the expression and co-incidence patterns of SP and/or CGRP in DRG neurons supplying the porcine descending colon

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Abstract

This study aimed at disclosing the PE influence on the expression of SP and/or CGRP in porcine DRG neurons supplying descending colon. In healthy pigs, SP was found in 37.8 ± 0.9% and CGRP in 54.3 ± 2.4% of all FB\(^+\) neurons and co-localization of them was observed in 32.3 ± 2.3% of FB\(^+\) cells. PE induced an increase in the number of SP-IR perikarya (till 59.2 ± 1.3%), while the number of CGRP-IR ones decreased (till 36.8 ± 0.7%). 22.2 ± 4.3% of FB\(^+\) cells were SP/CGRP-IR. These results are contradictory to data concerning the axotomy- and chemical inflammation-evoked changes in the expression of SP and CGRP in porcine DRG neurons.

Key words: proliferative enteropathy, neuronal plasticity, substance P (SP), calcitonin gene-related peptide (CGRP), dorsal root ganglia neurons, pig

Introduction

In addition to intrinsic primary afferent neurons, existence of which within the bowel wall has recently been unequivocally demonstrated (Furness et al. 1998), there are also extrinsic (spinal) primary afferent neurons (Berthoud et al. 2004) involved in the neural control of enteric functions, mediating both innocuous and noxious sensations. Recently, it has been found that porcine spinal primary sensory neurons respond to transection of their peripheral processes (Bossowska et al. 2004) or to chemical injury (chemically-evoked inflammation of the bowel wall; Bossowska et al. 2003) with profound changes in the synthesis rate and expression pattern of SP and CGRP. As some of these neurons may synthesize and utilize SP and/or CGRP as their messenger molecules released during “axon-reflexes” (Cooke 1998) and, furthermore, there is evidence that both substances may evoke slow EPSP in “second order” neurons involved in the intestinal secretion (Pan and Gershon 2000, Cooke 1998), it may be of interest to investigate putative changes in the expression pattern of both substances in extrinsic primary afferent neurons challenged by \textit{Lawsonia intracellularis}-infection.
Materials and Methods

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically diagnosed *Lawsonia intracellularis* infection (PE group) were used. All animals were subjected to laparotomy and injected with 5% aqueous solution of Fast Blue (FB) into the wall of the descending colon. After a three-week survival period, the animals were sacrificed with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland) and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Ten µm thick cryostat sections of the L1 – L3 and S3 DRG were subjected to routine double-labelling immunofluorescence using combinations of antisera raised in different species and directed towards SP (rat monoclonal) and CGRP (rabbit polyclonal). Anti-SP serum was visualized by species-specific secondary antiserum conjugated to FITC, while the rabbit anti-CGRP antibody was labeled with biotinylated donkey anti-rabbit IgG (both from Jackson Immunochemicals, USA). The latter antibody was then visualized by a streptavidin-CY3 complex (Jackson). Retrogradely labeled/double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each fifth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results and Discussion

Retrograde tract-tracing revealed that the vast majority (more than 80%) of descending colon-projecting extrinsic primary afferent neurons was present in

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Fig. 1. Control DRG; FB+ neuron (a) exhibit co-localized SP (b) and CGRP-IR (c). x 200.
Fig. 2. PE-affected DRG; SP (b) and CGRP (c) in a medium-sized, FB+ neuron (a). x 400.
Fig. 3. PE-affected DRG; large, retrogradely labeled perikaryon (a) contained both SP (b) and CGRP (c). x 200.
porcine $L_1-L_3$ and $S_3$ DRGs. The prevailing number of the neurons belonged to the class of small- and medium-sized nerve cells (Fig. 1). By means of double-immunofluorescence it has been found that in healthy animals, SP was found in $37.8 \pm 0.9\%$ of all $FB^+$ neurons, while CGRP was encountered in $54.3 \pm 2.4\%$ of retrogradely labeled cells. Co-localization of both substances was observed in $32.3 \pm 2.3\%$ of $FB^+$ cells (Fig. 1). PE induced an increase in the number of SP-IR perikarya (till $59.2 \pm 1.3\%$), while the number of CGRP-IR ones simultaneously decreased (till $36.8 \pm 0.7\%$). In contrast to control animals, only $22.2 \pm 4.3\%$ of $FB^+$ cells still exhibited co-localized SP and CGRP; however, these cells belonged to the class of medium-sized (Fig. 2) and large perikarya (Fig. 3). Results of the present study are thus contradictory to data concerning the mechanical injury – (a decrease in the number of SP-IR neurons and an increase in the number of CGRP-IR ones have been observed; Bossowska et al. 2004) and acute chemical inflammation-evoked changes (both substances were dramatically up-regulated; Bossowska et al. 2003) in the expression pattern of SP and CGRP in porcine descending colon-projecting DRG neurons. Therefore, the results of the present study suggest the activation of an adaptive mechanism(s) of porcine sensory neurons affected by PE that were different from those induced by transection of their peripheral processes or acute inflammation of their target tissue. Furthermore, assuming that SP is able to evoke slow EPSP also in porcine submucous (both outer and/or inner) plexus neurons, this tachykinin, released from activated extrinsic primary afferent c-fibres in an antidromic manner, may be implicated, together with SP released from intrinsic Dogiel type II primary afferent neurons (Brown and Timmermans 2004), to influence the electrolyte secretion in PE-affected colon.

**Acknowledgements**

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**References**


Influence of proliferative enteropathy on expression pattern of somatostatin (SOM) and galanin (GAL) in extrinsic primary afferent neurons supplying the porcine descending colon

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Abstract

The present study was aimed, by immunofluorescence and retrograde tracing at disclosing changes in the number of GAL- and/or SOM-IR afferent neurons resulting from a chronic, proliferative inflammation of the porcine descending colon. In intact DRGs, GAL was found in 1.5 ± 0.3% of all traced perikarya, while SOM was expressed by approximately 0.6 ± 0.1% of these cells. Within the PE-affected DRGs, the number of GAL- or SOM-IR neurons slightly increased, reaching 7.3 ± 0.6% and 0.8 ± 0.1% of all traced afferent cells, respectively. These data suggest an involvement of GAL, but not SOM, in adaptive events in DRG neurons afflicted by chronic inflammatory process.

Key words: proliferative enteropathy, neuronal plasticity, somatostatin, galanin, dorsal root ganglia neurons, pig

Introduction

In addition to IPANs (intrinsic primary afferent neurons, located within the bowel wall; for a review, see Clerc and Furness 2004), sensory modalities from the intestine are also transmitted by quite numerous EPANs (extrinsic primary afferent neurons located within the dorsal root ganglia or nodose/jugulare ganglion complex, a sensory component of the vagus; for a most recent data, see Berthoud et al. 2004). In the pig, EPANs connected with the descending colon were found in Th13 – L4 and S3 – S4 DRGs, being the most numerous in the L1 – L3 and S3 ganglia (Bossowska et al. 2003a). Furthermore, as it has been shown in the recent past, the transection of the peripheral processes of afferent neurons supplying this segment of the porcine gut (Bossowska et al. 2004), or an acute inflammation of their target tissues (Bossowska et al. 2003b), lead to profound changes in the expression pattern of GAL or SOM in retrogradely labeled perikarya. However, there is no data dealing with putative changes in the chemical coding of distal bowel-projecting EPANs, induced by a chronic inflammation. Therefore, the present study was aimed...
at disclosing supposed changes in the number of GAL- and/or SOM-immunoreactive (SOM-IR) afferent neurons, evoked by *Lawsonia intracellularis* infection, resulting in a chronic, proliferative inflammation of the bowel segment of interest.

**Materials and Methods**

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically diagnosed *Lawsonia intracellularis* infection (PE
group) were used. All animals were subjected to laparotomy and injected with 5% aqueous solution of Fast Blue (FB) into the wall of the descending colon. After a three-week survival period, the animals were sacrificed with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland; 90 mg/kg b.w.) and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Ten µm thick cryostat serial sections of the DRGs were subjected to routine double-labelling immunofluorescence using combinations of antisera raised in different species and directed towards GAL (rabbit polyclonal, 1:16000, Bachem, CH), and SOM (rat monoclonal, 1:350, Affinity, UK). Primary antisera were visualized by rat-specific secondary antisera conjugated to FITC or rabbit-specific antibodies conjugated to biotin (all from Jackson Immunochimicals, USA). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson). Retrogradely labeled/double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each fifth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results and Discussion

In intact L1–L3 and S3 DRG (the ganglia at these levels contained more than 80% of all retrogradely labeled, descending colon-projecting afferent neurons), GAL was found in 1.5 ± 0.3% of all traced perikarya (Fig. 1), while SOM was expressed by approximately 0.6 ± 0.1% of these cells (Fig. 2). Both substances were present in totally different populations of EPANs: while virtually all GAL-IR perikarya belonged to the class of small DRG neurons, immunoreactivity of SOM was confined to medium-sized perikarya. Within the DRGs collected from proliferative enteropathy-suffering animals, the number of GAL- or SOM-IR neurons slightly increased, reaching 7.3 ± 0.6% and 0.8 ± 0.1% of all traced EPANs, respectively (Figs 3, 4). This run I counter to the results obtained from animals with chemically induced acute inflammation, where GAL- and SOM-IR neurons constituted 55.8 ± 2.7% and 0.9 ± 0.3% of affected neurons, respectively (Bossowska et al. 2003b). Furthermore, data obtained in the present study are also contradictory to results of experiment based on transsection of the nervi colici caudales comprising peripheral processes of EPANs: it has been shown that this kind of mechanical injury elevated the number of GAL-IR cells up to 53.8 ± 1.5% of all traced perikarya, while only 1.13 ± 0.4% of colon-projecting afferent neurons expressed SOM immunoreactivity (Bossowska et al. 2004). Thus, the present results suggest an involvement of GAL in repair mechanism(s) of EPANs afflicted by bacterial inflammation; furthermore, the relatively low increase in the number of GAL-IR cells observed in PE-affected DRG may be a sign of an exhaustion of compensative potential of these cells during chronic challenge.

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References


In vitro effects of leukocidin LukE/LukD on the rabbit immunocompetent cells

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Abstract

Staphylococcus aureus bacteria produces leukocidins – biomponent toxins with destructive influence on mammalian leukocytes. The purpose of the studies was to determine the in vitro effects of leukocidin LukE/LukD on the phagocyte activity and proliferative ability of lymphocytes isolated from rabbit blood. The results have shown the modulatory influence of the toxin on the immunocompetent cells activity. Cytolytic concentrations of leukocidin induced immunosuppression of metabolic and potential killing activity of phagocytes. Subcytolytic concentrations of leukocidin caused stimulation of phagocyte activities. No modulatory influence on the proliferative ability of T and B lymphocytes was observed.

Key words: staphylococcal leukocidin, rabbit, phagocyte activity, lymphocyte proliferation

Introduction

Leukocidins (Luk) are bicomponent pore forming exotoxins produced by Staphylococcus aureus bacteria. The toxins are water soluble and consist of two protein subunits of S and F class. The up-to-date studies indicate that gamma-hemolysins exhibit leukotoxic and hemolytic effects, leukocidins have destructive influence on polymorphonuclear leukocytes, monocytes and macrophages isolated from higher vertebrates (Pedelacq et al. 1999). A few studies on the effect of leukocidins on leukocytes isolated from higher vertebrates were performed but there is little data on comparative in vitro studies on the influence of leukocidin LukE/LukD on rabbit lymphocytes. The purpose of the in vitro study was to determine the influence of leukocidin LukE/LukD at different concentrations on the metabolic activity (RBA) and potential killing activity (PKA) of polymorphonuclear (PMN) and mononuclear (MN) cells and proliferative ability of T and B lymphocytes isolated from rabbit blood.

Materials and Methods

Leukocidin LukE+LukD was added at concentrations 25000, 5000, 1000, 200, 40, 8, 1.6, 0.32 ng/ml RPMI 1640 medium and incubated 30 min. with the prepared lymphocyte suspensions. The metabolic activity (RBA) of blood PMN and MN cells described by Chung and Secombes (1988) and potential killing activity (PKA) of PMN and MN cells described by Rook et al. (1995) were measured. The blood T (stimulated by concanavalin A) and B (stimulated...
by bacterial lipopolysaccharide LPS) lymphocyte proliferation was determined by MTT assay (Mosmann 1983). The data was analysed statistically using program Statistica 5.0. P<0.05 was assumed as significant. Means and standard deviations are presented in the figures (n=10).

**Results**

The studies have shown the modulatory influence of leukocidin LukE/LukD on the activity of phagocytes isolated from rabbit blood. The highest suppression of RBA and PKA of PMN and MN cells was observed at concentrations 25000, 5000, 1000 and 200 ng/ml of the toxin (Fig. 1). Slight but statistically significant stimulation of phagocytic RBA and PKA was seen at subcytolytic concentrations (1.6, 0.32 ng/ml) of the toxin LukE/LukD. No modulatory influence of leukocidin LukE/LukD on proliferative ability of T and B lymphocytes was observed at any concentration used in the experiment (Fig. 2.).

![Fig. 1. In vitro influence of leukocidin LukE/LukD on metabolic activity (RBA) and potential killing activity (PKA) of PMN and MN cells isolated form rabbit blood (*Oryctolagus cuniculus*) (mean + SD, p<0.05, n=10).](image1)

![Fig. 2. In vitro influence of leukocidin LukE/LukD on proliferative ability of T and B lymphocytes isolated from rabbit blood (*Oryctolagus cuniculus*) (mean + SD, p<0.05, n=10).](image2)

Little is known on the in vivo local effects and general role of bicomponent toxins produced by *Staphylococcus aureus* in the pathogenesis of fish and mammalian diseases. Frequent isolation of different strains secreting leukocidins from mammalian necrotic lesions and abscesses suggests that these bicomponent toxins could be possibly important virulence factors in fish. Leukocidin LukE/LukD secreted by the Newman strain of *Staphylococcus aureus* is a bicomponent cytotoxin of molecular weight 32.2 kDa for S and 34.3 for F class protein subunits (Gravet et al. 1998). Some studies with the use of leukocidin were performed on mammalian phagocytic cells. Siwicki et al. (2004) observed the modulatory influence of leukocidin LukE/LukD on metabolic activity and killing activity of canine macrophages. The results of our in vitro studies have shown modulatory influence of leukocidin LukE/LukD on phagocyte activity isolated from rabbit blood. High cytolytic concentrations of leukocidin LukE+LukD diminished RBA and PKA of PMN and MN cells. The inhibitory effect was a direct result of lysis of phagocytes induced by leukocidin what was documented by cell viability results (25 000 ng/ml – 3%). Leukocidin induced pores cell membrane of PMN and MN cells consequently caused malfunction of ion exchange and cell integrity. Gravet et al. (1998) also noted the leukolytic effect of leukocidin LukE/LukD on PMN cells in rabbit and showed that in vitro the toxin is less cytotoxic in comparison to other staphylococcal leukotoxins in that animal species. Our in vitro studies revealed that T and B cells were not susceptible to modulatory influence of leukocidin LukE/LukD. Suppression of lymphocyte proliferation was not seen even at the highest concentration. On the other hand studies performed by Siwicki et al. (2003) have shown immunosuppressive influence of leukocidin LukE/LukD on the proliferative ability of canine blood lymphocytes. Lack of sensitivity of rabbit lymphocytes to leukocidin could be a result of presence of specific receptors inactivating the toxin or no specific receptor to bind the S component of leukocidin. Pfannenberg et al. (1975) demonstrated that partially purified leukocidin secreted by bovine P83 strain of *S. aureus* induced lysis of 25% of bovine lymphocytes in vitro but no morphological changes were seen during contrast – phase observation. Loeffler et al. (1986) using 51Cr release assay noted a slight cytotoxic influence of partially purified leukocidin produced by P83 *S. aureus* strain on bovine lymphocytes. The toxic effect of leukocidin on mammalian lymphocytes observed by these two authors could be a result of contamination with other staphylococcal products like alphanemolysin that normally induces destruction of lymphocytes. We need more in vitro and in vivo studies on the effects of other leukocidins using different animal

**Discussion**

Little is known on the in vivo local effects and general role of bicomponent toxins produced by *Staphylococcus aureus* in the pathogenesis of fish and mammalian diseases. Frequent isolation of different strains secreting leukocidins from mammalian necrotic lesions and abscesses suggests that these bicomponent toxins could be possibly important virulence factors in fish. Leukocidin LukE/LukD secreted by the Newman strain of *Staphylococcus aureus* is a bicomponent cytotoxin of molecular weight 32.2 kDa for S and 34.3 for F class protein subunits (Gravet et al. 1998). Some studies with the use of leukocidin were performed on mammalian phagocytic cells. Siwicki et al. (2004) observed the modulatory influence of leukocidin LukE/LukD on metabolic activity and killing activity of canine macrophages. The results of our in vitro studies have shown modulatory influence of leukocidin LukE/LukD on phagocyte activity isolated from rabbit blood. High cytolytic concentrations of leukocidin LukE+LukD diminished RBA and PKA of PMN and MN cells. The inhibitory effect was a direct result of lysis of phagocytes induced by leukocidin what was documented by cell viability results (25 000 ng/ml – 3%). Leukocidin induced pores cell membrane of PMN and MN cells consequently caused malfunction of ion exchange and cell integrity. Gravet et al. (1998) also noted the leukolytic effect of leukocidin LukE/LukD on PMN cells in rabbit and showed that in vitro the toxin is less cytotoxic in comparison to other staphylococcal leukotoxins in that animal species. Our in vitro studies revealed that T and B cells were not susceptible to modulatory influence of leukocidin LukE/LukD. Suppression of lymphocyte proliferation was not seen even at the highest concentration. On the other hand studies performed by Siwicki et al. (2003) have shown immunosuppressive influence of leukocidin LukE/LukD on the proliferative ability of canine blood lymphocytes. Lack of sensitivity of rabbit lymphocytes to leukocidin could be a result of presence of specific receptors inactivating the toxin or no specific receptor to bind the S component of leukocidin. Pfannenberg et al. (1975) demonstrated that partially purified leukocidin secreted by bovine P83 strain of *S. aureus* induced lysis of 25% of bovine lymphocytes in vitro but no morphological changes were seen during contrast – phase observation. Loeffler et al. (1986) using 51Cr release assay noted a slight cytotoxic influence of partially purified leukocidin produced by P83 *S. aureus* strain on bovine lymphocytes. The toxic effect of leukocidin on mammalian lymphocytes observed by these two authors could be a result of contamination with other staphylococcal products like alphanemolysin that normally induces destruction of lymphocytes. We need more in vitro and in vivo studies on the effects of other leukocidins using different animal
species that have not been tested yet to determine possible sensitive species and possibly work out methods for certain types of leukaemia treatment.

References


Increased expression of NADPH-d/nitric oxide synthase after facial axotomy; ultrastructural and light microscopic analysis

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Abstract

Four weeks after transection of the facial nerve in rats, NADPH-diaphorase (NADPH-d) histochemistry was performed to analyze the induction of nitric oxide synthase (NOS) in injured facial motoneurons. Light microscopic examination of the affected facial motor nucleus revealed a statistically significant increase in the total number of stained motor neurons with a significant increase in staining intensity. Electron microscopic analysis disclosed the NADPH-d precipitation associated with bands of the endoplasmic reticulum, nuclear envelope, Golgi apparatus and mitochondrial membranes.

Key words: facial nerve, axotomy, NADPH-diaphorase, light and electron microscopy

Introduction

Recently it became apparent that NOS, the enzyme synthesizing nitric oxide (NO), is dynamically expressed under pathological conditions (Schmidt et al. 1995) implying a role for NO in response to neuronal damage. Light microscopical studies revealed an increased NOS expression in motor (Yu 1994), sensory (Hokfelt et al. 1994) and neurosecretory (Hokfelt et al. 1994) neurons following traumatic injury. In our study we applied light and electron microscopy for a comparative assessment of the intensity of NADPH-d expression in control and axotomized rat facial motor neurons. While the application of the NADPH-d histochemical reaction at light microscopical level enabled cellular localization of NOS, the ultrastructural location of the enzyme in facial motoneurons reflecting the subcellular source of NO remains still unclear. Therefore, while using NADPH-d histochemistry for electron microscopy (Wolf et al. 1992), we investigated the ultrastructural distribution of NOS expressed in the neuronal compartment of the facial nucleus after facial nerve transection.

Materials and Methods

Animals. The experimental material consisted of eleven adult male Wistar rats (200 – 250 g) of our own breeding stock. All animals were treated in accordance to the “Principles of Laboratory Animal Care” (NIH publication No. 86-23, revised 1985). Following facial axotomy or sham operation the rats were allowed to survive twenty eight days. Four of them were processed for light microscopy and the remaining three for electron microscopy. The light microscopy control group included two sham-operated animals sacrificed twenty eight days after operation and two
intact rats. Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and subjected to unilateral transection of the left facial nerve (Yu 1994). In sham-operated rats the facial nerve was exposed but not transected. All the animals were perfusion fixed and processed for light and electron microscopic NADPH-d histochemistry (Calka et al. 1994).

Quantitative study. Cell counting was carried out on four representative sections per animal with application of a three grade scale for neurons: intensely, moderately and weakly stained. The total number of stained neurons as well as numbers of intensely, moderately and weakly stained cells were compared between the affected versus unaffected side. Statistical differences were analyzed using Student’s t test and considered significant if p<0.05.

Results

Light microscopy. Facial nuclei of all control, sham operated as well as experimental animals contained NADPH-d-positive motoneurons (Fig. 1a). An evident increase in the neuronal NADPH-d expression on the axotomized side was noticed (Fig. 1b). Among the motoneurons intensely, moderately and weakly stained profiles randomly distributed through the area of the nucleus were observed.

Electron microscopy. Electron microscopic examination of subcellular distribution of the NADPH-diaphorase activity in intact controls, sham-operated as well as axotomized animals revealed apparent histochemical staining of the facial motoneurons. The fine BSPT formazan precipitate labeled numerous mitochondria, bands of endoplasmic reticulum, nuclear envelope and membranes of the Golgi apparatus as well (Fig. 2a, b).

Cell counts. Following the nerve transection, there was a significant increase in number of intensely and moderately stained neurons on the affected side versus contralateral unaffected side. These changes were accompanied by a distinct, but not statistically significant, decrease in population of weakly stained neurons (Fig. 3) and a significant increase in the total number of stained motoneurons (Fig. 4) on the transected side comparing to the contralateral side.

Fig. 1. NADPH-d-positive staining (arrows) in control unaffected (a) and axotomized contralateral facial motoneurons (b). x 60.

Fig. 2. Electron micrographs (a, b) illustrating NADPH-d activity in axotomized facial motoneurons. Note the NADPH-d-labeled membranes of the Golgi apparatus (bold arrows), endoplasmic reticulum (arrowheads), mitochondria (empty arrows), and nuclear envelope (bended arrows). x 7000.
Fig. 3. Effect of facial axotomy on the relative number of NADPH-d-positive (intensely, moderately or weakly stained) neurons per section on both the axotomized and the unoperated contralateral side of the facial nucleus. Each group represents mean ± S.E.M. from 4 rats, 4 sections per animal. *Denotes significant difference (p<0.05) against the corresponding unoperated side by Student’s t-test. AX-axotomized, CON-control group.

Fig. 4. Effect of facial nerve transection on the number of NADPH-d-labeled facial motoneurons. Values shown represent means ± S.E.M. of the number of stained neurons per section. *Denotes significant difference (Student’s t-test) as compared to unoperated control, p<0.05.

Discussion

The present study demonstrates an increased expression of NADPH-d/NOS in facial motoneurons following axotomy. Although the functional implications of this phenomenon are unknown, earlier studies have shown that lesion-induced neuronal NOS expression is related to the death of injured neurons (Kristensson et al. 1994). Accordingly, inhibition of NOS could effectively reduce motoneuronal death (Wu et al. 1993) and promotes nerve regeneration (Zochonde et al. 1997). Recent reports indicate that nNOS activity is regulated by PSD-95 and CAPON (Che et al. 2000), and plays a pivotal role in regulation of synaptic plasticity and synaptogeneses. Thus, it is likely that axotomy-induced NOS expression observed in the present study might play an important role in the recovery of synaptic function. Our electron microscopical findings showing formazan precipitation in the nuclear envelope, endoplasmic reticulum, mitochondria and Golgi apparatus fully confirm light microscopical observations on high NOS activity in facial motoneurons.

References

The pathological investigation and analysis of the chicks cadmium chloride intoxication

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Abstract

In the experiment 216 one day old chicks were divided into 6 contrasting groups at random and 5 groups were fed on provender with cadmium chloride of $2 \times 10^{-7}$, $1 \times 10^{-6}$, $2 \times 10^{-6}$, $1 \times 10^{-5}$, $2 \times 10^{-5}$ respectively for 60 successive days. The observation records the changes of the 5 groups chicks in clinic presentation, pathological anatomy and pathological histology. The results indicated that the cadmium chloride intoxication led to growth restriction, serious anemia and caused the pathological changes of the chicks; livers, galls, stomachs and thyroid glands, especially the thyroid glands of the chicks were seriously harmed.

Key words: chick, cadmium chloride intoxication, growth control, anemia, pathomorphology

Introduction

The compounds of cadmium, e.g. CdO, CdS, Cd(NO$_3$)$_2$, Cd(CN)$_2$ and CdSO$_4$, etc. are of high toxicity. People can be poisoned by electroplate industry, the coloring manufacture of cadmium alloy, the electrode manufacturing of accumulator, the insecticide containing the element and the semiconductor and plastic manufacturing contacted with Cd compounds when taking cadmium compounds by accident or inhaling. No matter what form of the cadmium compound has been taken into the body, it can lead to poisoning, and the compounds will be distributed to every organ of the entire body. They are distributed mainly to the liver, kidney, lung and hypothyroid, and drained by kidneys. The compounds enter the body by intestine, and 85% of them drainage by intestine too (Shulin 1989). Poison can be classified as acute and chronic. Elicited by some intangible death of chickens and ducks in some regions we investigated toxicities of a series of metals and recorded the poisoning reaction- and histopathological changes in laboratory. The pathological investigation is the important basis in clinic diagnose and poisoning prevention. This article gives a brief report on the pathological changes as a result of the chicks cadmium chloride intoxication.

Materials

Animals: 216 chicks, male, the average weight was 38.75 g, from hennery of HuaYang ShuangLiu county.
Poison used in experiment: cadmium chloride, ShangHai Tingxin chemical plant (GB1285-94); Pass mark: 990801.
Apparatus used: Rotary Cutting Microtome, American AO Corporation

Methods

After one day observation of the newly break-shell chicks they were divided into 6 groups at random and...
were fed on provender with cadmium chloride of $2 \times 10^{-7}$, $1 \times 10^{-6}$, $2 \times 10^{-6}$, $1 \times 10^{-5}$, $2 \times 10^{-5}$ respectively. The feeding hours were as follows: 8 a.m., 2 p.m. and 8 p.m. without limit and with unlimited access to water every day. The weight of the chicks was estimated before adding feedstuffs at 8 a.m. Three chicks from each group were executed after picking blood sample every five days. Then they were dissected and pathological changes were recorded. Samples of liver, spleen, intestine, proventriculus, gizzard, thyroid, lymph nodes were fixed by 10% formalin solution, cut by paraffin sectioning and stained by haematoxylin and eosin, then examined under the microscope. The hemoglobin (Hb) was determined by Sahli method, and the red blood cell packed volume was measured by the Modified Wintrobe method (Ministry of Health P. R. China 1991).

## Results

**Clinical Presentation.** After feeding on provender with addition of cadmium chloride a large number of chicks showed low spirit, aversion to cold and poor appetite after twelve hours. The observed effects were dependent on the quantity of provender. The results of accumulation were distinct in different experimental groups. In the sixth group accidental death had taken place at the fifth day and all chicks were dead before the eighth day. In the fifth group color lightened with calcification. After twenty days, all five groups poisoned were white and the faeces were hoar and watery. These phenomena were more distinct at the twenty-fifth day; the beaks of the chicks pointed worse with time. All parameters were getting worse with time.

The Cadmium chloride intoxication affected the growth and the weight of chicks, the p<0.001. However, the fourth group showed the stimulating growth phenomena. The average weight of the first group was 282.29 g, the second one was 246.67 g, the third one was 208.33 g, the forth one was 262.85 g, the fifth one was 159.33 g on the thirtieth day; and on the sixtieth the average weight was 396.19 g (the first group), 346.19 g (the second group), 292.39 g (the third group), 368.39 g (the forth one), the fifth one was 222.22 g. The percent of dynamic restrain is shown in Fig. 1.

The effect of cadmium on the MCV (mean corpuscular volume) of RBC (red blood cell), on the MCH (mean corpuscular hemoglobin) of RBC, on the MCHC (mean corpuscular hemoglobin concentration) and on the RBC packed volume is shown in Table 1. The content of Hb in 100 ml blood is 12.6 g of the first group, 11.63 g of the second one, 11.88 g of the third one, 10.93 g of the fourth one, and 9.43 g of the fifth one on twenty day. After the sixtieth day, the content of Hb in 100 ml blood is 12.0 g of the first group, 10.56 g of the second one, 9.82 g of the third one, 8.63 g of the fourth one, 7.95 g of the fifth one, respectively.

**Macroscopical lesions.** The necropsy showed that 5% chicks did not absorb the yolk and had photoshop phenomena. The part of mucous membrane of gizzard broke off, necrosed and the cholecyst got bigger. The color of thyroid in other poisoned groups got lighter, and yellowish. Liver, spleen and proventriculus were congested and the mucous membrane of the proventriculus fell off easily; the small intestine bloated, and the mucous membrane disengaged; the liver and gall got bigger obviously and the gall took on dark green, the concentration of it was thicker than that of the control group. The blood of the most chicks was thick, while that of few was thin. With the poisoning time, the corneal layer of gizzard began to fall off on the fiftieth day and gizzard began to have nodular focal necrosis with calcification. After twenty days, all five groups showed weakness of legs.

**Microscopical lesions.** Blood smears from terminally affected chicks showed that RBC was distorsional and leucocyte membrane was dissolved. In addition we observed necrotic lesions in the thyroid and the proventricular mucosa etc. The experiment showed extensive necrosis in thyroid, superficial necrosis in mucous membrane of proventriculus; focal necrosis in gizzard. The jejunum, colon and rectum congested in different degrees, and the spleen congested lightly, the liver congested, hepatocytes necrosed in portal zone of the hepatic lobules, the others atrophied, vacuolized and deteriorated.

![Fig. 1. The influence of cadmium chloride on the weight of chickens.](image-url)

Explantion: the abscissa is time and the y-axis is the percent of restrain. The percent of restrain = (100- testing group/contrast group) × 100%.
Table 1. The effect of cadmium on blood parameters.

<table>
<thead>
<tr>
<th>Group</th>
<th>MCV(FL)</th>
<th>MCH(pg)</th>
<th>MCHC(g/L)</th>
<th>RBC packed volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>84.53 ± 3.93</td>
<td>30.15 ± 1.36</td>
<td>356.63 ± 18.96</td>
<td>34.49 ± 1.63</td>
</tr>
<tr>
<td>2</td>
<td>83.74 ± 3.12</td>
<td>28.46 ± 2.13</td>
<td>339.87 ± 19.26</td>
<td>32.66 ± 0.98</td>
</tr>
<tr>
<td>3</td>
<td>74.43 ± 3.31</td>
<td>25.59 ± 1.56</td>
<td>345.03 ± 19.26</td>
<td>31.56 ± 0.89</td>
</tr>
<tr>
<td>4</td>
<td>92.22 ± 3.03</td>
<td>26.15 ± 1.46</td>
<td>325.46 ± 30.16</td>
<td>30.05 ± 1.36</td>
</tr>
<tr>
<td>5</td>
<td>82.12 ± 3.26</td>
<td>20.69 ± 1.28</td>
<td>307.07 ± 23.63</td>
<td>28.30 ± 1.32</td>
</tr>
</tbody>
</table>

Explanation: MCV – mean corpuscular volume of RBC, MCH – mean corpuscular hemoglobin of RBC, MCHC – mean corpuscular hemoglobin concentration of RBC, RBC is red blood cell.

**Discussion**

The histopathological examination of cadmium chloride poisoned chicks revealed that the cadmium chloride intoxication will lead to growth restriction (Shulin 1989). There are four main reasons. First, cadmium chloride harms thyroid gland of the chicks and reduces the thyroxin coming from it, which restricts the growth of the chicks. Second, it can harm the enteron of the chicks and impair the chicks imbibing alimentation. Third, the CdCl₂ can restrain calcium imbibed by the chicks and accelerate venting of calcium from the chick, which also affects the growth of chicks (Zhang 1994). Fourth, the compound can harm liver and gall of the chicks. The mechanism of the cadmium chloride stimulating the growth of the chick individually is under discussion.

Cadmium chloride decreased the content of hemoglobin in 100 ml blood of the chick remarkably (p<0.01) leading to anemia. As it did not affect the red blood count of chick obviously (p>0.05), the cause of anemia was lack of iron. MCV and MCH decreased obviously (p<0.05), the PBC packed volume decreased and MCHC did not change remarkably. It was shown that the volume of the red blood cell was diminished and the content of Hb was decreased obviously, which was the signal of hypochromic microcytic anemia. The results of pathological analysis showed that the main reason of anemia had three points. First, the peptic glands of the chicks were harmed causing a decrease in secretion of both gastric juice and gastric acid, which resulted in the slowing of the motor activity of the stomach. This caused the food stagnating in stomach fermented and rotted, produced a great deal of gas and lactic acid etc, leading to the phenomena of small intestine bloat. It harmed stomach and intestine, and impaired the absorption of iron. Second, stomach and intestine were harmed by cadmium chloride in different degrees, resulting in hemorrhagic gastro-enteritis and petechiae in stomach and intestine of chick, which caused the reduction of iron imbibed. Third, cadmium chloride itself harmed blood corpuscle, eg. through blood corpuscle and leucocyte distortion, and the leucocyte membrane breaking and dissolving.

Besides, the cadmium compounds restricted the growth of animal and caused anemia probably because they were related to the secretion of growth hormone and thyroxin and they influenced the marrow cell. Cadmium compounds restrained the process of oxidatine phosphorylation in the mitochondria of hepatocytes. They influenced the activity of the enzymes such as amino acid decarboxidase, histidine enzyme, perocidare, dehydrogenase resulting in the mis-function of tissue metabolism. This mechanism is under the study.

**References**

Changes in density of sympathetic nerve terminals and steroidogenic activity of porcine ovaries after dexamethasone-induced polycystic ovarian syndrome

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Abstract

To establish the role of sympathetic nerves in the etiopathogenesis of ovarian cysts, we studied morphology and steroidogenic activity of the porcine ovaries after dexamethasone-induced polycystic status. In polycystic-changed ovaries, an increase in the density of TH/DβH- and/or NPY-IR nerve terminals were found; this was paralleled by significant changes in the content of progesterone, androstenedione and estradiol-17β, as well as in the expression rate of cytochromes P450_{scC} and P450_{arom} in the cysts and corpora lutea. These results suggest that sympathetic neurons supplying ovaries may participate in the etiopathogenesis of the ovarian cysts in gilts.

Key words: polycystic ovarian syndrome, ovary, neuropeptide Y, noradrenaline, steroidogenesis, dexamethasone, pig

Introduction

It is generally assumed that cystic ovarian disease (COD) is caused mainly by disturbances in the function hypothalamic-pituitary-ovarian axis, causing impairment of the synthesis, release, and/or storage of various hormones of this functional unit. However, it has also been suggested that an alternation in the activity of the sympathetic neurons innervating the ovary may contribute to the etiology and/or progression of cysts in women (Nakamura 1990) and rats (Parades et al. 1998). Therefore, we decided to study the possibility that cysts formation in gilts may be associated with a derangement of the sympathetic control of the ovary.

Materials and Methods

The principles of animal care (NIH publication No. 86-23, revised 1985) as well as the specific national law on the protection of animals were followed. In this experiment 12 crossbred adult (7-8 months) gilts were used. Animals were randomly assigned to one of two groups: I, experimental (n=6) and II, control (n=6). In the group I, cysts were
induced by injections of dexamethasone (DXM; Dexamethasone®, Norbrook Lab., Newry, UK, 3.3 µg/kg b.m., in total volume of 6 ml, i.m.), every 12 h on days 7-21 of the first studied estrous cycle. Using the same paradigm, the gilts of group II received 6 ml of saline. On day 11 of the next cycle, gilts were killed by electrical shock and exsanguination. Afterwards, the ovaries were dissected out, weighted, and the number of follicles, corpora lutea (CL) as well as cysts was counted. 10-µm-thick cryostat ovarian sections were stained by means of double-immunofluorescence to investigate the number and the distribution pattern of tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DβH) and/or neuropeptide Y-immunoreactive (NPY-IR) nerve fibers. The amount of progesterone (P4), androstendione (A4) and estradiol-17β (E2) was estimated by RIA in tissue probes taken out from CL, follicular and cystic wall, as well as in follicular and cystic fluids. Expression of cytochromes P450scc and P450arom in ovarian structures was determined by Western blot. Immunoblots were quantitated by scanning on Kodak 1D Image Analysis Software (USA). The mean (± SEM) weight of the ovaries and the number of ovarian structures was estimated per ovary, both in DXM-treated and control groups. Obtained data were subjected to one-way analysis of variance (ANOVA).

Results and discussion

The ovaries of the control animals were heavier (p<0.05) than those after DXM administration (9.8 ± 0.9 vs. 4.3 ± 0.6 g; respectively). In both the control and DXM-treated groups, the mean number of follicles with diameter 1 – 3 mm and CL were not significantly different (13.8 ± 2.5 vs. 7.75 ± 0.9 and 9.33 ± 1.0 vs. 5.5 ± 2.1; respectively). In the ovaries of the gilts treated with DXM, the follicles measuring 3 – 6 mm in diameter were not found, while 2.2 ± 0.4 of follicular cysts, 1 – 3 cm in diameter, was encountered per ovary. An increase in the number of TH/DβH- and/or NPY-IR nerves was observed in the vicinity of the follicles (Figs. 1b, 2b), CL, cysts (Fig. 2b) and blood vessels (Fig. 3b) in the experimental group, when compared to control animals (Figs. 1a, 2a, 3a). DXM injections lead to an increase (p<0.05) in the mean content of P4 and a decrease of A4 and E2 (p<0.05) in the cystic fluid, compared to 3 – 6 mm follicles in control group (P4: 347.2 ± 16.8 vs. 258.1 ± 17.5 ng/ml; A4: 5.7 ± 1.3 vs. 24.3 ± 4.2 ng/ml; E2: 2.8 ± 1.7 vs. 10.5 ± 1.5 ng/ml; respectively). The concentrations of P4, A4 and E2 in the cystic wall were higher (p<0.05 – 0.001) than those found in these follicles of control gilts (P4: 3398.7 ± 239.4 vs. 1266.1 ± 234.2 ng/g of tissue; A4: 277.5 ± 1.5 vs. 4.2 ± 0.9 ng/g of tissue; E2: 7.8 ± 1.6 vs. 2.7 ± 1.4 ng/g

Fig. 1. Cortex ovarii, DβH-IR nerve terminals running in a close neighborhood of primary and primordial follicles in intact (a) and DXM-treated animal (b). x 200.

Fig. 2. Cortex ovarii, noradrenergic nerve fibres running in the vicinity of follicles in control group (a) and a small cyst (b). F – follicle, C – cyst. x 200.

Fig. 3. DβH-IR perivascular nerve fibres supplying intraovarian artery in control (a) and DXM-treated animal (b). A – artery. x 200.
of tissue; respectively). In CL of the gilts receiving DXM, an increase \((p<0.001)\) in content of \(P_4\) and \(A_4\) was observed, when compared to that detected in control group \((P_4: 70038 \pm 1285 \text{ vs. } 6521 \pm 1664 \text{ ng/g of tissue}; A_4: 45.1 \pm 3.7 \text{ vs. } 1.2 \pm 0.2 \text{ ng/g of tissue}; \text{ respectively})\). Western blot analysis demonstrated that the expression of cytochromes \(P450_{\text{scC}}\) and \(P450_{\text{arom}}\) in CL of DXM-treated animals was higher \((130\% \text{ and } 104.7\% \text{ of control values}, \text{ respectively})\). In the cystic wall, the staining intensity of the \(P450_{\text{scC}}\) band was lowered \((by 8.2\%)\), whereas intensity of cytochrome \(P450_{\text{arom}}\) staining was enhanced by \(7.9\%)\, as compared to the \(3 - 6 \text{ mm follicles of control gilts}\).

Our study revealed that the formation of ovarian cysts caused a decrease in the ovarian weight, what correspond well with earlier observations in rats with COD \((\text{Lara et al. } 1993)\). In ovaries with DXM-induced polycystic status, a dramatic increase in the number of \(\text{TH/D}_\beta\text{H- and/or NPY-IR nerves was observed in close vicinity of follicles, CL, cysts and blood vessels. Increased density of sympathetic nerves, parallel to elevated content of noradrenaline (NA) was found in the polycystic-changed ovaries of women (Nakamura } 1990)\) and rats \((\text{Lara et al. } 1993)\). Thus, it appears possible that the increase in the \(P_4\), \(A_4\) and \(E_2\) contents in the cystic wall and CL, observed in the present experiment, may be a consequence of the increased density of sympathetic intraovarian nerves. It was reported that NA stimulated the \(P_4\) synthesis in the porcine granulosa \((\text{Wiesak et al. } 1990)\) and bovine luteal cells \((\text{Kotwica et al. } 2002)\). Moreover, expression of cytochrome \(P450_{\text{scC}}\) in the CL was considerably increased in ovaries investigated in the present study. It has been shown that NA activated cytochrome \(P450_{\text{scC}}\) in the bovine luteal cells \((\text{Kotwica et al. } 2002)\). In the present study, in the gilts treated with DXM, an increase in the \(E_2\) accumulation in the cystic wall and CL was associated with a higher content of \(P450_{\text{arom}}\) in these structures. However, as there is a lack of data dealing with the influence of NA on the content/activity of this enzyme, the relevance of this finding remains obscure yet. Increased \(E_2\) concentration in the polycystic ovaries can be explained by the stimulating effect NA on the production of \(P_4\) and \(A_4\) \((\text{precursors for estrogens})\), which was also elevated. Injections of DXM resulted in a decrease in the \(A_4\) and \(E_2\) concentrations in the cystic fluid. This observation was in agreement with earlier study in which a reduction of androgens and estrogens amounts in the cystic fluid was also observed \((\text{Babalola et al. } 1990)\). The present study revealed that in the polycystic-changed ovaries in gilts an increase in the number of the sympathetic nerve terminals was associated with changes of the steroidogenic activity, what indicates the important role of the sympathetic neurons in the etiology and/or progression of ovarian cysts.

References


The study on the NADPHd-positive innervation of the porcine mammary gland

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Abstract

The origin and distribution of NADPH-diaphorase-(NADPHd)-positive nerve fibres supplying the porcine mammary gland have been investigated. Retrograde tracing studies have revealed that NADPHd-positive innervation of the porcine last abdominal mamma originates from L1-L4 DRG. The NADPHd-positive nerves were found in the dermis of the nipple, with single nerve terminals penetrating into the epidermis. Smooth muscle cells (SMC) located in the nipple were supplied with few NADPHd-positive fibres. Small arteries of this region received abundant NADPHd innervation, while large arteries, veins and lactiferous ducts (LD) possessed relatively weaker supply. Similarly, the parenchyma was provided with moderate number of NADPHd-positive nerve fibres which surrounded SMC, blood vessels and LD.

Key words: NADPH-diaphorase, neurons, retrograde tracing, mammary gland, pig

Introduction

Accumulating evidence suggests that NO may participate in the regulation of morphological and functional features of the mammary gland (Onoda and Inano 1998). Although, application of the NADPHd histochemistry revealed NOS-positive, non-neuronal structures in the mammary gland of human (Thomsen 1995), goat (Lacasse et al. 1996), cow (Lacasse et al. 1996) and rat (Onoda and Inano 1998) there is a complete lack of information on the NO-ergic innervation of the gland. Therefore, while applying Fast Blue (FB) retrograde tracing method and NADPHd histochemistry, the origin and distribution of NOS nerve supply in the porcine mammary gland were the objective of this study.

Materials and Methods

Three sexually immature female pigs (3 months, 50 – 55 kg body weight) of the Large White Polish breed were anaesthetized with sodium pentobarbital (Vetbutal, Biovet, Poland; 20 mg/kg, i.v.). The retrograde tracer FB was injected into the nipple (10 µl) and parenchyma (20 µl) of the right last abdominal mamma. After three weeks, the animals were reanaesthetized and transcardially perfused with 4% buffered paraformaldehyde solution (pH 7.4). Subsequently, the right and left last abdominal mamma, and DRG including bilateral thoracic, lumbar and sacral ganglia were dissected out. The tissues were postfixed and transferred into 18% buffered sucrose solution (pH 7.4). The samples were cut into 10 µm thick
cryostat sections and processed for NADPHd histochemical staining procedure (Scherer-Siniger et al. 1983).

Results
Generally, the porcine mammary gland was moderately supplied with NADPHd-positive nerve fibres. The most abundant distribution of NADPHd-positive nerve fibres was observed in the dermis of the nipple (Fig. 1). These fibres occurred singly or in fascicles. Single nerve terminals penetrated into the epidermis as well. SMC located in the nipple were supplied with few NADPHd-positive nerve fibres running between and in parallel to myocytes. Small arteries of the nipple were abundantly innervated by the nerves; however, less numerous NADPHd-positive fibres were associated with larger arteries. Veins were also supplied with few NADPHd-positive nerves which were arranged mostly along the vessels. Additionally, some NADPHd-positive nerves were encountered around the LD.

The parenchyma was supplied with moderate number of NADPHd-positive nerve fibres. SMC located in the parenchyma were poorly innervated by these nerves (Fig. 2). Arteries were supplied with moderate number of NADPHd-positive nerve fibres that were mostly longitudinally arranged and some of them formed bundles. Veins were supplied with only single NADPHd-positive nerves. Some NADPHd-positive processes were observed around the LD and single fibres penetrated into the epithelium of the ducts.

Retrograde tracing studies have revealed that some DRG neurons projecting to the last right abdominal mamma were NADPHd-positive (Fig 3a, b). These neurons were located in right L1-L4 DRG.

Discussion
This is the first study reporting on the NADPHd-positive innervation of the porcine mammary gland. Although, Thomsen et al. (1995) observed NOS-I activity in vascular endothelial and myo-
oepithelial cells of human breast cancer and normal tissue, there is no data on expression of NOS in the neural supply of the gland. Additionally, NADPHd activity was revealed in the secretory epithelium of cow and goat mammary gland, but no specific staining was obtained using antibodies to NOS-I (Lacasse et al. 1996). On the other hand, Onoda and Inano (1998) not only revealed the NOS-I in the epithelial cells of the rat mammary parenchyma, but also proved identity of the NOS-immunocytochemical staining with NADPHd histochemistry.

Application of the retrograde tracing method revealed that neural perikarya supplying the mammary gland originate from the right L1-L4 DRG and some of them are NADPHd-positive. These results fully correspond to the former findings dealing with the distribution and chemical coding of DRG neurons supplying the porcine mammary gland (Franke-Radowiecka 2003). The acquired data concerning the origin of NADPHd innervation of the gland indicate that it may be sensory in nature; however, the distribution of the fibres may suggest its role in motor function and control of the local blood flow in the mammary gland, as well.

References

Proliferative enteropathy-induced *de novo* synthesis of NPY in intramural ganglia neurons of the porcine descending colon

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Abstract

This study investigated the influence of proliferative enteropathy (PE) on the distribution pattern of NPY-immunoreactive neurons in the descending colon. NPY was present in 0.3 ± 0.3%, 0.5 ± 0.3% and 0.7 ± 0.1% of neurons in the myenteric (MP), outer submucous (OSP) and inner submucous (ISP) plexus in control animals, respectively. In PE-influenced colon, NPY was found in 2.9 ± 0.7%, 1.1 ± 0.1% and 18.4 ± 2.7% of MP, OSP and ISP neurons, respectively. Furthermore, NPY-IR neur-oendocrine-like cells were observed in the colonic epithelium. The increase in the number of NPY-IR ISP neurons/intraepithelial neuroendocrine-like cells may suggest a neuro- and/or epithelioprotective role of NPY during chronic colitis.

Key words: proliferative enteropathy, neuronal plasticity, neuropeptide Y, enteric neurons, pig.

Introduction

Neuropeptide Y, a 36 amino-acid residues-long brain-gut neuropeptide (Tatemoto et al. 1982) is thought to be one of the most important peptidergic transmitters in both the sympathetic (Lindh et al. 1989, Klimaschewski et al. 1996) and parasympathetic nervous system (O’Donohue et al. 1985). On the other hand, under physiological conditions, it is virtually absent from sensory (Weihe 1990) and intrinsic enteric neurons. It should be stressed, however, that while NPY was observed in guinea-pig inhibitory circular muscle motor neurons with short projections and in descending interneurons subserving local reflexes (Furness 2000), no neurons in the porcine bowel were found to contain this peptide (Brown and Timmermans 2004). Thus, it is widely accepted that in the pig, the vast majority of NPY-IR nerve fibres within the wall of the small and large intestine are of extrinsic origin (for review, see Brown and Timmermans 2004). However, in a preliminary experiment concerning inflammation-induced plasticity of enteric neurons, we have unexpectedly encountered quite numerous population of NPY-IR cells within the inner submucous plexus. It should be noted that NPY has been proposed as a potent neuroprotective/neuroproliferative agent (Hansel et al. 2001). Therefore, we decided to study the dynamic of its presence in neurons/nerve fibres in the porcine proliferative enteropathy (PE)-affected descending colon.
Materials and Methods

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically diagnosed *Lawsonia intracellularis* infection (PE group) were used. The animals were housed and treated in accordance with the rules approved by the local Ethical Commission. All the animals were sacrificed with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland; 90 mg/kg b.w.) and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). In order to exclude any putative cross-reactivity with other antibodies, 10 µm thick cryostat sections of the descending colon wall were subjected to routine single-labelling immunofluorescence, using well-characterized polyclonal antiserum raised in rat and directed towards NPY (Affinity, UK; 1:240). Primary antiserum was visualized by species-specific secondary antibody conjugated to FITC (Jackson Immunochemicals, USA). Single-immunostained perikarya were evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each ganglionated plexus (i.e., the myenteric – (MP), outer submucous – (OSP), and inner submucous – (ISP) plexus), found in the section studied (6 sections per animal; only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results and Discussion

NPY was present in 0.3 ± 0.3%, 0.5 ± 0.3% and 0.7 ± 0.1% of neurons in the MP, OSP and ISP plexus.

Fig. 1. Expression of NPY in control and PE-influenced ISP (arrows). Note the *de novo* synthesis of NPY in a number of ISP neurons in pigs suffering from *adenomatosis*. x 200.
Fig. 2. *De novo* synthesis of NPY in neuroendocrine cells of porcine PE-influenced colon (arrows). x 200.
in the control animals, respectively, what corresponds well with previous studies (Timmermans et al. 1992). On the contrary, in PE-influenced colon, NPY was found in $2.9 \pm 0.7\%$, $1.1 \pm 0.1\%$ and $18.4 \pm 2.7\%$ of MP, OSP and ISP neurons, respectively (Fig. 1). Thus, the results obtained clearly suggest that this chronic, proliferative, bacteria-driven inflammation is not only able to induce the de novo synthesis of neuropeptide Y by a subset of enteric neurons, but also accomplishes this in a plexus-depending way. Interestingly, this process was neither accompanied by a boost in the density of intraganglionic or intramuscular NPY-IR nerve terminals nor by an increase in the number of such coded nerve fibres in the submucosal and mucosal layers. Hence, this may further, albeit indirectly, substantiate the hypothesis of neuroprotective function of NPY in challenged ISP neurons. Moreover, NPY-IR neuroendocrine-like cells were also observed in the colonic epithelium (Fig. 2), an event not reported in normal counterparts studied. Thus, an elevation in the number of NPY-IR ISP neurons/intraepithelial neuroendocrine-like cells may be suggestive for an additional epithelioprotective (epithelioproliferative?) role of NPY during chronic colitis.

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**References**

Co-incidence pattern of SP and CGRP in neural structures of the porcine descending colon affected by proliferative enteropathy

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Abstract

In control animals, SP-IR neurons constituted 3.7 ± 0.5%, 3.4 ± 0.4% and 6.6 ± 1.1%, while CGRP-IR perikarya amounted to 1.6 ± 0.2%, 1.1 ± 0.4% and 1.2 ± 0.5% of all neurons in myenteric (MP), outer (OSP) and inner submucous plexus (ISP), respectively. Co-localization was found in 26.1 ± 3%, 39.3 ± 10.7% and 23.3 ± 0.2% of all MP, OSP and ISP perikarya, respectively. In animals suffering from PE, SP-IR neurons contributed to 7.1 ± 0.8%, 9.1 ± 0.6% and 22.2 ± 1.6%, while CGRP-IR formed 4.0 ± 0.0%, 4.0 ± 0.8% and 11.7 ± 0.5% of all neurons found in MP, OSP and ISP, respectively. Co-localization was observed in 7.7 ± 0.6%, 23.8 ± 9.5% and 14.1 ± 4.1% of MP, OSP and ISP perikarya, respectively. These results may suggest a neuroprotective role of SP and CGRP.

Key words: proliferative enteropathy, neuronal plasticity, substance P (SP), calcitonin gene-related peptide (CGRP), enteric neurons, pig

Introduction

Sensory information from the intestinal lumen is conducted by two subsets of sensory neurons. First division of them is composed of intrinsic primary afferent neurons of the Dogiel II type, located in OSP and ISP (Clerc and Furness 2004), while the second subpopulation consists of extrinsic (spinal) primary afferent neurons located in DRGs (Berthoud et al. 2004). Both subsets of these neurons have been shown to contain SP and/or CGRP (Bossowska et al. 2003, Brown and Timmermans 2004). However, SP and/or CGRP have also been demonstrated in the secretomotor and excitatory motor neurons, both populations crucial for maintaining bowel homeostasis. As enteric neurons are now widely accepted to be particularly highly adaptive in their response to various pathological processes including inflammation (Sharkey and Kroese 2001), it appears to be of interest to study the expression pattern of SP and/or CGRP in neural structures of porcine descending colon affected by proliferative enteropathy (adenomatosis suis), a chronic proliferative inflammatory process evoked by Lawsonia intracellularis.

Materials and Methods

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically diagnosed Lawsonia intracellularis infection (PE group) were used. The animals were housed and treated in accordance with the rules approved by the local Ethical Commission. All the pigs were sacrificed...
with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland; 90 mg/kg b.w.) and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Ten µm thick cryostat sections of the descending colon wall were subjected to routine double-labelling immunofluorescence, using a mixture of antisera raised in different species and directed to substance P (rat monoclonal, Biogenesis, UK; 1:500) and CGRP (rabbit polyclonal, Biogenesis, UK; 1:8000). Primary antisera were visualiz-
ed by species-specific secondary antisera conjugated to FITC or biotin (all from Jackson Immunochemicals, USA). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson). Double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each ganglionated plexus found in section studied (6 sections per animal; only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Semi-quantitative evaluation of the density of nerve terminals within the muscular or mucosal layers has been based on counting of all terminals immunoreactive to given antigen per observation field. Nerve profiles were counted in 6 sections per animal, (in 6 fields per section) and obtained data were pooled and presented as mean. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

**Results and Discussion**

In the control animals, SP-IR neurons have been shown to constitute 3.7 ± 0.5%, 3.4 ± 0.4% and 6.6 ± 1.1% of all neurons in MP, OSP and ISP, while CGRP-IR perikarya amounted to 1.6 ± 0.2%, 1.1 ± 0.4% and 1.2 ± 0.5% of all MP, OSP and ISP neurons, respectively. Co-localization of both substances was found in 26.1 ± 3%, 39.3 ± 10.7% and 23.3 ± 0.2% of all MP, OSP and ISP perikarya, respectively. Furthermore, in the muscular layer of the normal colon, much more SP- (approximately 28.4 per field of observation) than CGRP-IR nerve profiles (2.3 per field) were observed (Figs. 1a-f). It has been shown that *Lawsonia intracellularis* infection evoked profound changes in the number of SP- and/or CGRP-IR neurons in particular plexuses: within MP, 7.1 ± 0.8% and 4.0 ± 0.0% of all neurons were SP- or CGRP-positive, respectively. In the OSP, SP was found in 9.1 ± 0.6% and CGRP – in 4.0 ± 0.8% of all ganglionic cells, while these peptides were present in 22.2 ± 1.6% and 11.7 ± 0.5% of all ISP perikarya, respectively. Co-localization of the substances studied was found in 7.7 ± 0.6%, 23.8 ± 9.5% and 14.1 ± 4.1% of MP, OSP and ISP somata, respectively (cf. Figs. 2a-f). The number of nerve fibres exhibiting immunoreactivity to the peptides studied was slightly lower in PE colon, when compared to the control intestine, however, a virtually total co-localization of both peptides has been observed. The present results may be indicative for a neuro- and/or epithelioprotective role of SP and CGRP (by intensification of mucous secretion?) during *Lawsonia intracellularis* infection, as may be judged from dramatic up-regulation in the expression rate of these substances within the inflamed bowel. However, the exact physiological relevance of adaptive changes observed remains to be elucidated in detail.

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**References**


Proliferative enteropathy (PE)-induced changes in the number of vasoactive intestinal polypeptide-immunoreactive (VIP-IR) neural elements in the porcine descending colon

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Abstract

In normal descending colon, VIP-IR varicose nerve terminals were moderately dense, while in PE-gut they were almost twice as numerous. VIP-IR neurons in control animals constituted approximately 0.9 ± 0.2%, 0.91 ± 0.4 and 7.7 ± 1.0% of all perikarya in the myenteric (MP), outer (OSP) and inner submucous plexus (ISP), respectively. In contrast, these neurons amounted to approximately 17.7 ± 1.6%, 11.8 ± 1.3% and 35 ± 3.3% of all ganglionic perikarya within MP, OSP and ISP of PE colon, respectively. As VIP is well known to be a potent neuroprotective agent, the up-regulation in its synthesis may reflect a repair mechanism developed by neurons affected by PE.

Key words: proliferative enteropathy, neuronal plasticity, vasoactive intestinal polypeptide (VIP), enteric neurons, pig

Introduction

Vasoactive intestinal polypeptide (VIP) is abundantly present within the porcine enteric nervous system, being confined to inhibitory motor, secretomotor and vasomotor neurons, located within all three ganglionic plexuses (for a review, see Brown and Timmermans 2004). Recently, the up-regulation of VIP within intrinsic enteric neurons have been shown to be a key-feature of adaptive changes in the inflamed bowel (Ekblad and Bauer 2004). Thus, in the recent past, VIP has been suggested to be one of the most potent neuroprotective agents for sympathetic (Klimaschewski 1997) as well as enteric neurons (Sandgren et al. 2003). It has been also proved to act as an important endogenous anti-inflammatory substance, which, on the one hand, down-regulates the synthesis rate of pro-inflammatory cytokines and, on the other hand, promotes the synthesis of anti-inflammatory interleukins, like IL-10, from, e.g. the myenteric ganglia (for review, see Ekblad and Bauer 2004, Mawe et al. 2004). Therefore, we decided to investigate the expression pattern of VIP in porcine descending colon affected by proliferative enteropathy, as it may be of interest to better understand the mechanisms of enteric neurons plasticity.

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Materials and Methods

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically diagnosed *Lawsonia intracellularis* infection (PE group) were used. The animals were housed and treated in accordance with the rules approved by the local Ethical Commission. All the animals were sacrificed with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland; 90 mg/kg b.w.) and perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).
Ten µm thick cryostat sections of the descending colon wall were subjected to routine double-labeling immunofluorescence, using combinations of anisera raised in different species and directed towards protein gene-product 9.5 (PGP9.5; mouse monoclonal, Sigma, PL, 1:2000) and VIP (rabbit polyclonal, Biogenesis, UK, 1:8000). Primary antisera were visualized by species-specific secondary antisera conjugated to FITC or biotin (all from Jackson Immunochimicals, USA). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson). Double-immunostained perikarya were evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each ganglionated plexus found in the section studied (6 sections per animal; only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

**Results and Discussion**

Within the muscular layer of the control gut segment, VIP-IR varicose nerve terminals were moderately dense (approximately 13 terminals per area of microscopic investigation), while in the PE-gut, they were almost twice as numerous (approx. 24 per area). In contrast, the number of VIP-IR nerve profiles within the colonic crypts slightly decreased, from approx. 42 per area in control specimens, to approx. 35 per area in PE-influenced colon. VIP-IR neurons in the control animals constituted approx. 0.9 ± 0.2%, 0.91 ± 0.4 and 7.7 ± 1.0% of all neurons in the myenteric (MP), outer (OSP) and inner submucous plexus (ISP), respectively. In contrast, a distinct increase in the number of VIP-IR perikarya was observed in chronically inflamed colon, where these neurons made up approx. 17.7 ± 1.6%, 11.8 ± 1.3% and 35 ± 3.3% of all ganglionic perikarya within the MP, OSP and ISP, respectively. The present results clearly demonstrate that proliferative enteropathy, induced by *Lawsonia intracellularis*, is able to up-regulate VIP synthesis in a dramatic manner, similar to that observed after axotomy or colchicine application (Ekblad et al. 1996), surgery (Schwarz et al. 1999) or intestinal hypertrophy (Ekblad et al. 1998). Thus, because VIP is well recognized as a potent neuroprotective agent (for review, see Ekblad and Bauer 2004), it appears possible that the up-regulation in its synthesis may reflect a repair mechanism developed by neurons affected by pathological processes accompanying *Lawsonia intracellularis* infection.

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Clinical evaluation of fillings made with glass-ionomer and composite materials in sandwich technique in canine molar teeth

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Abstract

The study concern 15 fillings of the carious defects in molar teeth in 15 dogs. The carious defects were located in grooves of the masticatory surface of molar teeth. The fillings were made in sandwich technique, with glass-ionomer material used as the base for the composite material. After 12 months from the manipulation the restorations have been evaluated according to modified Ryge’s scale. In this evaluation above mentioned materials obtained the results: marginal adaptation 95% – A, anatomical shape 95%, surface smoothness A – 100%, marginal discolouration A – 100%, colour assortment A – 60%.

Key words: caries, tooth, composit material, glass-ionomer, sandwich technique, dog

Introduction

The dental caries (caries dentis) occurs rarely in dogs. The medical treatment in case of the caries consists in mechanical ablation and following filling of the defect with suitable material (Bieniek and Bieniek 1993, Hale 1997). The sandwich technique, known also as second binding technique or double-layer, is wide used in the human and veterinary stomatology (Gończowski and Krupiński 2003, Janeczek et al. 2004). The present work presents the caries defects treatment and the filling way using glass-ionomer cement Kavitan® and composite XRV Herculite®, as well the point-evaluation of executed filling after 12 month interval.

Materials and Methods

The treatment was made on 12 dogs in various age and breeds. The symmetrical caries defects of two molar teeth from the the left and right mandible were detected in 3 dogs. All from the defects were located in grooves of the molar teeth masticatory surface. All de-
Effects were classified in the 1st degree (according to Black). After the dental calculus ablation the caries defects were performed next and they were filled. The operation was executed in the deep general anesthesia using xylasine (1 mg/kg) and atropine (0.02 mg/kg) i.m. for premedication. The introduction of general anesthesia was secured with ketamine (5 mg/kg) and diazepam (0.25 mg/kg) i.v. afterwards maintained with ketamine and diazepam i.v. in accordance with an effect production. The restoration treatment of caries defect was applied with the contra-angle hand piece NSK activated by Carlo de Giorgi microengine. The manipulations were executed in the following sequence: the preparation of caries defect, dentine and enamel were subjected to the dentine-enamel conditioner (10 sec.), the irrigation and desiccation of defect, the caries defect filling with glass-ionomer chemohardening material (Kavit®) – 6 minutes hardening time, 10 minutes cessation time, the dental surface was subjected to etcher (10 min.), 10 minutes cessation time, Opti Bond Solo plus® binding system application, polymerization, the filling covering with photohardening composite material XRV Herculite® in one or two layers, polymerization and filling preparation.

The glass-ionomer cement was prepared according to producer’s prescription. The polymerization process was made with halogen lamplight Hansadent Hilux 250. The filling has been reexaminated after one year period. The marginal adaptation, marginal discoloration, anatomical shape, surface smoothness and colour assortment have been also reevaluated according to modifited Ryge’s scale (Ruchała-Tyszler 2003).

Results

The above mentioned glass-ionomer cement was of ductile consistency enabling exact application into the cavity. The results of clinical examination after 12 months were fully satisfying (Tab. 1). Especially high mark was given to low appearance of marginal losses and marginal discoloration which characterize binding of the filling with tooth tissue and assure low level of secondary caries occurrence. The results of anatomical shape and surface smoothness were also positive. The esthetic of filling was a little worse but we have to notice that the filling was made in the rare exposed dog’ molar teeth. None of fillings was missed after 12th months, the secondary caries was not investigated.

Table 1. The results of clinical investigation after 12 months.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Anatomical shape</th>
<th>Marginal adaptation</th>
<th>Marginal discolouration</th>
<th>Smoothness of surface</th>
<th>Colour selection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>B</td>
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<td>Number</td>
<td>14</td>
<td>1</td>
<td>14</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

Discussion

The denatal caries are not popular in dogs. Hale (1998) reported caries in 5.3% from 435 investigated dogs. Bieniek and Bieniek (1993) observed caries in 6% of dogs.

The stomatological treatment of caries consist in the mechanical removal of pathological tissue until the caries defect is completely removed and the appropriate conditions for filling achieved (Hale 1998). Next the base material is applied into the defect. The constant ion-change process between hardened glass-ionomer, tooth tissue and saliva begin after the end of the hardning process. The chemical compound of fluorine releasing from the cement is important for the secondary caries prevention (Mount and Ngo 2003). The next of very important glass-ionomer materials propriety is very strong adhesion to the dentine, the low thermal expansibility index, relative neutrality for the dental pulp and high volume reduction of composite (Gaintantzopoulou at al. 1994). The compositive materials are characterized by the very high crush resistance and famous elastic proprieties. The preparation system used in our work and the following caries defect fillings are known as the conventional sandwich techniquie (Gończowski and Krupiński 2003). The method used in above-mentioned work allows for the optimal utilisation of the materials. Our work describes the reinvestigation 12 months after the treatment. The selected parameters which were classified according to modifited Ryge’s scale are generally use in the evaluation of fillings in human stomatology and allows for exact qualification of used materials proprieties in clinical treatment (Ruchała-Tyszler 2003).

Conclusion

The work proved that the sandwich technique using glass-ionomer and compositive materials is fully adequate for the canine molar teeth fillings. The modifited Ryge’s scale used in our clinical investigations gives strict information, which allows for the objective evaluation of filling. It is noticeable, the evaluation of selected parameters is possible in ambu-
latory conditions and it needs nothing except elementar stomatological equipment. The results of our investigation allow for statement that the materials used in our work may be also use successfully in animal stomatology.

**References**


The level of selected antioxidative indices and testosterone in the blood of rats injected with vitamin E and selenium under an increased ozone concentration in the air

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Abstract

In the blood of the ozone-exposed rats testosterone concentration was observed to decrease, whereas both the contents of glucose and malondialdehyde and the activity of glutathione peroxidase were found to increase. In animals exposed to ozone and injected with vitamin E and selenium the above-mentioned changes were significantly less intensive which suggests that those compounds reduce, at least to some extent, the effects of the harmful activity of oxidative stress.

Key words: rat, ozone, malondialdehyde, glutathione peroxidase, testosterone

Introduction

As a major component of photochemical smog ozone evokes oxidative stress in living organisms which is linked with the production of free oxygen radicals (Pryor et al. 1995, Salgo et al. 1995). Living organisms have been demonstrated to develop a number of defence mechanisms capable of preventing free-radical reactions, thus providing a balance between oxidative and antioxidative processes. One of the agents of these mechanisms is glutathione peroxidase (GPx) that protects cellular membranes by catalysing the reaction of \( \text{H}_2\text{O}_2 \) reduction. A key-component of that enzyme is selenium which is a part of the active centre of GPx (Arteel and Sies 2001). Selenium metabolism is closely linked to the metabolism of another recognised antioxidant, vitamin E. Its antioxidative properties are based on the reaction with free radicals which, by protecting a cell against the effect of the oxidative stress, simultaneously reduces an organism’s demand for selenium.

Although the available literature lacks the detailed data on the impact of ozone on the functioning of gonads in males, still it is known that free oxygen radicals may disturb their reproductive processes. Taking into account the metabolic relationships between vitamin E and selenium as well as the fact that ozone is often applied in medicine for therapeutic purposes, this study was undertaken to determine whether, and to what extent, selenium and vitamin E supplementation prevents the negative effects of the ozone activity on the testes.

Materials and Methods

The experiment was performed on 30 adult male Wistar Hannower rats with the average body weight...
of 405 ± 10 g and age of 12 months. All animals were randomly divided into 3 groups (10 rats each) denoted by the following abbreviations:

I (K) – control animals; II (OEv) – animals exposed to ozone and injected intramuscularly with vitamin E (22.5 mg/rat) and selenium (0.33 mg/rat) preparation Evetsel (Pliva, Kraków); injections were made for 25 days in 5-day intervals. Evetsel was administered to the animals 2 weeks prior to the experiment to increase and stabilise the levels of vitamin and selenium in their bodies; III (KO) – rats exposed to ozone without vitamin protection. All rats from groups II and III were exposed to 0.5 ± 0.2 ppm of ozone 5 h a day for the period of 25 days.

Ozone was generated from compressed air in an IMPOZ-4 ozoniser (Institute of Precise Mechanics, Warsaw) and supplied to a chemically-sealed (with neutral polyethylene foil) room to be mixed spontaneously with air. The concentration of ozone in the exposure chamber was controlled with the iodometric method (Saltzman et al. 1959). During ozonation the animals had free access to water but feed was removed due to an oxidative effect of ozone. Apart from the 5 hours of ozone-exposure the animals were kept under identical conditions in terms of air composition, temperature, and feeding regime. After termination of the experiment the maximal amount of blood was collected from all animals in halothane anaesthesia (Narcotan, Leciva, Czech Republic) by a heart puncture. Immediately after sampling the activity of glutathione peroxidase was determined in whole blood (Paglia and Valentine 1967), whereas concentrations of testosterone (Kotwica and Williams 1982), malondialdehyde (Ward et al. 1985), and glucose were assayed in blood plasma.

A statistical analysis of results involved calculations of arithmetic means, standard error of the mean and significance of differences compared to the control group.

The experimental animals were handled according to the provisions of the Act on Animal Protection as well as to recommendations of the Local Committee for Animal Experiments at the University of Warmia and Mazury in Olsztyń, Poland.

### Results

In blood of animals exposed to ozone (III KO) a statistically significant decrease in testosterone concentration, an increase in the contents of glucose and malondialdehyde (MDA) as well as an increased activity of glutathione peroxidase were observed (Table 1). In the animals exposed to ozone and injected with vitamin E and selenium (II OEv), the level of MDA was also statistically significantly higher, however, it was apparently lower compared to rats exposed to ozone only. In that group, an increasing tendency for glucose level and GPx activity with reference to the control group (I K) was observed.

### Discussion

In all ozonated animals, including those receiving vitamin E and selenium, the blood level of glucose was found to increase, which is a typical body response to stress. Under stressing conditions, the sympathetetic-adrenomedullary system is activated followed by the pituitary-corticomedullary system, which is connected through an increased demand of the body for directly available energetic substrates. An increased glucose concentration in blood is accompanied by an elevated activity of glutathione peroxidase and an increased level of malondialdehyde may indicate oxidative stress. At the same time, a high GPx activity proves the intensification of free-radical processes and antioxidative defence reactions of the organism (Arteel and Sies 2001). On the other hand, an elevated level of highly toxic malondialdehyde is linked with lipid peroxidation generated by free oxygen radicals with MDA being one of its main end-products (Pryor et al. 1995, Kim et al. 2000).

Oxidative stress produces changes in the spermatogenic epithelium and disturbs spermatogenesis (Sharma and Agarwal 1996), however, a decreased testosterone concentration is often observed upon different stressing conditions. It may also result from secretory disorders of the pituitary gland and hypothalamus and from changes in the gonads. An increased ozone concentration in the air (the state of

<table>
<thead>
<tr>
<th>Groups/indices</th>
<th>I K (n=10)</th>
<th>II OEv (n=10)</th>
<th>III O (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>testosterone [ng/ml]</td>
<td>1.01 ± 0.09</td>
<td>0.90 ± 0.31</td>
<td>0.73 ** ± 0.09</td>
</tr>
<tr>
<td>glucose [mg/dl]</td>
<td>77.02 ± 3.22</td>
<td>82.4 ± 5.09</td>
<td>92.42 * ± 6.47</td>
</tr>
<tr>
<td>malondialdehyde [µm/l]</td>
<td>9.18 ± 0.38</td>
<td>10.64 ** ± 0.24</td>
<td>12.26 ** ± 0.22</td>
</tr>
<tr>
<td>glutathione peroxidase [U/l]</td>
<td>2073 ± 165</td>
<td>2274 ± 87</td>
<td>2708* ± 168</td>
</tr>
</tbody>
</table>

Explanation: * p≤0.05 – significance in relation to the control group (I K), ** p≤0.01
oxidative stress) has been reported to diminish the activity of 17 beta-hydroxysteroid dehydrogenase and alkaline phosphatase in the gonad tissue in rats (Jedlińska-Krakowska 1998).

In the rats receiving injections of vitamin E and selenium the changes referring to ozone-exposure were apparently less intensive. An exception was a high, statistically significant level of malondialdehyde. The results suggest that Evetsel reduces, at least to some extent, the results of the detrimental activity of oxidative stress in male rats.

References


Lafora’s bodies in borzoi dog. Case report

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Abstract

Lafora bodies were diagnosed in female borzoi dog, 14 years old. There were no CNS clinical symptoms, except of peripheral neurogenic skeletal muscle atrophy and the pain. Post mortem examination revealed blood circulatory disturbances in the liver, lungs and spleen. The heart bicuspid and tricuspid valves had shown endocardiosis changes. The microscopic examination of the brain hemispheres and cerebellum revealed the presence of disseminated Lafora bodies. They were dark-blue in haematoxylin and eosin staining and PAS positive as well. The liver had slight features of the jaundice, with advanced hyperaemia and necrosis of hepatocytes. Lafora’s bodies were also found in degenerated/necrotic hepatocytes.

Key words: Lafora’s body, borzoi dog, histopathology, brain, liver

Introduction

The disease syndrome manifested by the presence of Lafora’s bodies in the brain is extremely rare in the dog (Davis at al. 1990, Kaiser et al. 1991). For years the etiopathology of this disease, which was observed in humans and animals, was the mystery. In the 80’s, Japanese authors presented the opinion that Lafora’s bodies appeared in the brains of aging animals (Kamiya and Suzuki 1989). Now it is documented that the syndrome has the inherited genetic background due to Lafora gene mutation and is manifested by extralysosomal storage of polyglucosan substance. In human medicine it was proved that Lafora’s disease is the result of autosomal recessive defect in carbohydrate metabolism (Serratosa at al. 1995). The stored material is present in the brain, liver, striated skeletal muscles and myocardium, and the skin tubular epithelium of apocrine sweat gland (Busard et al. 1994, Tamura et al. 1995, Thom and Revesz 1996). Lafora’s bodies are disseminated throughout the brain, seen in neuronal perikaryon and neuropil. These inclusions are rounded, in haematoxylin and eosin (HE) staining strongly basophilic and positive in periodic acid Schiff reaction (PAS). The presence of the inclusion bodies in the skin sweat glands argued for using the skin biopsy in the early procedure and in the differential diagnosis of Lafora’s disease in patients with neurological signs.

In this paper the results of post mortem examination of borzoi dog with Lafora’s disease are described.

Materials and Methods

Female dog, borzoi breed (Russian chart), 14 years old was presented for post mortem examination. For histopathological examination the following organ and tissue specimens were fixed in 10% buffered formalin: heart, liver, lung, spleen, kidney, skeletal muscle, brain and cerebellum, thyroid with para-
thyroid gland and adrenal glands. The paraffin embedded tissue sections were routinely stained with haematoxylin and eosin. Additionally cerebellum specimens were stained with PAS reagent.

Results and Discussion

The dog's owner, veterinary surgeon had informed, that the dog showed the symptoms of heart insufficiency since she had been born. The dog died with signs of leg muscle atrophy, mild icterus and lethargy. In post mortem examination the following macroscopic changes were found: the mucous membranes were yellowish stained, lungs were swollen and congested, both heart valves were thickened, the liver showed features of post hyperaemic lipid degeneration, kidney parenchyma was yellowish with disseminated cortical small scares, the brain and meninges were hyperaemic and leg skeletal muscles appeared atrophic. In the microscopic examination of internal organs the following changes were noted. In HE stained cerebellum specimens the numerous, dark-blue, round corpuscles, disseminated in the white substance were found (Fig. 1). Their diameter varied from point-like to the size of Purkinje cells nucleus. The corpuscles were PAS positive. The inclusions were present in the cytoplasm of Purkinje cells and in the cerebellum neuropil. The Purkinje cells showed signs of degeneration and necrobiosis. The brain had features of oedema (status cribrosus) and the degenerative changes in the neurons. In the brain cortex some focal calcium deposits were observed. The inclusion bodies were not so numerous, as was observed in the cerebellum. In the heart muscle focal purulent inflammation with necrotic focus in muscle cells were found. Occasionally in the myocardial fibres basophilic degeneration was observed. The microscopic changes in the liver were typical to chronic passive hyperaemia, followed by centrolobular trabecular atrophy with the bile retention in hepatocytes. The liver cells of the lobule periphery showed lipid infiltration. There were numerous areas of hepatocyte necrosis, in which the numerous round haematoxylin stained corpuscles were found (Fig. 2).

Fig. 1. Cerebellum of the dog with disseminated inclusion bodies typical for Lafora's disease. Haematoxylin and eosin staining, x 20.

Fig. 2. The liver of the dog with numerous Lafora's bodies seen in degenerated/necrotic hepatocytes, and the high degree of trabecular atrophy. Haematoxylin and eosin staining, x 20.

Fig. 3. Skeletal muscles of the dog with Lafora's disease with high degree of atrophic degenerative changes Haematoxylin and eosin staining, x 40.

In the lungs the high degree hyperaemia, proliferation of the interstitial tissue, focal calcium deposits, numerous syderocytes, dysplastic changes in cells of the bronchial epithelium and the focal oedema were observed. In the spleen the circulatory disturbances were manifested by the white pulp atrophy. In the kidneys interstitial and membranous-proliferative glomerular
Lafora's bodies are present in cells of the apocrine glands. The samples are taken from axillary region, because the skin biopsy is very useful in the clinical and laboratory differential diagnosis procedures. For the skin biopsy the specimen is unique. However, the central nervous system lesions were typical for Lafora's body syndrome – the dog clinical ailments, as was stated by the owner, were mainly reflected by circulatory and metabolic liver and striated muscle disturbances. The only neurological symptoms were lethargy and the skeletal muscle atrophy but the metabolic disturbances proved by the presence of Lafora's bodies were responsible also for the injury of parenchymatous organs and muscle cells. As compared to human clinical manifestation of Lafora's disease, in dogs case no epilepsy symptoms were observed. In the majority of human cases Lafora's disease is manifested by seizures typical for progressive myoclonous epilepsy and this symptom is considered as the significant for this disease syndrome. However, the similar brain lesions were described in the beagle dogs showing the signs of epilepsy (Montgomery, Lee 1983), but the cerebellar typical lesions were found in some sick dogs only. Among 68 beagle dogs, which had died with symptoms of myoclonous epilepsy, the cerebellar inclusions bodies localized in neurons, typical for human Lafora's body diseases were detected in six dogs only.

The presence of numerous basophilic inclusions in the cytoplasm of the liver cells or being free-laying in necrotic areas was found in the examined dog. These lesions were similar to changes described in human Lafora's disease. The description of Lafora's bodies in the liver (Yokota et al. 1987) as well as in tubular cells of skin glands (Busard et al. 1987, White and Gomez 1988) had made the liver and skin biopsy very useful in the clinical and laboratory differential diagnosis procedures. For the skin biopsy the specimens are taken from axillary region, because the Lafora's bodies are present in cells of the apocrine and eccrine glands located in this skin region. But the liver biopsy is considered to be the most reliable site for the early diagnosis of Lafora's disease, especially in juvenile human patients. There are no data concerning the application of the liver and skin biopsy in Lafora's disease of any animal species.

The microscopic changes found in other internal organs of the affected dog were not typical for the described disease syndrome, excluding the myocardium and skeletal muscles. The human Lafora's disease is characterized by the presence of typical cytoplasmic inclusions in the myocardial fibres, accompanying by their basophilic degeneration. In the dog myocardium the inclusions were very scarce and small but the degenerative changes were observed. The advanced histopathological changes, manifested by membranous-proliferative glomerular inflammation, followed by tubular degenerative and necrotic changes were found in the kidneys. These changes seemed to be the result of the long lasting generalized metabolic disturbances and liver damage which additionally were intensified by the dogs advanced age.

The above mentioned biopsy procedures are used in the routine diagnosis in the early stages of Lafora's disease in human medicine. Nowadays the biopsy techniques are well developed in veterinary medicine, so, may be used in Lafora's disease diagnosis when signs of myoclonus epilepsy are observed. The familiar character of Lafora's disease is the indication for having special attention in the animal breeding program. Very small number of publications on Lafora's disease cases in dogs diagnosed in various breeds suggest no breed dependent predisposition of this genetic anomaly inheritance.

References


Pathomorphological characteristic of supraspinal muscles in rabbits after short-term electrostimulation

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Abstract

This experiment was performed on developing rabbits (aged 3.5 months), divided into 2 groups (n=5): 1 – animals treated with electrostimulation for 2 h/day, 2 – control rabbits without electrostimulation of the supraspinal musculature. Stimulation was carried out using an electric stimulator SCOL-2, according to the method modified by Kowalski. The animals were sacrificed after 3 months of electrostimulation. The microscopic examination of supraspinal muscles of rabbits revealed the hypertrophy of muscle fibres, an increase in their congestion and a significant rise in the level of glycosaminoglycans. The obtained results indicate that the short-term electrostimulation has strengthened the structure of the muscles stabilizing the spine had strengthened.

Key words: scoliosis, lateral electrical surface stimulation (LESS), muscle, rabbit

Introduction

The idea of applying Lateral Electrical Surface Stimulation (LESS) in the treatment of idiopathic scoliosis (IS) is the stimulation of the supraspinal muscles area, which causes the impulsion of the preserved structures of spinal cord and mobilization of the supplementary system of control over the function at this level (Machida et al. 1994).

The effects of treating scoliosis in people with the application of the LESS method, in Poland also known by the name of the SCOL apparatus and in western countries by the name of the ScoliTron apparatus or by the name of Electro Spinal Orthosis – E.S.O., or the abbreviation of Surface Electrical Stimulation – SES, and also as Transcutaneous Electrical Stimulation – TCES (Wright et al. 1992, Machida et al., 1994), have been described for the last ten years. However, the long-term LESS method which has been applied so far, based on nine hours of therapy per day, has not proven effective enough and has produced undesired morphological lesions (Szarek et al. 2003). These observations served as the basis for establishing the author’s own Lateral Electrical Surface Stimulation programme, shortened to two hours a day (Kowalski 1999).

Materials and Methods

Ten male, pure-bred (New Zealand White), age-matched (born within 5 days) and clinically healthy rabbits were used in this study. The animals were kept indoors in the room with controlled tem-
perature (18°C) and humidity (70%). Each animal was placed in a metal cage (50 x 50 x 50 cm) and received dry feed and water ad libitum. A 30-day period was allowed for adaptation to new environment. At the beginning of the treatment, the rabbits were aged approximately 3.5 months and weighed between 2000 – 2200 g.

The experiment was conducted according to the previously described methodology, with the use of an electrical stimulator (SCOL-2, Elmech, Warsaw, Poland) of the technical specifications previously presented (Kowalski 1999, Szarek et al. 2003). The rabbits were randomly assigned to one of the two groups (each n=5): group 1, where the rabbits were treated for 2 hours/day and group 2 (control) – where animals were not electrostimulated.

Sections of right and left *musculus longissimus dorsi* (MLD): *musculus longissimus thoracis* (MLT) and *m. iliocostalis thoracis* (MIT) – muscles stabilizing rabbit spinal column were microscopically examined. The material was taken from areas targeted with electrostimulation on the right side and analogous areas on the left side of the spine. The material was fixed in 10% neutral formalin and paraffin slices were routinely stained with hematoxylin and eosin (HE). To show the glycosaminoglycane level, microscopic muscle preparations were stained by the PAS-method according to McManus. Digestion with diastase was used as a control sample. The analysis was carried out in compliance with the criteria given by Szarek et al. (1985).

### Results

In all rabbits from group 1 (animals subjected to LESS), wave-like muscle fibers were observed (Fig. 1). It affected small sections of fibers and was slightly more evident in the scraps derived from the muscle located nearer the electrodes.

Atrophy of fragments of muscle fibers in MLT on the right side (stimulated) was observed occasionally within two scraps (Fig. 2a). Atrophy of cross striation took place in two rabbits. Granular degeneration was present only in short sections of muscle fibers, in three scraps of MLT (Fig. 1a) and in one case of MIT. Occasionally the examined material revealed single vacuoles. Several tiny foci of necrosis were observed in one scrap of MLT on the right side.

Almost half of the examined muscles revealed a slight congestion. Additionally, regeneration of muscle fibres was seen in the analyzed material (Fig. 1). Proliferation of nuclei in the muscle fibres has been revealed in almost a half of the examined scraps, where occurred sporadically, and affected only small fragments of fibres. Infiltration of mononuclear cells were observed relatively rarely.

In the examined material of the left side of the rabbits from group 1 (not stimulated) pathomorphological lesions were observed similar to those observed on the right side, but no foci of atrophy and necrosis were found. The lesions visible here were of a vestigial character and they affected very small fragments of fibres.
The analyzed muscle fibres on the right side in rabbits from group 1 most frequently were characterized by a large and very large content of glycosaminoglycans. Positive PAS reaction was particularly clearly seen at the periphery of muscle fibres, while the atrophying fibres revealed excessive accumulation of glycogen (Fig. 2a). The level of glycosaminoglycans in muscle fibres on the left side in rabbits from group 1 was characterized as large. The polysaccharides content in these cases varied from average to very large.

In rabbits from group 2 (the control group), the microscopic structure of muscles appeared normal. Granular degeneration, proliferation of nuclei fibres and infiltration of mononuclear cells were observed only occasionally, in a small number of muscle fibres. Muscle fibres of animals from group 2 were predominantly characterized by a small amount of glycosaminoglycans; this substance was evenly distributed (Fig. 2b).

**Discussion**

The tension of supraspinal muscles stabilizing the spinal column depends on the spinal regulation (deep and superficial reflexes) and on complex mechanisms controlled by supraspinal nervous system centers (Wright et al. 1992, Machida et al. 1994). However, the reduction of tension of supraspinal muscles – conditions of dystonia – is widely accepted by the authors as the crucial factor in IS etiopathogenesis (Wright et al. 1992). Therefore, the role of LESS in the IS correction is the stimulation of the weakened neuromuscular system. As a result of this stimulation on the side of the developed scoliosis, the strengthening of the muscles stabilizing the spine occurs, and subsequently the correction (Kowalski 1999).

Our own research proved that short-term LESS occasionally caused unfavorable morphological disorders of small intensification within the area of musculus longissimus dorsi in the form of regressive lesions. However, there were frequently observed positive effects in supraspinal muscles: muscle fibre hypertrophy, the rise in their congestion and the rise in the polysaccharide level. These lesions indicated that the muscle tissue structure of the muscles stabilizing the spine had strengthened.

In the present experiment the new program of the LESS therapy, shortened to 2 hours a day, has proven to be devoid of unfavorable muscle disorders.

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NOS-, VIP- or GAL-immunoreactivity disappear from proliferative enteropathy-affected inferior mesenteric ganglion (IMG) neurons supplying porcine descending colon

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Abstract

This study investigated the influence of proliferative enteropathy (PE) on porcine descending colon-projecting IMG perikarya immunoreactive to NOS, VIP and/or GAL. Fast Blue (FB) tract-tracing combined with immunofluorescence revealed that in control animals, a low number of FB+ IMG neurons were NOS- (2.6 ± 0.8%), VIP- (0.9 ± 0.2%) or GAL- immunoreactive (IR) (2.3 ± 0.6%). In contrast, in animals suffering from PE none of retrogradely traced cells were IR to studied substances. These results suggest the existence of a Lawsonia intracellularis inflammation-specific response of affected prevertebral neurons.

Key words: proliferative enteropathy, neuronal plasticity, nitric oxide synthase (NOS), vasoactive intestinal polypeptide (VIP), galanin (GAL), inferior mesenteric ganglion, pig

Introduction

This study investigated the influence of proliferative enteropathy (PE), a chronic proliferative inflammatory process evoked by Lawsonia intracellularis, on neurochemical features of the porcine inferior mesenteric ganglia neurons, especially those exhibiting immunoreactivity to NOS, VIP and/or GAL, the substances previously suggested to be able to exert a neuroprotective action on injured sympathetic neurons (Klimaschewski et al. 1996; Majewski et al. 1996). However, while the preliminary data concerning the responses of IMG neurons supplying the distal colon in the pig to mechanical injury (axotomy) or to acute inflammation of the bowel wall have previously been presented (Majewski et al. 2002), virtually nothing is known about the influences of a chronic bacteria-induced colitis on the expression pattern of putative neuroprotective substances in affected sympathetic neurons.

Materials and Methods

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically...
diagnosed *Lawsonia intracellularis* infection (PE group) were used. All animals were subjected to laparotomy and injected with 5% aqueous solution of Fast Blue (FB) into the wall of the descending colon. After a three-week survival period the animals were sacrificed with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland; 90 mg/kg body weight) and transcardially perfused with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Ten µm thick cryostat sections of the IMG were subjected to routine double-labelling immunofluorescence, using combinations of anisera raised in differ-
ent species and directed towards NOS (mouse monoclonal, rabbit polyclonal), VIP (mouse monoclonal, rabbit polyclonal), GAL (rabbit polyclonal). Primary antisera were visualized by species-specific secondary antisera conjugated to FITC or biotin (all from Jackson Immunochemicals, USA). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson). Retrogradely labeled/double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each fifth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

**Results and Discussion**

Retrograde tract-tracing combined with double-immunofluorescence staining revealed a low number of retrogradely labelled perikarya containing immunoreactivity to NOS (2.6 ± 0.8%; Figs. 1, 2), VIP (0.9 ± 0.2%; Figs. 1, 2) or GAL (2.3 ± 0.6%) in the IMGs of control animals. Furthermore, dense, moderately dense or scarce nerve fibres exhibiting immunoreactivity to NOS, VIP or GAL, respectively, were observed in the control IMG (Figs. 1, 2). These fibres were patchily distributed throughout the ganglion, being more numerous around large, TH/DfH- and/or SOM-IR perikarya than around smaller, NPY-containing somata. When ganglia from PE-suffering animals were studied, it has been found that retrogradely labeled, PE-influenced IMG neurons were devoid of detectable amounts of any of the three substances studied (Fig. 3, 4). In contrast, the distribution pattern and the density of intraganglionic NOS-, VIP- or GAL-IR nerve fibres remained virtually unchanged. Hence, these results run counter to data dealing with axotomy- or acute chemical inflammation-evoked changes in the expression of above mentioned substances (Majewski et al. 2002). Thus, while a distinct up-regulation in the number of retrogradely labeled GAL-IR neurons was observed in the IMG affected by axotomy, numbers of NOS- and VIP-IR perikarya remained unchanged. On the other hand, an acute chemically-induced inflammation of the descending colon wall was not able to either up- or down-regulate the synthesis ratio of substances studied by ganglionic neurons. However, it resulted in a distinct increase in the density of intraganglionic terminals of viscerofugal enteric neurons (Majewski et al. 2002). Based on the data obtained, an existence of a *Lawsonia intracellularis* inflammation-specific response of affected prevertebral neurons may be suggested. Furthermore, it appears that NO and VIP does not fulfill the criterion of being neuroprotective agents in the porcine nervous system, at least in its sympathetic subdivision controlling the bowel-function. Further studies are needed to elucidate both the exact physiological relevance, as well as the exact mechanism(s) of these observations.

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Proliferative enteropathy (PE)-induced changes in the expression pattern of DβH, NPY and/or SOM in porcine IMG neurons supplying the descending colon

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Abstract

The present study aimed at disclosing PE-driven changes in the number of retrogradely labelled descending colon-projecting, noradrenergic (i.e., DβH-IR), NPY- and/or SOM-IR IMG neurons in the pig. In control animals, 89.6 ± 1.8% of all retrogradely labeled neurons exhibited DβH-IR; 57.7 ± 2.3% of them co-expressed SOM-IR, while 35.7 ± 3.7% of DβH-IR perikarya were NPY-positive. In PE-animals, 77.9 ± 1.3% of all retrogradely traced perikarya were DβH-IR, while 22.7 ± 3.4% of them were NPY-IR. Only 2.6 ± 0.3% of DβH-containing retrogradely labeled neurons were SOM-IR. Thus, it appears that the chronic form of the PE resulted in a down-regulation of synthesis rate of “main transmitters” in affected IMG neurons.

Key words: proliferative enteropathy, neuronal plasticity, noradrenaline, neuropeptide Y (NPY), somatostatin (SOM), inferior mesenteric ganglion, pig

Introduction

Inferior mesenteric ganglion (IMG) is the virtually sole source of sympathetic prevertebral neuronal input to the neural circuits controlling the porcine descending colon. As revealed previously (Pidsudko et al. 2001), the vast majority of normal colon-projecting neurons use different combination of noradrenaline (visualized by the presence of its synthesis-rate limiting enzymes, tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DβH)), NPY and/or SOM as their “main messenger” molecule(s). It is well-known that pathological processes including inflammation of the target tissue of affected cell are able to profoundly change biochemical pathways of affected sympathetic neurons (Klimaschewski et al. 1996, Zigmond 2000). However, there is lack of data dealing with bowel chronic proliferative inflammation as a factor influencing the homeostasis of sympathetic neurons involved in the control of the gut function(s). Therefore, the present study was aimed at disclosing putative Lawsonia intracellularis infection-driven (i.e., proliferative enteropathy (PE)-induced) changes in the relative frequency of retrogradely labelled, descending colon-projecting, noradrenergic (i.e., DβH-IR), NPY- and/or SOM-IR IMG neurons in the pig.

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Materials and Methods

Six immature female pigs (approximately 8 weeks old), three control (C group) and three with clinically diagnosed *Lawsonia intracellularis* infection (PE group) were used. The animals were housed and treated in accordance with the rules approved by the local Ethical Commission. All pigs were subjected to laparotomy and injected with 5% aqueous solution of Fast Blue (FB) into the wall of the descending colon. After a three-week survival period, the animals were sacrificed with an overdose of sodium pentobarbital (Vetbutal®, Biowet, Poland; 90 mg/kg b.w.) and transcardially perfused with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Ten μm thick cryostat sections of the IMG were subjected to routine double-labelling immunofluorescence, using different combinations of anisera raised in different species and directed towards TH (mouse monoclonal, rabbit polyclonal), DβH (mouse monoclonal, rabbit polyclonal), NPY (rat polyclonal, rabbit polyclonal) or SOM (rat monoclonal, rabbit polyclonal). Primary antisera were visualized by species-specific secondary antisera conjugated to FITC or biotin (all from Jackson Immunochemicals, USA). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson). Retrogradely labeled/double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each fifth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results and Discussion

In control animals, up to 90% of all retrogradely labeled neurons exhibited TH- and DβH-IR (89.6 ± 1.8%); 57.7 ± 2.3% of them co-expressed SOM-IR (Fig. 1), while 35.7 ± 3.7% of TH/DβH-IR perikarya were simultaneously NPY-positive (Fig. 2). In accordance with previous report (Pidsudko et al. 2001), virtually all the SOM-IR cells belonged to the class of large neurons, being approached by dense to moderately dense, patchily distributed SOM-IR nerve terminals; in contrast, smaller NPY-IR perikarya, located mainly at the ganglionic periphery, were spared by SOM-IR terminals. NPY-IR varicose nerve terminals were sporadically found in the IMG neuropil. In animals suffering from *Lawsonia intracellularis* infection, TH/DβH-IR neurons comprised 77.9 ± 1.3% of all FB-containing cell bodies; while the relative frequency of neurons containing simultaneously NPY reached approximately 23% (22.7 ± 3.4%; Fig. 3), there was a dramatic decrease in the number of noradrenergic SOM-IR perikarya – only 2.6 ± 0.3% of FB/DβH-containing neurons were simultaneously SOM-IR in affected IMG (Fig. 4). It has been found that in PE-suffering animals, the number of SOM-IR nerve terminals was slightly increased, whilst the delicate NPY-IR intraganglionic nerve fibres appeared to be stronger fluorescing, what may suggest an arrest in the orthodromic axoplasmonic transport/release of these neuropeptides by the affected neurons. Thus, as may be judged from the present results, the chronic form of the PE resulted in a drastic down-regulation of “main transmitter” synthesis rate in affected neurons, especially with regard to SOM. It may be thus indicative of a PE-evoked “exhaustion” of compensatory mechanism(s) in colon-projecting sympathetic neurons, what, in turn, may lead to an exacerbation in the deregulatory processes within the bowel. However, the exact mechanism of the process(es) described, as well as its/their pathophysiological relevance, remains to be elucidated.

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Figs. 1 and 2. In the IMG of control animals a large population of retrogradely labeled (1a, 2a), DβH-positive neurons (1b, 2b) were simultaneously SOM- (1c) or NPY-IR (2c). Only a few of FB+ cells were DβH-immunonegative (double-headed arrow in Fig. 2). Appropriate arrows/arrowheads point out to differently chemically coded IMG neurons. x 200.

Figs. 3 and 4. In PE-suffering animals the number of retrogradely labeled (3a) DβH/NPY-IR neurons was only slightly reduced (3b, c), while nearly all FB+ cells were devoid of SOM-immunoreactivity (4a-c). Appropriate arrows/arrowheads point out to differently chemically coded IMG neurons. x 200.
Effect of Methisoprinol, KLP-602 and NDV infection on the selected biochemical indices of the allantoic fluid of chicken embryos

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Abstract

The effect of Methisoprinol, KLP-602 and NDV infection on the reactivity of lymphocytes and selected biochemical parameters of allantoic fluid of chicken embryos was tested. Only KLP-602 positively affected the reactivity of lymphocytes. The administration of KLP-602 to infected with NDV embryos made lymphocytic reactivity increase to the level noted in the control group. The drug increased also the activity of ceruloplasmin in allantoic fluid of chicken embryos.

Key words: chicken embryos, NDV, Methisoprinol, KLP-602

Introduction

The process of introducing new drugs into the market includes preclinical tests performed on cell cultures and chicken embryos. Chicken embryos constitute good experimental material, because they contain immune system elements. Despite the fact that this system is morphologically immature and its functions are not fully developed yet, it allows to obtain positive results of in ovo inoculations (Sharma and Burmester 1982, Ahmad and Sharma 1993, Sharma 1997, Stone et al. 1997, Maś and Goddeeris 1999). The results of such studies suggest that drug administration to embryos may lead to changes in the biochemical parameters of their body fluids, and that simultaneous application of drugs and microbes can stimulate antiviral immunity.

Materials and Methods

The experiment was performed on chicken embryos aged 10 days and older. Ten days old embryos were inoculated by the allantoic cavity route. The drugs examined were introduced by the same route. Allantoic fluids were collected for biochemical analyses 6, 12, 24, 48 and 72 hours after inoculation.

Two antiviral agents KLP-602 (active substance – lysozyme dimer), manufactured by Nika Health Products, Ltd. USA at the following doses: 20, 10, 5, 2 and 1 mg/embryo, and Methisoprinol (active substance – isoprinozine), manufactured by Polfa Grodzisk, at the following doses: 50, 25, 12.5, 6 and 3 mg/embryo were used in the studies. A physiological buffer solution (PBS) was applied as a solvent.
Two strains of Newcastle disease virus (NDV) were used: mesogenic Roakin strain (EID₅₀=10⁻⁹.⁵/ml) and lentogenic inoculation LaSota strain (Sotasec vaccine, EID₅₀=10⁻⁹.⁵/ml).

In order to determine lymphocytic reactivity 10 days old chicken embryos were given the maximum tolerable doses of both drugs in the form of injections into the allantoic cavity. The other groups were inoculated by the same route with 10⁴ EID₅₀ of the LaSota strain of Newcastle disease virus, combined with the maximum tolerable dose of KLP-602 or Methisoprinol or without the drugs. The control group comprised chicken embryos treated with PBS. The inoculated embryos were incubated for five consecutive days. Then their lymphocytic reactivity was determined with a blastic transformation test on the day of inoculation (time 0), and after 72 and 120 hours (according to the method described by Małaczewska et al. 2003).

To determine biochemical parameters 10 days old chicken embryos were allocated to 9 groups, 25 embryos each. Then they were inoculated with: Methisoprinol (6 mg/embryo), KLP-602 (5 mg/embryo), LaSota strain of NDV (10⁵ EID₅₀) or the Roakin strain of NDV (10² EID₅₀), injected into the allantoic cavity (Table 1). The next two groups were inoculated with the LaSota strain and treated with Methisoprinol or KLP-602, and another two were inoculated with the Roakin strain and treated with both drugs. The control group was given PBS.

Allantoic fluids were collected 6, 12, 24, 48 and 72 hours after inoculation to determine ceruloplasmin activity by the spectrophotometric method described by Rice et al (1986), and lysozyme activity by the turbidimetric method described by Parry et al. (1965), modified by Siwicki and Anderson (1993).

The results obtained were subjected to a two-factor analysis of variance and Tukey test, determining means, standard deviations and significance of differences at p≤0.05 and p≤0.01.

**Results**

The maximum tolerable doses were: 6 mg/embryo of Methisoprinol and 5 mg/embryo of KLP-602, and such doses were applied in the experiment. Higher doses of both drugs were toxic to the embryos; their administration resulted in growth inhibition or even embryo death. Furthermore, high doses of Methisoprinol caused urate salt deposition on the embryos’ bodies, chorioallantoic membrane and in the kidneys.

The lymphocytes of 10 days old embryos of the control group incubated with phytohemagglutinin responded by slight simulation (IS was 1.5) and the level of this reactivity was increasing according to the age. On the 3rd day of incubation, IS was 3.2 (Table 1).

The activity of ceruloplasmin in the allantoic fluid increased as soon as after 6 hours from the administration of KLP-602 or inoculation with the LaSota strain or the Roakin strain of Newcastle disease virus, as well as after the application of the LaSota strain and Methisoprinol, and the Roakin strain and Methisoprinol or KLP-602. Methisoprinol applied alone had no effect on the activity of this acute phase protein (Table 2).

Table 1. Index of lymphocyte stimulation in chicken embryos under the influence of PHA.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time of the test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Control</td>
<td>1.5</td>
</tr>
<tr>
<td>KLP–602</td>
<td>–</td>
</tr>
<tr>
<td>Methisoprinol</td>
<td>–</td>
</tr>
<tr>
<td>LaSota + Methisopr.</td>
<td>–</td>
</tr>
<tr>
<td>LaSota + KLP</td>
<td>–</td>
</tr>
<tr>
<td>LaSota</td>
<td>–</td>
</tr>
</tbody>
</table>

The highest increase in lysozyme activity in the allantoic fluid was noted after 48 and 72 hours from inoculation with the Roakin strain or the LaSota strain of NDV, as well as after inoculation with the LaSota strain and simultaneous administration of Methisoprinol. When applied alone Methisoprinol cause a slight increase in lysozyme activity only in the 6th hour after injection. In successive hours the activity of this enzyme in this group of embryos was lower than in the control group (Table 3).

**Discussion**

The objective of the present study was to determine the effects of Methisoprinol and KLP-602 on the reactivity of embryo lymphocytes, and the activity of ceruloplasmin and lysozyme in the allantoic fluid.

The highest reactivity of lymphocytes was observed in the group receiving KLP-602 (IS=5.5), and the lowest – in the group of embryos inoculated with the LaSota strain of Newcastle disease virus and treated with Methisoprinol. The administration of KLP-602 to infected with NDV embryos made lymphocytic reactivity increase to the level noted in the control group.

Enhanced ceruloplasmin activity in the allantoic fluid of the embryos inoculated with both strains of NDV, as well as of those treated with the drugs examined, was observed starting from the 6th hour
Table 2. Ceruloplasmin activity in the allantoic fluid of chicken embryos (mg%).

<table>
<thead>
<tr>
<th>Group (administered drug, virus)</th>
<th>Sampling.</th>
<th>Time after inoculation (hrs)</th>
<th>Significance of differences between samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I 0  II 6  III 12  IV 24  V 48  VI 72</td>
<td></td>
</tr>
<tr>
<td>Control M</td>
<td>M</td>
<td>6.19 6.49&lt;sup&gt;a&lt;/sup&gt; 5.59 6.61 7.11 6.43</td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>SD</td>
<td>2.55 1.01 1.94 1.94 1.77 1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methisoprinol M</td>
<td>M</td>
<td>5.94 6.11&lt;sup&gt;c&lt;/sup&gt; 6.29 6.34 6.12 5.97</td>
<td>I&lt;II&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.95 2.22 1.62 1.67 1.45 1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLP-602 M</td>
<td>M</td>
<td>6.24 9.02&lt;sup&gt;d&lt;/sup&gt; 7.41 6.92 8.10 8.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>I&lt;II&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.57 1.14 2.09 0.85 0.40 1.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roakin M</td>
<td>M</td>
<td>6.31 8.05 7.01 9.17 7.93 6.62</td>
<td>I,VI&lt;IV&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>1.92 1.30 1.70 0.53 0.54 1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roakin+Methisoprinol M</td>
<td>M</td>
<td>5.81 9.09&lt;sup&gt;d&lt;/sup&gt; 6.96 8.54 7.27 7.16</td>
<td>I&lt;II&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>1.71 1.65 1.29 1.42 1.38 1.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roakin+KLP-602 M</td>
<td>M</td>
<td>6.68 8.4 7.36 9.00 8.17 7.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>I&lt;IV&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.86 0.64 1.23 1.16 1.17 1.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaSota M</td>
<td>M</td>
<td>5.94 9.01&lt;sup&gt;d&lt;/sup&gt; 7.35 7.61 7.20 6.90</td>
<td>I&lt;II&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>1.02 1.53 1.39 1.92 1.56 0.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaSota+Methisoprinol M</td>
<td>M</td>
<td>6.11 9.34&lt;sup&gt;b&lt;/sup&gt;d 6.39 6.30 6.04 6.37</td>
<td>I,II,III,IV,VI&lt;II&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>1.34 0.68 1.11 1.46 0.95 0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sota+KLP-602 M</td>
<td>M</td>
<td>6.05 6.81 6.80 6.58 5.73 5.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No statistically significant differences</td>
</tr>
<tr>
<td>SD</td>
<td>1.53 0.55 0.84 1.64 0.98 0.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Explanation:
*abcd – statistically significant differences at p≤0.05
**ABCD – statistically significant differences at p≤0.01

Table 3. Lysozyme activity in the allantoic fluid of chicken embryos (mg%).

<table>
<thead>
<tr>
<th>Group (administered drug, virus)</th>
<th>Sampling.</th>
<th>Time after inoculation (hrs)</th>
<th>Significance of differences between samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I 0  II 6  III 12  IV 24  V 48  VI 72</td>
<td></td>
</tr>
<tr>
<td>Control M</td>
<td>M</td>
<td>0.58 0.78&lt;sup&gt;c&lt;/sup&gt; 0.62&lt;sup&gt;ab&lt;/sup&gt; 1.02&lt;sup&gt;a&lt;/sup&gt; 1.58&lt;sup&gt;A&lt;/sup&gt; 2.37&lt;sup&gt;A&lt;/sup&gt;</td>
<td>I,II,III,IV&lt;VI&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.34 0.33 0.21 0.50 0.57 0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methisoprinol M</td>
<td>M</td>
<td>0.78 1.68&lt;sup&gt;A&lt;/sup&gt; 1.47 0.65&lt;sup&gt;Ac&lt;/sup&gt; 1.15&lt;sup&gt;A&lt;/sup&gt; 1.44&lt;sup&gt;A&lt;/sup&gt;</td>
<td>I&lt;II&lt;sup&gt;*, IV&lt;III,VI&lt;IV&lt;II&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.47 0.12 0.50 0.19 0.41 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roakin M</td>
<td>M</td>
<td>0.62 3.66&lt;sup&gt;b&lt;/sup&gt; 2.81&lt;sup&gt;b&lt;/sup&gt; 2.00&lt;sup&gt;d&lt;/sup&gt; 7.11&lt;sup&gt;BD&lt;/sup&gt; 7.66&lt;sup&gt;B&lt;/sup&gt;</td>
<td>I&lt;II,V,VI&lt;sup&gt;<strong>&lt;/sup&gt; II,III,IV&lt;V,VI&lt;sup&gt;</strong>&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.36 0.67 0.90 0.75 2.09 1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roakin+Methisoprinol M</td>
<td>M</td>
<td>0.51 1.58&lt;sup&gt;Ab&lt;/sup&gt; 2.20&lt;sup&gt;b&lt;/sup&gt; 2.17&lt;sup&gt;dB&lt;/sup&gt; 3.54&lt;sup&gt;Ca&lt;/sup&gt; 2.86&lt;sup&gt;A&lt;/sup&gt;</td>
<td>I&lt;II&lt;V,I,VI&lt;sup&gt;<strong>&lt;/sup&gt; II,III,IV&lt;V,VI&lt;sup&gt;</strong>&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.23 0.38 1.07 0.86 0.76 0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaSota M</td>
<td>M</td>
<td>1.02 2.81&lt;sup&gt;Db&lt;/sup&gt; 1.90 1.77&lt;sup&gt;d&lt;/sup&gt; 6.35&lt;sup&gt;BD&lt;/sup&gt; 6.42&lt;sup&gt;B&lt;/sup&gt;</td>
<td>I&lt;III&lt;IV&lt;,V,VI&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.13 1.23 0.75 0.46 1.80 1.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LaSota+Methisoprinol M</td>
<td>M</td>
<td>0.74 1.69&lt;sup&gt;A&lt;/sup&gt; 2.47&lt;sup&gt;b&lt;/sup&gt; 1.12 7.28&lt;sup&gt;BD&lt;/sup&gt; 7.64&lt;sup&gt;B&lt;/sup&gt;</td>
<td>I&lt;II&lt;sup&gt;*&lt;/sup&gt; I,II,III,IV&lt;,V,VI&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>0.28 0.30 0.92 0.37 0.93 1.53</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Explanation:
*abcd – statistically significant differences at p≤0.05
**ABCD – statistically significant differences at p≤0.01

after inoculation. Higher activity of this acute phase protein was observed until the end of the experiment, but a statistically significant increase in this parameter, compared with the control group, was recorded only in the 6<sup>th</sup> hour after inoculation and only in the group inoculated with the Roakin strain of Newcastle disease virus and treated with KLP-602. The activity of this enzyme in the control group was increasing in successive hours of observations.

Lysozyme activity in the allantoic fluid of the control embryos was increasing with their age. After inoculation with both strains of NDV it reached a significantly higher level than in the control group. Its highest activity was observed after 48 and 72 hours in the embryos inoculated with both strains of NDV and
in those inoculated with the LaSota strain and treated with Methisoprinol. In the groups receiving KLP-602 lysozyme activity was very high due to its external application, so, the data for these groups were ignored.

The available literature on the topic provides no information on the level of the above parameters in chicken embryos. Mazur-Gonkowska (2004), who analyzed acute phase proteins and non-specific immunity in turkeys inoculated with bacteria and viruses, reported similar values of the above parameters. However, a comparative analysis seems impossible in this case due to considerable differences in the type of experimental material (allantoic fluids of chicken embryos vs. blood serum of adult turkeys). This author observed a slight increase in the serum concentration of ceruloplasmin in turkeys inoculated with HE virus as late as 24 hours after inoculation. The serum activity of lysozyme in turkeys inoculated with this virus also increased 24 hours after inoculation reaching the highest level after 72 hours. Enhanced activity of this enzyme was also observed in the control group after 48 and 72 hours (data not shown).

It may be concluded that Methisoprinol and KLP-602 had some positive effects on chicken embryos in the aspects described above. This concerns first of all KLP-602, which had positively affected lymphocytic reactivity in chicken embryos under the influence of PHA, and on the development of immunocompetent organs (Małaczewska et al. 2003).

References


Levels of C-reactive protein and interferon gamma in pigs vaccinated with deleted vaccine against Aujeszky’s disease after experimental infection with virulent *Herpesvirus suis* type 1

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Department of Infectious and Invasive Diseases and 1Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Oczapowskiego St. 13, 10-719 Olsztyn, Poland

Abstract

The purpose of the present study was to evaluate the dynamics of the C-reactive protein (CRP) and interferon gamma (IFN-γ) level formation in sera of pigs vaccinated intramuscularly versus intradermally with a deleted vaccine against Aujeszky’s disease after experimental infection with virulent *Herpesvirus suis* typ 1 (SHV-1). Three groups, seven piglets each, were formed. At the age of 12 weeks two groups were vaccinated twice, 4 weeks apart, with deleted Porcilis Begonia vaccine (Intervet) at a dose of 2.0 ml (10^6.0 TCID_{50}) intramuscularly or 0.2 ml (10^5.0 TCID_{50}) intradermally. Pigs of control group were injected with 2.0 ml of PBS intramuscularly. Seventy days after the first vaccination all pigs were intranasally infected with Northern Ireland Aujeszky-3 strain of SHV-1 at a dose of 10^5.5 TCID_{50}. The increase of CRP level in pig sera from both vaccinated groups was statistically significantly lower (p<0.01) than in control group in the most terms of blood sampling, showing good protective action of the vaccine through quick stimulation of mechanisms of cell-mediated and humoral immunity. Intradermal vaccination, ensuring the high activity of lymphocytes T, induces higher level of IFN-γ after exposition to virulent NIA-3 strain of SHV-1, when compared to intramuscular vaccination.

Key words: Aujeszky’s disease, deleted vaccine, experimental infection, CRP, IFN-γ

Introduction

One of the components of the non-specific body response causing a restriction of inflammation, elimination of damaging or infectious factors and restoring the homeostasis is so called “acute phase response”. Changes in the concentration of several serum proteins, i.e. acute phase proteins (APP) took place during this process (Kostro et al. 1996). The most important signal inducing transcriptive factors of APP genes are proinflammatory cytokines – IL-1, IL-6 and TNFα, produced by various types of immune as well as epithelial cells and fibroblasts, as a result of activation by viruses, bacteria, fungi, substances releasing from destroyed tissues and several other factors (Kostro et al. 2003). In the pig, C-reactive protein (CRP), haptoglobin (Hp) and pig Major Acute Phase Protein (MAPP) are the main APP (Kostro et al.)
2002). Non-specific humoral immunity, as an answer to viral antigen, is however mainly represented by synthesis of interferons – INF (Gołąb et al. 2002). The mammalian cells are able to produce three main forms of interferons: α, β and γ (La Bonnardiere et al. 1994). Antiviral action of INF depends also on their influence on immunological system through stimulation of specific and non-specific mechanisms participating in the response to viral antigens. Production of INF plays also an extremely important role in protection against virulent Herpesvirus suis type 1 (SHV-1) (Chinsakchai and Molitor 1994). The most actively influencing the immune system is INF-γ, produced by activated lymphocyte T belonging to all subpopulations and NK cells (Chinsakchai and Molitor 1994, Gołąb et al. 2002).

The purpose of our study was to evaluate the dynamics in the formation of the levels of CRP and IFN-γ in pigs vaccinated intramuscularly versus intradermally with a deleted vaccine against Aujeszky’s disease after experimental infection with virulent SHV-1.

Materials and Methods

Animals. An experiment was carried out using 21 piglets, 8 weeks old, divided into three groups, 7 animals each, kept in the isolation units. Piglets were free from anti-gE antibodies checked by Pseudorabies Virus gp I Antibody Test Kit (Herd Chek Anti-PRV gp I, IDEXX Lab Inc, USA).

Vaccine and vaccination. Deleted, gE-and TK-negative, live attenuated Porcilis Begonia vaccine (Intervet, The Netherlands) with adjuvantive diluent Dilucav Forte was used. At the age of 12 weeks pigs were vaccinated twice, 4 weeks apart, at a dose of 2.0 ml (10^6.0 TCID_{50}) intramuscularly – group I or 0.2 ml (10^5.0 TCID_{50}) intradermally (I.D.) using needleless apparatus SERENA model SD 1-2, (Emaplast, Italy) – group II. Pigs of group III (control) were injected with 2.0 ml of PBS.

Experimental infection. Seventy days after the first vaccination all pigs were experimentally infected with virulent Northern Ireland Aujeszky-3 (NIA-3) strain of SHV-1 at a dose of 10^5.5 TCID_{50} by intranasal instilling of 0.5 ml of virus suspension into each nostril.

C-reactive protein (CRP). The levels of CRP in pig serum were determined by nephelometric method using Beckman Array 360 System apparatus (USA) and anti-human CRP antibodies (CRP kit p/n 449760) and human standards, according to the manufacturer recommendation and Heegaard et al. (1998) considerations. Blood samples were taken immediately before infection and every day in the period 1 to 7 days post infection (dpi).

Interferon gamma (IFN-γ). The levels of IFN-γ in pig serum were determined by immunoenzyme test using ready to use diagnostic Kit – Swine Interferon-γ (swIFN-γ) (BioSource International, Inc., USA). Blood samples were taken immediately before infection and on 1, 2, 3, 5, 7 and 14 dpi.

Statistical evaluation. The results were statistically evaluated using one-way analysis of variance (ANOVA) to compare several means (NIR test) at p≤0.05 and p≤0.01 and presented as mean and SD.

Results and Discussion

The fluctuations in the level of CRP in porcine sera after experimental infection is presented in Table 1. On 1 dpi highly statistically significant increase in the CRP level was observed in control group, as compared to both immunized groups. Statistically significant differences between immunized and control groups were observed until 5 dpi. The formation of CRP levels in pig sera of both vaccinated groups was very similar, only on 3 dpi statistically significantly higher level of CRP was noticed in group II, when compared to group I.

The formation of IFN-γ levels in pig sera after experimental infection is shown in Table 2. Analyzing the degree of non-specific immune response to vaccinal antigen, evaluated by the IFN-γ level in sera after experimental infection of piglets with virulent SHV-1, one can see that there were statistically significant increase in IFN-γ level on 3 and 5 dpi in pigs vaccinated I.D., when compared to pigs vaccinated I.M.

In this study the levels of CRP in pig sera after experimental infection with NIA-3 strain of SHV-1 were determined. CRP belongs to that kind of APP, whose level increases just 6-8 hours after affecting the organism by damaging stimulus and reaches its highest value during 24-48 hours (Kostro et al. 2003). In our study the increase of CRP level in pig sera from both vaccinated groups was statistically significantly lower than in control group in the most terms of blood sampling, showing good protective action of the vaccine through quick stimulation of mechanisms of cell-mediated and humoral immunity. The highest CRP level was observed in control group on 3 dpi, while in groups vaccinated I.M. and I.D. on 4 and 6 dpi, respectively. The obtained results were correlated with clinical signs observed at that time.

It is accepted, that acute phase response is parallel- ed by behavioral changes, like a decrease in the appetite and life activity, depression, elongation of the sleep time, slowing of digestion processes, as well as occurrence of fever as a result of disorders in the thermoregulation centre, induced by the action of IL-1, IL-6 and/or TNFα (Bigoszewski et al. 2001, Kostro...
Table 1. The CRP level (mg/l) in pig sera after experimental infection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>I (I.M.)</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>4.9</td>
</tr>
<tr>
<td>SD</td>
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<tr>
<td>II (I.D.)</td>
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<tr>
<td>X</td>
<td>3.3</td>
</tr>
<tr>
<td>SD</td>
<td>0.8</td>
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<tr>
<td>III (Control)</td>
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<td>X</td>
<td>5.1</td>
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<tr>
<td>SD</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Explanations: differences between groups A, B at p < 0.01; a, b at p < 0.05

Table 2. The IFN-γ (pg/ml) in pig sera after experimental infection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>I (I.M.)</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>12.23^Aa</td>
</tr>
<tr>
<td>SD</td>
<td>0.77</td>
</tr>
<tr>
<td>II (I.D.)</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>11.10^Ab</td>
</tr>
<tr>
<td>SD</td>
<td>0.71</td>
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<tr>
<td>III (Control)</td>
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</tr>
<tr>
<td>X</td>
<td>15.29^B</td>
</tr>
<tr>
<td>SD</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Explanations: differences between groups A, B, C at p < 0.01; a, b at p < 0.05

et al. 2003). In the control group, where intensification of clinical signs as well as increase of body temperature were observed from 3rd dpi up, simultaneously the highest CRP levels were noticed, while in both vaccinated groups, where clinical signs were weakly expressed, the levels of CRP in sera were also statistically significantly lower. Interferon plays an important role in e.g. increasing cytotoxicity of lymphocytes Tc and NK cells, MHC expression, macrophages activation and phagocytosis enhancement, as well as in the induction of expression of other cytokines, like IL-1, IL-6 or TNFα (Chinsakchai and Molitor 1994, Gołąb et al. 2002). In pigs infected with SHV-1 interferon appears in sera and urine 24 hours after infection, reaching its maximum level between 3 and 4 dpi. Then, its level gradually decreases (Wittmann et al. 1980, La Bonnardière et al. 1994). In our study considerably higher level of IFN-γ was observed in group II than in groups I and control. The increase of IFN-γ level was observed just on 1 dpi, but the highest value was noticed on 3 dpi in group of I.D. vaccinated pigs. Obtained results suggest, that I.D. vaccination, by ensuring the high activity of lymphocytes T, induces higher level of IFN-γ after exposition to virulent NIA-3 strain of SHV-1, when compared to I.M. vaccination. Thus, I.D. route of administration of deleted AD vaccine can be a good alternative and can be used in place of or parallelly to traditional I.M. route in AD “vaccination-eradication programme” in Poland.

References

Vimentin – the marker in dog kidney injury

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Abstract

The aim of this work was to perform the histopathological evaluation of 50 dog kidneys with special attention to vimentin immunocytochemical expression. In examined kidneys glomerulonephritis (50 cases), degeneration and/or necrosis of tubular epithelial cells (49 cases), acute or chronic interstitial nephritis with kidney fibrosis (39 cases) were diagnosed. Glomerular tuft atrophy and/or glomerular sclerosis was found in almost all kidneys. In 34 cases thickening of the blood vessel wall was also observed. In 47 cases vimentin expression was noted in low degree in podocytes of glomerular and basal membranes. In 49 cases the focal vimentin expression was found in tubular epithelial cells of kidney cortex and/or medulla. The weak expression of vimentin was also observed in the interstitium. In 46 dogs various degree of vimentin expressions were seen in blood vessel walls. Decrease in vimentin expression was correlated with glomerular damage and their appearance in tubular cells, as a symptom of the alteration of their cytoskeleton. The thickening of blood vessel walls seen in routine stained sections corresponded to changes in vimentin expressions. The vimentin expression is a very good marker of the early injury of cells in glomerules, tubules and blood vessels.

Key words: dog, kidney disease, vimentin expression

Introduction

There are many factors which play the important role in the physiological kidney function. Some authors considered that for normal kidney functions the most important among other factors are the normal blood supply and circulation.

The nephron is the kidney functional unit, so the primary glomerular diseases are responsible for secondary tubular damage. The primary glomerular diseases are caused by deposition of immunological complexes, thrombosis, emboli, and due to bacterial and viral infections. The long lasting noxious disease is followed by atrophy, or glomerular sclerosis, and secondary appearing regressive changes in tubular epithelium. The glomerular changes reciprocally may reflect in various tubular pathological changes with decreased blood supply. Kidney basal membranes play very important role in filtration process, cell adhesion, cell migration and differentiation, so their defects may be engaged in pathogenesis of glomerular and tubulo-interstitial diseases (Miner 1999).

Podocytes participate also in kidney filtration processes (Yaoita et al. 1999), so their alteration in diseased kidney is followed by hypertrophy of still healthy podocytes. These hypertrophic podocytes are more susceptible for pathological stimulation, so in consequence it leads to chronic kidney insufficiency (Yaoita et al. 2002). In Mammals, including Canidae glomelural podocytes express very strong vimentin immunocytochemical reaction. The intensity of this expression is also considered as the marker of their health status and functional ability (Yaoita et al. 1999).

The pathogenesis of extraglomerular kidney lesions are complicated and only partially recognized.
The noxious agents are causing tubular cell degeneration, necrosis and/or atrophy. The compensatory tubular mechanism is hypertrophy and regeneration of epithelium, mediated by various hormones and growth factors (Klahr and Morrissey 2000). Vimentin expression may indicate the proliferative podocyte activity, described in human and rat kidneys (Gröne et al. 1987).

The chronic kidney diseases with tubular atrophy are accompanied by proliferation of the interstitial tissue. According to recent data, fibroblasts of the interstitial tissue may originate from transformed tubular epithelial cells in diseased kidney (Klahr and Morrissey 2000, Okada et al. 2000, Stahl and Felsen 2001).

Vimentin has very strong expression in glomerular structures and endothelium and smooth muscles of the blood vessel wall in the healthy kidney. The results of research performed on homozygotic mice with “knock-out” vimentin gene indicated its crucial role in vascular adaptation capacity. The loss of this capacity was leading to the developing of so called end-stage kidney in those mice (Terzi et al. 1997).

The aim of this work was to perform the histopathological evaluation of dog kidneys, with special concern on vimentin immunocytochemical expression.

**Materials and Methods**

Kidneys were obtained from 50 dogs of both sexes, different breeds, from 4 months to 22 years old, sent for routine necropsy examination. The carcasses were selected according to anamnesis and post mortem lesions indicated for kidney diseases as the main cause of death. The time of collection of the material did not exceed 6 hours from the death of a dog. Both kidneys were fixed in 10% buffered formalin. From each kidney 2 – 4 specimens were collected, embedded in paraffin and stained with haematoxylin and eosin. Vimentin cytochemical expression was visualised on sections mounted on Silane-coated slides by using monoclonal anybodies clone V 9 (Novocastra).

The examined dogs were divided into 5 groups, according to the age of animals, as follows: I (0 – 1 year), II (1 – 5 years), III (>5 – 10 years), IV (>10 – 15 years) and V (>15 years).

**Results**

In dogs up to 1 year old focal glomerulonephritis was found. The inflammatory changes were accompanied by focal hydropic and parenchymatous degeneration seen in tubular epithelium. Vimentin expression was noted in some glomerular capsules, in podocytes of all glomerular and basal membranes. In 5 cases the focal vimentin expression was found in tubular epithelial cells of kidney cortex and medulla. The weak vimentin expression was also observed in the interstitium. In 4 dogs various degree expressions were seen in blood vessel walls.

In all dogs older then 1 year membrano-proliferative or proliferative glomerulonephritis was present. Glomerular tuft atrophy and/or glomerular sclerosis was found in almost all kidneys. In all the examined kidneys reversible injury and necrosis were seen in tubular epithelium. In almost all kidneys glomerular tuft atrophy and glomerular sclerosis were found. In some cases acute or chronic interstitial nephritis with kidney fibrosis was observed. Thickening of the blood vessel walls were found. Vimentin expression in Bowman’s capsule was seen focally. In all the examined kidneys vimentin expression was present in podocytes and in glomerular basement membranes, but in various degree of intensity. Vimentin expression in tubular epithelial cells was observed focally in the kidney cortex and in kidney medulla. In the interstitial tissue focal vimentin expression was noted. Vimentin expression in blood vessel walls was present and showed different intensity. In some cases vimentin expression was only in endothelial cells or completely absent in kidneys (Fig. 1, 2).

The histopathological lesions of kidneys observed in dogs 15-22 years of age were of the same type but more advanced, as in younger dogs. It was also manifested in changes of vimentin expression. Vimentin expression was found only in single glomerules, as in some blood vessel walls. In the majority of glomerules and blood vessel walls there was no vimentin expression. These vimentin expression alterations were accentuated by the appearing in multiple tubule cells of kidney cortex and medulla.

Histopathological lesions and vimentin expression in kidneys of all examinated cases are summarized in table 1.

<table>
<thead>
<tr>
<th>Part of nephron</th>
<th>Number of cases with histopathological lesions (HE)</th>
<th>Vimentin expression (IPOX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerules</td>
<td>50</td>
<td>47↓</td>
</tr>
<tr>
<td>Tubular epithelium cells</td>
<td>49</td>
<td>49↑</td>
</tr>
<tr>
<td>Blood vessel walls</td>
<td>34</td>
<td>46↓</td>
</tr>
</tbody>
</table>

Explanation:
↓ – reduced vimentin expression
↑ – increase in vimentin expression
Fig. 1. Vimentin expression found in tubular cells (→) and focally in blood vessel endothelium (→) in kidney of the IIIrd group. IPOX, x 100.

Fig. 2. High degree of vimentin expression visible in tubular cells, but the low degree of vimentin expression present in glomerules (→). There is no vimentine expression in blood vessel wall (→). IPOX, x 100.
Discussion

Vimentin expression in dog healthy nephron is limited to glomerules and it appears in tubular epithelium in chronic kidney diseases (Vilafranca et al. 1994). The tubular damage may be manifested by the increase in mitoses with transient expression of vimentin (Okoń 2003). Klahr and Morrissey (2000) observed that tubular cells undergoing early pathological changes transformed them into fibroblasts, which possessed expression of vimentin. The similar lesion was observed in tubular cells of rats exposed to carcinogens (Ward et al. 1994).

In all the examined kidneys of dogs up to 1 year membranous-proliferative glomerulonephritis was found. The tubular nephropathy was accompanied by the glomerular changes. Vimentin expression appears in degenerated tubules, in spite to glomerules, in which its expression decreased proportionally to the degree of glomerular damage. Vimentin expression in blood vessel walls was normal, indicating their good functional status (Terzi et al. 1997).

In the II nd group the histopathological changes were observed in all parts of the nephron, as well as in blood vessel walls. The degree of these changes correlated with vimentine expression. These findings were in accordance with previously presented data (Vilafranca et al. 1994, Terzi et al. 1997, Klahr and Morrissey 2000).

In the IIIrd and IVth groups the further enhancement of glomerulal pathological changes was manifested by appearing of so called semilunar crescents, which stimulated glomerular sclerosis or caused glomerular atrophy. These lesions were also responsible for the high degree of blood circulatory disturbances which were followed by the dystrophy of tubular epithelial cells. This was also accompanied by changes in vimentin expression. There was a decrease in vimentin expression correlating with glomerular damage and their appearance in tubular cells, as a symptom of the alteration of their cytoskeleton. The thickening of blood vessel walls seen in routine stained sections corresponded to changes in vimentin expressions. The alteration of vascular walls and interstitial fibrosis were the dominant type of lesions in the Vth group. The changes in vimentin expression were the most remarkable in those dogs kidneys, and were observed in nephrons and interstitial tissue and blood vessel walls.

In conclusion the results of the study have shown that kidney pathological changes increase with the age of a dog, but disease course is usually characterized by a silent, subclinical progression. The vimentin expression is a very good marker of the early cell alterations in glomerules, tubules and blood vessels. It is important to notice that the decrease in vimentin expression was seen in the blood vessel cells earlier than the development of the histopathological changes were noted. This observation suggests, that molecular disturbances present in the cell metabolism were followed by structural changes.

The results of the presented work may be applied in veterinary practice, as well as in comparative pathology research.

References


T-zone lymphoma in a dog. Preliminary morpho-immunohistochemical study

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Abstract

The author presents a case of canine T-zone lymphoma, correlating histopathological and immunohistochemical features. This lymphoid neoplasm, known in human medicine, is not contemplated in the actual World Health Organization (WHO) Classification of 2002, regarding the Haematopoietic Tumours of Domestic Animals.

Key words: dog, T-zone lymphoma, histopathology, immunohistochemistry

Introduction

As Pinkus and Said (2001) and Ralfkiaer et al. (2001) have shown, T-zone lymphomas are characterized by an infiltrate that involves lymph node’s paracortex. In human medicine they are included in the category of “Peripheral T-cell lymphoma, unspecified” and considered as low grade.

Materials and Methods

Haematoxylin and eosin stained sections of formalin fixed, paraffin embedded lymph node tissue of a four year old, mix breed, dog, with persistent submandibular lymphadenomegaly and no other signs, were examined to investigate the histopathological features of the lesion. Immunohistology, using the streptavidin-biotin-peroxidase method, was performed. As primary antibodies, mouse anti-human CD3 (T-cell marker, diluted 1:100, DAKO) and CD79α (B-cell and plasma cell marker, diluted 1:150, BIOGENEX) were used.

Results

Histologically, the architecture of the lymph node was effaced by a mass with a follicular (i.e. not diffuse) histological pattern consistent with follicles of small, dark, central areas surrounded by large, expanding, pale areas (Fig. 1-3). Cytologically, two types of cell population were identified. The pale areas were composed of small to intermediate, generally monomorphic, neoplastic lymphoid cells, with increased nuclear details, round to irregularly indented nuclei, irregularly thickened nuclear membranes, distinct chromocenters, irregular para-chromatin clearing, and pale cytoplasm. Intracellular boundaries were generally distinct. Mitoses were rare. The dark areas were composed of small, indented, dark, relatively mature, non-neoplastic lymphocytes (Fig. 4). Immunohistochemically, the neoplastic cells of the pale areas were CD3+ and CD79α-, demonstrating T-cell lineage (Fig. 5), whereas the non-neoplastic cells of the dark areas were CD79α+ and CD3-, demonstrating B-cell lineage (Fig. 6).
Fig. 1. The paracortex of the lymph node is effaced by a mass with a follicular (i.e. not diffuse) histological pattern consistent with follicles (spherical to oval structures) of small, dark, central areas surrounded by large, expanding, pale areas. Haematoxylin and eosin staining, x 1.25. Fig. 2. The benign mantle cells in the centre of the follicles are CD79α+ (brown); i.e. they are positive for a marker (CD79α) specific for the cells of the B lineage. Immunohistochemistry CD79α, x 1.25. Fig. 3. The neoplastic cells in the expanded paracortex (or T-zone area) are CD3+ (brown); i.e. they are positive for a marker (CD3) specific for the cells of the T lineage. Immunohistochemistry CD3, x 1.25. Fig. 4. Cytological detail of the same canine lymph node. In the follicular structures, two different cell populations are present. The malignant T cells of the pale areas (upper slide) are small to intermediate, generally monomorphic, with increased nuclear details, round to irregularly indented nuclei, irregularly thickened nuclear membranes, distinct chromocenters, irregular parachromatin clearing, and pale cytoplasm. Intracellular boundaries are generally distinct. The benign mantle cells of the dark areas are small, indented, dark, relatively mature, benign lymphocytes (low slide). Haematoxylin and eosin staining, x 40. Fig. 5. Detail of the follicular architecture of the mass. The malignant T cells of the expanded paracortex (or T-zone area) are CD3+. Immunohistochemistry CD3, x 40. Fig. 6. Detail of the follicular architecture of the mass. The non-neoplastic mantle cells (B cells) of the dark areas of the nodular (follicular) structures are CD79α+. Immunohistochemistry CD79α, x 40.
Discussion

In the first stages, follicular centre cell lymphomas (FCCL), mantle cell lymphomas (MCL), marginal zone lymphomas (MZL), and T-zone lymphomas assume a follicular histological pattern, characterized by the presence of spherical (nodular) structures.

Nevertheless, these spherical structures are differently defined in each particular neoplasm. In FCCL, MCL and T-zone lymphomas, the follicular histological pattern generally is consistent with a two layer spherical structure. In FCCL there is an expansion of the inner compartment of the follicle, because of proliferation of lighter staining, malignant, follicular centre cells (centrocytes and centroblasts). These are surrounded by a thinned perimeter of dark staining, benign mantle cells. In MCL there is an expansion of the outer compartment of the follicle, because of proliferation of dark staining, malignant mantle cells. These surround lighter staining, shrunken, almost depleted germinal centres. In T-zone lymphoma, there is an expansion of the compartment of the paracortex area, because of proliferation of lighter staining, malignant T-cells. These surround the dark staining, benign cells of the mantle zone, forcing them to usually aggregate as a single dense cluster at the centre of the follicle with consequent depletion of the follicular centre cells. Because the dark mantle cells appear at the centre rather than at the periphery of the lesion, as in follicular lymphomas, the appearance of these structures in T-zone lymphoma is referred to as “inverted follicles”. Finally, MZL has a characteristic follicular pattern consistent with a three layer spherical structure. In the centre there are the benign, lighter staining, follicular centre cells. These are surrounded by a rim of dark staining, benign, mantle cells. Around and outside of the mantle cells there is a continuous cuff of light staining, marginal zone cells.

The sections examined in this case appear with the follicular histological pattern of the T-zone lymphoma described in human medicine. Cytopathology and immunohistochemistry reinforce and confirm the diagnosis based on the histological pattern consistent with T-zone lymphoma in this dog, and this is the first time that this tumour is described in the veterinary medicine. More specific immunohistochemical studies, and molecular investigations need to be performed to better define the characteristic clinicopathological features of canine T-zone lymphoma and further updating of the WHO system 2002 is required.

References


The distribution and chemical coding of porcine urinary bladder trigone-projecting neurons located in prevertebral ganglia other than IMG

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Abstract

Combined retrograde tracing and double-labelling immunofluorescence were used to investigate the distribution and chemical coding of neurons in prevertebral ganglia supplying the urinary bladder trigone (UBT) in juvenile pigs (n=5). The urinary bladder trigone-projecting neurons (UBT-PN) were mainly located in the celiac-superior mesenteric ganglion complex (C-SMG), as well as in the ovarian (OG), aortico-renal (ARG) and adrenal (ADG) ganglia. Immunohistochemistry disclosed that the vast majority of UBT-PN were noradrenergic (i.e. TH-positive). Many noradrenergic neurons contained NPY or, less frequently, SOM and/or GAL. This study has revealed a relatively large population of differently coded UBT-PN located in porcine PVG other than the IMG. Thus, as may be judged from their somatotopic organization and neurochemical coding, sympathetic pathway to the UBT is more complex than suggested hitherto.

Key words: prevertebral ganglia, urinary bladder trigone, immunohistochemistry, retrograde tracing, neuropeptides, pig

Introduction

The function of the urinary bladder is to store urine and, at appropriate intervals, to evacuate it. What appears to be a relatively simple sequence of events is on deeper analysis a sophisticated process requiring close coordination between the different components of the lower urinary tract. It is well recognized yet that an intact sympathetic supply to the bladder is not essential for micturition to take place. The primary role of the sympathetic nervous system in lower urinary tract function is to provide an excitatory input to the bladder to maintain closure of the outlet. In addition, there is evidence that the sympathetic supply to the bladder serves to inhibit parasym pathetic activity during the accommodation phase of urine storage (De Groat and Steers 1988). The innervation of these organs has been found to originate from three sets of peripheral nerves: sacral parasympathetic (pelvic nerves), thoracolumbar sympathetic (hypogastric nerves and prevertebral ganglia – PVG), and sacral somatic (primarily the pudendal nerves).
(for references see Pidsudko 2004). Since our knowledge on the distribution and chemical coding of the urinary bladder trigone-projecting neurons in the pig is, at the moment, limited only to the IMG (Pidsudko 2000), we have combined retrograde tracing and double-immunolabelling to elucidate: 1) the involvement of PVG other than IMG in this neural pathway and 2) neurochemical features of their UBT-PN.

### Materials and Methods

The study was performed on 5 juvenile pigs of the Large White Polish breed. The animals were housed and treated in accordance with the rules approved by the local Ethical Commission. In the experimental animals, the fluorescent retrograde neuronal tracer Fast Blue (FB) was injected into both the left and right side of the urinary bladder trigone during laparatomy performed under pentobarbital anaesthesia. After a survival period of three weeks the pigs were reanaesthetised and transcardially perfused with 4% buffered paraformaldehyde. The collected prevertebral ganglia (i.e., C-SMG, ADG, ARG and OG) were postfixed by immersion in the same fixative for several hours and finally stored in 18% sucrose until sectioning. The left and right PVG were cut into 10 µm thick cryostat serial sections. FB-labelled cell counts were done prior to the immunohistochemistry. To determine the relative number of the UBT-PN, the neurons were counted in every fourth section from both the left and right ganglia in all the animals. Only neurons with a clearly visible nucleus were considered. All the sections containing retrogradely labelled neurons were processed for double-labelling immunofluorescence with antibodies listed in Table 1.

### Table 1. Antisera used in the study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Code</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DβH</td>
<td>rabbit</td>
<td>DZ 1020</td>
<td>1:500</td>
<td>Affiniti, UK</td>
</tr>
<tr>
<td>CGRP</td>
<td>rabbit</td>
<td>RPN 1842</td>
<td>1:1600</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>TH</td>
<td>mouse</td>
<td>1017381</td>
<td>1:40</td>
<td>Boehringer Mannheim, GER</td>
</tr>
<tr>
<td>GAL</td>
<td>rabbit</td>
<td>4600-5004</td>
<td>1:1600</td>
<td>Biogenesis, UK</td>
</tr>
<tr>
<td>SOM</td>
<td>rat</td>
<td>8330-0009</td>
<td>1:30</td>
<td>Biogenesis, UK</td>
</tr>
<tr>
<td>SP</td>
<td>rat</td>
<td>8450-0505</td>
<td>1:250</td>
<td>Biogenesis, UK</td>
</tr>
<tr>
<td>VIP</td>
<td>rabbit</td>
<td>20077</td>
<td>1:200</td>
<td>Incstar, MO, USA</td>
</tr>
<tr>
<td>NPY</td>
<td>rabbit</td>
<td>NA 1233</td>
<td>1:400</td>
<td>Affiniti, UK</td>
</tr>
<tr>
<td>NPY</td>
<td>rat</td>
<td>NZ 1115</td>
<td>1:200</td>
<td>Affiniti, UK</td>
</tr>
</tbody>
</table>

| FITC-conjug. goat anti-rabbit IgG | 1:400 | Jackson Immunores. Lab., USA |
| FITC-fluorolink. goat anti-mouse IgG | 1:400 | Jackson Immunores. Lab., USA |
| FITC-fluorolink. goat anti-rat IgG | 1:400 | Jackson Immunores. Lab., USA |
| Biotinylated goat anti-rabbit IgG | 1:400 | Dako, DK |
| Biotinylated goat anti-rat IgG | 1:400 | Amersham, UK |
| Biotinylated goat anti-mouse IgG | 1:400 | Amersham, UK |
| Cy3-conjugated streptavidin | 1:4000 | Dianova, Hamburg, GER |

Specificity of both the tracing and labelling techniques was checked as described previously (Pidsudko et al. 2001).

### Results and Discussion

The porcine PVG were found to contain many FB-positive (FB+) neurons projecting to the urinary bladder trigone which were distributed within both the left and right ganglia. The PVG complexes contained $1181 \pm 120$ (mean ± S.E.) of FB+ neurons. The majority of them (about 90% of all FB+ neurons) was localized in the OG. $921 \pm 98$ of FB+ neurons were found in OG, $216 \pm 19$ in ARG and $44 \pm 3$ in ADG, considering both the left and right PVG. Immunohistochemistry revealed that the vast majority of the FB+ UBT-PN were TH/DβH-IR (approx. 88%). A prominent proportion of these neurons contained also immunoreactivity to NPY (18%; Fig. 1.) and a smaller number was SOM- (5%; Fig. 2.) or GAL-IR (0.6%). The present study has shown that the efferent innervation of the porcine urinary bladder trigone originate not only from the IMG, but also from other PVGs. This corresponds well with findings obtained in laboratory and domestic animals, in which it has been shown that not only sympathetic chain ganglia, but also PVG, as sources of the efferent nerve supply, are crucial for the maintenance of the lower urinary tract functions (Downie et al. 1984, De Groat and Steers 1988). Immunohistochemistry has revealed that many of UBT-PN is noradrenergic in nature; however it is apparent that a lot of them contain neuropeptides as well. It should be stressed that some of them contain NPY- and, additionally, in a smaller proportion SOM- or GAL- IR (Jänig and McLachlan 1987). The precise role of these different sub-populations of neurons has
yet to be defined. The adrenergic activation of the smooth muscle of the urinary bladder was shown to be mediated via α-adrenoreceptors, and release both noradrenaline (NA; acting here as a relaxant agent) and a neurotransmitter that contracts these muscles. SOM-IR was seen in sparse to dense meshwork of nerve fibres throughout the pig urinary tract, but little is known of this peptide role. It is possible that neurons containing SOM have a local modulatory or inhibitory effect, as SOM is capable to powerfully blocking the release and interfere with the action of other transmitters (Jänig and McLachlan 1987, Pidsudko 2004). In summary, the porcine PVG have been found to contain many neurons projecting to the urinary bladder trigone. This study has also revealed a relatively large population of differently coded PVG UBT-PN, that are probably involved in the neural control of the urinary bladder.

Acknowledgements

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References


Caffeic acid feeding of pregnant mice influences the prenatal development of their offspring

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Abstract

The aim of the present study was to evaluate the effect of one of polyphenols, caffeic acid (CA), administered to the pregnant Balb/c mice on the embryos growth, angiogenic activity of embryos tissue, VEGF and bFGF production. The study was performed on 2-3 months old Balb/c mice fed during pregnancy caffeic acid (1 or 6 mg/day). The pregnancy was terminated at the 18th day, embryos were extracted, weighted and homogenized. Content of CA in embryos homogenates was estimated by HPLC. Concentration of angiogenic factors was tested by ELISA. Homogenates angiogenic activity was assessed in mice cutaneous angiogenesis assay. Feeding pregnant mice caffeic acid (6 mg/day) decreased the embryos weight and bFGF concentration (close to significance). CA did not decrease the angiogenic activity of embryos tissue, in higher dose this activity was elevated. The influence on VEGF concentration was not observed.

Key words: caffeic acid, pregnant mouse, angiogenesis, VEGF, bFGF, HPLC

Introduction

Common articles of consumption like tea, coffee, cocoa, or chocolate, are rich in methylxanthines and phenolic acids (i.e. caffeic acid, chlorogenic acid). Our previous studies have shown that these compounds exert antiangiogenic effect suppressing angiogenic factors production (VEGF, bFGF) and tumor-induced angiogenesis (Skopińska-Różewska et al. 1998, Barcz et al. 1998, Balan et al. 1999, Skopińska-Różewska et al. 2003). Moreover, we reported that pregnant mice chocolate feeding resulted in reduction of relative length of limbs and thigh bones in their progeny. VEGF content in offspring bones was also lowered (Skopiński et al. 2003). Human daily diet contains about 1 g of phenolic acids. We put forward a hypothesis that consumption of phenolic acids during pregnancy may influence embryogenesis. Therefore, the aim of the present study is to estimate the effect of one of polyphenols, caffeic...
acid, administered to the pregnant Balb/c mice on the embryos growth, angiogenic activity of embryos tissue and proangiogenic factors (VEGF, bFGF) production.

**Materials and Methods**

The study was performed on 2-3 months old inbred Balb/c mice fed during pregnancy (from 1st day to 18th day) 1 or 6 mg/day of caffeic acid (Sigma Aldrich, Poznań, Poland) served on wheat flakes. Control mice were fed only wheat flakes. Doses of caffeic acid used in this study were taken basing on our previous experiments (Bałan et al. 1999). The pregnancy was terminated on the 18th day using Morbital, embryos were extracted, weighted, suspended in phosphate buffer saline – 1 g/ml (PBS, Polfa, Kutno), homogenized using ultrasound sonicator (VirSonic, Virtis, USA) and frozen in -75°C for further evaluation. Experiments were approved by the Local Ethical Committee. Proangiogenic cytokines VEGF and bFGF concentration was estimated by the ELISA method in the embryos homogenates, according to the manufacturer’s instructions. The cutaneous angiogenesis assay was performed according to method modified by Sidky and Auerbach. Briefly, 0.05 ml embryos homogenate samples were injected intradermally into anaesthetized Balb/c mice. After 72 hours mice were sacrificed and new blood vessels were identified and counted in the dissection microscope (6 x magnification) in the central 1/3 of the microscopic field. The caffeic acid content in embryos homogenates was estimated by the high performance liquid chromatography (HPLC) system. Briefly, samples were diluted with 0.1 M NaOH, shaken on ultrasonic bath, passed through 0.45 µm filter and injected into HPLC system consisting of Luna C18 analytical column (Phenomenex, USA) with the mobile phase consisted of methanol-water-acetic acid delivered at the flow-rate of 1.0 ml/min, UV-Vis detector SPD-6 AV operated at 327 nm (Shimadzu, Germany) and a Rheodyne Model 7125 injection valve (Berkeley, USA) equipped with 20 µl loop. Quantitative results were obtained by

<table>
<thead>
<tr>
<th>Caffeic acid mg/day</th>
<th>Number of litters</th>
<th>Mean number of embryos/litter ± SE</th>
<th>Mean embryo weight/litter ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>6</td>
<td>8.7 ± 0.38</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>11.2 ± 0.95</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>6.7 ± 1.56 n.s.</td>
<td>0.42 ± 0.05 p&lt;0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Caffeic acid mg/day</th>
<th>Number of litters</th>
<th>Mean number of blood vessels ± SE</th>
<th>VEGF concentration (pg/ml) ± SE</th>
<th>bFGF concentration (pg/ml) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>6</td>
<td>15.7 ± 0.88 n=89</td>
<td>393 ± 45</td>
<td>897 ± 105</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>16.5 ± 0.92 n=72</td>
<td>326 ± 8.5 n.s.</td>
<td>978 ± 107</td>
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<tr>
<td>6</td>
<td>6</td>
<td>18.2 ± 0.51 n=60 p&lt;0.05</td>
<td>352 ± 65 n.s.</td>
<td>603 ± 105 0.05&lt;p&lt;0.1</td>
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<tr>
<th>Caffeic acid mg/day</th>
<th>Number of litters</th>
<th>Caffeic acid concentration (µg/g) ± SE</th>
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<tbody>
<tr>
<td>0 (control)</td>
<td>5</td>
<td>0.16 ± 0.04</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.45 ± 0.10</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1.01 ± 0.16</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>
comparing samples and standard (caffeic acid, Sigma-Aldrich, Poznań, Poland) peak areas.

**Results and Discussion**

Caffeic acid feeding exerted significant inhibitory effect on the embryos weight in both examined doses (1 and 6 mg/day). The mean number of embryos in litter was significantly elevated in dose of 1 mg/day of caffeic acid \((p<0.05)\) (Table 1). Caffeic acid feeding in dose 6 mg/day, stimulated the angiogenic activity of embryos homogenates \((p<0.05)\) (Table 2). The same dose decreased bFGF concentration, the difference was close to significance \((0.05<p<0.1)\). Caffeic acid in dose of 1 mg/day did not affect these parameters. The influence of both doses of caffeic acid on VEGF concentration was not observed (Table 2). The positive correlation between angiogenic activity of homogenates and caffeic acid concentration (HPLC analysis) was observed \((r=0.5819)\). Caffeic acid concentration in embryos homogenates was proportional to applied doses (Table 3).

In the available literature we did not find any data about the influence of phenolic acids on embryonic angiogenesis. In earlier study on the effect of chocolate feeding of pregnant mice, significant inhibition of embryos tissue angiogenic activity was observed (Skopińska-Różewska et al. 2003). Chocolate contains several active substances, among them methyloxanthines and polyphenols, so described by these authors inhibitory effect may depend on combined action of these compounds. In our study caffeic acid did not decrease the angiogenic activity of embryos tissue, in higher dose this activity was elevated. Moreover, caffeic acid did not influence VEGF concentration and bFGF concentration was not statistically significantly reduced. Some authors reported that phenolic acids, among them caffeic acid, stimulate angiogenic activity of human mononuclear leukocytes (MNL) (Glinkowska et al. 1997). The present study shows that caffeic acid influences prenatal development. But it is necessary to evaluate further the mechanism of caffeic acid action on embryogenesis and its influence on postnatal development.

**Acknowledgements**

This work was supported by KBN grant 3PO5EO3722.

**References**


In vitro influence of synthetic pyrethroid – cypermethrin on phagocytes of heterothermic and homeothermic animals

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Abstract

The in vitro effect of cypermethrin on fish and rabbit polymorphonuclear and mononuclear phagocytic cells was determined. Experimental studies were carried out on cells isolated from pronephros and spleen of carp and from the rabbit blood. Cypermethrin was added at concentrations of 0.0001, 0.0005, 0.001, 0.005 µg/ml medium to the fish phagocyte cell suspension, and to the rabbit phagocyte cell suspension was added at concentrations of 1, 2.5, 5, 10 µg/ml medium. The results indicate that cypermethrin in in vitro studies has a greater suppressive effect on carp than rabbit phagocytes.

Key words: cypermethrin, metabolic activity of phagocytes, carp, rabbit

Introduction

Pyrethroid compounds are commonly used in households to eradicate pests and insects. Cypermethrin – alpha cyano pyrethroid, is an example of a synthetic pyrethroid, that has short stability in the environment. Half-live period in soil is 2-4 weeks and 2 weeks in natural estuaries. Cypermethrin exhibits a very toxic influence on fish. The insecticide easily comes to tissues from water and its bioaccumulation index is about 1000. That group of chemical compounds is quite safe in use, quickly metabolised and excreted from the organism of higher vertebrates. Cypermethrin affects the nervous system mainly leading to disturbances in potassium, calcium and ion regulation in neurons.

The purpose of the studies was to determine in vitro the influence of cypermethrin on metabolic activity of carp (Cyprinus carpio L.) and rabbit (Oryctolagus cuniculus).

Materials and Methods

Pure cypermethrin was used in the study (Sigma, Aldrich). Material for the in vitro study was taken from five healthy carps and rabbits. Carps were killed by an anaesthetising overdose of 0.2% Propiscin preparation (IRS, Żabieniec, Poland). Pronephros and spleen were dissected mechanically and pressed through a nylon mesh. Cells were suspended in culture medium RPMI 1640 (Sigma, Aldrich). In order to isolate the phagocytes the suspension (mainly macrophage and neutrophils) was put on a gradient Gradisol G (Aqua-Medica, Łódź, Poland) and centrifuged at 400 g for 30 min. at a temperature of 4°C.
The phagocytes were collected and suspended in culture medium RPMI 1640. The blood was taken from rabbit ear veins. Isolation of blood phagocytes (mainly neutrophils) was performed using gradient Gradisol G solution.

Cypermethrin was added at concentrations of 0.0001, 0.0005, 0.001, 0.005 µg/ml medium to the cell suspension (3-5 x 10⁶ cells ml⁻¹) isolated from pronephros and spleen. The preparation was added to rabbit phagocytes isolated from the blood at concentrations of 1, 2.5, 5, 10 µg/ml medium.

In the purpose to determine the metabolic activity of phagocytes the RBA – Respiratory Burst Activity method described by Secombes (1990) was used. Each experimental and control determination was performed in six fold repetition.

The results were analysed statistically by ANOVA at a difference of means p≤0.05. The post-hoc Duncan test was performed to assess the differences of means.

**Results**

The results of the in vitro studies show the suppressive effect of cypermethrin on phagocytes isolated form carp and rabbit. After addition of cypermethrin at concentrations of 0.005, 0.001, 0.0005 µg/ml to the carp cell suspension, a statistically significant decrease of RBA test values was found (Fig. 1).

Various, a dose – dependent activity of phagocytes isolated from rabbit was found. Cypermethrin at a concentration of 5, 10 µg/ml medium causes suppression of metabolic and phagocytic activity on rabbit phagocytes (Fig. 2). After application of cypermethrin at a concentration of 2.5 µg/ml the parameters were not changed significantly in comparison to the control group. After administration of preparation in a concentration of 1 µg/ml statistically significant increase of metabolic activity of phagocytes isolated form rabbit blood was found.

**Discussion**

Several studies conducted in aquatic environmental have demonstrated that immune functions in fish collected from contaminated environments were severely impaired. The presented results showed that cypermethrin affects more suppressively on the metabolic activity of the cells isolated from pronephros and spleen of carp than on the metabolic activity of the cells isolated from rabbit blood. It is probably a consequence of higher sensitivity of fish to pyrethroids. Furthermore the results indicate that the modulatory influence of cypermethrin on phagocytic cells depends on the applied concentration (suppression at higher and stimulation at the lowest concentrations used). The results of the earlier studies also show that cypermethrin possess a strong immunotoxic effect on fish immunocompetent cells, manifested by a decreased number of antibody secreting cells – ASC (Rymuszka et al. 2002). These results are consistent with those obtained by other authors. Sopińska and Guz (1998) investigated the effect of permethrin (synthetic pyrethroid) on immune cells of carp. Permethrin decreases the phagocytic ability of neutrophils and macrophages residing in blood and pronephros. O’Halloran et al. (1996) showed the immunotoxic influence of esfenvalerate – a synthetic pyrethroid on rainbow trout lymphocyte T and B mitogenesis.

The pyrethroid insecticides are not free from adverse effects on immune system of higher vertebrates. The specific immunological effects include changes in the humoral response rate, thymus weight, and overall system resistance to infections (Tulinska et al. 1995, Madsen et al. 1996, Santoni et al. 1997). Cypermethrin and permethrin also decrease the division rates of T – and B – lymphocytes in laboratory cultures, suggesting a functional impairment of these cells (Stelzer and Gordon 1984, Blaylock et al. 1995).
In conclusion, the results of this study show that cypermethrin possess immunotoxic effect both on fish and rabbit phagocytes in vitro.

References


The influence of dexamethason on the ovarian and uterine structure and ER, PR and alfa-inhibin expression in pseudopregnant rabbits

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Abstract

Dexamethason given in therapeutic doses to pseudopregnant rabbits caused marked increase in the ovarian interstitial tissue transformation into luteinized cells. These changes were proved by the immunocytochemical expression of alfa-inhibin. The degree of interstitial luteinisation was proportional to the dose of dexamethason. Dexamethason effect was seen also in ovarian epithelium by the increase in their nuclear expression of ER. Simultaneously, this hormone caused changes in the uterine horn structures, with dose-dependent effect. It was manifested by epithelial dystrophy, loss of secretion ability and loss of nuclear ER/PR receptors expression in endometrial cells. In summary, dexamethason given in therapeutic doses to pseudopregnant rabbits caused significant changes in the ovarian and uterine microscopic structure, which were expressed by molecular cellular changes too.

Key words: pseudopregnant rabbit, dexamethasone, uterus, ovary, pathomorphology, immunocytochemistry

Introduction

Dexamethason is the popular corticosteroid hormone used in the therapy of human and animal diseases. According to the recently published data there may be the close interaction between adrenal and gonadal steroids (Doufourny and Skinner 2002). As well the research provided on the stress influence on the animals reproduction indicated the interaction between adrenal function and hypothalamus-pituitary secretion which play the basic role in regulating gonadal function (Plas-Roser and Aron 1981). It is also proved, that cortisol inhibits LH secretion.

The aim of the work was to investigate the structure of reproductive organs of pseudopregnant rabbits exposed to the therapeutic doses of dexamethason.

Materials and Methods

The experiment was performed on 9 female healthy conventional white Polish female rabbits, divided into 3 groups: A – 3 rabbits of the control
group; B – 3 rabbits, which received 0.5 mg/kg b.w. of
dexamethason (Dexafort, Intervet, Holland) i.m.; and
C – 3 rabbits, which received 1.5 mg/kg b.w. of
dexamethason i.m. After 16 days all the rabbits were
mated twice in 3 day intervals by vasectomised male
rabbit. All the rabbits were euthanized on the 22nd
day of the experiment (euthanasia was performed by i.v.
injection of barbiturates). The ovaries and uterine
horn specimens were fixed in 10% buffered formalin.
Paraffin sections were stained haematoxylin and cosin
(HE) and AB/PAS histochemical reaction. The paraffin
sections put on Silan covered glasses were used for
immunocytochemical reaction for ER receptors
(murine monoclonal antibodies IgG1 class, clone CC
4-5, Novocastra NCL-ER-LH 2), for PR receptors
(murine antibodies IgG1, clone 1 A6, Novocastra
NCL-PGR), and for alfa-inhibin expression (monoclonal antibodies inhibin alfa subunits R1 MCA 951 S, Serotec). The specimens of ovary and uterus were
routinely used for positive controls. The receptors ex-
pression was evaluated by the scale: 0 – negative, faint –
very low, + – low, ++ – weak, +++ – strong,

Explanation: 0 – negative, faint – very low expression, + – low expression, ++ – weak expression, +++ – strong expression,

Results

1. Microscopic structure of the ovary and uter-
ine horns in HE and AB/PAS staining methods.

1 A – rabbits of the control group. In HE stained
ovaries there were numerous corpus luteum antral
primary follicles, also with numerous apoptotic fol-
icles (Fig. 4). The main changes were localized in
ovarian interstitial tissue manifested by hyperplasia
and hypertrophy of theca interna cells and appearance
of the nests of luteinised interstitial cells. In uterine
horns endometrial proliferation, hyperaemia and
oedema with transformation of epithelial cells into se-
cretery phase were found. Glycosaminoglicans
(GAG) secretion was seen in the uterus surface and
by glandular epithelium (Fig.1).

1 B – experimental rabbits, which received 0.5
mg/kg b.w. i.m. of dexamethason. In ovaries of these
rabbits (Fig. 5) numerous antral follicles were
present but their oocytes were undergoing necrobiotic
changes. The same changes were observed in second-
ary follicles. However, on the first place changes were
observed in theca cells. The changes were manifested
by the appearing of very numerous polygonal cells
among normal theca cells. The theca externa/inter-
stitium cells also looked like luteinised cells, however,
corpora lutea were also present. In uterine horns atro-
phic changes were observed with the simultaneous de-
crease in GAG secretion (Fig. 2).

1 C – experimental rabbits, which received 1.5
mg/kg b.w. i.m. of dexamethason. In HE stained
ovaries the apoptotic changes were present almost in
all follicles. The follicular microysts formation were
observed too. The luteinisation of theca and inter-
stitial cells described in B group of experimental rab-
bits were of high degree in this group. Almost all of
the ovarian interstitium cells were transformed into
large polygonal cells with similar microscopic appear-
ance to the luteal cells (Fig. 6). The microscopic struc-
ture of uterine horns were similar to the rabbits of
B experimental group, however, hydropic degener-
ation of epithelial cells were additionally found. The
affected cells lost almost completely their secretory
ability, as was seen in AB/PAS reaction (Fig. 3).

2. The immunocytochemical expression of
alfa-inhibin, ER and PR receptors.

2 A – rabbits of the control group. In normal
ovary the follicular cells posses expression of alfa-in-
hibin in the cytoplasm. In the ovary of control
 pseudopregnant rabbit it was seen also in cytoplasm of
follicular cells, in various intensity of expression. The
faint alfa-inhibin expression was also observed in
single theca cells, as well as in small groups of theca
externa/interstitium cells. Some marginally localized
corpus luteum cells also showed faint expression of
alfa-inhibin (Fig. 7). The expression of ER nuclear
receptors was found in ovarian epithelial cells. The
faint ER expression was found in some follicular cells
of secondary follicles and cumulus oophorus cells.
Also very few cells of corpus luteum possessed faint
ER expression. In the uterus the faint ER expression
were seen in basal glandular cells, interstitial and my-
ometrium cells. The expression of PR nuclear recep-
tors was observed in follicular cells of secondary and
tertiary follicles. In the uterus very weak expression of
PR nuclear receptors was observed in glandular epi-
thelium.

2 B – experimental rabbits, which received 0.5
mg/kg b.w. i.m. of dexamethason. In comparison to

Table 1. Alfa-inhibin expression in ovaries of the control and the dexamethason-exposed pseudopregnant rabbits.

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>Follicular cells</th>
<th>Corpus luteum</th>
<th>Thela interna</th>
<th>Thela externa/interstitium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>++</td>
<td>+++ (cumulus oophorus cells)</td>
<td>faint</td>
</tr>
<tr>
<td>B</td>
<td>faint</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>faint</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

Explanation: 0 – negative, faint – very low expression, + – low expression, ++ – weak expression, +++ – strong expression,

+++ – very strong expression
Fig. 1. The uterine horn of the control pseudopregnant rabbit with well marked secretion of AB positive GAG in endometrial epithelium cells. AB/PAS reaction, x 10.

Fig. 2. The uterine horn of the pseudopregnant rabbit, which obtained 0.5 mg/kg b.w. i.m. of dexamethason. The endometrial proliferation and the decrease in AB positive GAG secretion in uterine epithelium are visible. AB/PAS reaction, x 10.

Fig. 3. The uterine horn of the pseudopregnant rabbit, which obtained 1.5 mg/kg b.w. i.m. of dexamethason. There are endometrial proliferation with the loss of secretory acivity and advanced hydropic degeneration of uterine epithelium cells. AB/PAS reaction, x 10.

Fig. 4. The ovary of the control pseudopregnant rabbit. Haematoxylin and eosin staining, x 10.

Fig. 5. The ovary of the pseudopregnant rabbit exposed to 0.5 mg/kg b.w. i.m. of dexamethason. The remarkable ovarian interstitial cells transformation and the follicular apoptosis are visible. Haematoxylin and eosin staining, x 10.

Fig. 6. The ovary of the pseudopregnant rabbit exposed to 1.5 mg/kg b.w. i.m. of dexamethasone. The transformed into luteal-like cells are almost all interstitial cells. Haematoxylin and eosin staining, x 10.

Fig. 7. The ovary of the pseudopregnant rabbit with follicular cell alfa -inhibin expression. IPOX, x 10.

Fig. 8. The ovary of the pseudopregnant rabbit exposed to 0.5 mg/kg b.w. i.m. of dexamethasone. The expression of alfa-inhibin is visible in follicular and in theca cells. IPOX, x 20.

Fig. 9. The ovary of the pseudopregnant rabbit exposed to 1.5 mg/kg b.w. i.m of dexamethason. The very advanced changes in alfa-inhibin expression are present in the stromal cells. The expression of various intensity is observed in almost whole ovarian interstitium cells. IPOX, x 20.
control rabbits, the expression of alfa-inhibin was increased in follicular cells of secondary and tertiary follicles. The increase in alfa-inhibin expression was also observed in theca interna and theca externa/interstitium cells (Fig. 8). In corpus luteum cells the increase in the expression of this cytoplasmic hormone was faint. Ovarian ER expression was of high intensity with concomitant hyperplasia of the ovarian epithelium. In the uterus there was not found ER expression. In the ovary, the faint expression of nuclear PR receptors was noted in cells which had features of luteinisation, and in nuclei of follicular cells of secondary follicles. There was no expression of PR nuclear receptors in the uterine horns of this experimental rabbit group.

2 C – experimental rabbits, which received dexamethason in doses of 1.5 mg/kg b.w. i.m. In this group of rabbits alfa-inhibin expression was significantly increasing in theca externa/interstitium cells, appearing in almost all ovarian interstitial cells but it was in various degree of expression (Fig. 9). The alfa-inhibin expression in the follicular and corpus luteum cells behaved similarly as in B experimental rabbit group. The ER expression in the ovarian epithelium was of very high intensity. In some follicular corona radiata cells low intensity of ER expression was observed. Similarly to B group of rabbits ER expression was not found in the uterus. The expression of nuclear PR receptor examined in ovaries and uterine horns were similar to the B experimental group of rabbits.

Discussion

The results of the present experiment indicated that both therapeutic dexamethason doses caused the changes in structure and function of the ovaries and uterus of pseudopregnant rabbits. The expression of the examined immunocytochemical markers that is: alfa-inhibin, ER and PR receptors, allows to conclude, that dexamethasone was responsible for significant functional changes in both examined reproductive organs. The most remarkable were the ovarian changes seen in alfa-inhibin expression. The physiological role of alfa-inhibin in the ovary is to regulate folliculogenesis by inhibition of FSH secretion (Erickson and Hsueh 1978). Alfa-inhibin present in follicular cells regulates also their oestrogen synthesis by autocrine mechanism. This cytoplasmic hormone is mainly secreted by follicular cells but may be present in small amount in theca and luteal cells. The effect of dexamethason on the ovarian functional morphology in pseudopregnant rabbit were manifested in considerable changes in alfa-inhibin expression which were increasing with the increase in hormone doses. The process of luteinisation in interstitial cells was observed also in control pseudopregnant rabbits but this process was significantly intensified with the dose-dependent effect in rabbits which received dexamethasone. In high dexamethasone therapeutic dose the process of interstitial cells luteinisation was found in almost all ovarian interstitium cells, however, of the various intensity. As the process of cell luteinisation is LH-dependent, it may be concluded that massive stroma cell luteinisation in pseudopregnant rabbits were under influence of this pituitary hormone. It is proved that alfa-inhibin may influence LH-dependent androgen synthesis in theca interna cells. So the increase in alfa-inhibin expression in theca/interstitial cells observed in pseudopregnant rabbits may reflect its stimulatory effect on androgen synthesis.

There is no answer, how long the observed changes in ovarian cells of pseudopregnant rabbit exposed to dexamethasone persisted, and if this process is totally reversible. The observed ovarian and uterine changes are responsible for the important side-effect of dexamethasone. This must be taken into account in veterinary clinical practice, as corticosteroids are the very popular hormones used in the therapy of different animal diseases. The further research is needed to elucidate the corticosteroids participation of eventual etiopathology of reproductive organ diseases.

The expression of ER and PR receptors in ovaries and uterus of both experimental pseudopregnant rabbit group proved the modulatory, dose-dependent dexamethasone effect. The increase in ER nuclear expression in ovarian epithelium cells after dexamethasone exposure may be interpreted as the oestrogenic effect of dexamethasone. In spite the effect of dexamethasone stimulation on the uterus was manifested by blockade of ER receptors, as compared to control rabbits.

The high intensity of nuclear PR expression was observed in ovarian interstitial luteinised cells of pseudopregnant rabbits which were exposed to both therapeutic dexamethasone doses.

The side-effect of dexamethason visible in epithelial and muscle cells is well known. The observed changes in rabbit uterus proved this type of dexamethason side-effect.

In summary, the results of the presented research proved the alteration of ovaries and uterus of pseudopregnant rabbits after exposure to dexamethasone given in therapeutic doses.

References


Short original article

Somatostatin-immunoreactive nerve structures in the ileum and large intestine of pigs undergoing dysentery

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Abstract

The present study investigated the chemical coding of nerve structures of the ileum and large intestine in pigs suffering from dysentery. Cryostat sections of intestines were processed for double-labelling immunohistochemistry using antisera against PGP 9.5 and SOM. Increased number of SOM-IR perikarya was encountered in dysenteric animals as compared to controls in both myenteric plexus (MP) of all intestine segments studied and in outer submucous plexus (OSP) of cecum and centripetal turns. In OSP of ileum and centrifugal turns the percentage of SOM-IR neurons didn’t change, whereas in OSP of descending colon number of SOM-IR perykarya decreased. Inner submucous plexus (ISP) of all intestines studied showed decreasing number of SOM-IR nerve cell bodies except ileum, where percentage of SOM-IR neurones was not changed. In all layers of intestines under investigation, namely: muscular coat, all plexuses (MP, OSP, ISP) and mucous membrane the density of observed SOM-IR nerve fibres decreased in dysenteric animals.

Key words: pig, dysentery, intestine innervation, somatostatin

Introduction

Among mammalian viscera, the gastrointestinal tract is unique with respect to the arrangement of its innervation. In contrast to many other organs, the innervation of the gut is accomplished by numerous intrinsic neurons forming intestinal plexuses. The morphology of this system, its neurochemistry and function is relatively well known, but information on neuronal elements containing this peptide in enteric nervous system in animals undergoing inflammation of the digestive tract, especially in pigs, is very limited. Therefore, the aim of the present study was to investigate immunohistochemical properties of SOM-IR nerve structures in the ileum and large intestine in pigs undergoing dysentery.

Materials and Methods

The study was performed on 9 five-months-old pigs divided into two groups. Control group (n=3) consisted of clinically healthy animals. Experimental ani-
mals (n=6) were infected per os with *Brachyspira hydysenteriae* bacterium. Then the animals showing distinct symptoms of disease were deeply anaesthetised and transcardially perfused with 4% paraformaldehyde. Then, the tissues (ileum, cecum, centripetal and centrifugal turns of the ascending and descending colon) were collected. The tissues were sectioned with a cryostat and processed for double-labelling immunohistochemistry using antisera against PGP 9.5 and SOM (for details see Kaleczyc et al. 2003). The sections were analysed using confocal microscope Bio-Rad MRA-2. Then, the percentage of SOM-IR perikarya was calculated as a fraction of total number of neurons labelled with PGP. All the animals were treated in accordance with the rules approved by the Local Ethical Commission (conforming to Principles of Laboratory Animals Care, NIH publication no. 86-23, revised 1985).

### Results

In the myenteric plexus (MP) of all intestines studied and in the outer submucous plexus (OSP) of the cecum and centripetal turns of dysenteric animals, an increased number of SOM-IR perikarya was found as compared to controls (Fig. 1a, 1b). In OSP of the ileum and centrifugal turns, the percentage of SOM-IR neurons not changed, whereas in OSP of the descending colon number of SOM-IR perikarya was decreased (Fig. 2a, 2b). The inner submucous plexus (ISP) of all intestines studied contained decreased number of SOM-IR nerve cell bodies (Fig. 3a, 3b) except ileum, where percentage of SOM-IR neurons was not changed. In all layers of intestines under study, namely: muscular coat, all plexuses (MP, OSP, ISP) (Fig. 1, 2, 3a, 1, 2, 3b) and mucous membrane, the density of SOM-IR nerve fibres was decreased in dysenteric animals. Exact data regarding percentage of SOM-IR neurones and density of nerve fibres are shown in Table 1.

### Discussion

This study has revealed changes in the number of SOM-IR structures (nerve fibres and perikarya) in porcine intestines of animals undergoing dysentery. Many studies dealing with innervation of the intestines disclosed the presence of SOM-IR structures in these organs. They were found in the digestive tract of humans (Keast et al. 1984), laboratory animals (Vinik et al. 1981) and pigs (Timmermans et al. 1990). Somatostatin plays an important, mainly inhibitory role in gastrointestinal motility and secretion.

### Table 1. Relative density of nerve fibres (NF) and percentage of the SOM –IR neurons (NCB) in intestines studied.

<table>
<thead>
<tr>
<th></th>
<th>muscular coat</th>
<th>myenteric plexus</th>
<th>outer submucous plexus</th>
<th>inner submucous plexus</th>
<th>mucous layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ileum control</td>
<td>NF +</td>
<td>+ + + + + + + +</td>
<td>+ +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>5.07 ± 0.98</td>
<td>7.32 ± 0.03</td>
<td>10.98 ± 0.78</td>
<td>-</td>
</tr>
<tr>
<td>dysenteric</td>
<td>NF + -</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>11.76 ± 1.18</td>
<td>7.51 ± 0.72</td>
<td>10.72 ± 1.28</td>
<td>-</td>
</tr>
<tr>
<td>cecum control</td>
<td>NF +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>3.71 ± 0.27</td>
<td>1.78 ± 0.17</td>
<td>1.17 ± 0.08</td>
<td>-</td>
</tr>
<tr>
<td>dysenteric</td>
<td>NF + -</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>6.38 ± 1.76</td>
<td>3.54 ± 0.08</td>
<td>0.54 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>centripetal turns</td>
<td>control</td>
<td>NF +</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>2.30 ± 0.21</td>
<td>0.50 ± 0.19</td>
<td>1.15 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>dysenteric</td>
<td>NF +</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>7.61 ± 0.96</td>
<td>5.61 ± 0.40</td>
<td>0.75 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>centrifugal turns</td>
<td>control</td>
<td>NF +</td>
<td>+ + + + + + + + + +</td>
<td>+ + + + + + + + + +</td>
<td>+ +</td>
</tr>
<tr>
<td>pigs</td>
<td>NCB -</td>
<td>2.77 ± 0.19</td>
<td>3.55 ± 0.24</td>
<td>2.83 ± 0.36</td>
<td>-</td>
</tr>
<tr>
<td>dysenteric</td>
<td>NF + -</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + + + +</td>
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<tr>
<td>descending colon</td>
<td>control</td>
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Fig 1. Myenteric plexus in the cecum of the control (a) and dysenteric pig (b).
Fig 2. Outer submucous plexus in centripetal turns of the control (a) and dysenteric pig (b).
Fig 3. Inner submucous plexus in the descending colon of the control (a) and dysenteric pig (b).
role in the regulation of many different intestinal functions including motility, secretion, blood flow, absorption and growth (Lamers 1987). SOM-IR structures were described also in inflamed intestines (Koch et al. 1988). Our findings are in accordance with results obtained by Koch et al. (1988) dealing with innervation of the intestine in idiopathic inflammatory bowel disease. Decreased number of SOM-IR nerve fibres in dysenteric porcine intestine and increased number of SOM-positive neurons can indicate an inhibition of SOM release from intestinal nerve endings which may escalate symptoms of diarrhea (lack of SOM neutralizes inhibitory effects of this peptide on enteric functions, especially on motility and absorption). This conclusion is strongly confirmed by application of SOM or its analogues in treatment of diarrhea with very positive results (Ruskone et al. 1982).

References


**In vitro immunosuppressive effects of oxytetracycline on phagocytic cells and lymphocytes isolated from rainbow trout (Oncorhynchus mykiss) and carp (Cyprinus carpio L.) head kidney**

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**Abstract**

The *in vitro* influence of oxytetracycline (OTC) on the metabolic activity and potential killing activity of phagocytes and the proliferative response on the mitogens of lymphocytes isolated from rainbow trout and carp head kidney were assessed. It was found that the cell ability to produce reactive oxygen species and potential killing activity were suppressed by OTC in the highest used concentrations (40-60 µg/ml), both in rainbow trout and carp. The suppressive effects of OTC were also seen on fish lymphocyte reactivity at drug concentrations from 20-30 µg/ml to 60 µg/ml.

**Key words:** oxytetracycline, fish, lymphocyte, phagocytic cells, immunosuppression

**Introduction**

Oxytetracycline (OTC) is an antibiotic with a broad spectrum of activity, often used in human and veterinary medicine. It belongs to the group of antibacterial drugs the most commonly used in farmed fish therapy, where was introduced in 1951. Since that time some negative aspects of its usage have been reported. It has been found to persist for several months in the fish farm sediments, where could be detected in an active form. That results in an induction of drug resistance in the sediment bacteria. Moreover some immunosuppressive effects of OTC were suggested in treated animals. The aim of the work was to assess the *in vitro* effects of oxytetracycline on the selected mechanisms of rainbow trout and carp leucocytes.

**Material and methods**

In the studies the principles of laboratory animal care and the national laws on the protection of animals were followed. The studies were conducted on...
ten healthy rainbow trouts (*Oncorhynchus mykiss*) and ten carps (*Cyprinus carpio* L.) weighing 300 ± 50 g. The rainbow trouts were maintained in 500 l tanks with aerated, flowing water at a mean temperature of 14°C and fed with diet Safir containing 45% of protein (Aller Aqua, Nożyńko) for over two months before sampling. The carps were maintained in 20 l tanks with aerated water at a mean temperature of 20°C and fed with diet AT Starter containing 35% of protein (Cargill, Siedlce) for four weeks before sampling. The fish were treated with a lethal dose of Propiscin (IRS, Żabieniec) and then organs, head kidneys, were taken. Isolation of leucocytes from fish head kidneys was performed by centrifugation on the density gradients according to Rowley (1986). Briefly, separating mixture was prepared by building up 2.5 ml of Gradisol L (1.077 g/ml, Aqua-Medica, Poland) on 2.5 ml of Gradisol G (1.115 g/ml, Aqua-Medica, Poland). After centrifugation (1000 x g, 40 min., 10°C) two bands were formed. The first band was rich with lymphocytes (80–85% of isolated cell population of the band), and the second one was rich with neutrophiles (85 – 95%). Phagocytic ability of phagocytes was measured by the respiratory burst activity (RBA) with oxygen burst activator PMA (phorbol 12-myristate acetate, Sigma) and potential killing activity (PKA) with *Staphylococcus aureus* 209P after 24 h exposure of the cells on oxytetracycline HCl (Pacific Pharmachem, Ltd.) at the concentrations of 1, 5, 10, 20, 30, 40, 60 µg/ml. To determine the influence of the drug on lymphocyte proliferative activity, the MTT test was used. The cells were exposed to the drug at the same concentrations as mentioned above for 72 h and stimulated with the mitogens – ConA (concanavalin A, Sigma) as a T cell activator, and LPS (lipopolysaccharide, Sigma) as a B cell activator. Statistical analysis of the results was performed by ANOVA. Differences between means were determined by Duncan test and considered statistically significant at p<0.05.

**Results and discussion**

The effects of OTC on metabolic activity of phagocytes, mainly neutrophiles, were examined by measuring NBT reduction. It was reported, that OTC inhibited respiratory burst activity of fish phagocytic cells at a concentration of 40 and 60 µg/ml. Similar results were seen when potential killing activity of the cells was tested (Fig. 1).

Because of the high activity of OTC in chelating Ca ions, both extra- and intracellular levels of calcium become lower in the presence of the drug. It may be the reason of lower activity of phagocytes, since Ca ions are of great importance for respiratory burst. It was also shown (Paape et al. 1991, Hoeben et al. 1997) that higher concentrations of OTC may cause morphological changes in cells, as smaller flexibility of cell membrane, a loss of pseudopodia and, in the result, worse ability to adhere to the base. OTC may affect the assembly of F-actin by interfering with calcium-dependent mechanisms involved in the formation of actin dimers and trimers (Hoeben et al. 1997). Neutrophiles are of special susceptibility on low calcium level, which stops phagosome maturing and their fusion with lisosomes (Kwiatkowska et al. 1999).

When incubating fish lymphocytes with OTC for 72 hours, lower proliferation was seen from 20 µg/ml in case of rainbow trout T cells (activated by ConA) and B cells (activated by LPS). Similarly, suppressed proliferation of carp T cells was observed from 20 µg/ml and 30 µg/ml of B cells (Fig. 2).

**Fig. 1.** *In vitro* effects of OTC on metabolic activity and potential killing activity of fish phagocytes (x ± SD, n=6, *statistically significant, p<0.05).*

**Fig. 2.** *In vitro* effects of OTC on fish lymphocyte proliferation (x ± SD, n=6, *statistically significant, p<0.05).*

Suppressive effects of OTC on proliferative activity of lymphocytes were also reported by others. Lunden et al. (2000) in *in vivo* study, after OTC treatment of
rainbow trout at a dose of 75 mg/kg b.w. per os for 10 days, have shown decrease of mitogen – stimulated proliferation by 40% and 63%, respectively, for B and T cells isolated from fish head kidney. They also observed strong immunosuppressive activity of OTC in in vitro studies. In that study drug concentrations from 0.1 µg/ml to 100 µg/ml inhibited T cell reaction on PHA. In case of B cells similar suppression was seen from a concentration of 1 µg/ml. Stronger than observed in our own study susceptibility of rainbow trout cells on OTC may have been a result of different used protocols, but probably age of used fish was the most important factor. In the mentioned study rainbow trout fry was used (mean b.w. 42 g) and the isolated cells were pooled, while our fish were older (mean b.w. 200 g) and all determinations were done individually for each fish.

Probably OTC can influence the cell proliferation in two ways. At first, as a calcium ion chelator, it reduces their concentration in cell surrounding, which makes impossible the calcium influx into the cells after mitogen stimulation and thus interrupts with DNA and RNA synthesis (Myers et al. 1995a). The second interaction can proceed at relatively low OTC concentrations. It is associated with suppression of mitochondrial protein synthesis which disturbs mitochondrial biogenesis (Kroon et al. 1983).

Changes in lymphocyte proliferative ability and their lower immunoglobulins production can also be an effect of modulation by OTC of IL-2 production. However, it is not well documented if modulated IL-2 secretion is the effect or the cause of weaker cell proliferation (Myers et al. 1995b).

References


Immunopathogenesis of herpesviruses: influence of channel catfish herpesvirus (CCHV) on macrophage and lymphocyte activity – *in vitro* comparative study

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Abstract

Several DNA viruses infecting fish have been studied for analysis the pathogenesis and for developing the effective methods of prevention and therapy. Also some of fish viruses have been used as models for studying disease mechanisms. In this paper, we present the *in vitro* influence of channel catfish herpesvirus (CCHV) on the phagocytic ability and potential killing activity of splenic phagocytes and proliferative response of pronephric lymphocytes stimulated by mitogens ConA isolated from european catfish (*Silurus glanis*) and carp (*Cyprinus carpio*). The results showed that channel catfish herpesvirus (CCHV) induced suppressive effect on the phagocytic ability and potential killing activity of splenic phagocytes and decreased the lymphocyte activity isolated from pronephros in european catfish, compared to the carp where the immunostimulating effect was observed. The analyses of this study suggested that CCHV inhibited of cell-mediated immunity in european catfish.

Key words: fish, CCHV, phagocyte activity, lymphocyte proliferation

Introduction

Interest in lower vertebrates viruses has recently increased for a variety of reasons despite the fact that none of them infect warm-blooded animals and man. Some of lower vertebrates viruses have been used as models for studying disease mechanisms. The family *Herpesviridae* contains the three subfamilies: *Alpha-herpesvirinae, Betaherpesvirinae, Gammaherpesvirinae* and the unassigned genus “Ictalurid herpes-like viruses” (Ward 1993, Essbauer and Ahne 2001). The *Herpesviridae* are the most extensively studied fish viruses. First of all because they are ubiquitous in aquatic organisms, since they have been isolated all over the world from both marine and freshwater fishes of different species, and is responsible for severe losses in aquaculture. Several viruses isolated from different species of fish have been classified as herpesviruses (Eaton et al. 1989, Jung 1995, Hedrick 2000, Essbauer and Ahne 2001). The icosahedral capsid is made of 162 capsomeres that are embedded in a protein matrix and are surrounded by an envelope, which
includes virus-encoded glycoproteins. The capsid contains a single linear double-stranded DNA molecule, which is replicated in the nuclei of the infected cells. In fish 25 types of herpes-like viruses have been detected but only few are well characterised. Channel catfish herpesvirus (CCHV) is one of the most pathogenic virus for catfish (Davison 1992). Experimental infection of catfish fingerlings with CCHV induces haemorrhagic, oedematous and anaemic disease and strong mortality (Davison 1992, Essbauer and Ahne 2001).

The aim of the present study was to determine the in vitro influence of channel catfish herpesvirus (CCHV) on the phagocytic ability of splenic phagocytes and proliferative response of pronephric lymphocytes stimulated by mitogen in European catfish (Silurus glanis) and carp (Cyprinus carpio).

**Materials and Methods**

The spleen and pronephros were isolated from 10 healthy European catfish and carp with mean weight of 100 g and the macrophages or lymphocytes were separated and cell culture prepared. Single cell suspension was obtained by placing the spleen and pronephros in medium RPMI-1640 and teasing it through a steel mesh. The macrophages were isolated by density-gradient centrifugation using Gradisol (Polfa) and lymphocytes were isolated by centrifugation using Histopaque-1077 (Sigma) gradient.

A modification of the Secombes (1990) method was used to study the respiratory burst activity of spleenic macrophages stimulated by phorbol myristate acetate (PMA, Sigma). Cells suspension (1x10⁶/ml of medium) were incubated with CCH virus (1x10⁵ p.f.u./ml of medium) for 2 h at 22°C. The plates were read on a micro-reader at OD 620 nm.

The proliferative response of the pronephric lymphocytes was determined by the MTT colorimetric assay method according to Carmichael et al. (1987) as modified for fish by Siwicki et al. (1999). Concanavalin A (ConA, Sigma) at a concentration of 64 fg/ml was used for stimulation of lymphocyte proliferation. The lymphocytes suspension (5 x 10⁶/ml of medium) were incubated 3 days with CCH virus (1 x 10⁵ p.f.u./ml of medium), with mitogen + CCH virus and only with mitogen. The plates were read on a micro-reader (OD 620 nm).

Statistical analyses were performed using the Student t-test. Differences in mean were considered statistically significant at p<0.05.

**Results and Discussion**

Herpesviruses are very pathogenic for lower vertebrates. Systemic infections of fish farming caused by herpesviruses have recently been recognized in Europe, Asia, USA and Australia. A unifying theme among the herpesviruses is the intimate interrelationship of virus infection with host cellular immunocompetence. Actually, we have a few informations about effect of herpesvirus on the cell-mediated immunity in fish. In present study the influence of the channel catfish herpes virus (CCHV) on spleen macrophage metabolism was assessed by examining their respiratory burst activity. The effects of CCHV on the respiratory burst activity in European catfish and carp are presented in Fig. 1. The results showed that channel catfish herpes virus significantly (p<0.05) decreased the macrophage activity level in European catfish, compared to the control and carp. In carp the stimulating influence of CCHV on the macrophage respiratory burst activity was observed.

![Fig. 1. In vitro influence of CCHV on the splenic macrophages respiratory burst activity (RBA) in European catfish and carp (mean ± SD, n=10).](image-url)

![Fig. 2. In vitro influence of CCHV on the proliferative response (LP) of pronephric lymphocytes stimulated by ConA (ConA), ConA and CCHV (ConA+CCHV), only by CCHV (CCHV) and in non-stimulated cells (Control) in European catfish and carp (mean ± SD, n=10).](image-url)
The proliferative ability of pronephric lymphocytes showed a similar pattern. The influence of channel catfish herpes virus on the proliferative response of pronephric lymphocytes are presented in Fig. 2. In European catfish the CCHV decreased the proliferative response of lymphocytes, compared to the carp and control. In carp the stimulating influence of CCHV on lymphocyte proliferation induced by ConA was observed.

A channel catfish herpes virus was tested in vitro on cell-mediated immunity in two species of fish: carp and European catfish. In our study, a strong suppressive influence of the CCHV on macrophage respiratory burst activity and lymphocyte proliferation was observed in European catfish, compared to the carp where CCHV increased the macrophage and lymphocyte activities. The effect of DNA viruses on the European catfish and carp leucocytes has been previously reported by Siwicki et al. (1999, 2000). The authors clearly demonstrated a suppressive effect of iridovirus on the phagocyte and lymphocyte activity and hypothesised that this suppression led to a reduction in the number of cells or to an impairment of phagocytic function. This preliminary in vitro study also demonstrated a strong inhibitory influence of the CCH virus, but on selected species. In carp the immunostimulatory influence was observed, compared to the European catfish where the strong suppression was observed. These results suggested that the CCHV suppressed the intracellular metabolism of the European catfish macrophages and lymphocytes and has immunomodulatory effect on the cell-mediated immunity dependent to the species of fish.

References
Pathogenesis of *Birnaviridae* – influence of infectious pancreatic necrosis virus (IPNV) on cell-mediated immunity, total Ig level and lysozyme activity in Salmonid

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Abstract

The *Birnaviridae* are the most extensively studied viruses in lower and higher vertebrates. Infectious pancreatic necrosis virus (IPNV) is the prototype of the *Birnaviridae*. In our study we examined the influence of IPNV on the splenic macrophage and lymphocyte activity, lysozyme activity and Ig level in serum of rainbow trout. Healthy fish a mean weight of 10 g were inoculated intraperitoneally with 50 µl of a viral suspension of the IPNV with a titre of $10^7$ TCID₅₀/ml, and 3 and 5 days after infection fish were scarified and dissected. Samples of pronephros and spleen were taken for histological and immunological assays. The results showed that IPNV strongly decreased pronephric macrophage and lymphocyte activity in infected fish, compared to the control. Also the lysozyme activity and total Ig levels were significantly decreased. The results showed strong suppressive effect of IPNV on immunocompetent cells and humoral defense mechanisms.

Key words: fish, IPNV, macrophage and lymphocyte activity, lysozyme and Ig levels

Introduction

The *Birnaviridae* are ubiquitous in aquatic organisms and they have been isolated all over the world from both marine and freshwater fish of different species. They are responsible for severe losses in aquaculture. The *Birnaviridae* dsRNA genome is easily purified and is resistant to common RNases. The birnaviruses have developed unusual coding strategies. The single-shelled, naked, icosahedral, viral particle of birnaviruses contains two segments (A and B) of double-stranded RNA which are circularized by VP1, a viral protein (genome-linked protein, VPg). Infectious pancreatic necrosis virus (IPNV) is the prototype of the *Birnaviridae* (Brown 1986). This group also includes the oyster virus (OV), the *Drosophila* X virus (DXV), the infectious bursal disease virus (IBDV) from poultry and a rotifer virus (RBV). These viruses differ from the bisegmented dsRNA viruses isolated from mammals which are tentatively classified as...
Picobirnaviruses, because of the size of their particle (55-65 nm) and their genome. The segments of IPNV and IBDV have been sequenced. The organization of these genomes is similar. Terminal repeats -AAGAG- are present at the 5’ and 3’ ends of the A segment and these are inverted on the B segment. These terminal sequences are identical for IPNV and IBDV and may play a role in replication or packaging. The motif GLPYIGKT (IPNV) and GLPYVGRT (IBDV), reminiscent of the GXXXGXKS/T ras-type GTP binding proteins found on VP1, may relate to guanylyl transerase activity demonstrated from both IPNV and IBDV (Dobos 1993). Hybrid arrested translation (HAT) shows that the protein order is 5’-VP2-NS-VP3-3’. The autocatalytic proteolytic enzyme is identified as NS and the active site maps to its carboxyl-terminus between amino acids 693 and 720 (Manning 1990) which results in the formation of VP1-pG and VP1-pGpG.

Very little information exists about the replication strategy of birnaviruses and their pathogenesis. VP1 is found in the viral particle both as a free form and bound to the RNA (Vpg) and is thought to be the viral RNA dependent RNA polymerase. However, attempts to search for consensus sequences in the VP1 protein fail to find any known motifs such as the GDD motif, characteristic for the RNA polymerases. Comparison with the mammalian Picobirnaviridae would be useful, but no sequences have been published yet. Also very few information exists about the influence of IPNV on the defence mechanisms in fish. In this study, we examine the effects of IPNV on the immunocompetent cells activity and nonspecific humoral defence mechanisms in rainbow trout (Oncorhyncus mykiss).

Materials and Methods

Two groups (50 fish per group) of healthy rainbow trout, with a mean body weight of 10g, were used in our study. The IPNV isolated from rainbow trout was used and virus stock was prepared in the chinook salmon embryo cell line (CHSE-214). When the cytopathic effect (CPE) was extensive, the cells were harvested and centrifuged to eliminate cell debris. The virus stock was titered and stored at -70°C until use. The fish from experimental group were inoculated intraperitoneally with 50 µl of a viral suspension of the IPNV with a titre of 10⁷ TCID₅₀/ml. The control group was inoculated with cell culture medium (RPMI-1640, Sigma). Fish were maintained in 500 l tanks with recirculation system of water at temperature 15°C. For the immunological and histological assays, 3 and 5 days after infection, 10 fish from each group were bled by syringe from the caudal vein and dissected. Simples of liver and spleen were taken for histological assays and pronephros for immunological study. The pronephros was removed and single cell suspension obtained by teasing the tissue in medium through a nylon mesh. The cells were then washed in heparinized Hank’s balanced salt solution (HBSS) and isolated by gradient-density centrifugation on a Percoll (Pharmacia, Upsala, Sweden).

A modification of the technique of Secombes (1990) was used to study the potential killing activity (PKA) and respiratory burst activity (RBA) of pronephric macrophages stimulated by phorbol myristate acetate (PMA, Sigma).

The lymphocyte proliferation (LP) was determined using the MTT colorimetric assay method of Carmichael et al. (1987). The cells were stimulated by concanavaline A (ConA, Sigma).

The lysozyme activity in serum was measured using a turbidimetric assay (Studnicka and Siwicki 1986) and total immunoglobulin (Ig) levels in serum were determined using colorimetric assay (Anderson and Siwicki 1994).

Results and Discussion

In the present experimental study, we continued the in vivo experiments to examine the effects of IPNV on macrophage and lymphocyte activity in rainbow trout. The respiratory burst activity (RBA) and potential killing activity (PKA) of pronephric macrophages isolated 3 and 5 days from fish infected and

<table>
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<td>PKA (OD 620 nm)</td>
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<td>LP (OD 620 nm)</td>
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<td>lysozyme activity (mg/l)</td>
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<td>total Ig level (g/l)</td>
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Explanation: * – statistically significant to the control p<0.05
control are presented in Table 1. The results of this experimental study showed the strong suppression effects of IPNV on the phagocytic ability and intracellular killing activity of pronephric macrophages. Also the 3 and 5 days after infection the proliferative response of pronephric lymphocytes stimulated by ConA was statistically lower (p<0.05), compared to the control IPNV-free fish (Table 1). The results presented that IPN virus has also decreasing influence on the proliferative response of pronephric lymphocytes.

The 3 and 5 days after infection, the lysozyme activity and total Ig levels in serum were significantly decreased (p<0.05), compared to the control fish (Table 1). The results showed that IPNV has influence on the lysozyme and Ig production by immunocompetence cells. The similar results were observed in rainbow trout naturally infected with IPNV (Siwicki et al. 1998). The present experimental in vivo study strongly suggested that infectious pancreatic necrosis virus has direct influence on the cell-mediated immunity and nonspecific humoral defence mechanisms in fish.

References

Chocolate feeding of pregnant mice resulted in epigallocatechin-related embryonic angiogenesis suppression and bone mineralization disorder


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Abstract

Our previous studies have shown inhibitory action of some phenolic compounds and theobromine on tumour and embryonic angiogenesis. We also observed significant inhibition of embryos growth and limbs and femoral bones shortening in 4 weeks old offspring of female mice fed chocolate during pregnancy and lactation. The aim of the present study was to evaluate: 1) the effect of chocolate given to mice during the pregnancy and lactation period on the angiogenic activity of 18 days embryos tissues (cutaneous angiogenesis test) and their epigallocatechin (EGC) content (HPLC method); 2) bones mineralization in 30 days old offspring by electron spin resonance spectroscopy (ESR). Results: 1) angiogenic activity of embryonic tissues correlated negatively with their epigallocatechin content; 2) crystallinity of femoral bones was higher in offspring of chocolate fed mothers than in control ones.

Key words: mice, pregnancy, chocolate, angiogenesis, EGC, bones mineralization

Introduction

Vascular endothelial growth factor (VEGF) is the key regulator of capillary invasion and cartilage remodeling during pre- and post-natal development. In mice, inhibition of VEGF led to inhibition of angiogenesis and to a decrease in the levels of chondrocytes and osteoblasts at the growth plates (Gerber and Ferrara 2000). We previously reported that feeding pregnant mice with chocolate resulted in a decrease in...
the relative length of limbs and femoral bones in 4 weeks old progeny as well as decrease in their VEGF content (Skopiński et al. 2003). The aim of the present study was to evaluate the mineralization process of these bones measuring crystallinity by electron spin resonance spectrometry (ESR). We also reported previously that embryonic tissues obtained from chocolate-fed mothers induced lower neovascular reaction in cutaneous angiogenesis test than embryonic tissues obtained from control group of pregnant mice (Skopińska-Różewska et al. 2003). The aim of the present study was to evaluate content of catechins in 18 days embryos and to establish its possible relation to angiogenic activity of their tissues.

### Materials and Methods

The study was performed on 2 months old female Balb/cxC3H F1 and female Balb/c mice fed during pregnancy and lactation, in addition to normal laboratory chow, 400 mg/day bitter chocolate (Wedel, Poland). Some mice were sacrificed by lethal dose of chloral hydrate on 18 day of pregnancy, embryos were extracted, suspended in PBS (1 g/1 ml), homogenized (VirSonic ultrasound sonicator) and frozen at -80°C. A high performance liquid chromatographic method for the simultaneous determination of four catechines (catechin, epigallocatechin, epigallocatechin gallate, epicatechin) was developed. The method was used to determine the levels of these catechins in the biological material and food sample. Analysis was carried out using a Phenomenex Luna C18 column with an isocratic solvent of methanol-water-acetic acid and UV detection (204 nm). Quantification was carried out by the external standard method. The cutaneous angiogenesis assay was performed to assess homogenates angiogenic activity (Rogala et al. 2001). Shortly, 0.05 ml homogenate samples (4-6) were injected intradermally to anaesthetized Balb/c mice. After 72 hours mice were sacrificed. Newly-formed blood vessels were identified and counted on the inner skin side, in dissection microscope, at the 6x magnification, in the central 1/3 of the microscopic field. The rest of progeny was sacrificed at the 4th week after birth. Femoral bones were separated into dia- and epiphyses. Evaluation of the amount of crystallinity of bone mineral was performed by electron spin resonance spectrometry, ESR (Ostrowski et al. 1980, Dziedzic-Gocławska et al. 1984), 6 weeks after irradiation with a dose of 100 kGy using 60Co source.

### Results and Discussion

The results of HPLC analysis are presented on the Table 1. Epigallocatechin content of embryos obtained from chocolate-fed mice was significantly higher than in corresponding controls, and significant negative correlation was found between epigallocatechin content of homogenates and their angiogenic activity (Fig. 1). Tissue concentrations of other catechins did not correlate negatively or positively with angiogenic activity. Evaluation of bone mineralization revealed that the value of crystallinity of compact bone of diaphyses was about 17% higher in chocolate group than in the controls, of cancellous bone of epiphyses was about 30% higher in chocolate group than in offspring of control mothers.

![Fig. 1. Negative correlation between angiogenic activity of embryos tissue homogenates and their EGC concentration.](image)

Our results of bone crystallinity measurements suggest that chocolate feeding of pregnant mice may disturb the processes of bone mineralization in offspring. It is in accordance to our previous findings, obtained from experiments performed on progeny of chocolate-fed mice, where we observed shortening of limbs and bones and lowering of their VEGF content (Skopiński et al. 2003).

The chocolate at dose 400 mg daily per mice during pregnancy significantly suppressed angiogenic
activity of 18 day embryos tissues (Skopińska-Różewska et al. 2003) and, as revealed by our present study, this effect correlated with epigallocatechin content. This daily dose corresponds to 200 g of bitter chocolate consumed by person of 70 kg body mass. We suppose, that malformations of limbs, reported in our previous work (Skopiński et al. 2003) and bone mineralization disorder observed in the present study might be connected with anti-angiogenic action of chocolate catechins, reported as anti-angiogenic agents in in vitro and in vivo models (Cao et al. 2002, Kondo et al. 2002). Attention should be made to possible harmful effects of catechines-rich food and beverages during pregnancy and lactation.

Acknowledgements

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References


Ultrastructural pattern of the muscle fibres of turkeys fed a diet containing oxidized fat and infected with the $O_{78}K_{80}H_{9}$ pathogenic serotype of *Escherichia coli*

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Abstract

This experiment involved 40 one-day-old BUT-9 turkeys. For the first 8 weeks, the birds were fed a mixture supplemented with a fat of peroxide value (PV) of 5 – 150 mEq O₂/kg and 10 turkeys from each PV level were taken and infected through the air sacs with a 1 ml dose of $O_{78}K_{80}H_{9}$ pathogenic serotype of *Escherichia coli* suspension representing the $8 \times 10^6$ bacteria count based on McFarland's scale. After a subsequent 4 days, all the birds were sacrificed and analysed macroscopically. Medial gluteal muscles from 5 turkey hens from each group were examined ultrastructurally.

It was found that the feeding turkey hens diets supplemented with fat of PV 150 mEq O₂/kg for 8 weeks resulted in the development of ultrastructural lesions most often in mitochondria, rarely in smooth and rough endoplasmic reticulum and sporadically in other organelles of the muscles. Such feeding also caused a tendency to steatosis. The birds fed diets supplemented with oxidized fat and then infected with *E. coli* exhibited a more explicit tendency to ultrastructural lesions in the above-mentioned organelles than the healthy birds.

Key words: turkey, oxidized fat, *Escherichia coli*, muscle

Introduction

Rapidly growing turkeys, genetically selected for high meat yield, were obtained at the cost of decreasing their natural immunity (Koncicki et al. 2001). Both the genetic basis and the intensive rearing conditions pose a threat to birds’ health (Jankowski et al. 2000, Koncicki et al. 2000). Peroxidized fat has a similar effect through its peroxide lipids which inhibit body metabolism. Studies in this respect were carried on...
out mainly on rats and, to a lower extent, on pigs, chickens or turkeys (Kanazawa et al. 1985, Dibner et al. 1996, Renerre et al. 1999, Szarek et al. 1999, Koncicki et al. 2000, 2001). Therefore, the aim of the study was to examine the muscle ultrastructural lesions in turkeys fed diets supplemented with different levels of peroxide fat and infected with Escherichia coli.

Materials and Methods

This study involved 40 one-day-old BUT-9 turkey chicks. The birds were divided into 4 groups (n=10) and fed ad libitum. The diet fed was described by Koncickiego et al. (2001). The feed was divided into week portions and supplemented with a mixture of rapeseed oil and poultry fat at the proportion of 66:34% and with a different peroxide value (PV): in groups I and II - below 5 mEq O₂/kg and in groups III and IV - 150 mEq O₂/kg. Until week 4 the diet was supplemented with 2% and then with 3% of oxidized fat (Jankowski et al. 2000).

The 8 weeks old turkeys in groups II and IV were infected through the air sacs with a 1 ml dose of the fat (Jankowski et al. 2000). The ultrathin sections were cut on a LKB ultratome for 1 h. Specimens were embedded in Epon O78K80H9 pathogenic serotype of infected through the air sacs with a 1 ml dose of the fat (Jankowski et al. 2000).

Discussion

Koncicki et al. (2001) showed the effect of oxidized fat on susceptibility of turkeys to infection with hae-
morrhagic enteritis virus (HEV). In this experiment, the greater spleen index in the birds fed diets supplemented with oxidized fat (1.77 – 2.61) in comparison to the control birds (1.07 and 1.55), showed that the presence of peroxide lipids in feed increases birds susceptibility to HEV infection. Additionally, it is known that the first effect of peroxide decomposition is the damage of intestinal mucosa which accelerates the absorption (Kazanawa et al. 1985).

In the previous experiments, the authors of this
paper observed a correlation between the body response of turkeys exposed to prolonged feeding diets supplemented with peroxide fat of the Lea number of 150 mEq O₂/kg and the infection with O₁₅₀K₈₀H₉ pathogenic serotype of *Escherichia coli*. This was reflected in the morphological lesions in mitochondria and smooth and rough endoplasmic reticulum as well as in vessel endothelium. The birds infected with this pathogen and fed diets supplemented with oxidized fat for 8 weeks showed a clearer tendency to ultrastructural lesions in these organelles than the healthy birds.

Previous papers indicated that diets supplemented with small amounts of oxidized fat and containing adequate levels of antioxidants (vitamin E and selenium) do not pose threat to birds’ health or evoke only subclinical lesions in their bodies (Lea et al. 1966, Renerre et al. 1999, Jankowski et al. 2000, Koncicki et al. 2000). However, larger doses have a negative effect on animals (Dibner et al. 1996, Szarek et al. 1999).

Based on the present experiment, feeding turkey hens diets supplemented with oxidized fat with a PV of 150 mEq O₂/kg for 8 weeks results in the development of ultrastructural lesions most often in mitochondria, rarely in smooth and rough endoplasmic reticulum and sporadically in other organelles in the medial gluteal muscle. It also causes a tendency to develop steatosis.

**References**


Ultrastructural characteristics of supraspinal muscles in rabbits after short-term electrostimulation

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Abstract

This study was conducted on ten rabbits, aged 3.5 months. The animals were divided into 2 groups (n=5 each). In group 1, the supraspinal muscles were electrostimulated for 2 h/day for three months (by means of SCOLL-2 apparatus) according to the Lateral Electrical Surface Stimulation (LESS) method modified by Kowalski. In group 2 (control rabbits) no electrostimulation was conducted.

An ultrastructural examination of the supraspinal muscles that were influenced by LESS showed generally a considerable increase in the number of mitochondria and their enlargement, and large clusters of the glycogen. These features indicated an intensified metabolism in muscle fibres caused by short-term electrostimulation. The disturbances in the course of the Z line were much less frequently observed. Other sub-cellular lesions in the stimulated muscles were observed relatively rarely.

Key words: scoliosis, lateral electrical surface stimulation (LESS), ultrastructural characteristics of muscle, rabbit

Introduction

Paediatric rehabilitation studies on the shortening of the period of use of the Lateral Electrical Surface Stimulation (LESS) method (from 8-10 h/day to 2 h/day) in the treatment of idiopathic scoliosis (IS) in children and adolescents gave positive preliminary results (Xiong et al. 1994). It proved the effectiveness of the method, particularly at an initial angle of the spinal scoliosis of less than 20°, according to Cobb’s method (Kowalski 1997). Nevertheless, since the negative side-effects of the long-term application of LESS on humans and animals are known (Kowalski 1997, Kowalski et al. 2002, Szarek et al. 2003) there is a need to examine the ultrastructure of the muscles that were subjected to electrostimulation, including a morphological examination at the sub-cellular level, in order to applying the modification of the method.

Thus in the light of the introduced facts, a study identifying the ultrastructural features of supraspinal muscles after short-term LESS is fully justified.
Materials and Methods

Ten male, pure-bred (New Zealand White), age-matched (born within 5 days) and clinically healthy rabbits, obtained from a farm in Gdańsk (G-1 on the list of Reproductive and Breeding Rabbit Farms of the Ministry of Agriculture and Food Economy, Poland) were used in the experiment. The animals were adapted for 30 days to the experiment conditions. Rabbits were kept indoors in a room with controlled temperature (18°C) and humidity (70%). Each animal was placed in a metal cage (50 x 50 x 50 cm) and received dry feed and water ad libitum. At the beginning of the LESS treatment, the rabbits were aged approximately 3.5 months and their average body mass was 2 100 g.

The study was conducted in two groups (n=5), where the rabbits in group 1 were subjected to LESS for 2 h/day, and those in group 2 (control) were not subjected to electrostimulation.

The ultrastructural examination was done on segments of *musculus longissimus dorsi* (MLD): *m. longissimus thoracis* (MLT) and *m. iliocostalis thoracis* (MIT) – the muscles which stabilise the rabbit’s spine. The samples were taken both from places which were subjected to LESS (on the right side) and from non-stimulated ones (on the left of the spine). The muscles were fixed in glutaraldehyde in phosphatic buffer of pH 7.2 and sealed in Epon 812. Semi-thin sections were stained according to the method of Levis and Knight’s (1977); the appropriate place for preparing ultrathin sections was established under a light microscope. Structural analysis was conducted using an Opton 900 PC electron microscope (FRG).

Results and Discussion

The ultrastructure of MLT in rabbits on the right side of the spine, which was subjected to LESS, was normal.

The most frequently observed mitochondria were spherical (Fig. 1, 2), and relatively often enlarged (Fig. 2). In all animals, the muscle fibres with a large number of mitochondria in sarcoplasm were found (Fig. 1, 3). Giant mitochondria were relatively rare, with transformation vesicle of the crests (Fig. 2). In the majority of the animals, large clusters of glycogen within the sarcoplasm were found (Fig. 2). In three rabbits, an irregular and sometimes zig-zagging course of the Z line was observed (Fig. 4, 5). In some of these cases, the Z line made thin processes running along the sarcomere (Fig. 4). Its atrophy was also observed, with accompanying enlargement of endoplasmic reticulum channels (Fig. 5, 6) and a loss of myosin and actin filament (Fig. 6). Occasionally, the thickening of the Z line took place (Fig. 1).

MLT on the left side in the rabbits subjected to LESS of the spine was normal as observed in an electron microscope. It is remarkable that the fibre structure of actin and myosin filaments was particularly distinct, with relatively frequent thickening of the Z line (Fig. 3, 7). The muscle fibres were usually rich in mitochondria (Fig. 3).

The ultrastructural examination of MLT on the right and left side in the control rabbits revealed the morphological features within the norm (Fig. 8).

The ultrastructural lesions observed in the supraspinal muscles in rabbits, caused by short-term LESS (proliferation of mitochondria, their enlargement, large clusters of glycogen in sarcoplasm, thickening of the limiting membrane) were positive. They are proof of intensified correct metabolism in the muscle fibres subjected to short-term LESS (Hadley-Miler et al. 1994). The character of the changed cell organelles indicates a correct muscle reaction to the training, caused by repeated electric stimuli with the frequency of 30-40 Hz that produces tetanic contraction (Wright et al. 1992). Similar results were observed by Bigard et al. (1993) in their experiments with monkeys.

It should be noted that short-term LESS causes sporadic low intensity disturbances; these included a loss of single filaments of myosin and actin, and an irregular course of the Z line. The intensity of those lesions did not indicate their negative effect on the physiology of muscles. Thus, the author's own re-
search emphasises the positive effect of short-term LESS on the muscle fibres of the supraspinal muscles, which stabilise and correct the spine.

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References


Pathomorphological pattern of the liver and kidney in polar fox fed a diet supplemented with a new generation of feed

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Abstract

In order to improve fox productivity, a new generation feed supplement containing strains of yeast (facilitating digestion), plant extract from Yucca schidigera (binding ammonia in the manure), lactic acid and zinc bioplex known under the commercial name as the DigDeo-Korektor (DDK) was added to their diet for a period of 3.5 months. The experiment was conducted on 60 young polar foxes in the period from weaning to pelting. The animals were divided into two groups (n=30) containing equal numbers of males and females. Group 1 was fed standard diet and group 2 received an addition of 5 g of DDK per day per animal. Based on the morphological examinations, it can be concluded that in foxes consuming their diets supplemented with the DDK, an increased blood supply to the organs, lower storage of lipid in the liver and an increased activity of the macrophage-histiocyte system were observed.

Key words: fox, DigDeo-Korektor, liver, kidney, pathomorphology

Introduction

The current fox rearing techniques aim at the maximum animal growth and thus producing the largest possible sizes of hide and best quality of fur. In achieving such results, feeding plays an important role, especially having appropriate protein, fat and energy levels in their diets (Skrede and Ahlstrom 1992, Ahlstrom 1995). Additionally fox breeders try to improve animal productivity by controlling the digestion processes. Such activities improve nutrient utilisation and eliminate pathogens. These are the methods for improvement of the sanitary state of foxes and increasing their performance.

Materials and Methods

The examination was conducted on 60 young polar foxes (30 females and 30 males), born at about the same time, in the period from weaning to pelting on a production farm. The experimental foxes were kept in cages designed for rearing young animals installed in one room. The animals were divided into two
groups (n=30) containing equal numbers of males and females. The foxes of group 1 were fed a standard diet and the animals of group 2 additionally received a new generation feed supplement containing strains of yeast (facilitating digestion), plant extract from Yucca schidigera (binding ammonia in the manure), lactic acid and zinc bioplex known under the commercial name as the DigDeo-Korektor (DDK) in the dose 5 g per day per animal. The feed was prepared from typical feed components by balancing the diets according to the requirements of the growing animals (Lorek et al. 2000, Szarek et al. 2003).

Animal body mass gain was analysed individually based on the measurement of the parameters taken every two weeks. The results were statistically analysed using a single-factor variance analysis in the orthogonal system (Ruszczyc 1981).

After 3.5 months of polar fox feeding in the above-described manner, the animals were sacrificed. Five males and 5 females were randomly selected from each of the two groups for morphological analyses (liver and kidney macro- and microscopic analyses). The experimental material was fixed in 10% neutralised formalin and the liver and kidney paraffin lyses (liver and kidney macro- and microscopic analyses). The experimental material was fixed in 10% neutralised formalin and the liver and kidney paraffin sections were stained with haematoxylin and eosin (HE) and the livers were additionally stained by Sudan III according to Lillie Ashburn.

Results and Discussion

The average fox body mass at the beginning of the experiment was 2.02 kg in group 1 and 1.92 kg in group 2. A similar difference was maintained until week 8 and after 10 weeks of the experiment the average fox body mass equalised and was 7.82 kg. By the end of the rearing period, the foxes from group 2 weighed slightly more. At the end of the experiment (after 14 weeks) the average body mass was 13.21 kg and 14.84, respectively. However, the differences in the average body masses were statistically insignificant.

Based on the macroscopic analysis, the foxes from group 1 were diagnosed for congestion of kidneys (in one case) and the animals from group 2 for congestion of liver (in two cases) and congestion of kidney (in one case).

Lipid infiltration was found in all the livers of group 1 foxes (Fig. 1). Relatively frequently fat droplets were larger than hepatocytes nuclei and they were found in 50%-70% of parenchymatous liver cells (Fig. 1). Parenchymatous degeneration of hepatocytes was found in 4 animals covering each time a small area (Fig. 1). Congestion of the liver with varied intensity was found in 50% of the analysed foxes (Fig. 1). Infiltration of mononuclear cells was found in the livers of three animals and proliferation of the stellate cells was observed in two animals.

Parenchymatous degeneration of the renal tubule epithelium was found in three cases of the paraffin liver sections of the foxes from group 1 (Fig. 2). In one animal, a few cells of the renal tubule epithelium had features of necrosis. Glomerulonephritis was observed in 2 foxes and congestion was observed in one animal (Fig. 2).

Fat droplets in hepatocyte cytoplasm (usually smaller than a cell nucleus) were found in the microscopic preparations of livers of all foxes fed with the DDK feed supplement (Fig. 3). It was lipid infiltration which covered from about 30% to 60% hepatocytes (Fig. 3). It was accompanied by parenchymatous degeneration most often in limited areas to a half of the analysed animals. Individual hepatocytes with the features of necrosis were seldom observed. In six foxes, congestion of the liver was observed, which most frequently was exhibited by dilatation of large blood vessels, filling them with blood cells and, occasionally, by excessive storage of blood morphotic elements in the Disse’s spaces. In one case, the described lesion was accompanied by a trail extravasation. The swelling of the walls of some blood vessels was observed in the livers of two foxes and hyperplasia of the connective tissue within the liver was found in two other foxes. Dispersed infiltration of mononuclear cells occurred with varied intensity in 7 animals. Infiltration of stellate cells occurred relatively frequently. In some cases, hypertrophy of these cells was found (Fig. 3). In the majority of animals, individual hepatocytes with mitotic divisions were observed.

In the kidneys of three foxes from group 2, foci of parenchymatous degeneration of the renal tubule epithelium were found and, in one case, hyaline degeneration was found. Only few tubules’ epithelium cells became necrotic. In 5 animals, kidney congestion was reported and, in one, extravasation was observed. In the kidney sections of 4 foxes, glomerulonephritis was observed. On occasion, it was accompanied by infiltration of macrophage-hisctiocyticals.

The experimental results indicate that the foxes fed diets supplemented with DDK had a better blood supply to the liver and kidney in comparison to the animals fed unsupplemented diets. Additionally, the effect of the analysed factors on the fox metabolism was visible. It resulted in a decrease of lipid infiltration by half. The size of the lipid droplets in hepatocyte cytoplasm was decreased and the number of lipid non-storing cells increased.

The DDK also stimulated the macrophage-histiocytical system. This was exhibited by cell infiltrations in livers and in kidneys as well as by hypertrophy of the stellate cells in liver.

Szarek et al. (2003) showed that the DDK administered to foxes in the same manner as in the described experiment induces a better blood supply of
Fig. 1. Liver of a polar fox from group 1 – lipid infiltration in the form of numerous and large lipid droplets in hepatocytes, parenchymatous degeneration and congestion. Haematoxylin and eosin staining, x 460.

Fig. 2. Kidney of a polar fox from group 1 – parenchymatous degeneration, glomerulonephritis. Haematoxylin and eosin staining, x 460.
Conclusions

Based on the morphological examinations, it can be concluded that diets supplemented with the Dig-Deo-Korektor increase blood supply to the organs, decrease storage of lipid in the liver and increase the activity of the macrophage-histiocytical system in polar foxes.

References

Pathomorphological pattern of the kidney in European sheatfish (*Silurus glanis*) after iridovirus infection and lysozyme dimer application in a bath

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Abstract

An experiment was conducted with european sheatfish (*Silurus glanis*) infected with iridovirus using fish which had been earlier immersed in lysozyme dimer (KLP-602) and then infected with iridovirus. The head kidney was evaluated microscopically and ultrastructurally. The results were compared to the observations of control fish. The lysozyme dimer was found to cause a rise in the activity of polymorphonuclear and mononuclear cells and inhibited the multiplication of iridoviruses.

Key words: sheatfish, kidney, iridovirus, lysozyme dimer

Introduction

The lysozyme dimer (KLP-602, Nika Health Products, USA) is an active and efficient product in the treatment of many diseases of various animal species (Klein et al. 1997, Kołodziejecyk et al. 2002).

Considering its immunostimulative and immunocorrective action (Siwicki et al. 1996, Kolman et al. 2002), the effect of dymerized lysozyme (KLP-602) on the morphology of european sheatfish kidney after infection with iridovirus was examined.

Materials and Methods

An experiment was conducted with fry of european sheatfish (*Silurus glanis*) with body weight of 80-100 g, obtained at the Experimental Farm IRŚ in Zatorze, Poland. The fish were adapted for 14 days to the experimental conditions. The examinations were carried out in plastic pools of 500-1000 l capacity with the closed water circulations and a temperature of 20-22°C. At the time of experiments fish were fed with commercial feed containing 35% of protein. The sheatfish were divided into three groups (n=12). The

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first group contained the control fish, not subjected to immersion in lysozyme dimer (KLP-602) and not infected with iridovirus. The sheatfish in the second group were not immersed in the lysozyme dimer but were infected with iridovirus (a virus isolated from sheatfish – 62.90 – was used), 0.1 ml of which was introduced to the organism intraperitoneally. The fish from the third group were immersed in a 0.2% solution of lysozyme dimer (KLP-602) for 0.5 h, and after 72 hours they were infected with iridovirus, like the fish in the previous group. The fish were subjected to clinical examination and anaesthetized by Propiscin (IRŚ Żabieniec) 96 hours later. During a microscopic evaluation, sections of head kidney were taken for microscopic and ultrastructural examination. The material for microscopic examination was embedded in paraffin and that for ultrastructural examination was embedded in Epon 812. Sections of paraffin were stained with hematoxylin and eosin, and the ultrathin ones were contrasted with uranyl acetate and lead citrate. The kidney was observed in an Opton 900 PC electron microscope, made in Germany.

Results

During the clinical observation, it was found that all the sheatfish in all groups behaved correctly throughout the time of the experiment. Their post mortem macroscopic pattern also did not deviate from standard.

The microscopic structure of the kidney of all the control fish in histological preparations was normal.

In the kidney of the control sheatfish, an ultrastructural pattern characteristic of the species under study was observed.

In the kidney of the sheatfish in group two, numerous epithelial cells with necrotic features were found (Fig. 1). Some epithelium cells contained an increased number of mitochondria and proliferation of rough endoplasmatic reticulum was observed in them. Myelin-like structures of various sizes were found with relatively high frequency in the cytoplasm of these cells. The presence of virions was observed in a considerable number of epithelial cells. They were usually situated in the nuclei cells, whose structure in such cases was damaged (Fig. 2). Only sporadically the virions were situated in the cytoplasm. They were gathered in large clusters, were usually mature and well-formed. Macrophages were found more frequently and plasmatic cells slightly more frequently than in the control fish.

Epithelial cells in the kidney of the third group became necrotic only sporadically. In many of them there was a complex rough endoplasmatic reticulum and in some of them, the smooth endoplasmatic reticulum became transformed into vesicles. Virions were present mainly in cytoplasm, where they formed small clusters (Fig. 3). They were found in the cells whose number was half as large as in the case of the previous group. Plasmatic cells and macrophage infiltrations, and sometimes granulocytes with mitotic

Fig. 1. Sheatfish 24 hours after an infection with iridovirus – necrosis of renal tubules epithelium. Haematoxylin and eosin staining, x 500.
Fig. 2. Sheatfish 24 hours after an infection with iridovirus – renal tubule epithelium cell with large clusters of virions present in the cell nucleus (arrows) and single virions present in cytoplasm (arrowheads). TEM, x 14 500.

Fig. 3. Sheatfish 24 hours after an infection with iridovirus, the fish was previously (72 hours earlier) immersed for 0.5 hour in a 0.2% aqueous solution of lysozyme dimer – renal tubule epithelium cells with a small number of virions present in cytoplasm (arrows) and single virions situated outside of the cell (arrowheads). TEM, x 14 500.
division, were found much more often than in the sheatfish of the second group.

Discussion

It was found that lysozyme dimer significantly increased the activity of polymorphonuclear and mononuclear cells (Siwicki et al. 1996). It accelerated the phagocytic activity of neutrophils and macrophages, lymphocyte proliferation, lysozyme and immunoglobulin levels in plasma. These processes were reflected in the experiment conducted for this study. The activity of these cells was more distinct in the sheatfish, which were subjected to the lysozyme dimer and iridoviruses than in the fish, which were not immersed in water with the addition of lysozyme dimer (KLP-602). In addition, the microscopic and ultrastructural image of the sheatfish kidney showed the inhibiting action of the lysozyme dimer (KLP-602) on the multiplication of iridoviruses and on their activity and the stimulation of the activity of plasmatic and phagocytic cells.

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References


Rhodococcus equi infection in foals – clinical and pathomorphological changes

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Abstract

Rhodococcus equi is one of the most important cause of disease in foals. Seldom it is observed in other animal species and in humans. Rhodococcosis is an endemic disease causing serious economical loses in a certain number of study farms in Poland. A number of inquiries devoted to this disease have been performed in Poland yet its occurrence is still not adequately explored. The aim of this study is to recognize the present situation of R. equi infection in selected study farms in Poland with a particular emphasis on the clinical course of the disease.

Key words: Rhodococcus equi, horses

Introduction

Rhodococcus equi is one of the most important cause of disease in foals seldom observed in other species of animals and in humans. The disease has worldwide distribution. Although all horse farms are likely to be infected with R. equi the clinical disease is enzootic and devastating on some farms, sporadic on others, and unrecognized on most (Takai et al. 1991).

Most commonly it is isolated from humans with acquired immunodeficiency syndrome (AIDS), but there were also cases of infection in renal transplant recipients, adult humans with hemolymphatic tumours (i.e. acute leukemia) or after corticosteroids therapy and in an immunocompetent persons as a posttraumatic infection (Prescott 1991).

Rhodococcus equi is ubiquitous in the soil. The main routes of infection are respiratory and alimentary tracts. The particular susceptibility of the foals is not clearly understood. Foals, whose maternal immunity wanes before generation of their own immune response, readily develop disease, if exposed aerogenously to sufficient numbers of Rhodococcus equi (Yager 1987). Therefore, management and environmental circumstances play a major role in determining the magnitude of this challenge and in the prevalence of the disease.

Although Rhodococcus equi infection can produce life-threatening pyogranulomatous pneumonia, most horses develop a protective immune response, which lasts throughout life (Kohler et al. 2003).

In 1-6 months old foals Rhodococcus equi is an important cause of pneumonia. About half pneumonic foals have also ulcerative colitis (necrotizing enterocolitis) or the other intestinal lesions (Yager 1987, Prescott 1991, Giguere and Prescott 1997, Hondalus 1997).

As it is known Rhodococcus equi infection could have some different clinical and pathomorphological forms. The aim of this study was to determine forms of infection, which are present in four farms with endemic rhodococcosis in Poland.
Materials and Methods

The study was performed in four farms in Poland on seventeen foals died with clinical signs of rhodoccosis. The foals were both sexes, 2-4 months old and of different breeds (xo – 3, xx – 8, oo – 1, cross-breed – 5). All animals were treated with different antibiotics (also erythromycin and rifampin). Clinical signs and dates were collected by interview with local veterinarians. Post mortem examination, histopathological and bacteriological diagnosis were performed in all cases. Histopathological examination (lungs and other pathological changed organs; paraffin slides stained haematoxylin and eosin) and bacteriological examinations (from suppurative changes; selective medium CAZ-NB agar) were conducted using routine methods.

Results

All seventeen foals suffered from pneumonia. They had dyspnoea and fever (39.5-41°C), rarely nasal discharge and cough. Four of them died after very short period (1-3 days) of clinical signs of disease. In fifteen cases there were solitary or numerous abscesses in lungs parenchyma (Fig. 1). The diameters of abscesses ranged usually from 0.1 to 3.0 cm, but the largest diameter measured 10 x 25 cm. Histopathologically, the lesions in lungs were predominantly pyogranulomatous. In one horse the giant cells were observed. Neutrophils were numerous; lymphocytes and plasma cells were present in moderate numbers. In six cases there were also inflammatory cell infiltrates in interstitial tissue of lungs. Other anathomopathological changes were also present, as follows: one case of pleuritis, one of pericarditis, twelve of hydropericardium. In one case (with clinically observed diarrhoea) there was colitis connected with other intestinal lesions (Fig. 2.). The pyogranulomatous changes have been observed in a colon (and R. equi was isolated). There was also clinically diagnosed corneal opacity (two cases) and edema of joints (two cases). Rhodococcus equi was isolated from nine cases (app. 53%).

Discussion

The Rhodococcus equi infection has been confirmed by bacteriological examination only from nine cases, but the clinical and anathomopathological symptoms were evident and similar in all foals. Problems with isolation of R. equi could be caused by long antibiotic treatment and difficult bacteriological
Fig. 2. Colon with abscesses caused by *R. equi*.

examination of suppurative changes. In some samples there was intensive growth of proteolytic bacteria (*Proteus spp.*). The predominant clinical symptom of the disease was pneumonia and it correlated very well with the results of post mortem examination. This is in accordance with previous publications (Prescott 1991, Giguere and Prescott 1997, Hondalus 1997). In our study the pathological changes in a digestive tract was diagnosed only in one case (app. 6%); however, the data from other publications differ and estimate its prevalence for 30-50% (Yager 1987, Prescott 1991, Giguere and Prescott 1997, Hondalus 1997). We did not observe other symptoms reported in the literature. Chaffin et al. (1995) described the cauda equina syndrome with urinary incontinence, discospondylitis and paravertebral abscess in a case of *Rhodococcus equi* infection in 4 months old male of Quarter Horse. In this case *Rhodococcus equi* was isolated from specimens of bone and fluid samples from abscess. Firth et al. (1993) reported osteomyelitis, connected with this infection in two foals. Giguere and Prescott (1997) described non septic polisynovitis, particularly of the tibiotalar and stifle joints and septic arthritis and/or osteomyelitis. In our material there were two clinical cases of joint edema.

Pedrizet and Scott (1987) described subcutaneous abscess formation and cellulitis in a three months old thoroughbred filly, but we have not proved such cases of infection.

Giguere and Prescott (1997) reported that some foals could have also uveitis or panophtalmitis, and in two horses there were clinical changes in cornea.

In conclusion, *Rhodococcus equi* infection in Polish horses features different morphological and clinical forms, just like the cases studied elsewhere. This preliminary study shows however that, contrary to other publications (Yager 1987, Prescott 1991, Giguere and Prescott 1997, Hondalus 1997), in the course of rhodoccosis the pathological changes in digestive tract are observed seldom.

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CGRP-immunoreactive structures in the porcine superior cervical ganglion (SCG) – decentralization/deafferentation-induced changes

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Abstract

The occurrence of CGRP-containing nerve structures was studied in intact and decentralized/deafferented porcine SCG. A low number of CGRP-IR perikarya, supplied by patchily distributed moderate to dense meshwork of varicose LENK-IR fibres, was found in the ganglion; number of these cells remained constant after decentralization/deafferentation. Three subpopulations of CGRP- and/or SP-IR nerve fibres have been found to exist in porcine SCG: SP/CGRP-IR, solely SP- and solely CGRP-IR (48%, 45% and 7% of all studied fibres, respectively). Virtually all SP- and SP/CGRP-IR nerves disappeared after the decentralization/deafferentation. However, solely CGRP-IR fibres were still present in SCG, what suggests their intraganglionic origin.

Key words: superior cervical ganglion (SCG), calcitonin gene-related peptide (CGRP), substance P (SP), decentralization, deafferentation, pig

Introduction

Calcitonin gene-related peptide (CGRP) was found to be widely distributed throughout both the central and peripheral nerve system. In the periphery, CGRP and substance P (SP) are thought to be the most important peptides involved in the transmission of sensory modalities. However, apart from the afferent collaterals present in the ganglionic neuropil (Heym et al. 1993), CGRP-IR postganglionic neurons have also been described in sympathetic ganglia of man (i.e. Tajti et al. 1999) and various mammals (for a most recent references, see Hisa et al. 1997). CGRP-IR sympathetic neurons have been found also in the porcine stellatum (Happola et al. 1993) and inferior mesentetric (Majewski and Heym 1992) ganglia. As there is no data concerning the distribution of CGRP-IR neurons/nerve fibres in the porcine SCG, the present study was aimed by means of double and triple-labelling immunofluorescence at unravelling the distribution and neurochemical coding of CGRP-IR structures in this ganglion of the pig.
Materials and Methods

Six immature female pigs, divided into two groups: intact animals (control group; C) and animals with cervical sympathetic trunk transected 1.5 cm below the SCG (decentralized/deafferented group, D), were used for the present study. Ten µm thick cryostat sections were incubated with two or three primary antibodies raised in different species, including rabbit anti-CGRP (1:4000, Affiniti, UK, Bachem, CH), rat anti-SP (1:350, Biogenesis, UK), rat anti-NPY (1:150, Affiniti, UK) and mouse anti-TH (1:60, Chemicon, USA) and mouse anti-LENK (1:600, Biogenesis, UK) antiserum. Double- or triple-labelled sections were incubated with goat anti-rabbit biotinylated IgG (DAKO, Dk) and antigen-antibody complexes visualized then by secondary antisera/reagents coupled to FITC, CY-3 or AMCA (all from Jackson, USA). Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

Results and Discussion

CGRP-IR but TH-, NPY- and LENK-immunonegative neurons were sporadically observed in the porcine SCG (Figs 1, 4, 5). These cells were associated with moderate to dense meshwork of LENK-IR, varicose nerve terminals (Fig. 4) which disappeared after cervical sympathetic trunk (CST) transection. SP-IR principal ganglionic neurons were found in porcine SCG neither in intact, nor in decentralized/deafferented ganglia (Fig. 3, 5). Similarly, no changes in the number and immunofluorescence intensity of CGRP-IR neurons were observed after CST transection. Double-immunofluorescence revealed three populations of CGRP- and/or SP-IR nerve fibres within the ganglion: SP/CGRP-IR, solely SP- and solely CGRP-IR (Fig. 3, constituting up to 48%, 45% and 7% of all fibres studied, respectively). While virtually all SP- and SP/CGRP-IR nerve fibres disappeared from the porcine SCG after the decentralization/deafferentation, a small population of solely CGRP-IR nerve terminals was still present in the ganglionic neuropil. Thus, the results dealing with the occurrence of CGRP-IR neurons in the porcine SCG are in line with data concerning the human, feline and canine ganglion, but are contradictory to the findings regarding rodents SCG (for a most recent references, see Hisa et al. 1997). As revealed by double- and triple-immunofluorescence labelling, sympathetic CGRP-IR neurons of the porcine SCG belonged to the subset of non-adrenergic neurons targeted by opioidergic nerve terminals in a very specific manner. This agreed well with neurochemical characteristics of the CGRP-IR nerve cells found in other species (i.e. cat, dog, for a most recent references, see Hisa et al. 1997), but not in human SCG, where a relatively large number of noradrenergic neurons contained immunoreactivity to CGRP0 (Baffi et al. 1992). Furthermore, as revealed by decentralization/deafferentation, virtually all of the solely CGRP-IR nerve terminals were of intraganglionic origin, while the SP/CGRP- and solely SP-IR nerve fibres were processes of spinal primary afferent neurons.

Acknowledgments

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References


Proliferative enteropathy (PE)-induced changes in the distribution pattern of vesicular acetylcholine transporter-immunoreactive (VAChT-IR) nerve terminals in the porcine inferior mesenteric ganglion (IMG)

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Abstract

This study was aimed at disclosing density of (both viscerofugal and preganglionic sympathetic) VAChT-IR nerve terminals in the IMG of pigs suffering from PE. In the control, healthy pigs virtually all IMG neurons were supplied by moderate to dense meshwork of VAChT-IR nerves. In PE-affected pigs, both the number and fluorescence intensity of these nerve terminals were distinctly increased. In conclusion, an increase in the number of VAChT-IR nerve fibres observed within the porcine IMG may reflect sprouting of viscerofugal cholinergic nerve terminals or may result from the inhibition of ACh release during PE.

Key words: proliferative enteropathy, noradrenaline, vesicular acetylcholine transporter (VAChT), inferior mesenteric ganglion (IMG), pig

Introduction

The present study focused on the cholinergic nerve terminals supplying descending colon-projecting, both DβH-IR and DβH-immunonegative neurons in the IMG of pigs undergoing proliferative enteropathy (PE, induced by Lawsonia intracellularis infection). Till now, the search of pre- and/or postganglionic cholinergic structures in the peripheral nerve system was based on the use of markers of acetylcholine (ACh) synthesis (choline acetyltransferase, ChAT, or vesicular acetylcholine transporter, VACHT). Since PE is known to be associated with a severe, chronic, proliferative inflammatory process of the bowel wall it may be of interest to investigate cholinergic nerve terminals in the porcine IMG to verify the assumption that the organization of the intestino-intestinal reflexes can be deregulated by this pathological process. In the present study, we decided to use the antibody against VAChT for the detection of the cholinergic structures within the porcine IMG, as it is more reliable marker than ChAT for axons/their arborizations originating from the spinal cord (intermediolateral nucleus, the source of sympathetic preganglionic fibres) or from the viscerofugal neurons located in the myenteric and submucosus plexuses (Brown and Timmermans 2004) of the porcine bowel.

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Materials and Methods

Nine immature female pigs (approximately 8 weeks old), three control (C group), three with clinically diagnosed *Lawsonia intracellularis* infection (PE) and three subjected to bilateral transection of the caudal colonic nerves, the sole pathway for all viscerofugal terminals projecting from the enteric ganglia to the IMG (A group) were used. All pigs were subjected to median laparotomy and injected with 5% aqueous solution of Fast Blue (FB) into the wall of the descending colon. After a three-week survival period, the animals of C and PE groups were sacrificed with an overdose of sodium pentobarbital (Vetbual, Biowet, Polad: 90 mg/kg b.w.) and transcardially perfused with freshly prepared 4% paraformaldehyde in 0.1 M phos-
phate buffer (pH 7.4). Pigs of A group were axotomized one week prior to the perfusion. Ten µm thick cryostat sections of the IMG were processed for a routine double-labelling immunofluorescence. Briefly, sections were incubated in the humid chamber with a mixture of two primary antibodies raised in different species (overnight, room temperature), including antisera to DβH (1:350; mouse monoclonal, Chemicon, USA) or TH (1:80; mouse monoclonal, Chemicon, USA) and VAChT (1:2400; rabbit polyclonal, Phoenix, USA). For visualization of complexes of the antigen-primary antibody, a mixture of directly labeled secondary antibodies either conjugated to FITC or CY3 (AffiniPure FITC-conjugated donkey anti-mouse Fab’ fragment and AffiniPure CY3-conjugated donkey anti-rabbit Fab’ fragment; both from Jackson Immunochemicals, USA; used in working dilution 1:1000) were used. Retrogradely labeled double-immunostained perikarya were then evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets. In order to estimate the relative number of FB+ cells apposed by VACHT-IR terminals, retrogradely labeled neurons were counted in each fifth section (only neurons with clearly visible nucleus were included) and presented as mean ± SEM. Pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

**Results and Discussion**

In the control ganglia, nearly all (90.5 ± 1.8%) retrogradely labeled neurons expressed co-localized TH (not shown) and DβH (Figs 1, 3). However, IMGs of animals suffering from PE showed a slight decrease in the number of noradrenergic perikarya (80%). In both, control and PE-affected animals, virtually all FB+ neurons were VACHT-negative, however, they were surrounded by moderate to dense meshwork of nerve terminals containing this substance (Figs 1-4). FB, VACHT-IR neuronal somata, sporadically observed in control and PE-affected ganglia were also supplied by VACHT-IR nerve terminals. In contrast to the unchanged number and properties of FB+ noradrenergic IMG neurons, an increase in the number of visible VACHT-IR fibres was found in PE-affected IMG (compare Figs. 1, 3 and 2, 4). Moreover, the intensity of immunofluorescence of these terminals was stronger as compared to that observed in the control ganglia (sections from both control and affected ganglia were stained with the same aliquot of antisera mixture, as well as were observed and photographed with the same camera settings). As revealed by the transsection of caudal colonic nerves (group A), the vast majority of VACHT-IR terminals within the porcine IMG originate from enteric ganglia. Thus, results of the present study suggest that an increase in the number of VACHT-IR nerve fibres observed within the porcine IMG reflects sprouting of viscerofugal cholinergic nerve terminals or may result from the inhibition of ACh release during *Lawsonia intracellularis* infection.

**Acknowledgments**

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**References**


Potential pathogens in intensive rearing of Wels catfish (*Silurus glanis* L.)

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Abstract

Seasonal occurrence of bacteria *Enterobacteriaceae, Aeromonas hydrophila, Pseudomonas fluorescens, Staphylococcus* spp. and fungi in post-cooling water, skin mucus and the alimentary tract contents in Wels catfish (*Silurus glanis* L.) of intensive fish cage breeding was determined. The microorganisms were found in the smallest amounts in mucus, whereas their largest amounts were found in the fish alimentary tract contents. The bacteria *Enterobacteriaceae* and fungi were the dominant microorganisms in water and alimentary tract contents, whereas in mucus fungi dominated. The microorganisms in question were usually the most numerous in summer and the least numerous in spring.

Key words: microorganism, post-cooling water, intensive fish rearing

Introduction

Fish are exposed to various microorganisms which are either saprophytic microflora of aquatic environment or get there from soil, waste or from air. Some of the microorganisms are pathogenic to fish, others are conditionally pathogenic, yet other are non-pathogenic. However, not always they can be classified as strictly saprophytic and pathogenic to fish (Kozińska et al. 2002). Quick development of pisciculture associated to intensive fish rearing has been noted for some recent years. In such conditions fish are much more vulnerable to diseases and the symptoms are much more intense than in fish living in the wild. The aim of the study was to determine potential pathogens present in water, in skin mucus and in the alimentary tract contents of Wels catfish (*Silurus glanis* L.) during intensive fish cage rearing in post-cooling waters.

Materials and Methods

The measurements were done with samples of water and fish (*Silurus glanis* L.) taken in the Centre of Fish Rearing in Ostrołęka where intensive rearing was carried out in fish cages placed in a canal, to which post-cooling water from a heat and power station was drained off.

Samples of water and fish were taken in three periods of 2003 – in spring (01 June), in summer (24 July) and in autumn (23 September). Samples of water

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were taken at two depths of the fish cage (0.3 m and 1.3 m), and the samples of fish, three individuals each time, were taken from the same place. Bacteria from the family Enterobacteriaceae, Aeromonas hydrophila, Pseudomonas fluorescens, Staphylococcus spp. and fungi were determined quantitatively and qualitatively in all the samples of water and skin mucus, as well as in the fish alimentary tract contents. Quantitative determinations were done by a culture method, on selective culture media (Table 1), in three parallel replications. The results were converted into colony forming units (cfu) in 1 cm\(^3\) of water, in 1 cm\(^2\) of skin mucus and in 1 g of fish alimentary tract contents. Physiological saline was used for diluting the samples (0.85\% NaCl).

Table 1. Rearing conditions of microorganisms determined in water and in fish.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Medium</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae</td>
<td>Endo</td>
<td>37\°C/24h</td>
</tr>
<tr>
<td>Aeromonas hydrophila</td>
<td>mA</td>
<td>37\°C/24h</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Kinga B</td>
<td>26\°C/48h</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>Chapmana</td>
<td>37\°C/48h</td>
</tr>
<tr>
<td>Fungi</td>
<td>Saboraunda</td>
<td>25\°C/5 days</td>
</tr>
</tbody>
</table>
Fig. 1. Mean numbers of potentially pathogenic microorganisms in post-cooling water \( p \) (cfu/cm\(^3\)), skin surface mucus (cfu/cm\(^2\)) and alimentary tract contents (cfu/g) in Wels catfish (\textit{Silurus glanis} L.) during intensive fish cage breeding.

**Discussion**

Data from literature suggest that because of a large amount of organic matter which gets to water during the intensive fish rearing an increased numbers of the microorganisms are found. They are mainly \textit{Aeromonas}, \textit{Pseudomonas}, \textit{Plesiomonas}, \textit{Acinetobacter} and the family \textit{Enterobacteriaceae} (Esteve 1991, Lewandowska et al. 2001, Zmysłowska et al. 2001). Contamination of the aquatic environment is reflected in the microbiological condition of fish. The results of own research have shown that bacteria \textit{Enterobacteriaceae} and fungi dominated in the post-cooling water and in the fish alimentary tract contents. Both groups indicate organic contamination of the allochtonic type. Bacteria \textit{Aeromonas hydrophila}, \textit{Pseudomonas fluorescens} and \textit{Staphylococcus} spp. were also found. These results confirm and supplement the research conducted by the authors who dealt with these issues (Zmysłowska 2000a, b, c, Lewandowska et al. 2001). Aquatic bacteria are highly changeable. In certain conditions saprophytic bacteria can become virulent and dangerous to fish. It is related to the factors, which make fish vulnerable to infections caused by relatively pathogenic, or even saprophytic bacteria; it is especially true about fish of reduced immunity.

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Antibiotic-resistant bacteria isolated from fish and from water during intensive fattening

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Abstract

The resistance of bacteria *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Enterobacteriaceae* isolated from water, the alimentary tract contents and skin mucus of catfish (*Silurus glanis* L.) to flumequine, oxytetracycline, novobiocin, trimethoprim, enrofloxacin, canamycin, oxolinic acid and neomycin was examined. The bacteria were the most resistant to novobiocin and the least resistant to canamycin. No difference was found in the resistance to medicine among the strains from various environments.

Key words: antibiotic-resistant bacteria, catfish (*Silurus glanis* L.), intensive fattening of fish

Introduction

Progressive intensification of fish production and an increase in the number of diseases caused by bacteria in fish are accompanied by the problem of the increasing resistance of bacteria to antibiotics, both in pathogens and in saprophytes. The issue is particularly important because of the transfer of the genes of resistance, both among pathogens and from saprophytes to pathogens.

The aim of the study was to determine the sensitivity of bacteria *Aeromonas hydrophila*, *Pseudomonas fluorescens* and those of the family *Enterobacteriaceae* isolated from water, alimentary tract contents and skin mucus of catfish (*Silurus glanis* L.) to antibiotics commonly used in fish diseases in Poland.

Materials and Methods

The samples of water, alimentary tract contents and skin mucus of catfish (*Silurus glanis* L.), which were taken in February, March and April 2004 in the Fish Breeding Centre in Ostrołęka were the material used in the experiment. In the course of the study the bacteria *Aeromonas hydrophila*, *Pseudomonas fluorescens* and those of the family *Enterobacteriaceae* were isolated. They were growing in the form of characteristic colonies on selective media (mA, King B and Endo, respectively) and their sensitivity to antibiotics was determined. The taxonomic identity was confirmed microscopically and biochemically.

The sensitivity of the isolates to antibiotics was determined by the method of diffusion disks on Muel-
ler-Hinton broth. The disks with the following antibiotic media were used in the experiment: flumequine (UB, 30 µg), oxytetracycline (OT, 30 µg), novobiocin (NV, 5 µg), thrimetoprim (W, 5 µg), enrofloxacin (ENR, 5 µg), canamycin (K, 30 µg), oxolinic acid (OA, 30 µg), neomycin (N, 30 µg).

Results

During the whole experiment 114 strains of bacteria were isolated. More than a half (60.53%) came from the aquatic environment and the others were isolated from the fish alimentary tract contents and from skin mucus. \textit{Aeromonas hydrophila} accounted for the majority of the isolates (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>UB</th>
<th>OT</th>
<th>NV</th>
<th>W</th>
<th>ENR</th>
<th>K</th>
<th>OA</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Aeromonas hydrophila}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>3</td>
<td>22</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>alimentary tract*</td>
<td>3</td>
<td>5</td>
<td>11</td>
<td>5</td>
<td>1</td>
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<td>4</td>
</tr>
<tr>
<td>skin mucus*</td>
<td>5</td>
<td>2</td>
<td>9</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
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<td></td>
<td></td>
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<td>3</td>
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<td>3</td>
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<td>1</td>
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</tr>
<tr>
<td>skin mucus</td>
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<td>6</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Enterobacteriaceae}</td>
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<td></td>
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</tr>
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<td>2</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>alimentary tract</td>
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<td>3</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>skin mucus</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>


Table 2. Number of antibiotics to which strain resistance was found.

<table>
<thead>
<tr>
<th>Number of antibiotics to which strain resistance was found</th>
<th>water</th>
<th>alimentary tract of catfish</th>
<th>skin mucus of catfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
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</tr>
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<td>7</td>
</tr>
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<td>–</td>
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</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Number of isolates</td>
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<td>21</td>
<td>24</td>
</tr>
<tr>
<td>% resistant isolates</td>
<td>98.55%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Fig. 1. Frequency of resistance to antibiotics (UB – flumequine, OT – oxytetracycline, NV – novobiocin, W – trimetoprim, ENR – enrofloxacin, K – canamycin, OA – oxolinic, N – neomycin) for bacterial strains.
Novobiocin was the antibiotic the resistance to which was found to occur the most frequently. The resistance to trimethoprim was also frequent. Canamycin and enrofloxacin were the antibiotics to which the absence of sensitivity was found the least frequently (Fig. 1). No difference was found in the resistance to the medicine among the strains from various environments.

Among 114 strains there was only one, isolated from water, which was sensitive to all the antibiotics used. Usually a resistance to one, two or three antibiotics was found. No strain was found to be resistant to more than five antibiotics (Table 2).

**Discussion**

The study revealed that the resistance to novobiocin and to trimethoprim existed in more isolates in comparison with other antibiotics (85.9% – 95.7% and 57.12% – 70.08% of isolates, respectively). The mechanism of resistance to these two antibiotics and to oxytetracycline may be the result of active removal of antibiotics with a protein acting as a pump (Markiewicz and Kwiatkowski 2001). A high frequency of resistance to antibiotics in the bacteria isolated from fish during intensive fattening indicates a role which may be played by fish breeding farms as reservoirs of genes of resistance, which may be transferred to other animals and to humans.

Like in the studies conducted by other authors (McPhearson et al. 1991, Miranda and Zamelman 2001) the bacterial strains were the most sensitive to canamycin and enrofloxacin.

Oxytetracycline is the most frequently used antibiotic in fish diseases in Poland. It is caused by its vast range action, a relative safety of use and low cost of production and sales. The resistance to oxytetracycline was as high as 52.36% among the strains isolated from the alimentary tract contents. The absence of sensitivity to this antibiotic was most often found in the rods *Aeromonas hydrophila*. These bacteria cause motile aeromonas septicemia, although those strains isolated from water, alimentary tract contents and from mucus are not necessarily virulent (McPhearson et al. 1991). Magee and Quinn (1991) found the resistance to oxytetracycline in the bacteria isolated from the environments where no antibiotics had been used, and Miranda and Zamelman (2001) – in bacteria isolated from the fish living in the wild. This indicates a possibility of transfer of the genes of resistance to this antibiotic.

**References**


Telomerase activity in squamous cell carcinoma in dogs

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Abstract

The eight cases of squamous cell carcinoma in dog were investigated for telomerase activity. The main value was 398.8 ± 98.9 U/µg protein. This is the first report about reference value of telomerase activity in canine squamous cell carcinoma.

Key words: telomerase, skin neoplasia, squamous cell carcinoma

Introduction

Telomeres are the regions of linear chromosomes essential for their stability. In the majority of normal somatic cells telomeres tend to successive cell division. Telomerase is a ribonucleoprotein enzyme which maintains the lengths of telomeres. It is present in fetal tissues, and shortly after birth only in immortal and germ line cells (Biller et al. 1998, Argyla et al. 2003). Telomerase activity can also be found in most malignant tumours in human and other mammals (Biller et al. 1998, Argyla et al. 2003). Information about values of telomerase activity in variety canine skin neoplasm in literature is very fragmentary. The aim of the study was to investigate telomerase activity in neoplastic tissue in dogs with squamous cell carcinoma (SCC).

Materials and Methods

The samples of skin tumours were obtained from dogs that were surgically treated. Immediately after surgical resection neoplastic tissues were divided in two parts: one for histopathological examination and other for telomerase activity determination. The diagnoses of SCC were based on histopathological examination of haematoxylin and eosin stained sections. The samples for telomerase activity determination were frozen in liquid nitrogen. When several samples were collected they were homogenized and examined of protein concentration. Telomerase activity was measured by the telomeric amplification protocol (TRAP) using TAgGG Telomerase PCR ELISA plus from Roche. As a control five samples of regular skin from dogs were used.

Results

The results of telomerase activity in SCC are shown in the Table 1. The activity of telomerase in samples of regular skin was less than 1 U/µg protein in all cases.

Discussion

From 1994 when Kim et al. (1994) announced results of their work about the method of measurement of telomerase activity in tissue samples (TRAP assay) many scientists have undertaken researches on this...
Table 1. Telomerase activity in neoplastic tissue of SCC.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Telomerase activity (U/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 years old, female, mix</td>
<td>560</td>
</tr>
<tr>
<td>11 years old, female, mix</td>
<td>354</td>
</tr>
<tr>
<td>9 years old, female, mix</td>
<td>527</td>
</tr>
<tr>
<td>6 years old, female, mix</td>
<td>343</td>
</tr>
<tr>
<td>8 years old, female, mix</td>
<td>278</td>
</tr>
<tr>
<td>10 years old, male, mix</td>
<td>339</td>
</tr>
<tr>
<td>7 years old, male, mix</td>
<td>379</td>
</tr>
<tr>
<td>7 years old, male, mix</td>
<td>410</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>398.8 ± 98.9</td>
</tr>
</tbody>
</table>

Many studies on telomerase activity in various human tissues have been published. Taylor at al. (1996) analysed telomerase activity in various types of skin diseases. They found it in 15 of 18 nonmetastatic cutaneous squamous cell carcinoma (SCC). Ueda (2000) showed telomerase activity in 6 of 6 SCC. In contrary Paris et al. (1999) reported telomerase activity only in 3 of 12 SCC. In conclusion, this studies show that although discrepancies exist, telomerase is frequently activated in sqamous cell carcinoma in human. Similar results of the researches of Ueda (2000) in human were obtained in our investigations in dogs where telomerase activity was found in all SCC samples. It is difficult to compare this data with the results concerning skin cancer in dogs obtained by other authors because they are very few. In all samples of regular skin telomerase activity did not exceed 1 U/µg protein. Our results show that telomerase activity may be useful indicator of malignancy in canine skin tumours. Its utility in differential diagnosis of sqamous cell carcinoma needs further investigations.

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