MODULATING EFFECT OF LYSOZYME DIMER (KLP-602) ON THE MORPHOLOGICAL PATTERN OF HEPATOPANCREAS OF SIBERIAN STURGEON (ACIPENSER BAERI, BRANDT 1869) FOLLOWING OXYTETRACYCLINE APPLICATION

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Abstract

Siberian sturgeons were given intraperitoneally 100 mg/kg b.m. of oxytetracycline (OTC) and 24 h after the injection they were immersed in lysozyme dimmer solution (KLP-602) at 100 µg/L of water for 30 min. The effect of OTC, applied separately and in combination with lysozyme dimer, on hepatopancreas morphology has been examined. The results of microscopic and ultrastructural examinations of the organ indicated the existence of morphological lesions following the administration of OTC: congestions and extravasations, parenchymal and lipid-related degeneration, and focal necrosis with relatively frequent infiltrations of lymphoid cells. Ultrastructural examination revealed damage to the mitochondria in hepatocytes. KLP-602 was found to have a protective effect in these changes. Immersion of the fish in an aqueous solution of lysozyme dimer was shown to have an immunomodulating effect, reducing the intensity of morphological changes that resulted from the administration of OTC.

Key words: Siberian sturgeon, oxytetracycline, lysozyme dimer, hepatopancreas, pathomorphology.

Acipenseridae fish (order Acipenseriformes, family Acipenseridae) are long-living and slow-growing fish, which do not reach their sexual maturity until the age of 11-13 years. This and strong anthropopressure, especially exerted during the past century, have greatly reduced population of the fish (10). Only in recent years, the rearing of the fish, including the Siberian sturgeon, has become more intensive (1, 2, 5). To this end, the breeding methods used in the rearing of trout and carp have been adopted (4, 7).

In consequence, Siberian sturgeons were transferred from the natural environment to closed circulation systems. Rearing under controlled conditions has revealed that the fish are susceptible to bacterial infections, which has resulted in considerable losses (3). Meanwhile, literature data on antibiotic therapy in cartilaginous fishes, including Siberian sturgeon, refer to a narrow range of research from few research centres (15-18). Hence, there is demand for effective methods of prevention and therapy of infectious diseases in sturgeon. Research aimed at developing such methods is all the more interesting that the coupled effect of concurrent antibiotic therapy and immunomodulation in Acipenseridae fish has not been fully explored. This has practical implications due to resistance to antibiotics in bacteria and, consequently, increasing difficulties in the application of antibiotic therapy. Another important fact is that there are no vaccines against some infectious diseases in fish, and immunomodulation may, in a way, fill the gaps in aquaculture therapy (9).

All this makes sturgeon a new model in experimental research, and it is becoming highly justified to try to determine whether immersion of Siberian sturgeon in lysozyme dimer (KLP-602) modulates morphological lesions developing in the hepatopancreas following administration of oxytetracycline.

Material and Methods

Fingerlings of Siberian sturgeon (Acipenser baeri, Brandt 1869) were used in the experiment. One hundred and twenty fish, aged 4 months, with body mass of 240 ± 10 g (Table 1) were obtained from the Inland Fisheries Institute, Olsztyn. They had not been subjected to antibiotic therapy or immunomodulation.

Before the experiment, the fish were adapted to the experimental conditions for 14 d. During that time,
their behaviour was regular from the clinical point of view and their condition was good. They were placed in four 180 l plastic tanks, in water at a temperature of 20 ±1°C, pH 7.5–8.0, and oxygen level of 7.5–8.0 mg/L. The amount of total ammonium nitrogen in the water did not exceed 0.2 mg/L. The fish were fed extruded fodder Nutra 2.0 (Skretting, Norway) at the dose of 1% of biomass/d (except from the day prior to administration of antibiotic). Continuous feeders were used. Both during the adaptation period and during the experiment, the fish were under veterinary control and were subjected to clinical examination.

Following the period of adaptation, the Siberian sturgeons were divided into four groups (n=30), anaesthetised in turn by sprinkling their gills with 0.2% solution of etomidate (Propiscin, Inland Fisheries Institute, Poland). After the fish were weighed and labelled, those in the groups 3 and 4 were given oxytetracycline (OTC) intraperitoneally at a dose of 100 mg/kg b.m. The preparation – TRIDOX L.A. (Eurovet Animal Health BV, The Netherlands) – was a suspension of OTC in oil, with a high concentration of 200 mg OTC/mL of the preparation. Twenty-four hours after OTC administration, the fish in the group 4 were immersed for 30 min in water with lysozyme dimer KLP-602 (BACHEM, Feinchemikalien AG, Switzerland), diluted to 100 µg/L of water. The fish in the group 2 were immersed only in an aqueous solution of KLP-602, and none of these procedures were applied to the fish in the control group 1.

Immediately following the immersion in the lysozyme and after 3, 7, 14, and 21 d, the fish were anaesthetised (six sturgeons in each group on each of those days) by sprinkling their gills with a Proposcin solution. The fish were put down and sections of the hepatopancreas were observed in all the Siberian sturgeons. Lipid droplets were usually observed all over a hepatocyte and pushed the nucleus to the perimeter. Sections of the hepatopancreas for ultrastructural examination were fixed in 7% glutaraldehyde in a phosphate buffer (pH 7.4). The samples were then postfixed in 2% osmium tetroxide in a phosphate buffer (pH 7.4). After being washed with phosphate buffer and Ringer’s solution, the sections were dehydrated in a range of alcohols and acetone. The material was sealed in Epon 812. Polymerisation was carried out at 45°C for 2 h and at 60°C for 48 h. Semi-thin sections were obtained from the blocks and were subsequently stained according to the method described by Levis and Knight (6). They were viewed under an optic microscope in order to establish the appropriate place for preparing ultrathin sections. The ultrastructural analysis was carried out with an Opton 900 PC TEM (Germany).

Results and Discussion

Clinical and macroscopic pattern. Sturgeons in the groups 1 and 2 were found to behave and react to external stimuli correctly. The fish in the groups 3 and 4, on the other hand, showed reduced movement activity during the first three days following the intraperitoneal injection of OTC. Such behaviour was recorded in some fish from the group 2 until day 7 of the experiment and, sporadically, until day 14.

The post-mortem examination revealed the regularity of the morphological structure of most sturgeons. Hepatopancreas congestion was observed only in 11 fish in the group 3.

Microscopic and ultrastructural pattern. Steatosis simplex (a physiological phenomenon) in the hepatopancreas was observed in all the Siberian sturgeons. Lipid droplets were usually observed all over a hepatocyte and pushed the nucleus to the perimeter. Their sizes were different (Figs 1-8), those in hepatocytes in the fish in the groups 1 and 2 were the smallest (Figs 1, 2). They took large shapes when sturgeons were given only OTC, with the condition unchanged for the entire period of the experiment (Fig. 3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of fish in the group</th>
<th>Oxytetracycline dose (mg/kg b.m. i.p.)</th>
<th>Dose of KLP-602*(30-min immersion 24 h after antibiotic administration) in µg/L</th>
<th>Day when hepatopancreas was taken for examination (after immersion in KLP-602*)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0, 3, 7, 14, 21</td>
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<tr>
<td>2</td>
<td>30</td>
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<td>3</td>
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<td>4</td>
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* the lysozyme dimer dose was applied as converted to the active substance.
Fig. 1. The liver of sturgeons from the group 1 (control). Regular structure. HE, 600x.

Fig. 2. The liver of sturgeons from the group 2 (lysozyme dimer). Infiltrations of lymphoid cells – arrows; 3 d after immersion in lysozyme dimer. HE, 600x.

Fig. 3. The liver of sturgeons from the group 3 (OTC). Congestion – asterisks (a, b), extravasation – short arrows (b-d), infiltration of lymphoid cells – arrows (a-d), parenchymatous degeneration of hepatocytes free from steatosis simplex (b), local necrosis – arrowheads (b-d); a – 4 d, b – 8 d, c – 15 d, d – 22 d after injection of OTC. HE, 600x.

Fig. 4. The liver of sturgeons from the group 4 (OTC and KLP-602). Congestion (asterisks) with small extravasation (short arrows) and infiltrations of single lymphoid cells (arrows); a – 3 d after immersion in lysozyme dimer, b – 7 d after immersion in lysozyme dimer. HE, 600x.
Some of the sections of the hepatopancreas were seen to contain (mainly near blood vessels) islets of parenchymal cells, free from steatosis simplex (Figs 3b, 4a, 4b). Parenchymatous degeneration was observed in such hepatocytes in the sturgeons from the group 3 in sections from each experimental period (Fig. 3b). This was also reflected in morphology of hepatocyte mitochondria (regardless of the extent of steatosis simplex). Damage in those substructures was observed to be the most intensive in fish in the group 3, where the mitochondria were swollen and without cristae (Fig. 7b). Changes in mitochondria were also recorded in hepatocytes of sturgeons from the group 4 (Figs 8a, 8b).

Focal necrosis was also observed in nearly all sturgeons in the group 3 (Figs 3b-3d) and was visible under both optic and electron microscopes (Figs 7a, 7b). This change was also recorded in sturgeons from the group 4. It was revealed only in a ultrastructural examination and it was shown to be present in a small number of hepatocytes in 2-3 fishes in each sampling (Fig. 8a).

Circulation disorders observed in the organ included congestion and extravasations. These were most commonly observed in sturgeons from the group 3 (26 fishes) – Figs 3a, 3b. In such cases, sporadic lesions in vascular endothelium were observed. However, circulation disorders were observed with a much lower intensity in the sturgeons from the group 4 (16 fishes), (Figs 4a, 4b).

Lymphoid cell infiltrations were observed in the fish relatively often and with varying intensity. In those given OTC varied infiltrations, from profuse (Fig. 3a) to single lymphoid cells (Fig. 3b) and none, were observed until day 8 following the injection. However, on day 15 and 22 following the injection, the infiltrations were sporadic and relatively small (Figs 3c, 3d). The hepatopancreas in the sturgeons from the group 4 frequently contained single lymphoid cells or their small clusters (Figs 4a, 4b).

Following the immersion in lysozyme dimer solution (group 2) and until day 14, relatively profuse infiltrations of lymphoid cells were prevalent (Fig. 2). A week later, only small numbers of such cells were observed.

A slight proliferation of mitochondria was observed in several fish in the groups 3 and 4. Hepatocytes with two nuclei were observed relatively often in sturgeons from the group 3.

The clinical, post-mortem, histopathological, and substructural examinations allowed determining the effects of the antibiotic (OTC) and immunomodulator (lysozyme dimer), taking into account their interactions. It is particularly noteworthy that the intraperitoneal dose of OTC administered to the sturgeons and used in experiments by other authors (13, 20) was 10 mg/kg of body mass and was ten times lower than those used in this experiment. Moreover, reports, which describe the effect of OTC on fish organism deal with model species of teleost fish (carp, European catfish, rainbow trout), therefore, a direct comparison of the morphological examination results is extremely difficult. The best example may be the results of morphological examinations of sturgeon liver, in which accumulation of lipids in the cytoplasm of hepatocytes is a physiological condition and steatosis simplex (referred to as ordinary) was observed in sturgeons in all experimental groups. However, there was some variability. It was the most intensive in the groups 3 and 4. This pattern indicates that OTC at 100 mg/kg of body mass deepens the process of lipid accumulation in hepatocytes. A similar reaction was observed by Soler et al. (11), who gave intramuscularly a bolus of OTC to tench at 100 mg/kg b.m. They observed histopathological lesions in the liver and recorded steatosis simplex 24 h after the antibiotic injection, which gradually withdrew. Moreover, Neskovic et al. (8) observed similar changes in carp (C. carpio) following immersion in another xenobiotic – atrazine.

It is noteworthy that parenchymatous degeneration was frequently observed in hepatocytes without steatosis simplex in sturgeons from the group 3. Moreover, the hepatic tissue of the sturgeons, which were given OTC was necrotised relatively frequently.

Studies conducted by Sopińska et al. (12) and Szarek et al. (14) also indicated the necrosis of hepatocytes in fish subjected to higher doses of xenobiotics – herbicides. The intensity of the change in sturgeons was mitigated by immersion of the fish in KLP-602. This is indicated by the fact that intraperitoneal administration of OTC to fish at 100 mg/kg b.m. without immersion in lysozyme dimer solution causes stronger retrogressive lesions in the hepatopancreas than when the two therapeutic agents are used in combination.

It should be pointed out that lymphoid cells are excited following immersion in a lysozyme dimer solution, what was reflected in the presence of infiltrations of these cells in the organ. The excitation was particularly clear until after two weeks following the sturgeon immersion in aqueous solution of KLP-602 and was more poorly expressed on day 22 of the experiment after OTC administration. On the other hand, the presence of the infiltration following OTC administration and then immersion in lysozyme dimer solution should be attributed not only to the stimulating effect of the dimer, but also as a reaction to the organ damage, especially when retrogressive lesions are present. This is indicated by comparison of the morphological structure of the liver in sturgeons treated with the two agents with the liver morphology in the fish treated with only one of them.

Moreover, when injected to rabbits, lysozyme dimer did not cause any morphological lesions in the liver (19).

These facts suggest that immersion of Siberian sturgeons in an aqueous solution of lysozyme dimer (at 100 µm/L of water) for 30 min has a protective effect, probably by immunomodulation, with respect to the morphological lesions resulting from injection of OTC at a dose of 100 mg/kg b.m.
**Fig. 5.** The liver of sturgeons from the group 1 (control). Fragment of hepatocytes with regular structure. 14,800x.

**Fig. 6.** The liver of sturgeons from the group 2 (lysozyme dimer). Fragment of hepatocytes with regular structure. 3 d after immersion in lysozyme dimer. 9,300x.

**Fig. 7.** The liver of sturgeons from the group 3 (OTC). Fragment of a hepatocyte with necrosis of cytoplasmatic structure (n), swollen and damaged mitochondria (asterisks), and maintained lipid drops; a – 15 d after OTC administration, b – 22 d after OTC administration. 14,800x.

**Fig. 8.** The liver of sturgeons from the group 4 (OTC and KLP-602). Fragments of hepatocytes with myelin-like structures (long arrows), damaged mitochondria (asterisks) and lysosomes (short arrows); a - 7 d after immersion in lysozyme dimer, 9,300x, b – 21 d after immersion in lysozyme dimer. 6,300x.
In conclusion, OTC administered intraperitoneally to Siberian sturgeons at the dose of 100 mg/kg b.m. causes retrogressive lesions in the hepatopancreas, as well as circulatory disorders and infiltration of lymphoid cells. These changes are visible as late as 22 d after OTC injection. Immersion of the fish in water with the addition of lysozyme dimer at a dose of 100 µg/L of water for 30 min alleviates the pathomorphological effects of the use of oxytetracycline.

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References