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RESEARCH ARTICLE

Morphological Pattern of Head Kidney in Siberian Sturgeon (*Acipenser baeri* Brandt 1869) Exposed to the Action of Dimerized Lysozyme (KLP-602) after the Application of Oxytetracycline

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ABSTRACT

The antibiotic treatment of cartilaginous fish, including Siberian sturgeon, is relatively poorly understood. The lack of research into simultaneous use of antibiotic and immunomodulation in the Acipenseridae has created the need for effective methods of prevention and treatment of the diseases affecting these species, especially in relation to intensive rearing and breeding with adapted technologies. The Siberian sturgeons administered oxytetracycline intraperitoneally @ 100 mg/kg BW and 24 hours post-injection the fish were immersed in lysozyme dimer @ 100 µg/l water for 30 minutes. The impact of oxytetracycline used together with or separately from lysozyme dimer on the morphology of head kidney in Siberian sturgeon was determined. The results of microscopic evaluation of this organ revealed the formation of morphological lesions resulting from the administration of the antibiotic: hyperaemia and regressive lesions in the epithelium of renal tubules - parenchymatous and vacuolar degeneration, necrosis and hyaline casts in the lumen of renal tubules. The domination of hematopoietic cells over lymphocytes, necrosis of hematopoietic cells, proliferation of lymphoid cells, presence of melano-macrophages and less-frequent macrophages were observed. It was shown that the immersion of Siberian sturgeons in lysozyme dimer had an immunomodulatory effect and decreased the severity of morphological lesions in the head kidney induced by the administration of oxytetracycline.

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INTRODUCTION

The Acipenseridae family (order *Acipenseriformes*) was subjected to strong anthropopressure in the last century which led to a significant reduction in the size of population. These fish are difficult to rear due to their slow growth and long time required to reach sexual maturity at the age of 11-13 years. In order to optimize breeding they have been transferred from the natural environment into closed water systems with the adaptation of breeding techniques used in trout and carp cultures (Kolman *et al.,* 1994). Gram-positive and Gram-negative bacteria pathogenic to fish with impaired immunity (due to, for instance, rearing conditions) have been isolated from water, mucus and chyme of Siberian sturgeon hybrids reared in the closed systems (Zmyslowska *et al.,* 2004; Krause *et al.,* 2006). Furthermore, the susceptibility of these fish to bacterial infections leading to substantial losses has been observed under controlled rearing conditions (Kolman *et al.,* 1999).

All of these factors have created the need for the development of effective prevention methods and treatments of infectious diseases affecting sturgeons. Moreover, the coupled impact of simultaneous antibiotic therapy and immunomodulation in the Acipenseridae has not yet been studied. It has a practical implication related to bacterial antibiotic resistance (Zmysłowska *et al.,* 2004; Haque *et al*., 2014) and increasing difficulties with the exclusive administration of antibiotics as well as the usage of these agents as growth promoters (Reda *et al.*, 2013). It is also important to note that there is a lack of vaccines against certain infectious fish diseases and, therefore, immunomodulation may somehow fill the gaps in treatment modalities used in the aquaculture (Sakai, 1999).

Studies of the impact of xenobiotics (i.e. a new generation of herbicides, fungicides and pyrethroids) on the structure of kidney cells (including the hematopoietic part) in some species of osteichthyes have found pathomorphological effects (Neskovic *et al.,* 1993; Szarek *et al.,* 2000; Cengiz, 2006; Velmurugan *et al.,* 2009, Xing H. *et al.*, 2012). The toxicity of these compounds most often affects the tissues in which the drugs reach high concentrations, i.e. muscles (Sharafati-Chaleshtori *at al.*, 2013) the liver and kidney and immunocompetent cells (Olatoye and Basiru, 2013). Considerable amounts of oxytetracycline (OTC) have been detected in the head kidney in fish, which partly explains its negative impact on the immune system (Rijkers *et al.,* 1980). Furthermore, in fish, OTC is slowly excreted, among others, in an unchanged form by the kidneys with urine (Posyniak, 2000). Apart from water temperature, the other parameters that may modify the availability of OTC include the pH of water, individual consumption of feed, the formation of OTC – metal ions complexes and the route of drug administration (e.g. with *per os* application, the fish are immersed in water, which is also a solution of a drug mixed with feed). In the case of trout, when OTC at 100 mg/kg BW was administered for 10 consecutive days, 33.1% of the total antibiotic dose was dispersed in water (Łapińska *et al.,* 2005). Moreover, OTC is very resistant to degradation in the environment, particularly with a lack of light and may be deposited in bottom sediments.

These considerations make sturgeon a new model for experimental studies and there is a need to determine whether the immersion of Siberian sturgeon on lysozyme dimer KLP-602 with prior administration of OTC has an impact on the morphological pattern of head kidney.

MATERIALS AND METHODS

Fingerling of Siberian sturgeon (*Acipenser baeri* Brandt 1869) was used in the experiment. 120 fish, aged 4 months, with a body mass of 240±10 g were obtained from the Inland Fisheries Institute, Olsztyn, Poland. They had not been subjected to antibiotic therapy or immunomodulation before.

Prior to experiment, the fish were adapted to the experimental conditions for 14 days. During that time, their behaviour was regular from the clinical point of view and their condition was good. They were placed in four plastic tanks, each with 180 l in capacity, in water at a temperature of 20±1^{o}C, pH: 7.5–8.0 and an 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 Siberian sturgeons were fed at the dose of 1% of biomass/day (except for the day prior to administration of the antibiotic) with extruded fodder Nutra 2.0 (Skretting, Norway) using continuous feeders. The fish were under veterinary control and subjected to clinical examination during both the adaptation period and the experiment.

Following the period of adaptation, the Siberian sturgeons were divided into 4 groups (n=30), (Table 1), anaesthetised in turn by sprinkling their gills with a preparation named Propiscin (0.2 % solution of etomidate), manufactured by the Inland Fisheries Institute, Zabieniec, Poland. After the fish were weighed and labelled, those in groups 3 and 4 were given oxytetracycline (OTC) intraperitoneally (*i.p.*) at 100 mg/kg of body mass. The preparation - TRIDOX L.A. produced by Eurovet Animal Health BV, The Netherlands – is a suspension of OTC in oil, with a high concentration of 200 mg OTC/1 ml of the preparation. 24 hours after OTC administration, the fish in the group 4 were immersed for 30 minutes in water with lysozyme dimer KLP-602 (produced by 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 those procedures were applied to the fish in the control group-1.

Immediately following the immersion in lysozyme dimer and after 3, 7, 14 and 21 days, the fish were anaesthetised (6 sturgeons in each group on each of those days) by sprinkling their gills with a Propiscin solution. The fish were put down and sections of head and kidney were taken for microscopic examination. The material was fixed in 7% neutralised formalin and sealed in paraffin blocks and the microtome sections were stained with haematoxylin and eosin (HE).

RESULTS

Clinical and macroscopic pattern: The sturgeons in the groups 1 and 2 showed clinically normal behaviour and reactions to external stimuli. Decreased mobility was observed in the sturgeons from the group 2 until day 7 and occasionally until day 14 of the experiment. In the groups 3 and 4 during three days after the intraperitoneal injection of OTC the fish were less mobile and the decreased mobility persisted until day 14 after the immersion in KLP-602.

Microscopic pattern: In the fish from the group 1, the morphological pattern of the head kidney was regular (Fig. 1). The proliferation of lymphoid cells which was particularly evident until day 7 after the immersion in lysozyme dimer was detected in almost half of the examined individuals from the group 2 (Fig. 2).

The haematopoietic cells in the head kidney in 67% of sturgeons from the group 3 were necrotized to a varied degree (Fig. 3a). The melano-macrophages (Fig. 3b, 3c, 3d) were quite often observed in the fish from this group and they were numerous until day 14 after the immersion in lysozyme dimer. The renal hyperaemia was detected in 7 individuals from this group (Fig. 3c). In 12 fish, the parenchymatous and vacuolar degeneration of epithelial cells and necrosis were reported within very few renal tubules observed in the organ examined; the hyaline casts were quite often present in the lumen of the renal tubules (Fig. 3d).

In the head kidney in the group 4, the haematopoietic cells often dominated over the lymphocytic cells. It was particularly evident in the first phase of the experiment where this finding was reported in four fish immediately after the immersion in lysozyme dimer (Fig. 4a) and in six fish at different time-points following the immersion. In three cases, the necrosis of individual haematopoietic cells was detected. The proliferation of lymphoid cells was occasionally reported. The melano-macrophages (Fig. 4b) were quite often observed in the head kidney of these fish and the macrophages were less common. These cells were

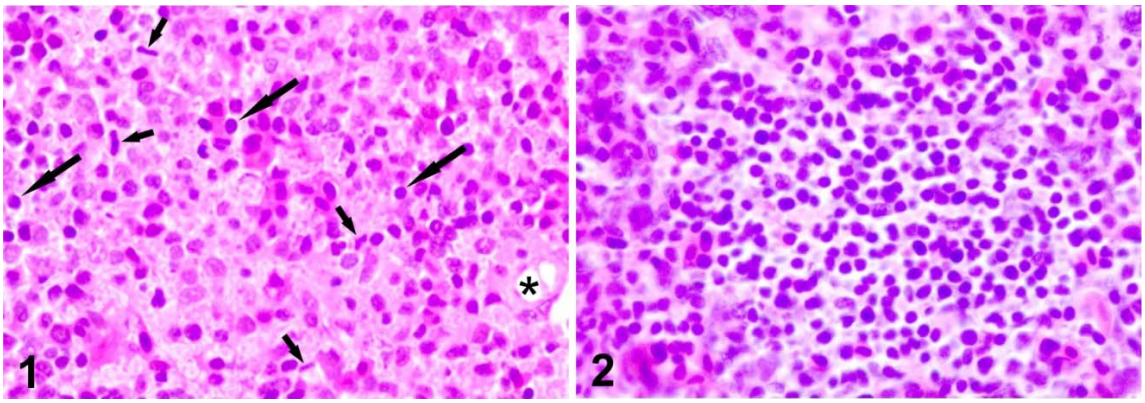

Fig. 1: The head kidney in the sturgeon from group 1 (control) with a regular pattern: lymphoid cells (long arrows), haematopoietic cells (short arrows), red blood cells and renal tubule (asterisk). HE stain. 400x.

Fig. 2: The head kidney in the sturgeon from group 2 seven days after the immersion in lysozyme dimer: proliferation of lymphoid cells in the central view with surrounding haematopoietic cells. HE stain. 400x.

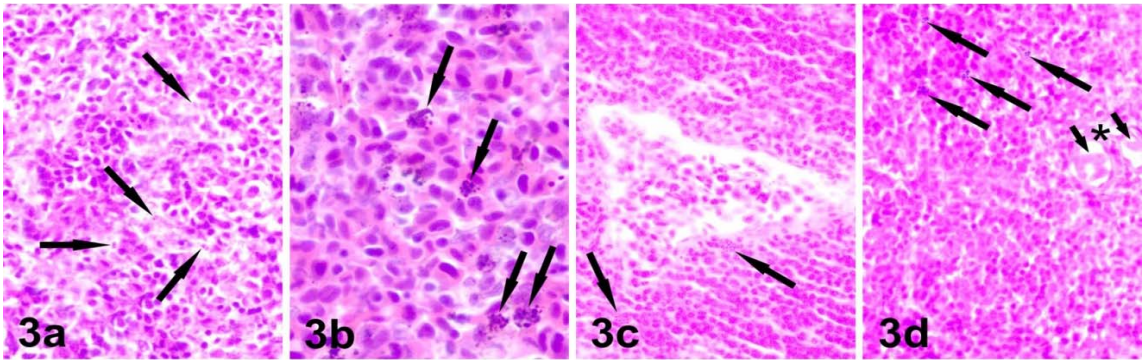

Fig. 3: The head kidney in the sturgeon from group 3: a- morphological pattern immediately after the immersion in lysozyme dimer: numerous haematopoietic cells, some of which were necrotic (arrows). 300x; b, c- 7 days after the immersion in lysozyme dimmer; b - numerous melano-macrophages (arrows) among predominant haematopoietic cells. 400x; c- hyperaemia, melano-macrophages (arrows). 200x; d- 14 days after the immersion in lysozyme dimer: melano-macrophages (long arrows), necrosis of the epithelium of renal tubule (asterisk) with hyaline casts in their lumen (short arrows). 180x. HE stain.

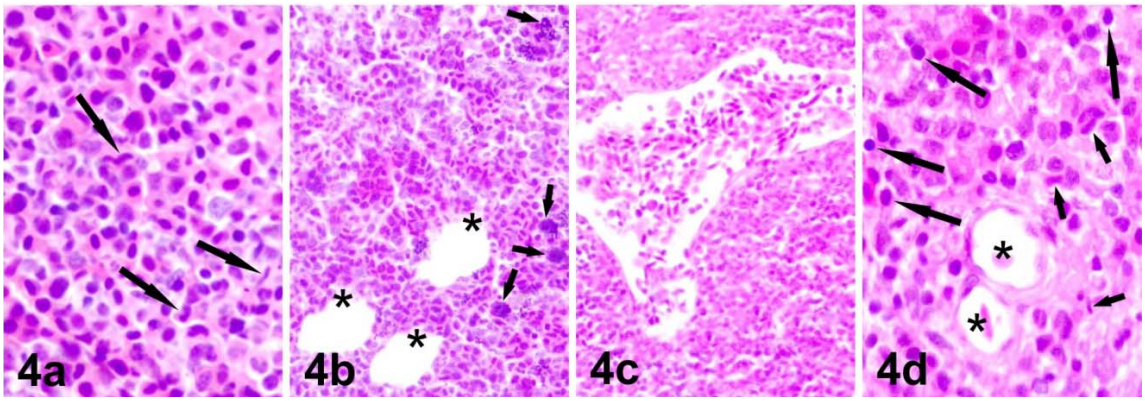

Fig. 4: The head kidney in the sturgeon from group 4: a- morphological pattern immediately after the immersion in lysozyme dimer: haematopoietic cells (arrows) were predominant in the parenchyma. 450x; b, c- 3 days after the immersion in lysozyme dimer. 200x; b- melano-macrophages (arrows) among haematopoietic cells, necrosis of the epithelium of renal tubules (asterisks); c– hyperaemia; d- 7 days after the immersion in lysozyme dimer: among lymphoid (long arrows) and haematopoietic cells (short arrows) the renal tubules with hyaline masses (asterisk), 450x. HE stain.

Table 1: Experiment layout

Group	Number of fish in the group	Oxytetracycline dose (mg/kg BW *i.p.*)	Dose of KLP-602* (30-min. immersion 24 h after antibiotic administration) in µg/l	Day when head kidney was taken for examination (after immersion in KLP-602*)
1	30	0	0	0, 3, 7, 14, 21
2	30	0	100	
3	30	100	0	
4	30	100	100	

* the lysozyme dimer dose was applied as converted to the active substance.

more numerous from day 7 after the immersion in lysozyme dimer and later their number was decreasing. Two sturgeons from the group 4 showed minor renal hyperaemia (Fig. 4c). In five fish from this group, the parenchymatous and vacuolar degeneration of the epithelial cells of renal tubules was observed and the presence of small hyaline masses was detected in the tubules and the necrosis of these structures was reported twice (Fig. 4b, Fig. 4d).

DISCUSSION

The studies enabled us to determine the impact of OTC and immunomodulator (lysozyme dimer) and their interaction on the morphology of head kidney in Siberian sturgeon. The innovative aspect concerns the dose of OTC applied and the way of application (100 mg/kg BW *i.p.).* The research into the use of OTC was also carried out by other authors, but the dose of the antibiotic was ten times lower than in our studies (Studnicka *et al.,* 2000) or the route of OTC administration was different (Soler *et al.,* 1996; Łapińska *et al.,* 2005).

In the examined sturgeons exposed to OTC, the necrosis of haemopoetic cells in the head kidney was detected (but to a lesser degree). A similar lesion, though more severe, was reported by Soler *et al.* (1996): in this case, the necrosis of haematopoietic tissue in tench *(T. tinca)* persisted until day 20 after the administration of OTC. Cengiz (2006) observed the pycnotic nuceli in the haematopoetic tissue in the kidney in carp (*C. carpio*) following the exposure to deltamethrin. The histopathological lesions in the pronephros in catfish (*S. glanis*) were also detected by Szarek *et al.,* (2000). In the examined sturgeons, the severity of this lesion was attenuated by the immersion of fish in KLP-602. It indicates that the intraperitoneal injection of OTC at 100 mg/kg BW without the immersion in lysozyme dimer results in more severe regressive lesions in the examined organs in comparison with these two factors being applied simultaneously.

In the sturgeons, lysozyme dimer stimulated the lymphoid cells, which resulted in their proliferation in the head kidney. This phenomenon was particularly evident until two weeks after the immersion in KLP-602 and became less visible in the third week after the administration of OTC. The presence of these cells after the application of OTC and the immersion in lysozyme dimer should be attributed not only to the stimulating effect of dimer but also to the reaction to organ damage, especially in relation to regressive lesions. This is further confirmed by a comparison of the morphological pattern of head kidney in the sturgeons exposed to both factors with the morphology of this organ in the individuals subjected only to one of these factors.

It should be noted that in the sturgeons from group 3 and to a lesser degree from group 4 the parenchymatous degeneration was detected in the epithelial cells within the renal tubules. In tench *(T. tinca)*, the necrosis of epithelial cells of the renal tubules that developed after the administration of OTC at 100 mg/kg BW *i.m.* was visible up to 7 days after the intramuscular injection (Soler *et al.,* 1996). These lesions were also observed by Neskovic *et al.,* (1993) in the carps immersed in water with atrazine. In African sharptooth catfish (*C. gariepinus*) exposed to cypermethrin at sub-lethal doses in water, epithelial hypertrophy, narrowing of the tubular lumen, atrophy of the glomerulus, broader Bowman's capsule, necrosis in the epithelial cells and pyknosis were observed (Velmurugan *et al.,* 2009). The latter lesions were noted by Xing *at al.* (2012) in common carp exposed to the action of atrazine and chlorpyrifos. It should be emphasized that the nephrotoxic action of xenobiotics in the initial phase of the studies and their impact on the pathomorphology of kidneys were confirmed by a lowered renal somatic index and observed histopathological lesions, such as shrinkage of the glomerulus and expansion of the Bowman's space reported in rare minnow (*G. rarus*) after the exposure to vinclozolin, which provided clear evidence of kidney dysfunction (Yang *et al.,* 2011). Similar regressive lesions in the excretory part of the kidney were detected in Nile tilapia (*O. niloticus*) exposed to cypermethrin (Korkmaz *et al.,* 2009). Velisek *et al.* (2010) reported that in carp (*C. carpio* L.) after the exposure to terbutrine (herbicide). The renal tubules were focally damaged and the small tubules with a thickened base membrane were surrounded by immense leukocyte infiltration. The alterations found in kidney of *Rasbora daniconius* exposed to lethal concentration of the paper mill effluent included hypertrophy of hematopoietic tissue, cell necrosis, blocked glomerulus with full of blood stain, dilated renal tubules with pyknotic nuclei and tubular necrosis (Pathan *et al*., 2010).

The *in vivo* and *in vitro* studies have shown that KLP-602 modulates impaired cellular immunity (due to the administration of antibiotics) and may be used to eliminate the immunosuppressive impact of xenobiotics on fish (Morand *et al.,* 1999; Rymuszka *et al.,* 2005; Studnicka *et al.,* 2000). Furthermore, Lydium KLP-602, as opposed to other modulators such as levamisole, ISC or chitosan, does not influence the growth of sturgeons (Kolman *et al.,* 1998).

These findings enable us to conclude that the immersion of Siberian sturgeons in a water solution of lysozyme dimer (at 100 μm/l of water) for 30 minutes has a protective action, most probably through an immunomodulatory mechanism, in relation to the morphological lesions induced by the intraperitoneal injection of OTC at 100 mg/kg BW.

Conclusion: Oxytetracycline at 100 mg/kg BW administered intraperitoneally to Siberian sturgeons induces the development of regressive lesions, circulation dysfunction and infiltration with lymphoid cells in the head kidney. These lesions are visible until day 22 following the injection of OTC. The immersion of fish in water with lysozyme dimer (KLP-602) at 100 μg/l for 30 minutes attenuates the pathomorphological effects of OTC administration.

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Author's contribution: JW - directly involved in all experimental work starting from the experiment layout preparation, through experimental and lab work till literature searching since this study is a part of dr Wojtacka's PhD thesis. JS was the supervisor of all experimental and lab work, directly involved in slides reading and describing, leading in macroscopic and microscopic evaluation and conclusion. ES helped in tissue taking and preparation during the experiment, taking care of the experiment procedure, digital preparation of the pictures, literature gathering.

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