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Original Scientific Paper

THE SIGNIFICANCE OF CERULOPLASMIN AND C-REACTIVE PROTEIN AS POSSIBLE MARKERS FOR THE DIAGNOSIS OF MYXOBOLOUS INFECTION CAUSED BY *Myxobolus cerebralis* IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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Summary

Myxobolus cerebralis is the etiological agent of one of the most devastating diseases (*Whirling disease*) in salmonids. It belongs to subphylum Myxozoa which possesses the highest susceptibility to the rainbow trout (*Oncorhynchus mykiss*). Acute phase proteins as an essential component of normal homeostasis are well defined and are presently used as a diagnostic and prognostic marker in human and animal pathology for evaluation of infection and inflammation. In piscine medicine the importance and the function of acute phase proteins still has not been fully determined. Furthermore, parasitic infections are more difficult to diagnose than bacterial and viral infections due to the lack of specific diagnostic tools therefore the change in the levels of some acute phase proteins could possibly be used as an alternative marker for myxobolosis. Further research is still needed to develop an understanding of the diagnostic significance of acute phase proteins in fish and the differentiation of their values in different types of disorders. The present study evaluated the modulation of ceruloplasmin and C-reactive protein following inflammatory stimulus by natural infection of *Myxobolus cerebralis* in rainbow trout (*Oncorhynchus mykiss*). In the research, biochemical tests and enzyme-linked immunosorbent assay were carried out. The test material was a diseased rainbow trout with a mortality rate of 45%. In the future, acute phase proteins have a real potential to become widely used as diagnostic tools in piscine medicine, as the evaluation of their levels can help better diagnosis and treatment, especially when

it occurs during illness. Measurement of C-reactive protein concentration in trout may be used useful as a bioindicator of the health condition of this species. Further research is still needed to develop an understanding of the diagnostic significance of acute phase proteins in fish and the differentiation of their values in different types of disorders.

Key words: C-reactive protein, ceruloplasmin, *Myxobolus cerebralis*, rainbow trout.

INTRODUCTION

Myxobolus cerebralis is a myxosporean parasite of salmonids that causes Whirling disease (WD) in farmed and wild populations. The disease, which occurs in juvenile fish, leads to skeletal deformities and neurological disturbances. The developmental cycle of *M. cerebralis* has two-host life cycle, tubificid oligochaete worm *Tubifex tubifex* and salmonid fishes. Triactinomyxon spores of *Myxobolus cerebralis* (*M. cerebralis*) can penetrate the final host through the skin, gills, mouth cavity and fins (Markiw, 1989; El-Matbouli et al., 1995). After attachment of the actinospore, the sporoplasms enter the epithelial cells of the fish and begin to multiply. Two days after that, the secondary sporoplasm cells migrate to the subcutaneous tissue, then into the cells of the peripheral nervous system, and within weeks reach the cartilage tissue (Sipos et al., 2018). Confirmatory diagnosis is based on either histology or molecular-based methods, with only one nested PCR technique has been approved. The acute phase response in higher vertebrates is more detailed and better studied than in lower vertebrates. The acute phase proteins (APPs) are substances synthesized during an acute phase response in the body, which may be the result of an infection, inflammation, stress or tissue damage (Cerón et al., 2005; Petersen et al., 2004). An APP whose blood concentration enhances by at least 25% during inflammatory processes are called “positive” APP and “negative” APP when they decrease in similar proportions (Gabay and Kushner, 1999) and furthermore, an increase in the concentration of “positive” APPs (such as C-reactive protein and ceruloplasmin) while “negative” APPs (such as albumin) levels decrease. Further, when the levels rise of 100-1000 folds within 1-2 days they are assigned to the highly positive APPs; at an increase of 5-10 times are called moderate APP and when increasing between 50 to 100 % are minor APP (Roy et al, 2017). Also, as a response to inflammatory stimuli, the concentration of negative APPs which are also described as acute booster reagents, decreases possibly as a result of provisional amplification in free hormones availability bound to these proteins (Ingenbleek and Young, 1994).

The formation and function of APP may differ depending on the animal species. In fish such as in mammals, hepatocytes are the prime source of APPs (Güleç and Cengizler, 2012). In fish, concentrations of petraxins, to which CRP belongs, following an acute phase response rise slightly or decrease (Ellis, 2001). CRP is the most important APP characteristic of vertebrates, including teleostean fishes, and is regarded as the basic APP for them, described in many species, including rainbow trout (*Onchorhynchus mykiss*), which takes part in bacterial agglutination, complement activation, activation of macrophages, inhibits the growth of some bacteria, emphasizes bacterial phagocytosis (Tîrziu, 2009). Also, Kodama et al. (1999) reveal that in rainbow trout CRP has immunostimulating activity for head kidney cells which leads to increased phagocytic and chemokinetic activity.
Ceruloplasmin (Cp) is an acute-phase protein whose concentration increases in response to infection, inflammation, or other tissue-damaging factors. The main source is hepatocytes, but it is also possible to synthesize at the sites of tissue damage (Szczubiał et al., 2012). It has the ability to suppress lipid oxidation, the ability to scavenge superoxide and release free copper ions, and also oxidize Fe_2^+ to Fe_3^+ (Tapryal et al., 2009). The changes in Cp concentration of trout could be of significance for early detection of myxobolosis caused by *Myxobolus cerebralis* and together with CRP results could be used as parameters for diagnosis before therapeutic procedures are undertaken. Therefore, the purpose of the present study was to observe changes in the CRP and ceruloplasmine concentrations in rainbow trout (*Oncorhynchus mykiss*) within a natural infection caused by *Myxobolus cerebralis* as well as possibility to use them as a marker for myxobolosis.

MATERIALS AND METHODS

The diseased rainbow trout (5-10 g) were obtained from a fish farm near Plovdiv, Bulgaria, that registered 45% mortality. The blood samples were taken from the tail at the back of the anal fin by completely severed using a scalpel blade. Biochemical parameters were measured on the veterinary biochemistry automated system Mindray BS-120 (Mindray Bio-Medical Electronics, China), using standard lab kits supplied by Biolabo SAS (France). The method of ceruloplasmin determination is based on oxidase activity that ceruloplasmin shows with p-phenylenediamine. A colored oxidation product is formed from the oxidation of PPD by Cp using Revin's method described by Bestujeva and Kolb. Fish high sensitivity C-Reactive Protein (hs-CRP) ELISA Kit were pre-coated with an antibody specific to hs-CRP. In sample wells, 10µl samples diluted in 40 µl sample dilution buffer are added, incubated at 37ºC for 30 min, and washed 5 times with wash solution. 50 µl CRP –

conjugated reagent was added to each well and plates were incubated for 30 min at 37ºC. Unbound conjugated was washed off and 50 µl Chromogen Solution A and 50 µl Chromogen Solution B was added to each well and incubated at 37°C for 15 minutes. The enzymatic reaction was stopped by adding 50 µl stop solution to each well. The optical density was measured at 450 nm.

The statistical analysis of the results was carried out using one way analysis of variance (ANOVA). The results obtained from the investigation were processed with software Statistica v.6.1 (StatSoft Inc.) and are presented as mean and standard error of the mean (Mean±Error). The statistical significance of parameters was determined by the Posthoc procedure LSD test at $P < 0.05$.

RESULTS AND DISCUSSION

Myxozoan infections differ in mode of infection, the route by which the parasite reaches its target, target tissue, and the appearance in a patient of disease. In certain cases, such as *M. cerebralis* infection, as the parasite migrates to protected pathways, the specific antibody response is delayed (Holzer et al., 2021) and also it is assumed that possibly the host cannot respond to the infection until the parasite has caused enough damage to the cartilage tissue to allow it to be exposed to the host‘s defenses. In fish, against internal and external changes, an immunological reaction occurs, which includes activation of both innate and acquired immune response (Gruys et al., 2005). The innate immune system of fish is involved in the response to inflammatory stimuli by activation of phagocytes, stimulating white blood cells activity and changing the concentrations of the acute phase proteins (Roy et al., 2017).

C-reactive protein is part of the innate immune defense system and has a key role in agglutination of foreign microbes, binding to phosphatidylcholine on microbes, activation of phagocytosis and the classical complement cascade (Nag et al., 2004). The results of Liu et al. (2004) indicates that the production of CRP in rainbow trout is detected mainly in hepatocytes, head kidney macrophages, spleen lymphocytes and peripheral blood lymphocytes. Unlike humans and mammals, the CRP levels in fish species are relatively high. In fish, pentraxins are commonly found to be in higher concentrations in serum are different for different fish species (Ellis, 2001), like as snapper (*Pagrus auratus*) - 54 µg/ml (Cook et al., 2003), plaice (*Pleuronectes plates*) – 55 µg/ml (Pathak and Agrawal, 2019), smooth dogfish (*Mustelus canis*) – 400 µg/ml (Robey et al., 1983), tongue sole (*Cynoglossus semilaevis*) - 7.6 µg/ml (Li et al., 2013). The normal serum ranges of CRP concentration in rainbow trout

between 30-88 μg/ml (Liu et al., 2004; Kodama et al., 2004; Pathak and Agrawal, 2019). In the current study, CRP level in diseased fish (481±5.11 μg/ml) was higher than healthy one (36.6±0.11 μg/ml) response to parasite's invasion. These data are consistent with earlier reports, where CRP level increased significantly at one weeks after invasion then decreased to below normal (Hoole et al., 2003; Roy et al., 2017). Saheb et al. (2020) reported that concentration of CRP elevates depending on the protozoa parasitic infection and the value can be used as a non-specific indicator of examination of the clinical presentation.

The effect of *M. cerebralis* of not only on CRP but in Cp levels was observed in present survey. In fish, the main function of Cp, like in mammals is copper transport in the blood (Sieroslawska et al., 2012). The role of ceruloplasmin is similar to that of interferon and transferrin; it inhibits bacterial development by depriving it of some nutrients, i.e. copper ions. It was noted that as an acute phase protein, Cp rises as a result of harmful factors. According to the our data, ceruloplasmin concentration of healthy rainbow trout (11-36 mg/dL) was closed to the previous reports 13,43-15,48 mg/% (Ispir and Dorucu, 2005; Yonar et al., 2010) while a multiple increase (145.39±23.17 mg/L) was found in trout infected with *M. cerebralis*. Some authors observe marked increase in ceruloplasmin activity after intoxication in common carp and Siberian sturgeon fry infested with *Diplostomum* sp. larvae, as well as in Siberian sturgeon after epin-solution (Kolman, 2001; Sieroslawska et al., 2012). *A. hydrophila* infection in tilapias resulted in a significant rise in the blood levels of ceruloplasmin (Charlie-Silva et al., 2019) as well as were elevated after infection with pathogens in the rainbow trout (Perrier et al., 1974), in European eel (*Anguilla anguilla*) infested with nematode (Terech-Majewska et al., 2015). Similarly, in the present study an increase in the concentration of Cp was noted in trout infected with *M. cerebralis*. In the study conducted by Kumar et al. (2017) it is revealed that the change in Cp levels is related to the degree of infection and its increase is most likely due to the response to parasite invasion targeting the liver to synthesize positive APPs to maintain a stable internal environment. Also, it can be assumed that Cp probably has an antiparasitic role in fish that needs to be studied in detail.

CONCLUSION

In the future, acute phase proteins have a real potential to become widely used as diagnostic tools in piscine medicine, as the evaluation of their levels can help better diagnosis and treatment, especially when it occurs during illness. Measurement of CRP concentration in trout may be used useful as a bioindicator of the health condition of this species.

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