UDK 57.044:577.3:639.3:612.062

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TISSUE-DEPENDENT CHANGES OF AMINOTRANSFERASES ACTIVITY IN GRAYLING (*THYMALLUS THYMALLUS* LINCK) AFTER CHLORAMINE-T DISINFECTION

In this study, we have tested use of Chloramine-T in dose 9 mg per L in disinfective procedures in the grayling (Thymallus thymallus Linck). To observe the effects of Chloramine-T at the therapeutic concentration, grayling, which is one of the most important salmonids in human diet was studied. Further, the aim was to evaluate the safety of this disinfective product (recently has been attracting researchers' attention for use in aquatic animals) for fish health using markers of aerobic and anaerobic capacity in the gills, muscle, hepatic, and cardiac tissues of grayling. The alanine (ALT) and aspartate aminotransferases (AST) activity were assessed in various tissues of grayling exposed to Chloramine-T and compared to untreated control. In the present study, ALT activity after exposition of Chloramine-T was significantly decreased by 10% ($p=0.005$) in the cardiac tissue of the fishes when compared to control value. Muscle and gill tissues also showed similar results as in cardiac tissue such as decrease of ALT activity by 18% and 6.4% ($p>0.05$), respectively. In case of hepatic tissue, ALT activity was non-significantly increased compared to control. The results clearly depicted that ALT activity was raised lowly in the cardiac tissue of grayling but this decreased activity was not tissue specific. AST activity was increased significantly in the hepatic tissue by 19% ($p=0.007$) but decreased in the cardiac tissue by 16.7% ($p=0.002$) as compared to control value during Chloramine-T disinfection. AST activity in the muscle tissue and gills after Chloramine-T exposition showed usual trend of decreased as compared to control value. In the present study, liver tissue showed the highest elevation of AST activity, while, in heart, the decrease of AST activity was observed. In case of muscle and gill tissues, non-statistically decreased AST activity was 20% and 7%, respectively. Considering all the tissues, here, the increase of AST activity was observed in the liver (by 19%, $p=0.007$) and the decrease in the gills, muscle and cardiac tissues under Chloramine-T exposure. The study also showed that hepatic and cardiac tissues were more sensitive to the changes than gills and muscle tissue. Therefore, these biochemical parameters can be considered as indicators for assessment of disinfective effects, although further studies are required for investigating the mechanism involved in this pattern. This study opens a new perspective on the investigation of toxic effects of Chloramine-T, mainly with respect to the biochemical parameters in various tissues of grayling.

Key words**:** *Chloramine-T, gills, muscle, liver, heart, alanine aminotransferase, aspartate aminotransferase*

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ТКАНЕСПЕЦИФИЧЕСКИЕ ИЗМЕНЕНИЯ АКТИВНОСТИ АМИНОТРАНСФЕРАЗ В ХАРИУСЕ (*THYMALLUS THYMALLUS* LINCK) ПОСЛЕ ДЕЗИНФИЦИРУЮЩИХ МЕРОПРИЯТИЙ С ХЛОРАМИНОМ-Т

Протестировано влияние дезинфектанта хлорамина-Т в дозе 9 мг/л на активность аланин- (АЛТ) и аспартатаминотрансферазы (АСТ) в жабрах, мышцах, печеночной и сердечной тканях хариуса. В настоящем исследовании активность АЛТ в сердечной ткани рыб после дезинфицирующих мероприятий с использованием хлорамина-Т значительно снизилась на 10 % ($p = 0,005$) по сравнению с величиной в контрольной группе рыб. Мышечная и жаберная ткань также показали аналогичные результаты, а именно: продемонстрировано снижение активности АЛТ на 18 и 6,4 %

(p>0,05) соответственно. Активность АСТ была значительно увеличена в печеночной ткани на 19 % (p = 0,007), но уменьшена в сердечной ткани на 16,7 % (p = 0,002) по сравнению с контрольной величиной. Активность АСТ в мышечной ткани и жабрах после экспозиции хлорамина-Т показала тенденцию к уменьшению по сравнению с контрольной группой рыб (уменьшение активности составляло 20 и 7 % соответственно). Учитывая все ткани, увеличение активности АСТ наблюдалось в печени (на 19 %, p = 0,007) и уменьшение – в жаберной, мышечной и сердечной тканях при воздействии хлорамина-Т. Наше исследование также показало, что печеночная и сердечная ткани остаются более чувствительными к воздействию хлорамина-Т, чем жабры и мышечная ткань. Поэтому эти биохимические параметры можно рассматривать как индикаторы для оценки дезинфекционных эффектов различных препаратов, хотя необходимы дальнейшие исследования для исследования механизмов, задействованных в этом процессе. Это исследование открывает новую перспективу для исследования токсических эффектов хлорамина-Т, главным образом в отношении биохимических параметров в различных тканях хариуса.

Ключевые слова: *хлорамин-Т, жабры, мышцы, печень, сердце, аланинаминотрансфераза, аспартатаминотрансфераза.*

Introduction

Chloramine-T is an organic N-chloramine with a slow-release mechanism involves the production of aqueous free-chlorine ($HOCl + OCl^-$) species that are quite toxic to aquatic life [12, 17]. Organic chloramines in general are thought to be considerably less toxic to aquatic life than the inorganic chloramines monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_3). Inorganic chloramines usually exist as monochloramine in aqueous solutions [17].

The toxicity of chloramine-T has been examined in a variety of fish species by several authors [3-6, 9, 14, 17]. Of the species tested, channel catfish, rainbow trout, and striped bass were similarly sensitive when tested in soft acidic water [3-5]. Chloramine-T 96-h LC_{50} values were 1.8 mg/L for channel catfish, 1.9 mg/L for rainbow trout, and 2.8 mg/L for striped bass (pH = = 6.5) [17]. The 96-h LC_{50} values in waters of pH 7.5 for channel catfish, rainbow trout, striped bass, and fathead minnow, and in water of pH 7.7 for harlequin fish were 3.8, 2.8, 6.3, 7.3, and 60 mg/L, respectively [17]. The 24-h LC_{50} for chloramine-T determined under a variety of conditions ranged from the low of 2.8 mg/L for rainbow trout to a high of 120 mg/L for harlequin fish in soft alkaline water (pH 8.0) [17].

Chloramine-T is easy to use and effective against many bacteria (both Gram-negative and -positive), viruses (enveloped and naked), fungi, algae, yeast, and parasites [8]. Chloramine-T is effective for the control of bacterial gill disease, proliferative gill disease, and flexibacteriosis. Bacterial gill disease is caused by a variety of Gram-negative bacteria (myxobacteria, aeromonads, and pseudomonads [8, 18].

The mode of action of chloramine-T is thought to be through oxidative processes, quickly destroying cell material or disrupting essential cellular processes. Microorganisms do not develop resistances to chloramine-T as often happens with antibiotics [8]. In intermittent exposures of rainbow trout to chloramine-T at the therapeutic concentration (10 mg/L [36 μM]), the fish exhibited behaviors that were consistent with respiratory distress (i.e., fish crowing at the surface and appeared to hyperventilate (study details not provided) [14]. Additional studies were performed to investigate the impact of a single exposure to chloramine-T. One-hour exposures of rainbow trout to chloramine-T (9 or 2 mg/L [30 or 7 μM]) or p-TSA (9 mg/L [50 μM]) through catheterized dorsal aorta resulted in a significant increase in both ventilation rates and PCO_2 levels. Both parameters returned to baseline levels within 90 minutes of removal from chloramine-T [8].

In this study, we have tested use of Chloramine-T in dose 9 mg per L in disinfective procedures in the grayling (*Thymallus thymallus* Linck). To observe the effects of Chloramine-T at the therapeutic concentration, grayling, which is one of the most important salmonids in human diet was studied. This study opens a new perspective on the investigation of toxic effects of

Chloramine-T, mainly with respect to the biochemical parameters in various tissues of grayling. Further, the aim was to evaluate the safety of this disinfective product (recently has been attracting researchers' attention for use in aquatic animals) for fish health using alanine and aspartate aminotransferases activity in the gills, muscle, hepatic, and cardiac tissues of grayling.

Materials and methods

Fish. Twenty clinically healthy grayling (*Thymallus thymallus*) were used in the experiments. The study was carried out in a Department of Salmonid Research, Inland Fisheries Institute (Rutki, Poland). Experiments were performed at a water temperature of 16±2°C and the pH was 7.5. The dissolved oxygen level was about 12 ppm with additional oxygen supply. All biochemical assays were carried out at Department of Zoology, Institute of Biology and Environmental Protection, Pomeranian University in Słupsk (Poland).

The fish were divided into two groups and held in 250-L square tanks (70 fish per tank) supplied with the same water as during the acclimation period (2 days). On alternate days, the water supply to each tank was stopped. In the disinfectant exposure, grayling (n=10) were exposed to Chloramine-T in final concentration 9 mg per L. Control group of grayling (n=10) were handled in the same way as Chloramine-T exposed groups. Fish were bathed for 20 min and repeated three times every 3 days. Two days after the last bathing fish were sampled. Fish were not anesthetized before tissue sampling.

Tissue isolation. Tissue samples were removed from grayling after decapitation. One grayling was used for each homogenate preparation. Briefly, muscle tissue were excised, weighted and washed in ice-cold buffer. The minced tissue was rinsed clear of blood with cold isolation buffer and homogenized in a homogenizer H500 with a motor-driven pestle on ice. The isolation buffer contained 100 mM tris-HCl; pH of 7.2 was adjusted with HCl.

Analytical methods. All enzymatic assays were carried out at 25±0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The enzymatic reactions were started by adding the homogenate suspension. The specific assay conditions are presented subsequently. Each sample was analyzed in triplicate. The protein concentration in each sample was determined according to Bradford (1976) using bovine serum albumin as a standard [7].

Assays of alanine aminotransferase (ALT, E.C. 2.6.1.2) and aspartate aminotransferase (AST, E.C. 2.6.1.1) activities. ALT and AST activity was analyzed spectrophotometrically by standard enzymatic method [15]. The ketoacids produced by the enzyme action reacts with 2,4-dinitrophenylhydrazine producing hydrazone complex measured calorimetrically at 530 nm. ALT and AST activities were expressed as μmol pyruvate per h per mg of protein.

Statistical analysis. The mean ± S.E.M. values was calculated for each group to determine the significance of inter group difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p>0.05$). Significance of differences between the oxidative stress biomarkers level (significance level, $p<0.05$) was examined using Mann-Whitney *U* test. Correlations between parameters at the set significance level were evaluated using Spearman's correlation analysis [29]. All statistical calculation was performed on separate data from each individual with STATISTICA 8.0 (StatSoft, Krakow, Poland).

Results and discussion. The present study is mainly concerned with comparative evaluation of enzymatic activities after disinfective procedure with Chloramine-T in different tissues, namely, liver, muscle, gill, and heart of grayling.

Alanine aminotransferase (ALT) plays a main role in synthesis and deamination of amino acids during stress imposed conditions for meeting the high energy demand of the organism [28]. Alanine aminotransferase activity in the muscle tissue, gills, hepatic and cardiac tissues of the trout disinfected by Chloramine-T is presented in Fig. 1.

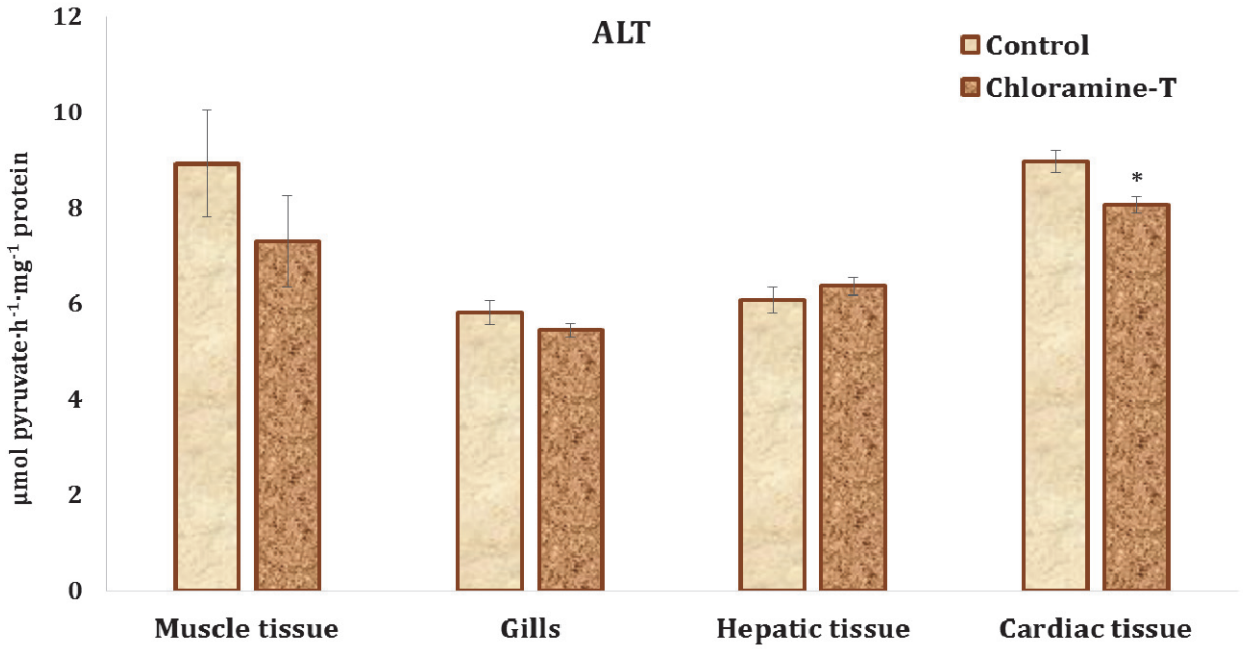

Fig. 1. Alanine aminotransferase (ALT) activity in the muscle tissue, gills, hepatic and cardiac tissues of the trout disinfected by Chloramine-T. Data are represented as mean ± S.E.M.
* – the significant difference was shown as $p<0.05$ when compared disinfected group (n=10) and unhandled group (n=10) values

In the present study, ALT activity after exposition of Chloramine-T was significantly decreased by 10% ($p=0.005$) in the cardiac tissue of the fishes when compared to control value (Fig. 1). Muscle and gill tissues also showed similar results as in cardiac tissue such as decrease of ALT activity by 18% and 6.4% ($p>0.05$), respectively. In case of hepatic tissue, ALT activity was non-significantly increased compared to control. The results clearly depicted that ALT activity was raised lowly in the cardiac tissue of grayling but this decreased activity was not tissue specific. The increased activity of ALT in different tissues of the test fishes indicated tissue damage which may be due to disturbance in normal physiological and biochemical processes such as Krebs' cycle, TCA cycle, and subsequent leakage of this enzyme from the tissue cytosol through membrane into the blood stream [16]. The oxaloacetic acid, pyruvate and α-ketoglutarate might have been channeled into the citric acid cycle. The glutamic acid formed from transamination may be subsequently deaminated leading to the formation of ammonia [2]. Several authors observed increased activity of ALT as a consequence of xenobiotics exposure in teleostean fishes. Enhanced activity of ALT provided the oxaloacetic acid and pyruvate to meet the increased energy demand during carbofuran imposed stress condition in the freshwater fish, *Clarias batrachus*. The activity levels of ALT, AST, glutamate dehydrogenase and glycogen phosphorylase a were found to increase in liver and muscle tissues during the exposure period [1]. However, in our study, ALT activity was reduced in comparison to the untreated control and approaching towards the control value in the hepatic tissue. In case of ALT, recovery pattern was in the following order: muscle tissue (18%) > cardiac tissue (10%) > gills (6.4%) (Fig. 1). Decreased ALT activity in the tissues of test fishes after disinfective procedure could represent an induction of adaptive repairing mechanism against the Chloramine-T toxicity.

Aspartate aminotransferase activity in the muscle tissue, gills, hepatic and cardiac tissues of the trout disinfected by Chloramine-T is presented in Fig. 2.

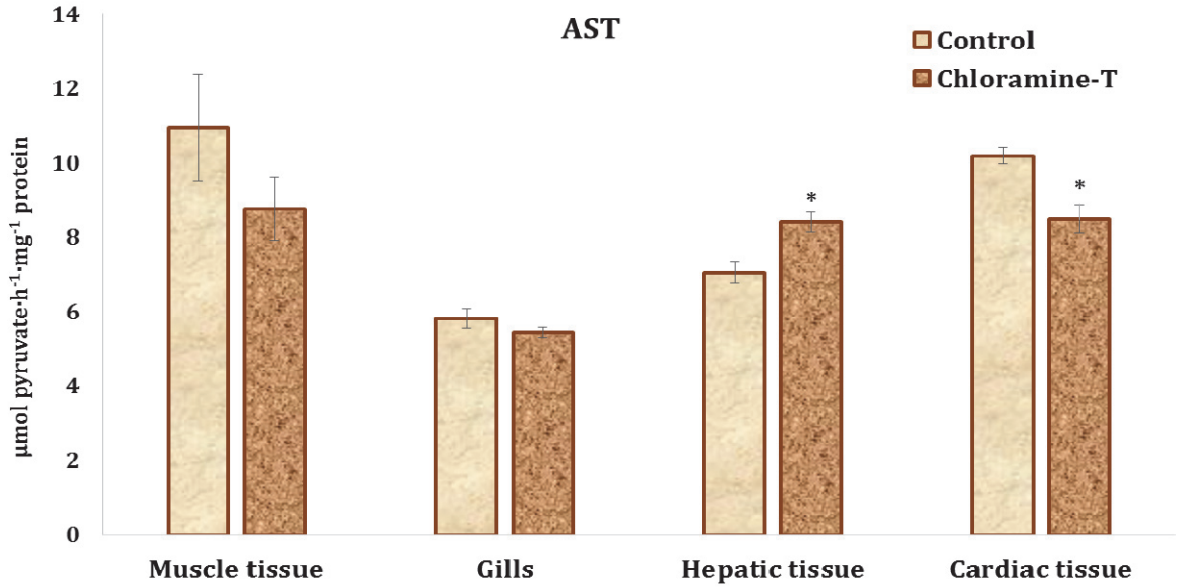

Fig. 2. Aspartate aminotransferase (AST) activity in the muscle tissue, gills, hepatic and cardiac tissues of the trout disinfected by Chloramine-T. Data are represented as mean ± S.E.M.
* – the significant difference was shown as $p<0.05$ when compared disinfected group (n=10) and unhandled group (n=10) values

Aspartate aminotransferase (AST), although a liver specific enzyme, is found in high amounts in skeletal muscle cells and promotes gluconeogenesis from amino acids in association with ALT [16]. AST activity was increased significantly in the hepatic tissue by 19% ($p=0.007$) but decreased in the cardiac tissue by 16.7% ($p=0.002$) as compared to control value during Chloramine-T disinfection (Fig. 2). AST activity in the muscle tissue and gills after Chloramine-T exposition showed usual trend of decreased as compared to control value. In the present study, liver tissue showed the highest elevation of AST activity, while, in heart, the decrease of AST activity was observed. In case of muscle and gill tissues, non-statistically decreased AST activity was 20% and 7%, respectively (Fig. 2). Considering all the tissues, here, the increase of AST activity was observed in the liver (by 19%, $p=0.007$) and the decrease in the gills, muscle and cardiac tissues under Chloramine-T exposure. The study also showed that hepatic and cardiac tissues were more sensitive to the changes than gills and muscle tissue. Enhanced AST activity in the liver of investigated fish under Chloramine-T exposure in the present study was an indication of damage either in tissues or organs leading to release of the enzyme into the serum or blood circulation and the presence of these metabolites acts as intermediate to the Krebs' cycle. The decrease pattern was in the following order: muscle tissue (20%) > cardiac tissue (16.7%) > gills (7%). Reduced activity of AST after disinfective procedure indicated counter response mechanism by the fishes to protect the permeability and integrity of membrane structure against Chloramine-T toxicity and to develop a compensatory response in the physiological system.

Any changes in responses of ALT and AST indicate tissue damage in organs. Due to its wide application in measuring contamination and an early warning of potentially hazardous alterations in contaminated aquatic organisms, it may be used as sensitive biomarkers in ecotoxicological studies [11]. In the study of Kumar and co-workers (2017), they have observed significant elevation of ALT and AST in liver, gill, brain, gonad and muscle of *Oreochromis mossambicus* collected from Bhima River. The main contaminating source is untreated wastewaters discharged from the twin industrialized cities like Pune and Pimpri Chinchwad (India). This elevation might be attributable due to increased proteolysis, enhanced protein catabolism, cellular alteration in the liver and gill structure, brain aberration, change in the gonad and muscle tissue and also due to imbalances in the

physiology and anatomy of the reservoir tissues [10]. Results of Levesque and co-workers (2002) suggest that fish challenged by environmental pollution may have a higher turnover of glucose and more glucose may be produced from non-carbohydrate substrates and used [11].

Metabolic enzymes such as aminotransferases and alkaline phosphatase (ALP) could be used as indicators of xenobiotic pollution in aquatic organisms and were recommended for environmental monitoring for investigating the mechanism involved in the recovery pattern. Samanta and co-workers (2014) have analysed the biochemical alterations in different tissues in two fish species, namely, *Anabas testudineus* and *Heteropneustes fossilis* exposed to glyphosate and subsequent recovery pattern in herbicide-free water when supplemented with Liv.52 (the approved ayurvedic medicine by drug regulatory authority, Department of AYUSH, Ministry of Health and Family Welfare, Government of India) on comparative basis. Their investigation demonstrated the alterations in biochemical enzyme activities of ALT, AST, and ALP in the fish tissues under the commercial herbicide formulation, Excel Mera 71, containing glyphosate which finally affected the fish health. The most severe alteration in enzyme activity was observed in liver and may be due to its prime role in detoxification of the compound. Although, after recovery, fishes showed positive trends of recovery and healing responses towards the normal enzyme status indicating adaptive response against the herbicidal toxicity [16].

Zhang and co-workers (2017) determine the ammonia and urea contents in body of Chinese loach, *Paramisgurnus dabryanus*, a freshwater omnivorous fish during ammonia loading and aerial exposure and provide some basic data for revealing the mechanism of ammonia detoxification in this species. *P. dabryanus* could survive in wet mud for quite long time during drought period. In this case, excretion of internal ammonia into surrounding water would be inhibited with the largest extent. The significant increase of ALT activity in plasma during aerial exposure, indicating that alanine synthesis through certain amino acid catabolism may be subsistent in *P. dabryanus*. In liver cell, ALT can transfer the amino from alanine to α-ketoglutaric acid, and transfer carbonyl from α-ketoglutaric acid to alanine, then alanine catabolize to pyruvic acid and α-ketoglutaric acid catalyse to glutamate. α-ketoglutaric acid of glutamate released through transamination with pyruvate can be completely oxidized to CO_2 and H_2O through the tricarboxylic acid cycle and the electron transport chain, producing ATP. In essence, a series of transamination is catabolized to alanine without releasing ammonia. From this perspective, certain amino acid catabolism leading to alanine formation may be an ammonia detoxification strategy or a process to reduce the production of internal ammonia at least. A few amino acid can be catabolized to alanine formation without releasing ammonia, for instance, a total of 10 mol of ATP are released through converting glutamate to alanine, the ATP yield would be higher if the starting substrate were arginine or porline [13, 27]. In *P. dabryanus*, the significant increase of ALT activity in plasma during aerial exposure, indicating that alanine synthesis through certain amino acid catabolism may be subsistent in this species [30].

In our previous study [19-26], we assessed the influence of chloramine-T on oxidative stress biomarkers and metabolic alterations in various tissues of grayling and rainbow trout (*Oncorhynchus mykiss* Walbaum). Chloramine-T bathing markedly decrease aldehydic and ketonic derivatives of oxidative protein, and aminotransferases activity only in rainbow trout liver, and their elevation is a compensatory mechanism to impaired metabolism. No significant changes were found in oxidative stress biomarkers between control and chloramine-treated brown trout. For grayling, Chloramine-T exposure caused significantly elevation in the levels of severe oxidative stress biomarkers in the liver. Increased aldehydic and ketonic derivatives of oxidative protein could modify lactate and pyruvate levels, aminotransferases and lactate dehydrogenase activities, principally causing increased enzymes activity due to oxidative stress in the liver of chloramine-exposed fish [26]. Our results also showed that chloramine-T bathing markedly increase aldehydic and ketonic derivatives of oxidative protein in hepatic tissue, while significantly decrease of carbonyl derivatives in cardiac tissue of grayling was observed [19, 22]. In the muscle tissue of

grayling, chloramine-T bathing markedly decrease lipid peroxidation with non-significant decrease of aldehydic and ketonic derivatives of oxidative proteins. However, reduced lipid peroxidation results in decrease of total antioxidant capacity. Moreover, decreased lipid peroxidation level causes decrease of aldehydic and ketonic derivatives of oxidatively modified proteins [23]. Our results also showed that Chloramine-T non-significantly decrease lipid peroxidation as well as aldehydic and ketonic derivatives of oxidative proteins in the gills of grayling. No statistically significant alterations in the activities of antioxidant defenses instead catalase and superoxide dismutase activity in the gill tissue of grayling disinfected by Chloramine-T were noted [21].

The effects of disinfection by Chloramine-T using oxidative stress biomarkers (levels of 2-thiobarbituric acid reactive substances and derivatives of oxidatively modified proteins) and biochemical enzymes' activity [alanine- and aspartate aminotransferases (ALT and AST), lactate dehydrogenase (LDH)] were assessed in the muscle tissue of rainbow trout [20]. Our results showed that Chloramine-T bathing caused the decrease of the lipid peroxidation as well as ALT and AST activity and significant decrease of LDH activity (by 339%, $p = 0.017$) compared to controls. Chloramine-T markedly affected on lactate and pyruvate metabolism and resulted to decrease of LDH activity. Correlative analysis revealed that the lipid peroxidation level is correlated with ALT and AST activity in the muscle tissue of unhandled control group. In the muscle tissue of trout disinfected by Chloramine-T, LDH activity is correlated positively with ALT and AST activity. Thus, the skeletal muscles of fish play an important role in the processing of lactate through the gluconeogenic and glycogenic pathways including a greater potential for biosynthesis [20, 24].

The effects of disinfection by Chloramine-T on the muscle tissue of grayling using oxidative stress biomarkers [levels of 2-thiobarbituric acid reactive substances (TBARS) and oxidative modified protein (OMP) derivatives] and antioxidant defense (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity) was studied in our previous study [25]. Our results showed that Chloramine-T bathing markedly decrease lipid peroxidation with non-significant decrease of aldehydic and ketonic derivatives of oxidatively modified proteins. However, reduced lipid peroxidation results in decrease of total antioxidant capacity. Moreover, decreased lipid peroxidation level causes decrease of aldehydic ($r = 0.854$, $p = 0.002$) and ketonic derivatives of oxidatively modified proteins ($r = 0.852$, $p = 0.002$). Fish developed tissue-specific enzyme responses, such as decrease in superoxide dismutase and catalase activity as well as total antioxidant capacity in muscle tissue with decrease of lipid peroxidation as response to the Chloramine-T disinfection. Correlative analysis has revealed positive correlations between oxidative stress biomarkers (aldehydic and ketonic derivatives of oxidatively modified proteins, TBARS as marker of lipid peroxidation) and antioxidant defenses [25].

Conclusions

The present investigation demonstrates the alterations in aminotransferases activities (ALT and AST) in the grayling tissues under the disinfective procedure with Chloramine-T in dose 9 mg per L. The most alterations in enzymes' activity were observed in liver and may be due to its prime role in detoxification of the compound. In addition, increased AST activity in the hepatic tissue may result from mobilization of aspartate for glucose production through the gluconeogenesis pathway to provide excess energy required to cope with stress. Therefore, these biochemical parameters can be considered as indicators for assessment of disinfective effects, although further studies are required for investigating the mechanism involved in this pattern.

This work was supported by grant of the Pomeranian University for Young Scientists.

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