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Ткаченко Г.М., Грудневская Й. Биомаркеры окислительного стресса в жабрах кумжи (*Salmo trutta m. fario*) вакцинированной против фурункулеза

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Oxidative stress biomarkers in gills of brown
trout (*Salmo trutta m. fario*) vaccinated
against furunculosis

Биомаркеры окислительного стресса были использованы для уточнения последствий вакцинации против возбудителя фурункулеза *Aeromonas* spp. на функционирование жаберной ткани кумжи на 21 день после иммунизации рыб. Уровень перекисного окисления липидов в жаберной ткани вакцинированной кумжи был существенно увеличен (на 179%, $p=0.000$) в сравнении с контролем. Вакцинация вызвала значительное увеличение уровня альдегидных и кетонных производных окислительно модифицированных белков на фоне значительного увеличения активности аминотрансфераз и лактатдегидрогеназы в жаберной ткани кумжи. Это, вероятно, является результатом окислительного стресса активированного иммунизацией. Изучение биохимических изменений в тканях вакцинированных рыб имеет важное значение для понимания сложных физиологических изменений, которые происходят после иммунизации, а также для улучшения методов в аквакультуре с целью максимизировать рост тканей и сохранения здоровья иммунизированных рыб

Ключевые слова: фурункулез, кумжа (*Salmo trutta m. fario*), перекисное окисление липидов

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Despite the importance and success of vaccination, little is known about the mechanisms of oxidative stress and antioxidant defenses in fish during vaccination. In the present study, we determined the influence of vaccination against furunculosis on level of oxidative stress biomarkers (lipid peroxidation and oxidatively modified protein levels) and biochemical enzymes activity (alanine and aspartate aminotransferases, lactate dehydrogenase) in gills of brown trout *Salmo trutta m. fario*. The level of lipid peroxidation in gills of brown trout treated by vaccine was significantly increased by 179% ($p=0.000$) compared to unhandled control. The content of aldehydic and ketonic derivatives of oxidatively modified proteins in the gills were significantly increased in the group vaccinated against furunculosis compared to unhandled group. Alanine (ALT), aspartate aminotransferases (AST), and lactate dehydrogenase (LDH) activities were significantly increased in the gills of vaccinated group compared to those in the control. Understanding the role of biochemical changes in the tissues of vaccinated trout has important implications for understanding of the complex physiological changes that occur in immunization but also for improving aquaculture practices to maximize tissues growth and health of vaccinated trout

Key words: furunculosis, brown trout (*Salmo trutta m. fario*), lipid peroxidation

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Introduction

Vaccination is now a part of routine husbandry management in many aquaculture systems, used as a means of controlling bacterial disease outbreaks, and the use of vaccines is steadily increasing as the diversity of species being farmed expands and new vaccines are developed for additional microbial agents. Both salmon and rainbow trout are vaccinated against three to five diseases during their production cycle, often with a multivalent vaccine, and productivity has increased as a result of vaccination [14].

Different kinds of vaccines have been investigated against *Aeromonas* spp. including whole cell, outer membrane proteins, extra-cellular proteins, lipopolysaccharides, biofilms and live *aeroA* mutant attenuated vaccines [13,15]. While each medicine probably are effective in the treatment of a particular disease, problems arise with the development of possible pathological side effects of immunization in fishes, as well as the emergence of antibiotic resistant pathogenic strains. Immunization with vaccines may lead in a strong local reaction and side effects, such as granuloma formations and pigmentation, sometimes leading to reduced growth of vaccinated fish and may also be regarded as an animal welfare problem [3].

Despite the importance and success of vaccination, little is known about the mechanisms of oxidative stress and antioxidant defenses in fish during vaccination. In the present study, we determined the influence of vaccination against furunculosis on level of oxidative stress biomarkers (lipid peroxidation and oxidatively modified protein levels) and biochemical enzymes activity (alanine and aspartate aminotransferases, lactate dehydrogenase) in the gills of brown trout *Salmo trutta m. fario*.

Materials and Methods

Experimental animals. Clinically healthy brown trout (*Salmo trutta m. fario*) were used in the experiments. The study was carried out in a Department of Salmonid Research, Inland Fisheries Institute near the village of Żukowo (Poland). Immersion vaccination of trout fry performed twice, in July (I stage, primary vaccination) and October (II stage, revaccination). Experiments were performed at a water temperature of $16.6 \pm 0.5^\circ\text{C}$ and the pH was 7.5 (I stage) and at a water temperature of $9.5 \pm 0.5^\circ\text{C}$ and the pH was 7.5 (II stage). The dissolved oxygen level was about 12 ppm with additional oxygen supply with a water flow of 25 L/min, and a photoperiod of 7 hours per day.

Experimental design. 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). Fish were vaccinated and grouped as follows: I) unhandled controls, II) vaccinated by vaccine against furunculosis. The vaccine against furunculosis is a vaccine containing an inactivated strain of *A. salmonicida* and *A. hydrofila* in concentration $1 \cdot 10^{10}$ colony forming units (CFU). The vaccine was produced in Department of Epizootology, Faculty of Veterinary Medicine, University of Warmia and Mazury (Olsztyn, Poland). Immersion solution contained 1 liter of vaccine per 10 liters of water. It was prepared immediately prior to vaccination. Immersion lasted from 60 to 120 seconds. The fish were kept for 21 days after revaccination.

Sampling. The animals were quickly captured and killed on 21 days post revaccination ($n=15$ in each group). Gills were removed *in situ*. Tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2) to a yield a 10% homogenate. Homogenates were centrifuged at 3,000g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -20°C until analyzed. Protein contents were determined using the method of Bradford (1976) with bovine serum albumin as a standard [1]. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ± 0.5 °C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate.

TBARS assay for lipid peroxidation. Lipid peroxidation level was determined by quantifying the concentration of 2-thiobarbituric acid reactive substances (TBARS) according to Kamyshnikov (2004) [4]. The TBARS level was expressed in nmol MDA per mg protein by using $1.56 \cdot 10^5$ mM⁻¹ cm⁻¹ as molar extinction coefficient.

Carbonyl derivatives of oxidatively modified protein (OMP) assay. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine as described by Levine et al. (1990) [5] and as modified by Dubinina et al. (1995) [2]. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP₃₇₀) and 430 nm (ketonic derivatives, OMP₄₃₀) and expressed in nmol per mg of tissue protein.

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 [10]. One unit of AST or ALT is defined as the liberation of 1 μmol of pyruvate per hour at 37°C incubation per mg protein.

Assay of Lactate dehydrogenase (LDH, E.C. 1.1.1.27). The colorimetric method of Sevela and Tovarek (1959) was used for the determination of LDH activity [11]. One unit of LDH is defined as the formation of 1 μmol of pyruvate per hour at 37°C incubation per mg protein.

Statistical analysis. Data were checked for assumptions of normality using the Kolmogorov-Smirnov one-sample test and Lilliefors tests ($p > 0.05$). Homogeneity of variance was checked using the Levene test. In order to find significant differences (significance level, $p < 0.05$) between control and vaccinated groups, Mann-Whitney *U* test was applied to the data [17]. Differences were considered significant at $p < 0.05$. All statistical analysis was performed by STATISTICA 10.0 software (StatSoft, Poland).

Results

In the present study, the level of lipid peroxidation in gills of brown trout treated by vaccine was significantly increased by 179% ($p=0.000$) compared to unhandled control (Fig. 1).

* the significant difference was shown as $p<0.05$ when compared unhandled group ($n=15$) and vaccinated group ($n=15$) values at 21 days after immunization

Fig. 1. The level of lipid peroxidation (nmol MDA per mg protein) in the gills of the brown trout treated by vaccine against furunculosis at 21 days after immunization

* the significant difference was shown as $p<0.05$ when compared unhandled group ($n=15$) and vaccinated group ($n=15$) values at 21 days after immunization

Fig. 2. Aldehydic and ketonic derivatives of oxydatively modified proteins in the gills of the brown trout treated by vaccine against furunculosis at 21 days after immunization

* the significant difference was shown as $p < 0.05$ when compared unhandled group ($n=15$) and vaccinated group ($n=15$) values at 21 days after immunization

Fig. 3. Alanine (ALT), aspartate aminotransferases (AST), and lactate dehydrogenase (LDH) activities in the gills of the brown trout treated by vaccine against furunculosis at 21 days after immunization

Reactive oxygen species (ROS) mediated reactions lead to the formation of protein carbonyl derivatives, which serves as a marker of ROS-mediated protein damage [12]. Highly ROS that are formed during normal metabolism and under conditions of oxidative stress are able to oxidize proteins or convert lipid and carbohydrate derivatives to compounds that react with functional groups on proteins [12]. The content of aldehydic and ketonic derivatives of oxidatively modified proteins in the gills were significantly increased (by 104%, $p=0.000$ and by 121%, $p=0.010$) in the group vaccinated against furunculosis compared to unhandled group (Fig. 2).

ALT, AST, and LDH activities were significantly increased in the gills of vaccinated group compared to those in the control (by 179%, $p=0.020$; by 114%, $p=0.001$; by 76%, $p=0.000$, respectively) (Fig. 3).

Discussion and Conclusions

The study shows a post-treatment changes in oxidative stress profile in the gills of brown trout treated by vaccine against furunculosis. The increase of lipid peroxidation, as well as aldehydic and ketonic derivatives of oxidatively modified protein was observed. The fish gills are important organs with multiple functions, including gas exchange, regulation of osmotic pressure, acid-base balance, ion transport and excretion of nitrogenous waste, which are closely related with the chloride cells of epithelial cells in the gills [6]. Gills are multifunctional – primarily a respiratory organ, they are also involved in the immune defense through the mucosa associated lymphoid tissues that harbour macrophages, neutrophils, lymphocytes and mast cells/eosinophilic

granulocytes [9]. Since the gills are in continuous close contact with the aquatic environment, perhaps the higher level of oxidative stress biomarkers in gills is due to their function. Fish gills are particularly sensitive to water quality, constituting the first target of pollutants, due to their anatomic location, direct contact with the water and quick absorption [8].

Response of oxidative stress biomarkers in different tissues of fish is dependent of immune system activation and ROS generation due to respiratory burst in response to microbe recognition induced by vaccination. Paiva and Bozza (2014) described the mechanisms by which ROS directly kill microbes or interfere with the immune response, the role of ROS in pathogenic viral, bacterial, and protozoan infections [7]. Phagocytes recognize microbes through many molecular patterns displayed by them and try to engulf them. Once a microbe is phagocytosed, the nature of the molecules recognized on microbe's surface dictates the treatment enacted within the phagosome. Respiratory burst, a process by which NADPH oxidase generates ROS in response to microbe recognition, is a possible outcome of this process and helps to get rid of many microbes [7]. Once a pathogen is phagocytosed, it must subvert the respiratory burst, withstand its oxidative power, or escape the phagosome to survive [7]. Microbe recognition sets the immune system in motion, and ROS are produced not only in the phagocyte respiratory burst but also in other cell compartments, such as mitochondria, as intermediaries in many signal transduction pathways, such as leukocyte pattern recognition receptor (PRR) signaling. The generation of ROS is a prerequisite to the formation of neutrophil extracellular traps (NETs); is actively involved in phagolysosomal formation and enzymatic degradation; autophagy; chemoattraction and inflammation; cell death of infection reservoirs; antigenic presentation, T-helper polarization, and lymphocyte proliferation; iron redistribution among tissues and cell compartment availability of iron [7]. ROS are used by cells of the adaptive immune system as regulators of signal transduction by cell surface receptors [16]. In our study, immunization against furunculosis induce increase the lipid and protein oxidation in gills of brown trout after vaccination (Figs 1 and 2) in response to oxidative stress induced by activation of immune system and respiratory burst activation.

Transaminases like ALT and AST play significant role in aminoacid and protein metabolism and they may release into the plasma following tissue damage and dysfunction. In the present study, the activities of both ALT and AST, as well as LDH were increased over a 21-day period after vaccination against furunculosis (Fig. 3). These results confirm our suggesting about respiratory burst through activation of phagocytic cells during vaccination against furunculosis. The significant increase of aminotransferases and LDH in the gills of vaccinated fish may indicate about susceptibility of tissue to ROS action and demonstrate the extent of oxidative damage during vaccination against furunculosis.

This study provides the identification of oxidative stress markers through an analysis of the lipid peroxidation and oxidatively modified proteins and their corresponding metabolic enzymes in the gills of brown trout after vaccination against furunculosis, shedding light on the molecular mechanisms triggered in brown trout during immunization against furunculosis and providing new molecular targets for further immunological research in fish. Understanding the role of biochemical changes in

the tissues of vaccinated trout has important implications for understanding of the complex physiological changes that occur in immunization but also for improving aquaculture practices to maximize tissues growth and health of vaccinated trout.

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