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Original article

Resistance of erythrocytes from Brown trout (*Salmo trutta* m. *trutta* L.) affected by ulcerative dermal necrosis syndrome

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Abstract

In the present work we evaluated the effect of ulcerative dermal necrosis (UDN) syndrome on resistance of erythrocytes to haemolytic agents and lipid peroxidation level in the blood from brown trout (*Salmo trutta* m. *trutta* L.). Results showed that lipid peroxidation increased in erythrocytes, as evidenced by high thiobarbituric acid reactive substance (TBARS) levels. Compared to control group, the resistance of erythrocytes to haemolytic agents was significantly lower in UDN-positive fish. Besides, UDN increased the percent of hemolysated erythrocytes subjected to the hydrochloric acid, urea and hydrogen peroxide. Results showed that UDN led to an oxidative stress in erythrocytes able to induce enhanced lipid peroxidation level, as suggested by TBARS level and decrease of erythrocytes resistance to haemolytic agents.

Key words: Brown trout, UDN syndrome, oxidative stress, erythrocytes, haemolysis.

Introduction

Over the past few years, ulcerative dermal necrosis (UDN) syndrome, a disease affecting the skin of mature wild salmonid fishes returning from the sea to spawn, had a serious impact on fisheries (Munro 1970, Johansson et al. 1982). It is one of the most destructive diseases among freshwater fish in the Pomeranian region and has spread though rivers, causing considerable loss.

Roberts (1993) reported that the disease is marked by little spots on the skin surface which penetrated into muscles. The initial signs of the disease are circles of pathologically changed epidermis. Subsequently, the intercellular spaces dilate and communicate with the exterior. The lesion is a progressive cytolytic necrosis of epidermal Malpighian cells, of the pemphigoid type which is restricted to sites of the head. On entry to fresh water, these rapidly ulcerate and may become infected with a number of opportunities pathogens, principal of which is the oomycete *Saprolegnia diclina*. Once this occurs the lesion extended by fungal activity death is due either to secondary bacterial infection of the ulcer or, more often, circulatory failure resulting from the osmotic haemodilution induced by the large area of ulceration (Roberts 1993). Histological changes include necrotising, granulomatous dermatitis and myositis associated with invasive, non-septate fungal hyphae (Ribelin and Migaki 1975, Bruno et al. 2007). The fungus may penetrate visceral organs, such as the kidney and liver, after it has spanned the musculature. The most severely affected fish frequently die before spawning (Bullock and Roberts 1979, Janssens et al. 2002, Bruno et al. 2007).

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Aetiology of the outbreaks of UDN syndrome remains unknown. There has been no conclusive evidence of the involvement of any particular organism as the primary pathogen (Roberts 1993). It is suggested that the fungal infections are triggered by metabolites of the necrotic epidermal cells (Khoo 2000). Skin samples were tested for standard virus isolation with negative results.

Law (2001) suggests that skin ulcers in fish can have many different etiologies, including infectious agents, toxins, physical and immunologic causes, nutritional and metabolic perturbations. Ulcerative lesions are likely to be initiated by a series of factors that lead ultimately to a breach of the normal barrier function of the skin. Kane et al. (2000) reported that lesions in fish are associated with a variety of organisms including parasites and bacterial, viral, and fungal infectious agents. Trauma, suboptimal water quality, and other abiotic stress factors may result in the loss of homeostasis.

Increased generation of reactive oxygen species (ROS) and lipid peroxidation (LPO) have been found to be involved in the pathogenesis of many diseases of known and unknown aetiology (Yagi 1993). The oxidative stress as one of the important mechanisms in the pathogenesis of many diseases. The oxidative stress has also been implicated to contribute to UDN-associated tissue injury in many organs of brown trout (Kurhalyuk et al. 2009, 2010).

Blood is the best indicators of the internal exposure of an individual to many diseases and influence of drug (Dykens et al. 1987). ROS may induce the lipid peroxidation leading to peroxidative damage of erythrocytes membranes (Yagi 1993). Therefore, the present attempt is to study the resistance of erythrocytes from brown trout (*Salmo trutta* m. *trutta* L.) with UDN syndrome to haemolytic agents.

Materials and Methods

Animals

Adult brown trout (*Salmo trutta* M. *trutta* L.) were collected from site on the river Słupia, Słupsk, Northern Poland. Fish-catching took place in close co-operation with Landscape Park "The valley of Słupia" as well as the Board of Polish Angling Association in Slupsk. Sea trouts were sampled from November to December, both in 2007 and 2008.

Chemicals

Thiobarbituric acid (TBA) was purchased from Sigma Chemical Co. Trichloroacetic acid (TCA), urea, 2,4-dinitrophenyl hydrazine, sodium azide and hydrogen peroxide were obtained from Fluka. All other chemicals were of analytical grade.

Experimental condition

The samples for analysis were collected from 30 and 47 healthy males and females (control group) as well as 35 males and 38 females of brown trout with UDN syndrome (study group) directly after catch. The trout from the Słupia River were caught by electro-fishing. The animals were quickly captured and killed after being anaesthetized. Specimens in each group were dissected. One fish was used for each preparation. After catching, microbiological tests were carried out (Szewczyk 2005). The cultivation of samples on the Aeromonas Isolation Agar for detecting of *Aeromonas spp.* suggested that *Aeromonas hydrophila complex* caused ulcerative dermal necrosis. The pathogen was isolated from the infected brown trout. Skin and gills samples were collected aseptically and washed three times with sterile saline. The organs were then put to buffer for obtaining the bacterial suspension (Dauber 1967) and the 0.2 mL of suspension was inoculated onto Aeromonas Isolation Agar with ampicillin at 37°C in triplicate. After 48 hours, green colony was re-isolated and subcultured on a new agar disc diffusion method (Bauer et al. 1966) on Mueller-Hinton agar supplemented with 1.5% NaCl, using the following antimicrobial agents: chlorphenicol (30 μg), erythromycin (15 μg), gentamicyn (10 μg), methicillin (5 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), rifampicin (5 μg), tetracycline (30 μg), and vancomycin (30 μg).

Isolation of erythrocytes

Blood samples were taken from the caudal vein using syringes in less than 1 min and transferred to tubes with EDTA, and held on ice until centrifugation at 3,000 g for 10 min. The plasma was removed; the erythrocytes were washed three times with five volumes of saline solution and centrifuged at 3,000 for 5 min.

Biochemical assays

Lipid peroxidation assay

Measurement of thiobarbituric acid reactive substrates (TBARS) continues to be a useful method for determination of extent of lipid peroxidation, as it is the most abundant aldehyde formed as a byproduct during this process. We measured the malondialdehyde (MDA) concentration in the erythrocytes by

a method with 2-tiobarbituric acid (Kamyshnikov 2004). Briefly, 0.1 mL of erythrocytes' suspension was added to 2 mL of distilled water. Suspension was mixed with 1 mL of thrichloroacetic acid and 1 mL of 2-tiobarbituric acid reagent. The mixture was heated in boiling water bath for 10 minutes. After cooling the mixture was centrifuged at 3,000 g for 10 minutes. The µmol of MDA (malondialdehyde) per l L of erythrocytes was calculated by using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as extinction coefficient. Lipid peroxides level in the blood was expressed in µmol of MDA per 1 L of erythrocytes.

Resistance of erythrocytes to haemolytic reagents

Acid resistance of erythrocytes

The acid resistance of erythrocytes was measured spectrophotometrically with a method with 0.1N HCl (Terskov and Gitelson 1957). The method is based on the measuring of the dynamics of erythrocytes disintegration under haemolytic reagent action. The time of haemolytic reagent action serves as the measure of erythrocytes resistance. Freshly collected blood samples were centrifuged at 3,000 rpm for 10 minutes. The sedimented cells were washed with saline solution. The process was repeated three times. Washed erythrocytes were dissolved with saline solution to prepare 1% erythrocytes solution. The assay mixture contained 10 mL of 1% erythrocytes solution and 0.1 mL HCl. The absorbance was read at 540 nm every 30 second after addition of HCl till the end of haemolysis. Difference of absorbance at the beginning and at the end of haemolysis was determined as 100%. Disintegration of erythrocytes (%) for every period of time was expressed as curve.

Osmotic resistance of erythrocytes

The osmotic resistance of erythrocytes was measured spectrophotometrically at the wavelength of 540 nm as described by Kamyshnikov (2004). The method is based on the determination of differences between osmotic resistance of erythrocytes to a mixture containing different concentration of sodium chloride and urea. Absorbance of mixture contained erythrocytes and 0.3 mol/L urea solution was determined as 100% (standard). The degree of haemolysis in every test tube (%) was calculated in respect to the absorbance of standard. Haemolysis of erythrocytes (%) in every test tube with different urea concentration was expressed as curve (Kamyshnikov 2004).

Peroxide resistance of erythrocytes

The peroxide resistance of erythrocytes was determined spectrophotometrically at 540 nm by monitoring the rate of erythrocytes disintegration by hydrogen peroxide as described by Gzhegotskyi et al. (2004). The mixture contained 0.25 mL of washed erythrocytes, 0.08 mL of 4 mM phosphate buffer (pH 7.4) with 4 mM sodium azide for catalase activity inhibition, and 0.17 mL of 30 µM hydrogen peroxide dissolved in phosphate buffer (pH 7.4). In the blank, hydrogen peroxide was substituted by phosphate buffer. Absorbance of mixture containing erythrocytes, distilled water and hydrogen peroxide was determined as 100%. The peroxide resistance of erythrocytes (hemolysed erythrocytes fraction) was expressed in %.

Statistical analysis

Results are expressed as mean ± SEM. Significance of differences in lipid oxidation data in the erythrocytes' samples of brown trout was examined using one-way ANOVA (significance level, $p < 0.05$), Levene's and Tukey's HSD test (test of reasonably important difference) (Zar 1999). All statistical calculations were performed with Statistica version 8.0.

Results

Figure 1 shows the intensity of LPO, measured as TBARS content in the erythrocytes of males and females from brown trout affected by UDN. UDN caused an elevation of LPO level in the erythrocytes of UDN-positive females by 32.8% ($p < 0.05$).

Erythrocytes are one of the most important indicators of the internal exposure of an individual to increased generation of ROS and lipid peroxidation.

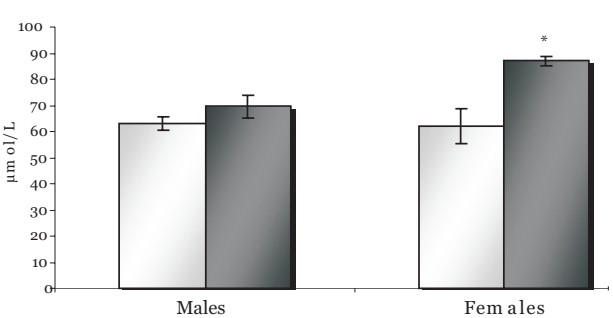

Fig. 1. Lipid peroxidation level, measured as malondialdehyde concentration in the erythrocytes of males and females from control (healthy specimens) and UDN-positive trout.
Each value represents the mean ± SEM.
* The significant change was shown as $p < 0.05$ as compared to the control (healthy specimens) group values.

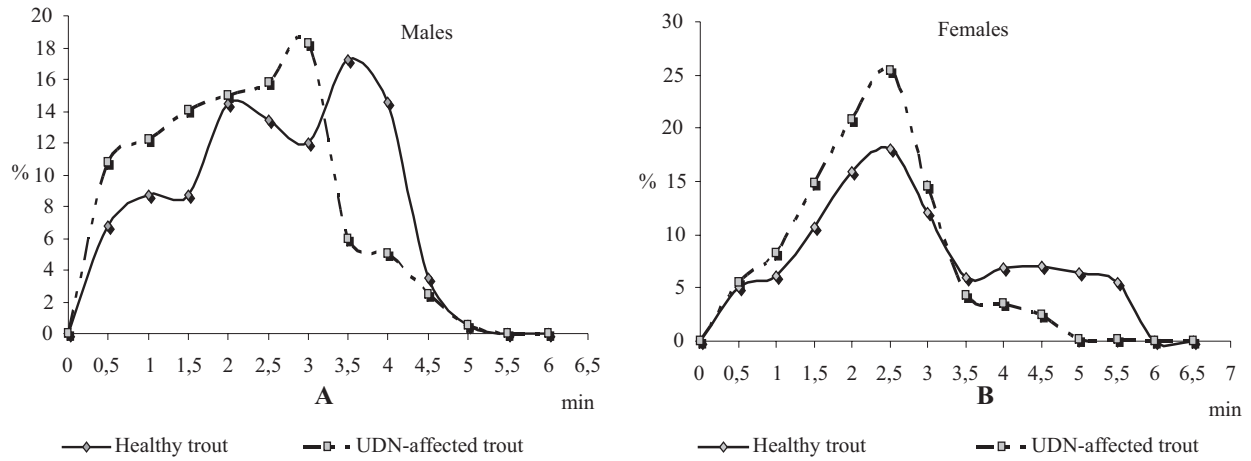

Fig. 2. Acid resistance of erythrocytes (% of hemolysated erythrocytes per minute) from healthy and UDN-positive males (A) and females (B) of brown trout.

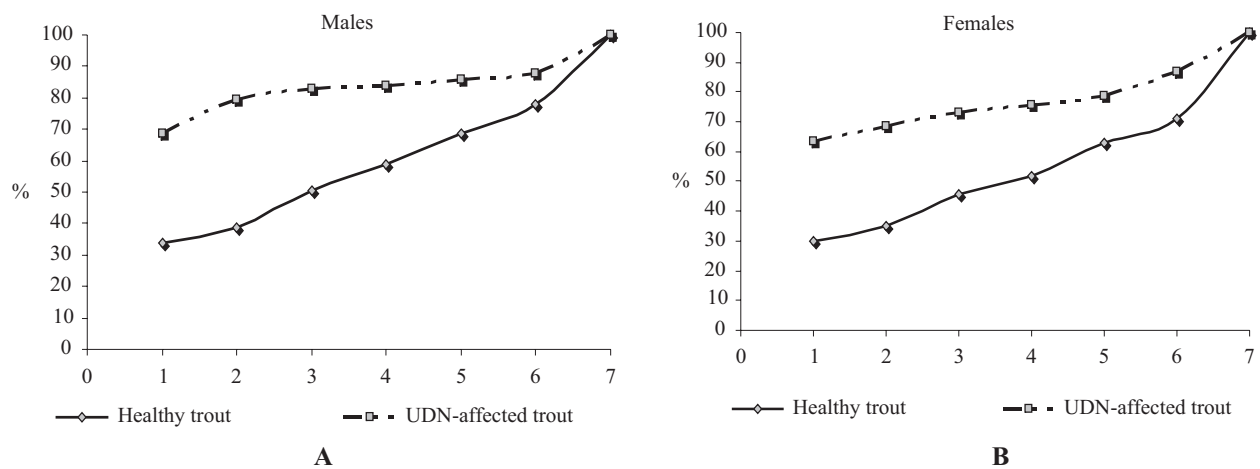

Fig. 3. Osmotic resistance of erythrocytes (% of hemolysated erythrocytes in different concentration of urea) from healthy and UDN-positive males (A) and females (B) of brown trout.
Horizontal axis: different urea concentration (1 – 0.12, 2 – 0.135, 3 – 0.15, 4 – 0.165, 5 – 0.18, 6 – 0.195, 7 – 0.3 mol/L).

Therefore, the next goal of our investigation was the measurement of erythrocytes resistance to haemolytic reagents in the blood from males and females of healthy specimens and UDN-affected trout (Figs. 2-4).

Acid resistance of erythrocytes (Fig. 2) was significantly lower in both males and females of UDN-positive fish compared to healthy specimens without dermal necrosis. Percent of hemolysated erythrocytes in the first minute of initiation of haemolysis was higher by 39.9% ($p < 0.05$) and 33.4% ($p < 0.05$) in both UDN-positive males and females compared to the healthy group of fish. The maximum percent of hemolysated erythrocytes per minute (18.23% for males and 25.45% for females of UDN-positive fish) was observed at 3 and 2.5 min after HCl adding to hemolysated erythrocytes, respectively. The highest percent of erythrocytes was hemolysated at 3 min (86% for males and 89% for females with UDN) compared to the healthy fish (96% for males at 4 min and 94% for females at 5 min).

Osmotic resistance of erythrocytes from healthy and UDN-positive trout is shown in Fig. 3. Percent of erythrocytes hemolysated under incubation with different concentration of urea was higher in the blood of trout with UDN syndrome. The resistance of erythrocytes from females remained higher than in male controls. The level of hemolysated erythrocytes at incubation with the lowest urea concentration (0.12 mol/L) was 2.02 and 2.11 times higher ($p < 0.001$) in males and females with UDN compared to the control group.

Resistance of erythrocytes from healthy and UDN-affected brown trout to hydrogen peroxide is shown in Fig. 4. Percent of haemolysed erythrocytes was higher in trout from UDN-positive group than in

both males and females from control group. Resistance of erythrocytes to hydrogen peroxide was found to be lower in both UDN-positive trout by 43% ($p < 0.05$) for males and 31.8% ($p < 0.001$) for females compared to the control group.

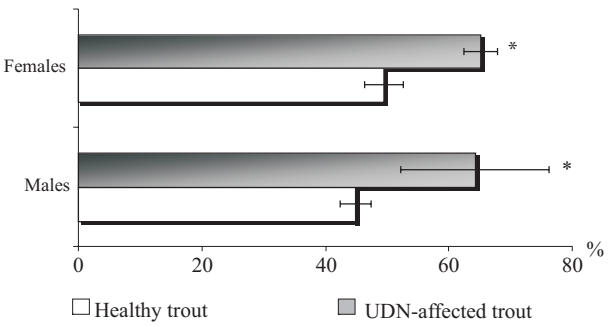

Fig. 4. Resistance of erythrocytes to hydrogen peroxide (% of hemolysated erythrocytes) from healthy and UDN-positive brown trout.
Each value represents the mean ± SEM.
* The significant change was shown as $p < 0.05$ as compared to the control (healthy specimens) group values.

Discussion

Oxidative stress causes responses of antioxidant defense system and lipid peroxidation which often act in concert. Our previous studies on pro- and antioxidative processes have revealed that some relationships have been found between oxidative stress markers and antioxidant enzyme activities in tissues of UDN-positive trout (Kurhalyuk et al. 2009, 2010). This study was aimed to check whether there are general patterns of relationships between oxidative stress indices in trout affected by UDN syndrome and physiological response of their erythrocytes.

Our previous studies showed that the blood and tissues of brown trout affected by UDN undergo lipid and protein oxidation due to the oxidizing effect of ROS. Lipid peroxidation assessed as TBARS, increased in most tissues of UDN-positive trout (Kurhalyuk et al. 2009, 2010). The present study showed that TBARS level in the erythrocytes of UDN-positive females was higher compared to control group (Fig. 1).

UDN caused accumulation of end-product of lipid peroxidation in tissues of UDN-affected trout and induced the increase of TBARS levels. Decrease in the cell antioxidant defence system was followed by the production of lipid and protein peroxidation products (Fridovich 1998). The peroxidation process is subsequent to the consumption of intracellular antioxidants (Yagi 1993). The intracellular concentration of reactive oxygen species can be increased either by overproduction of ROS or by an inability of the metabolic system to destroy them (Rikans and Hornbrook 1997, Limón-Pacheco and Gonsebatt 2009). High level of lipid and protein peroxidation also appeared as potential marker of oxidative stress induced by UDN. TBARS data confirmed that the peroxidation of lipids increase progressively to its maximum level in the male erythrocytes. This suggests that UDN induced the increase of TBARS level as potential marker of oxidative stress mediated by UDN.

The oxidative stress is one of the important mechanisms of ROS action (Nakazawa et al. 1996). Several studies have been focused on the possible toxic effects of ROS on membrane components and identified a correlation between these effects and oxidative damage (Halliwell and Gutteridge 1986, Yagi 1993, Fridovich 1998). These data suggest that altered lipid composition of membranes may results in altered membrane integrity, permeability, and function. These would increase the susceptibility to lipid and protein oxidation. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death (Nijveldt et al. 2001).

ROS are implicated as important pathologic mediators in many disorders. Increased generation of ROS and enhanced lipid peroxidation are considered responsible for the toxicity of a wide range of compounds (Halliwell and Gutteridge 1986, Cross et al. 1987). ROS subsequently attack numerous cell components, like membrane lipids, resulting in enhanced lipid peroxidation and cellular toxicity. Increased lipid peroxidation in UDN can be due to increased oxidative stress in the cell. Lipid peroxidation produces a progressive loss of membrane fluidity, thus reducing membrane potential and increasing its permeability to ions such as Ca^{2+} (Simonian and Coyle 1996).

Erythrocyte haemolysis test is far more affordable than having to rely on cell culture, whether cell lines or primary cells. A further advantage of this method is that it could presumably be applied to many fish species, and so provide information on the species of interest rather than on the species from which the cell line were once established (Janssens et al. 2002).

Our work clearly shows that erythrocytes from UDN-positive trout subjected to the haemolytic agents display higher level of haemolysis due to the oxidizing effect of the hydrochloric acid, urea and hydrogen peroxide compared to healthy specimens. Resistance of trout erythrocytes to haemolytic agents was significantly lower in both males and females of UDN-positive fish compared to specimens without UDN syndrome. Erythrocyte haemolysis is associated with peroxidation of erythrocytes membranes by UDN-induced oxidative stress.

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