



Is Zeolite a Detoxificant: Modelling of Ferrous Chloride/Zeolite Application of Aquatic Organisms on Rainbow Trout (*Oncorhynchus mykiss*) to Determine Its Effects on Oxidative Stress

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ABSTRACT

Populations of native fish and aquatic ecosystems have been negatively affected by the contamination of ground and surface waters as a result of various activities. Due to the ferrous chloride ($FeCl_2$), which is used as the reducing agent for the organic synthesis reactions in the contamination of water column and sediment, iron salts may be very toxic for some aquatic organism. In order to minimize these effects, natural products such as zeolite have been widely used recently. For this reason, rainbow trout (*Oncorhynchus mykiss*) were exposed to $FeCl_2$ and/or zeolite for 28 days and their oxidative stress responses were investigated. At the end of the treatment period, oxidative stress responses were determined with antioxidant enzyme activities in the samples taken from liver and kidneys. CAT, SOD, GPx and MDA values for kidney and liver tissues were found statistically important between control and treatment groups ($p < 0.05$). In this study, zeolite application provided lower values in terms of enzyme activities, and the protective effect of zeolite for aquatic organism was supported by biochemical parameters.

Keywords: Zeolite, fish, oxidative stress, enzyme, detoxificant

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Sucul Organizmalarda Demir Klorür/ Zeolit Uygulamasının Oksidatif Stres Üzerine Etkisinin Belirlenmesi İçin Gökkuşluğu Alabalığında (*Oncorhynchus mykiss*) Detoksifikan Modellemesi

Öz: Çeşitli aktiviteler sonucunda yer altı ve yer üstü sularının kirlenmesi ile yerli balık popülasyonları ve sucul ekosistemler olumsuz etkilenmektedir. Su kolonu ve sedimentte meydana gelen kirlilikte organik sentez reaksiyonlarının indirgenme maddesi olarak kullanılan demir klorür ($FeCl_2$) sonucunda demir tuzları bazı akuatik canlılar için toksik olabilmektedir. Bu etkileri minimize etmek adına özellikle son yıllarda zeolit gibi doğal ürünlerin kullanımı yaygınlaşmaktadır. Bu amaçla çalışmamızda, gökkuşluğu alabalıkları (*Oncorhynchus mykiss*) 28 gün boyunca $FeCl_2$ ve/veya zeolite maruz bırakılarak oksidatif stres cevapları araştırılmıştır. Deneme süresi sonunda karaciğer ve böbreklerden alınan örneklerde oksidatif stres cevapları antioksidan enzim aktiviteleri ile belirlenmiştir. Kontrol ve muamele gruplarında böbrek ve karaciğer dokuları için CAT, SOD, GPx ve MDA değerleri istatistiki olarak önemli bulunmuştur ($p < 0,05$). Bu çalışmada, enzim aktiviteleri açısından zeolit içeren gruplar daha düşük değerler vermiş ve zeolitin akuatik canlılar için koruyucu etkisi biyokimyasal parametrelerle desteklenmiştir.

Anahtar kelimeler: Zeolit, balık, oksidatif stres, enzim, detoksifikant

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Introduction

Fish are generally used as bio-indicators for the determination of the conditions and changes of the aquatic environment. Thus, the degree and the type of the responses that fish give to these changes in the ecosystem at different levels should be known (Fabacher and Little 2000; Adams 2002). Studies on the metabolic and enzymatic activities of aquatic organisms are

substantial and essential in terms of the determination of the ecological effects of the contaminants in fresh water and marine environment. Particularly enzymes, as physiological and biochemical indicators, are used for the determination of the health condition of aquatic organisms before seriously affected by these contaminants (Almeida et al. 2009; Alak et al. 2011).

Factors such as detergents, some organic materials, industrial wastes, petroleum and derivatives, artificial agricultural fertilizers, radioactivity, pesticides, inorganic salts, artificial organic chemical materials, heavy metals and waste heat in aquatic ecosystems are important contaminants that accumulate and disturbing the ecological balance in aquatic ecosystems (Hu 2000). Living organisms are provided with antioxidant defence system (ADS) in order to be protected from the detrimental effects of these toxic compounds. Antioxidant enzymes are key components induced by oxidative stress (Alak et al. 2011; Uçar et al. 2012). The presence of an atmosphere rich in oxygen has ensured the development of an endogenous antioxidant system against reactive oxygen species (ROS) and reactive nitrogen species (RNS). The decrease in the products of this O_2 metabolism controls the enzymatic (SOD), (CAT) and (GSH-Px) cellular defence mechanisms (Uçar et al. 2012). These enzymes are vital for the cellular balance; their induces are caused by the reaction to the contaminants and antioxidant enzyme activities and lipid peroxidation are substantial for the investigation of cellular damage in toxicological studies (Alak et al. 2011; Dogliotti et al. 2012).

Ferrous molecule (Fe^{+2}), which has important roles in the living organism system, is found in different forms in the nature and mostly taken in through drinking water and food. It is known that the increase of Fe molecule in the body increases the radical production and also has a role in the increment of the hydroxyl radical, which is effective particularly in the lipid peroxidation of $FeCl_2$, and hydroxyl- like radicals. Interest for the natural products has increased in order to prevent the negative effects of materials such as iron and aluminium in the regulation of water quality parameters, and studies on the utilization of alternative products have intensified. Among these products zeolites, which are found in large reserves in nature, are widely used for the elimination of heavy materials in water thanks to their sodium aluminosilicate and clay minerals, their ability of ion exchange and detraction of cations and their low cost (Nicula et al. 2010; Dayangaç et al. 2011; Çoğun and Şahin 2012).

Despite numerous studies on the usage of natural or synthetic products in the contamination, there is no study focusing on the materials of both group and on the determination of the oxidative stress response in the organism of aquatic system. The present study was modelled on rainbow trout, a locomotive species of culture fishing, in an attempt to scrutinise the topic in question and to constitute a reference for the future studies.

Materials and Methods

Fish maintenance and experimental design

Fish were obtained from Ataturk University, Faculty of Fisheries, Inland Water Fish Application and Research Center and the study was conducted at Fisheries Application and Research Center's Toxicology Experiment Unit during 28 days. Fiberglass tanks of 1 m diameter and 1 m depth with inclined tube drainage system and 40 rainbow trout (*Oncorhynchus mykiss*) of two years old and 165 ± 25 g weight were utilized in the research.

Filtered water was distributed to the tanks as no less than 0.5 l/min for kg fish. During the research, water temperature was measured as 11.5 ± 2.5 °C, pH was 7.4 and dissolved oxygen was 9.1 mg/l. Fish were randomly distributed to 8 tanks with 5 fish per tank. Two of the tanks were determined as control and the other 6 tanks were the treatment groups. $FeCl_2$ application dose LC5096 value was utilised and $\frac{1}{2}$ (0.002 mg/l) of this dose was applied to tanks (Billard and Roubaud 1985). Stock solutions of $FeCl_2$, obtained from a company (Sigma), were prepared with ultra distilled water and were applied to the tanks with determined water volume according to the experiment procedure of renewed environment in the concentration to form this dose once every 12 hours. Zeolite was determined as 1 g/l covering the tank floor (Nicula et al. 2010). Control and treatment groups were designed as ($FeCl_2$ 0.002 mg/l)(A), $FeCl_2$ +zeolite (0.002 mg/l+1 gr/l) (B), zeolite (1 gr/l) (C) and control (without $FeCl_2$ and/or zeolite) (D). Enzyme activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and also malondialdehyde (MDA) levels were determined in liver and kidney tissues for all groups.

Enzyme Analyses

At the end of the trial, treatment and control group fish were euthanized by cervical section, their liver and kidney tissues were taken and freezed in liquid nitrogen, and then tissue samples (liver and kidney) were waited at -86 °C. These tissue samples were waited in ice for 5-15 min at room temperature to thaw, afterwards, weighted on a precision scale between the range of 0.5-1 g and completely washed in 0.9% NaCl solution. KH_2PO_4 buffer solution of three times weight of the sample was added on the tissue samples splintered into small pieces. Samples were homogenised and centrifuged at 13000 rpm for an hour at 4 °C. Supernatants were taken and their enzyme activities were measured (Alak et al. 2013).

Superoxide dismutase (SOD) activity was measured by the method of Sun et al. (1998). The reaction mixture contained phosphate buffer (pH 6.5), nitro-blue tetrazolium (NBT) and enzyme fraction. The mixture was incubated for 20 min at

room temperature and then CuCl_2 was added for stop reaction and measured spectrophotometric at 560 nm. One enzyme unit was defined as SOD activity that inhibits 50% of NBT reduction. Catalase (CAT) catalyses the degradation of H_2O_2 . Its speed was spectrophotometrically measured by utilizing H_2O_2 's feature of absorbing the light at 240 nm (Aebi 1984). Glutathione peroxidase (GPx) catalyses the oxidation of reduced glutathione (GSH) by hydrogen peroxide to oxidised glutathione (GSSG). Hydrogen peroxide is degraded to GSH in t-butyl hydroperoxides environment by means of GSSG formed by GPx, glutathione reductase (GR) and NADPH. GPx activity was measured by reading the absorbance difference at 340 nm during the oxidation of NADPH to NADP^+ (Beutler 1975). Malondialdehyde seconder product is formed as a result of malondialdehyde (MDA) lipid peroxidation. The measurement is based upon the absorbance measurement of the pink complex at 532 nm, formed as a result of the incubation of MDA with thiobarbituric acid (TBA) at 95 °C. According to Bradford (1976), protein levels of each sample were determined spectrophotometrically at 595 nm wavelength were recorded by determining the bovine serum albumin (BSA) as the standard (Alak et al. 2013).

Statistical Analyses

The obtained data were expressed as mean \pm SEM. Statistical analysis of data was done using Duncan test and analyzed using SPSS version 10.0 software. A value of $p < 0.05$ was considered statistically significant.

Results

Heavy metals generally adhere to the functional groups of proteins such as imidazole, sulfhydryl, carboxyl, amino and peptide groups. In the enzyme-metal toxicity, toxic metal displaces a beneficial metal in the active area of the enzyme and becomes

effective by binding to the inactive area of the metal (Çoğun and Şahin 2012). Liver, placed at an important place between the general circulatory system and digestive tract, helps the foodstuffs to be properly metabolised and is provided with a group of mechanism that disposes potential harmful substances and detoxifies them. This organ also includes highly specific and selective transport mechanisms for necessary foodstuffs, and these foodstuffs not only provide the necessary energy but also supply physiologically important substances for the systemic necessities of the organism (Wepener et al. 2001). The effect of FeCl_2 and zeolite applications on the fish antioxidant enzyme activities is given in Table 1. It was determined that there were statistically important differences ($p < 0.05$) between de treatment and control groups in terms of CAT and SOD enzyme activities. Differences between the groups were found to be not important at GPx and MDA levels.

It was reported that kidneys are important organs that helps the disposal of metals in fish, and branchia and liver are other important organs in this process (Çoğun and Şahin 2012). In this study conducted with rainbow trout, groups of single zeolite and its combination with FeCl_2 for kidney tissue showed the lowest values in all research parameters (Table 2) and inter-group differences were found statistically important ($p < 0.05$). The decrease observed in the FeCl_2 + zeolite group is thought to be caused by the repression of FeCl_2 levels in water by zeolites (Nicula et al. 2010). Concerning the decreases determined in the enzyme activities and MDA levels of kidney tissue, it is thought that metal binding proteins have a part in the increase of reactive oxygen species (ROS) formed by interaction with FeCl_2 despite the fact that they are synthesised in kidneys (Kaya 2005; Çoğun and Şahin 2012). Depending on this increment, decreases are seen in the SOD activity levels of kidney and liver tissues, and these decreases are determined to be important ($p < 0.05$).

Table 1. The effects of different doses of FeCl_2 and/or zeolit on enzyme activities and MDA levels in liver tissue of *Oncorhynchus mykiss*

	GPx	SOD	CAT	MDA
A	0.01 \pm 0.00 ^a	0.14 \pm 0.04 ^c	46.78 \pm 5.25 ^a	0.45 \pm 0.00 ^a
B	0.01 \pm 0.00 ^a	0.63 \pm 0.15 ^{ab}	20.03 \pm 4.65 ^b	0.14 \pm 0.00 ^a
C	0.01 \pm 0.00 ^a	0.78 \pm 0.07 ^a	31.36 \pm 1.59 ^c	0.19 \pm 0.09 ^a
D	0.01 \pm 0.00 ^a	0.42 \pm 0.11 ^{bc}	17.12 \pm 0.82 ^c	0.32 \pm 0.02 ^a

(A:0.002 mg/lit FeCl_2 , B:0.002 mg/lit FeCl_2 +1 gr/lit zeolite, C:1 gr/lit zeolite D:Control (without FeCl_2 and/or zeolite)). All data points are the average of $n = 10 \pm \text{SD}$, Different superscript letters indicate statistically significant differences ($P < 0.05$) GPx CAT and SOD EU mgprotein⁻¹, MDA value nmol/ml

Table 2. The effects of different doses of FeCl₂ and/or zeolite on enzyme activities and MDA levels in kidney tissue of *Oncorhynchus mykiss*

	GPx	SOD	CAT	MDA
A	0.02±0.00 ^a	0.73±0.03 ^{ab}	48.80±14.00 ^b	0.47±0.18 ^a
B	0.01±0.0 ^b	0.06±0.01 ^c	9.37±2.25 ^c	0.26±0.06 ^{ab}
C	0.01±0.0 ^b	0.39±0.1 ^{bc}	8.16±1.57 ^c	0.08±0.00 ^b
D	0.02±0.0 ^a	1.33±0.4 ^a	92.89±28.41 ^a	0.38±0.04 ^a

(A:0.002 mg/lit FeCl₂, B:0.002 mg/lit FeCl₂+1 gr/lit zeolite, C:1 gr/lit zeolite D:Control (without FeCl₂ and/or zeolite)). All data points are the average of n = 10 ± SD, Different superscript letters indicate statistically significant differences (P < 0.05) GPx CAT and SOD EU mgprotein-1, MDA value nmol/ml

Discussion

Heavy metals accumulate in liver the most, because it is detoxification organ. In the present study, groups with zeolite showed a decrease in MDA and CAT enzyme activities compared to control and groups with FeCl₂, and this was attributed to the ion exchange ability of zeolite. Similarly, in a study on the protective effect of zeolite, it was reported that there were increases in the levels of some parameters (protein, RNA and glycogen level) and zeolite showed a protective effect (Balasubramanian and Kumar 2013).

Single and combination applications of zeolite and FeCl₂ caused increases in the CAT specific activities of fish liver, and higher values were recorded compared to the control (Table 1). Statistical analyses showed that inter-group differences were important (p<0.05).

Superoxide dismutase and catalase are the first and most important step in the defence against toxicity. It is known that enzymatic antioxidants taking part in the detoxification of H₂O₂, such as CAT, are higher in blood, bone marrow, mucous membranes, kidney and liver compared to other tissues, and show higher activity. Similar to our results, CAT activity was recorded to increase depending on the applications in the liver of tilapia (*Oreochromis niloticus*) by Peixoto et al. (2006) and green snakehead (*Channa punctatus*) by Sayeed et al. (2003). In this study, high CAT enzyme activity in the liver and kidney tissues arises from the presence of this enzyme in kidney and liver (Peixoto et al. 2006) indicated that catalase enzyme activity is exposed to both inhibition and induction against some contaminants, and that they are not among the effective biomarkers in toxicology studies.

Antioxidant enzyme activities, glutathione redox level and lipid peroxidation are generally preferred biomarkers in toxicology studies (Oruç et al. 2004). Particularly the intracellular changes at GSH level are accepted as important indicators of oxidative stress caused by contaminants in fish (Zhang et al. 2005; Çinkiloğlu 2007). Increases at GSH level under

contaminant effect are explained by the regulations and activations by enzymes taking part in GSH synthesis to replace the GSH level (Çinkiloğlu 2007). In this study, it is thought that decreases in the GPx in kidney and liver tissues under FeCl₂ compared to control are caused by O₂ increase (Matkovics et al. 1987; Yılmaz 2010).

MDA levels in the tissue groups with FeCl₂ show remarkable increases. In addition, it is thought that increases observed in the CAT enzyme activities of these groups can compensate the increase in lipid peroxidation (Kaya 2005). Since, it is known that FeCl₂ is effective in the increase of hydroxy and hydroxyl-like radicals effective in lipid peroxidation (Dayangaç et al. 2011). In zeolite groups, MDA level decreased compared to the control and groups with FeCl₂ alone, and this showed that zeolite protected these tissues against lipid peroxidation (Wu et al. 2013).

As a result of the study, regular follow up of the contamination in natural water environments has been deemed necessary based on the obtained data. Moreover, effect mechanisms of the alternative natural products used and their effects on aquatic organisms should be determined by biochemical and molecular based studies, thus generate a database. Based on the study results, zeolite can be said to show a protective effect. However, although there are studies in the literature that we can compare our research results, there is no study about the effect of zeolite on the antioxidant systems of fish. This study, in which single and combinational usage of two products used in water purification (FeCl₂ and zeolite) constitutes a substantial reference for future studies.

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