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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Vitamins A, E, C, β -carotene contents and MDA level of freshwater mussel, (*Unio elongatulus eucirrus* Bourguignat 1860) in the Karakaya Dam Lake

Keban Baraj Gölü tatlı su midyesi (*Unio elongatulus eucirrus* Bourguignat 1860)'nin, A, E, C vitamini, β karatone içeriği ve MDA değerleri

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Abstract: In this study, we aimed to determine the effect of domestic and agricultural discharge on the level of antioxidant vitamins A, E, C, β-carotene and malondialdehyde (MDA, an indicator of oxidative stress) in the muscle tissue of freshwater mussel as known bioindicator (*Unio elongatulus eucirrus*). The freshwater mussels were collected from Arguvan (uncontaminated reference site) and Battalgazi (exposed to discharge site) in the Karakaya Dam Lake. In order to examine the effect of this discharge on freshwater mussel collected from these two different regions, antioxidant vitamins A, E, C, β carotene and Malondialdehyde (MDA) levels were analyzed using High Performance Liquid Chromatography (HPLC). The comparison between groups from the different localities showed that concentrations of the vitamins A, E, C, β-carotene and MDA were found statistically significant according to between the localities ($p<0.05$) The results showed that vitamins levels were higher and MDA levels were lower in the reference site.

Keywords: Karakaya Dam Lake, freshwater mussel, antioxidant vitamins, MDA, HPLC, bioindicator

Öz: Bu çalışmada, biyoindikatör (***Unio elongatulus eucirrus***) olarak bilinen tatlı su midyesinin kas dokusunda evsel ve tarımsal deşarjın antioksidan vitaminler A, E, C, β karoten ve malondialdehit (MDA, oksidatif stresin bir göstergesi) düzeyine etkisini belirlemeyi amaçladık. Tatlı su midye örnekleri Karakaya Baraj Gölü'ndeki Arguvan (kirlenmemiş referans alanı) ve Battalgazi'den (deşarj alanına maruz kalan) toplanmıştır. Bu deşarjın bu iki farklı bölgeden toplanan tatlı su midyesi numuneleri üzerindeki etkisini incelemek için, antioksidan vitaminler A, E, C, β karoten ve Malondialdehit (MDA) seviyeleri Yüksek Performanslı Sıvı Kromatografisi (HPLC) kullanılarak analiz edildi. Bölgeler arası yapılan karşılaştırmada tatlı su midye dokularında tespit edilen antioksidan vitaminler ve MDA değişimleri arasında istatistiki olarak önemli bir farkın olduğu belirlenmiştir ($p<0,05$). Sonuçlar, referans bölgede vitamin seviyelerinin daha yüksek ve MDA seviyesinin daha düşük olduğunu gösterdi.

Anahtar kelimeler: Karakaya Baraj Gölü, tatlı su midyesi, antioksidan, vitaminler, MDA, HPLC, biyoindikatör

INTRODUCTION

Water pollution is an important part of environmental pollution and contaminants that occur cause damage to the ecological balance. Such pollutants affect the eggs of living resources and young individuals much more, endangering the survival of their generation and increasing the number of less sensitive and less valuable species that can survive in contaminated environments. As a result, biological diversity in the environment decreases and the number of individuals in dominant species increases and natural wealth disappears (Kanar, 2012). The mussels that are fed by filtering the particles in the water can accumulate the substances they take with the water for a long time (Pytharopoulou et al., 2008; Faria et al., 2014). For this reason, mussels have advantages such as being able to make comparisons during the evaluation of the information obtained, collecting information about the pollutants in the same region for long periods and being widely used in order to quickly evaluate the current situation of many pollutants in aquatic environments (Esen, 2006; Ergüden, 2012). *Unio elongatulus eucirrus* is densely distributed in Turkish rivers and freshwater reservoirs. Molluscs, which have an important place in the food chain, form the food of various water birds and aquatic mammals (sable, beaver, etc.), especially fish. In addition to being consumed by humans in some countries, they are also used in the breeding of some animals (fish, chicken, pig). For these reasons, every study on the nutritional value of mollusks gains importance (Ekin et al., 2010). In addition, the mussels are also a major contributor to filtration of pollutants in the aquatic environment and to increase water quality (Aksul et al., 2012).In this context, two different stations were determined on the Karakaya Dam Lake,

Arguvan, which was chosen as a reference, and Battalgazi, exposed to domestic and agricultural discharges. With the presence of toxic chemicals in their shells and tissues, they have a great role in reflecting the environmental quality of water basins and they have great ecological importance (Yalçın, 2006; Aksul et al., 2012; Şahin et al., 2016). Aquatic pollution is a major contributor to oxidative stress in fish and other aquatic creatures resulting from the redox cycling of pollution. Reactive oxygen species (ROS) mediated oxidation of membrane lipids results in the formation of lipid peroxidation products such as malondialdehyde (MDA) and isoprostanes. To cope with the continuous generation of ROS from normal aerobic metabolism, cells and tissues contain a series of cellular antioxidants with both enzymatic (e.g. catalase) and non-enzymatic (e.g. vitamin E, carotenoids) activities. Some non-enzymatic low molecular weight antioxidant compounds are consumed and may be below the normal ranges (Karatepe, 2004; Barım and Karatepe, 2010)

Vitamin C and vitamin E are widely known antioxidants. However, β-carotene, which is the precursor of vitamin A, also has many antioxidant properties. Therefore, vitamins A, C and E are emphasized as antioxidant vitamins. Antioxidant vitamins; It is known that they maintain the oxidant-antioxidant balance by using ways such as stopping and suppressing reactions, scavenging free radicals, repairing tissue damage, increasing antioxidant capacity. The MDA is not a specific or quantitative indicator of fatty acid oxidation, it is very important in that it correlates well with the degree of lipid peroxidation. Therefore, Malondialdehyde (MDA) is the main and most studied product of polyunsaturated fatty acid peroxidation (Karatepe, 2004; İriş and Çınar, 2019; Kızılkaya et al., 2021).

The present study aimed to determine whether non-enzymatic antioxidants (vitamin E, A, C and β-carotene) and MDA could be useful indicators of the aquatic pollution in *U. elongatulus eucirrus* samples collected from two different regions.

MATERIAL AND METHOD

Study area

Karakaya Dam Lake is the third largest dam lake in Turkey after Atatürk and Keban Dam Lakes. It has an extremely important place in terms of both electricity production and aquaculture. Although the Karakaya Dam Lake was put into service about 17 years ago, it is rapidly becoming polluted. The most important reason for this is the discharge of wastewater from the surrounding residential areas, city center and farmland into the streams that meet with the dam lake. In addition to the wastes of small and large-scale industrial establishments, especially large factories, agricultural wastes coming from the lands used as agricultural land around the lake are directly or indirectly discharged into the Karakaya Dam Lake (Özen, 2018). For these reasons, it is planned to monitor the pollution level of the dam, which has such an important socio-economically important place, and this level through mussels known as biomonitors. For this, the Arguvan region on the dam lake, one of which is not exposed to any pollution and selected as the reference region of our study, and our other sampling point was the Battalgazi region which constituted our sampling points (Figure 1).

Figure 1. Location of the study area and sampling the stations

Experimental design

The levels of vitamins A, E, C, β-carotene and MDA were determined in muscle tissues of the freshwater mussels. 47 freshwater mussels were collected only in March and April throughout the year 2015.The samples were collected from two stations Arguvan (uncontaminated, references); Battalgazi (exposed to discharge site); in Karakaya Dame Lake. The mussels were obtained in these stations. Mussel samples were transported to the laboratory in refrigerated tanks as soon as possible. Identification of the collected samples and all morphometric measurements including weight, shell length, shell height and shell width were done and recorded during the study. The tissues of the mussels were stored at -80 ^{o}C for biochemical analysis. Mussel's wet muscle tissue was thoroughly broken down in the homogenizer in order to determine the levels of vitamin A, E, C, β-carotene and MDA.

Determination of lipid soluble vitamins

Lipid soluble vitamins in tissues were assayed according to the method of Catignani and Bieri (1983). Approximately 0.3 grams of the crushed samples were weighed and taken into polyethylene tubes, 3 mL of Ethanol: Sulfuric acid (99: 1) and 1mL of water were added for precipitation of proteins. After thorough mixing with vortex, it was centrifuged at 4500 rpm for 5 minutes. Then 300 µL of n-hexane (0.05% butylated hydroxytoluene) was added to the centrifuged samples. With the addition of hexane, the lipid-soluble vitamins in the medium were extracted into the hexane phase. The tubes were mixed in a vortex and centrifuged again. At the end of the centrifuge, the hexane phase was carefully separated and taken into the glass tube. 300 µL of n-hexane was added to the sample again, mixed and centrifuged, and the n-hexane phase was combined with the hexane phase in the glass tube. The extracted hexane phase was carefully evaporated by using nitrogen gas. The residue from the hexane was dissolved in 100 µl of mobile phase (methanol/acetonitrile/chloroform, 47:42:11, v/v). 50 µL of this solution was taken and injected into HPLC.

Determination of vitamin C and MDA

MDA and vitamin C levels were assayed according to the method of Karatepe, with small modifications (Karatepe, 2004). Briefly, the 0.3 g tissues were weighed and taken into polyethylene tubes. Then the crushed tissues were treated with 1 ml of 0.5 M perchloric acid and 1 ml of water. The cells were scraped from the tubes and centrifuged for 5 min at ambient temperature. The 20 µL supernatant was taken and separated 17.5 % methanol (v/v) in 30 mM monobasic potassium phosphate buffer (pH 3.6) mobile phase.

Instrumentation

The liquid chromatographic system consisted of LC-20AD pumps, DGU-20A5 degasser, SIL 20A autosampler, CTO-10AS VP column oven, SPD-M20A DAD system. These apparatus were connected via a communication module (Model CBM-20A) and controlled by a Shimadzu LC Solution Workstation (Shimadzu, Kyoto, Japan). A Shimadzu Shim-pack vp-ODS column (150 L×4.6) was used.

Statistical analysis

All statistical analyzes in the study were performed using the SPSS (22.0)/PC package program. Data are presented as mean and standard error. The difference between the two means was determined by the Mann-Whitney U test (P<0.05).

RESULTS

Morphometric measurements and their standard deviations in freshwater mussels used in this study were calculated. Results were recorded on average weight, shell length, shell height and shell width. While the average weight of the mussels collected from the Battalgazi region was 51.07 ± 13.69 g, the shell length was 72.76 ± 7.41 mm, the shell height was 36.05 ± 3.68 mm, and the shell width was 29.36 ± 4.20 mm, the average weight of the mussels collected from the Arguvan region was recorded as 86.25 ± 16.19 g, shell length 85.00 ± 6.29 mm, shell height 41.77 ± 4.10 mm and shell width 34.15 ± 3.83 mm. The results of antioxidant vitamins A, E, C and β-carotene and MDA levels which are an indicator of lipid peroxidation analyzed in this study are given in (Table 1). As can be understood from the results, it was determined that the antioxidant vitamins A, E, C and β-carotene levels of the mussel samples collected from both stations were high in the Arguvan region, which was chosen as a reference, while the level of MDA, which was an indicator of oxidative stress, was low. On the other hand, MDA levels increased while antioxidant vitamin levels decreased in the Battalgazi region exposed to pollution. When the data obtained were evaluated statistically, the levels of antioxidant vitamins A, E, β -carotene ($p < 0.001$), vitamin C ($p < 0.01$) and MDA ($p < 0.05$) detected from both stations were found to be significant.

Table 1. Antioxidant vitamins and malondialdehyde levels detected in wet mussel samples (mean ± standard error)

Parameters	Sampling Areas		*P* value
	Battalgazi	Arguvan	
C Vitamin, mg/kg	0.32±0.06	0.58±0.04	0.0011
E Vitamin, mg/kg	7.04±0.74	20.65±2.73	0.0001
A Vitamin, mg/kg	0.15±0.02	0.41±0.05	0.0001
β-Carotene,mg/kg	26.18±4.11	58.73±9.01	0.0009
Malondialdehit,mg/kg	0.058±0.010	0.028±0.005	0.011

(Note: Significantly different; *p<0.05, **p<0.01, ***p<0.001).

DISCUSSION

The aim of this study was to evaluate the usefulness of antioxidant parameters in freshwater bivalve (*U. elongatulus eucirrus*) as biomarkers of exposure to pollutants and to examine their potential relevance in predicting toxicity. Since environmental pollutants toxically affect hemostatic tissues and organs, they also affect mechanisms such as the immune system (immune system). Examining several components that make up an integrated biological system, such as the immune system, can provide a precise and comprehensive measure of an organism's health status. This reflects the degree of pollutant-induced stress and thus may be an early indicator of disease susceptibility (Pipe et al., 1999). This determines the organism's capacity to survive against environmental pollutants (Dyrynda et al., 1998). Freshwater mussels are thought to be sensitive to the genotoxic effects of metals and complex pollutants (Bolognesi et al., 1996; Chandurvelan et al., 2013). Antioxidant defense systems control the damage of reactive oxygen species and free radicals in the body. Since these systems act on different free radicals and in different cells, they complement each other (Diplock, 1998). Cells are protected against oxidative stress caused by free radicals and peroxides through antioxidant defense systems under normal physiological conditions. In general, non-enzymatic antioxidants are more active outside the cell, while enzymatic antioxidants are more active inside the cell (Halliwell and Gutteridge, 2000). Antioxidant enzymes and lipid peroxidation triggered by the effect of pollutants are used as oxidative stress biomarkers (Cossu et al., 2000).

This study is the first comprehensive study to determine the levels of antioxidant vitamins A, E, C and β-carotene and MDA in the muscle tissue of freshwater mussels (*U. elongatulus eucirrus*) collected from Karakaya Dam Lake. As a result of the data of the study, it has been determined that there is a significant difference between the two areas exposed to pollution the reference, and it has been tried to be revealed by monitoring aquatic pollution through selected mussels as bioindicators. It was clearly seen that the levels of antioxidant vitamins were low and the level of MDA was high in the freshwater mussel samples collected from the polluted area. When we look at the studies carried out on mussels in order to

reveal the effect mechanism of pollution on antioxidant vitamins and enzymes, both at home and abroad, we see that the findings are parallel and confirm each other (Box et al., 2007; Vlahogianni et al., 2007; Faria et al., 2014). Ribera et al. (1991) aimed to reveal the relationship between free radical and lipid peroxidation in *Mytilus edulis* mussels. Mussels were exposed to compounds known for their ability to generate free radicals (carbon tetrachloride, CCl4) and reactive oxygen species via the redox cycle (menadione) and investigated the effects on digestive glands, gills and remaining tissues. They stated that lipid peroxidation parameters and free radicals (glutathione, vitamins A, E and C) were more affected by menadione exposure than CCl4. At the end of the study, they revealed the state of change in free radicals. Vitamin A and carotene have a number of biochemical functions, including oxyradical scavenging. These antioxidants levels were decreased in mussels with pollution exposure. Vitamin C is the other most important water-soluble antioxidant, acting either directly or by regenerating reduced tocopherol from tocopheroxy radical. Vitamin C levels were decreased in mussel tissues that were affected by pollution. Vitamin E is the major fat-soluble antioxidant present in the mussel membrane. It reacts directly with oxyradicals and singlet oxygen. The observed decreases in the muscle of mussels. The changes of free radical scavengers in all tissues with exposure to pollution, indicate that the molecules are involved in mechanisms of oxyradical detoxication and lipid peroxidation control *in vivo*. In the studies conducted by us and Ribera et al. (1991), the decrease in antioxidant enzyme levels in mussels exposed to oxidative stress showed similarity in the findings of both studies. Borkovic-Mitic et al. (2013) showed the differences in vitamin E concentrations in mussels that were observed in the samples obtained from the four sites on the Sava River. This may be due to higher levels of heavy metals (Cu, Cd and Mn). Significant negative correlations were established between the concentrations of vitamin E and Cu and Cd, and positive correlations between vitamin E and Mn. From the presented results it can be concluded, that investigated antioxidant enzymes and non-enzymatic components represent suitable biomarkers of metal pollution and that different tissues plays an active role in oxidative stress generation and antioxidant responses and can therefore be used as bioindicators of metal pollution. In our study of monitoring the ecological status of freshwater mussels, non-enzymatic antioxidant vitamin levels were also examined. At the end of the study, the amount of vitamin E in the mussel samples collected from the Battalgazi region, which was exposed to the discharge, was lower than the reference region. The findings of both studies were found to be similar.

CONCLUSION

It is known that antioxidants are widely used in biomonitoring studies. The high levels of these substances in polluted areas are linked to the key role they play in the detoxification of compounds that cause oxidative stress. Determining the toxic effects of pollutants on bivalves will help predict the toxic effects that pollutant levels will have on humans. It is planned to expand the study by considering other heavy metals, organic pollutants, and metallothionein proteins.

AUTHORSHIP CONTRIBUTIONS

Ayşe Gül ŞAHİN: Project administration, resources, funding acquisition, writing- reviewing and editing, Mustafa KARATEPE**:** Methodology, software, validation, writing-reviewing and editing.

CONFLICTS OF INTEREST

There was no financial or personal interest in our article titled "Vitamins A, E, C, β -carotene contents and MDA level of freshwater mussel, (*Unio elongatulus eucirrus* Bourguignat 1860) in the Karakaya Dam Lake".

ETHICS APPROVAL

Samples were provided within the scope of the TAGEM project titled "The effect of heavy metal pollution on biochemical parameters and meat quality in some fish species in Keban, Karakaya, and Atatürk Dam Lakes". Approval was granted by Elazığ Veterinary Control Institute Animal Experiments Local Ethics Committee (Date: 20.09.2016 /No: 16/02).

DATA AVAILABILITY

For questions regarding datasets, the corresponding author should be contacted.

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