

Cu/Zn superoxide dismutase enzyme immunoreactivity in the stomach tissue of rats fed with mussels (*Mytilus galloprovincialis*)

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ABSTRACT

Objectives: Mussels accumulate heavy metals in their tissues. Although there are few data about the toxicity of seafood that is exposed to environmental pollution, there are no animal studies about the gastric toxicity of mussels grown in the Dardanelles. The antioxidant Cu/Zn superoxide dismutase (Cu/Zn SOD) enzyme catalyzes the hydrogen peroxide dismutation of superoxide radicals and removes the effects of free radicals which cause oxidative stress. The purpose of the study was to demonstrate the Cu/Zn SOD in the stomach tissues of rats which are fed with mussels that are collected from the Çamburnu region of the Dardanelles.

Methods: A total of 24 male Wistar albino rats were randomly divided into four groups: Group 1 (n = 6), control group fed with standard rat food; Group 2 (n = 6), 75% mussels and 25% standard rat food daily; Group 3 (n = 6), 75% mussels and 25% standard rat food every two days; and Group 4 (n = 6), 75% mussels and 25% standard rat food every three days. To detect Cu/Zn SOD localization in the tissues, the LAB-SA Detection System was used.

Results: Cu/Zn SOD enzyme immunoreactivity was not detected in Group 1 and in samples without Cu/Zn SOD primer antibody. Cu/Zn SOD enzyme immunoreactivity was detected 82% in Group 2, 79% in Group 3, and 61% in Group 4. There was statistically significant difference between the Cu/Zn SOD immunoreactivity of epithelial cells in the gastric mucosa of the rats in the experimental and control groups ($p < 0.05$).

Conclusions: Determination of the increase of Cu/Zn SOD enzyme in the gastric mucosa of mussel-fed rats that we used in our study suggests that it triggers the antioxidant defense mechanism against mussel toxicity.

Keywords: Immunohistochemistry, Dardanelles, mussel, Cu/Zn superoxide dismutase, stomach

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Heavy metals, such as mercury (Hg), cadmium (Cd), lead (Pb), copper (Cu), nickel (Ni), zinc (Zn), chromium (Cr) and arsenic (As), have gained significance because they are toxic on certain concentrations and can increase their concentration during transition from one organism to another. Mussels are living organisms that are fed by filtering organic matter and phytoplankton in the water. The

mussels can also filter toxic substances during water filtration. Pollution travels along food chains and can harm all living things, including humans [1, 2]. In sea chestnuts growing in Dardanelles, the values of aluminium (Al), Zn, and iron (Fe) in samples taken from Gelibolu Hamzakoy station are high. Al and Fe values are higher in samples taken from Çardak region of the Dardanelles. Al, Fe and Zn values are higher in



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samples taken from Umurbey region of the Dardanelles. Al, Fe and Zn values are higher in samples taken from Çamburnu region of the Dardanelles [3]. International Agency for Research on Cancer (1987) has explained that heavy metals may affect and cause chronic degenerative changes and, in some cases, teratogenic and carcinogenic effects, especially by affecting the nervous system, liver and kidneys [4].

Heavy metals are toxic because they may have cumulative deleterious effects that can cause chronic degenerative changes [5], especially to the nervous system, liver, and kidneys, and, in some cases, they also have teratogenic and carcinogenic effects [4]. The mechanism of toxicity of some heavy metals still remains unknown, although enzymatic inhibition, impaired antioxidants metabolism, and oxidative stress may play a role. Heavy metals generate many of their adverse health effects through the formation of free radicals, resulting in DNA damage, lipid peroxidation, and depletion of protein sulfhydryls (e.g., glutathione) [6].

Cu/Zn superoxide dismutase (Cu/Zn SOD) enzyme is believed to play a major role in the first line of antioxidant defence [7]. SOD is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 . Peroxide can be destroyed by CAT or GPX reactions [8-10]. The antioxidant enzyme SOD, which provides an important means of cellular defence against free radical damage [11]. Antioxidant enzymes are capable of stabilizing, or

deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals. For the past decade, countless studies have been devoted to the beneficial effects of antioxidant enzymes. It has been found that a substantial link exists between free radicals and more than sixty different health conditions, including the aging process, cancer, diabetes, Alzheimer's disease, strokes, heart attacks and atherosclerosis. By reducing exposure to free radicals and increasing the intake of antioxidant enzyme rich foods or antioxidant enzyme supplements, our body's potential to reducing the risk of free radical related health problems is made more palpable [12].

The purpose of the study was to demonstrate Cu/Zn SOD immunoreactivity in the stomach tissues of rats which are fed with mussels that are collected from the Çamburnu region of the Dardanelles (Çanakkale, Turkey) (Figure 1).

METHODS

Animal Model

A total of 24 male Wistar albino rats, weighing 290-310 g, were used in the study. The study protocol was approved by the Çanakkale Onsekiz Mart University Ethical Council of Animal Research

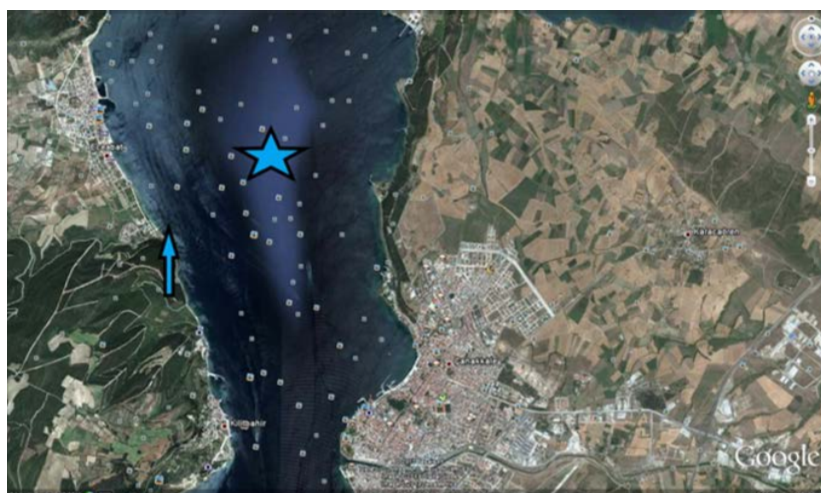


Figure 1. The area where the mussels are collected. Star shows Dardanelles. Arrow shows Çamburnu region (Çanakkale, Turkey).

(Protocol number-2010/09-03). The rats were kept for 30 days under appropriate conditions of temperature/humidity and a 12-h light cycle while being provided sufficient water and feed. The rats were randomly selected and divided into 4 groups: Group 1 (n = 6), control group fed with standard rat food; Group 2 (n = 6), 75% mussels and 25% standard rat food daily; Group 3 (n = 6), 75% mussels and 25% standard rat food every two days; and Group 4 (n = 6), 75% mussels and 25% standard rat food every three days.

Rats were fed twice daily for 30 days at 15% of their weight every morning and evening at the same time. The mussels given as food to the rats were removed from Çamburnu region in the Dardanelles (Figure 1). Average 100 ± 10 g weight were selected. After the beaks were overcooked, the meat broke off and the meat at 100 degrees was dried.

It was weighed into each rat's weight and 10 mg/kg intraperitoneal ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul, Turkey), and 20 mg/kg of xylazine 2% (Rompun, Bayer Turkey Pharmaceutical Ltd., Istanbul, Turkey) were anesthetized. The rats were anesthetized and then sacrificed. After the rats have received the stomachs other organs were also taken for further research.

Histological Evaluation

The stomach tissues were maintained in immunofix (Leica) for 24 hours for histopathological examination. The paraffin embedded stomach tissues were stained with hematoxylin and eosin (H&E) at a thickness of 5 microns. Immunohistochemical staining method was applied by cutting the paraffin embedded stomach tissues 3 microns in thickness.

The LAB-SA Detection System, (Histostain-Plus Bulk Kit, Invitrogen) was applied to determine immunohistochemical localization of Cu/Zn SOD enzyme in tissues. Sections taken from paraffin blocks were deparaffinized and rehydrated. Subsequently, tissue samples were resuspended in 0.2% Triton \times 100 (Santa Cruz Biotechnology) solution prepared with Phosphate Buffer Saline (PBS, Invitrogen) for 5 min. were kept. This allowed better passage of solutions from the pores in the cell and nucleus membranes. The tissue samples confined to the Pap pen were washed three times with PBS for 3 min. Subsequently, 3% H_2O_2 was applied to the sections to block endogenous

peroxidase activity. The sections were incubated in citrate buffer (0.1 M, pH: 6.0) in the microwave (800 watt, 10 min) for antigen retrieval, and the samples were washed with phosphate buffer solution (PBS, 0.1 M, pH: 7.2). After the samples had been incubated in the blocking buffer for 10 min, they were washed with PBS. Next, slides were incubated with polyclonal rabbit anti-superoxide dismutase (Cu/Zn SOD1, Enzo Life Sciences) antibody, which was diluted 1:50 for the stomach tissue, for an hour at room temperature in the humidity chamber, and they were then washed with PBS. Afterwards, biotinylated secondary antibody was applied to the samples for 30 min (Ultravision Detection System, Thermo Scientific, Fremont, USA). Then the samples were washed with PBS again and incubated with Broad Spectrum Antibody (Invitrogen, USA) for 30 min. After washing the samples, diaminobenzadine-tetrahydrochlorid (DAB, Invitrogen Corporation) was applied to them. Negative control was used to determine specific Cu/Zn SOD immunoreactivity, and hematoxylin stain was used as a nuclear counter stain.

Dye samples were evaluated on the Zeiss AXIO Scope 1 brand research microscope. Analysis of Cu/Zn SOD immunoreactive cells in the stomach tissue was performed using the Leica LAS V3.8 image analysis system. Five of the sections from the blocks containing the stomach tissues of all the rats in all groups were stained. From the stained sections, 1000 cells were counted and immunoreactive cells were identified among these cells. For this purpose; immunopositive cells / total cell count (1000) \times 100 % = % formula were used [13-15].

Statistical Analysis

SPSS 15 version was applied for the statistical evaluation of the results obtained with the applied formula. Kruskal-Wallis test was used for nonparametric tests to determine the differences between Cu/Zn SOD immunoreactivity groups. The difference between the groups was considered significant in the results of $p < 0.05$.

RESULTS

In immunohistochemical staining with Cu/Zn SOD, a significant difference was observed in the

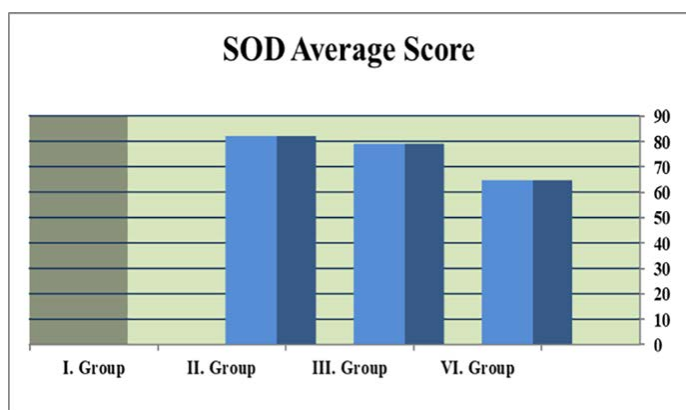


Figure 2. Cu/Zn SOD immunoreactivity ratios between control group and mussel groups.

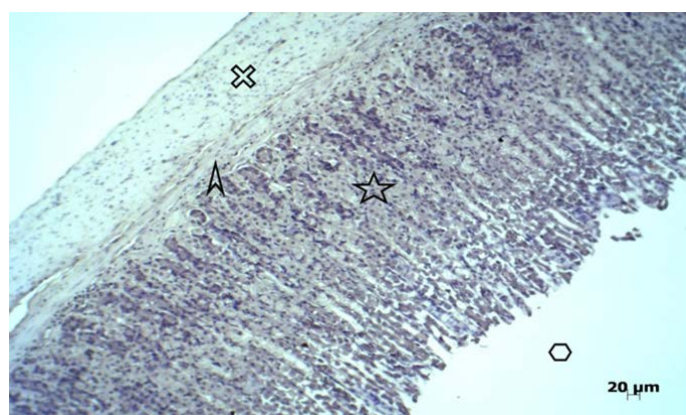


Figure 3. Group 1 (control group); standard rat diet was given every days. Rat stomach, (Cu/Zn SOD × 10, Bar = 20 µm). Hexagon = Gastric lumen, Star = Lamina propria mucosa, Pointed arrow = Lamina muscularis mucosa, Crossed = Tunica muscularis mucosa

gastric mucosal epithelial cells of the rats given mussels per day, every other day and every three days compared to rats fed with normal feed ($p < 0.05$) (Figure 2).

Cu/Zn SOD immunopositive cells could not be detected in epithelial cells of gastric mucosa of rats fed with standard rat diet (Group 1) (Figures 2 and 3). Dark brown staining in the cytoplasm of the cells was considered positive. Cu/Zn SOD immunopositive cells were found in 82% of the gastric mucosal epithelial cells of rats fed with mussels every day (Group 2)

(Figures 2 and 4). Cu/Zn SOD immunopositive cells were found in 79% of the gastric mucosal epithelial cells of rats fed with mussels every other day (Group 3) (Figures 2 and 5). Cu/Zn SOD immunopositive cells were found in 61% of the gastric mucosal epithelial cells of rats fed with mussels every three days (Group 4) (Figures 2 and 6). In the epithelial cells of the gastric mucosa of the rats fed with mussels every day, Cu/Zn SOD immunopositive cells could not be detected by negative staining (Figure 7).

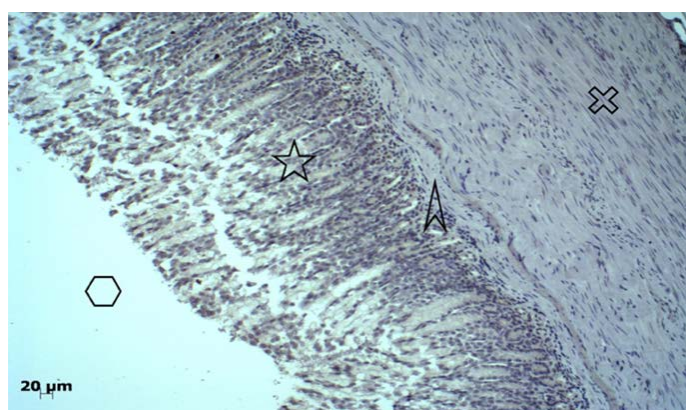


Figure 4. Group 2; 75% mussel + 25% standard rat diet standard rat feeds were given daily. Rat stomach, (Cu/Zn SOD × 10, Bar = 20 µm). Hexagon = Gastric lumen, Star = Lamina propria mucosa, Pointed arrow = Lamina muscularis mucosa, Crossed = Tunica muscularis mucosa, Arrows = Cu/Zn SOD positive gastric gland cells

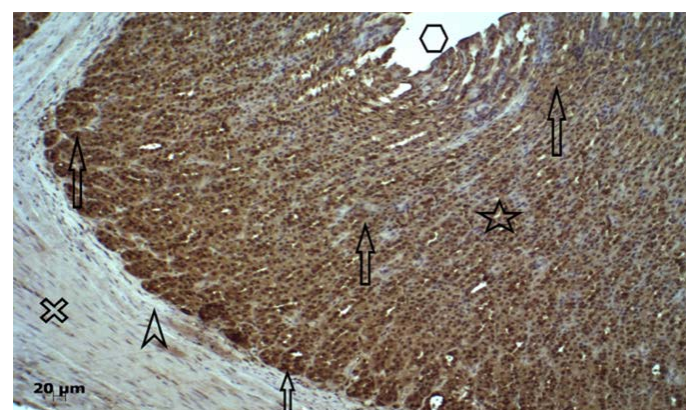


Figure 5. Group 3; 75% mussel + 25% standard rat diet was given every two days. Standard rat diet was given the other day. Rat stomach, (Cu/Zn SOD × 10, Bar = 20 µm). Hexagon = Gastric lumen, Star = Lamina propria mucosa, Pointed arrow = Lamina muscularis mucosa, Crossed = Tunica muscularis mucosa, Arrows = Cu/Zn SOD positive gastric gland cells

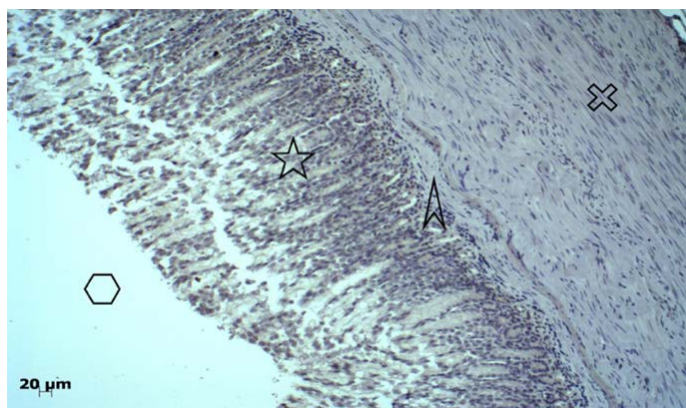


Figure 6. Group 4; 75% mussel+ 25% standard rat diet was given every three days. Standard rat diet was given the other two day. Rat stomach, (Cu/Zn SOD $\times 10$, Bar = 20 μm). Hexagon = Gastric lumen, Star = Lamina propria mucosa, Pointed arrow = Lamina muscularis mucosa, Crossed = Tunica muscularis mucosa, Arrows = Cu/Zn SOD positive gastric gland cells

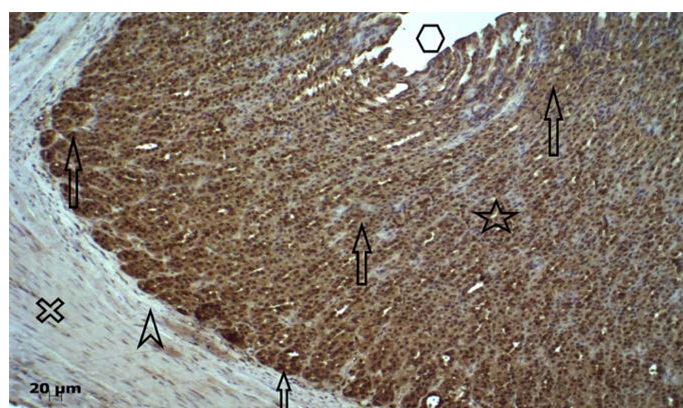


Figure 7. Group 2; 75% mussel + 25% standard rat diet standard rat feeds were given daily. Rat stomach, negative control, (Cu/Zn SOD $\times 5$, Bar = 20 μm). Hexagon = Gastric lumen, Star = Lamina propria mucosa, Pointed arrow = Lamina muscularis mucosa, Crossed = Tunica muscularis mucosa

DISCUSSION

We have found that the levels of heavy metals in bivalve and sea water in the Dardanelles throat are higher than acceptable levels in our previous researches [3, 16, 17]. All heavy metals are potentially harmful to most organisms at some level of exposure and absorption. Aquatic animals are also exposed to elevated levels of heavy metals. Some trace metals are essential in low concentrations for the metabolism of animals, but in the excess all trace metals are toxic [18]. International Agency for Research on Cancer has explained that heavy metals may affect and cause chronic degenerative changes and, in some cases, teratogenic and carcinogenic effects, especially by affecting the nervous system, liver and kidneys [4]. Toxic effects of heavy metals causes oxidative stress, mitochondrial damage, cellular death and apoptosis. In chronic Pb intoxication loss of kidney function, hypertension, anemia and hyperuricemia without tofus were reported [19]. Oxidative stress plays a major role in the pathogenic of many disorders including aging, cancer, diabetes, Alzheimer's, strokes, viral infections (that cause airway epithelial inflammation), neurodegenerative processes (including cell death, motor neuron diseases and axonal injury) and infarction, and brain edema. Antioxidant enzyme plays an important role in protecting oxidative injury to the body [20].

Mussels are living organisms that are fed by filtering organic matter and phytoplankton in the water. The mussels can also filter toxic substances during water filtration. Pollution travels along food chains and can harm all living things, including humans.

Oxygen species are key participants in damage caused by virus infections (that cause airway epithelial inflammation), progression to cancer (tumor invasion, and metastasis injuring local tissues), neurodegenerative processes (including cell death, motor neuron diseases and axonal injury), and both infarction and brain edema. Therefore, tissues must be protected from this oxidative injury by expression of stress-response genes and genes encoding antioxidant enzymes and activation of other related transcriptional regulatory proteins. Those abnormalities appeared in the cellular regulation and expression of antioxidant enzymes play a main role in cell division cycle and in the balance of life. This fact shows us the importance of the ROS scavenging and the antioxidant defence system in maintaining normal cellular physiology, facing diseases and promoting immunity. In fact, the regulation of gene expression by means of oxidants, antioxidants and the redox state, has emerged as a novel target that promises therapeutic implications [21].

Gezen stated that immunohistochemical staining methods are used to detect damage to cells and tissues

[22]. In this study, immunohistochemical staining method was used to detect Cu/Zn SOD enzyme production.

CONCLUSION

In this study, a high amount of Cu/Zn SOD was detected in the stomach tissues of the rats fed from the Çamburnu region from the Dardanelles. It is considered that the increase of Cu/Zn SOD production is thought to occur because heavy metal containing mussels may cause oxidative stresses. The first defense against free radicals in organism is the SOD enzyme, so Cu/Zn SOD production is thought to increase excessively.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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The authors disclosed that they did not receive any grant during conduction or writing of this study.

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Footnote

At the time of this research, the author (AM) worked at Department of Pathology of Çanakkale Onsekiz Mart University.

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