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Cu/Zn SOD Enzyme Immunoreactivity in the Stomach Tissue of Rats Feedind with Great Scallop *(Pecten maximus)*

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Abstract
Bivalve seafood grown in Dardanelles is exposed to environmental pollution for years. Especially heavy metal salts have accumulated in the flesh of marine species. Great scallop is consumed as food and it is a valuable product. There are no studies on the effects toxic of other living organisms consuming bivalve marine life in the Dardanelles. In this study, it was aimed to investigate the effects of great scallop on Cu/Zn SOD enzyme production. The great scallop given as food to the rats were removed from the Dardanelles Çardak region. Four groups of rats are included in the study, group 1 (n=6), control group fed with standard rat food, group 2 (n=6), 75% great scallop and 25% standard rat food daily, group 3 (n=6), 75% great scallop and 25% standard rat food every two days, group 4 (n=6), 75% great scallop and 25% standard rat food every three days. To detect Cu/Zn SOD localization in the tissues, the LAB-SA Detection System was used. Cu/Zn SOD immunoreactivity was detected of epithelial cells in the gastric mucosa of rats fed with great scallop. Cu/Zn SOD enzyme immunoreactivity was detected in gastric mucosal epithelial cells of great scallop-fed rats. Cu/Zn SOD enzyme immunoreactivity was observed in 91% in the second group, 88.67% in the third group and 68.50% in the fourth group. We think that the reason for the detection of Cu/Zn SOD enzyme immunoreactivity in the gastric mucosa of rats fed with sea tark collected from Dardanelles is due to the accumulation of heavy metal salts in great scallos.

Key words: Immunohistochemistry, great scallop, Dardanelles, Cu/Zn superoxide dismutase, stomach

1. Introduction

Aerobic organisms possess antioxidant defense systems that deal with reactive oxygen species (ROS) produced as a consequence of aerobic respiration and substrate oxidation. Small amounts of ROS, including hydroxyl radicals (•OH), superoxide anions (O_2 •2) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organisms in response to both external and internal stimuli (Hurst et.al., 1997; Jornot et al., 1988; Mills et. al., 1998). Low levels of ROS are indispensable in many biochemical processes, including intracellular messaging in the cell differentiation and cell progression or the arrest of growth, apoptosis (Ghosh and Myers 1998), immunity (Yin et.al., 1995), and defense

against micro-organisms (Bae et al., 1997; Lee et al., 1998). In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules (Chopra and Wallace 1998; Czene et. al., 1997; Wojtaszek 1997).

Superoxide dismutase 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 (Fridovich 1995; Sandalio et. al.,1997; Teixeira et. al., 1998).

Heavy metals, industrial and household wastes and pesticides are threats for the aquatic ecosystem. Polluted water sources are streaming into the seas and cause pollution in these systems. Dardanelles is exposed to pollution from the Marmara and Black Sea. Gezen et. al. (2011) has investigated the accumulation of heavy metals in the carpet shell clams, great scallops, sea snails and oysters from Umurbey region in the Dardanelles (Çanakkale, Turkey). In this research, Zn in carpet shell clams, Zn and Mn in great scallops, Zn in oysters, Al, Zn, Fe, Cu and Mn in sea snails found the metals as high. If the same zone is in seawater, the Zn level is high. In sea chestnuts growing in Dardanells, the values of Al, Zn, and Fe in samples taken from Gelibolu Hamzakoy station are high. Al and Fe values were higher in samples taken from Çardak region of the Dardanelles. Al, Fe and Zn values were higher in samples taken from Umurbey region of the Dardanelles. Al, Fe and Zn values were higher in mussels taken from Çamburnu region of the Dardanelles (Gezen et. al., 2011). Demir et al. (2011) has investigated the accumulation of heavy metals in the carpet shell clams, great scallops, sea snails and oysters from the Dardanelles Karacaören region. In this research, Al, Zn and Fe in carpet shell clams, Zn and Mn in great scallops, Zn in oysters, Al, Zn, Fe, Cu and Mn in sea snails found the metals as high.

The purpose of the study is to demonstrate Cu/Zn SOD enzyme immunoreactivity in the stomach tissues of rats which are fed with great scallop that are collected from the Çardak region of the Dardanelles (Çanakkale, Turkey).

2. MATERIAL and METHOD

2.1. Ethics Statement

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 Ethics Committee for Animal Research (Protocol number: 2010/09-03).

2.2. Animal Model

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. For the first study group (n: 6), was the control group; standard rat diet was given every days. For the second study group (n: 6), 75% great scallop + 25% standard rat diet standard rat feeds were given daily. For the third study group (n: 6), 75% great scallop + 25% standard rat diet was given every two days. Standard rat diet was given the other day. For the fourth group (n: 6), 75% great scallop + 25% standard rat diet was given every three days. Standard rat diet was given the other two day.

Rats were fed twice daily for 30 days at 15% of their weight every morning and evening at the same time. The great scallop given as food to the rats were removed from the Dardanelles Çardak region (Figure 1). Average 40-60 g weight were selected. After the beaks were overcooked, the meat broke off and the meat at 100 degrees was dried.

Figure 1. *The area where the great scallop are collected.* ***Arrow:*** *Çardak region (Çanakkale, Turkey),* ***Star:*** *Dardanelles*

It was weighed into each rat's weight and 10 mg/kg intraperitoenal ketamine hydrochloride (Ketalar, Eczacibasi, 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.

2.3. Histological evaluation

The stomach tissues were maintained in immunofix (Leica) for 24 hours for histopathological examination. 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 X 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, 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) X 100 % = ……..….. % formula were used (Tosun et. al., 2006; Bakır et. al., 1996; Avunduk et. al., 2000).

2.4. Statistical analysis

Data was analyzed using SPSS program, version 19.0 One-way analysis of variance (ANOVA), Tukey's test was used to analyze the data. The difference between the groups was considered significant in the results of $p < 0.05$.

3. Results

In immunohistochemical staining with Cu/Zn SOD, a significant difference was observed in the gastric epithelial cells of the rats given great scallop per day, every other day and every three days compared to rats fed with normal feed ($p<0.05$).

Dark brown staining in the cytoplasm of the cells was considered positive. Cu/Zn SOD immunoreactivity could not be detected in gastric mucosa cells of rats fed standard rat diet (Figure 2).

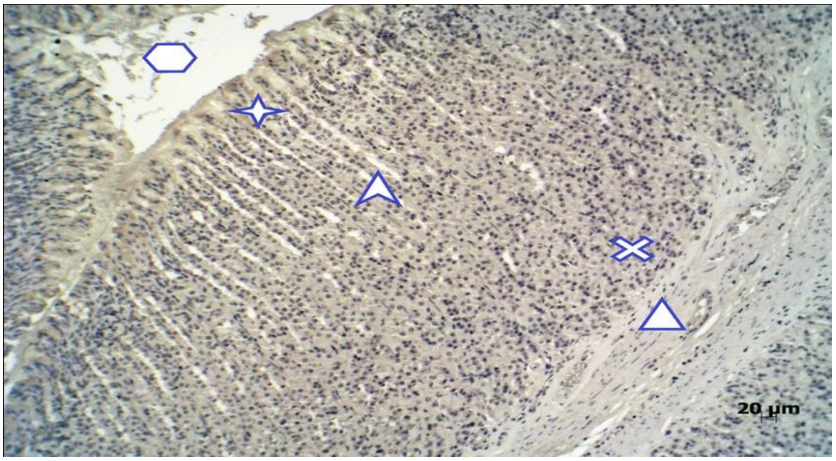

Figure 2. *For the first study group was the control group; standard rat diet was given every days. Rat stomach mucosa, (Cu/Zn SOD x 10).* ***Hexagon:*** *Gastric lumen;* ***Star:*** *Surface epithelial region,* ***Pointed arrow:*** *Neck region,* ***Crossed:*** *Basale region,* ***Arrow head:*** *Lamina muscularis mucosa*

In the second group, in the surface epithelial cell cytoplasm and stomach gland cells cytoplasm showed Cu/Zn SOD enzyme immunoreactivity (Figure 3).

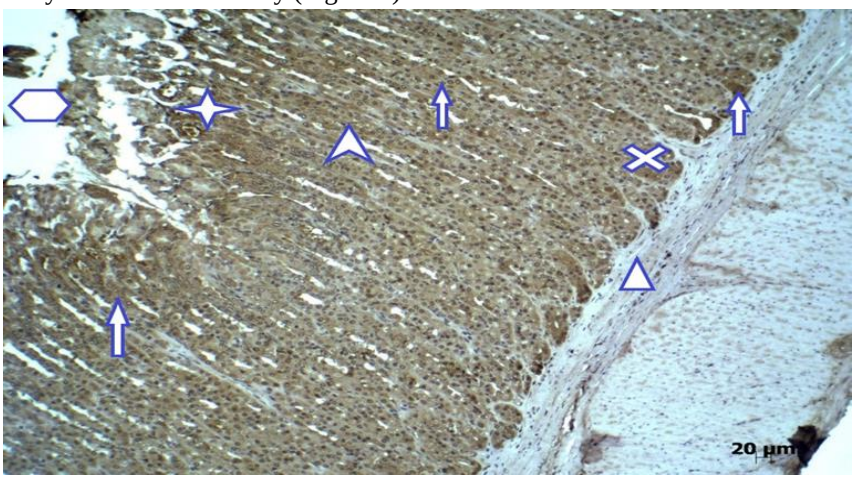

Figure 3. *For the second study group; 75% great scallop + 25% standard rat diet standard rat feeds were given daily. Rat stomach mucosa, (Cu/Zn SOD x10).* ***Hexagon:*** *Gastric lumen;* ***Star:*** *Surface epithelial region;* ***Pointed arrow:*** *Neck region;* ***Crossed:*** *Basale region;* ***Arrow head:*** *Lamina muscularis mucosa,* ***Arrows:*** *Cu/Zn SOD enzyme positive cells*

In the thirty group, in the surface epithelial cell cytoplasm and stomach gland cells cytoplasm showed Cu/Zn SOD enzyme immunoreactivity (Figure 4).

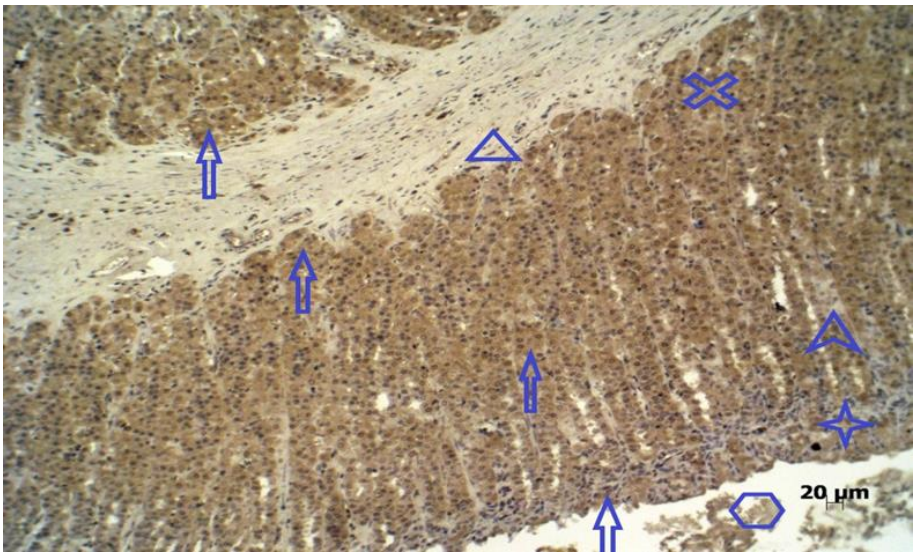

Figure 4. *For the third study group; 75% great scallop + 25% standard rat diet was given every two days. Standard rat diet was given the other day. Rat stomach mucosa, (Cu/Zn SOD x10).* ***Hexagon:*** *Gastric lumen;* ***Star:*** *Surface epithelial region;* ***Pointed arrow:*** *Neck region;* ***Crossed:*** *Basale region;* ***Arrow head:*** *Lamina muscularis mucosa,* ***Arrows:*** *Cu/Zn SOD enzyme positive cells*

In the fourty group, in the surface epithelial cell cytoplasm and stomach gland cells cytoplasm showed Cu/Zn SOD enzyme immunoreactivity (Figure 5).

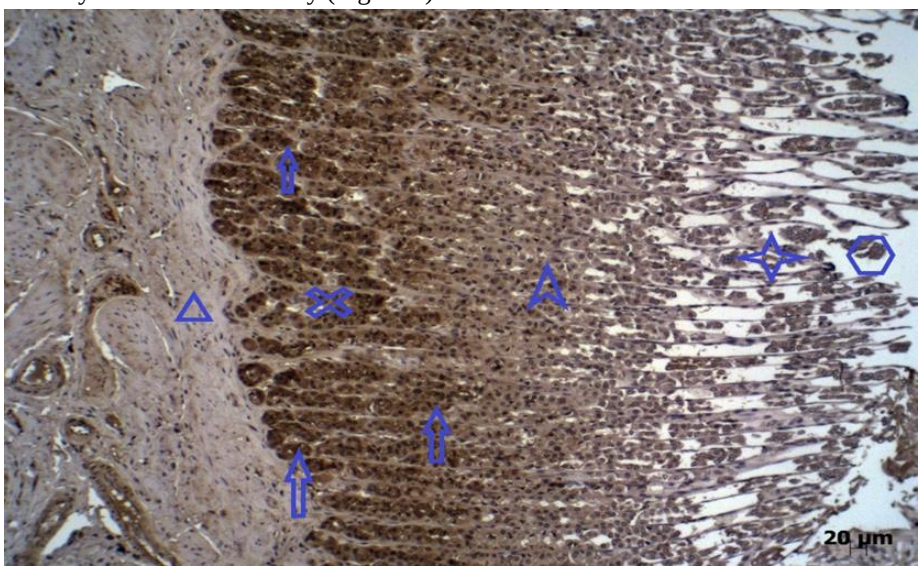

Figure 5. *For the fourth group; 75% great scallop + 25% standard rat diet was given every three days. Standard rat diet was given the other two day. Rat stomach mucosa, (Cu/Zn SOD x10).* ***Hexagon:*** *Gastric lumen;* ***Star:*** *Surface epithelial region;* ***Pointed arrow:*** *Neck region;* ***Crossed:*** *Basale region;* ***Arrow head:*** *Lamina muscularis mucosa,* ***Arrows:*** *Cu/Zn SOD enzyme positive cells*

In the epithelial cells of the gastric mucosa in the all group, Cu/Zn SOD immunopositive cells could not be detected by negative staining (Figure 6).

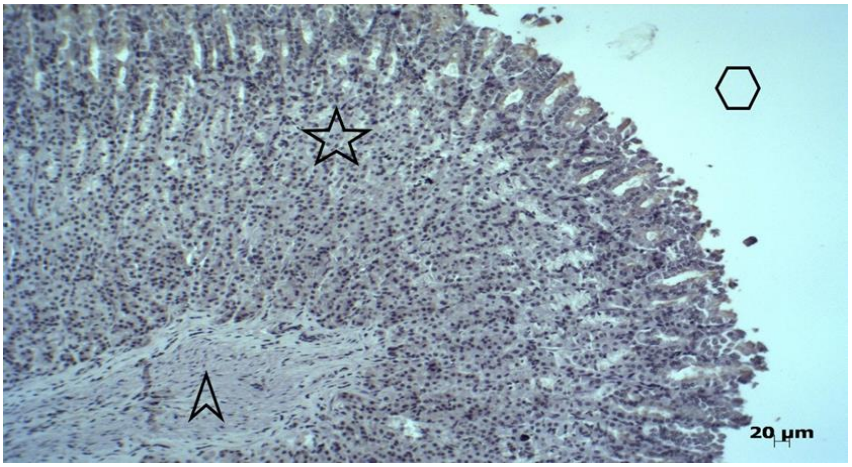

Figure 6. *For the second study group; 75% great scallop + 25% standard rat diet standard rat feeds were given daily. Rat stomach mucosa, negative control, (Cu/Zn SOD x10).* ***Star:*** *Lamina propria mucosa;* ***Pointed arrow:*** *Lamina muscularis mucosa;* ***Hexagon:*** *Gastric lumen*

Cu/Zn SOD enzyme staining in all epithelial and gland cells of the gastric mucosa of the rats in the second and third groups was severe, severe in the basal region in the fourth group, weak in the surface and neck cells.

The increase of Cu/Zn SOD enzyme in the gastric gland and surface epithelial cells of the stomach between the control group and the other groups was statistically significant. The Cu/Zn SOD enzyme immunoreactivity in the cytoplasm of mucus-secreting epithelial cells on the surface of the stomach and in the stomach gland cells of the lamina propria mucosa of the stomach was found 0% in the control group, 93.67% in the second group, 91.83% in the third group, 88.67% in the fourth group. There was no meaning between the second and third groups. Statistical significance was determined between the second and third group with the fourth group (Figure 7).

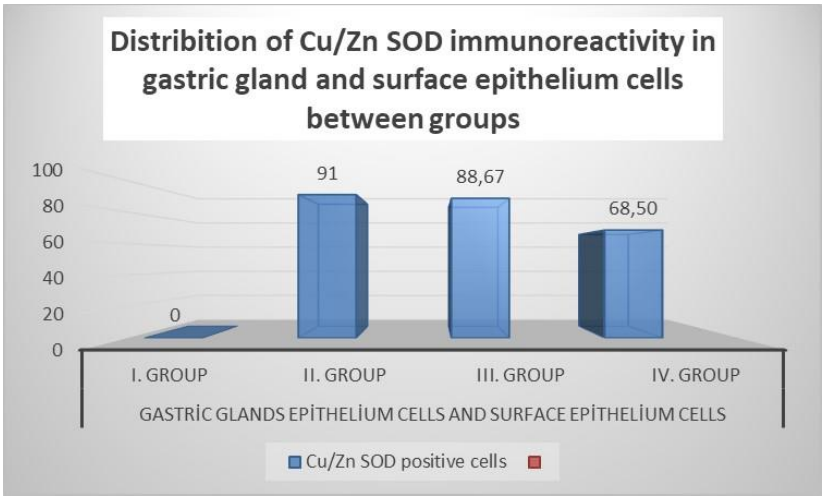

Figure 7. *Distribution of Cu/Zn SOD immunoreactivity total in gastric gland and surface epithelium cells between groups*

The increase of Cu/Zn SOD enzyme in the surface region of the stomach between the control group and the other groups was statistically significant. The Cu/Zn SOD enzyme immunoreactivity in the cytoplasm of mucus-secreting epithelial cells of the stomach was found 0% in the control group, 83% in the second group, 80.33% in the third group and 5.67% in the fourth group. There was no meaning between the second and third groups. Statistical significance was determined between the second and third groups and the fourth group (Figure 8).

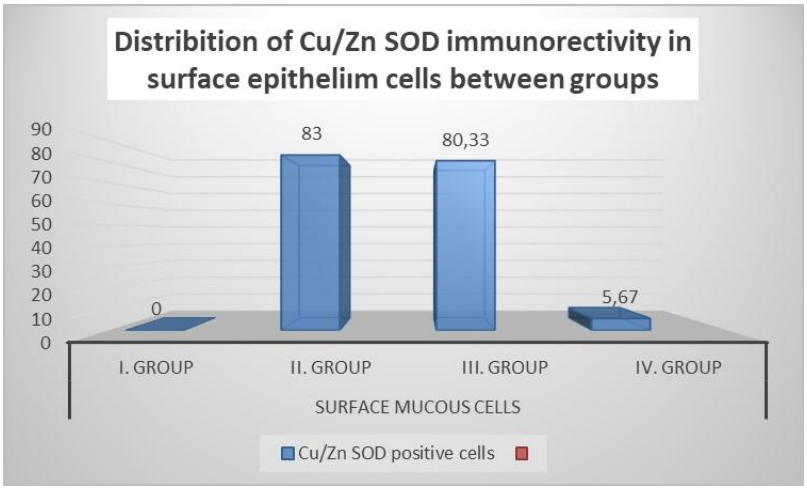

Figure 8. *Distribution of Cu/Zn SOD immunoreactivity in surface epithelium cells between groups*

Cu/Zn SOD enzyme increase was found to be statistically significant in the isthmus and neck region of the stomach between control group and other groups. The Cu/Zn SOD enzyme immunoreactivity in the cytoplasm of mucous neck cells and parietal cells in the neck region and the cytoplasm of stem cells and parietal cells in the isthmus region of the stomach was found 0% in the control group, 88.67% in the second group, 84.17% in the third group and 18.67% in the fourth group. There was no meaning between the second and third groups. Statistical significance was determined between the second and third groups and the fourth group (Figure 9).

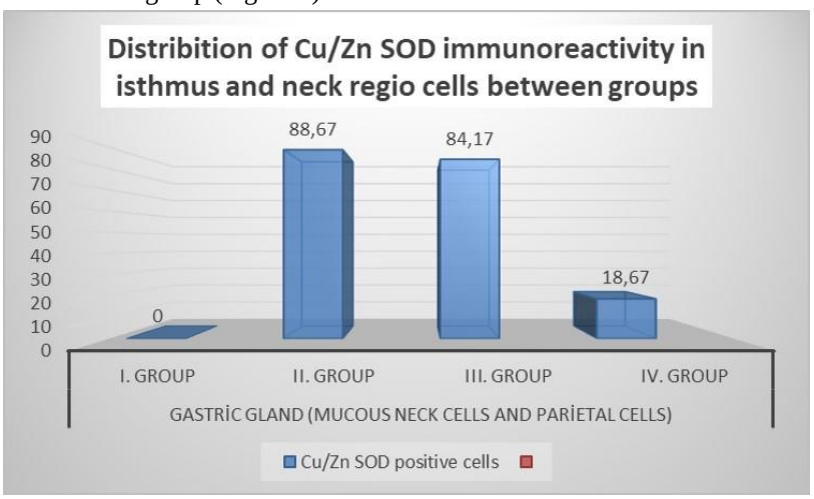

Figure 9: *Distribution of Cu/Zn SOD immunoreactivity in isthmus and neck regio cells between groups*

The increase of Cu/Zn SOD enzyme in the basal region cells of the stomach between the control group and the other groups was statistically significant. The Cu/Zn SOD enzyme immunoreactivity in the cytoplasm of the chief cells, endocrine cells and parietal cells in the basal region of the stomach was found 0% in the control group, 93.67% in the second group, 91.83% in the third group and 88.67% in the fourth group. There was no statistical significance between the second, third and fourth groups (Figure 10).

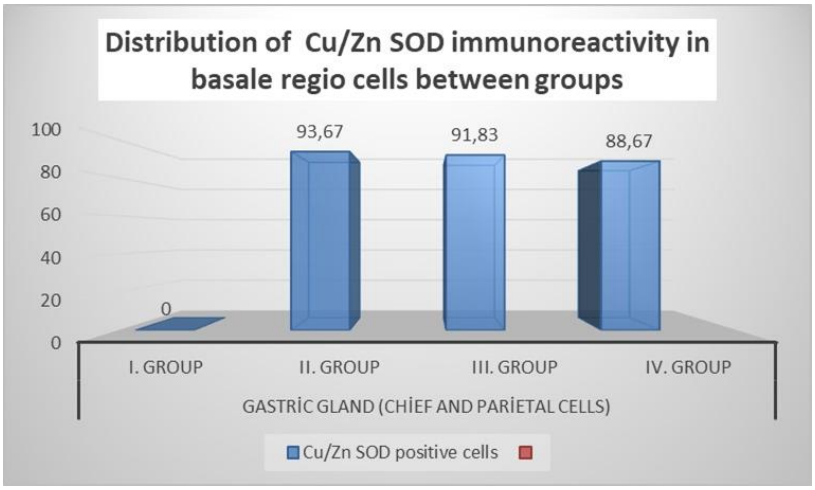

Figure 10. *Distribution of Cu/Zn SOD immunoreactivity in basale regio cells between groups*

4. Discussion

In our study, it was observed that great scallop-fed rats had an increase in Cu/Zn SOD enzyme due to increased great scallop consumption in gastric gland cells and surface epithelium cell cytoplasm. The Cu/Zn SOD enzyme immunoreactivity intensity was found to be more severe in basal area cells. Mucus secretion of surface epithelial cells and neck mucous cells suggests that heavy metal salts may less affect these cells. There are no studies showing that heavy metals cause Cu/Zn SOD enzyme increase in gastric mucosal cells in the literature. There are no studies showing that other researchers have caused heavy metals to cause Cu/Zn SOD increase.

Gezen (2017) stated that immunohistochemical staining methods are used to detect damage to cells and tissues. In this study, we used immunohistochemical analysis methods to detect Cu/Zn SOD enzyme.

All heavy metals are potentialy harmfull 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 fort the metabolism of animals, but in the excess all trace metala are toxic (Rainbow 1997). 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. Cu/Zn SOD is believed to play a major role in the first line of antioxidant defence (Prasad and Kundu, 1995). In this study Cu/Zn SOD enzyme was detected in stomach tissue of rats fed with great scallop collected from Çardak region of the Dardanelles. In this research, the detection of Cu/Zn SOD production in the stomach mucosa suggests that the great scallop may trigger oxidative stress.

The results of our research show that consumption of great scallop exposed to environmental pollution may lead to an increase in the oxidant. In addition to the findings of other researchers, we have also found that heavy metal salts cause histopathological changes in the stomach.

Footnotes

*At the time of this research, she was working at Department of Pathology of Çanakkale Onsekiz Mart University.

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