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Reactivity of TNF-α, NF-κB, and TGF-β in Liver Tissue of Rats Due to the Sand Mussel Consumption

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ABSTRACT: Toxic substances such as heavy metals have been reported to accumulate by aquatic organisms. In this study, it was aimed to investigate the histopathological changes that may occur in the liver parenchyma, the largest gland of the digestive system, by feeding the rats with sand mussel. The samples were analyzed for cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn) by ICP-OES. The liver tissue samples were stained with Hematoxylin-Eosin (H&E), and immuohitochemically staining inflamatory marker TNF-α, NF-κB, and fibrotic factor TGF-β. Heavy metal accumulation in sand mussel tissue suggests that it may triggers the toxicity in tissues with the consumption frequently that the hepatotoxic effect was quite severe especially in rats fed with sand mussel. Also immunoreactivity of TNF-α, TGF-β and NF-κB were observed in the liver cells of especially Group II (G2); were fed with 4/5 sand mussel + 1/5 standard rat food every day for 20 days. As a result, aquatic ecosystem can be affected with environmental pollution. It has been observed that crustaceans obtained from polluted ecosystems can cause especially digestive system organs damage in mammals.

Key words: heavy metal; hepatotoxicity; apoptosis; sand mussel; rat.

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NTRODUCTION

Especially heavy metal accumulation is an inevitable fact in aquatic organisms. Crustaceans have a good filter system and feed by a filtering system. It has a high potential for heavy metal deposition. In the present study, it was found that the amount of lead accumulated in bivalves in vivo is quite high (Sánchez-Marín et al., 2019). In vivo experimental study shows that cadmium poisoning causes a serious degeneration in kidney epithelial cells in addition to the expansion and adhesion of secondary lamellae in the gills, as well as in fish fed with cadmium-contaminated fish food (Beširović et al., 2011). The liver is often exposed to toxic substances and various drugs because of their anatomical localization, physiological and biochemical roles (Aksit and Bildik, 2014). It is known that different forms of this damage can occur with oxidative stress and subsequently free radicals. It has been shown in vivo and in vitro studies that toxic oxy and hydroxy radicals cause damage to the hepatocyte membranes by lipid peroxidation (Grunhage et al., 2003). The mechanism of DNA damage associated with oxidative stress results from the effects of endogenous or exogenous factors on the molecular integrity of the genetic material (Kciuk et al., 2020). DNA damage is a common phenomenon throughout the life of the cell that can lead to mutation, cancer, aging, and ultimately cell death. DNA is constantly exposed to changes throughout life by cellular metabolites (ROS; reactive oxygen species) and exogenous agents (Hajam et al., 2022).

Considering the environment and cooking techniques of clams, it is known that these creatures, which are cooked carelessly and collected from an unhealthy environment, can accumulate harmful pollutants, especially in their muscles and gills (Sanchez-Marin et al., 2019). Aquatic organisms that feed by filtering the water accumulate the harmful substances such as heavy metals and pesticides in their various tissues (Demir and Akkuş, 2018; Montazer and Ali, 2018).

These organisms collected from unhealthy environments threaten the human health. In a study, high concentrations of Pb and Cd were detected in mollusks from polluted areas (Koşer, 2020).

Heavy metals are the serious contaminants that can pass into aquatic organisms through the respiratory and digestive system, or through the skin and mucous membranes especially in organs related to metal metabolism and detoxification (Gintare and Gintaras, 2015; Muszynska and Labudda, 2019). In this study, organ toxicity related to seafood consumption frequency of rats with experimental feeding model was determined by histochemistry and immunohistochemical techniques.

Experimental procedures

The sand mussels were taken from the locations determined in Dardanelles (Lâpseki, Çardak, Çamburnu) from a depth of 1-2 meters. The samples were collected in June 2019 from 10-40 m depth by scuba diving from the determined location. Among the locations that study was carried out, the highest heavy metal level was detected in the sea water taken from Lâpseki and Çardak, and it showed parallelism with the heavy metal values in the muscle tissue of sand mussels collected from these regions. It suggests that heavy metals can trigger toxicity in vital organs such as the liver in humans, with the frequent consumption of marine based foods (Table 1). Then the samples were dried in an oven at 60-65 °C until a constant weight and then they were grinded into powder. In this study, 24 Wistar albino rats were used. All rats were housed in a 12-hour light and 12-hour dark with an average temperature of 22 ± 1°C and the humidity of 55 ± 5%. Rats were given *ad libitum*. Standard rat feed and sand mussel were given according to 15% of the weight of each rat for 20 days. The animals were randomly divided into 4 groups of 6 rats each, and they were treated as follows:

Table 1. Heavy metal concentrations of sand mussel muscle tissue (µg/g dry weight)

Heavy metals & Region	Cd	Pb	Cu	Zn
Camburnu	1.32±0.23	0.68±0.45	1.47±0.28	20.74±2.6
Lâpseki	1.54±0.5	0.81±0.47	1.53±0.3	23.74±3.87
Cardak	0.94±0.24	0.45±0.38	0.82±0.33	18.24±4.22
Average value	1.26±0.32	0.64±0.43	1.27±0.3	20.90±3.56
*Limit value (*µg/g) **WHO	1	1	5	20
Limit values (mg/L) *TSE	0.5	0.5	3	10

*Turkish Standards Institution
**World Health Organization

Group I (G1); were fed with standard rat food (control)

Group II (G2); were fed with 4/5 sand mussel + 1/5 standard rat food every day

Group III (G3); were fed with 4/5 sand mussel + 1/5 standard rat food every two days

Group IV (G4); were fed with 4/5 sand mussel + 1/5 standard rat food every three days

Heavy metal analysis

The samples were analyzed for cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn) by inductively coupled plasma-optical emission spectrometry (ICP-OES) (PERKIN ELMER-OPTIMA 800, USA). Sample preparation was performed using microwave-assisted acid digestion. Each sample (0.60 ± 0. 05 g) was mixed with 8 ml of 68% nitric acid and 4 ml of 30% hydrogen peroxide. The mixture was heated up to 120°C for about two hours. The solution was filtered with nitrocellulose membrane after cooling, and then transferred to acid-washed with deionized water (Kabengera et al., 2002).

Histopathological investigation

Liver tissue samples were fixed in a 10% neutral buffered formalin solution (Bio Optica, dilution: 1/7) for 24 hours. Tissue samples were passed through graded alcohols and dehydration. Then, the tissues passed through xylene to make them transparent and the alcohols of the tissues were removed. Paraffin infiltration was provided in tissue samples passed through xylene + paraffin and paraffin stages in oven at 60°C. Tissue samples removed from xylene were blocked using tissue embedding device. Tissue samples taken to the blocks were cut 4-5 microns thick in a microtome for routine histopathological investigation.

NF-κB, TNF-α, TGF-β antibody staining (IHC)

Immunohistochemically reactions were performed according to the Avidin/Biotin Method (ABC technique) (Hsu et al., 1981). First, the endogenous peroxidase was restricted by exposing specimens to 3% hydrogen peroxide in distilled water for 30 minutes. Then, the specimens were incubated with PBS in normal goat serum (DAKO X 0907, Carpinteria, CA). Following this step, the sections were incubated with a polyclonal rabbit anti-nuclear factor *kappa* B (NF-κB, abcam), anti- transforming growth factor (TGF-β,

dilution 1:100, abcam) and anti-tumor necrosis factor alpha (TNF-α, Abcam) for one hour. Then, the sections were incubated with biotinylated anti-mouse Immunoglobulin-G (DAKO LSAB 2 Kit, Invitrogen). 3, 3-Diaminobenzidene tetrahydrochloride (DAB, Invitrogen Corporation) solution was placed on the tissues as a chromogen substance and placed for 5 min in the dark. For background staining, it was kept in Mayer's Hematoxylin (**Öztürk et al., 2019**).

Evaluation of tissue samples and statistics

The immunoreactivity was evaluated with the H-score method, calculating the ratio of immunopositivity cells to all cells in the selected fields. Immunoreactive cells count were performed by a blinded observer and graded as follows: 0 was denoted no staining; 1 was denoted weak; 2 was denoted moderate; 3 was denoted strong in a specified field (Numata et al., 2013). SPSS 19 version was applied for statistical evaluation. Kruskal-Wallis Test, which is one of the nonparametric tests, will be used $p < 0.05$ for the difference between the groups.

RESULTS

H&E staining revealed no abnormal histopathological findings for the liver tissues of rats in the control group (Fig. 1). It was observed that the central vein and cord of hepatocytes forming the lobe were normal in the center of the classical liver lobule. No inflammatory cells were found in the liver parenchyma of the rats in the control group. In the liver tissues of rats fed with sand mussel, mononuclear cells caused inflammation in many areas, especially portal areas, and dilatation occurred in central vein and sinusoids (Fig. 1). In addition, it was observed the occurrence of congestion in central and portal veins, and pycnotic cells degeneration in hepatocytes. The hepatotoxic effect was severe, especially in rats given sand mussel every day (Fig. 1).

NF-κB, TNF-α, TGF-β immunoreactivity

In the immunohistochemical staining with NF-κB, a more severe positive immunoreactivity was observed in the liver of rats fed with sand mussel daily (G2) (Fig. 2). In this group, high immunoreactivity was observed around the central vein. Immunoreactivity were observed around the central vein with moderate intensity in G3 and G4. A statistically significant difference was observed between G1 and G2 ($p < 0.0001$). There was a low significance between G1 and G4 ($p < 0.05$) (Fig. 5).
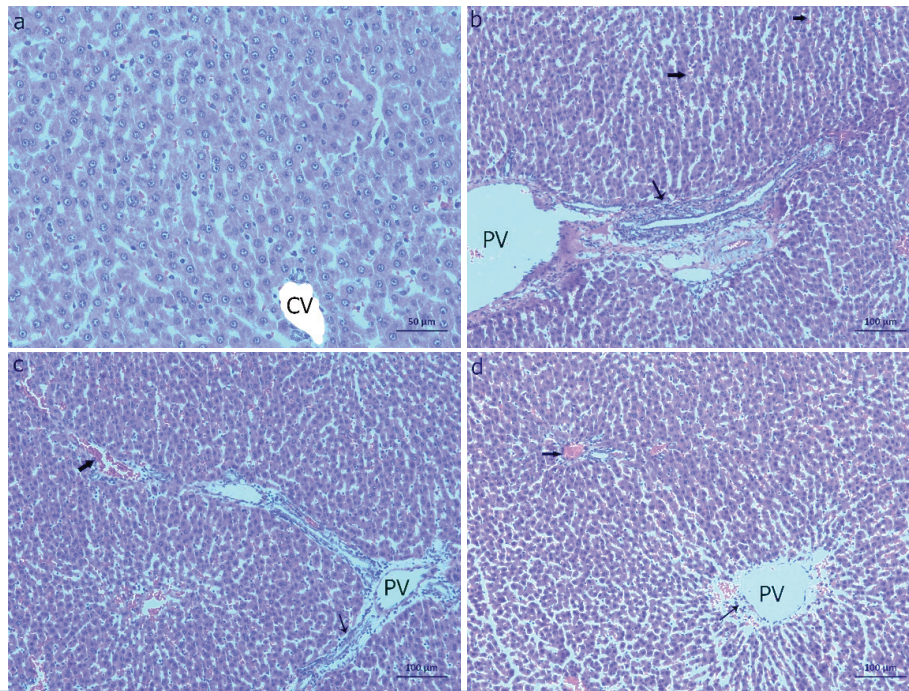
Figure 1. a. H&E staining of control group liver tissue (G1), b. Liver tissue fed with sand mussel every day (G2), c. Liver tissue fed with sand mussel every two days (G3), d. Liver tissue fed with sand mussel every three days (G4) (thick arrow: congestion, thin arrow: inflammation, PV: portal vein, CV: central vein)
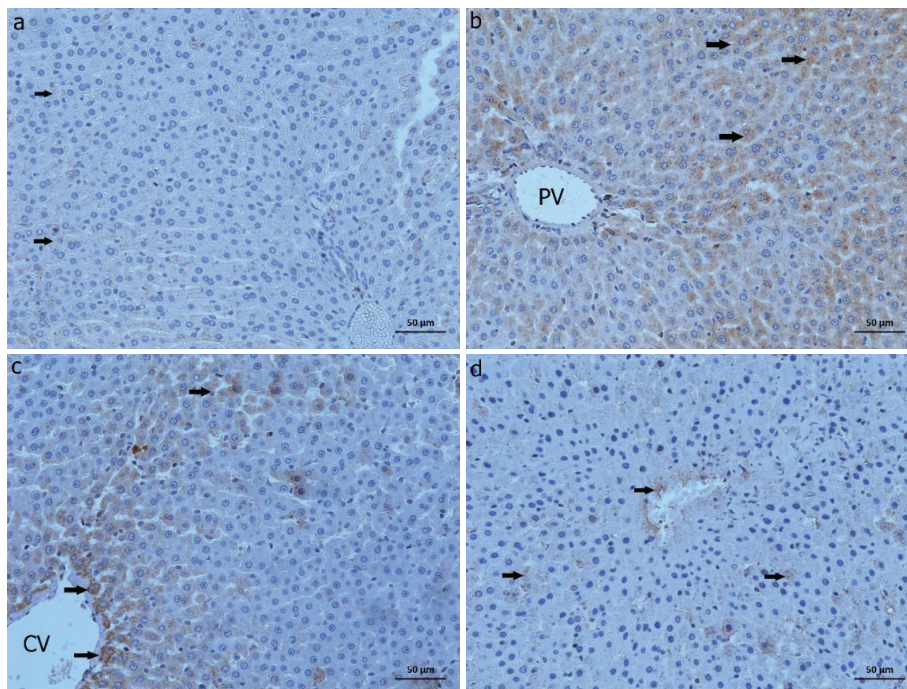
Figure 2. Immunohistochemical NF-κB staning for control (a, G1) and experimental groups (b-G2, c-G3, d-G4) (arrow: immunpositive cells)

TNF-α expression had a higher immunoreactivity in the liver due to the increase in G2, and staining was largely in the cell cytoplasm. The apoptosis was determined to be very high especially in G2 (Fig. 3)

In this study, it was observed that the rats fed with sand mussels increased TGF-β immunoreactivity (Fig. 4). When the control group and other groups were compared, a statistically significant difference was found (p<0.05). TGF-β immunoreactivity in the liver of rats, especially those fed with sand mussel every day, was more severe than other groups (Fig. 5).

ICP-OES results

It was determined that the Cd, Cu, Pb, and Zn values in water were above the upper limit values determined by the Turkish Standards Institute. Cu, Pb, Cd levels in water were found to be higher in Çardak and Zn in Çamburnu. The upper limit values determined by the Turkish Standards Institute and the World Health Organization are shown in Table 2.
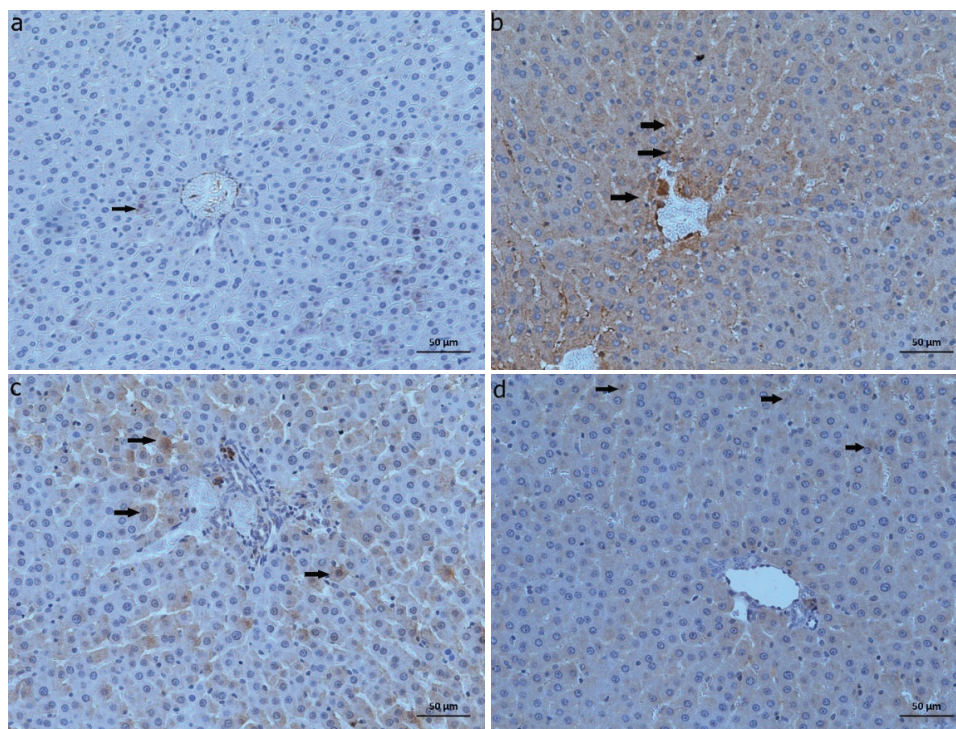
Figure 3. Immunohistochemical TNF-α staning for control (a, G1) and experimental groups (b-G2, c-G3, d-G4) (arrow: immunpositive cells)
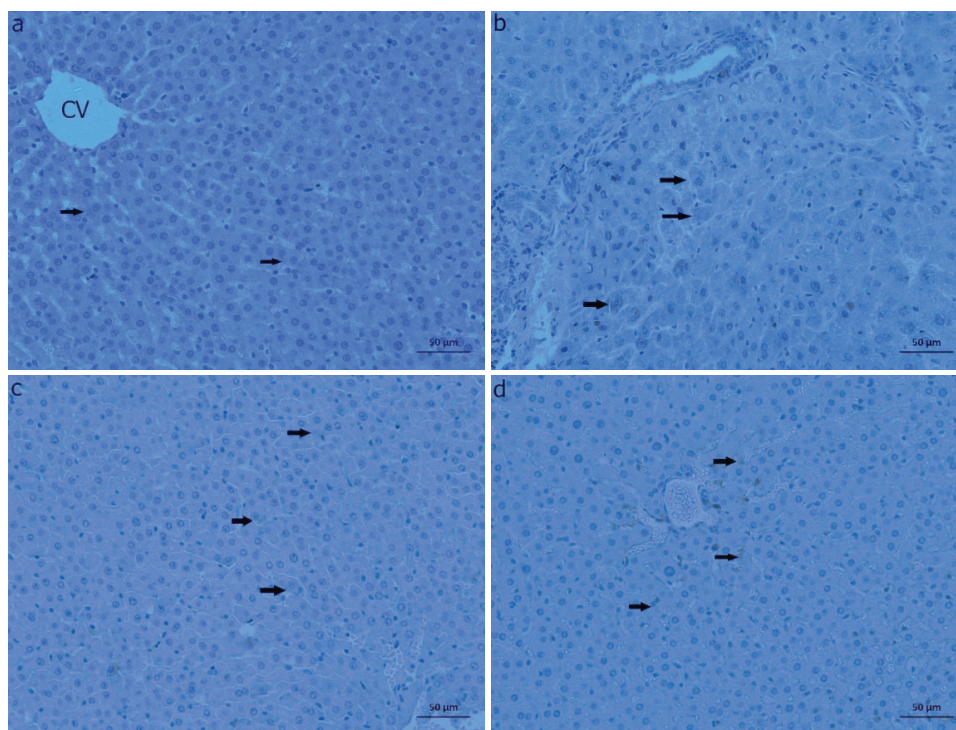
Figure 4. Control (a-G1) and experimental groups (b-G2, c-G3, d-G4) of liver tissue immunohistochemical staining of TGF-β, (arrow: immunoreactivity)
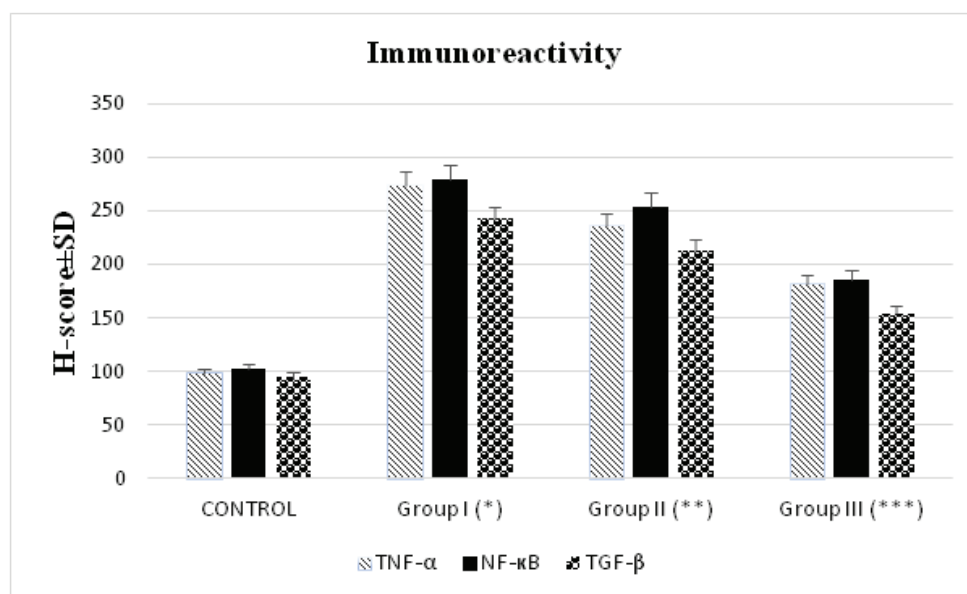
Figure 5. TGF-β, TNF-α and NF-κB immunoreactivity of control and all experiment groups. *p<0.0001 compared to the control group, **p<0.001 compared to the control group, ***p<0.05 compared to the control group. All experiment groups statistically significant difference were determined

Table 2. Heavy metal analysis of seawater

Heavy metals (µg/L)	Region Camburnu	Region Cardak	Region Lâpseki	Average value	Limit values (mg/L) *TSE	Limit values (mg/L) **WHO and USEPA
Cu	1.94±0.28	2.24±0.65	1.88±0.54	2.02±0.49	0.01	0.01
Cd	1.75±0.28	1.72±0.45	1.74±0.66	1.73±0.46	0.01	0.01
Pb	0.85±0.22	0.86±0.35	0.94±0.44	0.88±0.36	0.1	0.10
Zn	40.14±3.84	42.62±4.02	42.35±5.20	41.70±4.35	0.1	0.10

*Turkish Standards Institution
**World Health Organization & United States Environmental Protection Agency

DISCUSSION

In recent years, there has been a growing ecological and global health concern regarding environmental contamination by heavy metals. In addition, environmental problems that occur with the increase in industrial, agricultural, domestic and technological applications are increasing day by day (Tchouwou et al., 2012). Reported heavy metal sources in the environment are geogenic, industrial, agricultural, pharmaceutical, domestic wastewater and atmospheric sources (Zhou et al., 2021). Environmental pollution is high in point weld areas such as mining, foundries and smelters, and other metal-based industrial processes (Matthias and Shaughnessy, 2015). Cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn), are essential nutrients required for various biochemical and physiological functions. Inadequate supply of these micronutrients results in various diseases or symptoms (Aliasgharpour, 2020).

Coastal cities are an important source of pollutants for the Black Sea and the Marmara Sea. The aquatic ecosystem, which is increasingly polluted, is rapidly losing its ability to be a source of food. Aquatic organisms are very valuable and inexpensive sources of protein. However, to show the negative effects of the consumption of aquatic organisms grown in unhealthy conditions on human health, the tissue damage that may occur depending on the consumption frequency was determined in the experimental study. Thanks to the data obtained, the negative factors that occur in the experimental material provide a chance for a preliminary assessment in terms of food consumption awareness.

The Dardanelles is under the influence of the bidirectional current system. Heavy metal pollution carried to the Mediterranean by upstream flows from

the Dardanelles is more than the pollution carried to the Black Sea by downstream currents (Süren et al., 2007). Studies have shown that when the liver enters the degenerative process, TNF-α induces increased synthesis in hepatocytes and cytoplasm in Kupffer cells (Kalliolias and Ivashkiv, 2016).

NF-κB activation is triggered after activation at the TNF-α receptor in the cell death path. Thus, NF-κB goes to the nucleus and then NF-κB activates genes that try to block the apoptosis induced by TNF-α. In the resting cells, NF-κB is an inactive form in the cytoplasm. TNF-α-induced NF-κB activation regulates the expression of anti-apoptotic proteins such as members of the Bcl-2 family and prevents TNF-α-induced apoptosis. In addition, the NF-κB pathway is a pro-inflammatory signal pathway based on NF-κB activation. In this study, it was determined that consumption of clams caused deterioration of hepatocellular structure in the liver and TNF-α immunoreactivity increased as the dose increased. TNF-α, a cytokine secreted for protection, shows that the damaged structure of the liver can be cleared by apoptosis and inflammatory events, and regeneration can be triggered again.

When oxidative stress in tissues and related cell damage increase, activation of TNF-α receptor is followed by activation of nuclear factor NF-κB. Thus, NF-κB goes to the nucleus and then NF-κB activates genes that try to block TNF-induced apoptosis. In resting cells, NF-κB is an inactive form in the cytoplasm (Zinatizadeh et al., 2021; Tomita, 2016). The results of this study show that the expression of TNF-α and NF-kB increases in hepatocyte cytoplasm in parallel with its regulatory role in liver tissue, as in previous studies. The hepatotoxic effect increased with the increase in consumption of mussels. TNF-α and NF-κB expression were found to be at the highest level in the fourth group. In addition, it was shown that TGF-β, a marker of fibrosis, was higher in immunohistochemical staining in rat liver fed daily with sand mussel.

In the research conducted by Küçüksezgin et al., (2010) in the Gulf of Izmir, they found the cadmium accumulation, heterotopic and fecal coliform bacteria amount in Tapes decussatus varies seasonally. Crustaceans are organisms with a good filter system and they feed by filtering water. They have heavy metal deposition potentials. In the study, it was found that the amount of lead accumulated in bivalves in vivo is quite high (Sanchez-Marin et al., 2019). In addition, when the results of heavy metal analysis of sand mus-

sel collected seasonally from Şile coast (Black Sea) were observed, Zn is dense in that region (Ulusoy, 2010). Another study with clams about heavy metal accumulation, cadmium has been reported more intensely in kidney, digestive organs, and in muscles and gonads (Saavedra et al., 2008). When the effects of pectenotoxin-I (PTX1), a non-diarrheal toxin originating from mussels, on liver cells were investigated by fluorescence microscopy it was shown a decrease in number and loss in the radial arrangement of microtubules (Farabegoli et al., 2018). In addition, food poisoning related to mussel consumption is considerably high. These studies show that crustaceans toxic products increase oxidative stress parameters and trigger apoptosis. In this study, we showed the hepatotoxic effects histopathologically in the rat liver fed experimentally with sand mussel. The study results show that the degenerative effects in the liver as a result of feeding the sand mussel collected from the Dardanelles, where transit ship passages are intense. This toxicity can be related to the high amount of heavy metals obtained from seawater and aquatic organisms in previous studies (İrkin and Öztürk, 2021). The fact that the studies on this subject are quite limited and less comprehensive hinder the reliability of the results. For this reason, it is important to carry out more comprehensive in vivo and in vitro studies, taking into account the increasing food demand and decreasing resources.

In vivo experimental rat model, by growing the crustaceans in the environment, where Pb amount was high, the amount of Pb in blood, liver and kidney tissue were measured by giving 80% standard food and 20% crustacean as food. In the study by Sanchez-Marin et al., (2019) the results were not found at a level that could affect human health, since the duration of the experiment and the amount of toxic substances to which the crustaceans were exposed were quite low. However, in the present study, the amount of sand mussel consumed both as time and as feed was kept quite high. Therefore, it is suggested that it increases the amount of damage in the liver. Another study shows that cadmium poisoning causes a serious degeneration in kidney epithelial cells in addition to the expansion and adhesion of secondary lamellae in the gills, as well as in fish fed with cadmium-contaminated food (Besirovic et al., 2010). The results obtained in the study using critical reference values that can show heavy metal toxicity on aquatic fish showed that these values do not pose a toxicity hazard in the silverfish species. Therefore, periodic monitoring of

heavy metals in fish and water is very important, as aquatic ecosystems are easily exposed to pollutants and fish are generally consumed by humans (Yabanlı et al., 2014). Regardless of the marine based food, it is inevitable that they accumulate heavy metal in various tissues when they are found in contaminated environments. These unhealthy foods are not limited to crustaceans, but can also be seen in other marine based food.

CONCLUSIONS

The study results showed that the heavy metal accumulation in the sand mussel collected from the Dardanelles caused inflammation and degeneration in the liver tissue of rats. While searching for an alterna-tive food source, environmental factors should not be ignored and attention should be paid to consumption of clean and healthy products since it causes tissue damage in many systems especially in the digestive system organs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

A total of 24 male Wistar albino rats were used in the study. The study protocol was approved by the Canakkale Onsekiz Mart University Ethics Committee for Animal Research (Protocol number: 2021/02.02).

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