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Effect of Mediterranean Mussels *(Mytilus galloprovincialis)* From Polluted Areas on Hepatotoicity in Rats by Immunuhistochemical Method

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Research Article

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Abstract

Mussels *(Mytilus galloprovincialis*) are aquatic organisms that can accumulate all the factors that pollute the fresh and saltwater environment. Although the Dardanelles has been exposed to environmental pollution for many years, it is also an important region for crustaceans. We aimed to investigate the histopathological changes in the liver parenchyma which is an important gland of the digestive system by feeding these mussels to rats. Twenty-four male Wistar albino were used in the study. The first group (control): fed with standard rat feed, the second group (experiment 1): 4/5 mussel + 1/5 standard rat feed daily; third group (experiment 2): 4/5 mussel + 1/5 standard rat feed every other day, the fourth group (experiment 3): groups were formed with 4/5 mussel + 1/5 standard rat feed every three days. All liver tissue samples taken from the experimental and control groups were stained with hematoxylin-eosin, and immuohitochemically staining inflammatory marker TNF-α and NF-κB after routine histopathological follow-up and analyzed with a light microscope image analysis system. It was observed that mononuclear cells caused inflammation of portal areas, increased sinusoidal dilatation and congestion and degeneration due to vacuolization in hepatocytes in the liver parenchyma of mussel-fed rats. Besides, immunohistochemical staining, TNF-α, and NF-κB immunoreactivity were observed in the liver cells of especially in the second group of rats. As a result, it has been shown that the consumption of mussels obtained and marketed without considering environmental pollution may trigger important digestive system organs of liver diseases.

Keywords: Immunohistochemistry, inflammation, liver, hepatocyte degeneration, mussels.

Kirlenmiş Alanlardan Toplanan Akdeniz Midyesi'nin Ratlarda İmmünohistokimyasal Metotla Hepatotoksik Etkisi

Özet

Midye (*Mytilus galloprovincialis*), tatlı ve tuzlu su ortamını kirleten tüm faktörleri biriktirebilen sucul organizmalardır. Çanakkale Boğazı uzun yıllar boyunca çevre kirliliğine maruz kalsada, kabuklular için de önemli bir bölgedir. Bu midyelerin sindirim sisteminin önemli bir bezi olan karaciğer parankimindeki histopatolojik etkilerini araştırmayı amaçladık. Çalışmada 24 adet erkek Wistar albino türü rat kullanıldı. Birinci grup (kontrol): standart sıçan yemi ile beslendi, ikinci grup (deney 1): hergün 4/5 midye + 1/5 standart sıçan yemi; üçüncü grup (deney 2): 4/5 midye + 1/5 standart sıçan yemi 2 günde bir, dördüncü grup (deney 3): gruplar 4/5 midye + 1/5 standart sıçan yemi ile her üç günde bir oluşturuldu. Deney ve kontrol gruplarından alınan tüm karaciğer doku örnekleri, histopatolojik takipten sonra rutin hematoksilin-eozin ve enflamatuar belirteç olan TNF-α ve NF-κB ile immünohistokimyasal boyama yapıldı ve ışık mikroskobu görüntü analiz sistemi ile analiz edildi. Mononükleer hücrelerin portal alanlarda yangıya sebep olduğu, karaciğer parankimindeki hepatositlerde vakuolizasyonun dejenerasyon sonucu meydana geldiği, santral ven ve sinüzoidal dilatasyon ve konjesyonun olduğu gözlemlendi. Ek olarak, özellikle ikinci sıçan grubunun karaciğer hücrelerinde immünohistokimyasal boyama, TNF-α ve NF-κB şiddetli immünoreaktivitesi gözlendi. Sonuç olarak, çevre kirliliği dikkate alınmadan elde edilen ve pazarlanan midye tüketiminin sindirim sisteminin önemli bir organı olan karaciğer hastalıklarını tetikleyebileceği gösterilmiştir.

Anahtar kelimeler: İmmünohistokimya, inflamasyon, karaciğer, hepatosit hasarı, midye

INTRODUCTION

The relationship between healthy food and quality of life is gaining importance day by day. Accordingly, it increases consumers' orientation towards natural and delicious products that are not contaminated with drugs or chemicals. Despite all these positive thoughts, the increasing population every day and the increase in food consumption parallel to this increase in the orientation towards alternative food sources. Consumption in fish and shellfish occasionally bursts. Especially in coastal areas, there are seashells that have become food culture. Creatures such as mussels, oysters, and sea urchins are among the more preferred products as nutrients. Mussels are organisms that feed on organic and inorganic substances in the water. Mussels can also filter toxic substances during water filtration. Because of these properties, they can store harmful substances in water in their bodies al. (Figueras et al., 2019). The mussels are fed with microorganisms that secrete neurotoxins, especially in the summer months. Also, they filter the water and accumulate heavy metals, pesticides, and bacteria in the tissues. As a result of the pollution of seawater with environmental factors, toxin levels increase in the body of these creatures (Voudanta et al., 2016). Dardanelles is the marine area where the mussel population is the most and it is very prone to contamination in terms of location. The seas have indeed been polluted for years with domestic waste, pesticides, and industrial waste (Yigit et al., 2018).

Most of the liver studies have been conducted to examine regeneration as a result of either experimental or chemical damage (Palmes & Spiegel, 2004). TNF-α plays a role in the pathophysiology of TNF-α, viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease and, ischemia-reperfusion injury in the liver. Studies have shown that when the liver enters the degenerative process, TNF-α induces increased synthesis in hepatocytes and Kupffer cells in the cytoplasms (Yang & Seki, 2015) When oxidative stress in tissues and associated damage to cells increases, activation of the TNF-α receptor is followed by activation of the nuclear factor kappa B (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 (Karin, 2006; Schattenberg & Schuchmann, 2009).

Heavy metals are defined as metallic elements with a relatively high density than water (Fergusson, 1990). Heavy metals contain metalloids and heavy metals such as arsenic can have toxic effects even at low doses (Pourret & Hursthouse, 2019). In recent years, there has been an increasing ecological and global public health concern associated with environmental pollution by these metals. Besides, human exposure has increased significantly as their use has increased exponentially in some industrial, agricultural, domestic and technological applications (Tchounwou et al., 2012). In biological systems, it has been reported that heavy metals affect cellular organelles and components such as cell membranes, mitochondria, lysosomes, endoplasmic reticulum, nuclei, and enzymes involved in metabolism, detoxification, and damage repair (Wang & Shi, 2001). Metal ions have been found to interact with cell components such as DNA and nuclear proteins and cause conformational changes that can lead to DNA damage and cell cycle modulation, carcinogenesis, or apoptosis (Chang et al., 1996; Hubbard, 2005). Various forms of heavy metals, pesticides, viral and bacterial organisms that crustaceans filter and accumulate in their tissues also pass to mammals that consume these creatures (Gorinstein et al., 2008). As a result, it will inevitably produce important histopathological results in the liver. In this study, we aimed to draw attention to the consumption of seafood consumption in a healthier and more reliable environment by showing the changes in the liver tissue with histochemistry and immunohistochemical techniques by giving them to the rats with an experimental nutrition model in vivo.

MATREIALS and METHODS

The mussels used in the study were removed by diving from various locations determined in the Dardanelles of April- May 2019. Mussels were selected from the same region and those close to the same size. Mussel muscle tissue was assumed to be contaminated based on analysis results. The mussels were boiled in the shell and after the water was taken in the oven, they were turned into o pellet and fed to the subjects as feed. In this study, 24 male Wistar albino rats (250-300 g in weight) were used. All rats were housed in a 12-hour light and 12-hour dark environment with an average temperature of 22 ± 1°C, humidity 55 ± 5, ventilation and air conditioning system. Rats were given as

much water as they could drink. Standard rat food and mussel were given according to 15% of the weight of each rat in feeding planning (Gezen, 2018).

Experimental groups

The first group (Control, n = 6); Standard rat food,

The second group (Experiment 1, n = 6); 4/5 mussels + 1/5 standard rat food daily,

The third group (Experiment 2, n = 6); 4/5 mussels + 1/5 standard rat food every two days; other day standard rat food,

The fourth group (Experiment 3, n = 6); 4/5 mussels + 1/5 standard rat food every three days; the other two days are standard rat food; It was fed for four weeks.

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: 2020/04-07).

Histopathological Examination

30 days after the start of the study, rats were removed for this study after rats were sacrificed under ketas (150 mg/kg; i.p) and alfazyne 2% (25 mg/kg; i.p) anesthesia. Liver tissue samples from all groups were detected in a 10% neutral buffered formaldehyde solution (Bio Optica) for 24 hours. Tissue samples were passed through graded alcohols and their juices were removed. Then, the tissues passed through xylene were made transparent and the alcohols of the tissues were removed. Paraffin was allowed to enter into the tissue samples passed through xylene + paraffin and paraffin stages in a 60°C oven. Tissue samples removed from paraffin were blocked using a tissue embedding device. Tissue samples taken to the blocks were cut 4-5 microns thick in microtome for routine histopathological staining and taken into a water bath. The tissue samples opened here were taken on a normal slide and Hematoxylin-Eosin staining was applied.

Immunohistochemically Examination

Immunohistochemically reactions were performed according to the ABC technique described. Following this step, the sections were incubated with a polyclonal nuclear factor Kappa-B (NF-κB p50, Abcam), tumor necrosis factor (TNF-α, Abcam), then the sections were incubated with biotinylated anti-mouse Immunoglobulin-G (DAKO LSAB 2 Kit, Invitrogen). Following this step, the sections were incubated with the ABC complex (DAKO LSAB 2 Kit). For background staining, Mayer's Hematoxylin (Gürpınar et al., 2012; Öztürk et al., 2019).

Evaluation of tissue samples and statistics

During the evaluation of the results, the immunoreactivity was evaluated with the H-score method, calculating the ratio of immunopositivity cells to all cells in the selected fields. Immunoreactive cell count was performed by a blinded observer and graded as follows: 0 denoted no staining; 1 denoted weakly; 2 denoted moderate; 3 denoted strong staining in a specified field. The respective score was then calculated using the following formula: H-score = (% stained cells at 0) x 0 + (%stained cells at 1+) x 1 + (%stained cells at 2+) x 2 + (%stained cells at 3+) x3. The H-score value varies from 0 to 300. SPSS 19 version applied for statistical evaluation of the results obtained with this formula. To determine the differences NF-κB and TNF-α immunoreactivities between groups, the Kruskal –Wallis Test, which is one of the nonparametric tests, will be used. $P<0.05$ Difference between the groups will be considered significant.

RESULT and DISCUSSION

Histophatological Findings

Control Group: Hepatocellular damage, biliary tract damage, vascular damage, and sinusoid cell damage, and no histopathological findings of tumor cases were observed when staining liver tissue samples from rats fed with standard rat food every day with Hematoxylin and Eosin (Figure 1). Remark cord structure, sinusoid, and central vein, and portal vein, portal artery, and bile ducts located in the portal area were observed to have normal histological structure.

Experimental groups: The following histopathology table occurred in all experimental groups, respectively.

a- Hepatocellular Damage; Vacuolar degeneration, focal necrosis, inflammatory cells including neutrophils and eosinophils in the lobule and portal area were detected. Vacuolar degeneration in most of the hepatocytes manifested by diffuse swelling, pale staining of the cytoplasm, and the appearance

of the cytoplasmic residues around the nucleus. In some hepatocytes, the picnotic nucleus was observed, lobular inflammation in small foci, periductal inflammation with portal inflammation (Figure 1, Table 1).

Table 1. Histopathological evaluation of liver tissue samples.

	Groups			
Parameters	**Control**	**Experiment 1 (Every day giving mussel)**	**Experiment 2 (Two day giving mussel)**	**Experiment 3 (Three day giving mussel)**
Congestion	-	++++	++	+
Dilatation	+	++++	+++	++
Inflammation	-	+++	++	+

b- Vascular Damage; Central, portal, and sinusoidal dilatation, congestion in portal veins, central veins and sinusoid were observed. These histophatological changes occurred more severely in the first group of mussels given daily. In other groups, liver damage decreased due to mussel consumption (Figure 1).

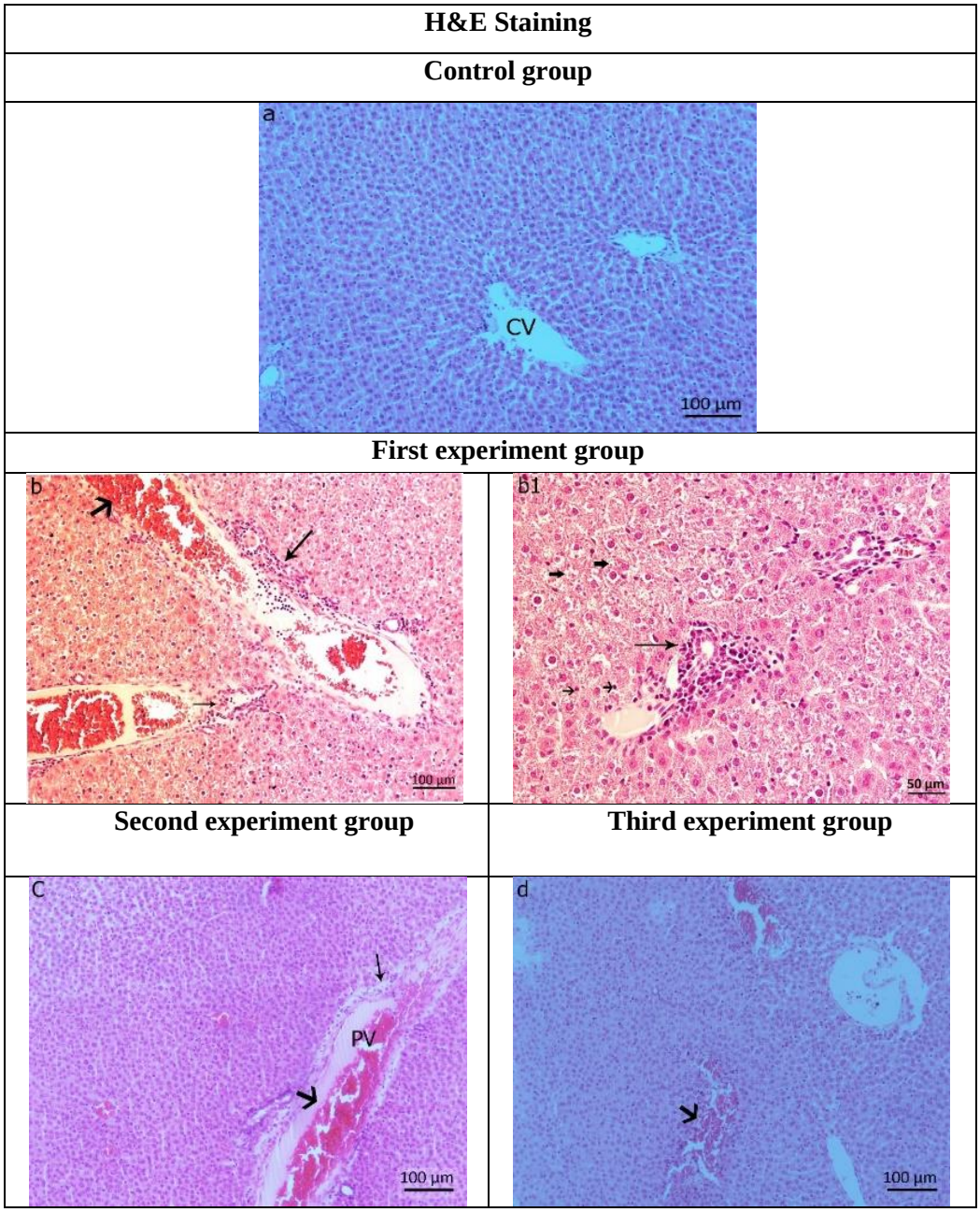


Figure 1. Control and experiment groups of liver tissue, H&E (CV: Central vein, long arrow: inflammation, short arrow: congestion, thick arrow: vacuolar degeneration).

NF- κB and TNF-α findings

Immunohistochemical staining results obtained from our study, we observed that NF-κB and TNF-α expression showed higher immunoreactivity in the liver due to the increase in the first experiment group, and staining was largely in the cell cytoplasm. The apoptotic mechanism was found to be very high in the liver tissues, especially in the first experimental group. Immunohistochemical staining with NF-κB, positive immunoreactivity was observed according to the amount of mussel given (Figure 2 and Figure 3). In the first experiment group, high reactivity was observed around the central vein. In the second and third groups, it was observed that the immunoreactivity around the central vein was moderate. A statistically significant difference was observed between the control and the first

experimental group of the subjects ($p<0.0001$). There was a weak significant difference between the control and third experiment group ($p<0.05$) (Table 2).

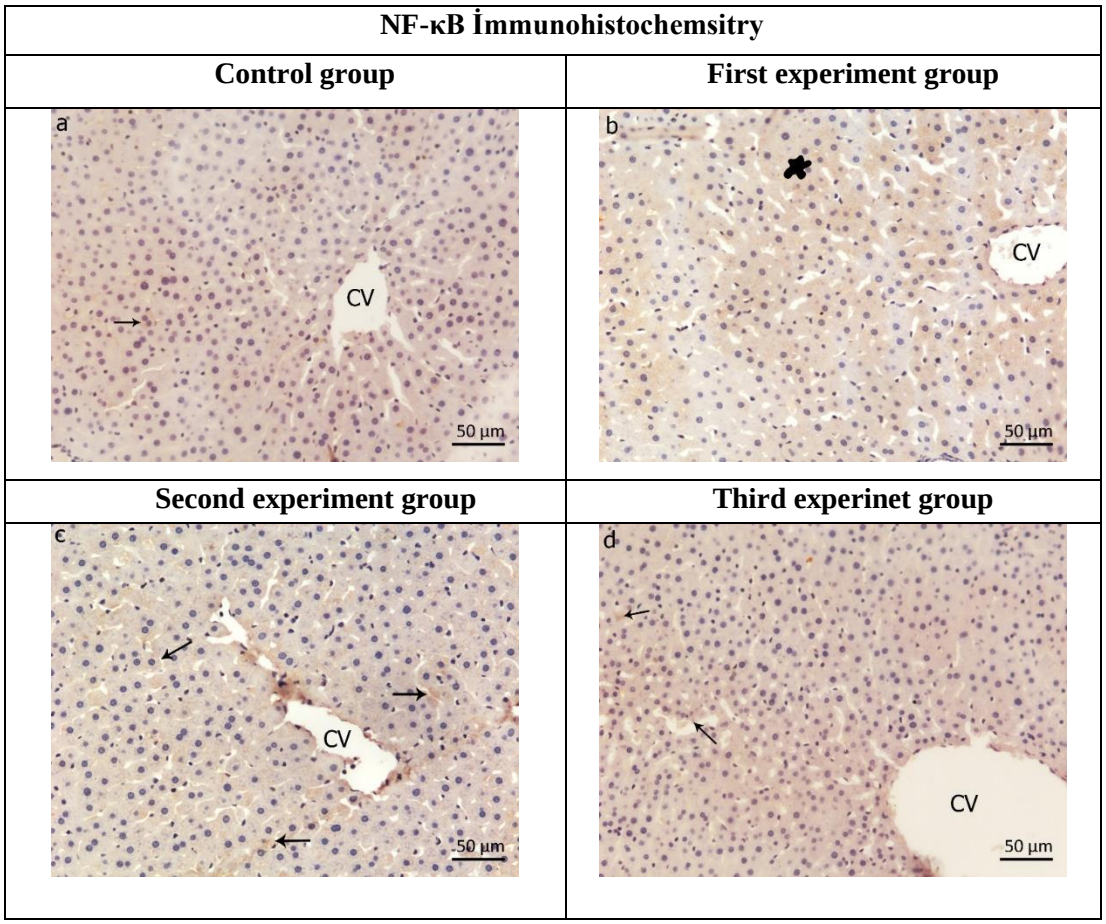


Figure 2. The immunohistochemical distribution of NF-κB in the control and experiment group of liver tissue (star and arrow: immunreactivity, CV: Central vein).

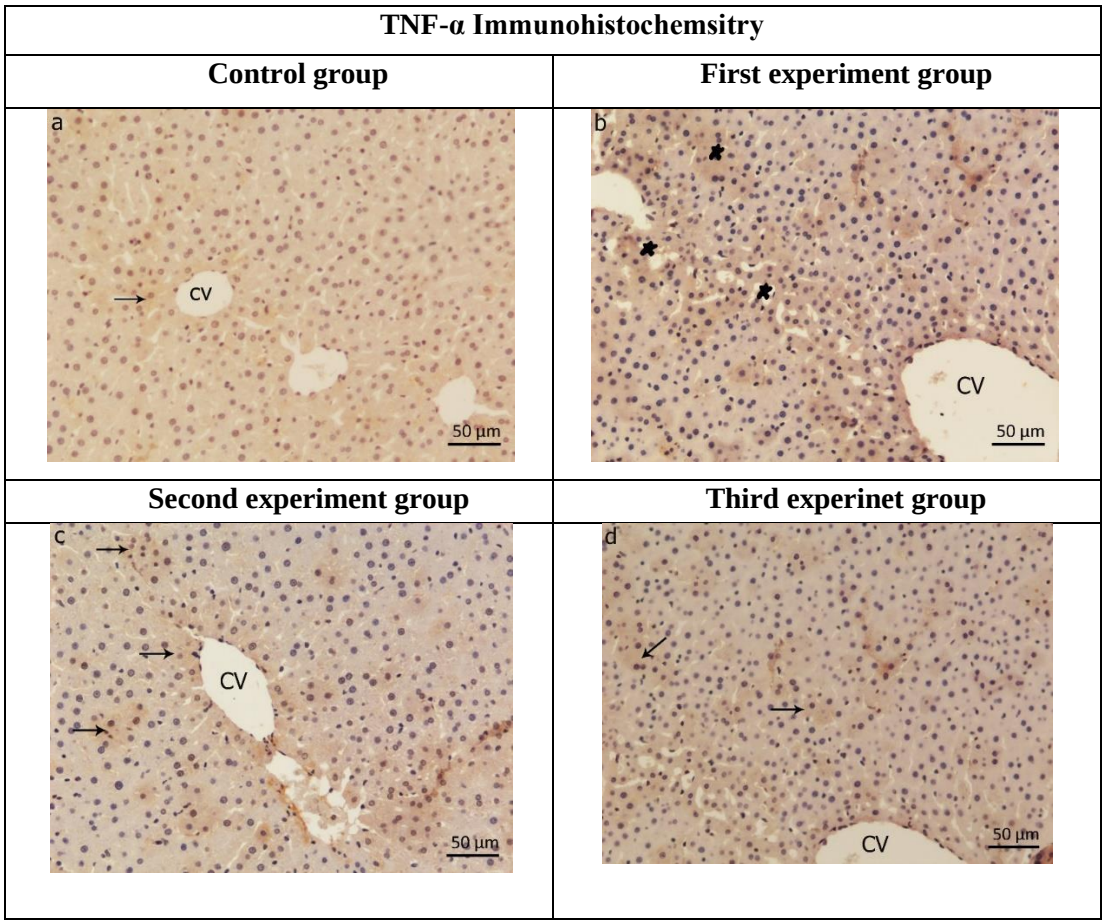
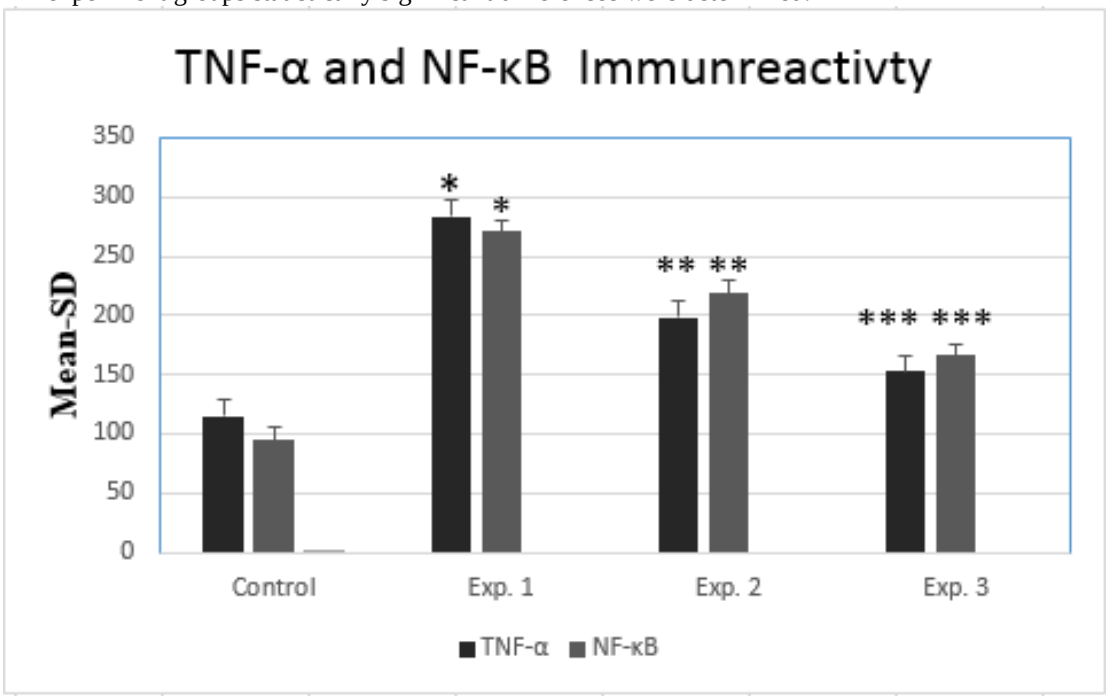


Figure 3. The immunohistochemical distribution of TNF-α in the control and experiment group of liver tissue (star and arrow: immunreactivity, CV: Central vein).

Table 2. TNF-α and NF-κB Immunoreactivty 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 differences were determined.



Heavy Metal Analysis Results

According to the research findings, considering the Cd, Pb, Cu, and Zn data in M. galloprovincialis, the places where the heavy metal concentration is the highest are Çamburnu, Yenikordon, and Çardak stations, respectively (Table 3).

Table 3. Heavy metal concentrations of *M. galloprovincialis* muscle tissue (µg/g dry weight.)

	Heavy metals			
Region	***Cd***	***Pb***	***Cu***	***Zn***
Çamburnu	1.22	0,56	1.35	18.74
Yenikordon	1.10	0,45	1.10	16.86
Çardak	0.84	0,42	0.72	16.24
Ortalama	1.05	0.47	1.05	17.28

There were differences and similarities between the groups in terms of histopathological damage in the liver tissues of the rats, which were collected with mussels collected from the Dardanelles. In our study, we researched Mediterranean mussels from shellfish, which have a high potential to cause liver damage to dietary habits. When mussels, which are both commercial and highly nutritious, are obtained from environmentally polluted environments, they can cause permanent damage to many organs, especially the digestive system (Gorinstein et al., 2008). Mussel dishes, which have become food culture especially in coastal areas and consumed frequently, can also cause permanent damage in one of the most important organs of our body, such as the liver. It supports this hypothesis in our research findings. The size of histopathological effects of mussels, which are frequently consumed and collected from unhealthy environments (exposed to industrial wastes), on the liver was found to be considerably large. Studies have reported that toxic substances such as heavy metals accumulate in fish and mussels due to marine pollution (Kayhan et al. 2006). In the researches, the presence of many heavy metals in single and bivalve seafood grown in the Dardanelles was determined (Ustunada et al., 2011). In our country, the annual pollution rate is quite high compared to the regions due to the transit ship passes of the Dardanelles and the Bosphorus. Again in the heavy metal detection studies carried out in the Mediterranean, trace elements such as cadmium, iron, and copper were found to accumulate in the bivalve (Göksu et al., 2005). Our findings show that, in the light of the data obtained from these studies, mussels can accumulate heavy metal and other pollutants in their tissues and create cytotoxic effects in humans and other organisms fed with them.

Long-term to heavy metals such as mercury (Hg), lead (Pb), chromium (Cr), cadmium (Cd), arsenic (As), copper (Cu), vanadium (V), nickel (Ni), and zinc (Zn) It has been reported that exposure may cause certain cancers in humans with chronic inflammation, cardiac, pulmonary and neurological effects (Mantovani et al., 2008; Nieboer et al., 2013). In our study, the detection of prominent foci of inflammation in the portal areas of the liver and parenchymal tissue was found similar to the findings of inflammation caused by the toxic effects of these investigators in people exposed to heavy metal. However, in our study, no findings related to liver cancer were encountered. Literature information has shown that heavy metals can cause cancer (Sá et al., 2016), but our study suggests that there is no cancer in the liver and that mussels are fed to rats for a short time, such as 30 days. Rats fed with mussels for a longer period may think that cancer may occur in the liver tissue.

Toxic liver diseases caused by drugs and chemicals mainly affect hepatocytes, bile ducts, vascular system, sinusoidal cells, and Kupffer cells, causing various morphological changes in the liver. Changing rates of hepatocyte damage, necrosis, lobular and portal inflammation are observed in hepatocellular damage. In its mildest form, balloon degeneration and apoptotic bodies, as well as focal necrosis foci, and inflammatory cells, including neutrophils and eosinophils, are observed in hepatocytes (Boone et al., 2005; David & Hamilton, 2010; Mantovani et al., 2008). In our study, balloon degeneration, portal and lobular fissures in hepatocytes are similar to the literature information seen in toxic liver cases. We detected necrosis, inflammation in the lobule and portal area. The fact that balloon degeneration is much more common in the group that feeds on mussels every day suggests that continuous mussel consumption causes more hepatocellular damage. In toxic hepatitis, cytotoxic hepatocellular damage can be seen as well as the cholestatic type (Bioulac-Sage & Balabaud, 2009; Lucena et al., 2008). Acute hepatocellular damage with cytotoxic effect may also be in mixed

form, where both cholestatic and cytotoxic damage can coexist. Cholestatic changes are accompanied by mild balloon degeneration, necrosis, and apoptosis (Schattenberg & Schuchmann, 2009). Our balloon degeneration and necrosis findings in our study suggest that mussels show cytotoxic effects in mixed form. We think that the source of histopathological damage occurring in the liver is due to the transition of heavy metals to mussels and toxic effects on the liver.

It has been shown that in liver injuries triggered by hemorrhagic shock, the protein supplements obtained from the crustaceans decrease the damage, and the level of inflammatory marker TNF α (Lee et al., 2012). However, it was shown that the severity of inflammation increased due to liver damage caused by shellfish collected and consumed without considering environmental pollution. Studies for its protective effects have also been shown in primary liver culture. When the protein products obtained from the crustaceans are given to the cells that are induced with carbon tetrachloride, it has been shown that the antioxidant level increases and the damage in the cells decreases (Chi et al., 2010). In another study, it was reported that pacific oyster extract decreased liver fibrosis, and inflammatory markers such as TGF-beta and NF-KB decreased (Zhou et al., 2015). In our study, it was determined that mussels caused degeneration of hepatocellular structure in the liver and that TNF-α immunoreactivity increased as the dose increased. TNF-α, a cytokine secreted for conservation purposes, shows that the liver's damaged structure can be cleaned by apoptosis and inflammatory events and re-trigger regeneration. When oxidative stress in tissues and associated damage to cells increases, activation of the TNF-alpha receptor is followed by activation of the nuclear factor kappa B (NF-kB). Thus, NF-κB goes to the nucleus, and then NF-κB activates genes that try to block TNF-induced apoptosis. In resting cells, NF-B is an inactive form in the cytoplasm (Karin, 2006; Schattenberg & Schuchmann, 2009). Our findings show that in previous studies, we observed that the expression of TNF-α and NF-κB increased in hepatocyte cytoplasms parallel to its regulatory role in liver tissue. As the hepatotoxic effect increased as consumption scallop increased, TNF-α and NF-κB expression was observed to be at the highest level in the first experimental group.

CONCLUSION

Our study results have confirmed the parameter results, which are encountered in many regions and countries, depending on the amount of consumption and environmental pollution. Heavy metal accumulation in mussels collected from the Dardanelles was observed to cause inflammation and degeneration in the liver. While searching for an alternative food source, environmental factors should not be ignored and it should be paid attention to the consumption of clean and healthy products since it causes tissue damage in many systems especially in the digestive system. Besides, before the consumption of mussels, especially heavy metal and other environmental pollution analysis should be done. Our people should be made aware of how much heavy metals they take with food accumulate in their organs and should be protected from possible liver diseases.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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