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ARAŞTIRMA / RESEARCH

Immunohistochemical investigation of histopathological changes in the kidney tissue of mussel-fed rats

Midye ile beslenen sıçanların böbrek dokusundaki histopatolojik değişikliklerin immünohistokimyasal yöntemle incelenmesi

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Abstract

Purpose: The aim of this study was to determine the histopathological changes that the mussels, which serve as important filters of the sea, cause in rat kidney tissue.
Materials and Methods: In our study, 24 female (Wistar albino 6-10 week old rats) rats were used. First group (n: 6): fed with standard rat food, second group (n: 6): fed everyday with 4/5 mussels + 1/5 standard rat food; third group (n: 6): fed 4/5 mussels + 1/5 standard rat food every two days; Fourth group (n: 6): were formed with 4/5 mussels + 1/5 standard rat food to be given every three days. After the routine histopathological follow-up, all kidney tissue samples taken and control groups were analyzed with Hematoxylin-Eosin staining, and the nephrotoxic effect was immunohistochemically with TGF-β and NF-κB and analyzed with a light microscope image analysis system.
Results: Mussel-fed rats were found to cause inflammation in the kidney tissue, dilatation in the distal and proximal tubules, shrinkage in the glomeruli and degeneration in the tubular epithelium. In immunohistochemical staining, TGF-β and NF-κB immunoreactivity increased due to cells leading to inflammation and apoptosis. The nephrotoxic effect was quite high especially in rats given daily mussels.
Conclusion: The mussel provided in unsanitary conditions may cause damage in kidney tissue from the excretory system organs due to excess consumption.

Keywords:. Nephrotoxicity, kidney, mussel, immunohistochemistry, histopathology.

Öz

Amaç: Bu çalışmada denizlerin önemli filtre işlevini gören midyelerin sıçan böbrek dokusu meydana getirdiği histopatolojik değişikleri belirlemeyi amaçladık.
Gereç ve Yöntem: Çalışmamızda Wistar albino cinsi 24 adet dişi sıçan (200-260 g) kullanıldı. Birinci grup (n:6): standart sıçan yemi ile beslenen, ikinci grup (n:6): 4/5 midye + 1/5 standart sıçan yemi ile her gün; üçüncü grup (n:6): 4/5 midye + 1/5 standart sıçan yemi ile gün aşırı; dördüncü grup (n:6): 4/5 midye + 1/5 standart sıçan yemi ile her üç günde bir verilecek şekilde gruplar oluşturuldu. Denek ve kontrol gruplarından alınan tüm böbrek doku örnekleri rutin histopatolojik takip yapıldıktan sonra Hematoksilen- Eozin boyamasıyla, nefrotoksik etki ise immünohistokimyasal olarak TGF-β ve NF-κB ile boyanarak ışık mikroskopta görüntü analiz sistemi ile analiz edildi.
Bulgular: Midye ile beslenen sıçanların böbrek dokusunda mononükleer hücre infltrasyonuna, distal ve proksimal tübüllerde dilatasyona, glomerüllerde büzüşmelere ve tübül epitelinde dejenerasyona neden olduğu görülmüştür. İmmünohistokimyasal boyamlarda da TGF-β ve NF-κB immünreaktivitesinin inflamasyona ve apoptoza giden hücrelere bağlı arttığı tespit edilmiştir. Özellikle her gün midye verilen sıçanlarda nefrotoksik etkinin oldukça fazla olduğu görüldü.
Sonuç: Sağlıksız koşullarda temin edilen midyelerin fazla tüketimine bağlı boşaltım sistemi organlarından böbrek dokusunda kronikleşebilecek hasarlar oluşturabileceği gösterilmiştir.

Anahtar kelimeler: Nefrotoksisite, böbrek, midye, immünohistokimya, histopatoloji

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INTRODUCTION

The main tasks of the kidney are to remove blood from metabolism residues by filtering the blood, adjusting the water and salt concentrations, plasma volume, electrolyte balance and controlling various hormones and blood pressure[1]. The structural proximal tubules of the kidney are very sensitive to toxic substances, ischemia and reperfusion damage[2]. In experimental studies, the creation of kidney damage with toxins is one of the most used methods[3]. Studies have reported that kidney damage occurs in humans due to chemical agents. The proximal tubule cells of the kidney are very sensitive to heavy metals and chemicals[4]. The emergence of toxic or heavy metals during chemical and industrial processes and their intensive use in agricultural areas can threaten the health of living organisms. Living organisms can face significant health problems by exposure to environmental pollutants directly from the digestive tract through the respiratory tract or through the food chain. Cadmium (Cd), a heavy metal, is produced as a metal accompanying zinc production. Until its use in zinc production was revealed, air, food and water did not mix significantly with natural processes[5]. Cadmium is also present in trace amounts in certain foods such as leafy vegetables, potatoes, grains and seeds, liver and kidney, and crustaceans and mollusks[6]. In addition, foodstuffs that are rich in cadmium can greatly increase the cadmium concentration in human bodies. Examples are liver, mushrooms, shellfish, mussels, cocoa powder and dried seaweed. Also Cd, Zn, Pb, and Ni have accumulated in the kidneys of mussels from these areas. When the concentrations of both essential and toxic metals in the mussel organs[7].

Nuclear factor κB (NF-κB) was initially explored as a B cell nuclear protein binding to the κ enhancer of the immunoglobulin κ light chain gene[8]. It later became clear that NF-κB is a ubiquitously expressed transcription factor that mediates signal-induced expression of numerous genes involved in different biological processes, including immune responses, inflammation, cell growth and survival[9]. Anti-inflammatory effect of TGF-β: TGF-β is one of the strongest immune-suppressive molecules known. TGF-β suppresses immune and inflammatory responses by suppressing the effector T (Th1 and Th2) and cytostatic T cells of the immune system and activating regulatory T-reg cells. Proapoptotic effect of TGF: In some cell types, TGF-β induces apoptosis by a mechanism that is not yet fully illuminated[10].

Water pollution occurs due to the reasons such as the discharge of domestic and industrial liquid wastes into water environments without treatment and the fertilizers used to increase productivity in agriculture and the transportation of medicines used for pesticides to water environments. Prevention of marine pollution is one of the important goals of humanity, especially due to the negative impact of industrial developments. Despite the success in maintaining a healthy environment, the pollution problem is far from being solved[11]. Meanwhile, studies on the dirty and unpolluted areas of the sea continue to be an important topic[12]. Seafood consumption has increased in recent years. Many patients added seafood, including mussels, to their diets. However, the benefits of these seafood supplements are not clearly known[13]. The bioactive compound content and antioxidant capacity of mussels in dirty and unpolluted areas differ significantly[14]. In addition, the effect of diets supplemented with mussels collected from these areas on laboratory animals is less well understood. For this reason, in our study, histopathological changes occurring in rat kidney tissue as a result of feeding the mussels collected from the Dardanelles, which is an important ship transit route, were shown by immunohistochemical methods.

MATERIALS AND METHODS

The study protocol was approved by the Canakkale Onsekiz Mart University Ethics Committee for Animal Research (Protocol number: 2020/04-07).

Animal Model

The mussels used in the research were taken from depth of 1-2 meters by diving from the locations (Çardak, Çamburnu) determined in the Dardanelles. The mussels collected from the same region and their average weights were close to each other and were fed to the working groups after they were boiled in the oven. In this study, 24 female rats (200-260 g) of Wistar albino type (6-10 weeks old rats) 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. The mussels collected at an average weight of 80 ± 10 g were broken after cooking and their meat was dried at 100 degrees. It was then turned into pellet feed. Standard rat food and mussel were given according to 15% of the weight of each rat in the feeding plan.

Group I (Control) (6); Group feeding on standard rat food every day,
Group II (6); A group fed 4/5 mussels + 1/5 standard rat food daily,
Group III (6); 4/5 mussels + 1/5 standard rat food every two days,
Group IV (6); The group fed 4/5 mussels + 1/5 standard rat food every three day.

The animal model of the study lasted four weeks. The time required for systemic damage to occur has been specified as 3-4 weeks in studies[15]. This type of study in vivo is not available in the literature. For this reason, the study was carried out by collecting mussels from regions where heavy metal analysis was performed before. Heavy metal concentrations in coastal areas and closed seas are higher than open seas have detected heavy metals in sea water and many molluscan species that growing in the Dardanelles[16-18].

Procedure

Histochemical staining

At the end of the experiment, the kidney tissue of all subjects were removed completely under anesthesia including Alfamine (90 mg/kg) (Ketamidor, Alfasan, The Netherlands) and Rompun (10 mg/kg) (Rompun, Bayer, Turkey) and placed in tissue transport cassettes and fixed for 48 periods in immunofix. Afterwards, tissue samples are passed through increasing grade alcohol solutions and purified from the water; alcohol in the tissues was cleaned with xylene and then blocked in paraffin in the oven. 3-5 micron thick sections were taken with microtome and placed on slide. Hematoxylin-Eosin and immunohistochemical stains were applied to the cut tissue samples[19].

Immunohistochemical staining

For immunohistochemical examination, 5 µm thick sections were taken from kidney tissue and sections were lowered after deparaffinization. The sections lowered into the water boiled for 20 minutes in the microwave oven in antigen retrival. After allowing to cool for 20 minutes at room temperature, the sections were washed with phosphate buffer solution (PBS). After this step, it was treated with 3% hydrogen peroxide (H_2O_2) prepared in methanol (Riedel-de Häen 24229) for 20 minutes to remove hydrogen peroxidase activity. Stirring in distilled water, the sections were washed with PBS (pH 7.6). To block non-specific antibody binding, 1% preimmune rabbit serum (Ultra V Block, LabVision, TA-015-UB) was applied to the sections. Sections were then incubated with primary antibody diluted 1/100 in the moist chamber for 1 hour. Polyclonal NF-ϰB (p50, Abcam, dilution 1:100), and mouse monoclonal TGF-β (Abcam, dilition 1:100) was used. Sections were kept in secondary antibody solution (Biotinylated Goat Anti-Mouse, LabVision, TM-015-BN) for 20 minutes after washing 3 times with PBS. 20 min streptavidin peroxidase solution (Streptavidin Peroxidase, LabVision, TS-015-HR) and 10 min 3-amino 9 ethyl carbazole (AEC) chromogen solution (LabVision, TA-002-HAC) were applied to the sections washed in PBS. After washing the sections with distilled water, Mayer's hematoxylin was applied for 5 minutes and contrast staining was performed and covered with closure medium (Mounting Medium, LabVision, TA-060-UG) and evaluated in the light microscope[20,21].

Statistical analysis

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[20]. SPSS 15 version will be applied for statistical evaluation of the results obtained with this formula. To determine the differences NF-ϰB and TGF-β immunoreactivities between groups, Kruskal –Wallis Test, which is one of the nonparametric tests, will be used. $P<0.05$ difference between the groups will be considered significant.

RESULTS

Histochemical findings

Among control group: In histological staining with hematoxylin - Eosin, no histopathological findings were found in the kidney tissues of the rats in the first group, which is the control. Cortex and medulla were observed normal.

In Gtoup II, in the kidney parenchyma of the two

group of daily mussel-fed groups, tubular dilatations and degenaration increased, blockages occurred in some tubules, glomerular shrinkage, and inflammatory fire was observed to be intensified. In addition, dilatations in the Bowman capsule and necrotic cells in tubular epithelial cells were observed. However, congestion was observed in the capillaries. Among Group III and IV, histopathological picture was mild in the kidney tissues of the subjects fed with mussels every two days and every three days, but tubular dilatations, congestion and inflammation continued. Also expansion in the bowman capsule with less glomerular shrinkage was observed in group III kidney cortex (Figure 1).

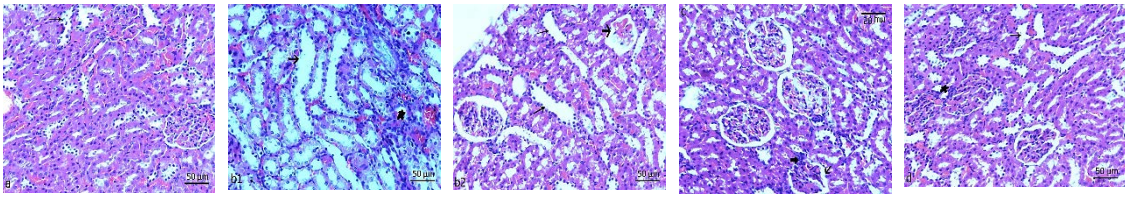

Figure 1. a-) Renal tissue of control group, b1-) Renal medulla of the second group of giving everyday mussel, (Star: inflamation), b2-) Renal cortex of the second group of giving everyday mussel (Short arrow: shrinked glomerule) c-) Renal tissue of the third group of giving every two days mussel (thick arrow: inflamation, thin arrow: shrinked glomerule, d-) Renal tissue of the fourth group of giving every three days mussel (Star: inflamation, arrow: tubular dilatation, H&E.

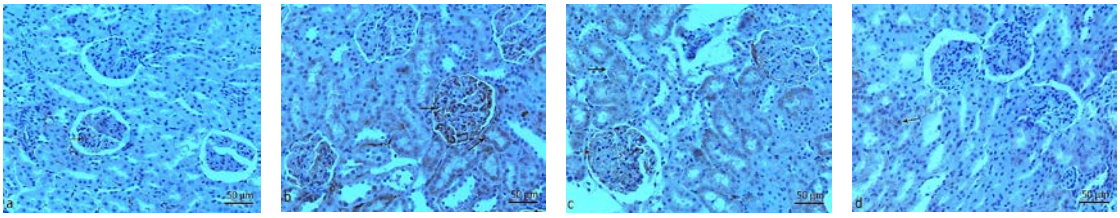

Figure 2. Control and experimental groups (b: group II, c: group III and d: group IV) of renal tissue immunohistochemical staining of NF-ϰB, X100, (Arrow: immunoreactivity).

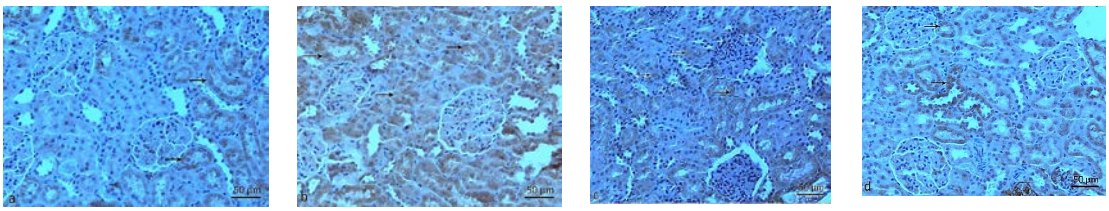

Figure 3. Control and experimental groups (b: group II, c: group III and d: group IV) of renal tissue immunohistochemical staining of TGF-β, X100, (Arrow: immunoreactivity).

Immunohistochemical findings

In immunohistochemical staining in the kidney tissues of the subjects, it was found that the immunoreactivity in the tissues of the group fed with mussels everyday (group II) showed a significant difference between the control (group I) and the other long term mussel groups (Group III and IV). Light immunoreactivity was observed in distal and proximal tubular epithelial cells in the control group. In third and fourth groups, immunoreactivity was moderate in cortex tubules, but it was also seen in collector tubule cells along the medullary beam. It was observed that the severity of immunoreactivity increased as the frequency of feeding with mussels increased. TGF-β and NF-ϰB immunoreactivity was found to be severe especially in the cortex of kidney tissues belonging to the second group (group II) (Figure 2, Figure 3). A statistically significant

difference was observed between the control and the second group than the other groups ($p<0.0001$). There was a significance of $p<0.05$ between the control and other groups. When the other groups are compared, the most significance was found between the second and fourth groups, and the least significance was found between the second and third groups (Chart 1).

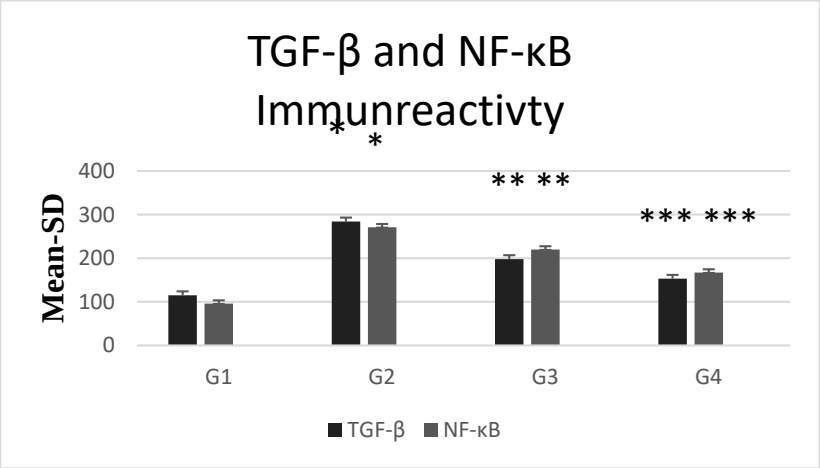

Chart 1. TGF-β and NF-κB Immunoreactivty of control and all experiment groups.
*p<0.0001 compared to the control group, **p<0.001 compared to the control group, ***p<0.05 compared to the control group. All experiment groups statistically significant difference were determined.

DISCUSSION

In parallel with the increase in the world population, the need for food is also increasing. This increasing need should be met with high nutritional values, quality and sustainable sources, and should be reliable in terms of food-borne risks. These criteria have increased the demand for seafood consumption, making it the fastest growing food industry in the world. Today, most of the seafood is still obtained from the sea by hunting. This is one of the oldest food sources in the diets of people without changing the way of consumption. Increasing environmental pollution in our country as in the whole world; In addition to suppressing the balance of life primarily, it also creates negative effects on living things with its elements entering the food chain[22]. We have reported histopathological changes in the rat kidney tissue in experimental modeling, based on the fact that sea crustaceans, which are among the important nutritional sources and which are among the top in terms of both economic and nutritional value, are obtained from contaminated environments.

Mytilus sp. are systematically used as preventive organisms to evaluate the side effects of the pharmaceuticals. Particularly, biological responses of pressurized water ecosystems to different types of organic, inorganic and pollutants are widely performed in the tissues and cells of mussels with using standard cellular, biochemical and molecular biological methods. Among the mussel cells, hemocytes are increasingly used as a non-destructive tool to predict their effects, both in field and laboratory studies. In addition to their ability to modulate specific biochemical pathways, usually due to the onset of various damage forms, they have great sensitivity and reproducibility[23-25]. In the experimental nutrition model, rats were fed with mussels that correspond to 15% of their weight, and the changes in the kidney tissue of the toxic products were examined that passed from the crustaceans to the rats through the food chain. Because mussels were directly exposed to various toxic substances in the previously designed models, and the results were reported.

In general, toxic substances and drugs used in the environment can cause serious damage to different organs of the body[26]. The sensitivity of organs to toxicity depends on several factors. The drug is defined as nephrotoxicity due to kidney disease or

dysfunction resulting from exposure to industrial and environmental chemicals[27]. The mechanism of kidney cell damage caused by drugs and chemicals is not exactly understood. The kidney is more sensitive to ischemic or toxic chemicals compared to other organs. Because the kidney has an important role in the conversion of chemicals into toxic reagents. The toxic substances formed cause oxidative damage by binding to cell macromolecules or by forming free radicals[28]. The kidney has a vital tissue integrity as an important urinary organ. It is also the organ that has the most interlocutor in toxic products taken into the body. Chemical and heavy metal analysis was not performed in the mussels collected because of the time and budget constraints for the study. However, by determining the pollution in the locations determined recently, it was determined which heavy metals increased in which season. Because, chemical products that are exposed to food, even with nutrition, and which can accumulate in the tissue such as heavy metals and cannot be thrown away for a long time can cause acute or chronic kidney disorders. In the results we observed based on long-term and frequent consumption of mussels, serious inflammatory inflammation and tissue damage were observed, especially in the functional units of the kidney. There fore more care should be taken in the consumption of shellfish, which have a feeding feature, and the products collected from healthy environments should be preferred.

In the researches, the presence of many heavy metals in single and bivalve seafood grown in the Dardanelles was determined[16]. 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. In heavy metal detection studies in the Mediterranean, trace elements such as cadmium, iron and copper accumulate in the bivalve[29]. In the study of Üstünada et al., *C. fragile subsp.* The values of the metals determined to accumulate in the fragile taxon, starting from the highest, are Cu, Zn, Pb and Cd, respectively. Again in this study, the savings were evaluated according to the stations and the highest values were determined as Eceabat, Cu in summer, Zn in Yenikordon in winter, Pb in spring and Cd in Gelibolu in spring[30]. As with most studies, the design of the current study is subject to limitations. The Dardanelles Strait covers a very long area and the association of mussels collected from the determined regions with the throat pollution obviously forced us a bit. However, we tried to minimize this limitation by taking previous study data as a reference. It is estimated that the damage caused by the mussels collected from the same region (from the Dardanelles) and fed as a nutrient to rats in the rat kidney tissue is due to the accumulation of heavy metal and toxic substances reported in these studies. Although the work has been carried out at different time intervals, the ship transitions in the Bosphorus continue to increase and sea pollution is increasing day by day[31].

In studies that have been exposed to heavy metals such as lead and copper for a long time and then analyzed by taking various tissues with mussels, it has been reported that these creatures accumulate these heavy metals in many tissues, including muscle, excretory or even genital organs[33,33]. In another experimental study, it was reported that atherosclerosis table develops in the vessels due to lipid accumulation in rats fed with mussels grown in contaminated environments[15]. It is seen that there is a parallelism between the information presented in these studies and our study results. Because the nutritional forms of the shellfish are filtering, the toxic products they are exposed to accumulate in their tissues and cause damage. Therefore, with the consumption of these creatures, these harmful substances can be transferred to the next step in the food chain, thus increasing the damage size.

In the study of the determination of the Cu/Zn SOD level in tissues with fish, the dynamic expressions of Cu/Zn SOD and Mn-SOD are generally increased in the liver, gill, kidney and spleen, mRNA expressions are arranged downward at onetime point in the kidney. Enzyme activities have been reported to increase after the A. hydrophila or liposaccharide challenge compared to control[34]. Immunohistochemical methods are an important method showing where and at what size tissue damage occurs. Since there are not many experimental studies with mussels, our findings are compared with the results of the study of liver and stomach tissues of these creatures. Stimulation of cell death in the absence of NF-κB activation is largely dependent on a prolonged activation of JNK[35]. In previous studies, as in many other tissues, it provides the dynamism necessary for cell renewal by controlling the genes that regulate the NF-κB proinflammatory and anti-apoptotic mechanism against tissues such as oxidative stress and other chemical stimuli in tissues such as kidney and liver. Changes in SOD activity in living organisms occur

due to oxidative stress factors, and such reactions occur to maintain tissue integrity[34]. We had little chance of comparing and criticizing our findings, as there is limited study data about the experimental model we have made. However, the data we have obtained show that the products consumed should be controlled in terms of health according to the increasing need for food and the rapid trend in the consumption of alternative products. And it will ensure that maximum attention is paid to the consumption of products that have the potential to affect human health through this type of food chain. In addition, the necessity of such studies with a larger budget and longer term is certain. The chemicals that mussels are exposed to can also affect the groups of animals that feed on them[36]. In our findings, NF-κB and TGF-β immunoreactivity due to mussel consumption was determined by severe staining in tubular epithelial cells every day.

In another study with mussels, iNOS immunoreactivity was found positive in the gastric mucosa of rats given mussel with the same amount and time[37]. For us, we stained with TGF-β and NF-kB, which are immune response markers for histopathological detection of kidney tissue damage, and the results showed a significant difference in the positivity of these antibodies in the kidney tissue of mussel fed rats every day. This, in turn, provided results that support the findings of inflammation, tubular degeneration and congestion that we achieved with routine staining. In particular, the integrity of the kidney tissue of rats fed with mussels every day has deteriorated too much.

This study results have actually confirmed the parameter results that are encountered in many regions and countries, and which vary 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 kidney. While searching for an alternative food source, environmental factors should not be ignored and it should be paid attention to consumption of clean and healthy products since it causes tissue damage in many systems, especially in the digestive and excretory system.

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