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Investigation of the Effect of Acrylamide on *Capoeta Capoeta* (Guldensttead 1773) by Histopathological, Electrophoretic and Biochemical Methods

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Abstract: The aim of this study was to investigate the effects of acrylamide on *Capoeta capoeta* (Guldensttead 1773) by histopathological, electrophoretic and biochemical methods. *Capoeta capoeta* caught from Kars stream were used in the study. The fish were divided into 5 groups, each containing 10 pieces, and placed in 300 liter tanks with tap water. Group 1 was kept as a negative control. 20 mg / kg cyclophosphamide given to group 2 (i.p. positive control group), 10 mg/L acrylamide given to group 3, 20 mg/L acrylamide given to group 4 and 30 mg/L acrylamide given to group 5. After all groups were kept in tanks for 4 days, blood and tissue samples taken from fish were investigated by histopathological, electrophoretic and biochemical methods. As a result of the analyzes serum AST and ALT levels were decreased in the other groups compared to the negative control group and serum TAS levels were significantly increased in the 30 mg/L acrylamide group compared to the negative control group ($P < 0.01$). Compared with the negative control group, TOS levels were increased in all groups. When the electropherogram obtained from SDS-PAGE was examined, it was determined increases and decreases at 21 kD, 27 kD, 36 kD, 42 kD, 48 kD, 54 kD protein expressions in groups with different concentrations compared to the negative control group. It was observed that protein expressions were inhibited especially in the group treated with 20 mg/L acrylamide. As a result of histopathological examinations; increased degenerations were detected in the gill and liver tissues of fish due to the concentration of acrylamide. As a result; acrylamide treatment caused toxic effects on *C. capoeta* after this varying time intervals and concentrations.

Keywords: Acrylamide, *Capoeta capoeta*, fish, Protein expression, AST, ALT, Histopathology.

1. INTRODUCTION

Acrylamide is crystalline, colorless, odorless, and soluble in water, acetone, methanol, and ethanol. The open molecule formula is C_3H_5NO (CH_2 = CH-$CONH_2$). Acrylamide is

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referred to as, propenamide, ethylene carboxamide, acrylic amide and vinyl amide and is used in polyacrylamide synthesis. Polyacrylamide is preferred in mineral, asphalt, paper production, crude oil processing, drinking and wastewater treatment, soil, and sand purification. It is also used in cosmetic industry, electrophoresis and molecular biology applications, photographic film and adhesive manufacturing, varnish and paint industry, and in the preparation of alloys in dentistry (Iarc, 1994; Europäische Kommission, 2002; Erdogan and Dastan, 2010).

When preparing foods, applications such as cooking, roasting and baking, temperature degrees ranging from 90-200 °C are used. These processes can lead to the formation of toxic compounds in foods. These toxic compounds include heterocyclic amines, polycyclic aromatic hydrocarbons, N-alkyl-N-nitrosamines and acrylamide (Claeys et al., 2005). Acrylamide is absorbed from the digestive tract and passed into the blood after oral administration. It disperses well in the body and crosses the barrier of placenta and passes into the fetus and milk (Tritscher, 2004).

Acrylamide is a substance that causes poisoning and increases the risk of cancer in humans. Acrylamide is in the group of suspected carcinogens in humans (2 / A). It causes cancer in laboratory animals given high concentrations orally. It has the potential carcinogenic in humans. In 2005, WHO and FAO suggested that some foods cooked at high temperatures contain significant levels of acrylamide and their consumption is risky for humans (FAO/WHO, 2012; Zhang et al., 2006).

The determination of acrylamide to be harmful to living things has necessitated a multidimensional approach. As a result of the literature reviews, no information was found about the histopathological, electrophoretic and biochemical effects of acrylamide on *Capoeta capoeta*. In this study it was aimed to investigate the effects of acrylamide on *Capoeta capoeta* by histopathological, electrophoretic and biochemical methods. In this study, the effects of higher levels of acrylamide than acceptable levels in aquatic environments will be revealed.

2. MATERIALS AND METHOD

2.1.Chemicals

Acrylamide and cyclophosphamide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2.Animals

* The study was conducted with permission from Kafkas University Animal Experiments Local Ethics Committee (KAU - HADYEK: 2014 / 023).

Fifty *Capoeta capoeta*, weighing 200-300 g caught from Kars Stream were used in the research. During the collection of fish, stream water's pH was 8.2-8.4, temperature was 16-18 and dissolved oxygen was 4.52-10.51 mg/L. Low-voltage shock and flip net were used to catch fish. After the fish were caught, they were placed in the water filled from this medium and oxygen-bonded drums. The fish brought to the laboratory were kept in aquariums with tap water for 10 days to adapt to the environment.

2.3. Experimental design

Fish were divided into groups, one containing 10 fish. Test concentrations were determined considering the lower and upper limit ranges used in previous studies (Petersen et al.,1987; Weideborg et al.,2001).

1. Group (n=10): Negative control group. Fish in this group were kept in a tank (300 L) with tap water and no treatment was performed.

2. Group (n=10): Positive control. The fish in this group were kept in a tank (300 L) with tap water and each fish was injected with 20 mg/kg cyclophosphamide intraperitoneally (i.p) (Grisolia et al., 2000).

3. Group (n=10): 10 mg/L concentration of acrylamide was added to 300-liter tanks containing tap water and the fish were kept in this environment.

4. Group (n=10): 20 mg/L concentration of acrylamide was added to 300-liter tanks containing tap water and the fish were kept in this environment.

5. Group (n=10): 30 mg/L concentration of acrylamide was added to 300-liter tanks containing tap water and the fish were kept in this environment.

The following investigations started after 4 days.

2.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Sds-Page) Method

Blood samples were taken from the dorsal veins of the fish and centrifuged at + 4 ℃ and 3000 rpm for 10 minutes to separate the serum and stored at - 20 ℃. Protein concentrations of serum samples were determined by the biuret method (Eisenthal et al., 1992) and SDS-PAGE was performed according to Laemmli and O'Farrell methods

(Laemmli, 1970; O'Farrell,1975). GangNam-Stain ™ Prestained Protein Ladder protein marker was used as the protein standard for electrophoresis process.

2.5. Biochemical Analysis Method

Serum aspartate aminotransferase (AST) (EnzyChrom™ Aspartate Transaminase Assay Kit, USA), alanine aminotransferase (ALT), (EnzyChrom™ Alanine Transaminase Assay Kit, USA), total antioxidant status (TAS) and total oxidant status (TOS) Assay kit (Rel Assay Diagnostics, Clinical Chemistry Solutions, Gaziantep, Turkey) levels, determined spectrophotometrically by the commercial kit (Erel, 2004, Erel, 2005).

2.6. Histopathological Method

Tissue samples taken from the specimens were fixed in 10% formaldehyde and then preparations were prepared by histopathological methods. These preparations were stained according to the hematoxylin-eosin staining method and examined under a light microscope (Luna, 1968).

2.7. Statistical Analysis Method

SPSS 22 package program was used for statistical evaluation of the data obtained from the study. One-way ANOVA was used for statistical analysis of the difference between group data. $P < 0.05$ was considered statistically significant.

3. RESULTS

There were no deaths in the control and experimental groups and no behavioral changes were observed in the samples.

3.1. Electrophoretic and Biochemical Analyzes

Serum AST enzyme levels were significantly decreased in animals treated with 30 mg/L acrylamide and 20 mg / kg cyclophosphamide compared to the negative control group ($P < 0.001$). It was determined that ALT enzyme levels were statistically decreased significantly in all experimental groups compared to the negative control group ($P < 0.001$). The TAS levels of the animals were found to be increased ($P < 0.01$) in the 30 mg/L administered group compared to the negative control group, whereas the increases and decreases in the other groups were not statistically significant. TOS levels were found to be increased in animals in all groups compared to the negative control group. The increase in 20 mg/L acrylamide group was statistically significant ($P < 0.001$) (Table 1).

Table 1: AST, ALT, TAS and TOS levels of the control group and animals treated with different concentrations of acrylamide.

	Control	10 mg/L	20 mg/L	30 mg/L	Cyclophos-phamide	P value
AST (ng/mL)	155 ± 18.3^{a}	134 ± 30.8^{a}	135 ± 22.5^{a}	60 ± 19.7^{b}	60 ± 16.5^{b}	0.001
ALT (ng/mL)	72.8 ± 14.9^{a}	38.5 ± 7.6^{b}	32.9 ± 11.2^{b}	31.1 ± 14.2^{b}	31.4 ± 17.6^{b}	0.001
TAS (mmol Trolox eq/L	4.4 ± 1.0^{bc}	3.5 ± 0.6^{c}	4.9 ± 1.8^{abc}	6.6 ± 1.3^{a}	6.0 ± 2.0^{ab}	0.001
TOS (µmol H_2O_2 eq/L)	28 ± 13.5^{c}	124 ± 25.2^{b}	168 ± 27.0^{a}	116 ± 24.8^{b}	87 ± 20.9^{b}	0.001

* Different letters on the same line indicate statistical significance.

When the electrophoregram obtained from SDS-PAGE was examined, it was found that there were variable increases and decreases in the protein expressions of 21 kD, 27 kD, 36 kD, 42 kD, 48 kD, and 54 kD in acrylamide groups compared to control groups. In particular, protein expressions were inhibited in the group treated with 20 mg/L acrylamide (Figure 1).

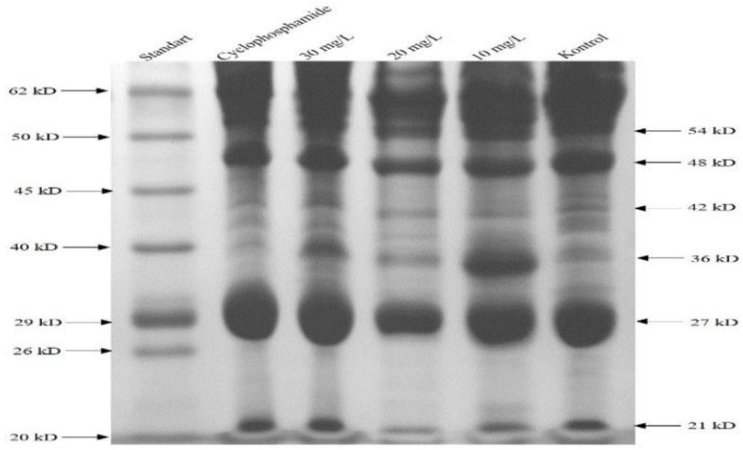
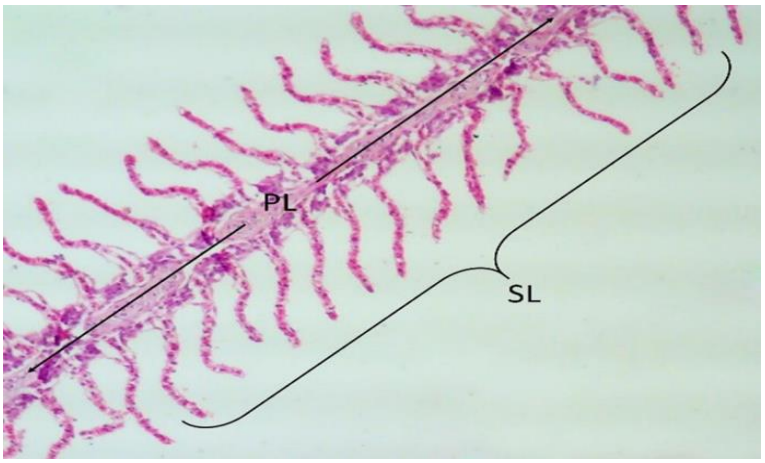


Figure 1: SDS-PAGE electropherogram of the control group and experimental groups.

3.2. Histopathological Analysis

3.2.1. Gills

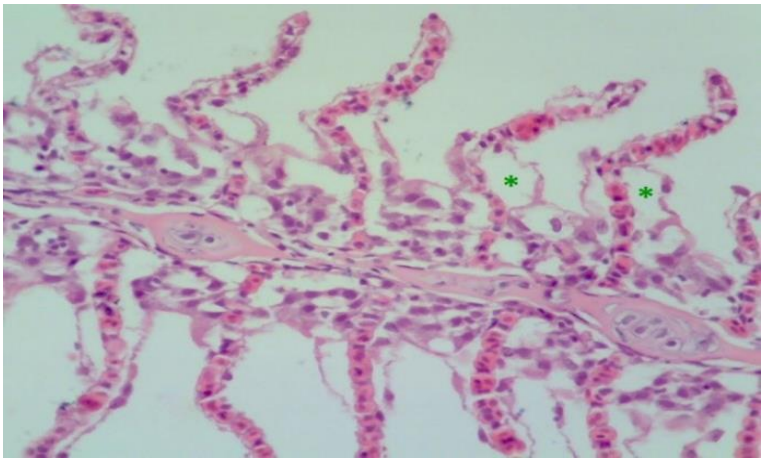
In the *Capoeta capoeta* control group, it was observed that the gill structure consisted of the secondary lamellae, which generally originated from the primary lamellae forming the main axis, and the epithelial covering surrounding the lamellae structure (Figure 2).



PL: primary lamellae, SL: secondary lamellae (H&E).

Figure 2. *Capoeta capoeta* control group, gill histology.

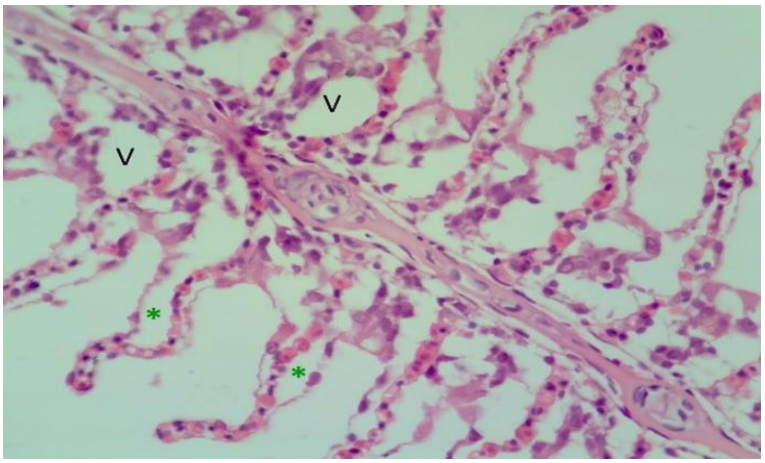
In the 10 mg/L concentrations acrylamide group, the gill structure was similar to the negative control group, but mild edema was detected (Figure 3).



*: edema (H&E).

Figure 3. *C. capoeta* low concentration group, gill histology

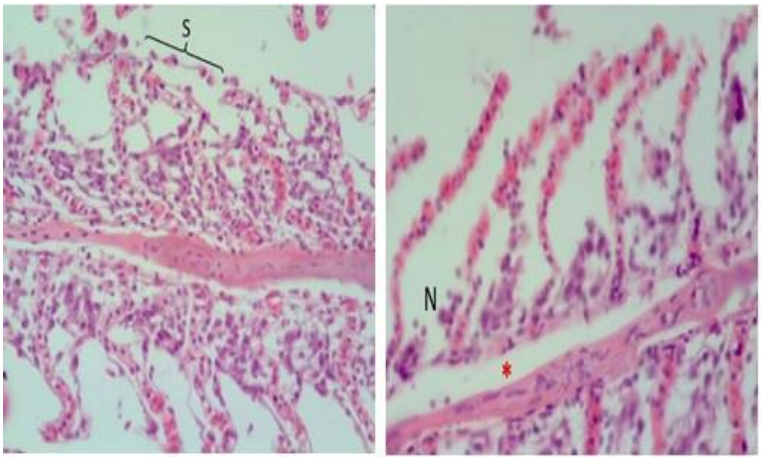
Unlike those exposed to acrylamide at a concentration of 10 mg/L, distinctive vacuolization was detected in the gills of fish given 20 mg/L acrylamide. In addition, edema was detected in the gills (Figure 4).



*: edema, V: vacuolization (H&E).

Figure 4. *C. capoeta* medium concentration group, gill histology.

In groups exposed to concentrations of 30 mg/L of acrylamide, the general appearance of secondary lamellae in the gill structure was irregular and epithelial separation was also determined. The significant separation was observed between the main axis of the cartilage of the primary lamellae and the epithelial layer. The presence of significant necrosis and deterioration of overall tissue integrity were detected in the epithelial layer covering the primary lamellae (Figures 5).



S: epithelial separation; *: separation, N: necrosis (H&E).

Figure. 5. *C. capoeta* high concentration group gill histology.

3.2.2. Liver

According to the observations in the negative control group, there was no lobular arrangement around the central vein in the *C. capoeta* liver parenchyma and series of hepatocytes lined around the central vein were seen with very thin sinusoids and bile ducts

located between them. Polygonal-shaped and large nucleus hepatocytes, which are the main cells of the parenchyma, were found to form fairly smooth cords (Figures 6a-b).

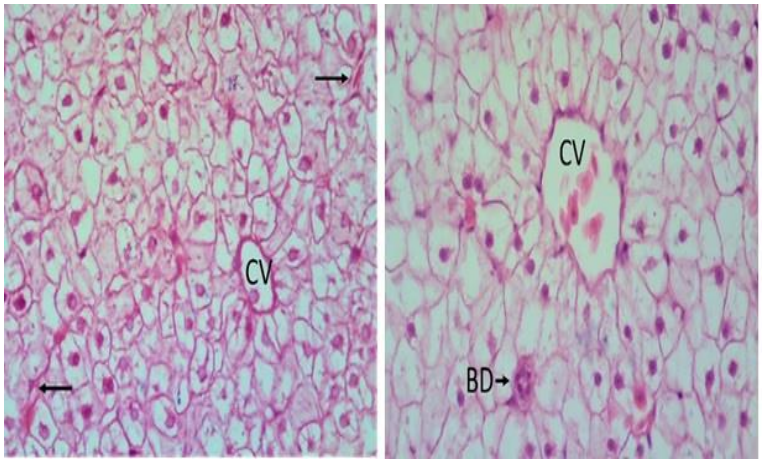


Figure 6a. *Capoeta capoeta* control group, liver histology. CV: central vein, arrows: sinusoids, **6b.** CV: central vein, BD: bile duct (H&E).

In the low concentration group (10 mg/L acrylamide) fish liver histology, sinusoid and central vein vasodilatation and increase in the number of erythrocytes, as well as the increased number of melanomacrophages to form clusters in the parenchyma was found to be the most prominent change (Figure 7).

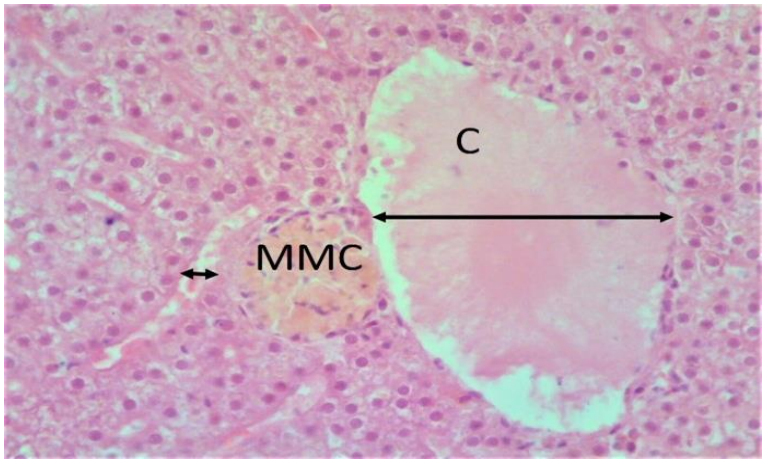


Figure 7. *C. capoeta* low concentration group, liver histology. ↔: vasodilatation, C: congestion, MMC: melanomacrophage cluster (H&E).

Acrylamide exposure to moderate concentration (20 mg/L acrylamide) revealed necrosis in addition to nucleic anomalies such as the pyknotic nucleus, karyorrhexic nucleus and karyolytic nucleus in hepatocytes in the liver parenchyma (Figures 8a-b).

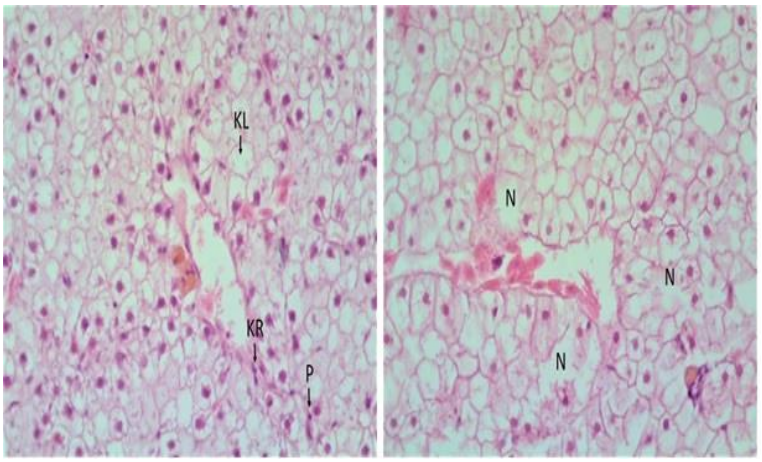


Figure 8a. *C. capoeta* medium concentration group, liver histology. P: pyknotic nucleus, KR: karyorrhexic nucleus, KL: karyolytic nucleus, **8b.** N: necrosis (H&E).

In the high concentration group (30 mg/L acrylamide), the incidence of necrosis in the liver parenchyma was found to increase and create large voids. Fibrosis was observed in hepatocytes within the parenchyma. Also, blood leakage into the parenchyma was detected due to deformations in the vessel walls (Figures 9a-b).

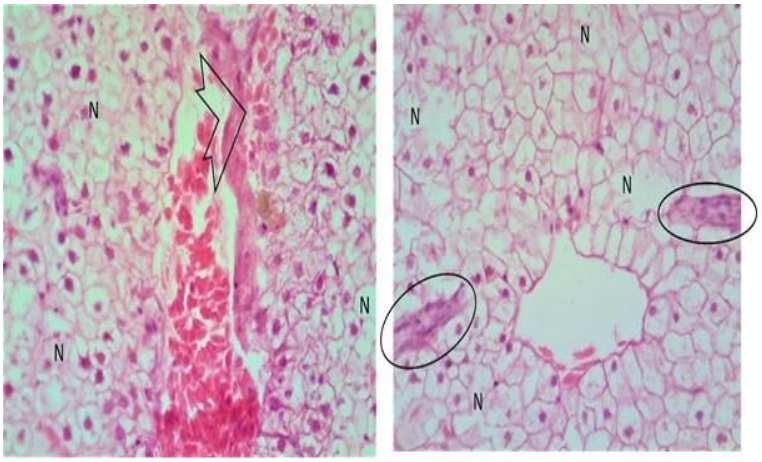


Figure 9a. *C. capoeta* high concentration group, liver histology.
⇨ : blood leakage into the parenchyma, N: necrosis, **9b.** Ellipse: fibrosis, N: necrosis (H&E).

4. DISCUSSION AND CONCLUSION

Experimental studies on various animals have shown that acrylamide is carcinogen and mutagen. Acylamide is also a possible carcinogen and mutagen in humans (Besaratinia et al., 2005).

Polyacrylamide is used in the production of paper, the composition of cosmetic products and the molecular biology laboratory (Manière et al., 2005). It was determined that acrylamide formed

spontaneously during high-temperature cooking of carbohydrate-rich foods (Stadler et al., 2002). The fact that it is taken into the body in various proportions by the food has increased the interest in acrylamide. In a study, glycidamide, considered to be the main metabolite of acrylamide, has been reported to have more genotoxic effects than acrylamide (Martins et al., 2006).

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) are two liver enzymes involved in glyconeogenesis and amino acid metabolism (Zareei et al., 2017). In particular, skeletal muscle, cardiac muscle, red blood cell, and liver cells carry high concentrations of AST enzyme. ALT is an enzyme found in primarily hepatocyte cytoplasm (Mayer et al., 2013). In the present study, it was determined that AST and ALT enzyme levels decreased in acrylamide treated fish. In fact, it is reported that the levels of these enzymes increase due to cell damage and toxicity due to the treatment of acrylamide (Nagi et al., 2014). HepG2 cells, a hepatocellular carcinoma cell line that maintains many functional properties of normal human hepatocytes, play an important role in determining human hepatocyte function (Mavri-Damelin et al., 2007). It has been reported that intracellular AST activity is inhibited as a result of changes in the structure of these cells due to the introduction of copper complexes into HepG2 cells (Jia et al., 2017). It has been suggested that the decrease in the level of aminotransferase due to acrylamide administration may result from the changes caused by the negative effect of acrylamide on HepG2 cells and/or excessive use of AST and ALT in meeting the energy need in the metabolic struggle against acrylamide toxicity (Larguinho et al., 2014a).

In toxicology studies conducted on different fish species, it was stated that the antioxidant level generally decreased and the oxidant level increased depending on the fish species and the substance applied. Depending on the glyphosate administration on Oncorhynchus mykiss (Nur and Deveci, 2018), on Cyprinus carpio administered with 2 mg / L and 3 mg / L tebuconazole, in the 3 mg / L group (Kaya et al., 2014), on Capoeta capoeta as a result of zinc sulfate application at a dose of 10 mg / L (Deveci et al.2015) and similarly on Capoeta capoeta administered with 0.01 mg / L and 0.02 mg / L glyphosate, in the group treated with 0.02 mg / L (Deveci et al., 2017) it is reported that the level of TAS decreases and the TOS level increases.

Experimental acrylamide toxicity studies reveal different results in terms of oxidative stress. It is stated that the reason for this is due to the different treatment ways and dosage of acrylamide (Çınar, 2010). Acrylamide treatment in rats was found to decrease antioxidant enzyme activity in the cerebral cortex and increase lipid peroxidation (Lakshmi et al., 2012). It was reported that there was no difference in MDA level compared to the control group, and GSH-Px antioxidant enzyme level decreased in rats (Çınar, 2010). It has been found that the treatment of acrylamide in rats increases the MDA level (El-Beltagi et al., 2016). It has been reported that glutathione S-transferase and superoxide dismutase activities increase in rats' plasma, liver, testis, brain, and kidney due to acrylamide treatment (El-Demerdash et al., 2006). It has been suggested that acrylamide causes an increase in MDA and GST enzyme activities in *Mytilus galloprovincialis* species (mussel) (Larguinho et al., 2014b). In this

study, an increase was observed in the TAS level of the animals in the group given only 30 mg/L of acrylamide, while an increase was found in the TOS level of the animals in all groups compared to the control group. It is reported that acrylamide can react with cellular nucleophiles having -SH, NH2 or -OH as well as reacting with GSH and forming glutathione S-conjugates (Awad et al., 1998). It is thought that the increase in TAS level due to acrylamide treatment may result from the formation of glutathione S-conjugates.

As a result of physiological stress, structural and functional changes occur in cellular proteins in living organisms (Shwetha et al., 2012). The change in protein fractions in the organism can be attributed to the deterioration of their structure or their possible overuse (Naveed et al., 2010). Proteins are used in the process of protecting the organism against oxidative stress caused by exposure to acrylamide. Acrylamide causes a significant decrease in the content of sulfhydryl groups and protein contents in different tissues (El-Demerdash et al., 2006). In this study, it was found that changes occurred in different protein expressions at different concentrations applied. An increase in 36 kD protein expression occurred in the group treated with 10 mg/L acrylamide, while protein expressions were inhibited in the group treated with 20 mg/L acrylamide.

Histopathological studies have shown that liver epithelial cells of rats are damaged significantly due to the treatment of acrylamide (Altinoz et al., 2015). Petersen et al. determined that acrylamide administration caused hyperplasia and metaplasia in the gills of the Rainbow trout (distal of secondary lamellae) (Petersen et al., 1987). The results of this research support the results of this study. In another study; Larguinho et al. treated concentrations of 1-10 mg/L of acrylamide to *Mytilus galloprovincialis.* They found that acrylamide has no histopathological effects on the gill (Larguinho et al., 2014b). In another study, it was reported that the liver parenchyma of *Carassius auratus* exposed to acrylamide is almost entirely composed of eosinophilic cells, and necrosis and focal hyperemia are seen) (Larguinho et al., 2014a). The results of these researches are in parallel with this study.

It can be said that the effects of acrylamide increase gradually depending on the concentration administered. This condition was detected in both the gill and liver tissues as a result of histopathological examinations. As a result, it can be suggested that acrylamide disrupts antioxidant/oxidant balance, affects liver enzyme activities and protein expression, and causes structural deterioration in gill and liver tissues in *Capoeta capoeta.*

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