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# Effects of Cyanide on Some Histological and Immunohistochemical Parameters of Common Carp (*Cyprinus carpio*)

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## ABSTRACT

In this study, Bcl 2 and Caspase 3 expressions and histomorphological changes were investigated in the liver, gill and skin tissues of carp (*Cyprinus carpio*) in which 0,1 mg/L and 0,2 mg/L concentrations of cyanide were added to their environment. It was determined that the lipid accumulation, lymphocyte infiltration, fibrosis and regeneration in the liver tissues; hyperplasia, cell aggregates and goblet cells in the skin epithelium and gill filaments of fish exposed to cyanide. As a result of the study, it was observed that Bcl-2 expressions decreased and caspase-3 expressions increased in all tissues of fish exposed to cyanide at concentrations of 0,1 mg/L and 0,2 mg/L. Changes in Bcl-2 and caspase-3 expression levels result in disruption of the apoptosis mechanism in the liver, gill and skin tissues. At the end of the study, it was concluded that the examined parameters were a good indicator for cyanide intoxication.

**Keywords:** *Cyprinus carpio*, cyanide, histology.

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**Sazan Balığında (*Cyprinus carpio*) Bazı Histolojik ve İmmunhistokimyasal Parametreleri Üzerine Siyanürün Etkileri**

**Öz:** Bu çalışmada bulundukları ortama 0,1 mg/L ve 0,2 mg/L konsantrasyonda siyanür eklenen sazan balıklarının (*Cyprinus carpio*) karaciğer, solungaç ve deri dokusu Bcl-2 ve kaspaz-3 ekspresyonları ve histomorfolojik açıdan değişimleri araştırılmıştır. Siyanüre maruz kalan balıkların karaciğer dokularında lipid birikimi, lenfosit infiltrasyonu, fibrosis ve rejenerasyon, deri epitelinde ve solungaç filamentlerinde ise hiperplazi, hücre agregatları ve goblet hücreleri tespit edilmiştir. Yapılan çalışma sonunda 0,1 mg/L ve 0,2 mg/L konsantrasyonlarda siyanüre maruz bırakılan sazan balıklarının incelenen tüm dokularında Bcl-2 ekspresyonlarının azaldığı, kaspaz-3 ekspresyonlarının ise arttığı görülmüştür. Bcl-2 ve kaspaz-3 ekspresyon düzeylerindeki değişimler ilgili dokulardaki apoptosis mekanizmasının bozulması sonucunu doğurmaktadır. Çalışma sonunda incelenen parametrelerin siyanür intoksikasyonu için iyi bir indikatör olduğu sonucuna ulaşılmıştır.

**Anahtar kelimeler:** *Cyprinus carpio,* siyanür, histoloji.

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## Introduction

Cyanide is an anion radical formed by the triple bond of a carbon and a nitrogen atom. Cyanide has both organic and inorganic compounds, the organic compounds are named as nitryl and has no toxic potential. But inorganic cyanide salts, like sodium cyanide and potassium cyanide have extremely high toxic potential. The most poisonous forms of cyanide are free cyanide (CN) and hydrogen cyanide (HCN) which is in gas form. Cyanide which is not carcinogen like heavy metals, causes sudden death when exposed to extremely overdose. In doses that do not result in death, it may leave permanent effects on metabolism.

Negative effects are seen on many animal species as a result of cyanide intoxication when industrially produced cyanide is transferred to environment. In literature the cited values are as

follows, on rabbits 30 min. $LC_{50}$ value is 188 ppm, on rats 60 min. $LC_{50}$ value is 143 ppm (Ballantyne 1983); on mouse 5 min. $LC_{50}$ value is 323 ppm (DiPasquale and Davis 1971). Of course, not all concentrations of cyanide are lethal in animals. Generally, 0.2 ppm is considered as the level in which toxic effect starts for animals (Sarı et al. 1999). But this concentration may cause lethal effect for aquatic organisms. It was reported in literature that even 0.01 ppm concentration causes lethal effect on fish (Blaha 1976).

Since cyanide can easily bond with metals, its use in gold and silver mining results in the damage of industrially produced cyanide to the aquatic environment. In aquatic environment, fish is the most affected living species by cyanide pollution. Fish is extremely vulnerable to pollutants since they take place in the last ring of food chain in aquatic environment. In literature, the lethal and toxic effects of cyanide for the fish has been widely studied. It is known that cyanide inhibits many enzyme systems of fish, destroys genetic structure, creates problems for their motive power and food intake (David and Kartheek 2016; David et al. 2008; Prashant 2011). Above all, this study will be the first about the effect of cyanide on histopathologic parameters of carps. From this point of view, in this study, taking into account the dangerous properties of cyanide, it was aimed to determine the toxic potential of cyanide in carp, a species with a wide ecological valence.

## Materials and Methods

### Test Environment, Fish Nutrition and Anesthesia

In this study sump systems, consisting of 5 aquariums whose dimensions are 30x40x60 cm, were used. The bottom aquarium in the system was used as a cleaning tank and there were no fish in it. The temperature of the aquariums was set to 22°C, and oxygen ratio of the water was never less than 6 mg/L. The fish used in this study were caught using tunnel net from the lake in Kütahya Dumlupınar University and then they were placed in aquarium units in the Kütahya Dumlupınar University Faculty of Arts and Sciences, Biology Department. After 15 days of acclimation period they were measured for their length and weight and placed in the aquarium randomly. 96 fish (48 for the 3-day experiment and 48 for the 15-day experiment) in total were used in the experiment. For each experimental period, 16 fish were determined as the control group and cyanide was not applied to these fish. As a source of cyanide, sodium cyanide (NaCN) was preferred. After adding the cyanide, the experiment continued for 3 days and 15 days. The water quality in the aquariums has been kept at a level that will not adversely affect the health of the fish (Lloyd 1992). No fish died during the experiment. At the end of the experimental period, the fish were taken from the aquariums and transferred to the anesthesia pool. As an anesthetic, clove oil, which has fewer negative effects than other chemical anesthetics, was used at a concentration of 600 mg/L (Han et al. 2016).

### Histopathological analyses

Liver, gill and skin tissues dissected from fish were first fixed in 10% neutral formaldehyde solution for 48 hours for microscopic examination. Tissues taken from the formaldehyde solution were passed through increasing degrees of alcohol (%70, %80, %90, %100) for to remove water. Afterwards, it was passed through xylol for transparency and kept in molten paraffin so that it can be cut in the microtome device. The tissues which took the shape of block were cut in a microtome device in 4 microns thick. Staining procedure was applied to prepared samples.

The parts cut and prepared in microtome device were evaluated and their pictures were taken with Leica DCM 4000 (Germany) computer aided imaging system, Leica Q Vin 3 software. Degeneration criteria chart was formed as a result of analysis carried out with Hematoxylin Eosin (H&E) staining method.

H&E staining scoring was formed as below:

0: No change
+1: Slight tissue change
+2: Moderate tissue change
+3: Severe tissue change (Murussi et al. 2016; Poleksic and Mitrovic-Tutundzic 1994).

For immunohistochemical staining (Bcl-2, Caspase-3) Ab-7973 Abcam for Bcl-2 and RB-1197-P0 Thermo Scientific antibodies for Caspase-3 were used. DAB staining was performed as chromogen for to identify positive cells. Hematoxylin was applied for background staining for one minute. The stained sections were passed through the increasing alcohol series and after the water was removed, they were kept in xylol for 5 minutes to make them transparent and covered with entellan. To define expression ratio and apoptotic index, cell count was carried out in five independent different regions with 20x zoom lens.

### Statistical analysis

SPSS 22 software was used to interpret and evaluate the gathered data. Graphs were created by calculating the mean and standard errors. To reveal statistical difference among groups One-Way ANOVA was used, and since it was supposed that there was homogeneous distribution among groups, Tukey test was used. To demonstrate the difference between 3-day and 15-day period experiments, Student t-test was

used. Results were analyzed at P<0,05 significance level.

## Results

### Histomorphological analyzes

When the findings obtained from the study were examined, it was determined that the pathological score level in the liver, gill and skin tissues of the fish exposed to cyanide for three and fifteen days increased significantly (Table 1).

With cyanide exposure in the liver, lipid accumulation in hepatocytes, lymphocyte infiltration, fibrosis, regeneration and loss of hepatic cord structures were observed (Figure 1).

In the gill tissue of cyanide exposed fish, hypertrophic and hyperplastic lamellae were formed, hyperplasia was seen in lamellae and cell aggregate accumulated (Figure 2).

### Bcl – 2 expressions

In this study, Bcl-2 expression decreased significantly with the presence ofcyanide in all three tissues whose histopathological analyzes were performed (Table 2). In particular, a statistically significant decrease was observed in all tissues of *C. carpio* exposed to cyanide at a concentration of 0.2 mg/L (P<0,05).

### Caspase-3 expressions

It was observed that Caspase-3 expressions in liver, gill and skin tissues of the fish used in this study increased significantly with cyanide exposure (Table 3). At the sametime, there is a direct correlation between increasing cyanide concentration and Caspase-3 expressions.

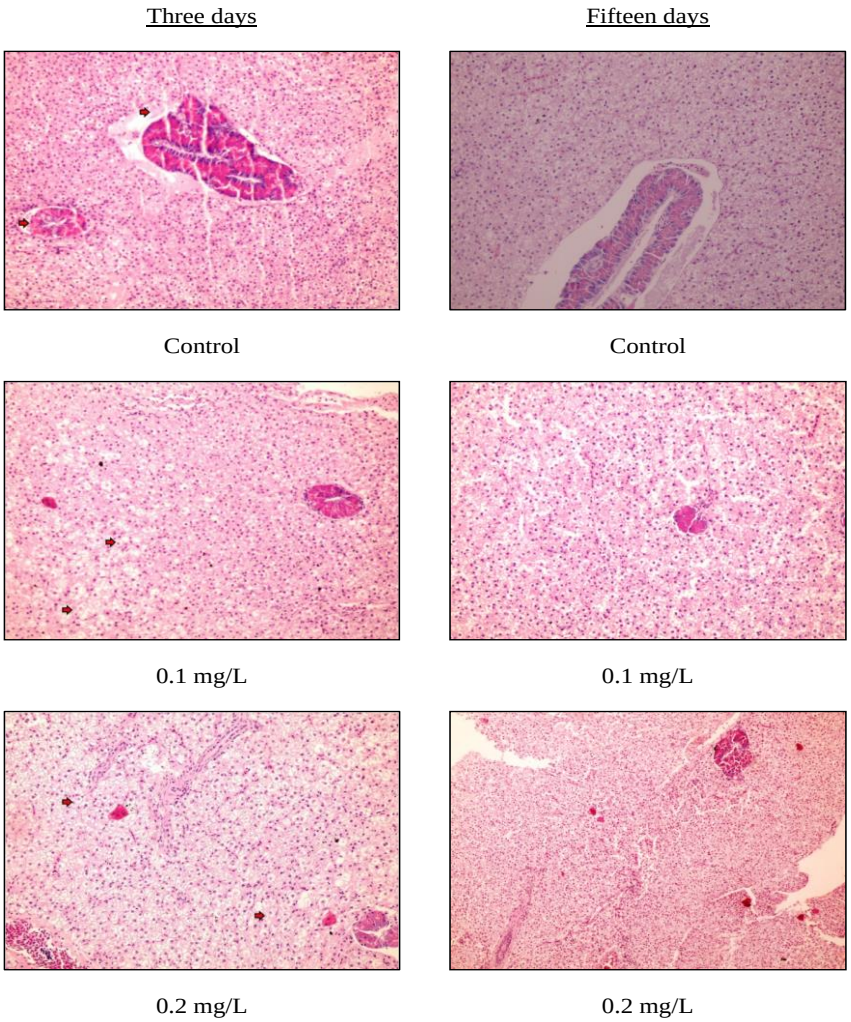


**Figure 1.** Histomorphological examinations of liver tissue (x200 magnification).

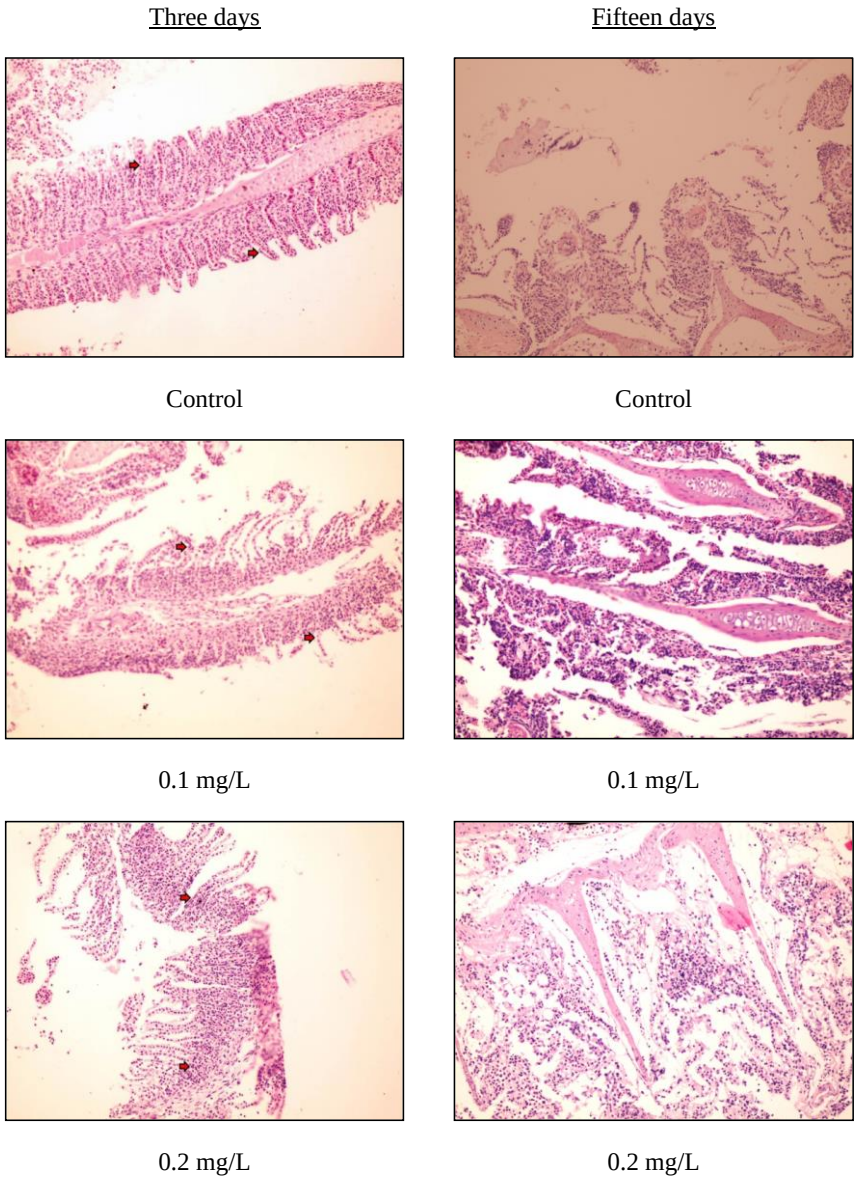


**Figure 2.** Histomorphological examinations of gill tissue (x200 magnification).

**Table 1.** H&E scores of fish used in the study

| | Hematoxylin & Eosin (score) | | | | | |
|---|---|---|---|---|---|---|
| | 3 Days | | | 15 Days | | |
| | C | 0.1 mg/L | 0.2 mg/L | C | 0.1 mg/L | 0.2 mg/L |
| Liver | 0.40±0.16$^{a}$ | 1.00±0.26$^{a}$ | 2.60±0.16$^{b}$ | 0.60±0.16$^{a}$ | 1.20±0.25$^{a}$ | 2.20±0.2$^{b}$ |
| Gill | 0$^{a}$ | 1.30±0.21$^{b}$ | 2.20±0.20$^{b}$ | 0.20±0.13$^{a}$ | 1.80±0.25$^{b}$ | 2.30±0.21$^{b}$ |
| Skin | 0$^{a}$ | 0.80±0.62$^{b}$ | 2.00±0.47$^{b}$ | 0.20±0.13$^{a}$ | 1.20±0.29$^{b}$ | 1.80±0.29$^{b}$ |

(C: Control; Values shown with different letters contain statistical significance)

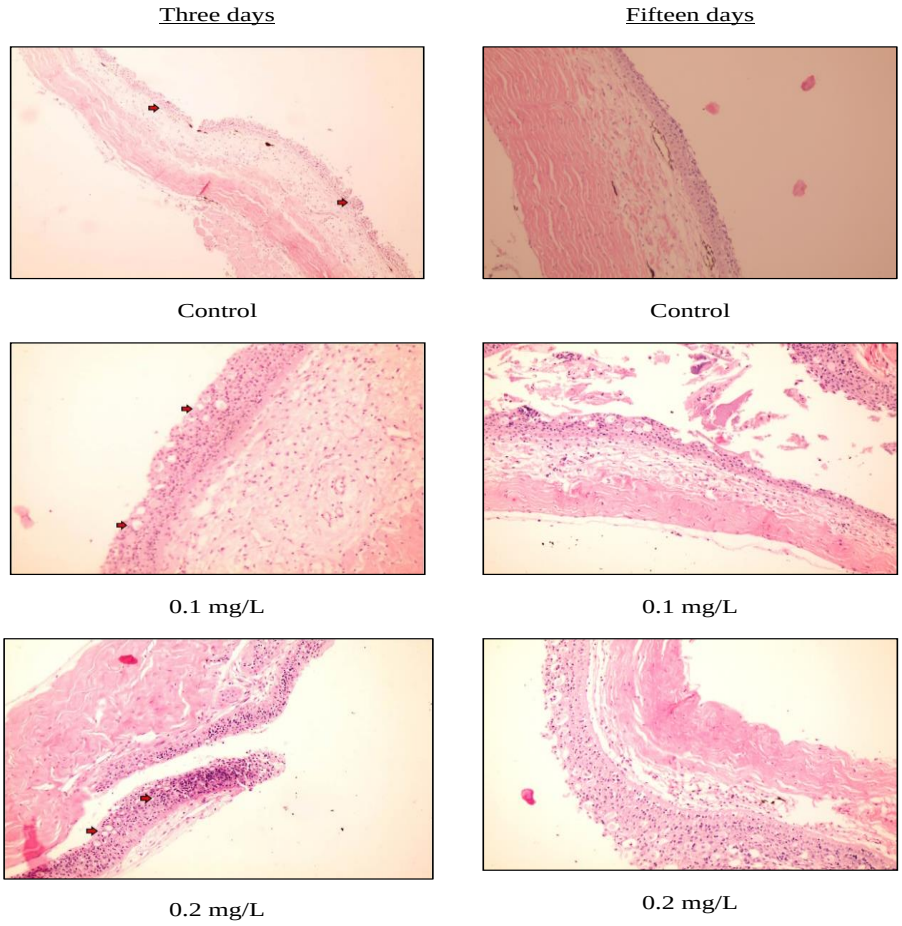


**Figure 3.** Histomorphological examinations of skin tissue (x200 magnification).

**Table 2.** Bcl – 2 expression percentages of fish used in the study

| | Bcl – 2 (%) | | | | | |
|---|---|---|---|---|---|---|
| | 3 Days | | | 15 Days | | |
| | C | 0.1 mg/L | 0.2 mg/L | C | 0.1 mg/L | 0.2 mg/L |
| Liver | 17.51±1.14$^{a}$ | 10.57±1.02$^{b}$ | 6.82±0.98$^{b}$ | 13.58±1.6$^{a}$ | 12.5±1.2$^{ab}$ | 5.99±0.66$^{b}$ |
| Gill | 25.95±2.84$^{a}$ | 22.74±3.08$^{ab}$ | 10.46±1.34$^{b}$ | 29.00±1.8$^{a}$ | 19.25±1.71$^{b}$ | 8.75±1.3$^{b}$ |
| Skin | 27.32±2.82$^{a}$ | 15.84±1.27$^{b}$ | 9.94±0.71$^{b}$ | 32.85±2.91$^{a}$ | 26.78±2.78$^{ab}$ | 13.44±1.84$^{b}$ |

(C: Control; Values shown with different letters contain statistical significance)

**Table 3.** Caspase - 3 expression percentages of fish used in the study

| | Caspase – 3 (%) | | | | | |
|---|---|---|---|---|---|---|
| | 3 Days | | | 15 Days | | |
| | C | 0.1 mg/L | 0.2 mg/L | C | 0.1 mg/L | 0.2 mg/L |
| Liver | 1.44±0.23$^{a}$ | 9.80±1.16$^{b}$ | 14.97±1.54$^{b}$ | 3.84±0.84$^{a}$ | 8.15±1.59$^{ab}$ | 15.21±1.79$^{b}$ |
| Gill | 8.00±1.01$^{a}$ | 11.28±1.68$^{ab}$ | 22.10±1.39$^{b}$ | 8.10±1.31$^{a}$ | 11.45±1.13$^{ab}$ | 27.35±1.51$^{b}$ |
| Skin | 7.22±1.4$^{a}$ | 14.12±1.73$^{b}$ | 19.5±1.4$^{c}$ | 8.18±1.23$^{a}$ | 18.54±1.15$^{b}$ | 22.81±1.47$^{b}$ |

(C: Control; Values shown with different letters contain statistical significance)

## DISCUSSION

As stated in the results, various changes were detected in all three tissues used in the studycompared to the control group. Hypertrophy is defined as the overgrowth of an organ or tissue, while hyperplasia is defined as the overdevelopment of the tissue by increasing the number of cells. David and Kartheek (2016), exposed *C. carpio* to 0,1 mg/L concentration of NaCN. They found similar pathologic findings as in our study like hyperplasia in liver tissue and

lymphocyte infiltration. Maceda-Veiga et al. (2013), reported that there was excessive metal accumulation in sewage contaminated water supply and this condition resulted negative effects like lymphocytic infiltration and lipid accumulation in liver histopathology of *Squalius laietanus*. Ben Ameur et al. (2012), reported that intense vacuolization, lipid accumulation in hepatocytes and necrosis in some parts of the tissue were detected in the livers of *Mugil cephalus* and *Dicentrarchus labrax*, which they caught from Bizerte Lagoon. Al-Ghanbusi et al. (2012), detected hypertrophy and hyperplasia in the gill tissues of *Aphanius dispar*, which they exposed to deltamethrin at different concentrations. At the same time, they found that some close secondary lamellae in the gills fused to each other with deltamethrin exposure. In an experiment with simazine exposure to *C. carpio*, hyperplasia was detected in the gill tissues. (Oropesa-Jimenez et al. 2005). Again, with the application of azadirachtin at different concentrations to *C. carpio*, an increase and hypertrophy of the epithelial cells of the secondary lamellae in the gill tissues were observed. In addition, hyperplasia and hypertrophy in mucus and chloride cells and aneurysm in the lamellae were detected (Murussi et al. 2016). Pathological alterations occurred in liver and gill tissues exposed to various chemicals are similar to the alterations occurred in gill tissues of fish exposed to cyanide in this study. At the same time, it shows that different chemicals effect on these organs extremely negatively. Pathological conditions such as hypertrophy and hyperplasia can be explained as the tissue overgrowth, creating a gap between the blood and the external environment, and trying to prevent the chemical from entering the metabolism (Cengiz 2006; Poleksic and Mitrovic-Tutundzic 1994). In the literature, it is seen that studies on the effects of cyanide on fish tissues are quite inadequate.

Bcl-2 is a protein found in mitochondrial external membrane which represses apoptosis. Bcl-2 inhibits caspase-mediated apoptosis pathway and necrosis which occurred as a result of oxidants and hypoxia (Green and John 1998; Arockiaraj et al. 2015). It is known that cyanide causes hypoxia and oxidative damage in living things. Therefore, it is important to know the expression of the Bcl-2 protein in the presence of cyanide. Vidal et al. (2008), carried out apoptosis dependent gene characterization of *C. carpio* and for the first time conducted the characterization of Bcl-2 protein. Cao et al. (2013) exposed *C. carpio* to fluoride for ninety days and observed the rate of apoptosis and Bcl-2 expression in the liver. It was reported that there was a positive correlation between fluoride exposure and increased apoptosis rate, and a negative correlation between Bcl-2 expression. (Cao et al. 2013). Yuan et al. (2016), identified and clarified Bcl-2 gene of *Ictalurus punctatus* for the first time and reported that in a condition of bacterial contamination and hypoxia Bcl-2 expression decreased. Studies show that Bcl-2 expression rates decrease in fish metabolism when exposed to any chemical or biological effects. This situation causes the acceleration of apoptosis in the tissue and accelerated apoptosis is a pathological condition. When the literature is examined, it can be said that there is no study on the relationship between cyanide exposure and Bcl-2 expression in fish tissues and this study is the first. Caspase-3 is an important apoptotic protease and enables apoptosis. The active Caspase-3 inhibits deoxyribonuclease enzyme and inactivates it and provides DNA fractionation. At the same time, it inhibits DNA repair and enables apoptosis irreversibly (Elvitigala et al. 2012). Jiang et al. (2015), exposed *C. carpio* var. Jian to copper and reported a significant increase of Caspase-3 expression in muscle tissue. Also reported that copper exposure increased apoptosis. Morcillo et al. (2016) investigated the expression rates of Caspase-3 in kidney and blood leukocytes after the treatment of *Sparus aurata* and *Dicentrarchus labrax* with various metals. As a result of their studies, it was determined that the Caspase-3 ratios of fish exposed to cadmium, mercury and arsenic concentrations at $EC_0$ and $EC_{50}$ ratios increased significantly. In the study, it was observed that lead also increased the expression of Caspase-3, but this situation was not statistically significant. The study reported increase of Caspase-3 expression resulted in increase of apoptosis (Morcillo et al. 2016). Kumaresan et al. (2016), defined caspase family in the tissues of *Channa striatus* and examined for the expression ratio of caspase family against bacterial and fungal threats. The highest expression of Caspase-3 in the studied species was detected in the spleen, a lymphoid organ. They reported that Caspase-3 expressions were significantly increased in bacterial and fungal threats, especially at 24 hours, compared to control groups (Kumaresan et al. 2016). When the results reported in the literature and obtained from this study are examined, it is concluded that Caspase-3 expressions increase when fish are exposed to a chemical or biological agent, and this may increase apoptosis in cells.

When all the data obtained from the study were examined, the following conclusions can be drawn:

It was determined that 0,1 mg/L and 0,2 mg/L concentrations of cyanide exposure caused various histomorphological differences in liver, gill, skin tissues of *C. carpio*, decreased Bcl-2 expressions and increased Caspase-3 expressions. These finding sheds light on the idea that studies at the cellular level should be done with electron microscopy in order to examine the damage caused by cyanide in more detail. It has been reported in the literature that histopathological parameters are suitable indicators for determining the toxic effects of pollutants on fish. In this study, it was observed that histopathological analyzes gave significant results.

In this study, *C. carpio* were used because they can live in all freshwater ecosystems and are resistant to pollutants. Considering the responses against pollutants may differ among living organisms and more vulnerable species may have negative effects with lower concentrations, so toxic effects of cyanide should also be investigated in different species. There is also a need to study the effects of different components of cyanide at different times.

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