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Research Article | **Araştırma Makalesi**

Histopathological Evaluation of Muscle Tissue of Horse Mackerel (*Trachurus trachurus*, Linnaeus, 1758) in Çanakkale Strait

Çanakkale Boğazındaki İstavritin (*Trachurus trachurus*, Linnaeus, 1758) Kas Dokusunun Histopatolojik Değerlendirilmesi

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Abstract: It is supported by studies that heavy metals and other water pollutants can reach humans through the food chain because of accumulation in organs of digestion, respiration, and muscles of fish, and may cause toxic effects depending on the amount of pollution and accumulation. This study was carried out by obtaining Horse mackerel samples from the Çanakkale Strait, which are fish species with high economic and nutritional value, as well as frequently consumed, seasonally (spring, summer, autumn, and winter). In the study, the tissues dissected from the dorsolateral muscles of the fresh fish were taken into Bouin's fixative, and sections were taken after routine histological follow-up. The findings revealed degenerative and inflammatory findings in the muscle tissues of fish caught especially in the autumn season. In addition, a statistically significant difference was found in autumn samples in terms of immunoreactivity ($p<0.05$).

Keywords

- Horse mackerel
- Çanakkale Strait
- Muscle tissue
- Histopathology

Özet: Ağır metaller ve diğer su kirleticilerin balıkların kas, sindirim, solunum sistemi organlarında birikmesi sonucu besin zinciri yoluyla insanlara ulaşabileceği, kirlilik ve birikim miktarına bağlı olarak toksik etkilere neden olabileceği çalışmalarla desteklenmektedir. Bu çalışma, Çanakkale Boğazı'ndan mevsimsel (ilkbahar, yaz, sonbahar ve kış) olarak avlanan, ekonomik ve besin değeri yüksek, ayrıca sık tüketilen bir balık türü olan istavrit örnekleri kullanılarak gerçekleştirilmiştir. Taze balığın dorsa-lateral kaslarından diseke edilen dokular fiksasyon için Bouin's fiksatifine konuldu ve rutin histolojik takipten sonra kesitler alındı. Bulgular, özellikle sonbahar mevsiminde yakalanan balıkların kas dokularında dejeneratif ve inflamatuar bulguları ortaya çıkardı. Ayrıca sonbahar örneklerinde immunoreaktivite açısından istatistiksel olarak anlamlı fark bulundu ($p<0.05$).

Anahtar kelimeler

- *Trachurus trachurus*
- Çanakkale Boğazı
- Kas dokusu
- Histopatoloji

1. INTRODUCTION

As a result of rapidly developing technological developments and industrialization, aquatic systems take their share from environmental pollution. Contamination of aquatic ecosystems by heavy metals and other pollutants has now become a global problem (Yılmaz, 2009). 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 resolved (Moore et al., 2006). Accumulation of heavy metals such as mercury, cadmium, and lead in the bodies of aquatic organisms, especially fish, can cause serious problems (Duruibe et al., 2007). Heavy metal toxicity can damage the lungs, kidneys, liver, and other vital organs, especially the nervous system (Tchounwou et al., 2002). Long-term exposure can affect the muscular and neurological process of targeted tissue damage (Thomas and Mohaideen, 2014). The aquatic ecosystem is very sensitive to pollutants such as heavy metals and the gradual increase in the levels of such metals in aquatic environments has become a primary concern. Especially fish are among the creatures that can be highly affected by heavy metals (Ayas et al., 2007).

Fish are indicator species for monitoring metal toxicity in water. Because heavy metal ions can accumulate in such creatures more easily compared to other foodstuffs (Igwilo et al., 2006). In addition, morphological, cytological, and histopathological changes occur in different organs of the body in response to water pollution (Deore and Wagh, 2012; Atli et al., 2015; Strzyzewska et al., 2016; Kaur et al., 2018). Heavy metals are directly associated with increased incidence of cancer, neuromuscular damage, reproductive defects, and hypersensitivity to various deadly diseases (Singla, 2015). This study was realized to show the horse mackerel (*Trachurus trachurus*) specimens caught seasonally from the Çanakkale Strait which is more affected by pollution and to what extent the damage encountered organs. In the findings obtained, results have been revealed on the possibility of long-term consumption of horse mackerel, which is hunted from the Çanakkale Strait and frequently consumed by humans, to cause health problems on humans through the food chain.

2. MATERIAL and METHODS

2.1. Histological methods

In the early hours of the morning, the fish that have just died from the pier where the fishing boats docked in the Çanakkale Strait were collected seasonally. A total of 40 fish, 10 for each season, were followed up for histological examination. The tissues dissected from the dorsa-lateral muscles of the fresh fish were taken into Bouin’s fixative and paraffin blocks were made after routine histological follow-up. Tissues were then fixed in Bouin's fixative for 24 hours. It was purified from water by passing through alcohol series and finally passed through xylene for transparency. For routine histopathological staining of tissues embedded in paraffin blocks, 4µ thick sections were taken in the microtome and routine Hematoxylin-Eosin (H&E) staining was performed (Çakına et al., 2021).

2.2. Immunohistochemical staining

Tissue samples were cut in a microtome with a thickness of 4 microns and taken into a water bath, and the tissue samples opened here were placed on special slides covered with Poly L-Lysine (Thermo Scientific) and adhered on a heating plate (Leica) at 40°C. All tissue samples were kept in an oven at 60°C for 1 hour, after dewaxing, they were passed through xylene twice and the paraffin was completely removed from the tissues, graded alcohols (absolute alcohol, 96% alcohol, 80% alcohol, 70% alcohol, 50% alcohol, 30% alcohol) tissue samples were both cleared of xylene and dewatered (dehydration). Tumor necrosis factor α (TNF-α), for inducible nitric oxide(iNOS) staining, is placed in heat-resistant plastic chalets containing citrate buffer (pH:6, dilution, 1:9) solution, 40 min. It was kept in a water bath (core) at 95°C for a period. Thus, the formaldehyde and paraffin used for the fixation of the tissues were completely cleaned from the tissue samples. Afterward, serum blocking solution (LAB-SA Detection System, Histostain-Plus Bulk Kit; solution A, Invitrogen) was added and left for 30 min. kept for a period. TNF-α (Ab1793) and iNOS (Ab15323) primary antibodies were administered. This procedure was done separately for each antibody. Tissue samples on which primary antibodies were added were incubated for 1 hour in a 37˚C oven. Then Seconder antibody solution (LAB-SA Detection System, Histostain-Plus Bulk Kit; A solution, Invitrogen) was added and 30 min. kept for a period. Then, enzyme conjugate solution (LAB-SA Detection System, Histostain-Plus Bulk Kit; A solution, Invitrogen) was added and 40 min. has been pending. 3,3´-Diaminobenzidene tetrahydrochloride (DAB, Invitrogen Corporation) solution as a chromogen for 5 minutes. After it was kept in the dark, it was kept in Mayer's Hematoxylin for 5 minutes for counter-staining and tap water for 10 minutes. has been washed. Finally, it was covered with a coverslip using entellan (Bio Mount, Bio-Optica) (Numata et al., 2013; Öztürk et al., 2019).

2.3. TUNEL assay

Terminal Transferase dUTP Nick End Labeling (TUNEL, ApopTag® Plus Peroxidase In Situ Apoptosis Kit) method, which allows staining apoptotic cells, was used to determine cell death. Sections were taken after deparaffinization were washed first in distilled water and then with PBS solution for 3x5 minutes. Then 20-µg/ml Proteinase-K diluted 1/500 with PBS solution for 15 minutes at room temperature. has been applied. 5 min after washing with PBS. After being treated with 3% H_2O_2, 3x5 min. washed with PBS. Samples were incubated with Equilibration buffer for 5 minutes at room temperature. After keeping it in a humid environment with TdT-enzyme at 37˚C for 1 hour, it is kept with Stop Wash Buffer for 10 minutes. and then 30 min with Antidioxygenin Peroxidase Conjugate. treated samples 3x5 min. washed with PBS. Afterward, dyeing was done with DAB and background staining was done with Mayer's Hematoxylin. TUNEL positive cells were detected by the blind method and the averages were evaluated statistically (Öztürk et al., 2019).

2.4. Evaluation of tissue samples and statistics

Five of the sections taken from the blocks containing the dorsal muscle tissues of all fish were stained. All stained tissue samples were evaluated under Zeiss AXIO Scope 1 brand research microscope and photographed with a digital camera (AxioCam ICc 3). TNF-α and iNOS immunoreactive cells were detected using the Leica LAS V3.8 image analysis system. Staining rate semiquantitative; 0 if less than 1% of cells stain; 1+ if 1-10% of cells have staining; 2+ if 11-50% of cells have staining; 3+ if 51-80% of cells have staining; It was evaluated as 4+ if more than 80% of the cells had staining. Also, staining intensity 0=no staining; 1=pale; 2=moderate; 3=intensively determined by the blind method. Then, the total score was calculated with the formula "(1+staining intensity/3) x staining rate" (Numata et al., 2013). The resulting data were compared with the One Way-ANOVA Tukey statistical test, and $p<0.05$ results were considered statistically significant.

3. RESULTS

Degenerative and inflammatory findings were observed in horse mackerel muscle tissues stained with H&E, especially in the autumn season. Polymorphonuclear leukocyte infiltration and necrotic muscle fibrils were determined to be common among the muscle fascicles of horse mackerel specimens caught in the autumn season. In other seasons, the histopathological picture is mild, and the damage size is the lowest in the winter season (Figure 1).

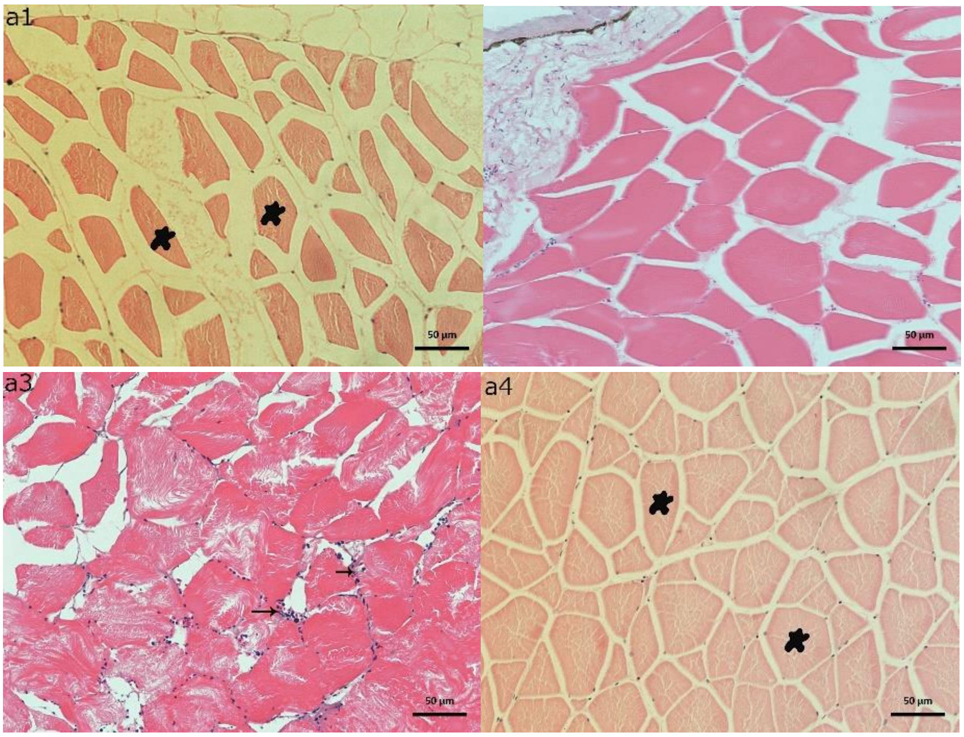

Figure 1. a1- Spring sample muscle tissue, transverse section, **a2-** Summer sample muscle tissue, longitudinal section, **a3-** Autumn sample muscle tissue, transverse section, **a4-** Winter sample muscle tissue, transverse section, H&E staining (arrow: polymorphonuclear leukocyte infiltration, star: muscle fascicle), 50 µm.

3.1. Immunohistochemical Findings

NO is a free radical with a very short half-life, which has been recognized in recent years and has proven to play an important role in many biological events such as smooth muscle relaxation, platelet aggregation, and neuronal impulse transmission. Determination of oxidative stress due to heavy metal accumulation in fish muscle tissue and its expression in muscle tissue damage were determined. It was determined that iNOS immunoreactivity was quite severe in the muscle tissue of horse mackerel in the autumn season. The severity of iNOS reactivity in the muscle tissue of the samples in other seasons was determined to be mild. There was a significant seasonal difference in tissue immunoreactivity ($p<0.05$) (Figure 2, Figure 4).

TNF-α immunoreactivity was found to be quite severe in the muscle tissue of horse mackerel specimens in the autumn season. The severity of TNF-α reactivity in the muscle tissue of the samples in other seasons was determined to be mild. There was a significant seasonal difference in tissue immunoreactivity ($p<0.05$) (Figure 3, Figure 4).

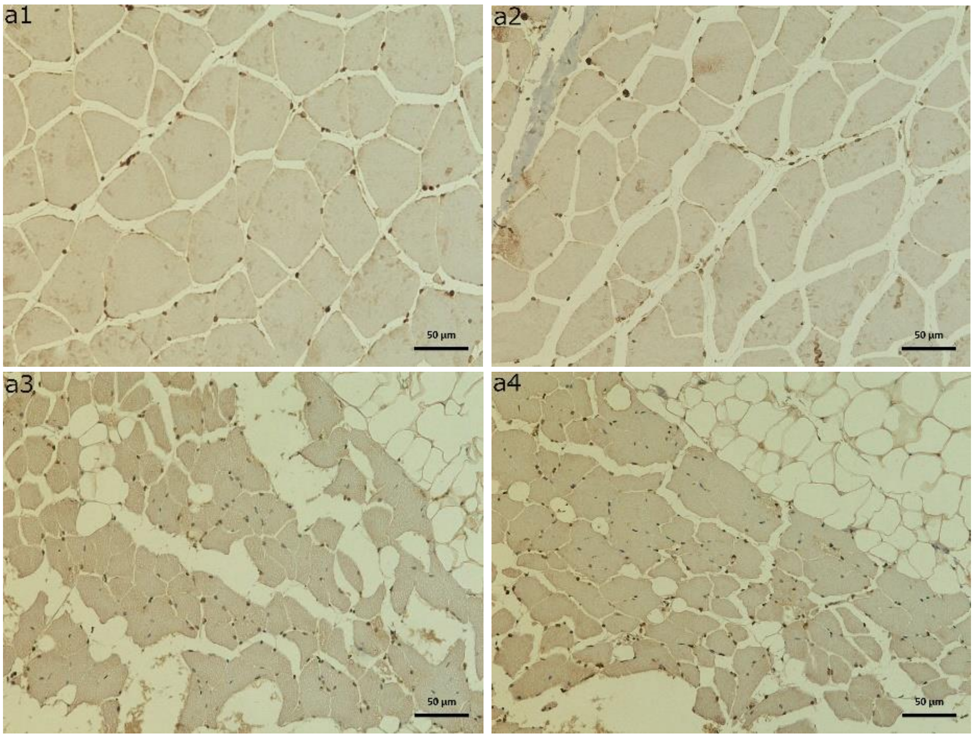

Figure 2. a1- Spring sample muscle tissue, **a2-** Summer sample muscle tissue, **a3-** Autumn sample muscle tissue, **a4-** Winter sample muscle tissue, **iNOS** reactivity, transverse section, 50 µm.

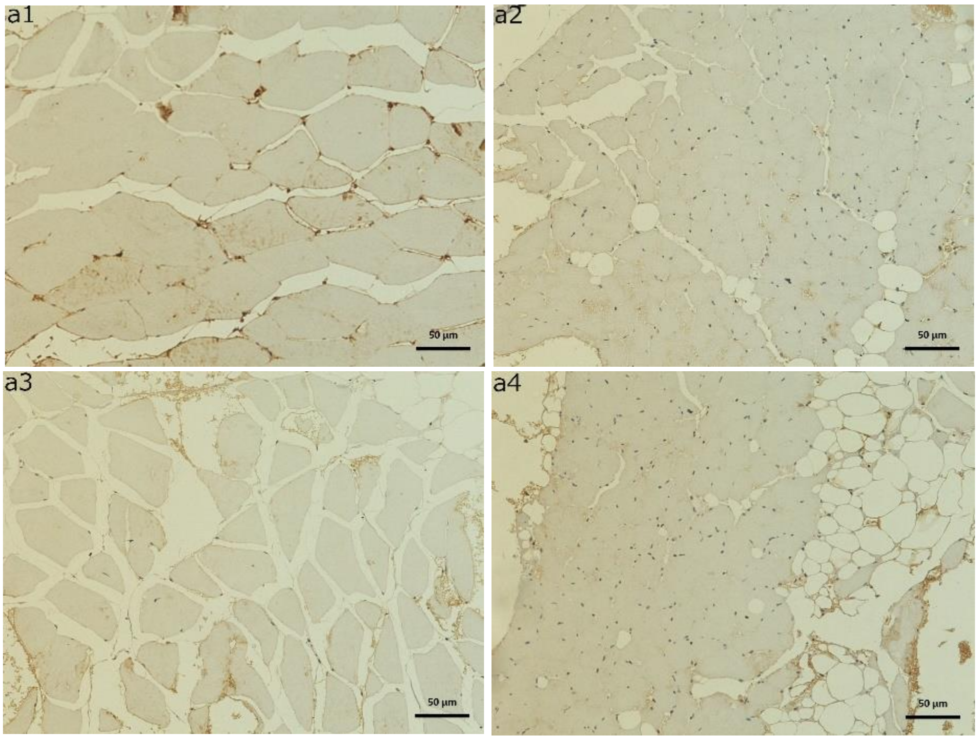

Figure 3. a1- Spring sample muscle tissue, **a2-** Summer sample muscle tissue, **a3-** Autumn sample muscle tissue, **a4-** Winter sample muscle tissue, **TNF- α** reactivity, transverse section, 50 µm.

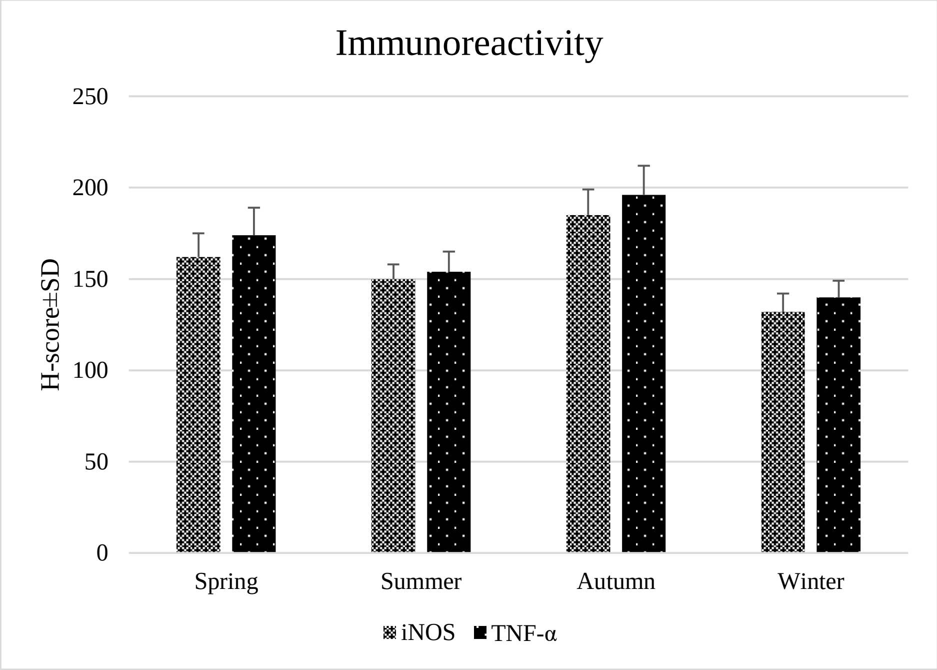

Figure 4. Distribution of iNOS and TNF-α in muscle tissue (n=8 for every sample seasonally).

3.2. TUNEL Findings

While the mechanism of programmed cell death usually occurs in embryonic tissues, disruption of oxidant-antioxidant balance and degeneration in the adult tissue are important processes that trigger apoptosis. Horse mackerel TUNEL reactivity was found to be more severe in autumn, while reactivity was mild in other seasons (Figure 5, Figure 6).

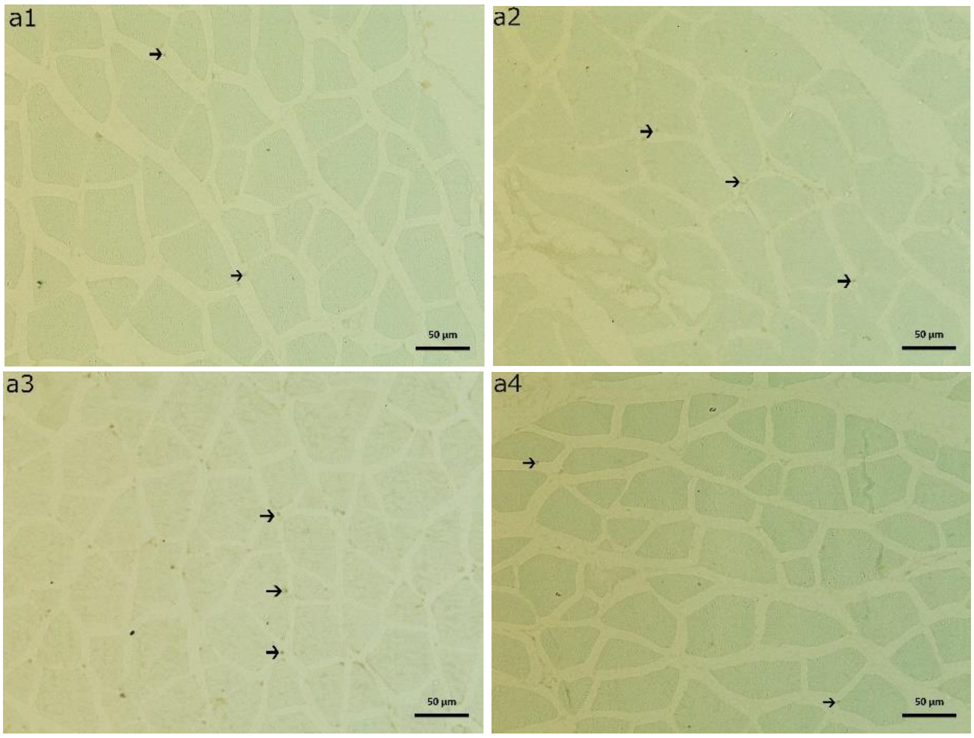

Figure 5. a1- Spring sample muscle tissue, **a2-** Summer sample muscle tissue, **a3-** Autumn sample muscle tissue, **a4-** Winter sample muscle tissue, **TUNEL** reactivity, transverse section, (arrow: apoptotic cells), 50 µm.

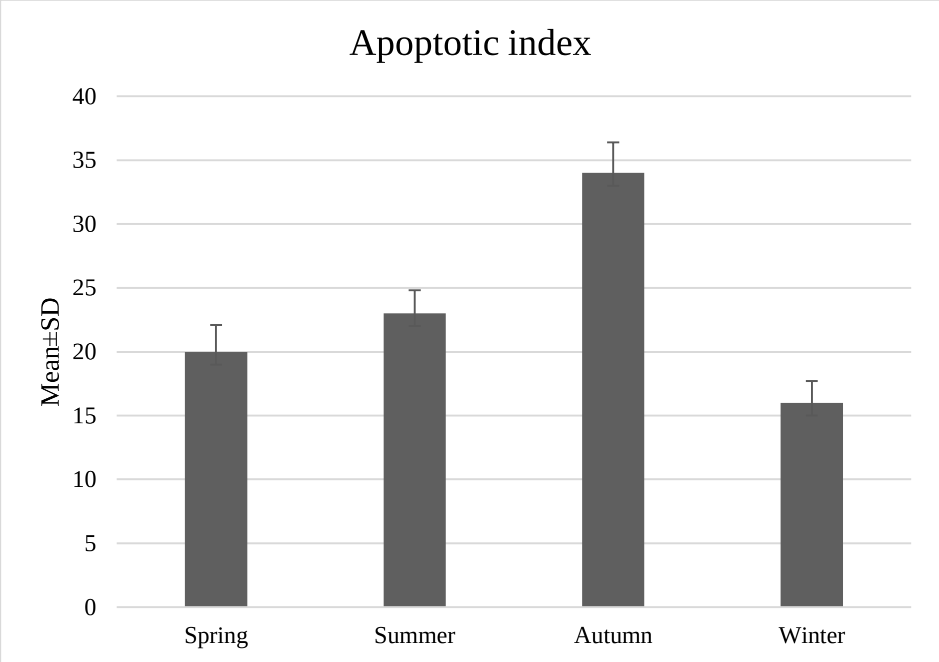

Figure 6. Apoptotic index values of muscle tissue (n=8 for every sample seasonally).

4. DISCUSSION

Today, there are almost no adequate medical and epidemiological studies on the negative effects of marine pollution on human health (Allen, 2011). Existing studies mostly focus on the formation of cancerous tissues because of the excessive accumulation of elements such as lead, cadmium, copper, nickel, and zinc (Yaman et al., 2007). This study was carried out by planning on the extent to which horse mackerel, which is one of the fish with high nutritional and economic value, is seasonally affected by pollution factors and whether it poses a threat to human health.

In studies, it has been stated that heavy metal accumulations in the seas disrupt the ecological system, this accumulation in seafood, which is a part of the food chain, cannot be ignored in terms of human health, and it may be more dangerous especially for children compared to adults (Yi et al., 2011). In a study conducted on the seawater of the Dardanelles Umurbey coast and some mollusks growing in this region (Gezen et al., 2011), because of examining the internal organs of clams, oysters, and sea snails. Zn and Mn in scallops, Zn in oysters, and Al, Zn, Fe, Cu, and Mn in sea snails were found above acceptable values. In research, the presence of many heavy metals has been detected in single and bivalve seafood grown in the Dardanelles Strait (Demir and Akkuş, 2011). In our country, the annual pollution rate is quite high compared to the regions due to the Çanakkale and Istanbul Strait transit ship passages. It is known that crustaceans accumulate pollution factors such as heavy metals with their advanced filter system. However, it is not known to what extent fish are affected by marine pollution and which fish are more affected. Support was provided with histopathological findings within the framework of the study plan on seafood, which is one of the ways to contribute to the literature and to threaten human health through the food chain. Our findings were directly on fish muscle tissue used by humans as a food source.

Histopathological biomarkers are used as a good indicator tool to reveal the effect and size of pollutants in fish. These markers have been successfully used to evaluate the effect on the vital organs of fish that respond well to toxic substances and stress (Abalaka, 2017; Dane and Şişman, 2017; Dane and Şişman, 2020). When the oxidative stress resulting from heavy metal exposure and the associated tissue damage were examined on muscle tissue, degeneration in the tissues of fish caught in the autumn season occurred more than in other seasons. In iNOS and TNF-alpha immunohistochemical staining, the immunoreactivity of muscle fascicles was more severe. These findings are like the results of other heavy metal studies (Jabeen and Chaudhry, 2010). Al-Khayat et al. (2018), Reddy and Rawat (2013) confirmed that histopathology is invaluable biomarker for genotoxic assessments. They also drew attention to the importance of histopathological biomarkers to determine the presence of pollutants in the aquatic ecosystem (Peebua et al., 2008; Jabeen and Chaudhry, 2010; Reddy and Rawat, 2013; Viana et al., 2013). This study revealed that the histopathological changes in the muscle tissue of the horse mackerel caught in the Dardanelles vary according to the seasons and that these pathological changes are also in the muscles, albeit to a lesser extent. These histological changes may be a direct or indirect indicator of the effects of genotoxic substances, heavy metals, pesticides, salts, industrial and domestic wastes discharged into the seas. In some studies, these histopathological changes in the muscles occur as a result of exposure to various toxic substances (Mansour and Sidky, 2003; Abbas and Ali, 2007; Kaur et al. 2018; Chang et al., 2019), and in some studies, Zn reports that similar effects occur in the presence of elements such as Cu and Pb (Padrilah et al., 2018; Reddy and Rawat, 2013; Drishya et al., 2016; Abalaka, 2017; El-Khayat et al., 2018). In our previous study findings for *Sardina pilchardus* (Irkin and Öztürk, 2021), elements such as Zn, Cu, Cd, and Pb were detected, albeit in low amounts, and it was observed that histopathological changes occurred more in the samples with high detection in the autumn season. It was determined by TUNEL staining that these heavy metals, which have genotoxic effects, also increased the apoptotic index. Although it shows that heavy metal accumulation is high in metabolic organs such as organs and the liver, it is consumed in large amounts by people in muscle tissue. accumulation has also been reported. We are of the opinion that it is necessary to evaluate the results in terms of health and to focus on more comprehensive studies.

CONCLUSION

The study is a pioneering study in terms of evaluating the muscle tissue of fish caught in the Çanakkale Strait and associating it with heavy metals. Other fish species should be evaluated with similar studies and the disadvantages of their consumption should be revealed. It should be determined whether fish, which is an important food, especially in coastal cities, are exposed to pollution at a level that threatens human health. In this regard, this study results show that there is no harm in consuming horse mackerel in the autumn season,

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AUTHORS CONTRIBUTIONS

Author LCİ and ŞÖ designed the study, LCİ and RA wrote the first draft of the manuscript, LCİ, ŞÖ performed and managed statistical analyses.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

For this type of study, formal consent is not required.

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