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Research Article

Investigation of Toxic Pathological Findings in European Seabass (*Dicentrarchus labrax* (Linnaeus, 1758)) Applied with High-dose Chlorine Dioxide

Eda Vural[1,(*)], Mustafa Kemal Ciftci[2]

[1]General Directorate of Agricultural Research and Policies, Mediterranean Research Production and Training Institute, Department of Aquatic Animal Health, Antalya, Türkiye
[2]Istanbul Gelisim University Research Information System, Faculty of Dentistry, Istanbul, Türkiye

Abstract

Aquatic species are important sources of high-quality protein and contribute significantly to global food security and national economies. In Türkiye, the European seabass (*Dicentrarchus labrax*) accounts for approximately 37% of marine aquaculture production and is widely cultured in coastal farming systems. However, diseases and environmental stressors remain major constraints that can significantly limit production in European seabass (*Dicentrarchus labrax*) aquaculture. Among these factors, water quality management is one of the most critical parameters affecting fish health and farm productivity. Physical approaches, such as filtration systems and ultraviolet (UV) treatment, are commonly used to maintain water quality; chemical disinfectants, such as ozone and chlorine, are also commonly used as essential complementary strategies. Nevertheless, these agents may be ineffective and can cause undesirable pathological effects in fish.Before the experiment, the 96-h median lethal concentration (LC_{50}) of Chlorine dioxide for European seabass was determined experimentally to be 1.33 mg/L. Based on this value, the present study aimed to evaluate the pathological effects of Chlorine dioxide at different concentrations (0.375, 0.750, 1.125, 1.5, 1.875, and 2.25 mg/L) in various tissues and organs (skin, gills, liver, heart, kidneys, and intestines) of 9–19-month-old European seabass. Fish were exposed to Chlorine dioxide for 7 days under semi-static conditions, with daily renewal of the disinfectant solution. In addition, oxidative stress and apoptosis-related biomarkers were evaluated in liver tissue. Macroscopically, haemorrhages were observed in the gills and liver. Histopathological examinations revealed dose-related pathological alterations, including degeneration, necrosis, and epithelial sloughing in the gills; hepatocyte swelling, vacuolization, and necrosis in the liver; and oedema in the brain. Histopathological changes were scored as 0 in the control group, and scores from 1 to 4 were assigned based on lesion severity. Scoring results for gill and liver tissues indicated that high-dose groups (1.5, 1.875, and 2.25 mg/L) had significantly higher lesion scores compared with the low-dose groups (0.375, 0.75, and 1.125 mg/L), demonstrating a significant increase in tissue damage with increasing dose ($p < 0.001$, $n = 10$ per group). ELISA analyses of oxidative stress and apoptosis biomarkers (Caspase-3 (pmol/L), Tumour Necrosis Factor-α (pmol/L), Cytochrome-C (pmol/L), Apo-1/Fas (pmol/L), Malondialdehyde (pmol/L), and P53/Tumour Protein P53 (pmol/L) showed no significant induction of oxidative stress or apoptotic responses in liver tissue following Chlorine dioxide exposure. In several cases, biomarker levels were significantly lower than those of the control group, indicating a suppression or reduction relative to baseline levels rather than activation of these pathways. In conclusion, Chlorine dioxide may be used as a disinfectant in marine aquaculture for European seabass at concentrations between 0.750 and 1.125 mg/L for short-term applications. Under the experimental conditions of this study, these concentrations did not induce significant oxidative stress or apoptosis, although dose-dependent histopathological alterations were observed at higher exposure levels.

Keywords: Chlorine dioxide, European seabass, Histopathology, Oxidative stress, Toxicity.

(*) Corresponding author:
Eda Vural
edavural28@gmail.com

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Introduction

The rapid increase in the world population and the insufficiency of agricultural production capacities to meet this increase have led to the transformation of aquaculture into a strategic sector. However, decreases in water quality, irregular nutrition, and stress factors weaken the fish's immune system and accelerate the spread of diseases, leading to serious economic losses (Bovenkerk and Meijboom 2012, Rahman et al 2019).

Under intensive production conditions, bacterial, parasitic and viral pathogens rapidly spread, leading to high mortality and significant economic losses

(Post 1987, Woo 2006, Dykova and Lom 2007, Kent et al 2009, Noga 2010). While disinfection is a critical component of biosecurity for pathogen control, the use of chemical agents should be applied with caution due to environmental and residue concerns (Grondel el al 1987, OIE 2009, Kumar et al 2022).

Chlorine dioxide (ClO_2) is a highly soluble oxidant that remains effective at low pH (Gordon and Rosenblatt 2005, Fan et al 2009). Unlike chlorine, it is a broad-spectrum disinfectant that does not produce by-products such as carcinogenic trihalomethanes and haloacids (Hoehn et al 2010).

Although ClO_2 is widely used as a disinfectant in aquaculture systems, information on its potential toxicopathological effects on marine fish, particularly European seabass, remains limited. Therefore, the present study aimed to investigate the toxicopathological effects of ClO_2 exposure in European seabass using experimental concentrations derived from a preliminary 96-h LC_{50} toxicity test. Fish were exposed to different concentrations of ClO_2 (0.375–2.25 mg/L) for a 7-day exposure period. Pathological effects of ClO_2 were investigated in European seabass tissues. During systemic necropsy, multiple organs were examined; however, detailed histopathological analyses focused on the gills, liver, and brain. Histopathological alterations were evaluated in gill, liver, and brain tissues, and oxidative stress and apoptosis biomarkers were analysed in liver tissue to assess the toxicological responses associated with ClO_2exposure.

Material and Methods

Experimental Procedure

Healthy European seabass (*Dicentrarchus labrax*), approximately 9 months old and weighing 60–80 g, were obtained from a commercial aquaculture facility. Before the experiment, fish were acclimated to laboratory conditions for two weeks. During this period, fish were maintained in aerated seawater tanks and fed a commercial diet.

During the experimental period, the average water temperature was approximately 18°C, dissolved oxygen was 7.15 mg/L, salinity averaged 38.31, and pH remained within the typical range for Mediterranean seawater.

A total of 70 fish were randomly distributed into seven experimental groups (n=10 per tank). Experimental concentrations were determined based on the results of a preliminary 96-h LC_{50} toxicity test. Following determination of the LC_{50} value (1.33 mg/L), experimental doses were selected as incremental fractions above and below this value at 0.375 mg/L intervals. Fish were randomly assigned to experimental groups to minimise potential selection bias. Each tank contained 80 L of seawater, and ClO_2 concentrations were adjusted daily.

In the preliminary LC_{50} determination study, a control group and three replicate ClO_2 exposure groups were established. Fish were exposed to ClO_2 for 96 hours under semi-static conditions with a 12 h light: 12 h dark photoperiod. ClO_2 solutions were freshly prepared daily and added to the tanks. Fish were not fed during the toxicity test. Mortality and behavioural changes were monitored at regular intervals, and the LC_{50} value was calculated using the Karber–Behrens method (OECD 2019).

Moribund fish observed during the experimental period were humanely euthanised using MS-222 (tricaine methanesulfonate, 250 mg/L). Fish found dead in the tanks were immediately removed and recorded. At the end of the experiment, all surviving fish were euthanised with MS-222 (250 mg/L) before the systemic necropsy.

Mortality developed in a dose- and time-dependent manner throughout the experimental period. In the high-dose groups (2.25, 1.875, and 1.5 mg/L), clinical signs appeared early, and all fish died during the experimental period. In the 2.25 mg/L group, mortality was complete within the first 30 minutes, whereas in the 1.875 and 1.5 mg/L groups, mortality occurred over a longer period, and all fish in these groups were lost later in the experiment (at 27 hours). A single moribund fish observed in the 1.500 mg/L group was humanely euthanised and included in the total mortality count.

In the low-dose group of 1.125 mg/L, mortality was distributed over time, occurring between 29 and 60 hours, and no live fish remained in the tank by the 60th hour. In contrast, no mortality was observed in the 0.750 mg/L, 0.375 mg/L, and control groups throughout the experimental period. All fish in these groups were euthanised at the end of the experiment using MS-222 (250 mg/L).

In total, 40 fish died during the experimental period, while 30 fish were euthanised at the end of the experiment. All fish (n=70) were subjected to systemic necropsy. During systemic necropsy, multiple organs, including skin, gills, liver, heart, kidneys, and intestines, were examined macroscopically and microscopically. However, no significant pathological alterations were detected in several tissues during preliminary evaluation. Therefore, detailed histopathological analyses focused on gill, liver, and brain tissues, which are among the most sensitive organs to waterborne toxicants and oxidative stress in fish.

Tissue samples from the gill, liver, and brain were collected for histopathological examination. In addition, a portion of liver tissue was separated for biochemical analyses, placed in sterile microcentrifuge tubes, and stored at −80°C until ELISA analysis.

Tissues were fixed in 10% buffered neutral formalin, processed routinely, and stained with Hematoxylin and Eosin (H&E) for microscopic examination.

Lipid accumulation in liver tissue was evaluated using Oil Red O and Sudan Black staining performed on cryosections prepared with a freezing microtome. Briefly, frozen liver sections were air-dried, fixed with formalin, and rinsed with 60% isopropanol before staining with freshly prepared Oil Red O solution for 15 minutes. After staining, sections were counterstained with hematoxylin and mounted using glycerin-based medium. Sudan Black staining was performed on separate sections according to the method described by Lillie and Ashburn (1945).

Histopathological changes were scored from 0 to 4 using a modified system adapted by Terzi and Çiftçi (2017). Detailed scoring criteria are presented in Tables 2–4.

Oxidative Stress and Apoptosis

Measurement of oxidative stress and apoptosis in tissue was performed using ELISA kits for Fish Caspase 3 (CASP3) (Cat. No E0218Fi), Fish TNF-α (Cat. No Q8JFG3), Fish Cytochrome-C (Cat. No MBS165577), Apo-1/Fas (Cat. No MERCK QIA26), MDA (MBS728071) and Fish P53/TP53 (MBS9351778) according to the manufacturers' instructions.

All assays were performed using standard curves generated from serial dilutions of known concentrations, and absorbance was measured at 450 nm with a microplate reader. The detection ranges and sensitivities of the kits were within the manufacturer's specified limits.

The concentrations of analytes were expressed in the units provided by each kit as follows: CASP3 (pmol/L), TNF-α (pg/mL), Cytochrome-c (ng/mL), Apo-1/Fas (ng/mL), MDA (nmol/mL), and P53 (ng/mL). All ELISA results are presented in the corresponding figures with their respective units clearly indicated.

Statistical Analysis

Data were analysed with IBM SPSS 22 (IBM Corp., Armonk, NY, USA). Prior to parametric tests, normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. ELISA data were analysed using one-way analysis of variance (ANOVA) followed by Duncan's multiple-comparison post hoc test. Histopathologic scoring was evaluated by the Kruskal-Wallis H test, and differences between groups were analysed by the Mann-Whitney U test. Results were presented as mean ± standard error of the mean (SE), and a value of $p < 0.05$ was considered statistically significant.

LC_{50} Value and Mortality

The LC_{50} value for ClO_2 in European seabass was calculated as 1.33 mg/L after 96 h of exposure. No mortality was observed in the control or low-dose groups (0.375 and 0.750 mg/L). Mortality occurred between 29 and 60 h of exposure in the 1.125 mg/L group. In the 1.5 and 1.875 mg/L groups, mortality occurred within 26–27 h, whereas complete mortality was observed within 30 min in the 2.25 mg/L group.

Results

Macroscopic Results

Macroscopically, hyperemia, haemorrhages, and opercular opening were observed in the gills of fish exposed to 1.875 and 2.25 mg/L ClO_2. In the tanks treated with 1.5, 1.875, and 2.25 mg/L ClO_2, the livers appeared pale and enlarged with blunt edges Figures 1-2.

Histopathological Results

No significant histopathological alterations were observed in the liver, gill, or brain tissues of fish in the control group. In contrast, histopathological changes were observed in the experimental groups with increasing ClO_2 dose. Histopathological findings in these tissues, together with the corresponding statistical analyses, are presented in Figures 3–16. In addition, liver sections were stained with Sudan Black and Oil Red O to evaluate lipid accumulation. Lesions were scored according to organ and treatment group. Statistical analyses revealed no significant

Table 1. Experimental design and group allocation of European seabass (*D. labrax*) in the present study.

Dose Group	Groups / Application	No implementation	0.375 mg/L ClO_2	0.750 mg/L ClO_2	1.125 mg/L ClO_2	1.5 mg/L ClO_2	1.875 mg/L ClO_2	2.250 mg/L ClO_2
Low Dose Groups	Control	X						
	Group I		X					
	Group II			X				
	Group III				X			
High Dose Groups	Group IV					X		
	Group V						X	
	Group VI							X

Table 2. Criteria for liver histopathological scoring in European seabass (*D. labrax*) in the present study.	
Score No	Histopathological Findings Observed in Liver
0	Mild fatty infiltration in hepatocytes, no change in the nucleus.
1	Hepatocytes swollen, with cytoplasmic vacuoles. The nuclei are pushed aside.
2	Hepatocytes with moderate vacuole formation and swelling. Necrotic hepatocytes with pushed aside nuclei and occasional pyknotic nuclei. Narrowing of sinusoids.
3	Diffuse vacuole formations and swelling in hepatocytes. Marked pyknotic changes in nuclei and necrosis of hepatocytes. Marked narrowing of sinusoids.
4	Severe vacuole formation in hepatocytes throughout the lobule. Necrotic hepatocytes with pyknotic nuclei are common. Sinusoids are lost.

Table 3. Criteria for gill histopathological scoring in European seabass (*D. labrax)* in the present study.	
Score No	Histopathological Findings Observed in the Gills
0	Lamellae and capillary vessels are normal, with no epithelial shedding.
1	Oedema, hyperemia and hypertrophy of the lamellae.
2	Marked oedema and thickening of the lamellae, swelling and localised shedding of the epithelium.
3	Severe oedema of the lamellae, mild telangiectasia, swelling of the nuclei of erythrocytes, necrosis and shedding of epithelium.
4	Severe hyperemia and haemorrhage in the lamellae, thickening and necrosis of the vessel walls, degeneration of the epithelium, diffuse haemorrhages, and shedding.

Table 4. Criteria for brain histopathological scoring in European seabass (*D. labrax*) in the present study.	
Score No	Histopathological Findings Observed in the Brain
0	There is no significant change in neuropil tissue.
1	Mild oedema in the perivascular spaces of vessels, around neurons and glia cells and in neuropil tissue.
2	Prominent oedema in the perivascular spaces of vessels, around neurons and glial cells and in neuropil tissue.
3	Severe oedema, hyperemia and degeneration of neurons in the perivascular spaces of vessels, around neurons and glial cells and in neuropil tissue.
4	Severe oedema and diffuse haemorrhages in the brain, degeneration and necrosis of neurons.

Figure 1. Heart, liver, muscle, intestine, gills, kidneys, and brain (from left to right) showing normal macroscopic appearance.

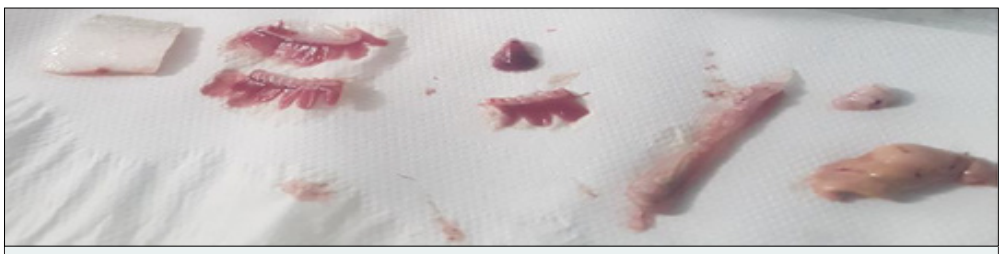

Figure 2. Muscle, gills, intestine, brain, and liver (from left to right) showing macroscopic appearance. Haemorrhages and erosion are observed in the gills, and pallor is observed in the liver.

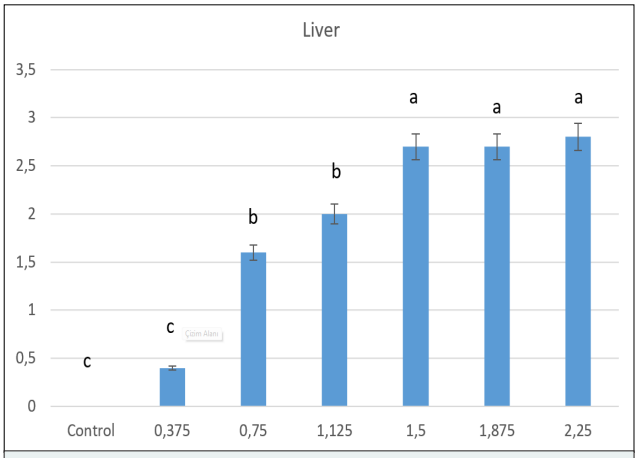

Figure 3. Distribution of mean liver histopathologic scores according to groups, statistical results ($p<0.001$).

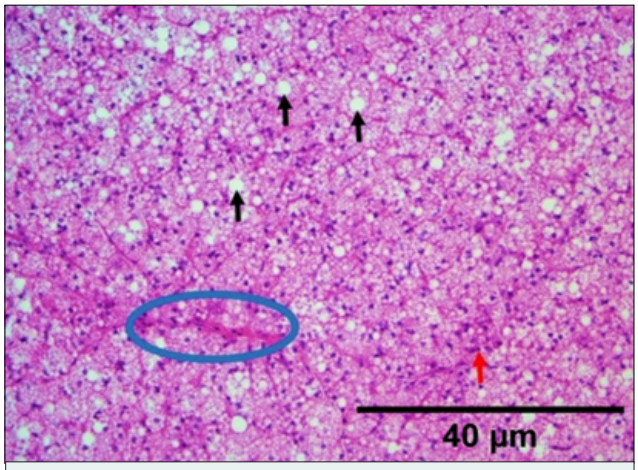

Figure 5. Liver micro photographs. Experimental Group II (0.75 mg/L dose). Moderate vacuole formations and swelling in hepatocytes (black arrows). Necrotic hepatocytes with pushed aside nuclei and occasional pyknotic nuclei (red arrow). Haemorrhage areas in the liver (circle) (score 2). Liver H&E staining, x40.

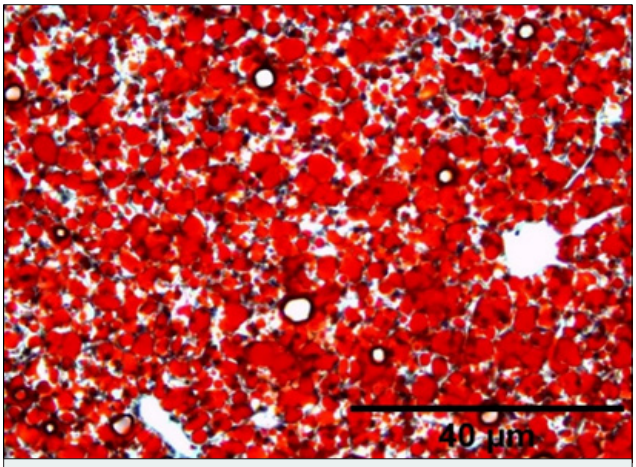

Figure 7. Experimental Group IV (1.5 mg/L dose). Diffuse fatty deposits in the liver. Diffuse fat droplets (red) in the cytoplasm of hepatocytes (score 4), Sudan Black staining, x40.

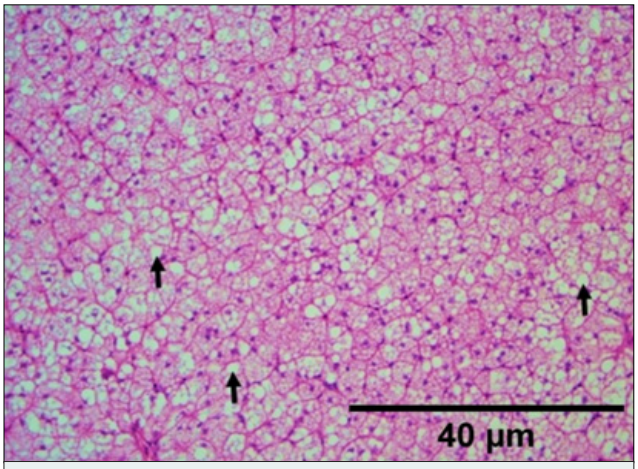

Figure 4. Liver micro photographs. Control Group. Mild vacuole formations (arrows) normally present in hepatocytes (score 0). Liver, H&E staining, x40.

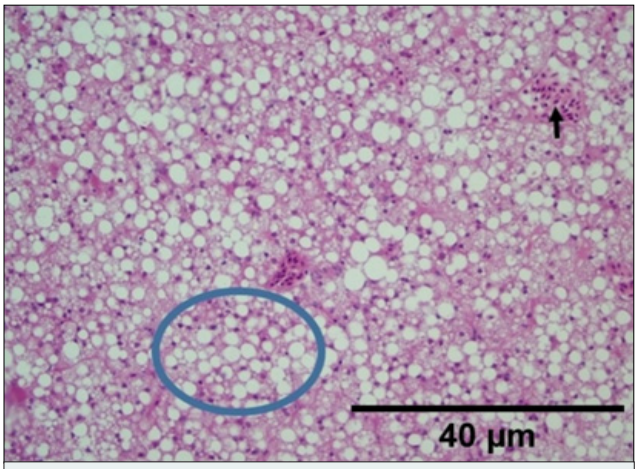

Figure 6. Liver micro photographs. Trial IV Group (1.5 mg/L dose). Severe vacuole formation in hepatocytes throughout the lobule (circle). Necrotic hepatocytes with picnotic nuclei are common, and sinusoids are lost. Vena centralis (arrow) (score 4). Liver, H&E staining, x40.

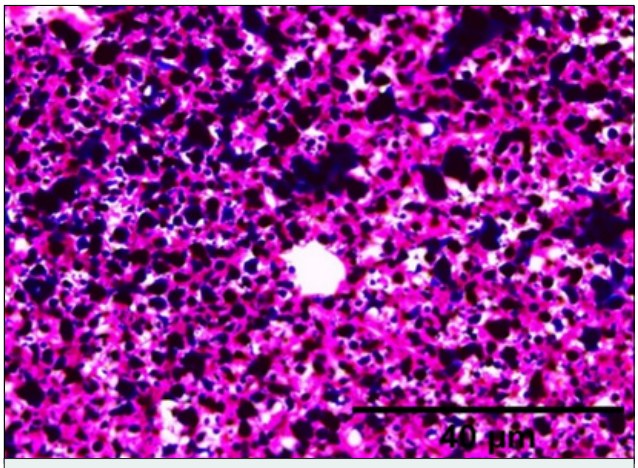

Figure 8. Trial Group V (1.875 mg/L dose). Diffuse fatty deposits in the liver. Oil droplets (black) in the cytoplasm of hepatocytes (score 4). Liver, Oil Red O staining, x40.

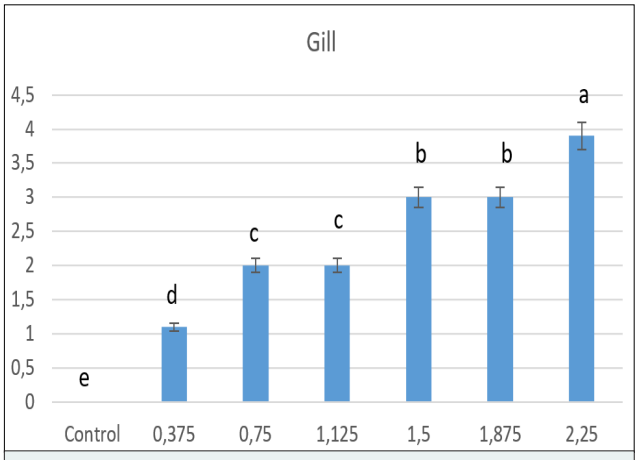

Figure 9. Distribution of gill histopathological scores according to groups and statistical results (p<0.001).

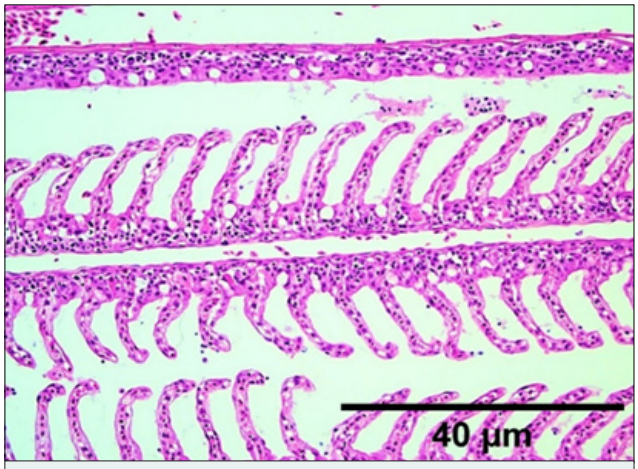

Figure 10. Control Group: Normal gill structure (score 0). Gill H&E staining, x40.

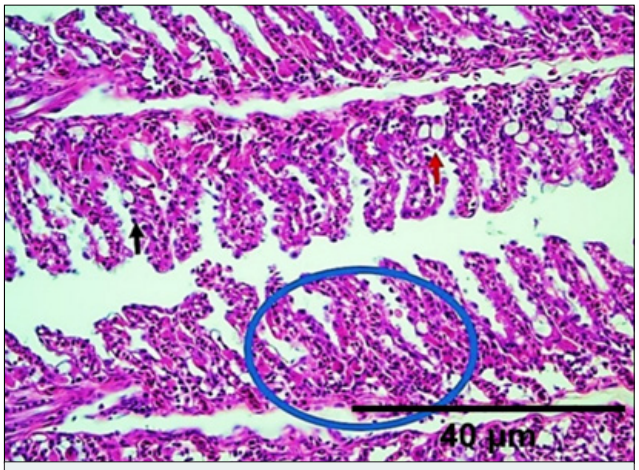

Figure 11. 0.75 mg/L dose trial: Marked oedema (red arrow), adhesions and thickening of the lamellae and necrosis in some areas (circle), swelling of the epithelium (black arrow), necrosis and shedding in some areas (score 2). Gill, H&E staining, x40.

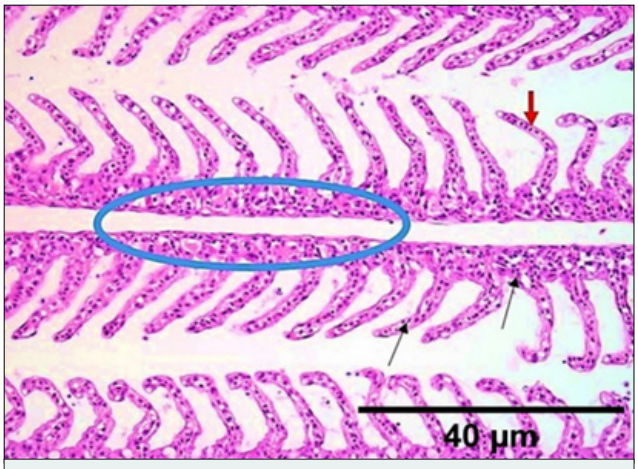

Figure 12. 1.125 mg/L dose trial, oedema (circle), necrosis of primary and secondary lamellae (black arrows), epithelial shedding (red arrow) (score 2). Gill, H&E staining, x40.

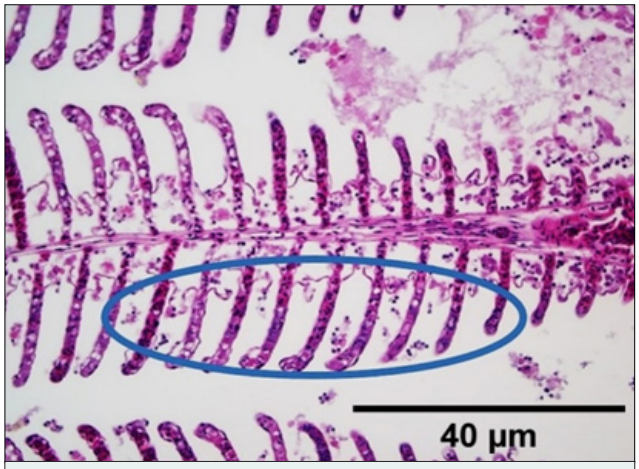

Figure 13. 2.25 mg/L dose. Widely necrosed and completely sloughed lamellae (circle), extensive haemorrhages with necrosis of vessel walls, erythrocytes with pyknotic nuclei (score 4). Gill, H&E staining, x40.

differences between the control group and the low-dose groups (0.375, 0.75, and 1.125 mg/L) (p>0.05). However, the high-dose groups (1.5, 1.875, and 2.25 mg/L) showed significantly higher lesion scores compared with both the control and low-dose groups (p<0.05) (Table 1).

Oxidative Stress and Apoptosis Findings

Oxidative stress and apoptosis test results are presented in (Figures 17-22). P53 levels (pmol/L) showed no significant difference between the control and 1.875 mg/L groups (p>0.05), and the remaining dose groups exhibited similar values without statistically significant differences (p>0.05) (Figure 17). Caspase-3 levels (pmol/L) did not differ significantly among the control, 0.75 mg/L, and 1.125 mg/L groups (p>0.05). The 0.375, 1.5, 1.875, and 2.25 mg/L groups also showed comparable levels, with no statistically significant differences within this subgroup (p>0.05) (Figure 18). Malondialdehyde (MDA) levels (pmol/L) were not significantly different between the control and 1.125 mg/L groups (p>0.05).

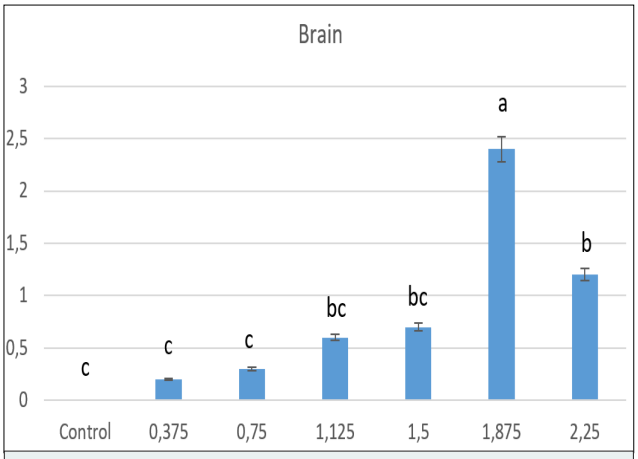

Figure 14. Distribution of brain histopathologic scores according to groups and statistical results (p<0.05).

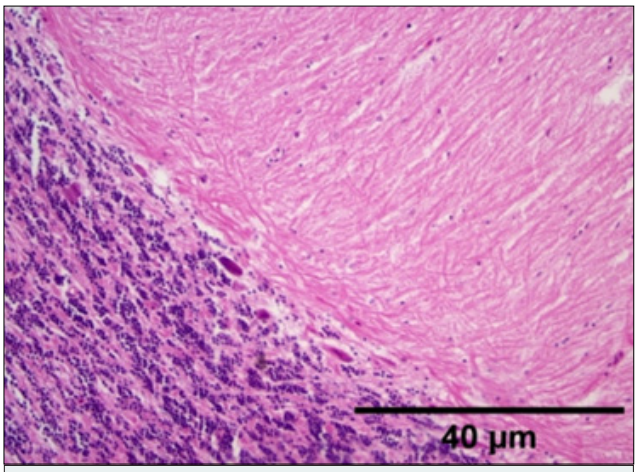

Figure 15. Control Group. Normal brain tissue (score 0). Brain, H&E staining, x40.

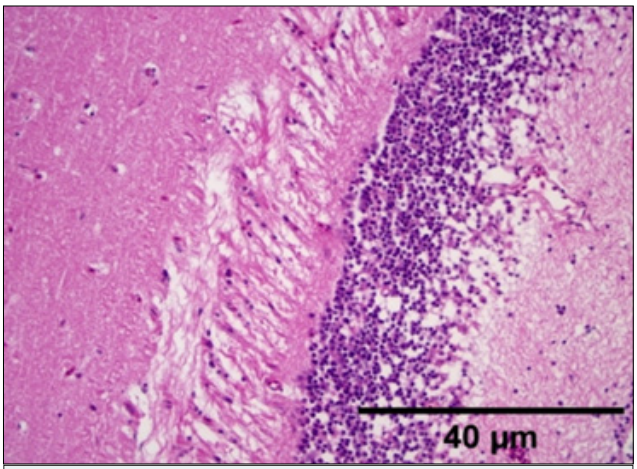

Figure 16. Experimental Group (1.5 mg/L). Oedema in the perivascular spaces of vessels, around neurons and glial cells and in neuropil tissue (score 1). Brain, H&E staining, x40.

However, significant differences were observed at other dose levels (p<0.05), while values within those respective groups remained statistically similar (p>0.05) (Figure 19). Apo-1/Fas levels (pmol/L) were significantly lower in all experimental groups compared with the control group (p<0.05), whereas no statistically significant differences were detected among the experimental dose groups (p>0.05) (Figure 20). TNF-α levels (pmol/L) were significantly decreased in all experimental groups compared with the control group (p<0.05). The 0.375, 0.75, and 1.125 mg/L groups did not differ significantly from each other (p>0.05), whereas a significant difference was detected between the 1.125 and 2.25 mg/L groups (p <0.05) (Figure 21). Cytochrome-c levels (pmol/L) were significantly lower in all experimental groups compared with the control group (p<0.05) (Figure 22).

Discussion

The P53 protein is an important regulator of the cell cycle and apoptosis. In the present study, P53 levels at the 1.875 mg/L dose were similar to those in the control group, whereas levels decreased in the remaining treatment groups. The observed reduction in P53 levels may suggest that ClO_2 exposure under the experimental conditions did not strongly activate classical apoptosis pathways. However, it should be clarified that ELISA measurements were expressed in pmol/L, as per kit instructions. However, ELISA measurements were expressed in pmol/L according to the kit instructions. It should also be considered that severe cellular damage or rapid toxic stress may suppress the expression or detectability of apoptosis-related regulatory proteins. Additionally, the sampling time point may have represented a later phase of tissue injury, after the initial peak of apoptotic signalling, which could explain the relatively low P53 levels detected in the exposed groups. In high-dose groups, tissues were obtained from fish that died between 0 and 60 h post-exposure, whereas in low-dose and control groups, all fish were sampled at the end of the 7-day exposure period. To our knowledge, there are currently no studies directly evaluating the relationship between ClO_2 exposure and P53 expression in fish tissues, highlighting the potential novelty of these findings (Fuchs and Steller 2011).

Caspase-3 is a key executioner enzyme involved in apoptotic pathways, particularly mitochondrial-mediated apoptosis (Fuchs and Steller 2011, Negroni et al 2015). In the present study, caspase-3 levels were decreased in the higher-dose groups (1.5, 1.875, and 2.25 mg/L) compared with the control. This decrease may indicate that ClO_2 exposure caused rapid cellular injury, leading to necrotic cell death rather than classical apoptotic mechanisms. Severe cellular injury may have limited the activation of classical apoptotic pathways and instead promoted

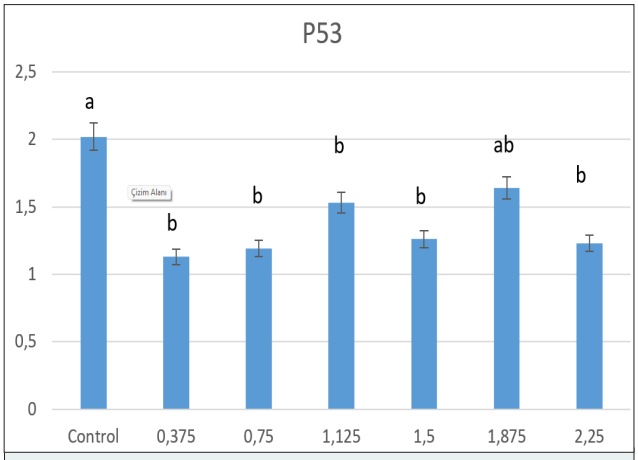

Figure 17. Distribution of P53 (pmol/L) levels in the liver tissue of European seabass exposed to different Chlorine dioxide concentrations. Data are expressed as mean ± SE (n = 10). Different letters indicate statistically significant differences among groups ($p < 0.05$).

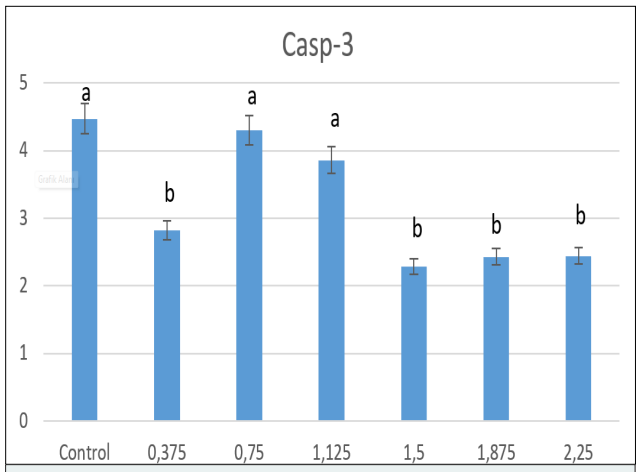

Figure 18. Distribution of Caspase-3 (pmol/L) levels in the liver tissue of European seabass exposed to different Chlorine dioxide concentrations. Data are expressed as mean ± SE (n = 10). Different letters indicate statistically significant differences among groups ($p < 0.05$).

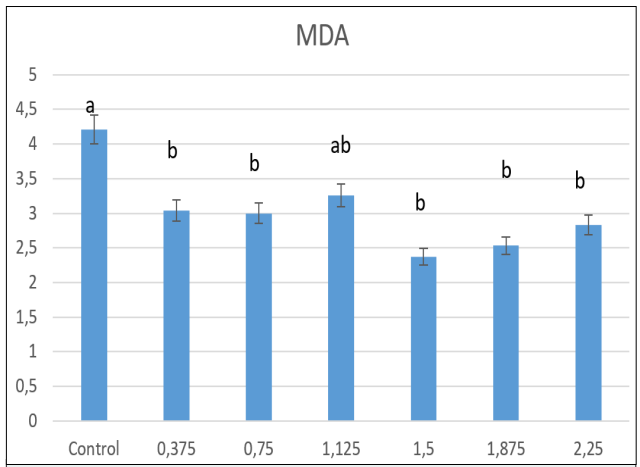

Figure 19. Distribution of MDA (pmol/L) levels in the liver tissue of European seabass exposed to different Chlorine dioxide concentrations. Data are expressed as mean ± SE (n = 10). Different letters indicate statistically significant differences among groups ($p < 0.05$).

alternative cell death mechanisms. Alternatively, sampling at the end of the exposure period may have occurred after the peak activation of apoptotic markers, resulting in lower detectable levels.

MDA levels were measured in the liver to evaluate oxidative stress. In the present study, MDA levels in the treatment groups were lower than in the control group. Rather than indicating the absence of oxidative processes, this finding suggests that ClO_2 exposure under the experimental conditions did not lead to detectable lipid peroxidation. ClO_2 is a well-known oxidizing agent, and previous studies have reported oxidative stress responses in aquatic organisms exposed to oxidants (Elia et al 2006). However, other antioxidant defense mechanisms, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), or glutathione (GSH), may have been active and were not measured—this should be clearly mentioned as a limitation. Therefore, other antioxidant defense mechanisms, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), or glutathione (GSH), may have contributed to maintaining oxidative balance but were not evaluated in this study.

The decrease in APO-1/Fas levels suggests that ClO_2 did not strongly activate the extrinsic apoptosis pathway in liver tissue. Similarly, the decreases observed in TNF-α and Cyt-C levels may indicate that mitochondrial apoptotic signalling was not prominently induced under the experimental conditions (Van Antwerp et al 1998, Aram et al 2017). Although several organs, including skin, heart, kidney, and intestine, were examined histologically, no remarkable pathological alterations were detected. In contrast, the most prominent lesions were observed in the gills, liver, and brain—organs particularly sensitive to waterborne toxicants due to their roles in respiration, detoxification, and neural regulation. Previous studies also report gill and liver tissues as primary targets of oxidizing disinfectants and environmental toxicants in fish (Ryu et al 1998, Perry and Gilmour 2002, Elia et al 2006, Heath 2018).

Histopathological examinations revealed dose-dependent alterations in target organs, particularly in the gills and liver, including epithelial degeneration, oedema, haemorrhage, and necrosis. In addition, hepatic lipid accumulation was observed in some exposure groups. Oil Red O staining revealed lipid droplets in hepatocyte cytoplasm, particularly in the 1.5 and 1.875 mg/L exposure groups, indicating fatty degeneration associated with Chlorine dioxide exposure (Beall and Ulsamer 1984, Haley et al 1995, Akamatsu et al 2012). These findings suggest that Chlorine dioxide may also induce metabolic disturbances in hepatocytes in addition to structural tissue damage. Similar pathological alterations in fish gill and liver tissues have been reported in studies evaluating oxidative

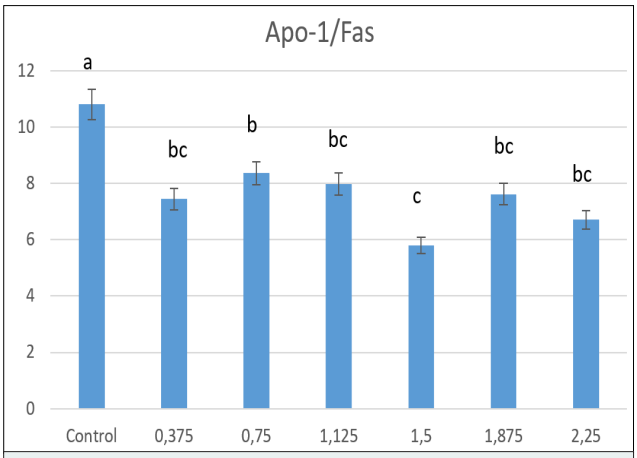

Figure 20 Distribution of Apo-1/Fas (pmol/L) levels in the liver tissue of European seabass exposed to different Chlorine dioxide concentrations. Data are expressed as mean ± SE (n = 10). Different letters indicate statistically significant differences among groups ($p < 0.05$).

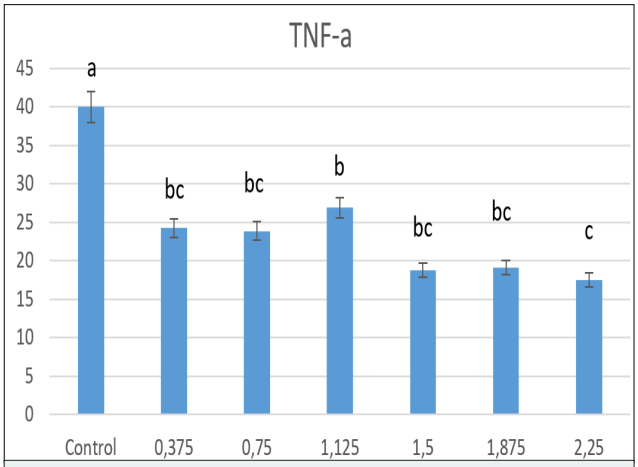

Figure 21. Distribution of TNF-α (pmol/L) levels in the liver tissue of European seabass exposed to different Chlorine dioxide concentrations. Data are expressed as mean ± SE (n = 10). Different letters indicate statistically significant differences among groups ($p < 0.05$).

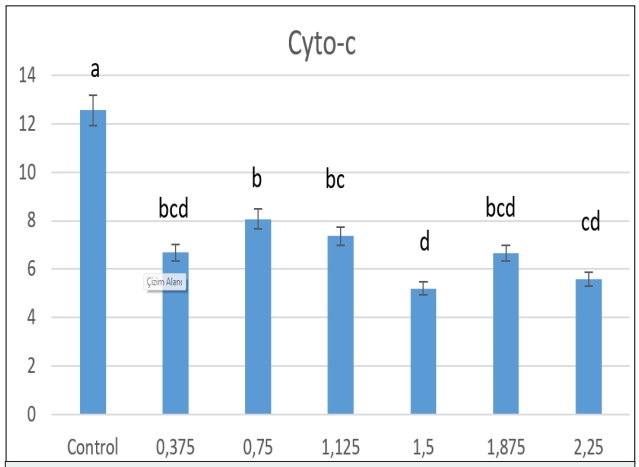

Figure 22. Distribution of Cytochrome-c (pmol/L) levels in the liver tissue of European seabass exposed to different Chlorine dioxide concentrations. Data are expressed as mean ± SE (n = 10). Different letters indicate statistically significant differences among groups ($p<0.05$).

disinfectants and environmental toxicants (Yonkos et al 200, Elia et al 2006).

In toxicological experiments involving aquatic disinfectants, both concentration and exposure duration may influence the severity of tissue damage. Therefore, cumulative exposure time may play an important role in the development of certain pathological alterations.

It should also be noted that the severity of some histopathological alterations in representative micrographs did not always follow a linear dose–response pattern. For example, the gill lesion in the 0.75 mg/L group appeared more pronounced than in the 1.125 mg/L group, likely due to variations in exposure duration rather than concentration alone. In this experiment, fish in some lower-dose groups remained exposed for a relatively longer period before trial termination, whereas higher-dose groups reached endpoints earlier due to toxicity-related effects. Therefore, cumulative exposure time may have contributed to the severity of some histopathological findings.

Our findings indicate that high-dose ClO_2 exposure caused significant pathological alterations in the gills, liver, and brain of European seabass. Histopathological findings, including degeneration, necrosis, oedema, and haemorrhage, were particularly evident in high-dose groups and intensified with increasing dose. Biochemical analyses indicated that oxidative stress and apoptosis markers, such as P53, Caspase-3, TNF-α, Cyt-C, APO-1/Fas, and MDA, did not increase compared to the control group; most parameters showed decreased levels. Taken together, these results suggest that ClO_2 toxicity in European seabass primarily involves acute cellular injury and necrotic tissue damage rather than classical apoptotic pathways.

Conclusion

Based on the experimental evidence presented above, the toxic–pathological effects of ClO_2 in European seabass were evaluated using histopathological and ELISA analyses. ClO_2 was applied at doses of 0.375–2.25 mg/L. The 96-h acute toxicity (LC_{50}) test yielded a value of 1.33 mg/L, indicating relatively high toxicity at doses ≥1.5 mg/L, where mortality and hemorrhagic lesions in the liver and gills were observed.

Histopathological examinations demonstrated that tissue damage increased with increasing dose, particularly in the gills and liver. Following the 96-h LC_{50} assessment, fish were exposed to selected sublethal concentrations of ClO_2 under repeated exposure conditions for 7 days to evaluate cumulative tissue responses. At lower doses (0.375–1.125 mg/L), only mild pathological findings were observed, whereas higher doses (≥1.5 mg/L) caused pronounced

degenerative and hemorrhagic lesions.

Biochemical and ELISA analyses supported these observations. Alterations in parameters such as P53 and Caspase-3 (pmol/L) were mainly observed in the higher-dose groups (≥1.5 mg/L), whereas the lower-dose groups showed values similar to those of the control group.

ClO_2 is known to exhibit species-specific toxicity depending on dose, body size, and age (Svecevičius 2005, Zhang et al 2017). Although slight pathological changes were detected at 0.375–0.750 mg/L in the present study, these findings may be related to the 7-day repeated exposure design, which represents a more intensive exposure scenario than routine aquaculture practices.

Therefore, under practical aquaculture conditions where continuous daily application is uncommon, ClO_2 concentrations of approximately 0.750–1.125 mg/L may represent a relatively safe range for short-term water disinfection. It should be noted that chronic exposure and antioxidant response markers were not evaluated, which remains a limitation. However, further studies are needed to investigate chronic low-dose exposure, exposure duration, antioxidant responses, species- and size-dependent differences in toxicity, and optimised dosing strategies for safe aquaculture applications.

These findings contribute to understanding ClO_2 toxicity in marine fish and provide experimental data that may support the development of safer disinfection protocols in aquaculture systems.

Overall, the present study provides experimental evidence on the dose-dependent toxic effects of ClO_2 in European seabass and highlights the importance of carefully controlled application levels to minimise potential health risks in aquaculture environments.

Declarations

Competing Interests
Authors declares that there are no conflicts of interest related to the publication of this article.

Availability of Data and Materials
The data that support the findings of this study are available on request from the corresponding author.

Ethical Statement
Prior to the commencement of the study, ethical approval was obtained from the Local Animal Experiments Ethics Committee of the Mediterranean Fisheries Research, Production and Training Institute, under decision number 2020/03 dated 13 April 2020.

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Author Contributions
Motivation / Concept: EV; Design: EV; Control/Supervision: MKC; Data Collection and Processing: EV, MKC; Analysis and Interpretation: EV; Literature Review: EV; Writing the Article: EV; Critical Review: EV, MKC

ORCID
EV: https://orcid.org/0000-0002-9128-6109
MKC: https://orcid.org/0000-0003-4476-2114

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