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EVALUATION OF OXIDATIVE/NITROSATIVE STRESS BIOMARKERS IN KIDNEY, HEART AND MUSCLE TISSUES OF *Oncorhynchus mykiss* (WALBAUM, 1792) TREATED WITH MERCURY CHLORIDE ($HgCl_2$)

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Abstract: The widespread presence of mercury (Hg) and its derivatives in aquatic environments necessitates the investigation of their toxic effects on fish and the potential risks they pose to the health of local populations. The objective of this study was to investigate the toxic effects of mercury chloride ($HgCl_2$) on *Oncorhynchus mykiss* (Walbaum. 1792) by examining the dynamics of oxidative and nitrosative stress in muscle, kidney, and heart tissues. The rainbow trout used in the study had an average weight of 125.65±8.30 g. Five experimental groups were created, including a control group and groups exposed to 25% LC50 concentration of $HgCl_2$ for 7 and 14 days, as well as groups exposed to 50% LC50 concentration of $HgCl_2$ for 7 and 14 days. A total of 120 fish, with 40 fish assigned to each tissue (muscle, kidney, and heart), were used in the study. To evaluate oxidative/nitrosative stress, various parameters including $ONOO^-$, TOS, TAS, OSI, and MDA levels were measured. Statistical analysis revealed a significant difference between the experimental groups in terms of TAS, TOS, OSI, $ONOO^-$, and MDA levels in all three tissues (muscle, kidney, and heart) ($P<0.05$). In conclusion, the findings of this study demonstrate that $HgCl_2$ induces stress and toxicity in rainbow trout, as evidenced by increased levels of MDA and $ONOO^-$, which are stress indicators in the fish.

Keywords: Mercury chloride, rainbow trout, stress, muscle, kidney, heart

1. INTRODUCTION

Mercury chloride, also known as mercuric chloride or corrosive sublimate, is a white crystalline solid. It is highly toxic and is classified as a hazardous substance. The chemical formula for mercury chloride is $HgCl_2$, indicating that it contains one mercury atom and two chlorine atoms. Historically, $HgCl_2$ has been used in various applications such as disinfectants, fungicides, and pesticides. $HgCl_2$ can cause acute toxicity in aquatic organisms, resulting in impaired respiration, decreased growth, altered behavior, and increased mortality. $HgCl_2$ is known to bioaccumulate in aquatic organisms, especially in fish and shellfish. It can accumulate in their tissues over time, posing risks to higher trophic levels and potentially impacting human health through consumption (Jaishankar et al., 2014; Moiseenko & Gashkina, 2016; Pandey et al., 2005). The presence and harmful effects of metals in aquatic environments can be primarily attributed to the extensive industrialization and unregulated use of pesticides. These activities greatly contribute to the discharge of effluent containing high levels of metals and the runoff of metal-contaminated substances into nearby surface water bodies (Gobi et al., 2018; Owokotomo et al., 2020). Consequently, there has been a growing interest in conducting ecological and human health risk assessments to mitigate the risks and minimize the adverse consequences for local populations (Jerome et al., 2020). Mercury (Hg) compounds are a group of toxic heavy metals that are extensively dispersed and have profound impacts on the growth and maturation of aquatic organisms, even at minimal and substantial levels of concentration (Jebara et al., 2021). Furthermore, fish are exposed to mercury through contamination of inland water bodies, which undoubtedly has detrimental effects on fish health, resulting in decreased fish quality and reduced fish production. In the aquatic environment, mercury exists in different chemical forms, primarily as inorganic species including elemental mercury (Hg^0) and divalent mercury (Hg^{2+}), as well as organic species such as methylmercury (MeHg) (Beckers &

Rinklebe, 2017). In fish tissues, mercury is typically found in the form of methylmercury, with the primary source being inorganic mercury. Fish are widely recognized as valuable indicators of water quality in aquatic environments. This is mainly attributed to their extensive mobility, diverse feeding habits across different trophic levels, relatively long life, ease of sampling and identification in the field, and significant public awareness they generate (Whitfield & Elliott, 2002). There are numerous parameters that serve as indicators of oxidative stress and antioxidant status. Since measuring and evaluating all of these parameters simultaneously can be challenging, there has been a search for a general laboratory parameter that can serve as an overall indicator. With the development of new methods, it has become possible to measure Total Antioxidant Status (TAS) and Total Oxidant Status (TOS). The accuracy and validity of these parameters have been demonstrated, leading to the production of commercial assay kits. Additionally, an oxidative stress index (OSI) can be calculated based on these two parameters. The significance of OSI lies in its ability to represent the overall balance between oxidative stress and antioxidant status by neutralizing reactive increases in both directions.

Through the values of TAS, TOS, and OSI, information can be obtained and interpretations can be made regarding the oxidant-antioxidant capacity status in the etiology of many diseases within the organism. The measurement of TAS and TOS provides a useful means of assessing the overall antioxidant system, which consists of multiple components, and evaluating oxidative stress. Malondialdehyde (MDA) is a product of lipid peroxidation. Its level increases in proportion to the increase in oxidative stress. Therefore, MDA level is used as an indicator of oxidative stress. Cellular membrane damage is initiated by lipid peroxidation, which can be induced by various factors such as pesticides, metals, xenobiotics, and antibiotics (Gamble et al., 1995; Atessahin et al., 2005; Gnanasoundari & Pari, 2006; Pari & Gnanasoundari, 2006; Yonar et al., 2011).

In this study, the objective was to investigate the alterations in nitrosative and oxidative stress ($ONOO^-$, TOS, TAS, OSI, and MDA) parameters in the muscle, kidney, and heart tissues of rainbow trout exposed to $HgCl_2$. By examining the combination of these processes with Hg-induced oxidative and nitrosative stress biomarkers, the aim was to provide insights into the mechanisms underlying oxidative damage. Rainbow trout was selected as the experimental species due to several advantageous characteristics it possesses. These include its resilience to low concentrations of heavy metals, its capability to accumulate heavy metals within its body, easy availability, adaptability to laboratory conditions, and sufficient tissue or organ samples for analysis.

2. MATERIALS AND METHODS

2.1. Chemicals and fish

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. and Merck. *Oncorhynchus mykiss* (total *n*= 120), weighing 125.65±8.30 g (mean ± SD) were sourced from local fish culture pools in Elazığ, Turkey. The fish were kept in aquaria with a capacity of 60 L, where freshwater was continuously aerated to maintain dissolved oxygen levels between 7.5 and 8.0 mg L-1. The temperature was maintained at 20 ± 1°C, and the pH was maintained at 7.4 ± 0.2. A 12:12 light-dark cycle was established as the photoperiod. Prior to the start of the experiment, the fish underwent a 14-day acclimatization period and were fed commercial fish food twice daily.

2.2. Experimental setup

The entire experiment was conducted independently on two occasions, resulting in a total of two replicates. Each replicate consisted of eight fish, leading to a total of 120 fish used in the study. The fish were divided into 5 groups as the control group, the groups exposed to mercury chloride for 7 and 14 days with 25% and 50% of the lethal dose. The sublethal concentrations were chosen according to the HgCl2 96-h LC50 value previously de-termined for *O. mykiss* (551 µg/L) (Taysi et al., 2021). Following the euthanasia of the fish, appropriate autopsies were conducted, and the gill tissues were stored at -80°C until further use. The frozen gill tissue samples were individually homogenized in a 1:10 (w/v) ratio using a 10 mM Tris-buffer (pH 7.4) containing 0.1 mM NaCl, 1% Triton X-100, 0.2% SDS, and 2.5 mM ethylenediaminetetraacetic acid.

2.3. Total Antioxidant Status (TAS) measurement

The powerful and harmful free radicals' total antioxidant capacity (Total Antioxidant Status: TAS) against the organism can be measured using a fully automated method developed by Erel. The antioxidant molecules present in the sample convert the dark blue-green colored ABTS (2-2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) solution into a colorless ABTS form. The change in color intensity,

which can be measured spectrophotometrically, is associated with the total amount of antioxidant molecules present in the sample. The absorbance change is measured at 660 nm based on the varying color intensity. TAS measurement is calibrated using a stable antioxidant standard solution traditionally named Trolox Equivalent, which is an analog of vitamin E. The results are reported in mmol Trolox Equivalent / Liter (Erel, 2004).

2.4. Total Oxidant Status (TOS) measurement

The total oxidant capacity (Total Oxidant Status: TOS) of the organism can be measured using a fully automated colorimetric method developed by Erel. The oxidants present in the sample with measured oxidative capacity oxidize the ferrous ion chelate complex to ferric ions. In an acidic environment, ferric ions form a colored complex with a chromogen. The color intensity, which can be measured spectrophotometrically, is associated with the total amount of oxidant molecules present in the sample. The absorbance change is measured at 530 nm based on the varying color state. TOS measurement is calibrated using hydrogen peroxide. The results are reported in micromolar hydrogen peroxide equivalent / Liter (Erel, 2005).

2.5. Calculation of Oxidative Stress Index (OSI)

The Oxidative Stress Index (OSI) can be calculated by dividing Total Oxidant Status (TOS) by Total Antioxidant Status (TAS). In response to an increase in oxidative molecules, the increase in antioxidant molecules can mask the evaluation of oxidative damage based solely on TOS measurement. By comparing the changes in the TOS/TAS ratio, the misleading reactive antioxidant response that could be misleading for oxidative damage can be eliminated, allowing for an accurate comparison (Sirmatel et al., 2007).

2.6. Measurement of Malondialdehyde (MDA)

The amount of Malondialdehyde can be determined using a spectrophotometric method based on the reaction between MDA and thiobarbituric acid (TBA), as described by Draper & Hadley (1990). In this method, the color formation resulting from the reaction of MDA with TBA is evaluated through spectrophotometric measurement (Draper & Hadley, 1990).

2.7. Determination of $ONOO^-$

Assessment of nitrosative stress status in kidney, heart and muscle tissues was achieved by determination of $ONOO^-$. ONOO- mediated nitration of phenol was quantified following the procedure described by Vanuffelen et al., (1998). Ten microliter of serum was added to 5 mM phenol in 50 mM sodium phosphate buffer (pH 7.4) to get a final volume of 2 mL. After 2 hours incubation in dark place at 37ºC, 15 µL of 0.1M NaOH was added and the absorbance, at wavelength of 412 nm, of the samples were immediately recorded. The calculation of nitrophenol yield was based on the molar absorptivity (ε) value of 4400 M/cm (Al-Nimer et al., 2012).

2.8. Statistical Analysis

In order to analyze the data obtained from the control and experimental groups, means and standard errors were calculated for each group. The statistical significance of the differences between these groups was assessed using analysis of variance (one-way ANOVA) followed by Duncan's post-hoc test. The SPSS ver. 21.0 computer program (IBM Corp. Released 2012) was utilized for these statistical analyses. P-values below 0.05 were considered to indicate statistical significance.

3. RESULTS

The control and experimental fish exhibited typical feeding behavior throughout the experiment. Moreover, no indications of respiratory distress, such as rapid breathing, heightened gill cover movements, or floating at the water surface, were observed. There were no fatalities recorded in either group during the course of the experiment. The effects of $HgCl_2$ oxidative and nitrosative stress parameters of the fish are given in Table 1, Table 2 and Table 3. The level of MDA was significantly increased in the kidney and heart samples of the groups treated only with 25% LC50 14 days and 50% LC50 14 days, in the muscle samples of the groups treated only with 25% LC50 14 days. The level of $ONOO^-$ was significantly increased in the kidney, muscle and heart samples of the all groups treated. The level of OSI was significantly decreased in the kidney, muscle and heart samples of the groups treated only with 25% LC50 14 days. The level of TOS was significantly decreased in the kidney and heart samples of the groups treated only with 25% LC50 14 days. The level of TAS was significantly decreased in the kidney, muscle and heart

Table 1. The kidney MDA (nmol/mg pro) level, TAS (mmol/L), TOS (μmol/L), OSI and $ONOO^-$ (mmol/L) activity in the control and experimental groups (*n*=8 fish for each group).

Groups	TAS	TOS	OSI	$ONOO^-$	MDA
Control	2.35 ± 0.44 [a]	3.18 ± 0.74 [a]	92.53 ±20.19 [a]	42.19 ± 8.30 [a]	71.60 ± 8.29 [a]
25% LC50 7 Days	2.42 ± 0.27[a]	3.26 ± 0.59 [a]	93.17 ± 19.52 [a]	44.28 ± 7.11 [a]	69.58 ± 9.11 [a]
50% LC50 7 Days	2.56 ± 0.51 [a]	2.95 ± 0.37 [a]	93.08 ±15.40 [a]	53.86 ± 4.87 [a]	73.82 ± 6.52 [a]
25% LC50 14 Days	1.77 ± 0.36 [b]	1.32 ± 0.70 [b]	68.89 ±19.04 [b]	75.53 ± 6.19 [b]	102.03 ± 14.59 [c]
50% LC50 14 Days	2.05 ± 0.29 [a]	3.78 ± 0.53 [a]	85.22±16.3 [a]	69.05 ± 8.10 [b]	85.26 ± 10.57 [b]

* The discrepancy between average values represented by different letters within the same line is statistically significant (P<0.05).

Table 2. The muscle MDA (nmol/mg pro) level, TAS (mmol/L), TOS (μmol/L), OSI and $ONOO^-$ (mmol/L) activity in the control and experimental groups (*n*=8 fish for each group).

Groups	TAS	TOS	OSI	$ONOO^-$	MDA
Control	1.96 ± 0.17 [a]	3.29 ± 0.55 [a]	31.59 ± 4.10 [a]	35.17 ± 4.19 [a]	53.29 ± 5.18 [a]
25% LC50 7 Days	1.87 ± 0.12 [a]	3.42 ± 0.72 [a]	33.18 ± 5.27 [a]	43.35 ± 3.58 [a]	55.34 ± 4.90 [a]
50% LC50 7 Days	1.90 ± 0.13 [a]	3.84 ± 0.64 [a]	34.57 ± 4.53 [a]	56.48 ± 5.07 [b]	56.42 ± 6.73 [a]
25% LC50 14 Days	1.13 ± 0.09 [c]	2.03 ± 0.86 [a]	19.72 ± 6.17 [b]	58.76 ± 6.11 [b]	80.12 ± 9.21 [b]
50% LC50 14 Days	1.55 ± 0.14 [b]	3.15 ± 0.41 [a]	27.44 ± 5.21 [a]	62.87 ± 5.27 [b]	58.38 ± 8.06 [a]

* The discrepancy between average values represented by different letters within the same line is statistically significant (P<0.05).

Table 3. The heart MDA (nmol/mg pro) level, TAS (mmol/L), TOS (μmol/L), OSI and $ONOO^-$ (mmol/L) activity in the control and experimental groups (*n*=8 fish for each group).

Groups	TAS	TOS	OSI	$ONOO^-$	MDA
Control	1.90 ± 0.16 [a]	3.74 ± 0.52 [a]	182.30 ± 28.17 [a]	50.19 ± 4.23 [a]	62.17 ± 13.76 [a]
25% LC50 7 Days	1.92 ± 0.12 [a]	3.70 ± 0.41 [a]	184.11 ± 22.48 [a]	58.50 ± 3.61 [a]	63.01 ± 15.41 [a]
50% LC50 7 Days	1.89 ± 0.10 [a]	3.81 ± 0.89 [a]	185.20 ± 36.19 [a]	62.13 ± 5.41 [a]	60.86 ± 12.10 [a]
25% LC50 14 Days	4.71 ± 0.67 [c]	2.13 ± 0.72 [b]	131.71 ± 45.42 [b]	89.70 ± 5.20 [b]	87.09 ± 20.25 [c]
50% LC50 14 Days	2.40 ± 0.32 [b]	3.28 ± 0.51 [a]	176.53 ± 33.59 [a]	76.66 ± 4.31 [b]	70.26 ± 11.35 [b]

*The discrepancy between average values represented by different letters within the same line is statistically significant (P<0.05).

samples of the groups treated only with 25% LC50 14 days and 50% LC50 14 days.

4. DISCUSSION

$HgCl_2$ is a toxic compound that can have detrimental effects on various organisms, including fish. When it comes to the effects of $HgCl_2$ on heart tissue in fish, research indicates several potential impacts. $HgCl_2$ has been shown to induce cardiotoxic effects in fish. Studies have demonstrated that exposure to $HgCl_2$ can lead to structural damage and functional alterations in fish heart tissue. This damage can include cellular degeneration, necrosis (tissue death), and disruption of normal cardiac function (Monteiro et al., 2017; Naija & Yalcin, 2023; Vijayakumar et al., 2014). Mercury chloride exposure can affect the contractility of the fish heart muscle. This compound has been found to interfere with the normal regulation of calcium ions, which are essential for proper contraction and relaxation of cardiac muscle fibers. As a result, the fish heart may experience reduced contractile strength, leading to impaired pumping ability (Ferreira et al., 2022; Monteiro et al., 2017). $HgCl_2$ exposure has been associated with the development of arrhythmias in fish. Arrhythmias are abnormal heart rhythms that can disrupt the coordinated pumping of the heart. Studies have shown that mercury chloride can interfere with the electrical signaling pathways in the fish heart, leading to irregular heartbeats and potentially compromising overall cardiac function (Hodkovicova et al., 2022; Kannan et al., 2021). $HgCl_2$ is known to induce oxidative stress in various tissues, including the heart. When fish are exposed to this compound, it can lead to an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms. Increased levels of ROS can cause oxidative damage to cellular components, including heart tissue, which can contribute to cardiac dysfunction (Leitão et al., 2022; Mamdouh et al., 2021; Wu et al., 2022). The data presented in this study show that $HgCl_2$ causes a significant increase in the MDA and $ONOO^-$ levels of rainbow trout. The increased MDA and $ONOO^-$ levels observed in this study may have been caused by free radicals produced through $HgCl_2$ administration.

When fish are exposed to $HgCl_2$, it can be

absorbed into their bodies through the gills or ingestion of contaminated food. Once inside the fish, mercury chloride can accumulate in different organs, including the kidneys. The kidneys play a crucial role in maintaining the internal balance of a fish's body, regulating water and electrolyte levels, and filtering waste products. Exposure to $HgCl_2$ can disrupt the normal functioning of the kidneys and lead to various adverse effects (Lee et al., 2020). Mercury chloride can induce structural alterations in the kidney tissue of fish. This can include degeneration, necrosis (cell death), and disruption of the cellular architecture in the renal tubules and glomeruli (Adams et al., 2010; Moss et al., 2020; Rogers et al., 2023). $HgCl_2$ exposure can impair the normal functioning of the fish's kidneys, leading to decreased filtration capacity, reduced urine production, and altered electrolyte balance. These effects can disrupt the fish's ability to regulate its internal environment effectively (Ayyat et al., 2020; Khan et al., 2017). $HgCl_2$ is known to induce oxidative stress in fish. It can generate reactive oxygen species (ROS) and impair the antioxidant defense mechanisms in the kidneys. This oxidative stress can cause damage to the cellular components of the kidney tissue, including lipids, proteins, and DNA (Jang et al., 2020; Waheed et al., 2020).

$HgCl_2$ exposure can cause histopathological changes in fish muscle tissue. These changes may include muscle fiber degeneration, necrosis, inflammation, and fibrosis. The severity of these effects can depend on the concentration and duration of exposure to mercury chloride (Lee et al., 2020; Mahboub et al., 2021; Mamdouh et al., 2021). Mercury chloride can induce oxidative stress in fish muscle tissue. It leads to an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms in the cells. This oxidative stress can result in damage to proteins, lipids, and DNA within the muscle tissue (Mahboub et al., 2022; Waheed et al., 2020). $HgCl_2$ can interact with proteins in fish muscle tissue, leading to protein denaturation. This can disrupt the structure and function of proteins, affecting the integrity and contractile properties of the muscle tissue (Nong et al., 2021).

5. CONCLUSIONS

Current research shows that mercury chloride has harmful effects on trout and causes oxidative stress. Oxidative stress and biochemical damages can occur in aquatic organisms exposed to mercury chloride ($HgCl_2$) and other oxidant stressors. These effects are well-documented in scientific literature and have raised concerns about the impacts on aquatic ecosystems. By gaining a deeper understanding of these processes, researchers can develop more effective strategies for monitoring and mitigating the impacts of oxidant stress on aquatic ecosystems. Additionally, such research can contribute to the development of guidelines and regulations to minimize the exposure of aquatic organisms to pollutants and prevent ecological damage. Overall, continued scientific investigation is crucial for comprehending the specific oxidative stress responses and toxic effects of pollutants like mercury on aquatic organisms and ecosystems.

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