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Sub-chronic impact of nickel (Ni) on glutathione peroxidase and metallothionein gene expression in *Cyprinus carpio* L.1785 (Common carp)

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Abstract

Nickel (Ni) is considered a toxic heavy metal that causes serious diseases in humans and animals. Therefore, this study was conducted on common carp, which is considered an essential food item for humans. The present study aimed to investigate the effect of nickel exposure on the gene expression of glutathione peroxidase and metallothionein in common carp (Cyprinus carpio L.1785 (Common carp)), focusing on the effects observed after 3 and 6 weeks of exposure. The experimental fish's morphometric features were noted. All fish were stocked, regardless of gender. Fish were subjected to varying concentrations of 20, 40, 68, 80, and 100 mg/L to obtain the LC50 for Ni during 96 hours which was 80 mg/L through 96 hours. Additionally, the fish were exposed to two safe concentrations, 1 ppm and 5 ppm for 3 and 6 weeks. The results were as follows: Glutathione peroxidase GPX results were significantly higher in all treatment groups as compared to control groups. The treated groups exhibited higher MT concentrations than the control groups. Increased levels of glutathione peroxidase (GPX) and metallothionein (MT) gene expression indicate metal pollution and abiotic stresses in blood.

***Key words*:** *Cyprinus carpio*, nickel, metallothionein, glutathione peroxidase, gene expression

التأثير شبه المزمن للنيكل (Ni) على التعبير الجيني للميتالوثيونين والجلوتاثيون بيروكسيديز في أسماك الكارب الشائع *Cyprinus carpio* L.1785

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الخلاصة

يعتبر النيكل من المعادن الثقيلة السامة التي تسبب أمراض خطيرة للإنسان والحيوان، ولذلك أجريت هذه الدراسة على أسماك الشبوط الشائع والتي تعتبر من المواد الغذائية الأساسية للإنسان. هدفت الدراسة الحالية إلى التحقيق في تأثير التعرض للنيكل على التعبير عن جينات الجلوتاثيون بيروكسيديزو الميتالوثيونين ، مع التركيز على التأثيرات التي تظهر بعد ثلاثة إلى ستة أسابيع من التعرض. وقد تم الحصول على صغار أسماك الكارب الشائع من مفرخة أسماك قريبة. وقد تم تسجيل السمات الشكلية للأسماك التجريبية. وقد تم تخزين جميع الأسماك بغض النظر عن الجنس. تعرضت الأسماك لتراكيز متفاوتة 20 و40 و68 و80 و100 مجم / لتر للحصول على LC50 للنيكل خلال 96 ساعة، والتي كانت 80 مجم / لتر خلال 96

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ساعة. وقد تعرضت الأسماك لتركيزين آمنين، 1 جزء في المليون و5 جزء في المليون. بعد ثلاثة وستة أسابيع، كانت النتائج على النحو التالي: كانت نتائج الجلوتاثيون بيروكسيديز GPx أعلى بشكل ملحوظ في جميع مجموعات العلاج مقارنة بمجموعة الضبط كانت تركيزات MT في المجموعات المعالجة أعلى من مجموعة الضبط. تشير المستويات المرتفعة من التعبير عن جين الجلوتاثيون بيروكسيديز (GPX) والميتالوثيونين (MT) إلى تلوث المعادن والضغوط غير الحيوية في الدم.

Introduction

A heavy metal is defined as any metallic chemical element that has a high density and can be deadly or dangerous in small quantities is referred to as a heavy metal. The crust of the planet naturally contains heavy metals and these metals cannot be destroyed or degraded. Although heavy metals (HM) are naturally occurring trace elements in aquatic environments, their concentrations have risen due to activities such as , agriculture, industrial waste, and geochemical structure.[1]. They enter the aquatic ecosystem by a range of releases from domestic, industrial, and agricultural operations. Heavy metals tend to collect and have negative impacts on living things since they are permanent and non-biodegradable [2]. Because they are not removed from aquatic systems by natural methods heavy metals are significant pollutants for fish [3] and aquatic organisms.

Heavy metals often contain a combination of essential and non-essential elements. It is therefore impossible to determine their true impact on biological processes by evaluating their harmful effects separately [4]. Following a prolonged exposure to copper, the liver of common carp displayed hepatic, degenerative, and necrotic hepatocyte cells together with mildly inflammatory cells and an accumulation of cholesterol inside the cells. [5].
The effect of mercury chloride on a few biochemical and immunological indicators in common cap, *Cyprinus carpio*, was investigated by [6] . In summary, the study showed that mercury chloride is very toxic to common carp and exhibits immune-suppressive properties.
Commonly, both natural and artificial sources discharge nickel into soil and water systems .nickel can be released into the environment, for example, through the tannery industry's effluent, production of nickel alloys, pigment business, and the weathering and pedogenesis of mafic and ultramafic rocks and the soils that result from them [7]. Due to this metal's inability to degrade in the environment, pollution from it is becoming a growing concern, especially in developing nations [8]. Metallothionien exhibit a significant expression response to heavy metals, making them potential biomarkers for assessing the aquatic environment's heavy metal contamination. With reverse transcription polymerase chain reaction, it would be possible to assess the identification of minute alterations in biomarkers at the gene level RT-qPCR. In both lab and field settings, the MT gene expression has frequently been employed as a molecular biomarker for metal exposure [9]. Metallothioneins as a defense to get rid of the excess heavy metal load. Aquatic creatures' primary element detoxifiers are metallothioneins. It is well-established that organisms in environments with high metal concentrations overexpress MT. The MT-a protein, which is produced by the gene, scavenges excess metal burden. Consequently, it serves as a valuable indicator for researching how heavy metals affect aquatic animals [10]. Enzyme-Linked Immunosorbent Assays (ELISA) has emerged as the recommended technique for MT detection, despite the fact that a variety of assays are available to monitor the amount and type of metallothionein (MT) from environmental samples or in biomedical assays. Elisa assay is inexpensive, straightforward, specific, and effective [11]. Glutathione peroxidases are a crucial enzyme family that helps prevent oxidative damage in living things [12]. The GPX family, along with superoxide dismutase (SOD), catalase (CAT), and peroxidase (PRX), is essential for ROS detoxification in fish and other aquatic species [13]. According to [14]. During the process of oxidizing glutathione (GSH), glutathione peroxidase shields cells from reactive oxygen

species (ROS; H2O2 reduction), organic lipid peroxides, and cholesterol esters. The expression of the GPX gene is upregulated in response to a wide range of abiotic stressors. Glutathione peroxidase a crucial peroxidase believed to protect erythrocytes from damage caused by from H2O2 damage (GPX). Hydroperoxides and hydrogen peroxide can be reduced through their catalytic action on glutathione. Hence, it is believed that this enzyme might shield tissues from lipid peroxidation-induced oxidative damage. Glutathione peroxidase activity can be induced by environmental contaminants [15].

Materials and Method

Cyprinus carpio L. Acclimatization and Experimental design

The fish utilized in this investigation were common carp (Cyprinus carpio L. 1785), which were measured the weight using a sensitive balance after being obtained from nearby fish hatchery incubators (Al Hilla City). The experimental fish used in this study were characterized by a standard length of 15-20 cm and an average body weight ranging from 50-80 grams. The fish were acclimated in 40 L aquariums measuring 60 x 40 x 50 cm for 14 days prior to the experiment. The aquariums were aerated continuously and maintained under a 16-hour/8-hour light/dark photoperiod. During the acclimation period and throughout the experiments, the water in the aquariums was changed every 48 hours (just in the sub-chronic period). Fish were kept in dechlorinated water contained within glass aquariums. Tap water left in the aquaria for 72 hours to remove any chlorine residue. Observations of the aquaria were undertaken daily during the acclimatization period to remove wounded, diseased, and dead individuals from an environment free of metabolic waste. During the experiment, every 24 hours, tank water was replaced and a fresh solution of nickel was added [16]. The nickel concentrations were kept constant while the dissolved oxygen level was optimized using an air pump.

Laboratory conditions

A mercury thermometer with a scale of 0-100 °C was used to measure the temperature of fish aquaria. pH value of water was evaluated using a portable pH meter following calibration with a buffer solution (pH=7). Dissolved Oxygen was Measured by using D.O meter (lovibond senso direct) model, in mg/l units.

Estimation the Concentrations of Nickel

Nickel sulfate was used in this experiment, and the concentrations of nickel were determined using the following equation: In mg / liter.

Concentration 1 × Volume 1 = Concentration 2 × Volume 2

Acute Toxicity

The common carp (*C. carpio*) was given five different concentrations of Ni were examined during the acute toxicity tests. used 20, 40, 68, 80, and 100 mg/L to obtain the LC50 for Ni during 96 hr according to [15]. Recorded the median lethal concentration of Ni was 80 mg/L during 96 hours. After 24, 48, 72, and 96 hours, the mortality rate was computed, and dead fish were removed as soon as they were found. The experiment involved six groups, each comprising ten fish. For the acute toxicity assessment, the fish were placed in 40-liter glass aquariums filled with water. They were maintained in these conditions for a period of 96 hours, during which no food was provided. The exposed fish samples were divided into five groups for each concentration with the first serving as a control. To prevent the toxicant employed in the experiment from oxidizing, feeding was stopped 24 hours before the start of the experiment. After 24, 48, 72, and 96 hours, fish mortality was measured. In terms of regulating organisms, fish samples were visually checked daily and judged at end point when no respiratory movement or unusual swimming in reaction to mild touching.

Median Lethal Concentration (LC50)

Based on analysis method reported by Chatterjee, et al. [17] the LC50 was calculated for nickel, and MS Excel 2007 was used to find the regression equation (Y=mortality; X=concentrations). The median lethal concentration of nickel was 80 mg/L through 96 hours.

Sub Chronic Toxicity Test

The fish were subjected to two nickel safe concentrations, determined by dividing the 96 LC50 values determined in this study by 1/15th and 1/7th, respectively [18]. In sub-chronic test the water aquaria were divided into two groups, in the first group, the fish were exposed to 5 and 16mg/L for Ni for 3 to 6 weeks; while the second group was left as a control group. Fish are anesthetized to keep animals alive while blood is properly drawn. To prevents hemolysis blood was drawn from the heart via a heart puncture and placed in EDTA tubes [19].

Metallothionein concentration

Using Fish Metallothionein Elisa Kit / BT LAB / China, the MT levels were measured. While there are several assays that can be used to monitor the quantity and type of metallothionein (MT) in environmental samples or biomedical assays. Enzyme-Linked Immunosorbent Assays (ELISA) has become the standard method for MT detection [11].

Gene Expression of MT and GPX

Primers used in the study were according to their reference sequence from the molecular diversity preservation international (MDPI) and shown in table (1).

RNA extraction from the blood sample

Used TransZol Up Plus RNA kit (TransGen, biotech. ER501-01)

RT- qPCR (Real Time PCR /QIAGEN / USA)

Real-time quantitative PCR (RT-PCR, 7000 system software) was used to quantify all of the genes (MT-gene, GPX, and housekeeping gene) forward and reverse primers were designed using Primer Express 3.0 (Table 1). A melting curve analysis was conducted at 45-95 C to evaluate the specificity of the target amplification. Every target gene's Ct value was normalized to that of b-actin. Using the$2^{-\Delta\Delta ct}$ technique, the relative expression levels of MT, GPX, and β-actin by target genes were ascertained [20, 21].

Table 1: Forward and reverse primers for genes expression estimation.

Primers		Sequence (5′→3′ direction)	Ref.
MT1	Forward	ATGGATCCTTGCGATTGCGCCA	[22]
	Reverse	CGAACAGGTTCACATAGGTGA	
GPx	Forward	AGGCACAACAGTCAGGGATT	[23]
	Reverse	CGGACTTCAGAGACAGCAGA	
B-Actin	Forward	TGACCCACACTGTACCCATC	[23]
	Reverse	CGGACAATTTCACTCTCGGC	

Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests were employed to examine the normality distribution of data. The study variables were expressed as Mean ± standard deviation. One-way and two-way ANOVA were employed to assess significant differences among means of study groups and followed by Tukey's test as post hoc was. The differences were considered significant when P value ≤ 0.05. The statistical analysis was performed using GraphPad Prism 9.5 [24].

Result and Discussion

Physical and Chemical Parameters of Aquaria

Water temperature, pH, and dissolved oxygen (DO) were among the chemical and physical parameters measured in the lab to verify the quality of water is suitable for fish growth. Table (2) shows the results of the physical and chemical properties of water in the glass aquarian that was used in the experiment.

Table 2: Physical and chemical properties of water in glass aquarian

Physical and Chemical Properties	Range
Temperature (°C)	20- 28
Dissolved Oxygen (D.O) (mg/l)	5.5-7.9
Hydrogen Ion Concentration(pH)	6.9-7.6

Median Lethal Concentration (LC50) of Nickel

The toxicity of Ni to common carp is dose-dependent, as their toxicity to common carp increased with increasing particle concentrations. Figures (1) for Ni show that at 100 mg/L, all the animals died within 96 hours. The LC50 was determined for Ni to be 80 mg/L.

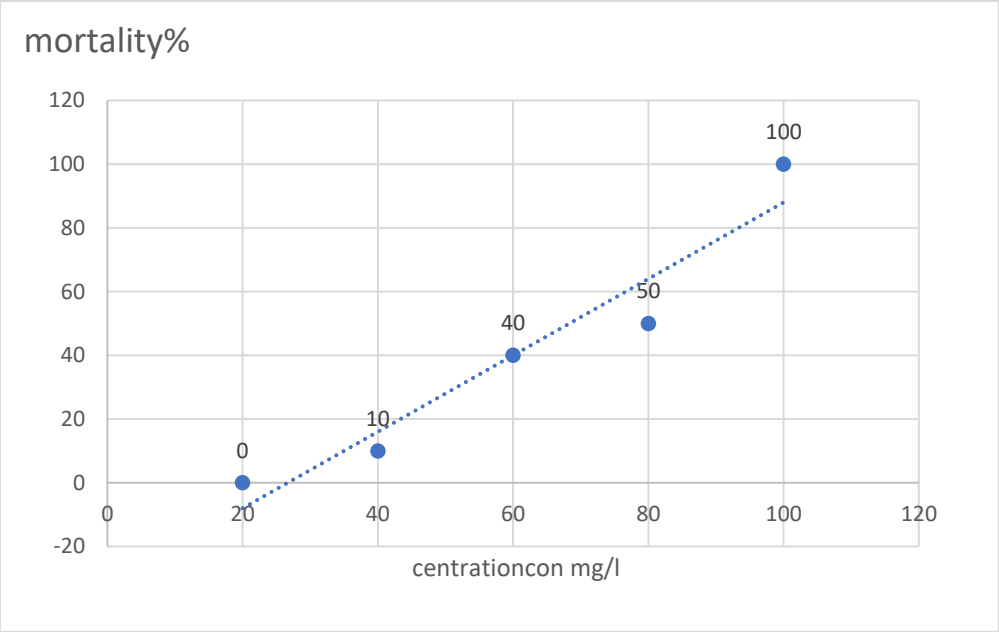

Figure 1: LC50 (96 hrs.) of Ni for *C. carpio*

Glutathione peroxidase (GPX)

The level of GPX in the blood was found to be elevated gradually during the exposure period with Ni 1ppm and 5ppm compared with control group were recorded highest value 576 (U/L) of GPX at 5ppm after 3 weeks. While, after 6 weeks the values were higher and almost equal between the two concentrations (1 and 5 ppm) 620 and 618 (U/L) according to (figure 2). These results are in agreement with Li *et al* [25], which underscores the enzyme's role in preventing lipid peroxidation. They also reflect similar findings with an increased level of glutathione peroxidase in microcystin-induced toxicity. Heavy metal exposure in Cyprinus carpio results in elevated levels of GPX. Also, these results agreed with Lenártová *et al* [26], suggest that If the fish consume oxidants that boost glutathione peroxidase activity, the liver is one of the primary target organs they aim at. This is likely a result of the fish's adaptive response to the oxidative stress they've experienced. Glutathione peroxidase being most critical peroxidase, has an essential role in protecting the erythrocytes from the damage caused by H2O2 [10].

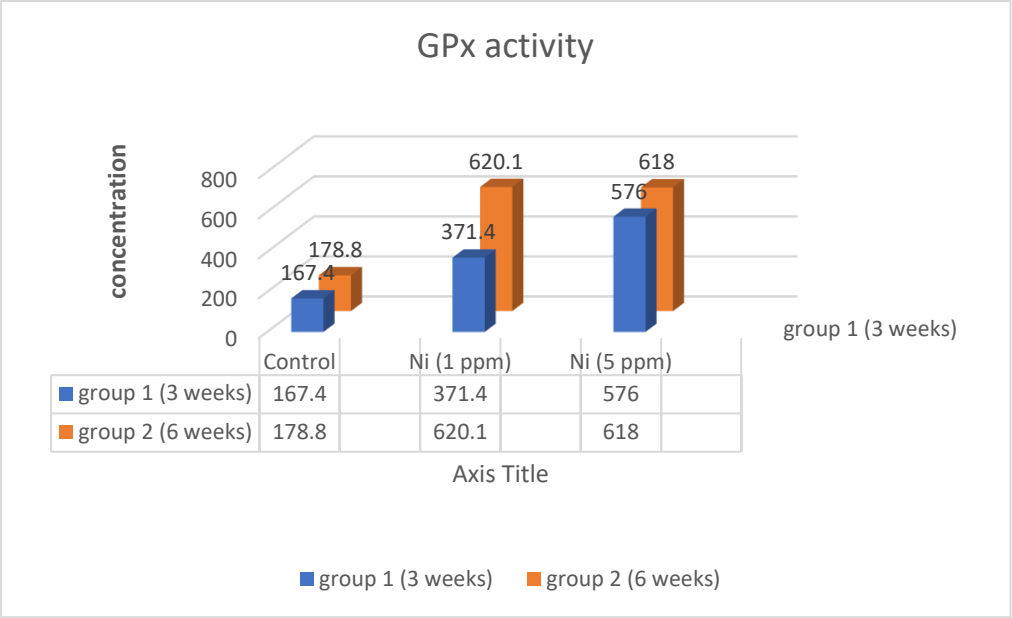

Figure 2: Effect of nickel on glutathione peroxidase activity after 3 and 6 weeks in common carp

Metallothionein concentration

Elisa Assay

Figure 3 shows the results of the level of MT increased in Ni 1 and 5 ppm through 3 weeks and 6 weeks compared with control groups. After 3 weeks, the highest value of MT was 2.43 ng at Ni 5 ppm, and the lowest value was 2.1 ng at Ni 1 ppm, compared with the control value of 1.2 ng. After 6 weeks, the highest value of MT was 2.649 ng at Ni 5ppm compared with the control group, while the lowest value of MT was 2.33 ng less than the control group. These results agreed with Chu *et al.* [27] were found MTs are easily induced by heavy metals because of the metal responsive elements in the promoter region of MT genes. Since metallothionein's MTs have been shown to exhibit a distinctly enhanced induction following heavy metal exposure in a wide range of investigated organisms, MTs have garnered considerable attention as potential biomarkers to indicate the presence of heavy metal pollution[28].

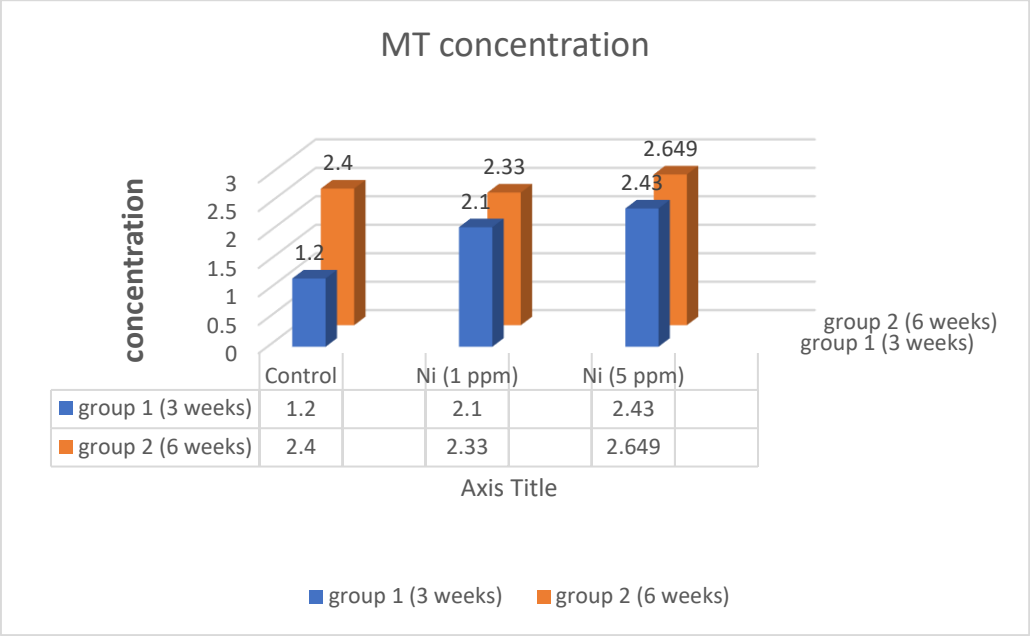

Figure 3: Effect of nickel on metallothionein concentration after 3 and 6 weeks

Glutathione peroxidase gene expression

Figure 4 illustrates the expression pattern of the GPX gene in the blood of fish. The maximum fold expression was observed in 3 weeks (1.125) at Ni 5 ppm, and the minimum fold expression was (1.121) at Ni 1 ppm. While, in 6 weeks, the maximum fold expression was (3.942) at Ni 1 ppm and the minimum fold expression was (1.933) at Ni 5 ppm. The current study was supported by Kim *et al,* [29] which proposed that by modifying their gene expression in response to oxidative stress caused by heavy metals, GPX and glutathione reductase may be engaged in cellular defense systems.

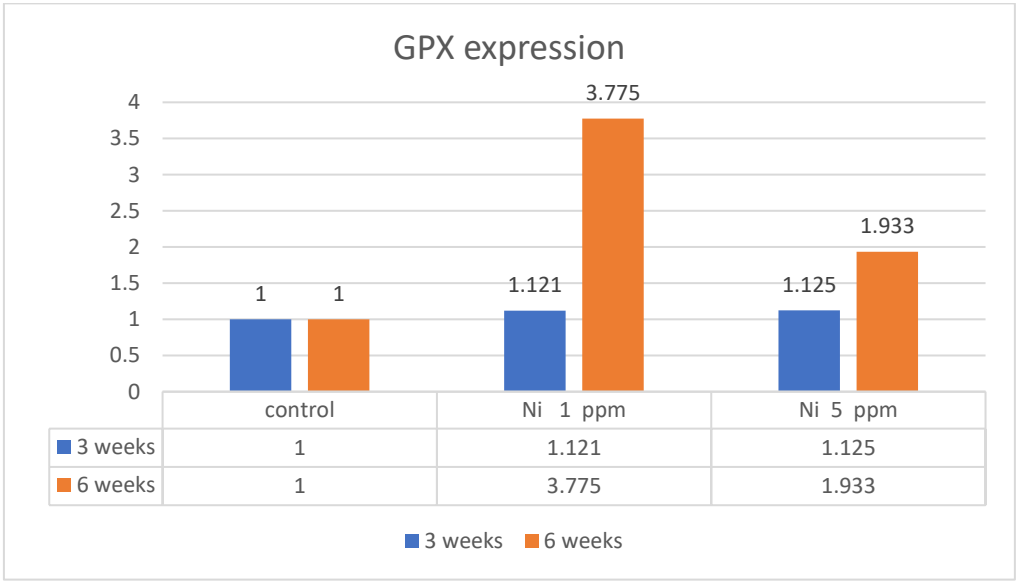

Figure 4: Effect of nickel on glutathione peroxidase expression after 3 and 6 weeks

Metallothionein gene expression

The results of metallothionein observed in figure (5) after 3 weeks, the maximum fold expression was (2.195) at Ni 5 ppm, and at 1 ppm of Ni, no expression showed. After 6 weeks the maximum fold change was (2.8) observed at Ni 5 ppm and the minimum fold expression was (1.243) at Ni 1ppm. These results agreed with Abumourad *et al* 2013 [30]

when study Heavy Metal Pollution and Metallothionein Expression: A Survey on Egyptian Tilapia Farms It was found that exposure to heavy metals in fish increases the gene expression of metallothionein. Also, Banday *et al,* [14] confirm these findings when they are discovered. The involvement of metals in causing toxicity is confirmed by the expression of the genes for GPX and MT. The C. carpio obtained from Dal Lake exhibited greater immunological challenges and was more affected. Increased level of metallothionein levels in common carp due to nickel exposure [31].

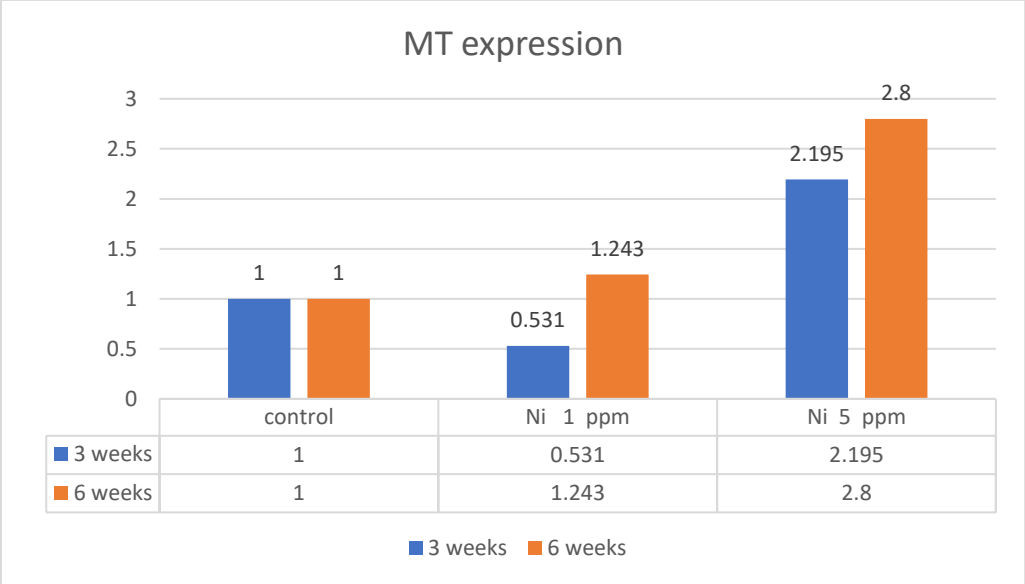

Figure 5: Effect of nickel on metallothionein expression after 3 and 6 weeks

Conclusion

The elevated expression of MT and GPX genes in the immune organs of *C. carpio* is an indispensable molecular and genotoxic biomarker. These various measurements can serve as valuable indicators for evaluating both the initial health status and physiological condition of aquatic organisms. Additionally, they may be instrumental in detecting alterations in the immune function of fish. Hence, they could be employed as sensitive tools in monitoring the effects of heavy metal-loaded effluents on aquatic ecosystems. Heavy metal intoxication causes immunotoxic and genotoxic changes. The findings could be used to develop standards for protecting water bodies from eco-toxicants. As a result, C. carpio is an appropriate biological indicator of water contamination.

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