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Research Article

Protective effect of curcumin-coated chitosan-alginate nanoparticles against oxidative stress induced by nickel oxide nanoparticles in the Gold fish (*Carassius auratus*)

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Abstract

Metal oxide nanoparticles are the most widely used compounds among nanoparticles, and the increasing use of nickel oxide (NiO) nanoparticles requires a better understanding of their toxic potential impact on various organisms. Although aquatic organisms are predicted to be the main targets of nanoparticles released into the environment, the toxicity of nickel (Ni), especially NiO nanoparticles, in aquatic organisms has not been well studied. This study aims to investigate the oxidant effect of NiO nanoparticles as well as the antioxidant effect of curcumin-coated chitosan-alginate nanoparticles in the Gold fish (*Carassius auratus*). In this study, five groups of fish (mean weight 8.65±0.45 g; mean length 5.58±0.33 cm) were used for experiments within 4-week treatments. The study groups were included: (i) control group, (ii) treatment group of NiO nanoparticles, and (iii) 3 treatment groups of NiO nanoparticles+ curcumin-loaded alginate-chitosan nanoparticles. At the end of the study period, liver samples were isolated and collected for oxidative damage. Total antioxidant levels, MDA, glutathione, and antioxidant enzymes–catalase (CAT), glutathione-S-transferase (GST), and superoxide dismutase (SOD) – were measured in all groups. The results showed that NiO nanoparticles decreased the level of total antioxidants while increased the level of MDA in the NiO nanoparticle treatment group, but the groups that received NiO nanoparticles + curcumin-loaded alginate-chitosan nanoparticles had better antioxidant parameters. These results showed strong evidence of induction of cellular oxidative stress due to exposure to NiO nanoparticles as well as the protective effect of curcumin-loaded alginate-chitosan nanoparticles.

Introduction

New scientific innovations in the field of engineered nanoparticles (<100 nm diameter in at least one dimension) have led to widespread applications in electronics, chemicals, environmental protection, medical imaging, disease diagnosis, cancer treatment, gene therapy, etc. Nanomaterials may enter the body of living organisms through the lungs (respiratory tract), skin (dermal contact), and gastrointestinal tract (gut) either intentionally or unintentionally. In recent years, water resources have received more attention to meet the nutritional demands of human societies. Aquatic organisms, as one of the human food sources, accumulate heavy metals in their bodies from water (bioaccumulation) and these substances are then transferred to higher levels and eventually to humans during the biological cycles. Metallic elements are found throughout the earth's crust and fish are directly exposed to them due to the high solubility of these compounds (Muyssen *et al.*, 2004). The most important metals in fish toxicology are aluminum, chromium, iron, nickel, copper, zinc, arsenic, cadmium, mercury, and lead. Nickel compounds are commonly considered to be genetic, immune, and carcinogenic toxins for living organisms. Nickel is widely distributed in the environment and its concentration depends on fossil fuels. Nickel absorption and accumulation in various organs lead to metabolic changes, tissue metal content disruption, and lipid peroxidation. Although nickel and its compounds are widely used as industrial catalysts, there is less known about its toxicity and pathological effects, particularly NiO nanoparticles, in aquatic organisms. Kovrižnych *et al*. (2014) studied the long-term (30-day) toxicity of NiO nanoparticles for adult zebrafish (*Danio rerio*). The results showed that the acute toxicity of NiO nanoparticles is low, but long-term contact with this compound can lead to its accumulation in the tissue and increase the toxicity. Nickel in plasma is transported by binding to albumin, α-2-macroglobulin, small polypeptides, and amino acids. The low molecular weight of the nickel-L-histidine complex makes it easy to cross the biological membrane (Paquin *et al.* 2000). This element causes damage by making irreversible connections with macromolecules and therefore disrupts the biological activity of cells. After entering the body, nickel can cause damage to various tissues including the liver, kidney, lung, and testis (Ezekwe and Edoghotu., 2015).

Nanotechnology utilizes the physiochemical and biological properties of substances in sizes less than 100 nm in various sciences and industries. Nanoparticles do not seem to have much difficulty in crossing physiological barriers in the body due to their extremely small size and therefore are effectively distributed through the vascular system in the tissues, especially the liver. The small size of nanoparticles plays an important role in the toxicity of these substances. Nanoparticles can pass through the cell membrane and enter the cell, causing direct cell damage. Nanoparticles have received a great deal of attention from researchers for their several applications over the past decade (Wells *et al.*, 2000). In nanoparticles, reducing the particle size increases the particle surface

area-to-volume ratio, sharply increasing the catalytic properties of metal nanoparticles known as the most important effect of this phenomenon. As the size of the particles decreases and their surface area-to-volume ratio increases, their biochemical reactivity increases, leading to an increase in the production of free radicals. Kazemian and Bakhshi (2020) indicated that zinc (Zn) nanoparticles in aqueous environments with high concentrations and increasing the duration of exposure to these nanoparticles increase oxidative stress and have inappropriate effects on liver enzymes and these parameters undergo severe changes. Smirnova *et al.* (2023) have shown that curcumin protects against metal-induced lipid peroxidation and mitigates adverse effects on the antioxidant system. Also showed that curcumin imparts promising metal toxicity-ameliorative effects that are related to its intrinsic antioxidant activity.
Mohammed *et al.* (2023) reported that nano-chitosan and nano-sodium alginate were evaluated as edible coating to improve the microbiological quality of Ras and soft white cheeses during ripening. Just and Slater (2024) rvaluated the Potential of alginate, chitosan and polyethylene glycol as substances for colloidal drug delivery as determined by protein release and digestion. Fabrikov *et al*. (2024) researched antimicrobial and antioxidant activity of encapsulated tea polyphenols in chitosan/alginate-coated zein nanoparticles: a possible supplement against fish pathogens in aquaculture.

Nowadays, the literature globally focuses on considering the imbalance in producing and eliminating free radicals and disrupting the natural redox of body cells, which causes oxidative damage and several diseases in organisms. For example, clinical research has shown that compounds with antioxidant activities have promising impacts in reducing the consequences of reduced cellular disorders due to various diseases, including diabetes, neuropathy, and retinopathy. Therefore, the aim of this research is to investigate the cytotoxic effect of nickel nanoparticles on biochemical parameters as well as the protective effect of chitosan-alginate nanoparticles containing curcumin in the goldfish, which can open a new idea in relation to the challenges in aquatic environments.

Materials and methods

Synthesis of chitosan-alginate nanoparticles

To prepare the alginate solution, 0.06 g of alginate powder was initially dissolved in 20 mL ofDW. The alginate solution must be mixed several times with a magnetic stirrer to dissolve well. The resulting solution can then be brought to the desired pH using a pH meter. To that end, acidic (CH_3COOH) or basic (NaOH) solutions are dropwise added to the prepared alginate solution until the pH meter shows pH=5.1.

First, 0.0064 g of chitosan powder was dissolved in acetic acid solution to prepare chitosan nanoparticles. About 100 µL of glycolic acetic acid was added to 10 mL of distilled water to prepare the acetic acid solution, and about 8 mL of which was dissolved in chitosan powder. Then the obtained solution can be brought to the desired pH (pH=5.4).

To produce curcumin-loaded alginate-chitosan nanocapsules, 0.06 g of alginate

was first dissolved in about 20 mL of distilled water and then brought to a pH of 5.1. Then 0.0064 g of chitosan powder was dissolved in 8 mL of 1% acetic acid solution. Moreover, the pH of the resultant solution was calibrated to be 5.4, and also about 0.0134 g of calcium chloride salt was dissolved in 4 mL of distilled water. To prepare nanocapsules, the alginate solution was first poured into a beaker and with a magnet was placed on a stirrer. The calcium chloride solution was then added dropwise to the alginate solution for about 30 min at 500 rpm. After that, about 500 mg of curcumin was added to the chitosan solution, and the chitosan containing curcumin was added dropwise to them for about an hour and finally placed on the stirrer for 1 h until it was well encapsulated. The resultant solution was then centrifuged at 12,000 rpm for 15 min, and after centrifugation, the supernatant and jelly-like deposit were easily separated and used for subsequent steps.

Studied groups

The commercial feed available to the fish was used for 2 weeks before the start of the experiment to adapt *Carassius auratus* (*C. auratus*) to the diets and laboratory. After this adaptation period, 5 continuously aerated aquariums were used for experiments, in each of which 12 goldfish were placed. Water temperature and pH in this experiment were 20.2±0.5 and 8.2±0.4, respectively, and were kept completely constant during the experiment.

Nickel and curcumin nanoparticle treatments were performed over 4 weeks. The treatments included:

1. Control group; fish received no treatment and were kept in the same environmental conditions as the treatment groups.
2. Fish received nickel nanoparticles for 4 weeks.
3. Fish received nickel nanoparticles + curcumin-loaded alginate-chitosan nanoparticles for 4 weeks (Treatment 1).
4. Fish received only curcumin-loaded alginate-chitosan nanoparticles for the first 2 weeks, and received nickel nanoparticles + curcumin-loaded alginate-chitosan nanoparticles for 2 weeks later (Treatment 2).
5. Fish received nickel nanoparticles + curcumin-loaded alginate-chitosan nanoparticles for the first 2 weeks and received only curcumin-loaded alginate-chitosan nanoparticles for 2 weeks later (Treatment 3).

At the end of the period, liver samples were quickly isolated and collected to investigate oxidative damage and stored in the freezer until testing.

Tissue homogenization

A homogenizer was used to homogenize the liver tissue of fish in this study. After weighting liver tissues, they were transferred to a Falcon tube and 1 mL of Tris-HCl buffer with a pH of 7.4 was then added to every 100 mg of tissue. After homogenization, they were placed inside the centrifuge at 12,000 rpm and a temperature of +4°C for 20 min. Afterward, the above transparent liquid was separated and kept in the freezer at -20°C for antioxidant analysis until the experiments.

Measuring total antioxidant levels

In this study, the level of total antioxidants in the studied groups was measured using the FRAP technique (antioxidant power of samples to reduce ferric to ferrous (Benzie and Strain., 1996). Through this technique, the sample antioxidant levels were measured according to their power in the reduction of ferric ions to ferrous ions. The main material used in the FRAP technique is called TPTZ. This compound has a strong affinity for bonding with ferric ions and therefore forms the Fe^{3+}–TPTZ complex (Fig. 1). The complex is naturally colorless, but if the ferric ion in the solution is reduced to the ferrous ion by the antioxidants, the complex will quickly turn blue. The total antioxidant activities were estimated by measuring the intensity of this dye with spectrophotometer at 593 nm.

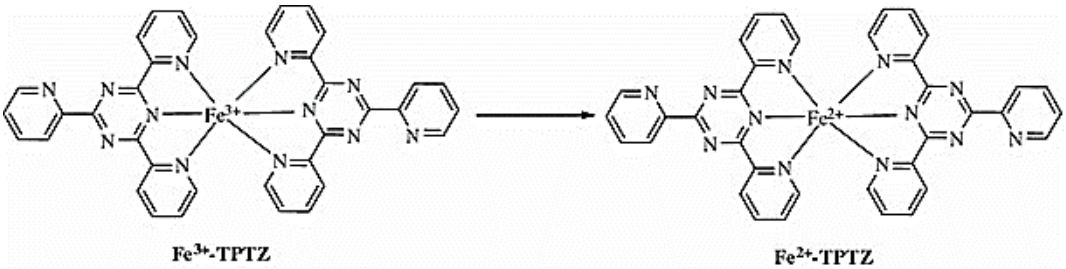

Figure 1: Reduction of ferric ion to ferrous in FRAP technique.

Measurement method

About 50 µL of the sample was added to 1.5 mL of the FRAP working solution and placed in the water bath at 37°C for 10 min. The absorbance of the samples were read by spectrophotometry at 593 nm. It should be noted that the blank solution also contains 1 mL of FRAP-ready solution plus 50 µl of distilled water. To calculate the value of total antioxidants in the studied samples, the standard diagram of $FeCl_2.4H_2O$ was used and reported in µmol/L.

Measuring malondialdehyde levels

Malondialdehyde (MDA) levels are measured as an appropriate indicator to evaluate the oxidative status. The main primary products of lipid peroxidation, such as LOOH, are unstable compounds that break down during several stages of chemical reactions to other secondary compounds, such as aldehydes and ketones. Since MDA is more stable than other aldehyde compounds, it is used as a proper diagnostic tool to measure lipid peroxidation. The thiobarbituric acid (TBA) method is the most common and simplest method used to measure MDA under high-temperature conditions (approximately 100°C) and acidic media. The reaction of MDA with TBA produces 2 mol of H_2O and 1 mol of MDA-TBA. The MDA-TBA complex has a pale pink color that can be seen with the naked eye (Zeb and Ullah, 2016) (Fig. 2).

MDA levels were measured in the studied groups using a spectrophotometric method according to the Buege and Aust method (Buege and Aust,1978). MDA reagents include thiobarbituric acid (TBA),

trichloroacetic acid (TCA), and hydrochloric acid (HCL) in distilled water.

Measurement method

About 50 µL of the sample was added to 1950 µL of MDA reagent and placed in 100°C water for 15 min. After cooling, the solution was centrifuged at 2500 g for 10 min. Then 1 mL of the supernatant was removed from the centrifuged sample and its absorbance was read at 535 nm. Furthermore, the blank solution containing 50 µL of distilled water and 1950 µL of MDA reagent were mixed and tested as above. MDA concentration was calculated using the extinction coefficient of 156 $mM^{-1}Cm^{-1}$ according toBeer–Lambert law (A=εdc) and was reported as nmol/mL.

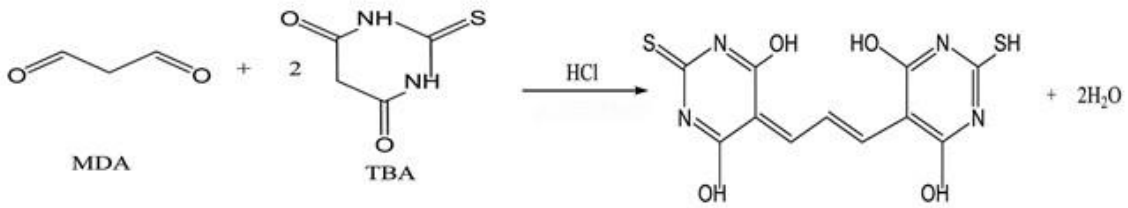

Figure 2: Reaction of malondialdehyde with TBA reagent.

Measuring catalase activity

This enzyme was assayed according to Aebi (1984) method by spectrophotometry using a hydrogen peroxide substrate. The buffer used in this experiment was 50 mM potassium phosphate.

Measurement method

The reaction was performed in a 1 mL cuvette. The volume of the reaction mixture was 1 mL containing 332 µL of 30 mM hydrogen peroxide, 666 µL of phosphate buffer, and 2 µL of sample. The reaction was started by adding a substrate and the adsorption variations were measured for 1 min at 240 nm and 25°C. Enzyme activity per unit time per mL of sample was calculated according to the following equation 1. By definition, a unit of catalase is an enzyme that decomposes 1 µmol of H_2O_2 in 1 min at 25°C.

$$U/gHb = \frac{\Delta OD \times Vt}{\varepsilon \times Vs \times C} \quad (1)$$

Where, ΔOD = absorption coefficient variations per min; Vs=sample value; Vt = total reaction volume; C = protein concentration (mg/mL); ε=absorption coefficient. The absorption coefficient used in this experiment is related to H_2O_2, which is 39.4 $M^{-1}Cm^{-1}$.

Measuring glutathione-S-transferase activity

Glutathione-S-transferase activity was measured by a modified method of Habig *et al*. (1974). Potassium phosphate buffer with a concentration of 100 mM and pH of 5.6 was used in this method.

Measurement method

The final solution for absorbance reading by spectrophotometer contains 2850 mL of potassium phosphate buffer, 50 µL of homogenized sample, and 50 µL of GSH, which after blanking of 50 µL of CDNB solution was added to the sample cuvettes. The sample adsorption variations were measured at 340 nm and 25°C for 3 min and then the adsorption variations per minute

were calculated. The specific activity of the enzyme was calculated using the molar absorption coefficients of 1-chloro-2&4-dinitrobenzene at pH = 6.5 based on the wavelength used which is equivalent to 9.6 $mM^{-1}cm^{-1}$ and was expressed in U/mL. The amount of enzyme activity was calculated according to the following equation 2:

$$U/ml = \frac{\Delta OD \times Vt}{\varepsilon \times Vs} \qquad (2)$$

Measuring myeloperoxidase activity

Method of Bradley *et al*. (1982) was used to measure myeloperoxidase activity in the studied groups.

Measurement method

Dianisidine reagent and H_2O_2 were reached to 3 mL with phosphate buffer in cuvette. After blanking the spectrophotometer, the sample was added to the cuvette containing the assay reagent. The adsorption variations at 460 nm were then read for 2 min and those were used to calculate the enzyme activity. It should be noted that the blank solution was used instead of the sample containing phosphate buffer. The amount of enzyme activity was calculated according to the following equation 3:

$$U/L = \frac{\Delta OD \times Vt}{\varepsilon \times Vs} \qquad (3)$$

In this experiment, the adsorption coefficient of dianisidine was used to calculate the activity of the enzyme, the value of which is considered to be 11.3 $mM^{-1}Cm^{-1}$.

Measuring glutathione

Tietz method was used to measure glutathione (Paglia and Valentine, 1967). For this experiment, 100 mM potassium phosphate buffer was used. The standard glutathione solution was prepared in concentrations of 12.5 to 200 µM and used to draw the standard glutathione diagram. Glutathione concentration was calculated in µM mg protein.

The used solutions:

The steps for the preparation of 0.1 M potassium phosphate buffer (pH=7.5) are as follows:

a. 1.79 g of sodium dihydrogen phosphate was dissolved in 50 mL of distilled water.
b. 0.38 g of potassium dihydrogen hydrogen phosphate was dissolved in 50 mL of distilled water.
c. The above two solutions were poured into a beaker with dissolved in a ratio to reach pH = 7.5.
d. 100 mL of 0.1 M potassium phosphate buffer+0.234 g of EDTA was used.
e. 0.01189 g of DTNB was dissolved in 5 mL of distilled water.
f. 5 mL of solution (e) was added to 33 mL of solution (d).

Measurement method

A mixture of 1520 µL of solution (f) with 100 µL of distilled water was prepared and the spectrophotometer was zeroed. To read the standard absorption of 12.5-200, 1520 µL of solution (f) and 100 µL of standard solution were added to each tube and the absorbance was read at 412nm. About 100 µL of diluted TCA reagent (solution e) was added to 100 µL of the liver sample, and after vortex, the sample was centrifuged at 10,000 rpm for 3 min. 1520 µL (solution e) was added to 100 µL of the supernatant and read at 412 nm (Fig. 3).

Measuring superoxide dismutase

This enzyme was assayed according to method of Sun *et al*. (1988).

Measurement method

The final volume of the reaction mixture was 3 mL. The final concentrations of the materials used in the reaction were 0.1 mM xanthine, 0.1 mM EDTA, 50 mg/L bovine serum albumin, 25 µM NBT, 9.9 nM xanthine oxidase, and 40 mM Na_2CO_3 (pH=10.2). In each tube, 2.45 mL of SOD assay reagent (prepared above) and 0.5 mL of liver sample or water as blank and standard Cu-ZnSOD solution with concentration of 25 ng were added.

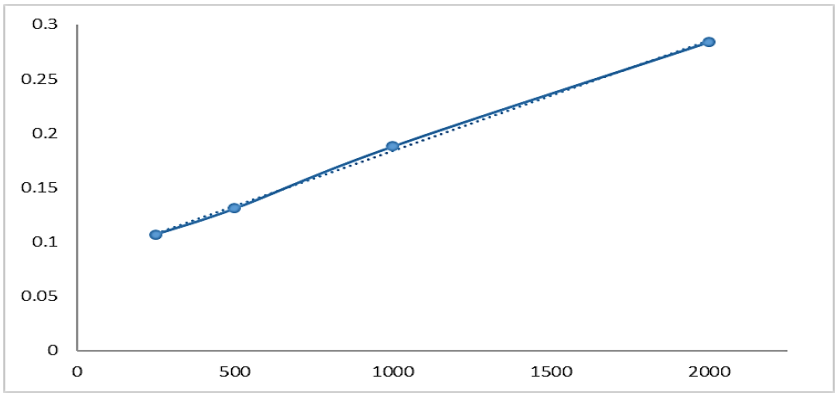

Figure 3: Glutathione standard diagram.

The reaction was started by adding 50 µL of xanthine oxidase solution to the tubes placed in a 25°C water bath. After 20 min, by adding 1 mL of 0.8 mmol/L $CuCl_2$ solution to each tube, the reaction was stopped and the changes in formazan adsorption formed at 560 nm were read and the inhibition percentage was calculated as follows 4:

$$\text{Inhibition\%} = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100\% \quad (4)$$

The standard inhibition of 25 ng SOD solution was used to calculate, in which one unit of SOD is the amount of protein that inhibits NBT reduction by up to 50%. The normality of data was first examined using the Shapiro-Wilk test. The data were statistically analyzed using SPSS software.

Data analysis

The results of the experiments were analyzed using SPSS software. ANOVA test was used to analyze the mean difference of quantitative results between the respective groups. The activity diagram of each enzyme was plotted by Excel software. Tukey post hoc test was used to compare the studied groups.

Results

Effect of NiO and curcumin nanoparticles on total antioxidant levels

The total antioxidant level in the study groups was measured using the FRAP method and the results are shown in Figure 4. This value was 5.76±0.71 in the control group, 3.00±70.59 in the NiO nanoparticle group, 5.10±0.75 in Treatment 1, 5.51±0.51 in Treatment 2, and 5.00±32.50 in

Treatment 3. According to the ANOVA test, it was found that there is a significant difference between the studied groups at the significance level of $p<0.001$. According to the Tukey post hoc test, it was found that the level of total antioxidants in the group treated with NiO nanoparticles had a significant decrease compared to the control group ($p<0.001$). In Treatment 1, which received NiO nanoparticles and curcumin nanoparticles simultaneously, the level of total antioxidant was lower than in the control group, but it was not significant ($p>0.001$). In Treatment 2 and 3, which simultaneously received curcumin nanoparticles but NiO nanoparticles at the same time for a shorter period, the level of total antioxidant did not show a significant difference with the control group group ($p<0.001$).

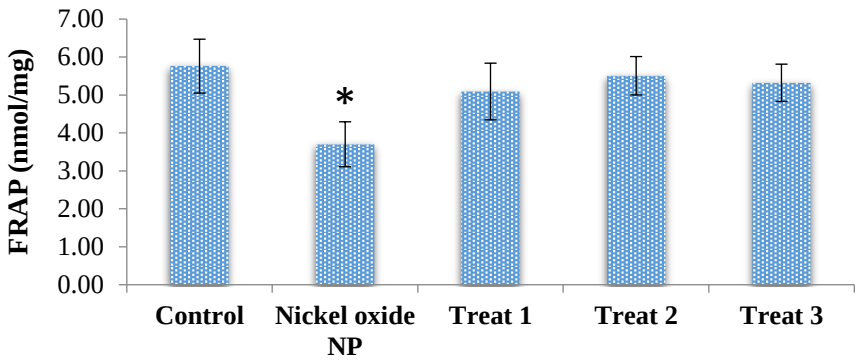

Figure 4: Total antioxidant levels in the study groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

Effect of NiO and curcumin nanoparticles on MDA level

MDA levels were measured in the study groups and the results are shown in Figure 5. The level of MDA was 3.36±0.59 nmol/mg in the control group, 5.91±0.40 in the group treated with only NiO nanoparticles, 4.02±0.49 in Treatment 1, 3.77±0.55 in Treatment 2, 3.85±0.37 in Treatment 3. According to the ANOVA test, it was found that there is a significant difference between the studied groups at the level of $p<0.001$. According to the Tukey post hoc test, it was found that the level of MDA in the group treated with NiO nanoparticles had a significant increase compared to the control group. In Treatment 1, which received NiO nanoparticles and curcumin nanoparticles simultaneously, the level of MDA showed a significant decrease compared to the group treated with NiO nanoparticles group ($p<0.001$).

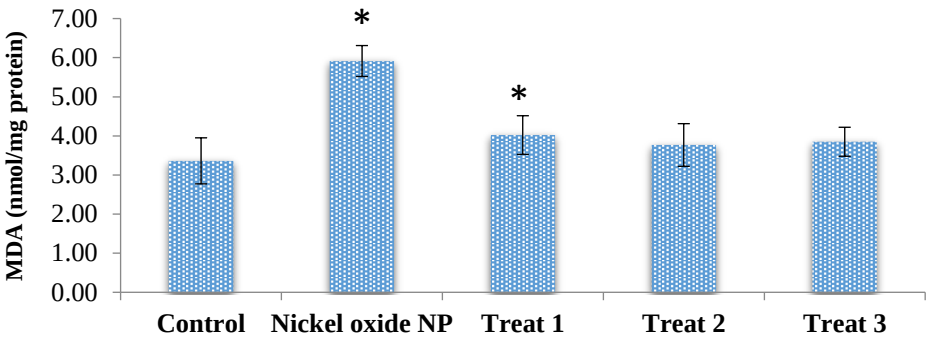

Figure 5: MDA level in the study groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

Although the MDA level of this group showed a significant decrease compared to the group treated with NiO nanoparticles, but still had a higher level compared to the control group (p <0.001). In Treatment 2 and Treatment 3, which received curcumin nanoparticles simultaneously with NiO nanoparticles in a shorter duration, the level of MDA showed a significant decrease compared to the group treated with NiO nanoparticles but did not show a significant difference compared to the control group. In other words, in two groups that were exposed to NiO nanoparticles for a shorter period, lipid oxidation was more effectively inhibited by curcumin nanoparticles.

Effect of NiO and curcumin nanoparticles on GSH levels

GSH levels were measured in the study groups and the results are shown in Figure 6. The level of GSH was 23.38±1.35 in the control group, 15.45±1.15 in the group treated with NiO nanoparticles, 17.10±41.37 in Treatment 1, 22.02±1.51 in Treatment 2, and 21.93±1.55 in Treatment 3. According to the ANOVA test, it was found that there is a significant difference between the studied groups at the significance level of <0.001. According to the Tukey post hoc test, it was found that GSH levels in the group treated with NiO nanoparticles had a significant decrease compared to the control group. In Treatment 1, which received NiO nanoparticles and curcumin nanoparticles simultaneously, GSH levels showed a significant increase compared to the group treated with NiO nanoparticles. Although the GSH level of this group showed a significant increase compared to the group treated with NiO nanoparticles, it still had a lower level compared to the control group and even Treatment 2 and Treatment 3 (p<0.001). In Treatment 2 and Treatment 3, which simultaneously received curcumin nanoparticles but NiO nanoparticles with a shorter duration, the level of GSH also increased compared to the group treated with NiO nanoparticles but did not show a significant difference compared to the control group. In other words, in two groups that were exposed to NiO nanoparticles for a shorter period, the reduction of GSH level was more effectively inhibited by curcumin nanoparticles.

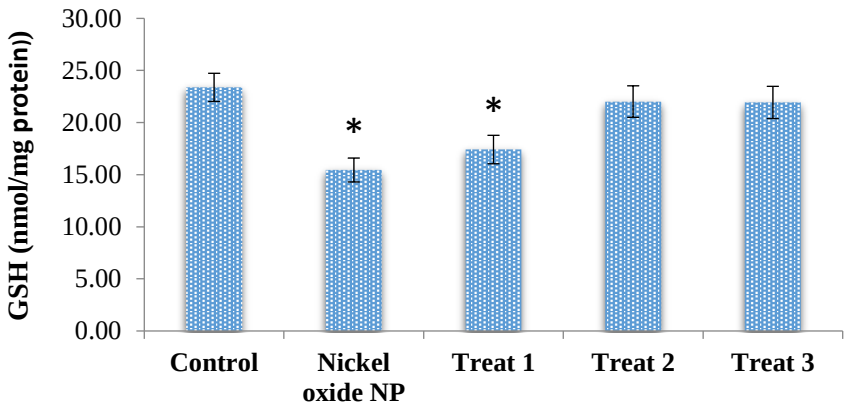

Figure 6: GSH levels in the study groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

Effect of NiO and curcumin nanoparticles on catalase activity

Catalase activity was measured in the study groups and the results are shown in Figure 7. This value was 23.03±1.80 in the control group, 16.22±1.42 in the group treated by NiO nanoparticle, 20.20±99.02 in Treatment 1, 22.43±2.20 in Treatment 2, and 22.10±1.96. According to the ANOVA test, it was found that there is a significant difference between the studied groups at $p<0.001$. According to the Tukey post hoc test, it was found that catalase activity in the group treated with NiO nanoparticles had a significant decrease compared to the control group. In Treatment 1, which received NiO nanoparticles and curcumin nanoparticles simultaneously, catalase activity was lower than the control group, but this reduction was not significant. In Treatment 2 and Treatment 3, which received curcumin nanoparticles simultaneously but NiO nanoparticles for a shorter period, the catalase activity did not show a significant difference with the control group. Finally, treatment groups 1, 2, and 3 had significantly more catalase activity than fish that received only NiO nanoparticles.

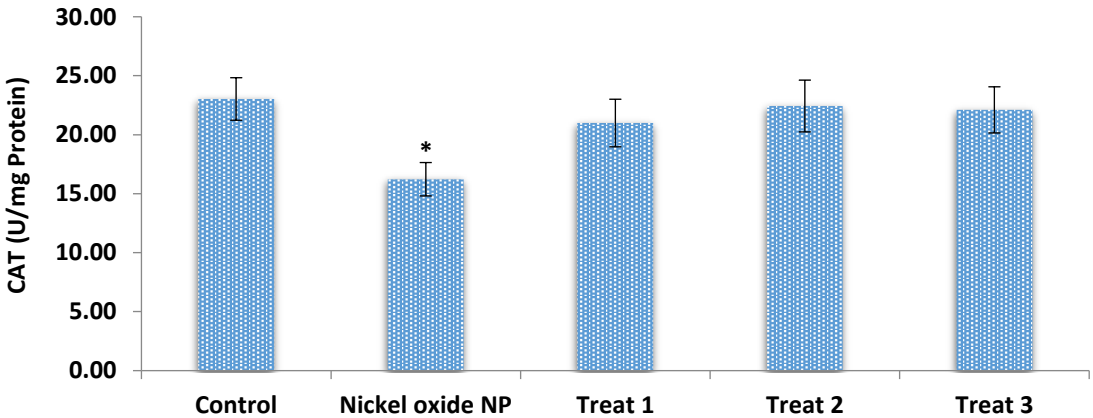

Figure 7: Catalase activity in the study groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

Effect of NiO and curcumin nanoparticles on myeloperoxidase activity

Myeloperoxidase activity was measured in the study groups and the results are shown in Figure 8. This value was 46.87±3.23 in the control group, 22.71±2.75 in the group received NiO nanoparticle, 45.31±2.71 in Treatment 1, 44.20±2.79 in Treatment 2, 45.83±2.05 in Treatment 3. According to the ANOVA test, it was found that there is a significant difference between the studied groups at the level of $p<0.001$. According to the Tukey post hoc test, it was found that myeloperoxidase activity in the group treated with NiO nanoparticles had a significant decrease compared to the control group. In Treatment 1, which received NiO nanoparticles and curcumin nanoparticles simultaneously, myeloperoxidase activity was lower than the control group, but this reduction was not significant. In Treatment 2 and Treatment 3, which simultaneously received curcumin nanoparticles but NiO nanoparticles for a shorter period, myeloperoxidase activity did not show a significant difference with the control group. Finally, treatment groups 1, 2, and 3 had significantly higher myeloperoxidase activity than fish that received only NiO nanoparticles.

Effect of NiO and curcumin nanoparticles on GST activity

GST activity was measured in the study groups and the results are shown in Figure 9. The level of GST was 329.63±30.80 in the control group, 561.30±3195 in the group treated with NiO nanoparticles, 520.47±33.38 in Treatment 1, 365.88±24.12 in Treatment 2, and 505.43±47.21 in Treatment 3. According to the ANOVA test, it was found that there is a significant difference between the studied groups at the level of $p<0.001$. According to the Tukey post hoc test, it was found that GST levels in the group treated with NiO nanoparticles had a significant increase compared to the control group. In Treatment 1 and Treatment 3, which received NiO nanoparticles and curcumin nanoparticles simultaneously, the GST level showed a significant increase compared to the control group but showed a significant decrease compared to the group treated with NiO nanoparticles ($p<0.001$). In Treatment 2, which simultaneously received curcumin nanoparticles but NiO nanoparticles with a shorter duration, the level of GST also decreased compared to the group treated with NiO nanoparticles but did not show a significant difference compared to the control group. In other words, in Treatment 2, which was exposed to NiO nanoparticles for a shorter period, the change in GST activity was more effectively inhibited by curcumin nanoparticles.

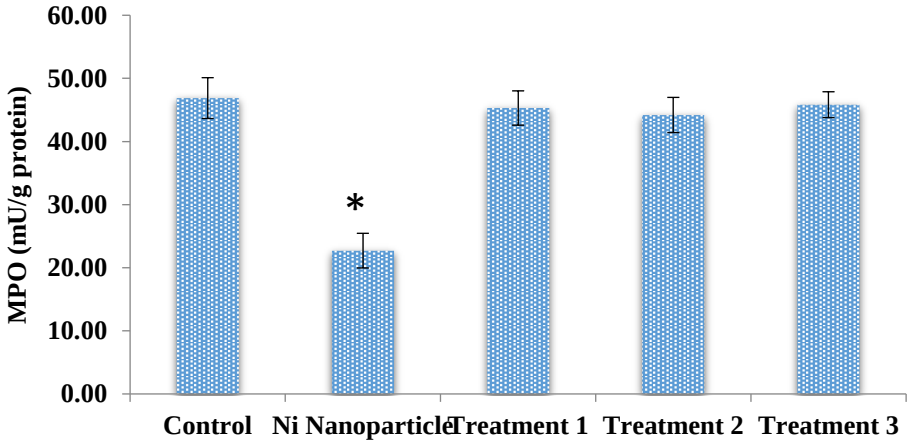

Figure 8: Myeloperoxidase activity in the study groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

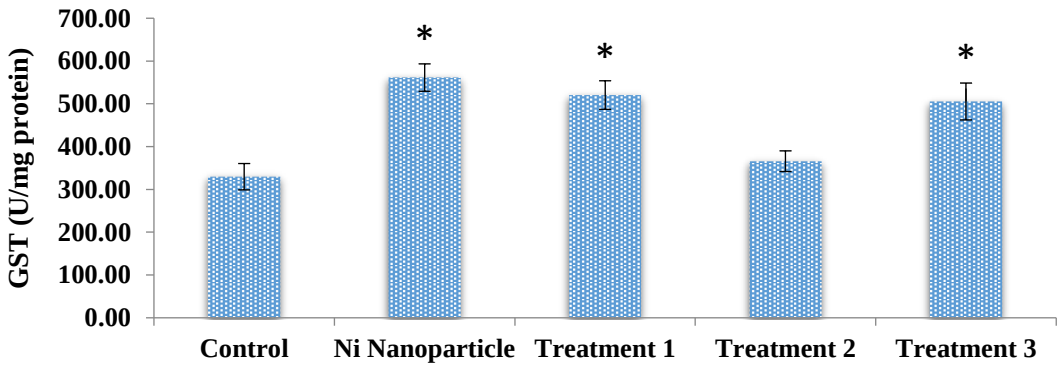

Figure 9: GST activity in the studied groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

Effect of NiO and curcumin nanoparticles on SOD activity

SOD activity was measured in the study groups and the results were presented in Figure 10. The level of SOD was 65.84±3.27 in the control group, 100.38±8.18 in the group treated with NiO nanoparticles, 88.06±6.82 in Treatment 1, 69.61±4.20 in Treatment 2, and 74.16±6.10 in Treatment 3. According to the ANOVA test, it was found that there is a significant difference between the studied groups at $p<0.001$. According to the Tukey post hoc test, it was found that SOD activity in the group treated with NiO nanoparticles showed a significant increase compared to the other groups. In Treatment 2, which received NiO nanoparticles with less period and curcumin nanoparticles at the same time, SOD activity did not show a significant difference compared to the control group and Treatment 3, but showed a significant decrease compared to other groups ($p<0.001$). In Treatment 1, which simultaneously received curcumin nanoparticles but NiO nanoparticles with a shorter duration, SOD activity also decreased compared to the group treated with NiO nanoparticles but did not show a significant increase compared to the other groups. In other words, in Treatment 2, which was exposed to NiO nanoparticles for a shorter period, the change in SOD activity was more effectively inhibited by curcumin nanoparticles (Table 1).

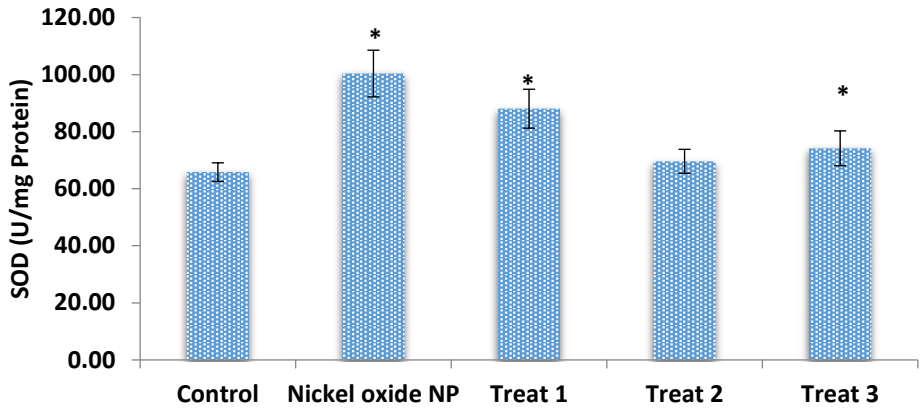

Figure 10: SOD activity in the study groups. The presence of a star in the column indicates a significant difference between the investigated treatments.

Table 1: Comparison of related studies conducted by different researchers.

Number	Researchers	Year	Research content	Result
1	Kovrižnych *et al*.	2014	studied the long-term (30-day) toxicity of NiO nanoparticles for adult zebrafish (*Danio rerio*).	The acute toxicity of NiO nanoparticles is low, but long-term contact with this compound can lead to its accumulation in the tissue and increase the toxicity.
2	Kazemian and Bakhshi	2020	Studied the effect of Zn nanoparticles in aqueous environments with high concentrations in Koi (*Cyprinus carpio).*	Increasing the duration of exposure to these nanoparticles increase oxidative stress and have inappropriate effects on liver enzymes and these parameters undergo severe changes.
3	Mohammed *et al.*	2023	reported that nano-chitosan and nano-sodium alginate were evaluated as edible coating to improve the microbiological quality of Ras and soft white cheeses during ripening.	Nano-chitosan showed maximum antibacterial and antifungal activities with an inhibition zone of 29 mm for *Staphylococcus aureus* and 12 mm for *Aspergillus niger*.
4	Just and Slater	2024	They rvaluated the Potential of alginate, chitosan and polyethylene glycol as substances for colloidal drug delivery as determined by protein release and digestion.	They reported that the feeding simulation trial showed that an oil-coated diet containing alginate-chitosan as a protective matrix can be used to protect the model protein during feeding (release to the water) and against the harmful milieu of the fish stomach.
5	Fabrikov *et al.*	2024	researched antimicrobial and antioxidant activity of encapsulated tea polyphenols in chitosan/alginate-coated zein nanoparticles: a possible supplement against fish pathogens in aquaculture.	These results supported encapsulation as a good strategy for tea polyphenols, as it allows maintaining significant levels of antioxidant activity and increasing the potential for antimicrobial activity, in addition to increasing protection against sources of degradation.

Discussion

The level of total antioxidants and the activity of various antioxidant enzymes in the liver tissue of fish exposed to NiO nanoparticles as well as the protective role of curcumin nanoparticles were investigated in this study. The effects of curcumin on hepatotoxicity due to environmental or occupational toxins have been confirmed and attributed to its inherent antioxidant, anti-inflammatory, anti-cholestatic, anti-fibrogenic, and anti-carcinogenic properties. Thus, curcumin protects the liver against damage and fibrogenesis by suppressing hepatitis, reducing hepatic oxidative stress, and increasing the expression of xenobiotic detoxification enzymes.

Since in the present study, NiO nanoparticles were provided to fish through the diet, it seems that the contaminant was absorbed from the intestine and reached various parts of the body as well as the liver through the bloodstream. The presence of these compounds in cells can increase the production of free radicals and eventually cell destruction and loss of normal function of the tissue or organ. The liver is an important organ in detoxifying compounds entering the body, so the liver is a tissue prone to oxidative damage induced by metals in aqueous media.

By directly interacting its thiol (-SH) group with ROS, glutathione can act as a non-enzymatic antioxidant, or it can be involved in the enzymatic detoxification reaction for reactive oxygen species as a cofactor or coenzyme because it is a cysteine-containing tripeptide that has reactive-SH with the ability to reduce. Therefore, glutathione can be considered as another marker of oxidative stress. Accordingly, the results also showed that liver tissue in fish exposed to NiO nanoparticles had a significant reduction in glutathione levels. This significant decrease in glutathione levels may be due to the ability of NiO nanoparticles to bind to the thiol group in glutathione and increase ROS production by this nanoparticle (Das *et al*., 2008).

The results of this study showed that the addition of curcumin nanoparticles to the diet of fish strengthens the antioxidant system of liver tissue and prevents the oxidation of macromolecules such as proteins and lipids. As shown in this study, the use of curcumin nanoparticles significantly prevented the increase in MDA levels observed in fish treated with NiO nanoparticles. Therefore, the addition of curcumin as nanocapsules to the diet contaminated with NiO nanoparticles resulted in the relative protection of liver tissue from damage caused by NiO nanoparticles. Such an improvement in the antioxidant level of fish liver tissue may indicate the protective role and antioxidant properties of curcumin nanoparticles.

SOD is an enzyme bound to oxyradicals and is responsible for dismutation of superoxide radicals to O_2 and H_2O_2. This enzyme is very sensitive to the stress caused by pollutants and can be used as a signal of oxidative stress for early warning of environmental pollution (Linhua *et al*., 2009).

The results of the present study showed that curcumin nanoparticles significantly inhibit the reduction of SOD activity. The

highest inhibitory effect on inhibition of SOD activity in treatment with NiO nanoparticles was observed in the groups that received curcumin nanoparticles as pretreatment. Since NiO nanoparticles result in the production of ROS in the liver, changes in SOD activity can indicate oxidative stress and tissue damage. The increase in SOD activity may be due to the synthesis of new enzymes or an increase in pre-existing enzyme levels.

CAT and peroxidase are key enzymes in antioxidant defense systems for converting H_2O_2 free radicals to water and oxygen. The catalase-peroxidase-SOD system provides the first defense against oxidative toxicity at the cellular level. Several studies have shown that the antioxidant defense system can develop significantly under a certain amount of stress at a given time. In this study, a changing trend was observed in the antioxidant content, so that a more inhibitory effect was observed in 4-week treatments of NiO nanoparticles compared to 2-week treatment with NiO nanoparticles.

Therefore, the more exposure time of NiO nanoparticles, the more destructive effects will occur. On the other hand, the use of curcumin nanoparticles effectively counteracted the oxidative damage so that the antioxidant activity was observed in the control group. The results showed that the protective effect of curcumin nanoparticles is enhanced by its pretreatment. It is predicted that curcumin nanoparticles may be able to counteract the oxidative damage caused by NiO nanoparticles in two ways: (i) scanning reactive oxygen species, or (ii) chelating NiO. In addition to its direct antioxidant activity, curcumin can act indirectly as an antioxidant by enhancing glutathione synthesis, which is known as a substrate for GPx.

Kazemian and Bakhshi (2020) indicated that zinc (Zn) nanoparticles in aqueous environments with high concentrations and increasing the duration of exposure to these nanoparticles increase oxidative stress and have inappropriate effects on liver enzymes and these parameters undergo severe changes. Smirnova *et al.* (2023) have shown that curcumin protects against metal-induced lipid peroxidation and mitigates adverse effects on the antioxidant system. Also showed that curcumin imparts promising metal toxicity-ameliorative effects that are related to its intrinsic antioxidant activity.

Mohammed *et al.* (2023) reported that nano-chitosan and nano-sodium alginate were evaluated as edible coating to improve the microbiological quality of Ras and soft white cheeses during ripening. Nano-chitosan showed maximum antibacterial and antifungal activities with an inhibition zone of 29 mm for *Staphylococcus aureus* and 12 mm for *Aspergillus niger*.

Just and Slater (2024) rvaluated the Potential of alginate, chitosan and polyethylene glycol as substances for colloidal drug delivery as determined by protein release and digestion. They reported that the feeding simulation trial showed that an oil-coated diet containing alginate-chitosan as a protective matrix can be used to protect the model protein during feeding (release to the water) and against the harmful milieu of the fish stomach. Different combinations of the investigated encapsulation substances can be used to achieve optimal encapsulation and

protective characteristics depending on the application objective.

Fabrikov *et al*. (2024) researched antimicrobial and antioxidant activity of encapsulated tea polyphenols in chitosan/alginate-coated zein nanoparticles: a possible supplement against fish pathogens in aquaculture. These results supported encapsulation as a good strategy for tea polyphenols, as it allows maintaining significant levels of antioxidant activity and increasing the potential for antimicrobial activity, in addition to increasing protection against sources of degradation.

It should be noted that the combination of curcumin nanoparticles with NiO nanoparticles in the diet reduces the severity of oxidative tissue damage to the liver of fish in experimental groups (Treatments 1, 2, and 3) compared to the NiO treatment. As well as, by comparing Treatments 1, 2, and 3, it can be concluded that long-term exposure to NiO nanoparticles has intensified oxidative damage to the liver tissue of fish. In other words, the group that was pretreated with curcumin nanoparticles and exposed to NiO nanoparticles for a shorter period, was well protected from oxidative damage.

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