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Inhibitory effect of nano selenium on the recurrence of *Aeromonas hydrophila* bacteria in *Cyprinus carpio*

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ABSTRACT

The recurrence of bacterial infection in fish is related to several factors, including the immune status and oxidative state. A total number of 240 *Cyprinus carpio* was divided into four treatments; control (T1) fed on free diet and three treatments (T2–4) fed on supplemented diet with nanoparticles of selenium Se NPs (1 mg/kg fish feed) for 10, 20, and 30 days, respectively. Supplemented fish with Se NPs for 30 days (T4) had significantly higher total protein and globulin content (5.83 and 3.1 g/dl, respectively) compared to the control indicating an enhanced immune status. Fish fed on Se NPs supplementation for 30 days (T4) recorded significantly higher anti-inflammatory cytokine "interleukin (IL)-10", while the pro-inflammatory cytokines "tumour necrosis factor (TNF)–α, IL-1β, and IL-8" reduced significantly compared to the control. Antioxidant enzymes (glutathione peroxidase GPx, catalase CAT, and superoxide dismutase SOD) were gradually and significantly enhanced in time-dependent manner. Genotoxicity (DNA damages, DNA fragmentation, and presence of micronuclei) was low in supplemented fish while challenged fish performed in an opposite manner. However, Se NPs supplementation had overcome those withdraws causing a decrease in the signs of genotoxicity. Se NPs improved the immunity, antioxidant, and histopathological status of *C. carpio* showing enhancements in disease resistance, as reducing the chance of recurrence of *Aeromonas hydrophila* infection and genotoxicity.

INTRODUCTION

Aquaculture produces about 42.2% of the global world's production of fish in 2016 which represents 50 % of the fish consumption**;** fish and fish products are the key to meet the need for protein concerning human nutrition in Egypt **(FAO, 2018).**

Selenium (Se) is a vital trace mineral that plays an important role in animal metabolism saving the antioxidant state and immune status (**Yu *et al.*, 2014; Biller-Takahashi *et al.*, 2015; Zhang *et al.*, 2017).** The nutritional requirements of selenium were estimated in different fish species; Nile tilapia (*Oreochromis niloticus*; 1.06–2.06 mg/kg) **(Lee *et al.*, 2016),** and the gibel carp (*Carassius auratus gibelio var.* CAS III; 0.73–1.19 mg/kg) **(Zhu *et al.*, 2017).** To optimize the antioxidative state of rainbow trout *(oncorhynchus mykiss)* fry, the supplementation of Se was recommended to be added to the plant-based diets **(Fontagné-Dicharry *et al.* 2015).** Under stress conditions, damages of the cell membrane, protein, and DNA of the animal cells are due to the formation of free radicals **(Halliwell, 2006; Kumar *et al.*, 2018).** As a part of antioxidant enzymes, Se protects the cell membranes and cellular components from the impacts of oxidative stresses **(Liu *et al* 2004).** Se nanoparticles (Se NPs) is less toxic and safer than inorganic Se **(Forootanfar *et al.*, 2014; Kumar *et al.* 2018),** it also enhances the immune responses and antioxidant activity in common carp *(Cyprinus carpio)* **(Saffari *et al.*, 2018)** and *O. mykiss* **Kohshahi *et al.* (2019).**

Fish pathologists are using tremendous amounts of antibiotics and disinfectants to control fish diseases that resulted in the emergence of antibiotic-resistant bacteria **(Abutbul *et al.*, 2004; Sherif *et al.*, 2021a).** Additionally, Se NPs supplementation enhances fish resistance against bacterial infection; **Xia *et al.* (2019)** noticed that zebrafish *(Danio rerio)* fed on Se NPs at a dose of 10 μg/g had a higher survival rate compared to the control group after challenging against *A. hydrophila.*

To study the genotoxicity of any material in water and/or fish, chromosomes are the best choice **(Parveen & Shadab 2012)** and the comet assay is a reliable test to confirm DNA damage **(de Andrade *et al.*, 2004).**

The purpose of this study is to assess the potential role of Se NPs in decreasing the recurrence of bacterial infection in *C. carpio* through studying the influence of Se NPs on the immunity, antioxidant activity, and genotoxicity besides a trial of isolation of pathogenic bacteria from survived fish after different periods.

MATERIALS AND METHODS

2.1 Fish sampling, accommodation, and experimental design

A total number of 260 *Cyprinus carpio* (5 ± 0.1 g b.w.) was collected from a local private freshwater fish farm at Tolompate 7 Village, Kafrelsheikh Governorate, Egypt, and were stocked in the wet laboratory of Animal Health Research Institute (AHRI). Fish were acclimated in a fibre-glass tank (3 × 1.5 × 1 m) for two weeks. Afterwards, 20 fish specimens were subjected to clinical and post-mortem analyses following the methods described by **Austin and Austin (2012)** to make sure that the fish were free of any diseases. Fish (n=240) were divided equally and randomly into four treatments (T1– 4); each treatment had three replicates (20 fish / glass aquarium). The first presented the

control (T1) fed on a free diet, while the second (T2), the third (T3), and the fourth (T4) were fed on supplemented-Se NPs diet at a dose of 1 mg/kg fish feed for 10, 20, and 30 days, respectively. Fish were fed on a formulated diet (CP 38% and digestible energy 2954 Kcal/Kg) twice daily with 5% of their weights for six days per week. Water parameters of tank and glass aquaria (110 × 50 × 50 cm) were maintained in suitable condition for fish culture (temperature 28±1 °C, dissolved oxygen ≥ 5.5 mg/l, pH 7.8 and salinity ≤ 0.3 g/l), also one-third of water was daily exchanged with fresh water to keep water quality and remove solid discharges.

2.2 Source of nano-selenium spheres and diet preparation

Se NPs were manufactured following the methods described by **Zommara (2007)** and **Prokisch *et al.* (2008)** using lactic acid bacteria (LAB-Se, Lactomicrosel®). Briefly, Se NPs were manufactured from pure yoghurt cultures of *Streptococcus thermophilus* (CNCM I-1670) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (NCAIM B 02206)**.** The size of the obtained Se NPs was within the range of 100–500 nm, the characterization of Se NPs was performed using inductively coupled plasma mass spectrometer (ICP-MS) (X series, THERMO FISHER SCIENTIFIC, Germany) **(Eszenyi *et al.*, 2011; Prokisch *et al.*, 2011).**

Firstly, Se NPs were ultrasonically distributed in Milli-Q water (1 mg/ml) according to the procedure developed by **Lammel and Sturve (2018),** Thereafter, fish food (pellet form) was soaked and fully homogenated till paste formation. Then, the gelatine was added to feed past/ Se NPs mixture to improve feed consistency (Canal Aqua Cure, Egypt) and left to dry at room temperature then was evenly cut into small size.

2.3 Bacterial infection

After the feeding trial, fish (50 fish/treatment) was experimentally subjected to bacterial infection with *Aeromonas hydrophila* (AHRAS2, accession number in NCBI is MW092007. Fish was injected via intraperitoneal route with LD_{50} (2.4×10^5 CFU) according to methods described by **Schaperclaus *et al.* (1992).** The number of dead fish was recorded for 14 days, and cumulative mortality rate (CMR) was measured using the following equation:

CMR (%) = (number of deaths in a specific period ⁄ total population during that period) × 100.

After 14 days of bacterial infection, the survived fish was bacteriologically examined for *A. hydrophila.* Three attempts of bacterial isolation were performed with a week interval. Bacterial isolation was done using five fish randomly selected from each treatment group**.** Samples for bacteriological analyses were collected from skin lesions, kidneys, heart, liver, spleen, and gills and inoculated onto brain heart infusion agar (BHIB) and incubated at 28°C for 24 h (**Tonguthai *et al.*, 1999**). Pure colonies streaked onto Rimler's- Shotts medium (RS medium), *Aeromonas* selective agar base with ampicillin supplement, XLD media. All cultures were incubated at 28 °C for 24 h, and

characterization of isolated bacteria was recorded. Phenotypic characterization of the bacterial isolates was confirmed according to **Bergey (1994)**, **Elmer *et al.* (1997)** and **Madigan and Martinko (2005)**. All isolates were identified biochemically by using API 20E strips **(Bio-merieux, 1984**).

2.4 Cytokines of the experimental *C. carpio*

Interleukin 1β (IL-1β), TNF-α, and IL-10 were measured in the serum of *C. carpio* by ELISA (enzyme-linked immunosorbent assay) using a solid-phase sandwich ELISA test kit obtained from (My BioSource Co., San Diego, California, USA). The procedures were done following the manufacturer's instructions.

2.5 Total protein and globulin fractions

The concentration of serum total protein (TP) **(Weichsellbaum, 1946)** and albumin (ALB) **(Doumas *et al.*, 1971)** were measured by colorimetric methods. While serum globulin concentrations (GLO) were calculated by subtracting the concentration of TP from ALB concentration. The electrophoretic pattern of serum protein fractions was measured using polyacrylamide gel columns **(Maurer, 2011)**. Moreover, the gel was scanned and read according to **Glick (1968).**

2.6 The activity of antioxidant enzymes

The activity of glutathione peroxidase (GPx) (EC 1.11.1.9) in the liver of *C. carpio* was determined according to the method described by **Anderson and Greenwald (1985)**. The contents of reaction mixture were 1.44 ml of 0.05 M PBS (pH 7.0), 0.1 ml of 1 mM EDTA, 0.1 mM sodium azide, 0.05 ml of glutathione reductase (GR; 1 U/ml), 0.1 ml of 1 mM glutathione (GSH), 0.1 ml of 2 mM NADPH, 0.01 ml of 0.25 mM H_2O_2 and 0.1 ml of 10 % PMS in a total volume of 2 ml. The disappearance of NADPH was recorded using a spectrophotometer at 340 nm. Enzyme activity was expressed as nmol NADP reduced/min/ mg protein using a molar extinction coefficient of 6.22 × 103/M/cm. Catalase CAT (EC 1.11.1.6) activity in the liver of *C. carpio* was determined spectrophotometrically at 240 nm and calculated as μmol H_2O_2 decomposed/ mg protein/min following a method developed by **Anderson and Greenwald (1985).** Superoxide dismutase SOD (EC 1.15.1.1) activity in hepatic tissue was measured using the auto-oxidation principle of pyrogallol, which is inhibited in the presence of SOD. The optical density change was determined kinetically for 2 min at 420 nm, at 10-second intervals, according to the method mentioned by **Beutler (1984).** Activities of antioxidant enzymes were measured as U mg/protein.

2.7 Genotoxicity of the experimental *C. carpio*

2.7.1 The comet assay

Comet assay was performed referring to the procedure developed by **Blasiak *et al.* (2004)**. Hepatic cells of fish were mixed with low-melting-point agarose (ratio of1:10v/v), then pipetted to precoated slides with normal-melting-point agarose. The slides were kept flat at 4°C for 30 min in dark condition. The third layer of low melting point agarose was pipetted onto the slides and was left to solidify (4°C for 30 min). The

slides were shifted to pre-chilled lysis solution, kept for 60 min at 4°C, after that, slides were immersed in freshly prepared alkaline unwinding solution at room temperature in the dark for 60 min. Slides were exposed to electrophoresis run at 0.8 V/cm, 300mAmps at 4°C for 30 min. The slides were rinsed in neutralizing solution followed by immersion in 70% ethanol and then air-dried. The slides were stained with ethidium bromide and then visualized using Zeiss epifluorescence microscope (510–560 nm, barrier filter 590 nm) with a magnification power of ×400. 100 cells per fish were scored. DNA damages were analysed using software (Comet Score, TriTek corp., Sumerduck, VA22742; **Collins *et al*. (1997).**

2.7.2 DNA fragmentation assay

The rates of DNA fragmentation were done following the methods used by **Yawata *et al.* (1998)**. Briefly, hepatic tissues of *C. carpio,* treated with Se NPs and/ or infected with pathogenic bacteria, were homogenized in saline (0.09% v/v). The harvested cells (plus floating cells) were washed with Dulbecco`s phosphate-buffered saline then, cells were lysed using the lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), and 0.5% Triton X-100] for 30 min on ice. Lysates products were vortexed and cleared by centrifugation at 10,000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml ethidium bromide.

2.7.3 Micronuclei Test

The presence of micronuclei was confirmed according to the method described by **AL-Sabti and Metcalfe (1995)**. Briefly, the peripheral blood was obtained from the caudal vein of each fish, then smeared immediately after mixing with foetal calf serum and fixed in absolute methanol, and hence, dried in air. The slides were stained with Giemsa stain and observed under a microscope (Magnification 1000 X). Finally, the frequency of micronuclei was calculated in 2000 cells per individuals by using the following formula:

MN frequency (%) = (Number of cells with Micronuclei / Total number of cells counted) × 100

2.8 Histopathological Investigations

For histopathological analyses of experimental fish, samples were collected from three tissues liver, kidney, and spleen before and after the bacterial challenge test. Formalin-fixed paraffin-embedded sections were processed routinely for H&E staining according to methods described by **Suvarna *et al.* (2012).**

2.9 Statistical Analyses:

The impacts of Se NPs in *C. carpio* were statically analyzed with SPSS software for windows, SPSS Inc., Chicago, IL, USA **(SPSS 2004)** using analysis of variance (ANOVA). All values were expressed as the mean ± SE (standard error). Duncan's

multiple range test **(Duncan 1955)** was used to determine differences among treatments at a significance level of 0.05.

2.9 The applied biosafety measures

This study followed the biosafety measures concerning the pathogen safety data sheets: Infectious substances- *A. hydrophila,* Pathogen Regulation Directorate, **Public Health Agency of Canada (2010).**

RESULTS

During the experimental period, the water parameters were maintained in a suitable range for *C. carpio*; water temperature 28±1 °C, dissolved oxygen ≥ 5.6±0.5 mg/l, pH 8.2±0.6 and salinity ≤ 0.3 g/l.

3.1 Challenge experimental fish with *A. hydrophila* bacteria

A fifty *C. carpio* from each treatment was experimentally infected with LD_{50} of *A. hydrophia* to assess the immunostimulant properties of Se NPs. The survival of fish (Table 1) was gradually and positively increased with Se NPs. In Table (1), challenged *C. carpio* in T4, fed on Se NPs for 30 days resisted the bacterial infection and their survival rate was higher (37 out of 50) compared with the control (T1) (24 out 50 fish) ($p < 0.05$). A five *C. carpio* from each treatment was bacterially examined for the presence of *A. hydrophia* after a two-week period of the challenge test. The isolation rate of *A. hydrophia* was gradually decreased in a time-dependent of Se NPs supplementation. In T4, which received Se NPs for 30 days, *A. hydrophia* was not isolated after 4 weeks of infection.

Table 1: Mortality and infection rates of *C. carpio* Challenge with *A. hydrophila*

Item	T1 (Control)		T2 (10 day)		T3 (20 day)		T4 (30 day)	
	no.	%	no.	%	no.	%	no.	%
Fish	50	-	50	-	50	-	50	-
CMR	26	52	24	48	20	40	13	26
Sur.	24	48	26	52	30	60	37	74
Exa.	15	-	15	-	15	-	15	-
Inf. Post 3 weeks	3	60	3	60	3	60	2	40
Inf. Post 4 weeks	3	60	3	60	1	20	0	0
Inf. Post 5 weeks	2	40	1	20	1	20	0	0

Treatments; T1: control; T2: supplemented Se NPs 10 days; T3: supplemented Se NPs 20 days; T4: supplemented Se NPs 30 days. no.: fish number; CMR: cummlative mortality rate; Sur.: survived fish; Exa: examined fish in last 3 weeks; Inf: infection rate.

3.2 Alteration of inflammatory cytokines in experimental fish

As shown in Fig. (1), the cytokines were significantly affected with the addition of Se NPs to the *C. carpio* diet. Anti-inflammatory cytokine IL-10 was significantly increased (P< 0.05) in serum of *C. carpio* in T2, T3, and T4 treatments compared with the control 14.01, 15.51, and 19.89 pg/mg, respectively. Pro-inflammatory cytokines TNF –α, IL-1β, and IL-8 were significantly decreased with time in T2-T4 treatments compared to the control treatment (4, 1.55, and 3 pg/mg, respectively).

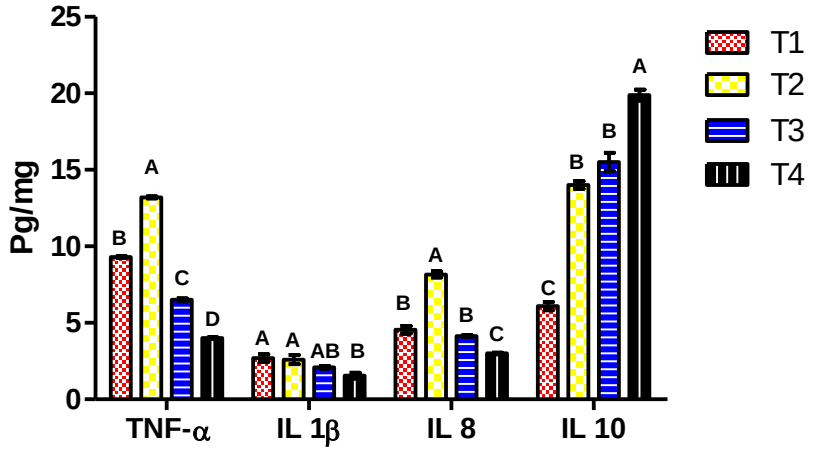

Fig. 1: Cytokines changes in serum of *C. carpio*.

Treatments; T1: control; T2: supplemented Se NPs 10 days; T3: supplemented Se NPs 20 days; T4: supplemented Se NPs 30 days. Different letters mean that treatments are significantly different at (P< 0.05). Values are expressed as the mean ± SE.

3.3 Serum protein and antioxidant enzymes changes

Serum total protein (TP), albumin (ALB), globulin (GLO), and GLO fractions (Alpha, beta, and gamma) were only and significantly improved (P< 0.05) in T4 in which fish fed on Se NPs supplementation for 30 days (Table 2). The control (T1) treatment differed insignificantly from (P< 0.05) treatments fed on a diet supplemented with Se NPs for 10 or 20 days.

Table 2: Total protein and globulin fractions in *C. carpio* blood.

Item	**T1** (Control)	**T2** (10 day)	**T3** (20 day)	**T4** (30 day)
TP (g/dl)	$4.3^{B} \pm 1.5$	$4.72^{B} \pm 0.06$	$4.6^{B} \pm 0.21$	$5.83^{A} \pm 0.27$
ALB (g/dl)	$2.17^{B} \pm 0.03$	$2.27^{B} \pm 0.09$	$2.16^{B} \pm 0.07$	$2.73^{A} \pm 0.12$
GLO (g/dl)	$2.13^{B} \pm 0.13$	$2.45^{B} \pm 0.05$	$2.43^{B} \pm 0.14$	$3.1^{A} \pm 0.17$
Alpha (g/dl)	$0.55^{B} \pm 0.03$	$0.61^{B} \pm 0.01$	$0.67^{B} \pm 0.08$	$0.95^{A} \pm 0.04$
Beta (g/dl)	$0.47^{B} \pm 0.07$	$0.53^{AB} \pm 0.02$	$0.48^{B} \pm 0.06$	$0.69^{A} \pm 0.05$
Gamma (g/dl)	$1.12^{B} \pm 0.04$	$1.3^{AB} \pm 0.09$	$1.29^{AB} \pm 0.1$	$1.47^{A} \pm 0.09$

Treatments; T1: control; T2: supplemented Se NPs 10 days; T3: supplemented Se NPs 20 days; T4: supplemented Se NPs 30 days. TP: total protein; ALB: albumin; GLO: globulin. Values are presented as the mean ± SE. Treatments with different letters within the same row are significantly different at P≤.05.

In Fig. (2), the antioxidant enzymes GPx, CAT, and SOD activity in the hepatic tissues of the experimental fish were significantly and gradually increased with the supplementation period of Se NPs, *C. carpio*. T4, fed on supplementation for 30 days, had a significantly higher antioxidant activity compared to the control fish.

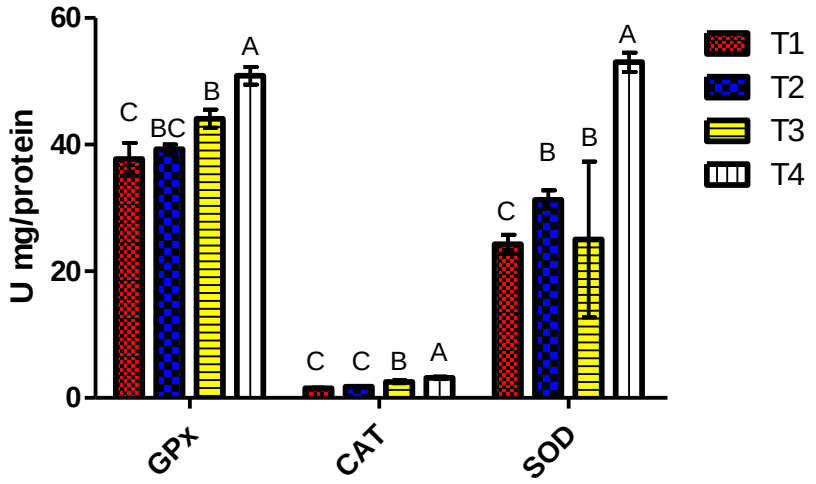

Fig. 2: Antioxidant activities in the liver tissues of C. carpio.

(n= 3/replicate) GPx: Glutathione peroxidase enzyme; CAT: catalase enzyme; SOD: Superoxide dismutase enzyme. Treatments; T1: control; T2: supplemented Se NPs 10 days; T3: supplemented Se NPs 20 days; T4: supplemented Se NPs 30 days. Different letters mean that treatments are significantly different at (P< 0.05). Values are presented as the mean ± SE.

3.4 Genotoxicity of *C. carpio* fed on Se NPs supplementation

Determination of the DNA damage in fish liver is summarized in Table (3) and Figs. **(**3, 4, 5). The results found that control liver and nano-Se liver tissues, treated groups at several time intervals (10, 20, and 30 days), were significantly decreased (P< 0.05) in DNA damage values compared to the positive control as exposed to bacterial infection. However, the DNA damage rates were significantly increased in the liver of fish treated with bacteria. On the contrary, the DNA damage rates were decreased significantly in the liver tissues exposed to bacterial infection and treated with Se NPs at different time intervals in which the 30 days treatment (T4) was the most effective time.

Determination of the DNA fragmentation rates in the fish liver is summarized in Table (4) and Figs. (3, 4). Results revealed that the DNA fragmentation rates were significantly increased (P<0.001) in liver of fish treated exaction with bacteria. On the contrary, the DNA fragmentation rates were significantly decreased in liver tissues

exposed to bacterial infection and treated with Se NPs at different time intervals in which the 30 days treatment was the most effective time.

The results of micronuclei in blood cells of *C.carpio* of different treatments are summarized in Table (5). According to the results, the micronuclei frequency in fishes treated with nano selenium at different times didn't show any significant difference comparing to the control except at day 30 which slightly increased more than the control. In fishes exposed to bacterial infection, the micronuclei frequency increased significantly compared to the control ($P \leq 0.05$). On the contrary, all treatments exposed to bacterial infection and treated with Se NPs at different time intervals (10, 20, and 30 days) were significantly decreased ($P < 0.05$) in micronuclei frequency values compared to the positive control exposed to bacterial infection in which the 30 days treatment was the most effective time.

Table 3: Visual score of DNA damage in hepatic tissues of *C. carpio* supplemented with Se NPs and/ or bacterial infection.

Treatment	No. of cells		Class**				DNA damaged cells % (Mean±SEM)
	Analyzed*	Comets	0	1	2	3	
Un infected fish							
T1	400	30	370	27	3	0	7.52 ± 0.65^{d}
T2	400	31	369	25	4	2	7.75 ± 0.86^{d}
T3	400	35	365	24	7	4	8.76 ± 1.25^{cd}
T4	400	37	363	22	9	6	9.27 ± 1.11^{c}
Infected fish							
T1	400	98	302	31	28	39	24.50 ± 1.19^{a}
T2	400	69	331	28	25	16	17.25 ± 1.13^{b}
T3	400	58	342	21	24	13	14.50 ± 1.04^{bc}
T4	400	41	359	17	14	10	10.25 ± 1.12^{c}

*: Number of cells examined per a group, **: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. Treatments; T1: control; T2: supplemented Se NPs 10 days; T3: supplemented Se NPs 20 days; T4: supplemented Se NPs 30 days. Values are presented as the mean ± SE. Treatments with different letters within the same row are significantly different at $P \leq .05$.

Table 4: DNA fragmentation detected in hepatic tissues of *C. carpio* supplemented with Se NPs and/ or bacterial infection.

Treatment	DNA Fragmentation %	Change	Inhibition
Un infected fish			
T1	8.2±0.25^{d}	0	0
T2	8.1±0.32^{d}	0.1	100.49
T3	9.3±0.56^{d}	1.1	94.61
T4	10.2±0.63^{cd}	2	90.20
Infected fish			
T1	28.6±0.82^{a}	20.4	0.00
T2	21.5±0.48^{b}	13.3	34.80
T3	17.9±0.67^{bc}	9.7	52.45
T4	12.4±0.35^{c}	4.2	79.41

Treatments; T1: control; T2: fed Se NPs 10 days; T3: fed Se NPs 20 days; T4: fed Se NPs 30 days. Values are presented as the mean ± SE.Means with different superscripts (a and b) between locations in the same column are significantly different at P<0.05.

Fig. 3: Visual score using comet assay of normal DNA (class 0) and DNA damage (classes 1, 2 and 3) in hepatic tissues of fish supplemented with Se NPs and/ or bacterial infection.

Table 5: Micronuclei frequencies (%) in blood erythrocytes of *C. carpio* supplemented with Se NPs and/or bacterial infection.

Treatment	Total number of counted cells/group	MN frequency (%)
Un infected fish		
T1	10000	0.09±0.02 ^{f}
T2	10000	0.11±0.02 ^{f}
T3	10000	0.14±0.03 ^{ef}
T4	10000	0.20±0.02 ^{de}
Infected fish		
T1	10000	0.71±0.02 ^{a}
T2	10000	0.57±0.03 ^{b}
T3	10000	0.38±0.01 ^{c}
T4	10000	0.23±0.01 ^{d}

Treatments; T1: control; T2: supplemented Se NPs 10 days; T3: supplemented Se NPs 20 days; T4: supplemented Se NPs 30 days. Treatments with different letters within the same row are significantly different at P≤.05. Values are presented as the mean ± SE.

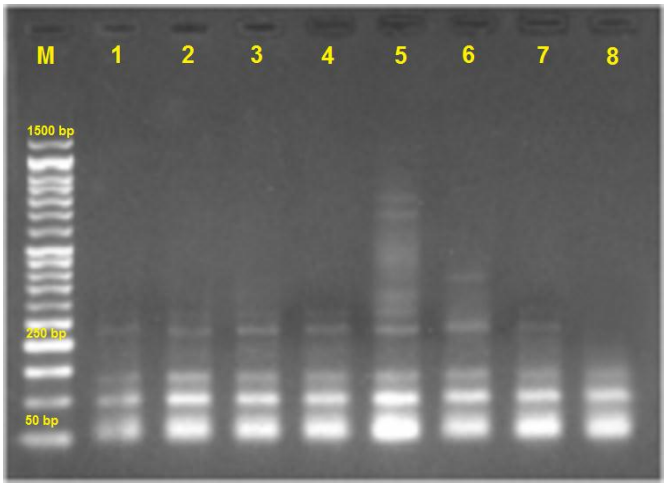

Fig. 4: DNA fragmentation detected with Agarose gel in hepatic tissues of *C. carpio* treated with Se NPs and/ or bacterial infection.

M: represent DNA marker, Lanes 1: Control treatment (-ve), Lane 2: represents fish supplemented with Se NPs 10 days, Lane 3: represents fish supplemented with Se NPs 20 days, Lane 4: represents fish supplemented with Se NPs 30 days, Lane 5: represents fish exposed to bacterial infection, Lanes 6-7: represent fish similar to those in lanes 2-4 plus bacterial infection.

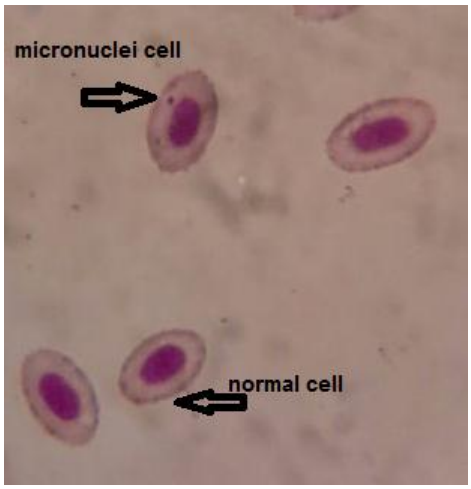

Fig. 5: Erythrocytes of *C. carpio* showing micronucleus induced by bacterial infection.

4. Histopathological analyses of *C. carpio* fed on Se NPs supplementation

Three tissues liver, kidney, and spleen were histopathologically examined for any alterations accompanied by the Se NPs supplementation and *A. hydrophila* infection. Hepatic tissue of *C. carpio* showed no alteration after feeding Se NPs for different periods 10, 20, and 30 days.

Hepatic tissue of *C. carpio* challenged with *A. hydrophila* showed necrotic areas (Fig. 6a), these lesions were decreased in *C. carpio* supplemented with Se NPs in time-dependent manners as T4 (Fig. 6c) was less pronounced than T2 (Fig. 6b). Renal tissue of challenged *C. carpio* (Fig. 7a) was heavily infiltrated with inflammatory cells and Se NPs supplementation for 30 days T4 (Fig. 7c) had the same change; while *C. carpio* supplemented with Se NPs for 10 days T2 (Fig. 7b) had a significant reduction in cellular infiltrations compared to the control treatment. Melano-macrophages centre was markedly proliferated in spleen tissues of challenged fish (Fig. 8a) and supplemented with Se NPs (Figs. 8b,8c).

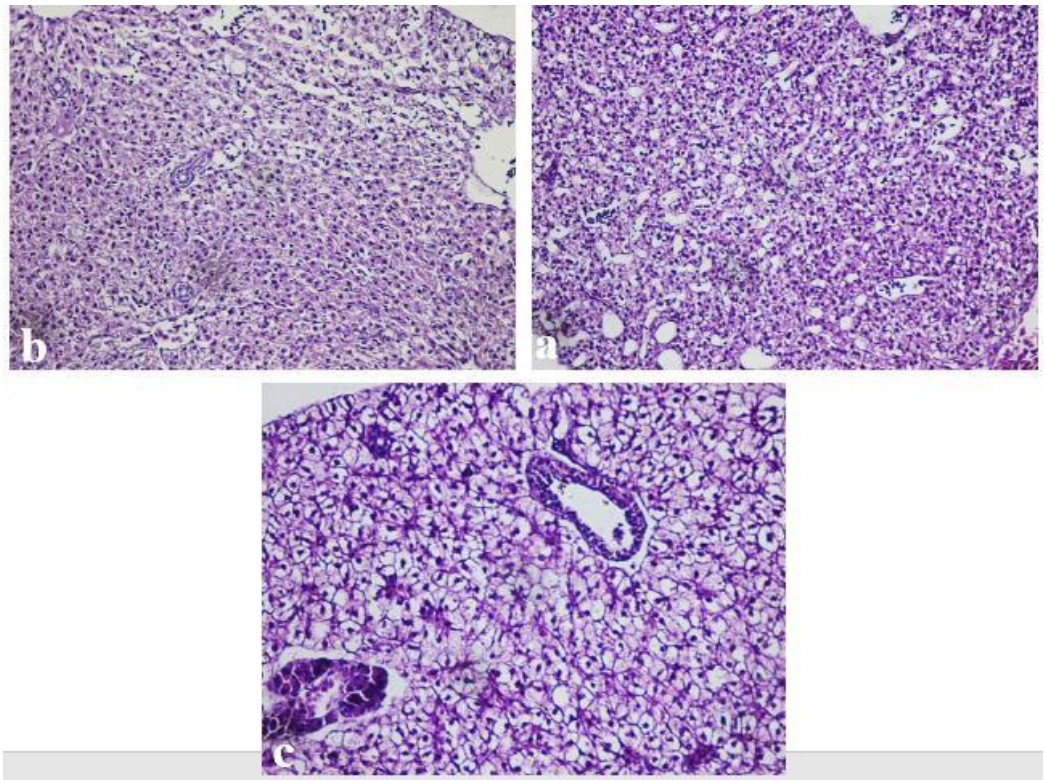

Fig. 6: Hepatic tissue with marked degeneration in A. hydrophila (T1) (a), with gradual reduction in the degeneration severity in Se NPs treated (T2) (b), and mild degree of degeneration in Se NPs supplemented (T4) (c). H&E X 400.

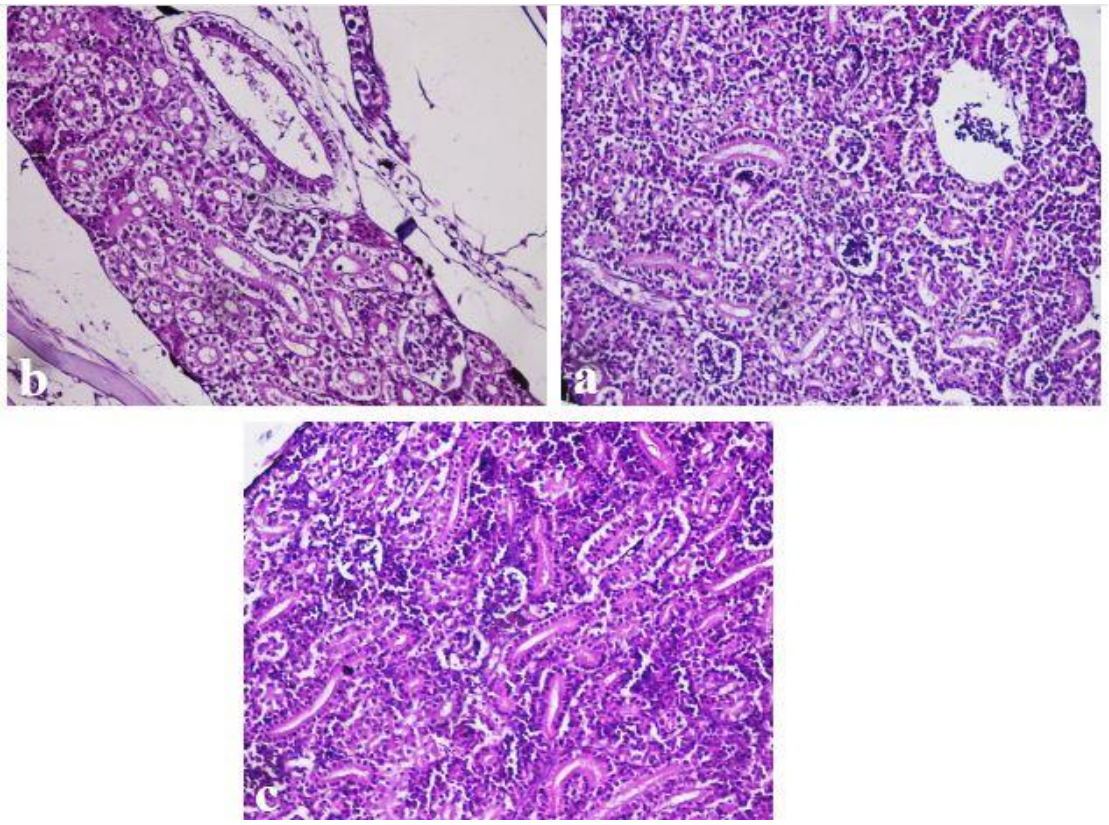

Fig. 7: Renal tissue with heavy interstitial inflammatory cells infiltration associated with glomerular degeneration in *A. hydrophila* (T1) (a). Absence of inflammatory cells infiltration in Se NPs supplemented (T2) (b), while massive inflammatory cells infiltration in Se NPs supplemented (T4) (c). H&E X 400

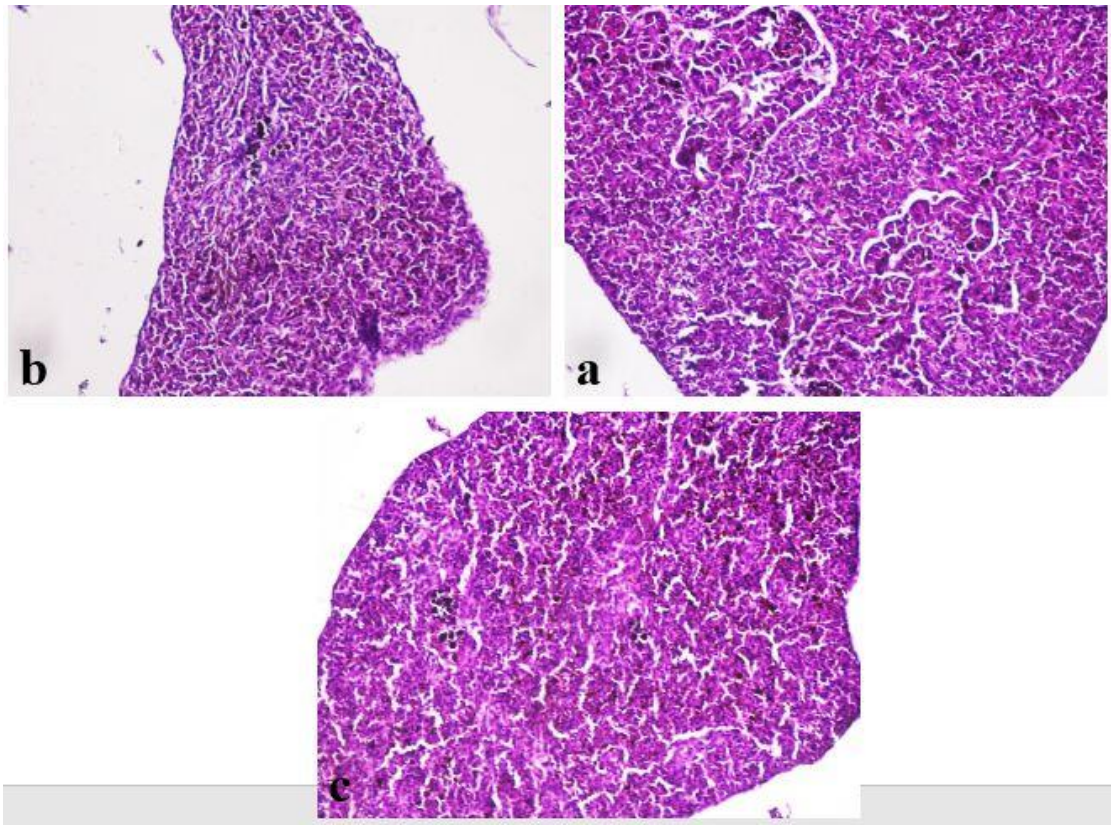

Fig. 8: Spleen with marked activation of melanomacrophages in *A. hydrophila* (T1) (a), Se NPs supplemented (T2) (b), and Se NPs supplemented (T4) (c) groups. H&E X 400

DISCUSSION

In the last decade, many researchers **(Abdel-Tawwab *et al.*, 2018; Nikapitiya *et al.*, 2018; Sherif *et al.*, 2019)** stated that nano-materials became widely used in fish production for their positive properties (immunostimulant and diseases resistant) and also, less toxic compared with their organic and/or inorganic sources**.**

The pilot challenge with pathogenic agents is the most reliable test for judging the immune system **(Köllner *et al.*, 2002).** In a time-dependent manner, *C. carpio* fed on Se NPs supplemented diet (1 mg/kg feed) had a lower mortality rate than the control treatment, fish fed on Se NPs supplementation had improved disease resistance against the experimental challenge with *A. hydrophila*, particularly with those in T4 (fed on Se NPs for 30 days) which had 13 out 50 survival rate. Similarly, **Jobling (2012)** observed that in many fish species a dose of 0.15 to 0.7 mg/kg of nano-selenium meets the optimum requirement needed for the normal growth performance without any nutritional disorders. Supporting our findings, *C. carpio* feeding a diet supplemented with β-1, 3 glucan binding protein-based selenium nanowire (0.5 mg, 1 mg, and 2 mg for 30 days) could resist aquatic pathogen infection; namely, *A. hydrophila, Vibrio parahaemolyticus* and *V. alginolyticus,* and showed a high surviving rate **(Iswarya *et al.*, 2018)**.

Furthermore, with the same dose, **Kumar *et al.* (2018)** stated that *Pangasinodon hypophthalmus* could resist the experimental infection with *A. veronii biovar sobria* showing a low cumulative mortality rate. Furthermore, the immune stimulant, antioxidant, bioavailability, and low toxicity properties of Se NPs (0.7 mg/kg) could counteract the bacterial challenge in *O. niloticus* **(Neamat-Allah *et al.*, 2019)** and *C. carpio* **(Saffari *et al.*, 2018)**.

In this study, *A. hydrophila* could not be isolated from challenged *C. carpio* at the third week after experimental infection in T4 (fed on Se NPs 1 mg/kg feed for 30 days). In accordance, **Neamat-Allah *et al.* (2019)** stated that the mortality of *O. niloticus* which was challenged with *S. iniae* ceased at 5th, 12th, and 14th days in, Se NPs, Se, and control, respectively, with mortality rates of 93.33%,73.33%, and 26.66%, respectively. This indicates that Se NPs speed the recovery and elimination of pathogenic agents.

The anti-inflammatory cytokine IL-10 was significantly increased ($P< 0.05$) in *C. carpio* fed on Se NPs whereas, pro-inflammatory cytokines TNF-α, IL-1β, and IL-8 were significantly lowered with time compared to the control treatment. In accordance, the pro-inflammatory cytokines, IL-8, TNF-α, and transforming growth factor-β were significantly ($P<0.05$) decreased at 0.67–1.46 mg/kg; whereas, IL-10 was down-regulated with lower supplementation doses < 0.67 mg/kg **(Jingyuan *et al.*, 2020)**. In the same way, IL-10 was decreased in zebrafish and chinook salmon (*Oncorhynchus tshawytscha*) that received lower selenium supplementation diets **(Lulijwa *et al.*, 2019; Wang *et al.*, 2020).** In contrast, the anti-inflammatory cytokine in fish is up-regulated in response to inflammation and adverse conditions **(Saber *et al.*, 2019; Jiang *et al.*, 2020),** these observations are not in contrast with the present findings, since Se NPs enhanced the immune responses (anti-inflammatory cytokines) and it did not initiate an inflammation (low pro-inflammatory cytokines).

The resistance of fish against bacterial infections relies on their immune status. Therefore, biochemical parameters in plasma **(Davis 2004, Sherif *et al.*, 2021b)**, total protein, and albumin **(Ortuno *et al.*, 2001)** are good tools to assess the immunity of fish. The current findings testified that the TP, ALB, GLO, and GLO fractions (Alpha, beta, and gamma) were improved in T4 (fed Se NPs supplementation1 mg/kg feed for 30 days) while, no changes was recorded in T2 or T3 (fed on Se NPs for 10 and 20 days, respectively). Fish fed on Se had improvements in the serum levels of total protein, globulin, and albumin since Se is a part of selenoprotein that helps in albumin syntheses **(Suzuki *et al.*, 2010; Ashouri *et al.*, 2015; Mansour *et al.*, 2017),** total protein and albumin in plasma also improved with Se NPs additions **(Jingyuan *et al.*, 2020)**. Nevertheless, the decrease in the serum protein contents was observed in fish infected with *S. iniae* due to immunosuppression and/or hepatic dysfunction **(Badr *et al.*, 2012).**

Study findings showed an enhancement in a time-dependent manner in the antioxidant activity (CAT, GPx, and SOD) of liver of *C. carpio* fed on Se NPs (1 mg/kg feed). In agreement, the activities and gene expression of hepatic antioxidant enzymes

(GPx, CAT, and SOD), as well as reduced glutathione (GSH) level, were enhanced at a dose of Se 1.06 ($P < 0.05$) **(Kumar *et al.*, 2018; Jingyuan *et al.*, 2020)**. Noticeably, the Se is an active component of glutathione peroxidase enzymes that eliminates the adverse impacts of ROS with the **(Reeves & Hoffmann, 2009)**; while, Se NPs had higher activities of antioxidant enzymes (GPx, NO, SOD, and CAT) than other Se forms organic or inorganic in the experimental fish (**Sarkar *et al.*, 2015; Saffari *et al.*, 2018)**.

When the antioxidant capacity fails to neutralize the accumulated ROS in cells, oxidative stress emerges, resulting in DNA damages, including base pairs aberrations in **(He *et al.*, 2018; Kassotis *et al.*, 2018)**. The content of the antioxidant enzymes is an important indicator for the health of fish and the integrity of their body tissues and cells. Cytogenetic analysis of chromosomes can be used to assess the biological effects of any genotoxic substance on fish **(Frenzilli *et al.*, 2009).** Our findings concerning the activity of antioxidant enzymes indicated that Se NPs protect the fish cells from genotoxicity, since comet assay, DNA fragmentation rates, and the presence of micronuclei in blood cells decreased significantly ($P < 0.05$) in fish received Se NPs (1 mg / kg feed) mainly in T4 (30 days treatment was the most effective period). Antioxidant enzymes GPx, CAT, and SOD catalyse the conversion of hydrogen peroxide and fatty acid hydro-peroxides into the water and fatty acid alcohol protecting cell membranes against oxidative damage **(Watanabe *et al.*, 1997)**, and protecting the cell membranes, DNA, proteins, and lipids against oxidative stress **(Hodgson *et al.*, 2006)**. It is worth noting that, using Se NPs is safe (low toxicity) and more bioavailable than the other forms of Se (organic and inorganic) **(Wang *et al.*, 2007)**.

In Fig. (6a), a bacterial infection in *C. carpio* resulted in severe degeneration and areas of necrosis even for the complete absence of hepatocytes, bacterial toxins caused upregulation of NO and TNF-α expression. Those reactions stimulate inflammatory responses with further hepatic damages **(Shimohashi *et al.*, 2000)** and necrosis **(Shobana *et al.*, 2018).** In accordance, **Chopra *et al.* (2000)** stated that cytotoxic enterotoxin of *A. hydrophila* upregulated pro-inflammatory TNF-α and (Cox2 and Bcl-2). Those cytokines mediate tissue degeneration and necrosis **(Song *et al.*, 2014).** In the present results, the enhancements in histopathological features in *C. carpio* supplemented with Se NPs (Figs. 6b, 6c) could be attributed to immunological and antioxidant properties of Se. Similarly, Se transfers into selenoproteins and stimulates the anti-inflammatory and anti-apoptotic effect with down regulation of TNF-α with further down-regulation of cyclooxygenase (Cox2) and B-cell lymphoma (Bcl2) **(Huang *et al.*, 2012; Qian *et al.*, 2019; Fan *et al.*, 2020)**. In addition, **Avery and Hoffmann (2018)** denoted that the Se deficiency is accompanied with up-regulating some inflammatory responses.

In Fig. (7a), the fish kidney was infiltrated with inflammatory cells after *A. hydrophila* infection, which is explained by the findings of **Majumdar *et al.* (2007)** who stated that *A. hydrophila* enhances macrophages which are the first line of defense against

the microbial challenge. In the present results, fish supplemented with Se NPs (T2) for 10 days (Fig. 7b) had low cellular infiltration in kidney tissue compared with those supplemented for longer periods (30 days) in T4 (Fig. 7c). Nano-particles affect and modulate the neutrophil's functions in a time-dependent and concentration manner **(Collins & Meyer 2009; Griffitt *et al*., 2009; Combs *et al*., 2011)**. Different findings were reported by **Jovanovic *et al.* (2011)** who mentioned that high levels of nanoparticle accumulation in tissues resulted in a reduction of inflammatory cell infiltration and subsequently immune response against bacterial infection. In agreement, **do Carmo *et al.* (2018)** and **Shobana *et al.* (2018)** observed degeneration and necrosis in renal tubule and glomeruli with hypotrophy.

As shown in Fig. (8a), a marked proliferation of melano-macrophages in the spleen of *C. carpio* was experimentally infected with *A. hydrophila*. Spleen tissue formed of melanomacrophage centres which are an aggregation of highly pigmented macrophages playing an immunological role **(Kipp *et al.*, 2009)**. Changes in the number, size, and cellular content occurred in response to microbial challenge **(Daeron, 1997).** Similar changes in the spleen (marked proliferation of melano-macrophages) were observed in *C. carpio* fed on a diet supplemented with Se NPs for 10 days (Fig. 8b) and 30 days (Fig. 8c). Those histopathological alterations were due to the accumulation of Se NPs in the spleen which is one of the prediction sites of bioaccumulation **(Gutscher *et al*., 2009).**

CONCLUSION

Feeding on a diet supplemented with selenium (nano-size) improves the immune and antioxidative status of *C. carpio*. Serum total protein, globulin, levels of anti-inflammatory (IL-10), as well as antioxidant enzymes (GPx, CAT, and SOD) in serum were gradually and significantly enhanced with fish fed on Se NPs (1 mg / kg fish feed). Due to those improvements, *C. carpio* could resist bacterial infection as no bacteria were isolated from fish fed for 30 days on supplementation at the third week after experimental infection. *C. carpio*, fed Se NPs for 30 days, showed lower histopathological lesions, DNA damages, and micronuclei even after bacterial infection. Thus, it could be safe to feed common carp (*C. carp*) with Se NPs without any threat to fish health.

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