Toxicity effects of multi-walled carbon nanotubes (MWCNTs) nanomaterial on the common carp (Cyprinus carpio L. 1758) in laboratory conditions

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\textbf{Abstract}

Over the past decade, the usages of carbon nanotubes in various industries have been increased. Multi-walled carbon nanotubes (MWCNTs) are special form of carbon nanotubes which are used as nano-absorbents for various purposes of different industries due to their high surface to volume ratio. In aquatic environments these active nano-agents can easily absorb and accumulate in animal cells and tissues due to their tiny sizes and induce toxicity effects on bio-organisms mainly via pro-oxidants production. The present study assayed MWCNTs toxicity effects on anti-oxidative enzymes activities, serum hormonal and biochemical stress biomarkers, hematological parameters, histopathology and growth performance of the common carp Cyprinus carpio. Experiment was conducted in five treatments including 0 (control), 5, 10, 15, 20 mg/l MWCNTs in triplicate and each of the experimental tanks consisted of a 400-l recirculating system, stocked with, 20 fish (12 ± 2 g) for 28 days. The results indicated that by increasing the concentrations of the MWCNTs weight gain, specific growth rate and survival rate parameters were decreased. The findings showed that cortisol secretion, blood glucose level and anti-oxidative enzymes activities were increased with the increase of MWCNTs concentrations in the treatments. Histopathology results depicted that 15 and 20 mg/l MWCNTs caused hyperplasia, telangiectasia, apoptosis, and necrosis damages in gills and also, apoptosis, sinusoidal spaces, fibrosis, hepatocyte degeneration and necrosis in the liver of C. carpio. Despite these findings, further researches on effects of nanomaterials on aquatic organisms and ecosystems are essential to protect these environments against the newly found nanomaterials hazards.

\section{1. Introduction}

Nowadays, different contaminants from natural or artificial resources threaten human health and environmental security (Zhang et al., 2020). Some nanomaterials (NMs) are part of these contaminants that widely used for various purposes of different industries. NMs are materials that at least, one of their dimensions is in range of 1–100 nm and some of them have relative high surface-to-volume ratio which are known as nano-absorbents (Qu et al., 2013). Carbon nanotubes (CNTs) are main groups of these nano-absorbents and commonly have adverse effects to aquatic animals and environments and a large quantity of these NMs enters the environment due to the increasing production and applications, yearly (Zhang et al., 2020). These NMs have toxicity to animal cells and tissues depends on their tube size and length and aquatic environments are the ultimate sink for these nano-contaminants, via either direct discharge or hydrological processes (Lee et al., 2015). In various industries, the long tubes structures of these materials are used and they have less toxic to animal cells, but after releasing and entering to the aquatic environments, in different processes their carbon bonds are broken along the nano-tubes over time and their structures are changed to small, active and toxic nano-agents. CNTs NMs release into the aquatic ecosystems through domestic and industrial wastewaters and induce adverse effects on aquatic organisms (Wu et al., 2019). Animal cells and tissues can easily absorb these NMs due to their tiny sizes and induce sustained inflammation, fibrosis, cancer, geno-toxicity (Kobayashi et al., 2017). Also, penetration of CNTs through the cellular lipid bilayer membrane results in oxidative stress that may lead to the inflammation and also result in cytotoxicity (Mohanta et al., 2019). CNTs accumulate in the zebra fish embryos over a 5-day period and cause moderate toxicity (Wang et al., 2016), also Jackson et al. (2013) reported that CNTs NMs are hazardous to aquatic organisms and specially invertebrates as bio-indicators. Mwangi et al.
Experimental animals

Fish species are widely used to evaluate the health of aquatic ecosystems because pollutants and contaminants build up in the food chain and are responsible for adverse effects and death in aquatic ecosystems (Rezaei Tavabe et al., 2016; Rezaei Tavabe et al., 2017). Cyprinid species are appropriate biological models for contaminants in aquatic ecosystems due to their high resistance to environmental conditions. The common carp, *Cyprinus carpio* due to its high tolerance to adverse environmental conditions can exist in polluted aquatic environment and it is one of the best species as bio-monitor for various contaminants in the aquatic ecosystems (Rafiee et al., 2015; Vali et al., 2020). Freshwater animals can be used for controlling pollution in three ways, involving three different time frames: (1) evaluation of water quality condition and its variations during certain times, (2) monitoring the health of populations of fish in the field or in a hatchery, (3) providing an early warning system for potential harm to the aquatic environment (Yang, 2014). The main objective of the present study was assessment of multi-walled carbon nanotubes (MWCNTs) toxicity on oxidative enzymes activities, serum stress biomarkers, hematology parameters, and histopathology of liver and gills tissues in the common carp *Cyprinus carpio*.

2. Materials and methodology

2.1. MWCNTs nanomaterials

MWCNTs NMs were obtained from Sigma-Aldrich Company (USA) and according to the information provided by the company, purity of the sample was 95% and it was produced by chemical vapor deposition method. SEM (Tescan mira 3, CZE) images of the NMs were prepared (Fig. 1) and average diameter of tubes was 38 nm. In accordance to Cimbaluk et al. (2018) the obtained NMs used to prepare a stock solution in distilled water in concentration of 1 g/l and then were sonicated for 15 min in ultrasonic bath (ULTRA 8020, James Co., UK) for dispersion.

2.2. Experimental animals

Fish (*n = 350*) were obtained as mixed both sexes from a private center in Babolsar, Mazandaran province, Iran. Obtained Fish (12 ± 2 g) were stocked in a 1000-l fiberglass tanks for adaptation approximately two weeks prior to the study.

2.3. Experimental set up and design

According to MWCNTs monitoring in aquatic environments and different wastewaters, these NMs concentrations are very variable and up to about 20 mg/l (Nezhadheydari et al., 2019). So, the present research was conducted in five treatments including 0 (control), 5, 10, 15, 20 mg/l MWCNTs in triplicate. Each experimental tank consisted of a 400-l recycle closed system (without water exchange during the experiment) with top filter, stocked with, 20 fish for 28 days experiment period. During the research, photoperiod was set at 12 h light and water temperature was maintained at 28 ± 1 °C, in accordance with recommendations of Tavabe et al. (2013) and Rezaei Tavabe et al. (2015). The fish were fed ad libitum with a commercial formulated pellet (Faradaneh Co., Shahr-e-Kord, Iran) twice a day (at 7.00 am and 7.00 pm). Other water physico-chemical parameters of tanks water including pH (7–7.6), dissolved oxygen (6 ± 1 mg/l), ammonia-N (< 0.2 mg/l), and nitrite-N (< 0.1 mg/l) were adjusted during the research period. Water quality parameters such as dissolved oxygen and pH parameters were measured by digital multi-meter device (HI-9828 Multi-meter, Hanna Co., USA) and also nitrite and ammonia parameters measured by specific kits and rechecked by spectrophotometry device (Cary UV–Vis 4000, Agilent Co., USA).

2.4. Growth indices

At the end of the trial period, feeding was stopped for 24 h then all the fish of each tank were moved in a solution of 7 g/l clove extract for 10 min. After anesthesia, the weight of the liver and the weight and length of the fish were used to determine the indices. Weight gain (WG), Condition factors (CF), Hepato-somatic index (HSI), Specific growth rate (SGR) and survival indices were calculated according to the below standard formulas used by Rezaei Tavabe et al. (2018):

- **WG%** = [(final weight–initial weight)/initial weight] × 100
- **SGR** = (ln(final weight in grams) − ln(initial weight in grams)) /Culture period (days) × 100
- **CF** = [Final weight (g)/Final length (cm)³] × 100
- **HSI** = Liver weight (g)/Body weight (g) × 100
- **Survival (%)** = (final number of fish/initial number of fish) × 100

2.5. Hematology parameters assay

After taking blood samples from caudal tail vessels using 23-gauge needles and collecting in heparinized Eppendorf tubes of the fish, hematology parameters including red blood cell (RBC) and white blood cell (WBC) counts were done by a Neubauer crystalline counting chamber. Hemoglobin content (Hb) was measured by the cyanmethemoglobin method at 546 nm on a spectrophotometer (Unico 1100RS, USA) and the hematocrit value (Hct) was determined using micro-hemocytocapillary tubes centrifuged at 1680 × g for 5 min (Klontz, 1994). Also, the mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated using the following equations (Benfey and Sutterlin, 1984):

- **MCV (nm³)** = Hct (%) × 100/RBC (×10⁶/μl)
- **MCHC (g/dl) =** Hb (g/dl) × 100/Hct (%)
- **MCH (g/dl) =** Hb (g/dl) × 10/RBC (×10⁶/μl)

2.6. Oxidative stress assay

Five fish per treatment were randomly collected from each tank at the end of research period for biochemical analysis. Gill and liver tissues were removed and weighted then immediately snap-frozen in liquid nitrogen and stored at −20 °C until needed. The frozen tissues were rinsed in 9-fold chilled 100 mmol/l, pH 7.8 sodium phosphate buffer solution and homogenized by a hand-driven glass homogenizer. The homogenates were centrifuged at 10000 × g at 4 °C for 20 min and the supernatant was stored in Eppendorf tubes at 4 °C. The liver supernatant was diluted with 9-fold chilled sodium phosphate buffer
solution to 1%. The prepared supernatants were analyzed for antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT) activities to determine possible effects on oxidative stress. The SOD activity was assayed in accordance to Hao et al. (2009), based on its ability to inhibit the reduction of nitro-blue tetrazolium (NBT) by superoxide radicals generated by xanthine/xanthine oxidase, which exposes its presence by inhibiting the reduction of NBT using the colorimetric activity kit (Kit number: EIASODC Invitrogen, Thermo Fisher Scientific Co., USA). One unit of SOD activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT reduction under the experimental conditions and the specific enzyme activity was expressed as units per gram fresh weight of tissue per hour. Also, The CAT enzyme activity was determined by measuring the initial rate of the decrease in absorbance at 240 nm as a consequence of H2O2 consumption over 1 min. Activity was expressed as a unit (one activity unit defined as absorbance at 240 nm changes 0.01 per min) per gram fresh weight of tissue in accordance to Hao et al. (2009).

2.7. Serum stress biomarkers measurement

The blood samples aliquots were transferred into Eppendorf tubes without anticoagulant and stored for 6 h at 4 °C. After blood clotting, samples were centrifuged (at 3000 g for 5 min, 4 °C) and serum was separated and stored at −80 °C until analysis. Plasma cortisol levels were determined by radioimmunoassay in samples. The kits were prepared from Bayer (1 vial of ACS: 180 Cortisol lite Reagent, 1 vial of ACS: 180 Cortisol Solid Phase) and then for assay, reagents and protocols were used in accordance to method described by Pankhurst and Sharples (1992). And also, glucose levels were determined in accordance to Sabzi et al. (2017).

2.8. Histo-pathological analysis

For histo-pathological analysis, liver and gills tissues samples of experimental fish were removed and fixed by 10% formalin solution buffered with sodium phosphate (Merck Co., Germany) for 24 h, at 4 °C and then washed off with Phosphate Buffer Saline (PBS, pH 7.4). After dehydration processing, sections of each tissue were cut into 5 μm thickness and stained with Hematoxyline and Eosin (H&E, Merck KGaA, Germany) and examined under the light microscope (Scopepad-LX116, Labex Co., India).

2.9. Ethics statement

The authors confirm that the trial protocol was approved by the Ethics Committee for the Animal Research, University of Tehran (Committee ID: IR.UT.REC); none of the fish suffered starvation, trauma or electrical shock and all animals were completely anesthetized before tissue and blood sampling.

2.10. Data procedures

The data were normalized by Shapiro-Wilk test and the means of the five treatments to each biomarker were compared by one-way ANOVA and significant differences among the means were found ($P < 0.05$) by Duncan's test in SPSS version 24 (IBM, USA).

3. Results

3.1. Growth indices

Although, at the end of research period, the fish final weight, HSI and CF parameters were not significantly ($P < 0.05$) different among the treatments, but WG and SGR indices had different values. The lowest WG (58.3 ± 2.5%) and SGR (1.64 ± 0.18%) were recorded in the 20 mg/l MWCNTs treatment. Survival rate decreased with increasing concentrations in the treatments such that the highest survival (93.3%) was for the control treatment and the least (45%) was recorded in the 20 mg/l treatment (Table 1).
3.2. Hematology parameters assay

According to the hematology data, relative indices including MCH, MCHC, and MCV didn’t show any significant differences among the treatments but WBC, RBC, Hb, and Htc parameters had significantly different. By increasing MWCNTs concentrations in the treatments, although RBC, Hb, and Hct parameters approximately showed decreasing trend, but WBC parameter was increased in *C. carpio* blood (Table 2).

3.3. Oxidative stress assay

Anti-oxidative enzymes activities including superoxide dismutase (SOD) and catalase (CAT) in liver and gills tissues of *C. carpio* were assayed as bio-marker to determine MWCNTs oxidative stress. The results showed that SOD activity of *Cyprinus carpio* liver was higher in the 5 and 10 mg/l of MWCNTs which were lower than the 15 and 20 mg/l MWCNTs concentrations, while in gills the SOD activity increased in all concentrations of MWCNTs tested in relation to the control (Fig. 2).

In liver, catalase enzyme activity in all experimental treatments was same and showed significant differences versus the control treatment. In gills, also the control treatment was different in comparison to the other treatment and the greatest catalase enzyme activity was recorded in the 20 mg/l MWCNTs treatment (Fig. 3).

3.4. Serum stress biomarkers

With increasing MWCNTs concentrations in the treatments, cortisol hormone secretion and glucose level in *C. carpio* serum also increased. Significant differences between the control treatment and the other treatments of cortisol and glucose in serum indicates that the MWCNTs NMs are stressful for *C. carpio*. In the 20 mg/l MWCNTs treatment, cortisol hormone secretion and glucose concentration in the serum were 78 ng/ml and 139 mg/dl respectively (Fig. 4).

3.5. Gills and liver tissues histopathology

The treatments 15 and 20 mg/l MWCNTs NMs in *C. carpio* gills tissue caused hyperplasia of the epithelial cells (HP), telangiectasia (T), apoptosis (A), and necrosis (N) damages (Fig. 5). Also, these concentrations of MWCNTs showed apoptosis (A), sinusoidal spaces (SS), fibrosis (F), and hepatocyte degeneration (HD) and necrosis (N) lesions in the liver tissue (Fig. 6).

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### Table 1

<table>
<thead>
<tr>
<th>MWCNTs concentrations (mg/l)</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>WG (%)</th>
<th>Survival rate (%)</th>
<th>SGR (%)</th>
<th>Condition factor (CF)</th>
<th>HSI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12 ± 2</td>
<td>22 ± 2</td>
<td>83.3 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.3%</td>
<td>2.18 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.2</td>
<td>9.5 ± 2.1</td>
</tr>
<tr>
<td>5</td>
<td>12 ± 2</td>
<td>22 ± 2</td>
<td>83.3 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.3%</td>
<td>2.18 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.3</td>
<td>9.6 ± 2.5</td>
</tr>
<tr>
<td>10</td>
<td>12 ± 2</td>
<td>20 ± 1</td>
<td>66.6 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85%</td>
<td>1.82 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.2</td>
<td>8.5 ± 2.8</td>
</tr>
<tr>
<td>15</td>
<td>12 ± 2</td>
<td>20 ± 2</td>
<td>66.6 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.3%</td>
<td>1.82 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.3</td>
<td>8.6 ± 2.2</td>
</tr>
<tr>
<td>20</td>
<td>12 ± 2</td>
<td>19 ± 1</td>
<td>58.3 ± 2.5</td>
<td>45%</td>
<td>1.64 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.2</td>
<td>8.5 ± 2.1</td>
</tr>
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</table>

The comparisons are intergroup and means with different superscript letters in same columns are significantly different (*P* < 0.05).

### Table 2

<table>
<thead>
<tr>
<th>MWCNTs concentrations (mg/l)</th>
<th>WBC (×10&lt;sup&gt;3&lt;/sup&gt;/μl)</th>
<th>RBC (×10&lt;sup&gt;6&lt;/sup&gt;/μl)</th>
<th>Hb (g/dl)</th>
<th>Htc (%)</th>
<th>MCH (pg/cell)</th>
<th>MCHC (g/dl)</th>
<th>MCV (nm&lt;sup&gt;3&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.22 ± 2.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.41 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.62 ± 0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.01 ± 1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.24 ± 6.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.26 ± 3.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>156.19 ± 13.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>33.19 ± 2.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.40 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.60 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.19 ± 1.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.12 ± 5.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.81 ± 2.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151.26 ± 14.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>10</td>
<td>35.79 ± 2.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.42 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.65 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.97 ± 1.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>60.79 ± 4.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.36 ± 3.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>147.07 ± 15.38&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>15</td>
<td>40.08 ± 1.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.28 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.45 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.08 ± 1.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.11 ± 4.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.44 ± 5.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>141.35 ± 10.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>41.62 ± 1.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.26 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.12 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.22 ± 0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.34 ± 5.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.16 ± 5.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>144.52 ± 13.65&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

The comparisons are intergroup and means with different superscript letters in same columns are significantly different (*P* < 0.05).
4. Discussion

The present study indicated that *C. carpio* growth indices, oxidative enzymes activities, hematology parameters, serum bio-chemical stress factors, liver and gills tissues histopathology and survival rate were strongly affected by MWCNTs NMs. After 28 days of the research period, growth indices of *C. carpio* were decreased by increasing the concentrations of the MWCNTs in the tanks. Various pollutants in chronic conditions reduce aquatic animal's growth performances in environment (Rezaei Tavabe et al., 2019). Pollutants alter the biochemical and hormonal systems of the organism's body to deal with the stressor; consequently, in challenge with the stress, growth indices are reduced (Vali et al., 2020). Because, under stress condition, organism body metabolism and allocated energy to maintenance are increased. On the other hand, MWCNTs NMs showed dose-dependent toxicity to *C. carpio*, so that by increasing its concentration, superoxide dismutase (SOD) and catalase enzymes activities were strongly increased. NMs enhance the formation of reactive oxygen species (ROS), which ROS as pro-oxidants cause oxidative stress and oxidation and peroxidation of proteins, lipids, and DNA, which can lead to significant cellular damage and even tissue or organ necrosis and failure (Tripathy, 2016). Oxidative stress has been defined as a disturbance in the balance between the production of ROS, or free radicals and antioxidant defenses, which may lead to aquatic organism's tissue injury (Rezaei Tavabe et al., 2019). Wang et al. (2015) indicated that exposure of goldfish (*Carassius auratus*) to different CNTs NMs caused obvious changes in antioxidant enzymes activities such as SOD and catalase in the liver. SOD is an enzyme that alternately catalyzes the produced ROS into ordinary molecular oxygen and hydrogen peroxide, so the enzyme activity is an appropriate biomarker for oxidative stress in aquatic organisms (Walters et al., 2016). Saria et al. (2014) confirmed effects of MWCNTs toxicity on SOD and catalase enzymes activities in the African frog (*Xenopus laevis*) tadpoles. Also, increased activities of these enzymes were shown in zebrafish (*Danio rerio*) (Souza et al., 2019), and spotted snakehead (*Channa punctatus*) (Amjad et al., 2018) in dose-dependent manner. Our results are in good accordance with previous studies and depicted that catalase and SOD enzymes activities in *C. carpio* liver and gills tissues increased in exposure to MWCNTs NMs.

The stress reactions in fish involves a series of physiological and biochemical responses that enables the fish to overcome the effects of the stressor. Cortisol hormone secretion and glucose level in fish blood serum are the most important stress biochemical biomarkers (Nolan et al., 1999). At the present study these factors levels in *C. carpio* blood serum had direct relations to MWCNTs concentrations in the treatments. Cortisol secretion and hyperglycemia are the main biochemical processes in response to stress factors (Sreenivasula Reddy et al., 2011).
Fig. 5. Effects MWCNTs concentrations on gills tissue of C. carpio. a: control treatment, gills showing secondary lamellae (SL), epithelial cells (EC) and mucous cells (MC). b: 5 mg/l treatment showing hyperplasia of the epithelial cells (HP). c: 10 mg/l treatment demonstrating hyperplasia of the epithelial cells (HP), epithelium lifting (EL). d: 15 mg/l treatment, showing hyperplasia of the epithelial cells (HP) and telangiectasia (T). e: 20 mg/l treatment, demonstrating telangiectasia (T), apoptosis (A), and necrosis (N). Tissue sections were stained with hematoxylin and eosin, and analyzed at 10 × magnification, Scale bar = 0.5 mm.
Fig. 6. Effects MWCNTs concentrations on liver tissue of *C. carpio*. a: control treatment with normal hepatocytes (NH). b: 5 mg/l treatment with normal hepatocytes (NH). c: 10 mg/l treatment, showing apoptosis (A), and fibrosis (F). d: 15 mg/l treatment, depicting sinusoidal spaces (SS), fibrosis (F), and hepatocyte degeneration (HD). e: 20 mg/l treatment, demonstrating hepatocyte degeneration (HD), and necrosis (N). Tissue sections were stained with hematoxylin and eosin, and analyzed at 40 × magnification, Scale bar = 0.1 mm.
Cortisol is released in response to adrenocorticotropic hormone which activates the central nervous system and induces an increase in glucose levels to provide energy to combat the effects of the stressor (Canli et al., 2018). Once NMs enter the body of bio-organisms, they cause stress and activation of different stress responses mechanisms (Walters et al., 2016). Canli et al. (2018) indicated Al2O3, CuO, TiO2 nano-particle effects on the same fish cortisol secretion reaction. The present study findings indicated that the MWCNTs are toxic and stressful for C. carpio, so that in 20 mg/l MWCNTs concentration, cortisol hormone secretion and glucose concentration in the fish serum were 78 ng/ml and 139 mg/dl respectively; while, normal range of cortisol for C. carpio is about 10–30 ng/ml (Ruane et al., 2002) and glucose normal level in different fishes plasma is about 15–61 mg/dl (Martínez-Porchas et al., 2009).

A basic knowledge of the hematology represents a valuable guide to assess the condition of aquatic organisms and it is widely used as an indicator of environmental stress (Fazio et al., 2015). So, it is well know that certain hematology parameters serve as reliable indicators of fish health. Stress induces changes in blood cell numbers and activities. An increase in red blood cell (RBC) count and hemoglobin level and decrease in white blood cell (WBC) count usually has been reported in fish subjected to acute stress (Fazio et al., 2015). According to the hematology results of the present study, relative indices including MCH, MCHC, and MCV didn't show any significant differences among the treatments but WBC, RBC, Hb, and Hct parameters had significantly differences. So that by increasing MWCNTs concentrations and stress in the treatments, RBC, Hb, and Hct parameters approximately showed decreasing trend, but WBC parameter was increased in C. carpio blood. Numerous studies about effects of different NMs on fish hematology in challenge to stressors have been conducted (Chen et al., 2013), but their results are inconsistent. It seems that in fishes, acute stress increases RBC and decreases WBC in a short-time period, whereas this trend is reversed in chronic stress during long-time period. Depending on the stress intensity and time and due to the continuous activation of the hypothalamic-pituitary-adrenal (HPA) axis, responses of blood factors changes (Tort, 2011). Spleen is a major storage organ for blood cells and it is known to contract in teleost fish during stress condition, as a strategy to improve blood capacity to phagocyte stress factor and carry oxygen under the high energy demand condition (Ruane et al., 2000). The type of hematological parameters reactions and their changing procedures depends on the nature, severity and duration of the stressor. At the end of the present study period, by increasing MWCNTs concentrations in chronic condition, WBC parameter was increased in C. carpio blood, while other parameters such as RBC, Hb, and Hct parameters were decreased. Probably, an increase in white blood cells to phagocytose nanomaterials. The increase in WBC is probably due to phagocytosis of MWCNTs NMs; whereas the reduction of RBC is associated with oxidative stress of the NMs (Rifkind and Nagababu, 2013).

At the present study, by increasing MWCNTs concentrations in the treatments, hyperplasia of the epithelial cells, telangiectasia, apoptosis, and necrosis damages were appeared in C. carpio gills. Also, this nanomaterial especially in 15 and 20 g/l MWCNTs concentrations showed apoptosis, sinusoidal spaces, fibrosis, and hepatocyte degeneration and necrosis lesions in the liver tissue. The histological damages may be ascribed to direct toxic effects of nano-toxictants on liver cells because it is the chief site of detoxification and hence a sink for potential toxicants (Maftuch et al., 2018). On the other hand, gills are heavily influen by pollutants and stressors because of its direct contact to the water environment (Forouhar Vajargah et al., 2018). Gimbakul et al. (2018) indicated that MWCNTs can penetrate to Danio rerio and Astyanax altiparanae fishes cells and tissues in acute and sub-chronic condition and induce cytotoxicity, neurotoxicity and geno-toxicity. These NMs also induce apoptosis and antioxidant gene expression in the gills and liver tissues of Oreias latipes fish and also gills were more sensitive to MWCNT toxicity than the other organs (Lee et al., 2015). Qu et al. (2014) demonstrated that MWCNT as a combined factor intensifies the bioaccumulation of heavy metals and tissue damages in the freshwater fish Carassius auratus. In environment, depending on the stressor intensity, stress duration and fish age, the fish gradually weaken and eventually perish. The results depicted that liver and gills histopathology of C. carpio are appropriate biomarker to indicate NMs contamination and monitoring in aquatic ecosystems.

5. Conclusion

Aquatic environments are the ultimate destination for NMs accumulation and bio-monitoring of these nano-toxicants materials such as MWCNTs through bio-organisms in these environments is very important. The present study showed MWCNTs adverse effects to C. carpio oxidative stress, hematology parameters, hormonal responses, blood glucose level, and histopathology of gills and liver tissues as dose-dependent. The findings indicated that by increasing MWCNTs concentrations in the treatments, antioxidant enzymes activities, cortisol hormone secretion and glucose concentration in C. carpio serum also increased to deal with the effects of the NMs stressor. On the other hand, with the increase in the level of MWCNTs NMs weight gain, specific growth rate and survival rate were decreased. Histopathology sections depicted that MWCNTs 15 and 20 mg/l concentrations caused hyperplasia, telangiectasia, apoptosis, and necrosis damages in gills and also, apoptosis, sinusoidal spaces, fibrosis, hepatocyte degeneration and necrosis lesions in the liver of C. carpio. Generally, according to the research outputs, even the lowest concentration of MWCNTs tested (5 mg/l) caused changes in biomarkers in C. carpio liver, gills and blood and this issue is very worrying of aquatic environments protection. Therefore, the treatment of wastewaters containing MWCNTs NMs and prevention of these wastewaters entrance into aquatic environments must be done. Also, further researches on the effects of other nanomaterials on aquatic organisms and environments are essential to protect the aquatic ecosystems against these new materials.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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