HISTOPATHOLOGICAL CHANGES (GILLS AND LIVER) AND CLINICAL SIGNS OF COMMON CARP, CYPRINUS CARPIO L. EXPOSED TO GRAPHENE NANOPARTICLES

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ABSTRACT
This study was conducted to investigate the effect of graphene nanoparticles on histopathological changes and clinical signs of common carp *Cyprinus carpio*. A total of 48 fish were used in the experiment with an average weight 68.44 g. Fish were exposed to graphene nanoparticles at concentrations of 10 and 20 mg / L for the T1 and T2 respectively, as well as to control group (without graphene nanoparticles). Fish exposed to graphene showed no abnormal behavior and no mortality satisfactory clinical signs in the experimental fish. Histopathological changes of gills showed fusion with shorting of the secondary lamellae, lifting up secondary lamellae beside necrosis and hyperplasia of the epithelial cells of secondary lamellae in gills. On the other hand, there was necrosis of hepatocytes, dilation of sinusoids, hydrobic degeneration, hepatocellular vacuolation, hypertrophy and hydrobic of degeneration in liver. The present study was suggested to investigate of the graphene nanoparticles effects on histopathological changes and clinical signs to clarify a clear representation about on fish and its danger of its accumulation in aquatic environment.
Key words: common carp, graphene, nanoparticles , histopathology

التغيرات النسجية المرضية في غلاصم وكبد سمكة الكارب الشائع Cyprinus carpio المعرضة الى جسيمات الكرافين

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المستخلص

أجريت الدراسة الحالية لمعرفة مدى تأثير الجسيمات النانوية للجرافين على التغيرات النسجية في سمكة الكارب الشائع Cyprinus carpio. أستعمل 48 نموذجا من أسماك الكارب الشائع بمعدل أوزان 66.48 غم. عرضت الاسماك الى الجرافين بتراكيز 10 و20 ملغم/لتر للمعاملتين الاولى والثانية على التوالي فضلا عن مجموعة السيطرة الخالية من الجرافين . بينت نتائج الدراسة الحالية ألى عدم ظهور أي سلوك غير طبيعي للأسماك, كما لم تظهر علامات سريرية مرضية في أسماك التجربة ، أوضح الفحص النسجي المرضي الى وجود اتحاد الصفائح الثانوية للغلاصم, قصر الصفائح الثانوية مع فرط تنسج ونخر الصفيحة الثانوية في الغلاصم في المعاملة الأولى والثانية, كما لوحظ نخر الخلايا الكبدية, وأتساع الجيبانيات الكبدية وتفجي الكبد, فرط التحلل وتخلخل خلايا الكبدية .

الكلمات المفتاحية : الكارب الشائع ، الكرافين ، الفحص النسجي

INTRODUCTION

The term "Nano" used to describe the size measurement; a nanometer (nm) is a millionth of a millimeter. Any material size is between 0.1 and 100 nm describe as nanoparticle (NP) (5).The application of nanomaterials is very novel in scientific study field, nanotechnologies and Nano sciences are fast developing disciplines and extremely promising in industrial and innovation research, nanomaterials displays various multidisciplinary activities in both of aquaculture and agriculture sectors (5)**.** In 2004 graphene was discovered, made from carbon with single layer of atoms similar to honeycomb- shaped network and paying great attention in last years (9, 26). Increasing in production of graphenes and their derivatives by companies recently lead to suspect released of these materials into different environmental area (18). Toxicity mechanisms of graphenes, fullerenes and carbon nanotubes (CNTs) are not clear to researchers (31) owing to differences in size, shape, charge, surface area, and aggregation chemistry (11), which make it hard to prophesy the toxicity effects of nanomaterials. Histopathological alterations regarded as important tool of sub-lethal toxicity used to rapid detect effects of chronic exposure, irritants, in different organs and tissues (13). The exposure of fish to nanomaterial is led to induce different lesions in various organs (14 , 23 , 28). Gills (21 , 32) and liver are appropriate organs for histopathological investigation with the purpose of detects the effect of contamination. Fish liver histopathology is an indicator of chemical toxicity and useful way to study the effects of exposure of aquatic animals to toxins present in the aquatic environment (10). Several behavioral changes reflects toxicity of nanoparticles such as low activity, increased feeding time, stayed closer together, and less explorative, changes in hopping and appendage movement, increase or decrease heart rate etc. (19 , 23). However, the mechanism of graphennnanoparticles toxicity is not clear to researchers and due to few studies showed the impacts of these materials on fish. Therefore, the purpose of present study was to explain the effects of graphene nanoparticleson histopathological changes and clinical signs of *Cyprinus carpio*.

MATERIALS AND METHODS

Experimental fish

Healthy fish of 100 *C. carpio* with an average weight 66.48g used in this experiment were obtained from a commercial farm (Al-Talibi fish farm, Babylon) and were acclimated to laboratory conditions for 15 days before beginning of the experiment, fed with commercial diets .

Experimental design

Preparation of grapheme suspension

Graphene (Fig.1) was purchased from Areej Alfurat Bureau added to deionized water and submitted to bath sonication for 10 minute and this suspension regarded as stock solution then from this suspension multiple exposure concentrations prepared (10 and 20 mg/l) with size 6-8 nm.

Limit test of grapheme

Using the techniques described in OECD Guideline (27), a limit test should be accomplished at 100 mg (graphene)/l for 7 fish under optimum environmental conditions for 96 hr. to determine the LC50.

Fish exposure

Graphene exposure was performed in glass containers filled with 28 L water with constant aeration without feeding. The graphene concentrations were selected based on former acute toxicity tests in which no mortality was detected for concentrations up to 100 mg / . Thus, sub-lethal concentrations of 0 (control), 10 (T1) and 20 (T2) mg/L were evaluated in following assays (duplicate in each exposure, 16 fish per treatment). Fish were exposed to graphene for 10 days, clinical signs and histopathological changes were studied.

Clinical signs

During the experiment period the observation of toxic symptoms such as stress, movement, respiration, swimming, responses to the outer effects and mortality were investigated.

Histopathological studies

Histopathological changes were studied in fish that exposed to graphene. After dissection, samples of gills and liver were collected per fish and fixed in 10% formalin for 24h, dehydrated in graded ethanol concentrations and embedded in paraffin wax. Sagittal sections (5μm of thickness) were cut and

mounted on glass slides. Sections were deparaffinized in xylene, hydrated in ethanol, stained with hematoxylin – eosin (HE) and were examined using light microscope (20).

Figure 1. Show grapheme nanoparticles in fish aquarium

RESULTS AND DISCUSSION
Clinical signs
Results showed no mortalities or abnormal clinical signs for all treatments. Changes in swimming behavior caused by sub-lethal exposure to toxic contaminants may impair the ability of intake food (17). Swimming behavior is the most frequently assessed behavioral response during toxicity investigations of fish. Swimming capacity and swimming activity, are commonly used to assess contaminant-related changes in locomotion (16).There is no data available on effect of graphene in behavioral of fish. In present study, during 10 days of exposure to graphene at concentrations of 10 and 20 mg/l fish do not revealed any abnormal signs such as swimming imbalance, operculum movement, irritation, jumping, etc. Mattsson et al. (23) showed that Crucian carp *Carassius carassius* received nanoparticles 24 and 27 nm, 130 mg per feeding through a natural three trophic level food chain (algae, zooplankton and fish) revealed lower activity, increased feeding time, stayed closer together, and were less explorative. Rainbow trout *Oncorhynchus mykiss* exposed to copper cu NPs for hr. at concentration of 50 mg/l exhibited no significant difference $P > 0.05$ in behaviors compared to negative controls (38).Recent studies have demonstrated NPs can absorb chemical and biological material in suspension (4 , 12 , 41)and it is possible that adsorption of grapheme NPs lower bioavailability and/or also the bioreactivity (38). Therefore we can conclude that NPs size, dose, and exposure time and/or route are important with respect to behavioral effects,

but the underlying mechanisms are yet to be determined.

Histopathological studies
A. Gills
Histopathological sections of gill do not reveal any histological alteration in secondary and primary lamella. Fish in the negative control group showed normal structure of primary and secondary lamellae (Fig. 2 A, B, C and D), all structures of gills were normal such as extracellular matrix , chondrocyte, epithelial cell , mucus cell , chloride cell , pillar cell as well as blood capillary, while when fish exposed to 10 mg/l of graphene (T1) (Fig. 3 A,B,C and D) for 10 days multiple histopathological changes was noticed such as complete fusion of the secondary lamellae, shorting of the secondary lamellae as well as lifting of the secondary lamellae (arrows) with hyperplasia. fish exposed to 20 mg/L graphene (T2) (Fig. 4 A,B,C and D) for 10 days were showed histopathological changes such as hyperplasia with complete fusion of the secondary lamellae and dilation of the central venous with blood congestion.
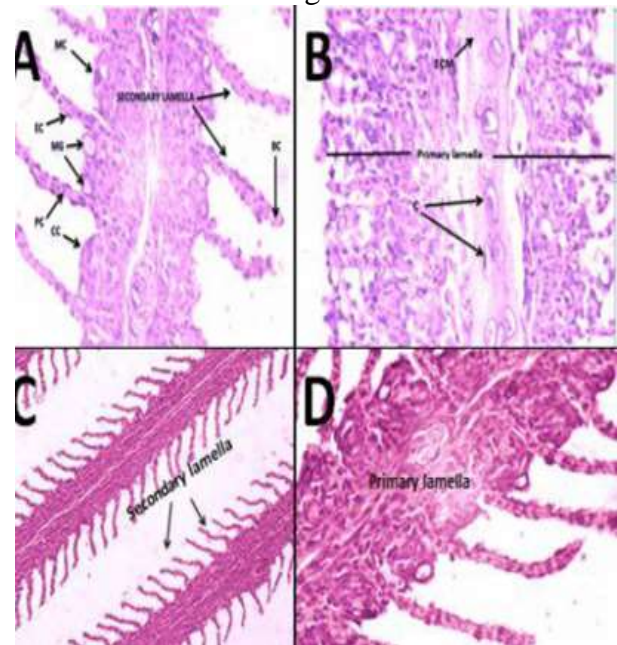

Figure 2. Histological section showing normal structure of gill in negative control group. A.blood capillary (BC), epithelial cell (EP), pillar cell (PC), chloride cell (CC), mucus cell (MC) and mucus gland (MG). B. Extracellular matrix (ECM), chondrocyte (C). C. secondary lamella D. Primary lamella (HE x40).
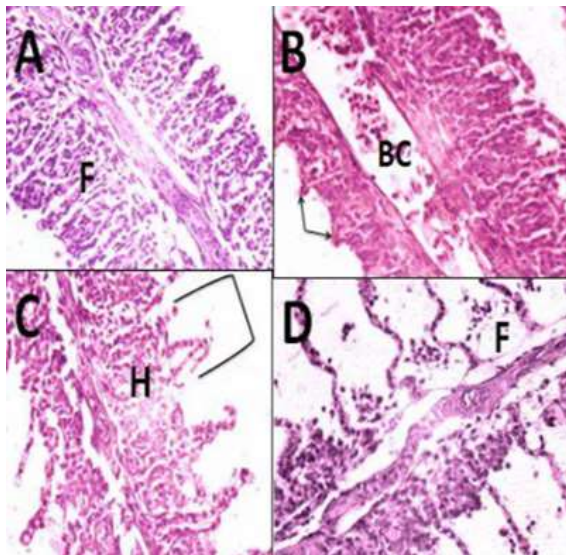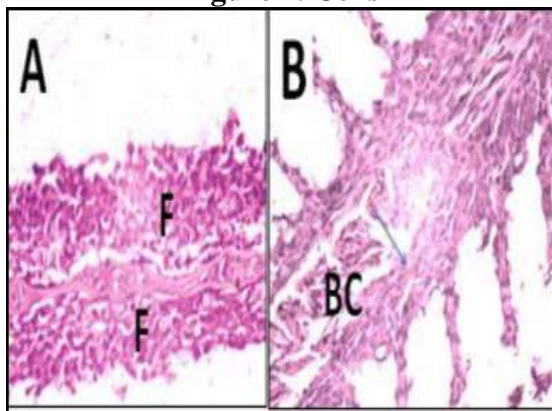
Figure 3. Histological section in gill of *C. carpio* exposed to 10 mg/l graphene exhibited (A) complete fusion of the secondary lamellae (F). (B) Blood congestion (BC) with shorting of the secondary lamellae (arrows). (C) Lifting up of the secondary lamellae (arrows) with hyperplasia (H). (D) Necrosis of the secondary lamella (F) (HE x40).

Figure 4. Cells

exposed to 20 mg/l of graphene expression multiple histological changes. (A) secondary lamellae showing hyperplasia with complete fusion of the secondary lamellae (F). (B) Showing dilation of the central venous with blood congestion (BC) (HE x40).

B. Liver

The morphological structure of liver in the control group was normal, as observed in Fig. 5. Hepatocytes are polygonal and have a distinctive central nucleus, also normal pancreatic tissue and central vein was noticed. But when *C.carpio* exposed to 10 mg/l of graphene (T1) for 10 days (Fig. 6 A,B,C and D) was showed multiple histopathological

changes such as necrosis of hepatocyte, dilation of sinusoids , hydropic degeneration and hepatocellular vacuolation.Exposure of fish to 20 mg/l graphene (T2) for 10 days (Fig. 7 A,B,C and D) resulted in hypertrophy and vacuolation degeneration of hepatocytes , infiltration of MNCs priporal region with vacuolation degeneration of hepatocytes , focal area of necrosis (N). Fish exposed to 20 mg/L graphene (T2) showed several histological structure as well as infiltration of MNCs around pancreatic tissue .
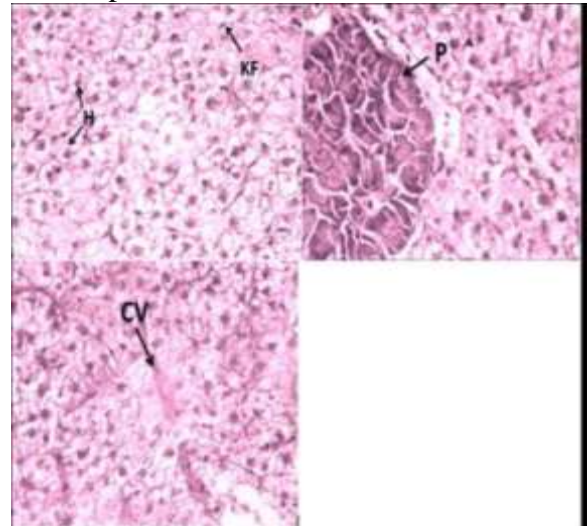

Figure 5. Histological section of liver in negative control in *C.carpio* with normal morphological structure including central vein (CV), hepatocyte (H), kuppffer cell (KF) and pancreatic tissue (P) (HE x40).
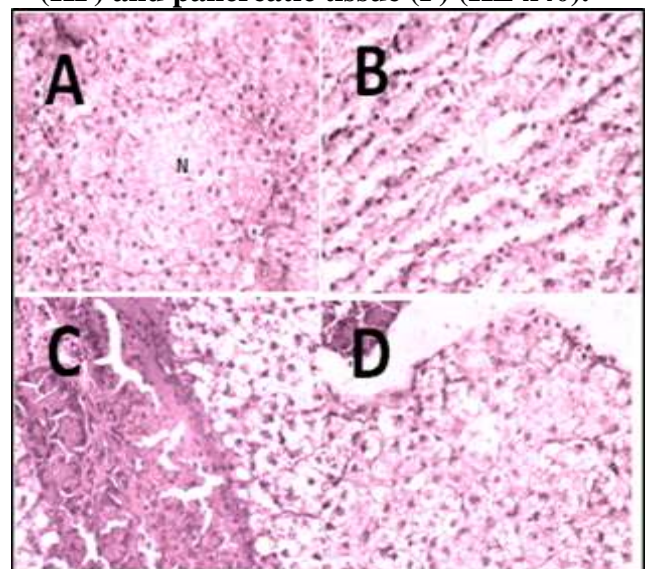

Figure 6. Histological section of liver in *C.carpio* exposed to 10 mg/L of graphene for 10 days. A. necrosis (N) of hepatocytes.B. Dilation of sinusoids.C. hydrobic degeneration. D. hepatocellular vacuolation (HE x40).
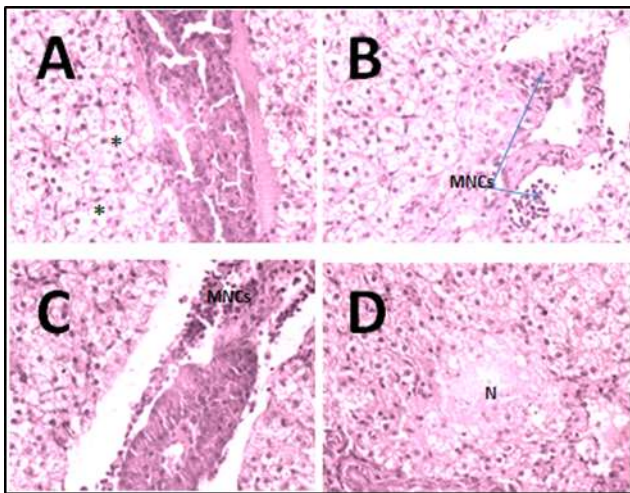
Figure 7. Histological section of liver in *C.carpio* exposed to 20 mg/L of graphene for 10 days. A. hypertrophy and vacuolation degeneration of hepatocytes.B. Infiltration of MNCs periportal region with vacuolation degeneration of hepatocytes.C. Focal area of necrosis (N).D. infiltration of MNCs around pancreatic tissue (H&E x40).

Results of gill and liver morphology of *C.carpio* showed normal in all the unexposed control groups. Results showed that all tissues were affected by graphene nanoparticles and caused various histopathological changes. Few related studies have investigated the effects of exposure to graphene NPFs on histopathological changes in fish. Graphene oxide caused changes in gill cells, these changes were linked to the organism's defense mechanism, which prevents grapheme oxide GO from reaching the sensitive gill epithelium (36).de Souza-Filho et al. (7) observed that acute exposure to Single Walled Carbon Nanotubes (SWCNT) caused lesions on zebra fish *Danio rerio* gills due to physical irritation and the occlusion of surface tissue. Similar damages have been reported in zebra fish when exposed to graphene oxide (15 days) at 2, 10 and 20 mg /l (37). Smith et al. (36) showed that chronic exposure of rainbow trout to SWCNTs resulted in pathologies such as edema, altered mucocytes and hyperplasia. Likewise, Al Subiai et al. (3) conclude that exposure to(C60) caused significant degenerative changes and necrosis in gill tissue of the mussel *Mytilus* sp. Gills are responsible for gas exchange, carbon dioxide release, and the exchange of salt and water, and play a major role in the excretion of nitrogenous waste products, primarily ammonia (25). Structural damage induced by GO therefore makes the fish vulnerable to osmotic deregulation as well as respiratory difficulties (37). In another study, GO administration was found to induce dose-dependent lung toxicity, granulomatous abrasions and injuries, and inflammatory cell penetration (30).It has been demonstrated that the increased mucus layer and cell hyperplasia may constitute a barrier for NP uptake by the gills (34). Additionally, fish gills serve as the primary tissue that makes contact with exogenous toxicants in the aquatic environment; thus, some branchial impairments with reference to toxicity may influence oxygen consumptionand disrupt osmoregulation (40). Shortening and fusion of gill lamellae and epithelial hyperplasia reduce contact of gills with water which results in reduced gas and ion exchange (28). According to various authors, epithelial lifting usually results from edema of the secondary lamellae (8 , 29). Nanoparticles inhibit ion transport by the branchial Na+ and K+-ATPase, which results in osmotic imbalance (2 , 35). Graphene NPs made dilation of capillaries and congestion of erythrocytes. According to Martinez et al. (22), these abnormalities demonstrate injuries to pillar cells and blood vessels and increase of the blood flow in lamellae. Our results showed that lesions on liver tissues after long exposure to GO could affect organ function. Similar liver histopathological changes have been reported in zebra fish exposed to graphene oxide (15 days) at concentration of 2, 10 and 20 mg /l (37). Same result was presented by Chen *et al*. (6) after exposure in vivo of zebra fish to GO; this group reported hepatocytes with vacuoles and a loose arrangement, necrosis and the disintegration of cell boundaries, all of which became more severe with increasing GO concentration. Tabish et al. (39) showed that exposure of common carp to graphene foam (7 days) 5, 10 and 15 mg/l caused degeneration of hepatocytes, pyknosis, karyolysis, and karyorrhexis in nuclei of hepatocytes, degeneration of the central vein in the liver. Abnormal accumulation of triglycerides and other neutral lipids may cause formation of vacuoles in hepatocytes and can be accompanied by pathological lesions such as

necrosis (14). Vacuolation of hepatocytes and the presence of pyknotic nuclei are indicative of the early stages of necrosis (2 , 15). Hepatocytes of fish exposed to the highest concentrations of both nanoparticles decreased in size and showed karyolysis (28). Histological hepatic changes are varied based on the types of nanoparticles, its concentration, fish species, and time exposed to it besides other items (35). Exposed to concentration of 3, 300 and 1000 mg// of Nano silver solution for eight weeks revealed histopathological liver changes as local congestion in the hepatic parenchyma, decreasing in the size and diameter of the hepatocytes and massive destination in the hepatic sinusoids sizes (24). Linhua et al. (15) reported that the livers and gills of some fish exposed to 100 and 200 mg/l TiO2-NPs showed cytoplasm vacuolation and apoptosis including necrotic cell bodies and nuclear fragments which appeared to be apoptotic bodies, and a few foci of lipidosis with minor fatty change in liver and increase in the incidence of thickening, edema, fusion and hyperplasia in the gill lamellae and filaments. Pournori et al. (33) demonstrated that fish fed on dietary nucleotides exposed to water borne silver nanoparticles resulted in necrosis of epithelial cells, blood accumulates in the bottom of the blade gill, swelling and fluid in the secondary blades, increased secretion of eosinophilic and hyperplasia with lift in epithelial cells in gills and bleeding and pigmentation, fibrosis of hepatocyte cells, vacuolization of cytoplasm, necrosis of hepatocytes area, dual-core, the pyknotic nuclei, nuclear degeneration and hypertrophy were observed in liver. Abarghoei et al. (1) showed that exposure of golden carp to silver nanoparticles (14 days) resulted areas of hyperplasia, edema and lifting of the gill epithelium, and lamellar fusion in gills besides hemosiderosis, hemorrhage, hydropic swelling, and pyknotic nuclei in liver. Exposure of *C.carpio* to graphene nanoparticles for 10 days led to severe histopathological changes in gills such as necrosis and fusion of gill lamellae.However tere was no behavioral alteration and mortalities during experimental period.

Conflict of interest
There exists no conflict of interest.
Authors contribution
All the authors have contributed equally in terms of giving their technical knowledge to frame the article.

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