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Quantification histopathological analysis in the gills of carp fish exposed to sub lethal concentration of nano zinc oxide

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Abstract

Nano-zinc oxide ranks third in the world in production, as it is used in many industries, enters the aquatic environment directly or indirectly, and is considered one of the most toxic substances for aquatic organisms. Therefore, these studies aimed to determine the toxic effect of the sublethal concentration of N-ZnO on the gills of carp fish that were treated for 7, 14, 21, 28, 35, and 42 days and using a semi-quantitative evaluation protocol for histopathological alteration. The histopathological alteration involved circulatory changes, cell growth disturbances, and morphological changes, the most severe lesion occurred on day 35 of treatment, and the occurrence of necrosis and death of the gill tissue at day 42 of the treatment, which was more significant for morphological changes when compared with the other lesion and period. The results showed that the histopathological changes on the seventh day of treatment were severe lesions, while in the rest of the treatments, they were irreversible lesions. It is concluded from this study that the gills are a good bio-indicator for evaluating the pollution status of the aquatic environment and that the sub-lethal concentration of N-ZnO leads to pathological changes in the gills and the possibility of using semi-quantitative assessment and statistical analysis to give significance for the most severe pathological lesions.

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Introduction

It has been established that nanotechnology is a significant advanced technology that can be applied to a variety of human activities. It is utilized in agriculture, manufacturing, and diagnostic, medical and scaffold therapy, leishmania, curcumin, frac, staff (1-5). The ecology is negatively impacted by the advancement of nanotechnologies. Fish and other aquatic life are negatively impacted by the introduction of nanomaterials into the environment change in the biological activity of toxic compounds or foodstuffs (6). Metal oxide and NPs may leak into natural bodies of water in their life cycles (production, storage, transportation, storage, consumption, reproduction or disposal), (7) it has a toxic impact on aquatic organisms. Therefore, there is an urgent need for information on the ecological risks of metal oxide NPs (8-11). Nano Zinc oxide (N-ZnO) are one of the metal oxide that consider the third most produced nanomaterials after Silicon Oxide SiO_2 and titanium oxide nanoparticles,the quantity of N-ZnO produced is estimated at 550 tons (12,13), used as antimicrobial, anticancer, sunscreen and cosmetic (14-16), zinc oxide nanoparticles are classified as extremely toxic to aquatic organisms. N-ZnO has a ubiquitous feature of as small size and has a large specific surface area (17), so it absorbed by the body 15-20 times compared to bulk particles and accumulation in the different tissues and cause variable physiological and cellular metabolic disturbances. Al-Taee and Al-Hamdani (18) obtained a 24-hour lethal concentration of 9 mg/l for N-ZnO treated of *Cyprinus*

carpio, it causes malformation and suppressor in fish embryonic development and decrease hatching rate (19,20) with adverse behavioral effects on *Oreochromis niloticus* (21). Histopathological changes are one of the most important biomarkers for pollution and imbalance of physical and chemical properties of the aquatic environment (22), and since gills are the most in contact with the aquatic environment, therefore, semi quantitative analysis of histopathological alteration is very important to estimate the toxic effect of nano-zinc oxide in *C. carpio*.

Materials and methods

Ethical approve

Scientific ethical committee on animal experimentation at College of Veterinary of Medicine, University of Mosul, give the approve to conduct this study under the licenses number UM.VET.2021.45.

Experimental fish

C. carpio were obtained from fish pond in the College of Agriculture, University of Dohuk. A total 60 fish with an average length 25 ± 5cm and weight 75± 10 gm placed in polythene bags which were filled with sufficient oxygenated water transport to fish lab in the College of Veterinary Medicine, University of Mosul. Fish adopt for at least two weeks, so that they acclimation to aquarium environment with daily renewed oxygenated freshwater at concentration 5 mg/l, water temperature 22°C and pH= 7.5 with 12 hours regular for light and dark cycle.

Nano ZnO preparation

N-ZnO at size 30 nm were purchased from Shijiazhuang Sun Power- China and the stock solution prepare by dissolving 9 mg/l in the water with magnetic stirrer for 30 min (23).

Experimental design

Fish were randomly dividing in to the control group (fish were place in water without N-ZnO) and treated group were fish expose to the sub lethal concentration of N-ZnO 9 mg/l (11), after 7, 14, 21, 28, 35 and 42 days from experimental beginning the gill were dissected from five fish under general anesthesia MS-222 at 150 mg/l (24). The gills were placed in formalin 10% for fixative and staining technique with hematoxylin and eosin (25). The histopathological alteration classified in to three stages according to the severity modify from Flores-Lopes and Thomaz (26) (Table 1).

Table 1: Categories and stage of severity of histopathological lesion in fish exposed to N-ZnO

Stages	Categories	Lesions
1	Cell growth disturbance	hyperplasia and hyper trophy of gill epithelium
	Circulatory disturbances	Congestion, edema, dilatation of blood vessels and infiltration of inflammatory cells
	Morphological alteration	Secondary gill lamellae were shortening, curling, fusion and disorganization
2	Cell growth disturbance	hyperplasia and hyper trophy of chloride cells and mucus cells (empty from mucus)
	Circulatory disturbances	Hemorrhage and blood vessels injury
	Morphological alteration	Rapture and damage of the epithelium cells
3	Cell growth disturbance	Telangiectasia in the gill lamellae
	Circulatory disturbances	Aneurysm
	Morphological alteration	Cellular degeneration and necrosis

Semi-quantitative histopathological analysis

Bernath *et al.* (27) explain the semi-quantitative analysis for ten gill filaments from each blinded slide, the importance factors (IF) of histopathological alterations have range (1= reversible alteration) or range (3 = irreversible alteration), each one of these histopathological changes has score value (SV), 0=no alteration occurrence, 2=mild alteration occurrence, 4= moderate alteration occurrence, 6=severe alteration occurrence. An alteration index (AI)=IF×SV, and calculate gills index (IG) by summing all alteration index. The severity of AI classified according to range value determined by Poleksic and Mitrovic (28) (Table 2).

Table 2: Ranging value of the Alteration Index severity in the gills

Severity of gills damage	Ranging value
Normal gill structure	0 – 10
Slight lesions	11 – 20
Moderate lesions	21 – 50
Severe lesions	51 – 100
Irreversible lesions	> 100

Statistical analysis

The data of this study was statistical analysis by fisher test significant at $P \leq 0.05$ and at $P \leq 0.01$. Both these analyses were completed using SAS program (29).

Results

Microscopic examination

A serious histopathological lesions may be occurrence in the gills architecture of fish exposed to sub lethal concentration N-ZnO which are variable in the severity according to the duration of exposure, most important were mild circulatory and morphological development as hemorrhage and curling in the secondary lamellae with hydropic in the chloride cells in fish treated for 7day (Figure 1), while at the 14 and 21day of exposure there was congestion in the blood vessels in the primary gill filament with moderate morphological alteration represented by shortening with clown shape like appearance in the apex of secondary gill filaments, fusion and hydropic degeneration in the apex of primary gill filaments (Figure 2). The histopathological lesions in the gills of fish exposed to N-ZnO for 28 days represented by cell growth disturbances as hyper trophy of mucus cells which appear empty, edema and congestion (Figure 3), with shortening and loss secondary gill filament (Figure 4). Severe morphological, circulatory and cells growth disturbances occurrence in the gill tissue of fish exposed to N-ZnO toxicity for 35 days, the microscopic examination in the (Figure 5) show curling and lifting epithelial cells with sever congestion and hyper trophy of pillar and chloride cells and infiltration of inflammatory cells as well as disrupting cartilaginous core and hemorrhage with hydopic degeneration in the epithelial will be occurrence (Figure 6). Continuing exposure of fish to toxicity of zinc oxide nanoparticles for 42 days leads to severe hydropic degeneration in the pillar cells (Figure 7) with necrotic gill tissue and loss architecture of gill arch (Figures 8 and 9) respectively.

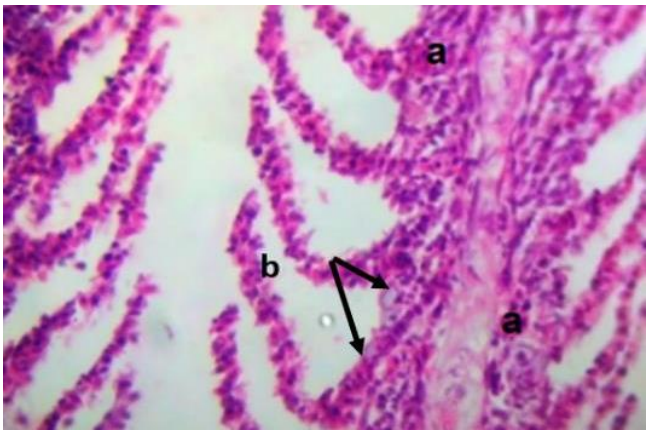

Figure 1: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 7 day show the hemorrhage (a) and curling in the secondary gill filament (b) with hydropic in the chloride cells (black row). H&E, 420X.

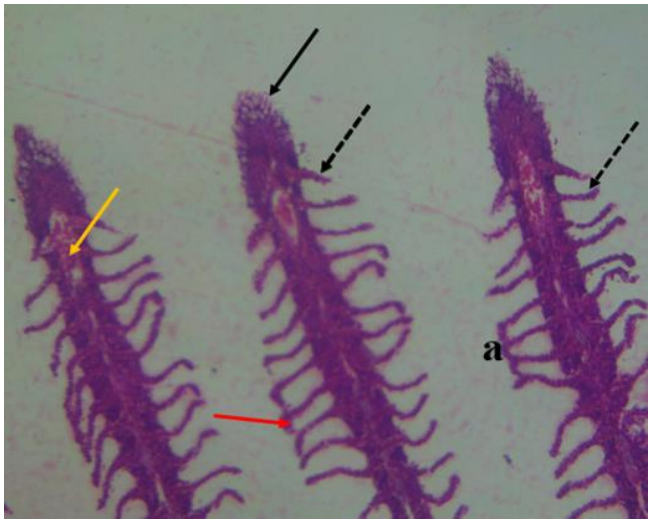

Figure 2: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 14 and 21 days show the congestion (yellow row) hydropic degeneration in the apex primary gill filaments (black row), shortening of the secondary gill filaments (black dot row), clown like appearance (red row) with fusion (a), H&E, 350X.

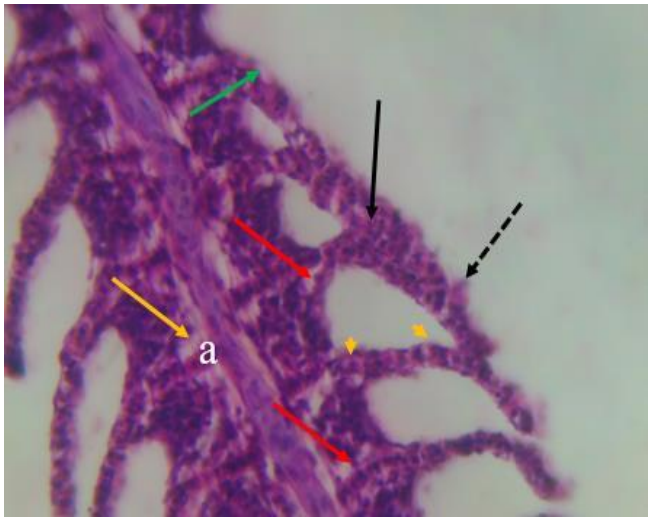

Figure 3: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 28 day show the congestion (b) hydropic degeneration in the epithelial cells (green row), mild lifting epithelial cells (black dot row), fusion in the secondary gill lamellae (black row), hyper trophy in both pillar cells (yellow head row) and mucus cells (red row), H&E, 420X.

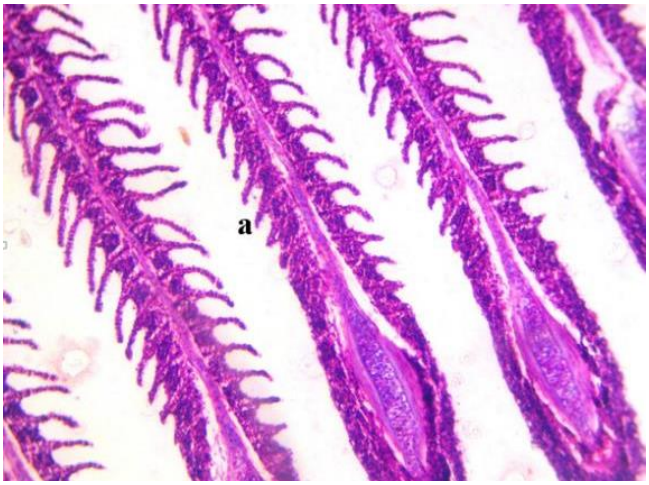

Figure 4: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 28 day show shortening and loss secondary gill filaments (a), H&E, 350X.

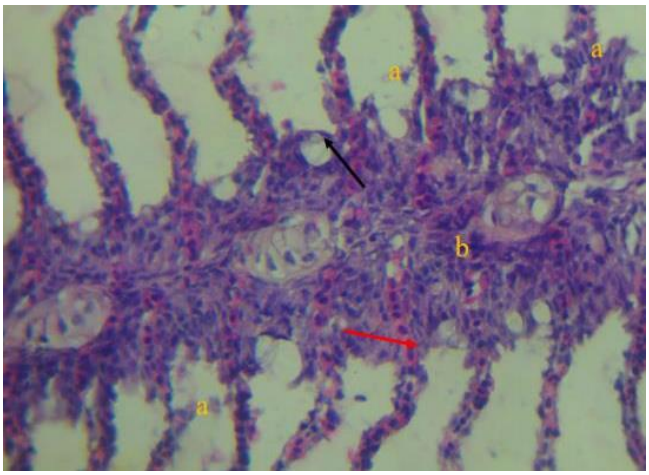

Figure 5: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 35 days show moderate lifting cells (a) infiltration of inflammatory cells (b), hyper trophy in both of pillar cells (red row) chloride cells (black row), H&E, 440X.

Semi -quantitative analysis

The quantitative assessment method is a scientific protocol by conducting statistical analysis to find out the most significant variation between histopathological lesions (Table 3), the histopathological lesions classified in to three categories: (I) circulatory alteration involve hemorrhage which is significant ($P<0.05$) at 35 day from fish exposure to toxicity at ratio 66.67% while the ratio of edema and infiltration of inflammatory cells are 42.86% and 56.25% respectively is more significant ($P<0.01$) at 42 day from fish exposure to N-ZnO toxicity, (II) in the cell disturbances the ratio of hyper trophy42.86% is significant at 35 day from treated fish, in the (III) morphological alteration all ratio of pathological lesions are highly significant in group of fish exposure to N-ZnO toxicity for 42 day

The result of statistical analysis revealed that the ratio of Alteration Index in gills of fish exposed to toxicity for at least 35 day is 657.14% and it is highly significant, while the severity of lesions ranging from sever lesions in gills of fish treated for 7 days to irreversible lesions at the 14, 21, 28, 35 and 42 days from the time of the experiment.

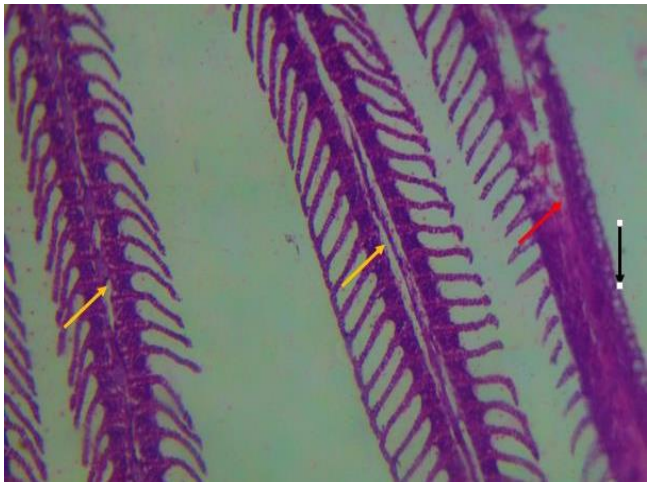

Figure 6: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 35 day show disrupting cartilaginous core (yellow rod row), hemorrhage (red row) with hydopic degeneration in the epithelial (black row), H&E, 350X.

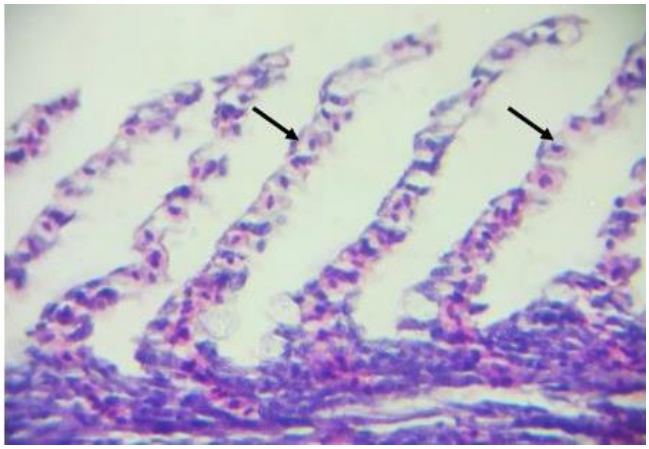

Figure 7: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 42 day show sever hydropic degeneration in the pillar cells (black row), H&E, 440X.

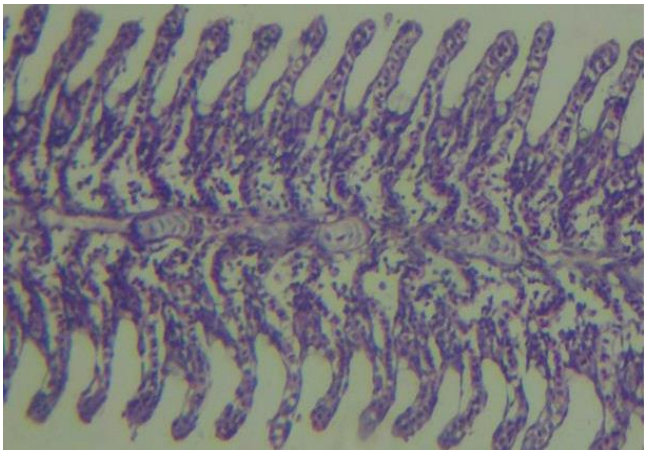

Figure 8: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 42 day dead tissue and necrosis, H&E, 420X.

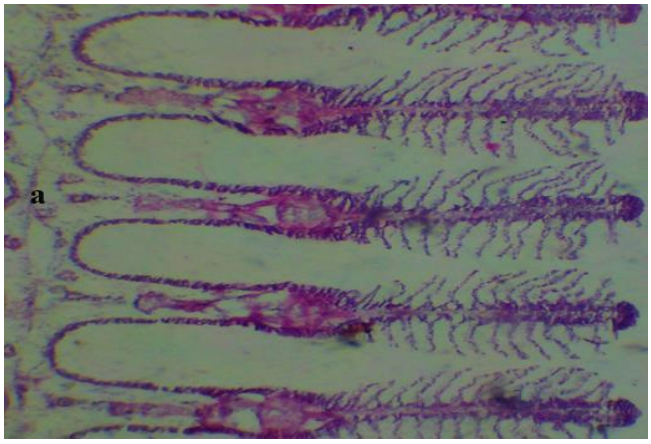

Figure 9: Microscopic examination in gill of *C. carpio* exposed to sub lethal concentration of N-Zno 9 mg/l for 42 days show loss gills arch architecture (a) H&E, 125X.

Table 3: Semi quantitative analysis of gill histopathological alteration in fish exposed to N-ZnO toxicity

Categories	Description	Duration of exposure to N-ZnO toxicity (day)						Chi2
		D7	D14	D21	D28	D35	D42	
Circulatory disturbances	hemorrhage	2	0	0	0	4	0	13.42*
	%	33.33	0.00	0.00	0.00	66.67	0.00	
	congestion	0	2	2	4	0	0	9.99NS
	%	0	25	25	50	0	0	
	edema	0	0	0	4	4	6	15.14**
	%	0.00	0.00	0.00	28.57	28.57	42.86	
	Inflammatory cells infiltration	0	2	2	4	6	18	40.00**
	%	0.00	6.25	6.25	12.50	18.75	56.25	
Cell growth disturbances	Hydropic degeneration	2	4	4	4	6	937.5	2.62^{NS}
	%	0.21	0.42	0.42	0.42	0.63	97.91	
	Hyper trophy	0	2	2	4	6	0	11.71*
	%	0.00	14.29	14.29	28.57	42.86	0.00	
Morphological alteration	Curling and clown	6	6	12	12	12	18	9.28^{NS}
	%	9.09	9.09	18.18	18.18	18.18	27.27	
	Shortening	0	6	6	12	12	18	22.00**
	%	0.00	11.11	11.11	22.22	22.22	33.33	
	Loss	0	0	0	6	12	18	48.00**
	%	0.00	0.00	0.00	16.67	33.33	50.00	
	Fusion	0	6	6	6	12	18	24.00**
	%	0.00	12.50	12.50	12.50	25.00	37.50	
	Lifting cells	0	0	0	6	12	18	48.00**
	%	0.00	0.00	0.00	16.67	33.33	50.00	
	Cartilage disruption	0	0	0	0	12	18	65.59**
	%	0.00	0.00	0.00	0.00	40.00	60.00	
	Necrosis and dead tissue	0	0	0	0	0	18	90.00**
	%	0.00	0.00	0.00	0.00	0.00	100.00	
Alteration Index %		10	28	36	62	92	56	88.34**
		71.43	200.00	257.14	442.86	657.14	400.00	

* Refer to significant difference between groups at P<0.05. ** refer to high significant difference between groups at P<0.01. NS: refer to non-significant difference between groups.

Discussion

Nanoparticles (NP) are being produced and used at an increasing rate, it is intermediate phase between bulk and molecules particles, and they are more toxic to human and environment health than bulk materials due to their increased surface reactivity and small size, which enable them to enter and concentrate inside of cells (30,31). Zinc oxide (ZnO) nanoparticles is one of the most nanomaterials, used in many in application and dustries such as sunscreens, cosmetics, ceramics, UV filters and paints photocatalysis, so it impacts the aquatic environment both directly and indirectly, and cause eco- toxicity and affected public health (32,33). Gills is a vital organ for osmoregulation and fish respiration (34), it is the first organ that direct contact with the aquatic environment and stress agents, so it is represented a good biomarker for ecosystem quality, in these study the microscopic examination exhibit progressive structural distortions as hemorrhage, curling and shortening in the secondary gill filament, clown like appearance with hydropic degeneration in the chloride cells at the 7,14 and 21 day exposures were observed due to N-ZnO toxicities, the lesions at the 28,35 and 42 days become more severity related to long exposure to toxic agent, this result agreements with Jones *et al.* (34). The ability of N-ZnO to induce oxidative stress is the main toxic mechanisms in the tissue (35-37) which is interferes with cell wall permeability and biological function of the organelles and decline the activity of Na^+/K^+-ATPase cause cellular ionic imbalances, decline ATP which is the key for the pathological events which lead to disrupting of Na^+/K^+ pump (increase influx of Ca^{+2}, H_2O and Na^+ with efflux K^+). If the causative agent persists these molecular events lead to tissue necrosis and death (38,39), histological alteration come as compensatory, defense and adaptive mechanisms to reduce the effects of toxicant or come as pathological events as increase the distances which is result from cell hyper trophy or infiltration of inflammatory cells these will lead to decrease gas and oxygen exchange and then hypoxia will be occur in the tissue and organs which is the key pathway for cell injury (40,41). These alterations were subsequently quantified by a semi-quantitative analysis in order to detect differences in the intensity of the mentioned alterations these result agreements with Marinovic *et al.* (22). It is concluded from this study that the sub-lethal concentration of nano-zinc oxide leads to histopathological changes in the gills, which vary in the intensity according to the duration of exposure.

Conclusion

The histopathological alteration in the gill recommended that sub-lethal concentration of ZnO nanoparticles could cause significant injury that would finally lead to high mortality in fish at elevated toxicant concentration in the aquatic environment. The cellular level histopathological alterations in the common carp's gills and their direct link with the concentration and exposure time of N- ZnO suggested that these alteration in the gill's architecture could be used as a bio-indicator for the ZnO NPs induced toxicity. The findings suggest that gill can be useful target organs and that common carp can be utilized as a test fish to determine the toxicity of ZnO NPs in freshwater.

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Conflict of Interest

No conflict interests

Reference

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التحليل المرضي النسجي الكمي في غلاصم اسماك الكارب والمعاملة بالتركيز دون المميت الوسطي لأوكسيد الخارصين النانوية

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الخلاصة

يحتل أوكسيد الخارصين النانوية المرتبة الثالثة عالميا بالإنتاج فهو يدخل في صناعات عديدة، وبهذا يصل الى البيئة المائية بصورة مباشرة أو غير مباشرة ويعتبر من أخطر المواد السمية على الأحياء المائية. لذا هدفت هذه الدراسة لتحديد التأثير السمي للتركيز دون المميت الوسطي لأوكسيد الخارصين النانوية على غلاصم اسماك الكارب والتي تمت معاملتها لمدة٧و١٤و٢١و٢٨و٣٥و ٤٢ يوم وباستخدام بروتوكول التقييم شبه الكمي للتغيرات المرضية النسجية. شملت التغييرات المرضية النسجية التغييرات الوعائية واضطرابات نمو الخلية والتغييرات الشكلية وكانت أكثرها شدة عند اليوم ٣٥ من المعاملة وحدوث نخر وموت نسيج

الغلاصم عند اليوم ٤٢ من المعاملة والتي كانت اكثر معنوية للتغيرات الشكلية عند مقارنتها مع بقية التغيرات والمدة الزمنية وأظهرت النتائج بان التغييرات المرضية النسجية عند اليوم السابع من المعاملة هي آفات شديدة بينما في بقية المعاملات كانت آفات غير رجعية يستنتج من هذه الدراسة بان الغلاصم مؤشر حيوي جيد لتقيم حالة تلوث البيئة المائية وان التركيز دون المميت الوسطي لأوكسيد الخارصين النانوية يؤدي الى تغيرات مرضية نسيجة في الغلاصم وإمكانية استخدام التقييم شبه لكمي والتحليل الإحصائي لإعطاء معنوية للآفات المرضية الأكثران شدة.