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Assessment of Hepatotoxicity Induced by Aluminum Oxide Nanoparticles in *Oreochromis niloticus* Using Integrated Biomarkers: Exposure and Recovery

Ehab Massoud[1,2] · Attalla El-Kott[3,4] · Kareem Morsy[3,5] · Amr Adel Abdel-Khalek[5]

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Abstract

The hepatotoxic impacts of 2, 4, and 8 mg/L of Al_2O_3 nanoparticles (31.4 ± 4.8 nm) were evaluated in *Oreochromis niloticus* after 7 days of exposure and 15 days of recovery periods. The biochemical analysis of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase in plasma showed significant increases in both 4 and 8 mg/L Al_2O_3 NPs exposed groups. The antioxidant biomarkers showed concentration-dependent elevations in catalase, superoxide dismutase, glutathione peroxidase activities, and thiobarbituric acid reactive substances levels. Glutathione reduced contents showed significant reductions in both 4 and 8 mg/L Al_2O_3 nanoparticles exposed groups. Several hepatic histopathological alterations were recorded ranging from adaptive responses (e.g. melanomacrophages aggregation) to permanent damage (e.g. necrosis). The recovery period using toxicant-free water led to an obvious reduction in the Al content in liver, liver and antioxidant enzymes in addition to regressive histopathological alterations based on the frequency of alterations occurrence and the extent of affected areas.

Keywords Aluminum oxide NPs · Hepatotoxicity · Liver enzymes · Antioxidant mechanism · Histopathology · *Oreochromis niloticus*

Introduction

The special properties of nanoparticles are the key reason why they enter many industries and continue to reach different water habitats (Xia et al. 2013). Therefore, a growing field of toxicology, nanotoxicology, was developed to discuss specifically the adverse effects of nano-sized materials on the living organism (Arora et al. 2012). Aluminum oxide nanoparticles (Al_2O_3 NPs), are among the most processed and industrially used NPs in cosmetics, catalysis, clothing, biosensors, drug delivery, and wastewater treatment (Abdel-Khalek et al. 2020a). The leading causes of nanotoxicity including Al_2O_3 NPs toxicity are their relatively high surface area, nano-sized, and highly reactive properties. All those unique properties facilitate the entrance of Al_2O_3 NPs into aquatic biota through gills, skin, and gastrointestinal tract after that to different tissues (especially hepatic tissue) via blood circulation. The exposure to 40 nm-sized Al_2O_3 NPs has been shown to induce histological modification of the liver tissue of *Oreochromis mossambicus* (Murali et al. 2017). Several studies have displayed that direct exposure to Al_2O_3 NPs negatively affected aquatic biota and may lead to health problems such as alterations in serum biochemistry (Canli et al. 2018), oxidative damage (Abdel-Khalek et al. 2020a), histopathological changes in vital tissues (Murali et al. 2017). Several biomarkers could be integrated to identify the targeting toxicity to a certain organ and the level of adverse effects (Abdel-Khalek 2016; Canli et al. 2018). Biochemical analysis of plasma constitutes is a useful tool in the physio-pathological assessment in many aquatic organisms

✉ Amr Adel Abdel-Khalek
ecoamr@gmail.com

1 Biology Department, Faculty of Science and Arts in Dahran Aljnoub, King Khalid University, Abha, Saudi Arabia

2 Agriculture Research Centre, Soil, Water and Environment Research Institute, Giza, Egypt

3 Biology Department, Faculty of Science, King Khalid University, Abha, Saudi Arabia

4 Zoology Department, Faculty of Science, Damanhour University, Damanhour, Egypt

5 Zoology Department, Faculty of Science, Cairo University, Cairo, Egypt

(Paunovic et al. 2017). The high levels of liver enzymes in plasma indicate liver malfunction, hepatocyte damage, and leakage of liver enzymes into the bloodstream. Moreover, destruction of the cell membrane of hepatocytes reported by many authors as a result of a disturbance in antioxidant defense components and elevated reactive oxygen species (ROS) production in tissues (Chen et al. 2016). When the generated ROS exceeds the ability of the antioxidant defense system to remove it, excessive ROS can induce oxidative stress that provokes lipid peroxidation (Souza et al. 2019). Al_2O_3 NPs (16.7 nm) were reported to induce irreversible alterations in the antioxidant defense system of several tissues of the freshwater fish, *O. mossambicus* (Vidya and Chitra 2018a). Moreover, histopathological biomarkers are a sensitive tool in nanotoxicological studies as it demonstrates the direct impact of NPs on the normal architecture of cells and reflect the overall health status of tissues (Murali et al. 2017). We hypothesized that the recognized biochemical and histological alterations associated with Al_2O_3 NPs exposure may result indirectly from ROS induction and directly by physical NPs-induced damage. Therefore, the present work aimed to evaluate the hepatotoxic effects of different sub-lethal concentrations of Al_2O_3 NPs using integrated biochemical, antioxidant, and histopathological biomarkers. Furthermore, the persistence hepato-toxicological effects of Al_2O_3 NPs were evaluated by withdrawing the treatment in toxicant-free water for 15 days.

Materials and Methods

The nano-powders of Al_2O_3 (product number 544833) were purchased from Sigma-Aldrich, St. Louis, MO, USA. As provided by the manufacturer the molecular weight is 101.96, the surface area is larger than 40 m^2/g, and the particle size was less than 50 nm. In addition to the information provided by the manufacturer, the characterization of these Al_2O_3 nanopowders was detailed described by Abdel-Khalek et al. (2020a) with confirmation of their particle sizes (31.4 ± 4.8 nm) using field emission transmission electron microscopy (FETEM, JEM-2100F, JEOL Inc., Japan) at accelerating voltage of 200 kV; average hydrodynamic size in water (80.8 nm) by Nano-zetasizer-HT, Malvern Instrument, UK; zeta potential (38.2 mV) using Malvern Zetasizer Nano ZS instrument.

The experimental male fish, *Oreochromis niloticus*, were purchased from a private fish farm located in El-Ismailia governorate, Egypt with an average total length and total body weight ranges from 11.3 to 13.2 cm and 32–38.7 g, respectively. The transportation process of all fish was done in the early morning using large plastic containers with portable oxygen pumps. Fish were maintained for three weeks in glass aquaria (40 × 70 × 26 cm) with 50 L of aerated, dechlorinated tap water with 7 fish per aquarium. Water temperature (25 ± 1 °C), dissolved oxygen (6.4–7.5 mg L^{-1}), and pH (7.3–7.5) were checked daily and kept at the same ranges in all aquaria. With commercial food pellets (20% crude protein, 4% crude fat, 5% crude fiber, 12% crude ash, and 10% crude moisture), fish were fed daily during the acclimatization period. During this period, dead fish, debris, and 40% of water were renewed daily. The excess food pellets and wastes were removed using a suction pump.

The used sub-lethal concentrations (2, 4, and 8 mg/L) of Al_2O_3 NPs were prepared by dissolving the dry nano-powder into the dechlorinated water (pH 7.3–7.5), then ultrasonicated for 60 min. (100 W, 40 kHz) using an ultrasonic homogenizer (BioLogics, Inc., Manassas, VA, USA), to increase their dispersion in water. The selected sub-lethal concentrations were according to Canli et al. (2018) [tested 1, 5, 25 mg/L Al_2O_3 NPs and they recorded that the concentration of Al_2O_3 NPs up to 25 mg/L is a sub-lethal concentration for *O. niloticus* and the highest concentration had eliciting effects] and Abdel-Khalek et al. (2020a) [the work of our lab. showed that 10 mg/l of Al_2O_3 NPs is a sub-lethal concentration for *O. niloticus* with no recorded mortality after 7 days of exposure and had eliciting effects].

Fifty-six of acclimatized fish were divided into 4 groups (n = 14 fish per group divided into 2 aquaria): control (dechlorinated water), 2 mg/L Al_2O_3 NPs exposed fish group, 4 mg/L Al_2O_3 NPs exposed fish group, and 8 mg/L Al_2O_3 NPs exposed fish group. The exposure period was 7 days followed by 15 days of a recovery period (Al_2O_3 NPs-free water). Seven fish were sampled (using clove oil as an anesthetic agent 25 mg/L) from each group during the experimental periods as there is no fish mortality was recorded in both exposure and recovery periods. The actual concentrations of Al_2O_3 NPs were 89 ± 2% of nominal concentrations as confirmed by inductively coupled plasma (ICP-AES), Thermo Sci, model: iCAP6000 series according to APHA (2005). Water was partially changed daily (40% of each aquarium changed and re-loaded with Al_2O_3 NPs) and about an hour before any water renewing, fish were permitted to feed to avoid NPs adsorption on the food pellets and decreasing the nominal concentrations.

The concentrations of Al were measured in hepatic tissues (7 specimens/group) according to APHA (2005) using inductively coupled plasma (ICP-AES), Thermo Sci, model: iCAP6000 series with a limit of detection equal 0.1 µg/l for Al. Acid digestion of liver tissues was done according to Neugebauer et al. (2000). The samples were dried in an 80°Coven for 8 h till complete drying. A concentrated mixture of nitric acid and perchloric acid (4:1,v-v) was used to digest the tissue samples. The digested samples were placed on a heat plate then the temperature rose gradually to 100°Cuntil reaching clear solutions. In a volumetric flask (25 mL), the resulted clear solutions were transferred and

diluted to known volume by de-ionized water. The concentrations of Al metal were expressed as mg/kg dry weight. A standard reference material (Lake Superior fish 1946 NIST, National Institute of Standards and Technology, USA) has been used to verify the measuring process and the recovery ranges were 95%–101%.

Using heparinized syringes, blood samples were withdrawn from the caudal vein of the sampled fish (7 fish/group). The obtained blood samples were centrifuged at 3000 r.p.m. for 15 min to separate plasma samples. Based on the activities of three liver enzymes (AST, ALT, and ALP), the liver functions were evaluated according to the colorimetric method of Reitman and Frankel (1957) for AST and ALT while alkaline phosphatase (ALP) activity was estimated according to Belfield and Goldberg (1971). All activities were expressed as U/L.

The dissected liver tissues were homogenized (1 g tissue in 5 ml specific cold buffer solution for each biomarker) according to the instructions of used biodiagnostic kits, Biodiagnostic Dokki, Giza, Egypt. The produced homogenates were stored at -80 °C after the centrifugation for 15 min. at 4000 r.p.m. in a cooling centrifuge (4 °C). The activities of the CAT enzyme were determined based on stopping the enzymatic reaction between CAT and a specific volume of H_2O_2 (after one minute) using a catalase inhibitor. The colored chromophore (resulted from the reaction between remaining H_2O_2; 3, 5-Dichloro-2-hydroxybenzene sulfonic acid; 4-aminophenazone; peroxidase) was inversely proportional (510 nm) to the amount of catalase as described by Aebi (1984). The homogenizing solution of the CAT kit was 50 mM potassium phosphate, pH 7.4. 1 mM EDTA, and 1 mL/L Triton X100. Based on the ability of SOD to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye, the activity of this enzyme could be assessed as designated by Nishikimi et al. (1972). The inhibition rate of SOD was directly proportional to the color change at 560 nm. The specific homogenizing buffer for the SOD kit was 100 mM potassium phosphate, pH 7.0, containing 2 mM EDTA. GPx enzyme could reduce organic peroxide into oxidized glutathione (GSSG) which is recycled to its reduced form in the presence of glutathione reductase. Therefore, the activity of GPx was determined (indirectly) using the method described by Paglia and Valentine (1967). The oxidation of NADPH to $NADP^+$ is combined by an absorbance reduction at 340 nm providing a spectrophotometric means for monitoring the activity of GPx. The used GPx homogenizing buffer was consistent from 50 mM phosphate buffer, pH 7.0, containing 5 mM EDTA and 1 mM 2-mercaptoethanol. The activities of CAT, SOD, and GPx were expressed as U/g proteins. Depending on the method described by Beutler et al. (1963), GSH could be colorimetrically estimated (at 405 nm) based on the ability of GSH to reduce 5, 5′-dithiobis 2-nitrobenzoic acid to a yellow-colored product. The used homogenizing buffer was 50 mM potassium phosphate, pH7.5, 1 mM EDTA and the measurement unit is mmol/g protein. The level of TBARS (an indication of lipid peroxidation process) was assessed according to Ohkawa et al. (1979), in which the TBARS reacts with thiobarbituric acid producing a colored product. The color intensity (at 534 nm) was equivalent to TBARS level and expressed as nmole/g tissue.

Liver tissues were isolated and washed in a saline solution then preserved in Bouin's fixative. All tissues were processed, sectioned at 4 µm, and then stained using hematoxylin and eosin according to Bernet et al. (1999). Liver tissues from 7 different fish were sectioned per group (2 slides/fish; n = 14) and the observed alterations were recorded by light microscopy to calculate the % of each alteration in each studied fish group after the exposure and recovery periods.

To evaluate the statistically significant difference ($P < 0.05$) between different groups, data were statistically analyzed using analysis of variance (ANOVA) and Duncan's multiple range test as showed by different case letters in the descending order A, B, and C. Data also were statistically analyzed using t-test to show the significant difference ($P < 0.05$) between the exposure and recovery periods. The results were expressed as mean ± S.E. The analysis was done using Statistical Processor Systems Support, SPSS software, version 16.0, IBM, Chicago, IL, USA.

Results and Discussion

Regarding the accumulation potency of aluminum oxide NPs in the liver, there was a concentration-dependent accumulation pattern with a significant difference ($P < 0.05$) among all studied fish groups (Table 1). Although the accumulated Al metal was significantly decreased after 15 days of a recovery period in all studied groups there were measurable amounts of aluminum still accumulated in the hepatic tissues. Owing to their nano-size, Al_2O_3NPs can invade the body through different mechanisms and migrate to various tissues with different amounts. The biodistribution studies on several NPs have shown that the liver tissue is the main target tissue for those studied NPs (Paunovic et al. 2017). The concentration-dependent Al bioaccumulation in liver tissues of the present study is in agreement with Murali et al. (2017) who reported a dose-dependent hepatic accumulation of aluminum metal in fish that was exposed to variable concentrations of Al_2O_3 NPs for 96 h. Also, this observation may be due to the excessive production rate of metallothionein that effectively combines with metals to minimize their potential damages (Abdel-Khalek et al. 2020b). Besides, the liver is an active metabolic organ that plays an important role in the detoxification of metals via conjugation of metal with sulfur protein forming M-S-protein complexes (Abdel-Khalek 2015).

Table 1 Aluminum concentrations in liver tissues of *O. niloticus* after 7 days of exposure to Al_2O_3 NPs and 15 days of a recovery period in free-toxicant water. N = 14 for each group

	Aluminum concentrations in liver tissues (mg/kg dry wt.)		
	Exposure period (7 days)	Recovery period (15 days)	P_t
Control	N.D	N.D	–
Al_2O_3 NPs (2 mg/L)	80.37**C** ± 4.7[a]	50.65**C** ± 5.02[b]	**p < 0.05**
Al_2O_3 NPs (4 mg/L)	150.22**B** ± 8.89[a]	90.62**B** ± 7.22[b]	**p < 0.05**
Al_2O_3 NPs (8 mg/L)	369.47**A** ± 20.81[a]	206.87**A** ± 13.83[b]	**p < 0.05**
P_f	**p < 0.05**	**p < 0.05**	

Data are represented as means ± SE. The capital letters represent Duncan's test (p < 0.05) between different groups of the same period compared with the control group. Columns with the same letters are not significantly different; otherwise, they do. The small superscript letters represent Duncan's test (p < 0.05) between exposure and recovery periods of the same group. Rows with the same letters are not significantly different; otherwise, they do

N.D. = not detect (limit of detection is 0.1 µg/l)

All studied liver enzymes (Fig. 1) were significantly elevated after 7 days of exposure to 4 and 8 mg/L of Al_2O_3 NPs. While the 2 mg/L Al_2O_3 NPs exposed group showed insignificant changes in all studied enzymes except the ALT enzyme showed a slight increase compared with the control group. The effects of the recovery period in reducing the activities of those enzymes were obvious in all studied groups except for 8 mg/L Al_2O_3 NPs exposed group. The excessive accumulation of aluminum NPs in the liver may cause metallic damage and develop leaky hepatocellular membranes. These findings are consistent with Paunovic et al. (2017) who reported obvious hepatotoxic effects of several metallic NPs (Ag NPs, Au NPs, Fe NPs, Ti NPs, Zn NPs, and Pt NPs) on liver enzymes. Also, the elevated liver enzymes in the plasma of *O. niloticus* were recorded after 14 days of exposure to Fe_2O_3 and Al_2O_3 NPs individually and in a mixture (Abdel-Khalek et al. 2020a). The elevated levels of studied enzymes named AST, ALT, and ALP are believed to derive from liver damage caused by Al_2O_3 NPs, which allowed the release of intracellular hepatocellular enzymes into the bloodstream. The insignificant change in the studied liver enzymes (AST and ALP) of all samples collected from the 2 mg/L Al_2O_3 NPs exposed group confirmed that this concentration could not affect the permeability of the hepatocytes and unable to disturb the integrity of cell membranes (as confirmed by the histological results of the present work).

As shown in Fig. 2, the activities of all studied antioxidant enzymes were significantly elevated in all studied groups (except GPx of 2 mg/L Al_2O_3 NPs exposed group). After the recovery period, the activities of those enzymes were sharply decreased compared to the exposure period and some enzyme activities (ex. CAT of 2 and 4 mg/L Al_2O_3 NPs exposed group) became insignificant with the control group. Regarding the non-enzymatic biomarkers (Fig. 2) for antioxidant status, a significant decrease in GSH concentration was recorded in both 4 and 8 mg/L Al_2O_3 NPs exposed groups. While the TBARS level was significantly increased in all studied groups with a maximum elevation in the 8 mg/L Al_2O_3 NPs exposed group. After 15 days of free-toxicant water exposure, the adverse effects of Al_2O_3 NPs on the GSH and TBARS levels were reduced in comparison to

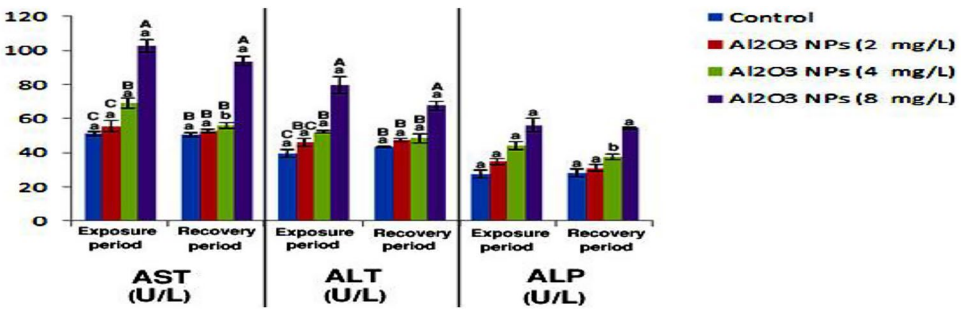

Fig. 1 The activities of AST, ALT, and ALP enzymes in the plasma of *O. niloticus* after 7 days of exposure to Al_2O_3 NPs and 15 days of a recovery period in free-toxicant water. Data are represented as means of seven samples in each group ± SE. The capital letters represent Duncan's test ($P < 0.05$) between different groups of the same period compared with the control group. Columns with the same letters are not significantly different; otherwise, they do. The small letters represent Duncan's test ($P < 0.05$) between exposure and recovery periods of the same group. Columns with the same letters are not significantly different; otherwise, they do

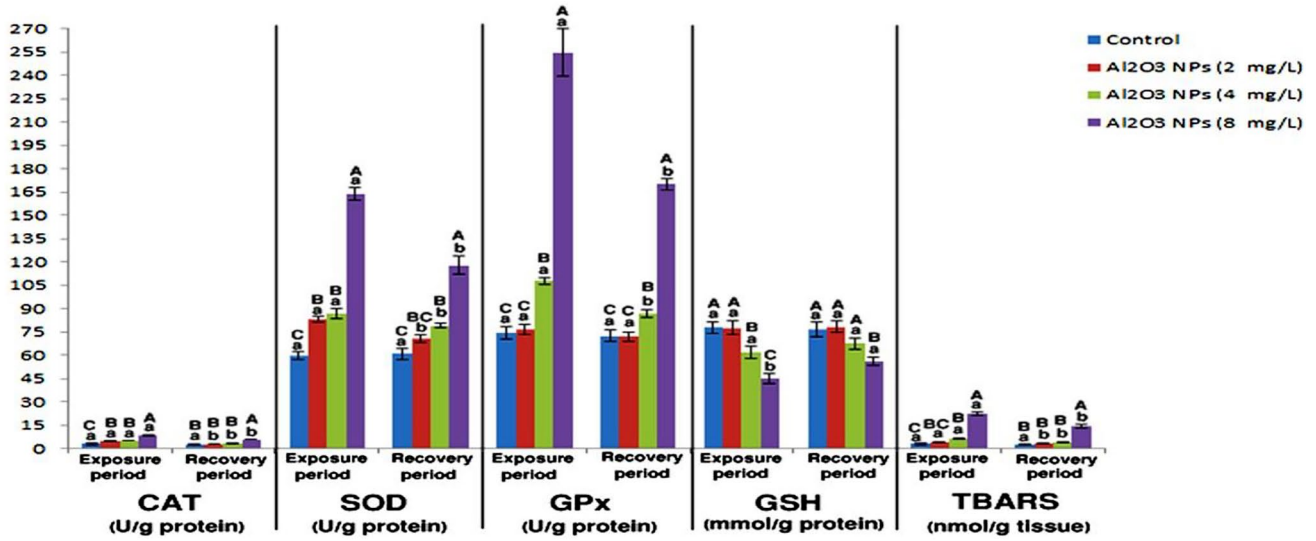

Fig. 2 The studied antioxidant biomarkers (CAT, SOD, GPx, GSH, and TBARS) in the liver of *O. niloticus* after 7 days of exposure to Al_2O_3 NPs and 15 days of a recovery period in free-toxicant water.—Data are represented as means of seven samples in each group ± SE. The capital letters represent Duncan's test ($p < 0.05$) between different groups of the same period compared with the control group. Columns with the same letters are not significantly different; otherwise, they do. The small letters represent Duncan's test ($p < 0.05$) between exposure and recovery periods of the same group. Columns with the same letters are not significantly different; otherwise, they do

the control group whereas such effects are still observed to some extent in the 8 mg/L Al_2O_3 NPs exposed group. The monitoring of the interactive antioxidant compounds signifies the potency of many toxic compounds, as many metal NPs invoke their toxicological impacts by disrupting the antioxidant mechanism and generating excessive amounts of ROS (Wu and Zhou 2013). The present results indicated the activation of the first antioxidant defense mechanism as confirmed by the elevation of the CAT-SOD defense system. As reported by Qu et al. (2014), the SOD and CAT enzymes are the first line of defense against oxidative damage. SOD plays a crucial role in transforming toxic superoxide anions into H_2O_2 which is a less reactive compound. The produced H_2O_2 is converted to non-reactive substances, such as H_2O and O_2 in the presence of the CAT enzyme as a co-working enzyme. The significant elevation in both related enzymes (SOD and CAT) after Al_2O_3 NPs exposure revealed the increased expression of these enzymes to overcome the overproduction of ROS. As shown in the study of Abdel-Khalek et al. (2018), GPx is an important enzyme for detoxifying H_2O_2 and organic peroxides. The present elevation in GPx activities confirmed by Hao and Chen (2012) who reported an elevation in GPx of the liver and gills tissues of *Cyprinus carpio* after the exposure to ZnO NPs.

The elevation of SOD and CAT activities without any significant change in the GPx activity after the exposure to 2 mg/L Al_2O_3 NPs revealed that both SOD and CAT were able to scavenge the produced ROS, and the role of GPx was no longer required. This hypothesis is in agreement with Souza et al. (2019) who showed similar observations in zebrafish after sub-lethal exposure to graphene oxide. The recorded elevation in GPx activities was synchronous with a sharp decrease in the concentration of GSH, signposted that the GPx-GSH antioxidant process occurred with inadequate GSH restoration. The decline of GSH was supported by Jozefczak et al. (2012) who revealed this decline to the consumption of GSH by ROS and the conjugation between metal to GSH forming GS-metal complex. While the elevation in TBARS contents indicated that the initialization of all antioxidant battery could not prevent lipid peroxidation. Vidya and Chitra (2018a) reported that Al_2O_3 NPs of 16.7 nm particle size induced irreversible changes in the antioxidant biomarkers of gill, liver and brain tissues of *Oreochromis mossambicus* fish which disagree with the significant changes recorded in the present work after the recovery period.

The histological sections collected from the control fish group showed normal hepatocytes with homogenous cytoplasm and a centralized nucleus (Fig. 3). The histopathological sections of Al_2O_3 NPs exposed groups showed several alterations in hepatic tissues as degeneration of hepatocytes, cytoplasmic vacuolization, irregular arrange of hepatocytes, infiltration of red blood cells, congestion of central vein, pyknotic nuclei, and melanomacrophage aggregation (Fig. 3). The percentage appearance of the recorded histological injuries (Table 2) revealed that the recorded histopathological damages were concentration-dependent. After the recovery period, complete healing of many recorded lesions was not observed which means that the recorded histopathological alterations were irreversible or needs more

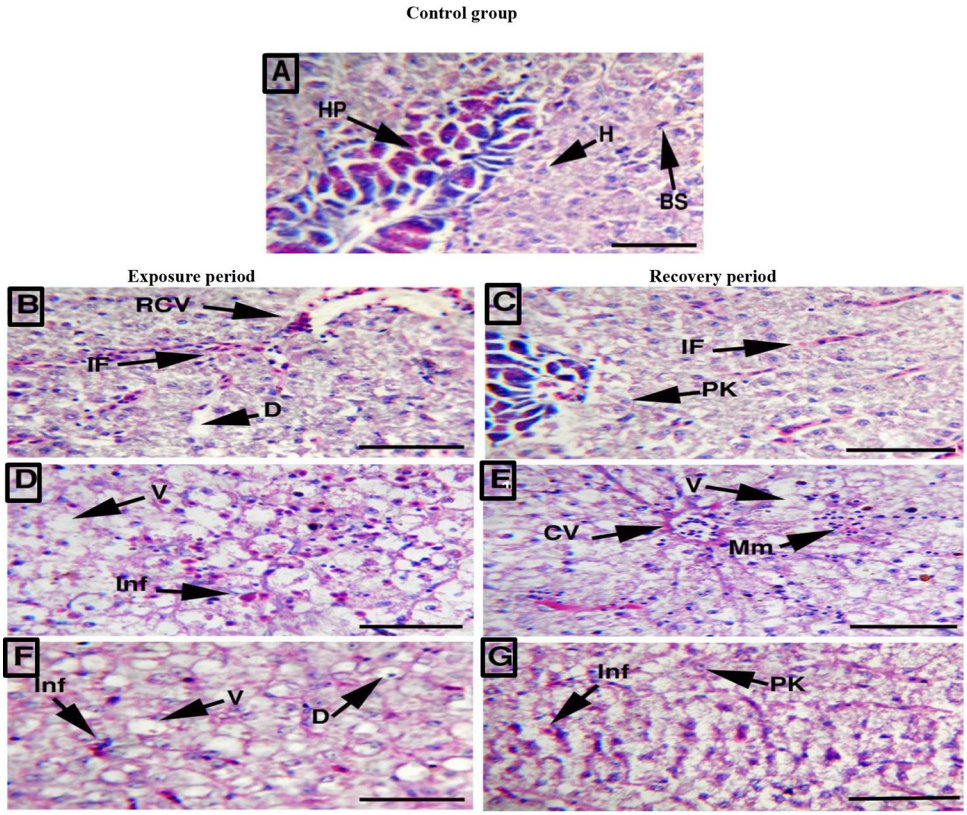

Fig. 3 Representative histopathological alterations in liver tissues of *O. niloticus* after 7 days of exposure to Al_2O_3 NPs and 15 days of a recovery period in free-toxicant water. *H* hepatocytes; *HP* hepatopancreatic tissue; *BS* blood sinusoid; *RCV* rupture of central vein; *IF* infiltration of red blood cells; *D* degeneration of hepatocytes; *PK* pyknotic nuclei; *V* vacuolization; *Inf* inflammatory cells; *CV* Central vein; *Mm* melanomacrophage aggregation. Scale bar = 100 µm

Table 2 The percentage of the recorded histopathological alterations in liver tissues of *O. niloticus* after 7 days of exposure to Al2O3 NPs and 15 days of a recovery period in free-toxicant water. N = 14 specimens/group

Recorded alterations	Control group		Al_2O_3 NPs (2 mg/L)		Al_2O_3 NPs (4 mg/L)		Al_2O_3 NPs (8 mg/L)	
	Exposure period	Recovery period	Exposure period	Recovery period	Exposure period	Recovery period	Exposure period	Recovery period
Irregular arrange of hepatocytes	3/14(21.4%)	2/14(14.3%)	4/14(28.6%)	4/14(28.6%)	6/14(42.9%)	5/14(35.7%)	9/14 (64.3%)	10/14(71.4%)
Degeneration of hepatocytes	0/14(0%)	0/14(0%)	2/14(14.3%)	2/14(14.3%)	5/14(35.7%)	5/14(35.7%)	10/14(71.4%)	10/14(64.3%)
Vacuolization	1/14(7.1%)	1/14(7.1%)	4/14(28.6%)	3/14(21.4%)	7/14(50%)	7/14(50%)	12/14(85.7%)	10/14(71.4%)
Infiltration RBCs	2/14(14.3%)	1/14(7.1%)	4/14(28.6%)	3/14(21.4%)	7/14(50%)	6/14(42.9%)	13/14(92.9%)	12/14(85.7%)
Congestion of central vein	1/14(7.1%)	1/14(7.1%)	3/14(21.4%)	3/14(21.4%)	6/14(42.9%)	6/14(42.9%)	8/14(57.1%)	8/14(57.1%)
Pyknotic nuclei	1/14(7.1%)	2/14(14.3%)	5/14(35.7%)	4/14(28.6%)	8/14(57.1%)	6/14(42.9%)	11/14(78.6%)	11/14(78.6%)
Melanomacrophages	0/14(0%)	0/14(0%)	2/14(14.3%)	2/14(14.3%)	5/14(35.7%)	4/14 (28.6%)	7/14 5(0%)	6/14 (42.9%)
Total recorded alterations	8/98 (8.2%)	7/98 (7.1%)	24/98 (24.5%)	21/98 (21.4%)	44/98 (44.9%)	39/98 (39.8%)	70/98 (71.4%)	67/98 (68.4%)

time to reach a complete recovery. The cytoplasmic vacuolization, congestion in blood vessels, and necrotic damage were recorded by Murali et al. (2017) in the *O. mossambicus* after 96 h. of sub-lethal exposure to the Al_2O_3 NPs of 40 nm size. The increased vacuolization was recorded before as a sign of metabolic dysfunction, excessive fat accumulation in cytoplasm, a disorder in proteins, and carbohydrates synthesis (Ciji and Nandan 2014). The congestion in the central

vein and RBCs infiltration through hepatocytes indicated a weakened hepatic blood vessel. Degenerative-necrotic damage of hepatocytes displayed severe injuries in the integrity of cell membranes that is related to NPs-induced oxidative stress (Wu and Zhou 2013). These recorded alterations and melanomacrophage aggregation showed that Al_2O_3 NPs were markedly stored in the liver (confirmed by the bioaccumulation results of the present study) and behaved as an injurious factor that induces direct degenerative and hemorrhagic damages. These findings are agreed with Vidya and Chitra (2018b) who recorded irreversible histological damages in liver tissues of *O. mossambicus* after exposure to 4 mg/L of Al_2O_3 NPs (16.7 nm) for short-term (96 h) and long-term (60 days) durations.

Conclusion

Based on the integrated biomarkers of the current study, the exposure to Al_2O_3 NPs induced concentration-dependent hepatotoxicity in *O. niloticus*. When fish submitted to free-toxicant water to recover after the exposure periods, the accumulated Al metal in hepatic tissues, liver enzymes (except for 8 mg/L exposed group), and antioxidant capacity showed a significant enhancement and the hepatotoxicity recorded in the exposure period became less critical. These findings are agreed with Souza et al. (2019) who stated that antioxidant defense components may be elevated a few hours after toxicants exposure and these components can restore their normal level when the fish return to their initial conditions. The enhanced biochemical and oxidative stress biomarkers after the recovery period is consistent with the hypothesis of the present study indicating the effectiveness of withdrawal treatment in a free-toxicant water. Based on the frequencies of the histopathological alterations observed in the exposure period, these alterations were not completely repaired after the recovery period but the size of the affected areas was more or less reduced. The incomplete recovery of the histological structure of the liver could explain the elevated liver enzymes in the 8 mg/L exposed group after the recovery period as the incomplete recovery of the hepatocytes facilitates the leakage of liver enzymes into the bloodstream.

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Authors' Contributions Abdel-Khalek AA and Massoud E. conceived and designed research. Abdel-Khalek AA and Morsy K.conducted experiments. Abdel-Khalek AA, El-Kott A., and Morsy K. analyzed data. Abdel-Khalek AA wrote the manuscript. All authors read and approved the manuscript.

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Availability of Data and Materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards:

Conflict of interest The authors declare that they have no competing interests.

Consent to Participate All authors read and approved the final manuscript.

References

Abdel-Khalek AA (2015) Antioxidant responses and nuclear deformations in freshwater fish, *Oreochromis niloticus*, facing degraded environmental conditions. Bull Environ Contam Toxicol 94(6):701–708

Abdel-Khalek AA (2016) Comparative evaluation of genotoxic effects induced by CuO bulk and nano-particles in Nile tilapia, *Oreochromis niloticus*. Water Air Soil Pollut 227:35

Abdel-Khalek AA, Elhaddad E, Mamdouh S, Marie M-AS (2018) The chronic exposure to discharges of Sabal drain induces oxidative stress and histopathological alterations in *Oreochromis niloticus*. Bull Environ Contam Toxicol 101(1):92–98

Abdel-Khalek AA, Badran SR, Marie M-AS (2020a) The effective adsorbent capacity of rice husk to iron and aluminum oxides nanoparticles using *Oreochromis niloticus* as a bioindicator: biochemical and oxidative stress biomarkers. Environ Sci Pollut Res 27:23159–23171

Abdel-Khalek AA, Zayed HS, Elsayad SM, Zaghloul KH (2020b) Assessment of metal pollution impacts on *Tilapia zillii* and *Mugil cephalus* inhabiting Qaroun and Wadi El-Rayan lakes, Egypt, using integrated biomarkers. Environ Sci Pollut Res 27:26773–26785

Aebi H (1984) Catalase in vitro. Method Enzymol 105:121–126

American Public Health Association (APHA) (2005) Standard methods for the examination of water and wastewater. American Water Works Association, New York

Arora S, Rajwade JM, Paknikar KM (2012) Nanotoxicology and in vitro studies: the need of the hour. Toxicol Appl Pharmacol 258:151–165

Belfield A, Goldberg DM (1971) Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. Enzyme 12:561–573

Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T (1999) Histopathology in fish: proposal for a protocol to assess aquatic pollution. J Fish Dis 22:25–34

Beutler E, Duron O, Kelly MB (1963) Improved method for the determination of blood glutathione. J Lab Clin Med 61:882–888

Canli EG, Dogan A, Canli M (2018) Serum biomarker levels alter following nanoparticle (Al_2O_3, CuO, TiO_2) exposures in freshwater fish (*Oreochromis niloticus*). Environ Toxicol Pharmacol 62:181–187

Ciji PP, Nandan SB (2014) Toxicity of copper and zinc to *Puntius parrah* (day, 1865). Mar Environ Res 93:38–46

Jozefczak M, Remans T, Vangronsveld J, Cuypers A (2012) Glutathione is a key player in metal-induced oxidative stress defenses. Int J Mol Sci 13(12):3145–3175

Hao L, Chen L (2012) Oxidative stress responses in different organs of carp (*Cyprinus carpio*) with exposure to ZnO nanoparticles. Ecotoxicol Environ Saf 80:103–110

Murali M, Suganthi P, Athif P, Sadiq Bukhari A, Syed Mohamed HE, Basu H, Singhal RK (2017) Histological alterations in the hepatic tissues of Al_2O_3 nanoparticles exposed freshwater fish *Oreochromis mossambicus*. J Trace Elem Med Biol 44:125–131

Neugebauer EA, Sans Cartier GL, Wakeford BJ (2000) Methods for the determination of metals in wildlife tissues using various atomic absorption spectrophotometry techniques (Technical Report Series No. 337E). Canadian wildlife service, Headquarters, Hull, Québec, Canada

Nishikimi M, Appaji N, Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 46:849–854

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158–169

Paunovic J, Vucevic D, Radosavljevic T, Pantic S, Nikolovski D, Dugalic S, Pantic I (2017) Effects of metallic nanoparticles on physiological liver functions. Rev Adv Mater Sci 49:123–128

Qu R, Feng M, Wang X, Qin L, Wang C, Wang Z, Wang L (2014) Metal accumulation and oxidative stress biomarkers in liver of freshwater fish *Carassius auratus* following in vivo exposure to waterborne zinc under different pH values. Aquat Toxicol 150:9–16

Reitman S, Frankel S (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 28:56–63

Souza JP, Mansano AS, Venturini FP, Santos F, Zucolotto V (2019) Antioxidant metabolism of zebrafish after sub-lethal exposure to graphene oxide and recovery. Fish Physiol Biochem 45:1289–1297

Vidya PV (1852a) Chitra KC (2018a) Aluminium oxide nanoparticles induced irreversible alterations in the antioxidant system and marker enzymes activities in the fish *Oreochromis mossambicus* (Peters. Eur J Biomed Pharm Sci 5(2):1162–1170

Vidya PV (1852b) Chitra KC (2018b) Aluminium oxide nanoparticles induced irrevocable damages in gill, liver and brain tissues of the freshwater Fish, *Oreochromis mossambicus* (Peters. Int J Fish Aquac Res 3(2):13–17

Wu Y, Zhou Q (2013) Silver nanoparticles cause oxidative damage and histological changes in medaka (*Oryzias latipes*) after 14 days of exposure. Environ Toxicol Chem 32:165–173

Xia J, Zhao HZ, Lu GH (2013) Effects of selected metal oxide nanoparticles on multiple biomarkers in *Carassius auratus*. Biomed Environ Sci 9:742–749

Zhao J, Wang Z, Liuc X, Xiea X, Zhang K, Xing B (2011) Distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) and their potential toxicity. J Hazard Mater 197:304–310