Impact of Copper Oxide Nanoparticles on freshwater crayfish, *Procambarus clarkia.* A combined histopathological, Biochemical and genotoxicological study

Mahmoud Abd El-Atti[1], Mahmoud M. A. Desouky*[1,2], Amaal Mohamadien[1,3] and Radwa M. Said[2]

1- Department of Zoology, Faculty of Science, Zagazig University, Zagazig, Egypt.
2- Al Jouf University, Sakaka, KSA
3- Department of Biology, Faculty of Science, Taif University, KSA.
* Corresponding author. E mail: mdesouky0@yahoo.com

ABSTRACT

Copper oxide nanoparticles (CuO NPs) are commonly used in many industrial and biomedical applications. Excessive usage of these products may lead to their discharge into aquatic ecosystems causing harmful ecotoxicological hazards. The present study was performed to assess the impact of CuO NPs on mortality, bioaccumulation, oxidative stress, histopathology of the hepatopancreas and DNA damage of the red swamp crayfish *Procambarus clarkii*. The assessment of such effectsof Cu NPs may be useful for understanding the mechanism of their toxicity and evaluation of the possibility of using nanoparticles-induced alterations in the investigated organism as pollution biomarkers. For these purposes, adult crayfishes were exposed to 25, 125 and 250 mg/l of CuO NPs for 28 days. Percentages of mortalities were 0%, 6.7% and 36.7%, for application of these concentrations respectively. Bioaccumulation analysis revealed that the highest accumulation levels of copper were observed in gills followed by hepatopancreas while the lowest was detected in muscles. Biochemical analysis showed that exposure of *P. clarkii* to the above concentrations of CuO NPs caused significant increase in the activities of lipid peroxidation (LPO), Catalase (CAT), Glutathione-S-transferase (GST) and Metallothioneins (MTs) in hepatopancreas. On the other hand, levels of glutathione (GSH), total lipids (TL) and total proteins (TP) were significantly decreased. Light microscopical examinations of the hepatopancreas exposed to CuO NPs revealed lumen dilatation, increased yellowish brown granules, vacuolation, nuclear pyknosis and cellular lysis in the hepatopancreatic epithelial cells. At the electron microscopiclevel, vacuolated cytoplasm with densely stained secretory granules, fragmented microvilli of absorptive cells, ruptured RER, and deformed mitochondria were noticed. Comet assay analysis showed that high concentrations of these nanoparticles caused nuclear DNA damage. In conclusion, CuO NPs induced many histopathological and biochemical alterations in *P. clarkia* which are clear enough to be used as biomarkers for CuO NPs exposure.

Keywords: *Procambarus clarkii,*CuO NPs, Bioaccumulation, antioxidant enzymes, DNA damage,Histopathology.

INTRODUCTION

Nanomaterials have received growing interests as a result of their unique chemical and physical features, including uniform pore sizes, high surface area, high reactivity and diverse biological activity (Sahooli *et al*., 2012; Khashan *et al*., 2016). Due to rapid development of nanotechnology, nanomaterials have been used enormously in commercial, industrial and medical products (Laurent *et al*., 2008, Joh *et al*., 2011; Tang *et al*., 2012). In order to efficiently detect and manage potential environmental effects from nanomaterialexposure, it is important to study the environmental, health and safety sides at an early stage of nanomaterial development and use (Oberdörster *et al*., 2005). The aquatic

ecotoxicology of nanomaterials is a quite new and evolving field which will be important for future generations (Farlow, 2014).

Concern has especially increased in the case of metal oxide nanoparticles, because these particles have wide industrial applications in photocatalytic water purification systems and solar cells (Hagfeldt & Graetzel, 1995 ; Usui *et al*., 2004) .They are extensively used in medical applications including disinfection and as antimicrobials (Katwal *et al*., 2015).Among metal oxide nanoparticles are copper oxide nanoparticles (CuO NPs) which attracted significant attention because of their antimicrobial and biocide properties and their usage in many industrial and biomedical applications (Perreault *et al.,* 2012; Nations *et al*., 2015). Although CuO NPs have demonstrated their use in biomedical applications; the major disadvantage for their use on the medical field is due to their possibly toxic effects for mammalian cells as well as for vertebrates and invertebrates (Isani *et al.,* 2013; Ostaszewska *et al.,* 2015). CuO NPs revealed different toxic *in vitro* and *in vivo*activities, when tested on mammalian cells and on various animal models (Khatri *et al.,* 2013). They caused inhibition in growth in common carp *Cyprinus carpio* and malformations in the liver and retina of zebrafish embryos and larvae (Sun *et al.,* 2015).Zhang *et al.* (2014) revealed also that copper oxide nanoparticles caused cellular abnormalities in the form of mitochondrial and DNA damage.

On the other hand, the red swamp crayfish, *Procambarus clarkii* is a native species in Mexico and USA and it was introduced to Egypt in the early 1980′s for aquaculture but escaped and spread via the River Nile (Ibrahim *et al.,* 1995). It is able to tolerate polluted environments and has been used as an indicator of metal pollution in numerous studies of aquatic environments. Moreover, its central position in aquatic food webs makes it a potential vector of contaminants to high trophic levels (Serrano *et al.,* 2000). Therefore, the aim of the present study was to investigate the effects of CuO NPs on *P. clarkii* and to critically evaluate its utility as a bioindicator for CuO NPs toxicity in the aquatic environment.

MATERIALS AND METHODS

1. Experimental design

CuO NPs (Average size 100-140 nm and with a purity > 99%)was purchased from Lab Chemical Trading Co., Cairo, Egypt. Suspension of CuO NPs was prepared with distilled water and dispersed with bath sonicator for 20 min. The stock solution was diluted to desired concentrations before treatment. The prepared concentrations were 25, 125 and 250 mg/l.

During April 2018, the red swamp crayfish, *P. clarkii* (about 120 individuals) were collected with 0.7 cm diagonal net size from Bany-Helal irrigation Canal, Miniet Alkamh, Sharkia Governorate, Egypt. The collected specimens were transferred alive to the laboratory and maintained in glass aquaria (40 x 40 x 40 cm).The average water quality parameters were: total ammonia, 0.28 ± 0.03; temperature, 27.8 ± 0.28; dissolved oxygen, 6.05 ± 0.35; pH, 7.6 ± 0.19. All these values were within the acceptable ranges according to Boyd (1984). Suffocation was avoided by ensuring a water depth of 7 - 10 cm. Mature males weighing 25 – 34 g and 9 – 12 cm long were acclimated to indoor laboratory conditions for one week. Ten mature animals were stocked inside each aquarium and treated with either 0.0 (control), 25, 125, or 250 mg/l CuO NPs for 28 days in triplicates. Crayfish were fed with carrot and minced meat up to apparent satiation thrice a day at 9:00 , 13:00, and 17:00 h. Light regime was maintained to be 12:12 h using light tubes. Aquaria's water was renewed daily with aerated tape water and re-dosed with CuONPs. At the end of the experiment, crayfish were collected from each aquarium and counted.

Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii*.
A combined histopathological, Biochemical and genotoxicological study

2. Bioaccumulation analysis

Tissues of the hepatopancreas, gills and muscles were dissected out and oven-dried for 72 hours at 80°C. The tissues were ground to a fine powder using mortar and weighed to obtain ~0.1 g. Tissues were placed in an Xpress vessel containing 5 ml of acid digestion mixture (3 ml HNO_3: 2 ml $HClO_4$). Chemical digestion was performed using the MARS Xpress Microwave. Measurements were conducted by "Buck scientific 210VGP Atomic Absorption Spectrophotometer" at the Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.

3. Antioxidant biomarkers

Lipid peroxidation (LPO), catalase (CAT), glutathione-S-transferase (GST), glutathione (GSH) and metallothioneins (MTs) were analyzed according to the corresponding assay kit protocol (Bio Vision-Milpitas, CA, USA). Total proteins were measured using total proteins kit according to Biuret method. Total lipids were colourimetrically determined by using the method of Smedes and Thomasen (1996).

4. Light microscopic preparations

Specimens of hepatopancreas of both control and CuO NPs-exposed animals were dissected out, fixed in 10% formalin and dehydrated via an ascending series of ethanol, embedded in paraffin wax, sectioned at 4-6 μm thick and stained with hematoxylin and eosin.

5. Transmission electron microscopic preparations

Small pieces of hepatopancreas were dissected out from control and CuO NPs-exposed animals, fixed in 2% glutaraldehyde in 0.1 M phosphate buffer and post-fixed in 1% OsO_4 for 60 min. Samples were then dehydrated in an ascending ethanol series and finally embedded in Araldite Epon. Ultra-thin sections were contrasted with uranyl acetate, lead citrate stains, and examined using aJeol Transmission Electron Microscope at the Regional Centre for Mycology and Biotechnology, El-Azhar University, Nasr city, Cairo, Egypt.

6. Comet Assay

DNA damage analysis of the hepatopancreatic cells was carried out by Single Cell Gel Electrophoresis developed bySingh *et al.* (1988).The hepatopancreas was dissected out, minced in a 20 mM solution of EDTA in PBS and centrifuged at 3000 rpm for eight minutes. Ten μl of supernatant were drawn off and mixed with 90 μl of low melting agar. One hundredμl solutions were placed on a pre-treated comet assay slide. These slides were placed in an alkaline solution (pH 13) to unwind the DNA for 30 minutes. Slides were then placed in the Comet Assay Electrophoresis System (Trevigen) in electrophoresis buffer (pH >13) for 30 minutes, placed in 70% alcohol for five minutes and allowed to air dry overnight. Extracted DNA on the slides was stained with SYBR-green, which emits fluorescent light within the 425-500 nm regions. The slides were analysed under a mercury lit epifluorescent microscope. A Magnifier SP Digital Camera was used. Length of DNA (comet) migration was measured using Image-Pro Plus software. All nuclei and their comets on the slides were measured (~100 cell). DNA damage was determined by measuring the length of DNA migration, the percentage of migrated DNA and finally measuring tail moment.

7. Statistical analysis

SPSS program version 20 (SPSS, Richmond, VA, USA) as described by Dytham(2011)was used to calculate the appropriate statistical tests.Prior to statistical analysis, all data were tested for normality and homogeneity using the Kolmogorov–Smirnov

Mahmoud Abd El-Atti *et al.*

and Bartlett's tests. One-way ANOVA was used to compare the effect of CuONPs concentrations. Two-way ANOVA was used to explore the effect of CuO NPs concentrations and exposure periods in different organs. Significant differences among treatments at $P < 0.05$ were performed using Duncan test as a post-hoc test.

RESULTS

1. Crayfish Mortality.

Figure (1) represents percentages mortality of red swamp crayfish *P. clarkii* treated with different concentrations of CuO NPs for 28 days. Percentages of dead crayfish after exposure to 125 and 250 mg/l of CuO NPs were 6.7% and 36.7%, respectively. No mortality was recorded after exposure to 0.0 or 25 mg/l of CuO NPs.
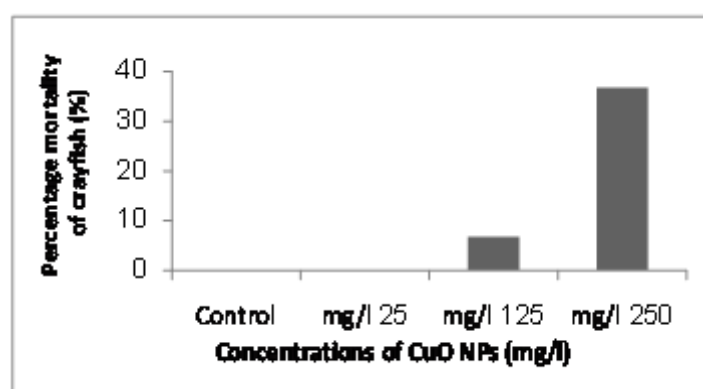
Fig. (1): Effect of different concentrations of CuO NPs on mortality of *P. clarkii* after 28 days of exposure.

2. Bioaccumulation of CuO NPs in different organs of *P. clarkii*

The accumulation of Cu in various tissues of CuO NPs-exposed animals for 28 days is shown in Table (1) and Figure(2). Cu accumulation was significantly affected by CuO NPs concentrations, exposure period, and the target organs. The highest Cu accumulation was recorded in gills followed by hepatopancreas and muscles, respectively. The highest Cu residues were retained in gills and hepatopancreas after 7 days of exposure to 250 mg/l of CuO NPs (100.31 and 2.54 µg/g dry weight, respectively). The amount of Cu in both organs increased significantly ($P < 0.05$) to be 197.67 and 14.2 µg/g dry weight, respectively after 28 days of exposure (Figs 2a & b).Cu accumulation in muscles was 1.09, 1.48, and 1.82 µg/g on the day 7, increased to be 1.59, 3.34, and 4.79 µg/g dry weights after exposure to 25, 125 and 250 mg/l CuO NPs for 28, respectively (Fig. 2c).

Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii.* A combined histopathological, Biochemical and genotoxicological study

Table 1. Concentrations of copper (µg/g dry weight) accumulated in selected organs of *P. clarkii* after exposure to different concentrations of CuO NPs in different exposure periods.

Organ	The used concentrations (mg/l)	Concentrations of CuO NPs (µg/g dry w.) accumulated in different organs of *P. clarkii* at different exposure periods (days)			
		7	**14**	**21**	**28**
Muscles	**Control**	1.08 ± 0.24^a	0.68 ± 0.24^a	0.67 ± 0.24^a	0.52 ± 0.24^a
	25 mg/l	1.09 ± 0.08^b	1.31 ± 0.06^b	1.72 ± 0.04^b	1.59 ± 0.30^b
	125 mg/l	1.48 ± 0.21^b	2.73 ± 0.23^c	3.20 ± 0.07^c	3.34 ± 0.30^c
	250 mg/l	1.82 ± 0.23^b	$2.2\pm0.11^{b,c}$	$2.35\pm0.05^{b,c}$	4.79 ± 0.28^d
	Two-way ANOVA **Time exposure** **CuO NPs concentration** **Concentrations x exposure time**	**F-Value** 38.31 96.2 8.12		**P-value** 0.000 0.000 0.005	
Hepatopancreas	**Control**	2.05 ± 0.17^a	2.11 ± 0.17^a	1.81 ± 0.17^a	1.87 ± 0.17^a
	25 mg/l	$3.35\pm0.31^{a,b}$	3.82 ± 1.37^b	$5.04\pm0.33^{b,c}$	6.04 ± 0.20^c
	125 mg/l	2.98 ± 1.03^b	6.99 ± 0.55^c	$5.05\pm0.85^{b,c}$	6.18 ± 1.01^c
	250 mg/l	2.54 ± 0.64^b	6.84 ± 0.86^c	7.39 ± 0.68^c	14.20 ± 0.31^c
	Two-way ANOVA **Time exposure** **CuO NPs concentration** **Concentrations x exposure time**	**F-Value** 13.53 40.33 37.60		**P-value** 0.000 0.000 0.005	
Gills	**Control**	7.82 ± 0.67^a	5.75 ± 0.67^a	4.74 ± 0.67^a	2.53 ± 0.67^a
	25 mg/l	$22.91\pm1.98^{a,b}$	25.12 ± 0.75^b	23.64 ± 1.25^b	26.73 ± 4.06^b
	125 mg/l	$53.17\pm1.24^{b,c}$	$110.78\pm2.23^{c,d}$	$106.82\pm1.12^{c,d}$	$118.31\pm4.33^{c,d}$
	250 mg/l	$100.31\pm17.1^{c,d}$	191.11 ± 2.42^d	174.35 ± 2.15^d	197.68 ± 1.83^d
	Two-way ANOVA **Time exposure** **CuO NPs concentration** **Concentrations x exposure time**	**F-Value** 90.98 1622.6 31.56		**P-value** 0.000 0.000 0.000	

- Valuesare mean of 3 samples ± SD.

-Mean values with different alphabetical superscripts for each parameter among different concentrations are statistically significant differed at P<0.05 (Two way ANOVA and subsequent post hoc multiple comparison with Duncan`s Multiple Range Test).

-Mean values with the same alphabetical superscripts are not statistically significant at P < 0.05.
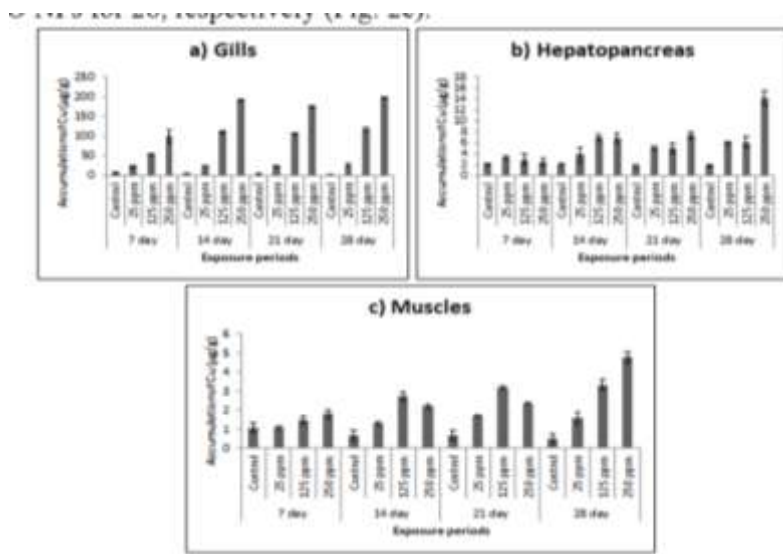
Fig. 2. Accumulation of Cu (µg.g^{-1}) in a) Gills, b) Hepatopancreas, and c) Muscles of *P. clarkii* exposed to different concentrations of CuO NPs for 28 days.

3. Biochemical biomarkers in hepatopancreas of *P. clarkii* exposed to different concentrations of CuO NPs.

Table (2) and Figure (3) showed that the levels of LPO, CAT, GPX and MTS enzymes in hepatopancreas of crayfish treated with 125 and 250 mg/l of CuO NPs were significantly higher than that of control group. On the other hand, levels of GSH, total proteins and total lipids were significantly decreased ($P < 0.05$) in hepatopancreas of crayfish after 28 days of exposure. After exposure to125 and 250 mg/l on CuO NPs,LPO, CAT, GPX and MTs were increased by 76.2 % - 260.7% ; 78.5%-137.9% ; 13.4%-20.7% and 52.9%-120.7%, respectively; while GSH levels were reduced to -23.4% and - 40.4% after treatment with 125 and 250 mg/l, respectively (Fig. 3).
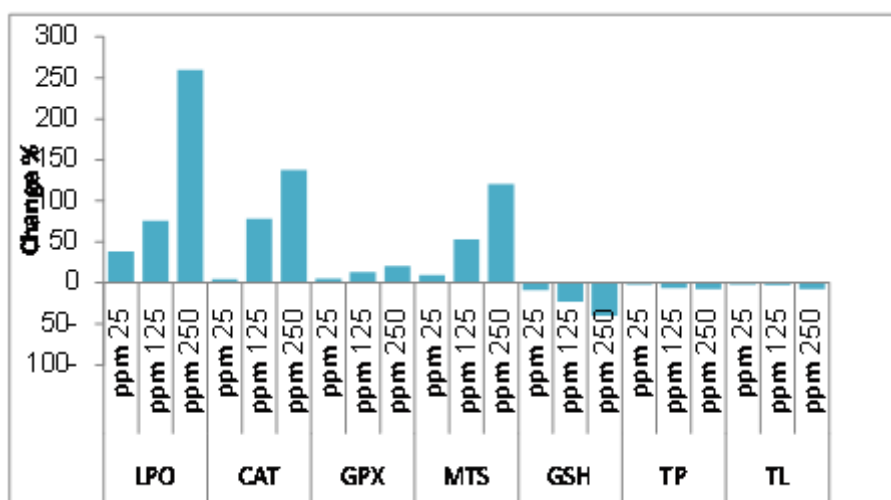
Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii.*
A combined histopathological, Biochemical and genotoxicological study

Fig (3): Percentage changes in the hepatopancreas biochemical biomarkers in *P. clarkii* exposed to CuO NPs for 28 days.

Table 2 Biochemicalbiomarkers in hepatopancreas of *P. clarkii* treated with different concentrations of CuONP for 28 days

Parameter	Concentrations of CuO NPs (mg/l)				F-value	P-value
	0.0 Control	25	125	250		
LPO(nmol/mg)	2.14±0.64[a]	2.96±0.06[b]	3.77±0.18[c]	7.72±1.9[c]	29.2	0.005
CAT(MU/mg)	2.61±0.21[a]	2.73±0.07[a]	4.66±0.30[b]	6.21±0.74[c]	31.6	0.003
GPX(MU/mg)	22.91±1.11[a]	24.08±0.06[b]	25.98±0.18[b]	27.66±1.32[c]	10.8	0.02
GSH (µg/mg)	0.47±0.06[a]	0.43±0.08[a]	0.36±0.01[c]	0.28±0.04[c]	7.04	0.04
MTs (µg/mg)	1.21±0.06[a]	1.33±0.17[a,b]	1.85±0.21[a,b]	2.67±0.31[b,c]	20.3	0.007
TP(mg/gm)	123.52±0.82[a]	120.3±2.73[a]	115.16±2.76[b]	113.92±1.95[c,d]	9.3	0.03
TL(mg/gm)	60.01±0.77[a]	58.75±0.70[a]	58.37±1.58[b]	55.27±0.53[a,b]	13.8	0.1

-Each value is mean of 4 samples ± SD.
-Means values with different alphabetical superscripts at each row are significantly differed at P<0.05 (one way ANOVA and subsequent post hoc multiple comparison with Duncan`s Multiple Range Test).
 -Mean values with the same alphabetical superscripts are not statistically significant at P < 0.05.

4. Histopathology of hepatopancreas
4.1. Normal hepatopancreas
Hepatopancreas is formed of numerous digestive tubules(Plate 1 a) which communicate with the lumen of the mid gut and end blindly in the gland itself. Each tubule has a central lumen and its wall is formed of three types of cells: absorptive, secretory and fibrillar cells (Plate 1 b). The absorptive cell is the most numerous cell type with basely located nucleus and the cytoplasm comprises an apical small vacuole. The secretory cell has a basely located nucleus and a large central vacuole filled with acidophilic secretions. The fibrillar cell is small, darkly stained with a large basely located nucleus (Plate 1 b).

Under EM, the apical surface of the absorptive cell has numerous microvilli (Plate 2 a) while its basal portion contains a spherical nucleus, Golgi complex, and parallel tubules of RER (Plate, 2 b). The secretory cells contain very thin layer of perinuclear RER, a small number of mitochondria and a central large vacuole (Plate 2 c). The fibrillar cell has a basely located nucleus and massive RER that fills almost the entire cell (Plate 2 d).

4.2. CuO NPs-Treated hepatopancreas
Light micrographs showed that treatment with 25 mg/l of CuO NPs resulted in the presence of yellowish brown granules in vacuoles of both secretory and absorptive cells as well as inflammatory infiltration with haemocytes between the tubules (Plate 1 c & d). Exposure to 125 mg/l of CuO NPs caused lumen dilatation of the tubules, extensive vacuolation and presence of numerous yellowish brown granules in vacuoles of secretory and absorptive cells. Lysis of epithelial cells was also observed (Plate 1 e & f). After treatment with 250 mg/l of CuO NPs, many tubular disruptions were noted (Plate 1 g) as tubular destruction, lysis of epithelial cells, appearance of pyknotic nuclei, haemocytes infiltration and presence of extensive number of yellowish brown granules in the tubular epithelial cells (Plate 1 h).

TEM Examination revealed many ultrastructural alterations in hepatopancreatic cells exposed to 250 mg/l of CuO NPs for 28 days. The microvilli of absorptive cells became fragmented and their cytoplasm lost its identity and being highly vacuolated (Plate 2 e & f).

The nucleus became pyknoticand itschromatin materials appear as electron dense aggregates along the inner surface of nuclear membrane (Plate 2 f). Presence of dense granules was also noticed (Plate 2 g). RER of the fibrillar cell became dilated and had disorganized arrangement and the nucleus became pyknotic (Plate 2 h).
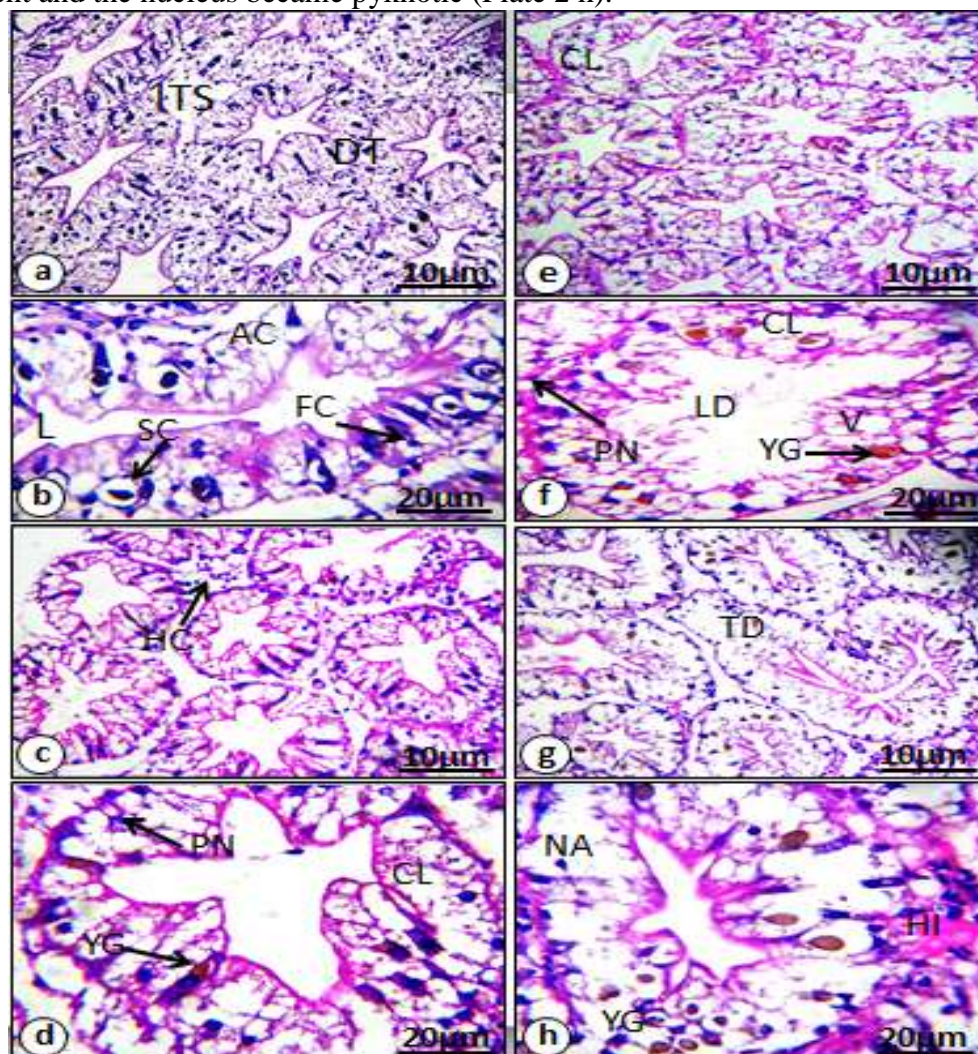
Plate 1: Light micrographs of the hepatopancreatic tubules of *P. clarkii*. (a): T.S. of control tubules with normal structures of the cells (X100). (b): T.S. of the control tubules showing different types of hepatopancreatic cells (X400). (c&d): T.S of hepatopancreatic tubules after exposure to 25 mg/l CuO NPs for 28 day showing presence of yellowish brown granules (X100 & 400). (e&f): T.S of hepatopancreatic tubules after exposure to 125 mg/l CuO NPs for 28 day showing lumen dilatation and extensive vacuolation (X 100 & 400). (g& h): T.S of hepatopancreatic tubules after exposure to 250 mg/l CuO NPs for 28 day showing distinct cellular destruction and lysis of the cytoplasm (X 100 & 400). AC: Absorptive cell; CL: Cell lysis; DT: Digestive tubules; FC: Fibrillar cell; HC: Haemocytes   HI: Haemocytes infiltration; ITS:Intertubularspace; L: Lumen LD: Lumen dilatation; NA: Necrotic Area; PN: Pyknotic nuclei; SC: Secretory cell; TD: Tubular disruption; V: Vacuolation; YG: Yellow granules.

Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii.* A combined histopathological, Biochemical and genotoxicological study
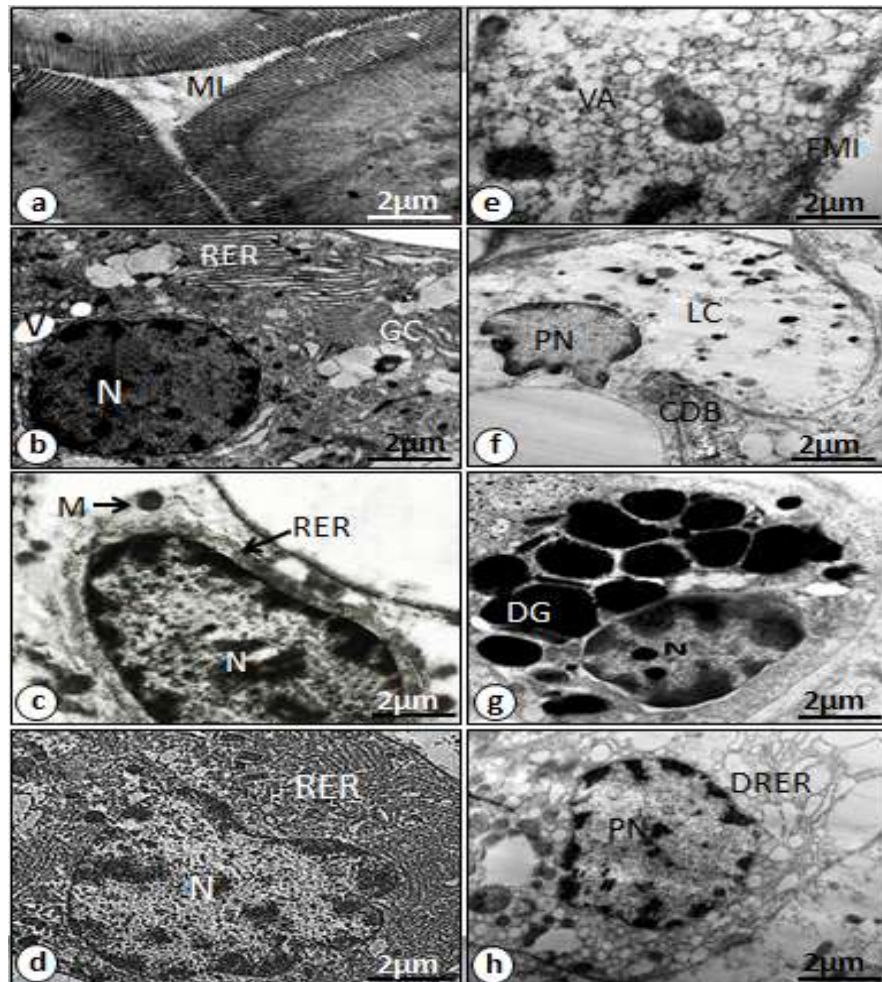
Plate 2:Electron micrographsofhepatopancreatic cells of *P.clarkii*. (a): Apical portion of absorptive cell with numerous microvilli and number of mitochondria (X15,000). (b): Basal portion of absorptive cell with spherical nucleus and rough endoplasmic reticulum (X 8,000). (c): Secretory cell with thin layer of rough endoplasmic reticulum around the nucleus (X 12000).(d): Fibrillar cell with extensive rough endoplasmic reticulum (X 5,000). (e): Apical portion of absorptive cell after exposure to 250 mg/l CuO NPS for 28 day showing fragmented microvilli, swelled mitochondria and vacuolated cytoplasm (X12,000). (f and g): Basal portion of absorptive cell after exposure to 250 mg/l CuO NPS for 28 day showing lysis of cytoplasm and nuclear pyknosis and presence of dense granules around the nucleus (X 6000&10000). (h): Fibrillar cell after exposure to 250 mg/l CuO NPS for 28 day showing vacuolated cytoplasm, the RER become dilated and had disorganized arrangement and pyknotic nucleus (X8,000).CDB: Cellular debris ;DG: Dense granules ;DRER: Dilatation of cisternae of RER ; FMI: Fragmented microvilli ; GC: Golgi complex ;LC: Lytic cytoplasm; MV: Microvilli ; M:Mitochondria;N: Nucleus ;PN: Pyknotic nuclei ;RER: Rough endoplasmic reticulum ; VA: Vacuolated cytoplasm .

5. DNA damage

DNA damage was analyzed by the Comet assay technique. The mean of tail lengths of the comet reflects approximately the increasing patterns of DNA damage. Figure(3) shows that CuO NPs treated groups produced concentration-dependent increases in DNA damage in hepatopancreatic nuclei of *P. clarkii*. The significant increase in tail lengths revealed DNA damage caused by CuO NPs compared to control. Figure (4) illustrated a significant increase ($P <0.05$) in tail length after exposure to 125, 250 mg/l CuO NPs, where tail length is 11.84-20.1 PX, respectively.
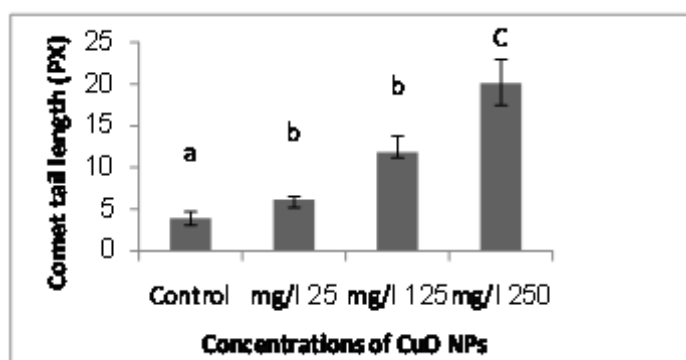
Fig. 4: CuO NPs-induced DNA damage in hepatopancreatic nuclei of *P. clarkii* .The results are expressed as means ± SD from three replicates, Letters a, b and c show differences between concentrations at agiven tissue. Data shown with different letters are significant at the p < 0.05 level.

DISCUSSION

The investigation of the impacts of nanomaterials on the aquatic ecosystem,that ultimately receives overflow and wastewater from domestic and industrial sources,becomes a topic of interest. These concerns have prompted number of studies to examine the release of these materials from nano-functionalised products. Nano CuO is one of the most interesting innovations in medical care, since they decrease microbial infections within hospitals(Lazary *et al.,* 2014).In order to evaluate the possibility of using nanoparticles-induced alterations in the tested organism as biomarkers of nanoparticles water pollution,the present study focuses on the impact of CuO NPs on different biological aspects of *P.clarkii*.

The present study documented that the crayfish mortality increased with increasing concentrations of these nanoparticles. Crayfish mortality after exposure to 25, 125 and 250 mg/l CuO NPs were 0%, 6.7% and 36.7%, respectively.The mortalities in crayfish exposed to CuO NPs may be due to the direct toxic effects of these nanoparticles on gill epithelium, resulting in hypoxia and osmoregulatory stresses. Moreover, nanoparticles may cause many biochemical changes inside the crayfish body that may inhibit the vital activities and immune system of the crayfish making them more susceptible to death (Jitka *et al.,* 2017).

Results in the present study showed that the highest accumulation of copper was observed in gills followed by hepatopancreas while the lowest accumulation was detected in muscle tissues and accumulation levels showed a dose and time-dependent. This finding was in agreement with Griffitt *et al.* (2007) who reported that gills of *Danio rerio* were the primary target organ for CuO NPs accumulations. Similarly, Shaw *et al.,* (2012) found that accumulation of copper increased in gills of *Oncorhynchus mykiss* exposed to 20 and 100 µg/L Cu NPs compared to control levels. Contrarily, in *Oreochromis niloticus* exposed to

Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii.*
A combined histopathological, Biochemical and genotoxicological study

20 µg/L Cu NPs for 15 days, accumulation of copper was higher in liver followed by gills then muscle tissues (Mustafa *et al.,* 2017). Rise in CuO NPs accumulation in the gills may be related the formation of metal complex with the mucus on the gill, due to its direct exposure to toxicant in water and acts as a reservoir of metal accumulation (Oliveira-Filho *et al.,* 2010).Elevated accumulation levels of CuO NPs in the hepatopancreas of *P. clarkii* revealed that this organ plays a vital role in pollutant storage, redistribution and detoxification(Jaiswal and Sanojini, 1990). Muscle has the lowest amount of Cu accumulation because it is not an active organ in accumulating metals (Alam *et al.,* 2002).

Biochemical investigations have been used for assessing the nutritional and health status, effects of stressors, and the adaptive capacity of organisms to the external environment (Schreck and Moyle, 1990; Abdel-Tawwab, 2016). Many biochemical parameters are estimated in the present work to be used as biomarkers. These include: total protein, total lipids as well as non-enzymatic and enzymatic antioxidants.

Proteins are important biochemical components that play an essential role in metabolic pathways and biochemical reactions. Under extreme stress conditions, protein supply energy in metabolic pathways and biochemical reactions. Therefore, an assessment of the total protein contents could be used as a diagnostic tool for determining the physiological status of an organism (Prasath and Arivoli, 2008). The current study clearly showed that total proteins decreased significantly in the hepatopancreas of red swamp crayfish, *P. clarkii* treated with CuO NPs. This decrease may be resulted from high protein hydrolytic activity due to elevation of protease enzyme in hepatopancreatic tissues.Similarly, Hamdi (2001) reported a reduction in total proteins of hepatopancreas of red swamp crayfish, *P. clarkii* exposed to Malathion. This reduction may be due to the enhanced proteolytic activity in this organ under stress, destruction of cells and consequent impairment in protein synthesis (Bradbury *et al.,* 1987). In contrast, Muralisankar *et al.* (2015) found that total protein increased in hepatopancreas of *Macrobrachium rosenbergii* after treatment with 40 mg kg^{-1}.Cu NPs.

Lipids are important metabolites for locomotory and reproductive activities. The storage of lipid reserves mostly triglyceride and cholesterol acts as a main source for energy utilization (Derise and Druilhet, 1988).The present study showed that CuO NPs significantly decreased total lipids in hepatopancreas of crayfish, *P. clarkii*. This decline may be due to an increase in hormonal secretions that enhance metabolic rate which in turn reduces the metabolic reserve of the triglyceride (Turner and Bargnara, 1976). The decline in total lipids may also be due to the imposition of high energy demands to counter the toxic stress. These results are in agreement with Abd El-Atti (2002) who found that cadmium, copper, zinc and mercury decreased total lipids in the crayfish *P. clarkii*.

Many types of environmental contaminants exert toxicity associated to oxidative stress and can produce oxidative damage in aquatic organisms (Lushchak *et al.,* 2005; Stara *et al.,* 2013& 2014). The evaluation of oxidative stress markers is critical to the investigation of oxidative stress in organisms and pro-oxidant activity can be used to evaluate water pollution (Slaninova *et al.,* 2009). The steady-state concentration of the markers of oxidative stress is a balance between production and removal, producing a steady-state of reactive oxygen species level (Dalibor *et al*., 2014).

Lipid peroxidation (LPO) is the first sign of oxidative stress.The onset of LPO within biological membranes is associated with changes in their physicochemical properties and with alteration of biological function of lipids and proteins. It is considered as the main molecular mechanisms involved in the oxidative damage to cell structures (Javed *et al.*, 2016). LPO is the main contributor to the loss of cell function under oxidative stress (Huang *et al.,* 2003). The responses of LPO may vary with the concentration of chemicals and exposure time (Rua*set al.*, 2008). The present study showed clearly that LPO in

hepatopancreas of *P. clarkii* increased significantly after treatments with CuO NPs. Likewise, Muralisankar *et al*. (2015) found that LPO level increased in hepatopancreas of *Macrobrachium rosenbergii* after treatment with Cu-NPs.This increase in LPO levels lead to excess ROS generation due to a less effective antioxidant defense system (Javed*et al.,* 2016).

Oxidative substances in cells may lead to elevation in antioxidants of both enzymatic and non-enzymatic components as a defense mechanism (Venancio *et al.,* 2013). Antioxidant enzyme activities are found broadly distributed in tissues of aquatic organisms, with higher activity in the digestive gland of invertebrate organisms. Assaying antioxidant enzymes can indicate the antioxidant status of the organisms, working as a potential biomarker for contaminant-mediated oxidative stress. Moreover, they are useful biomarkers reflecting not only an exposure to pollutants but also their toxicity (Valavanidis *et al.* 2006).

Catalase (CAT) and Glutathione peroxidase (GPx) are antioxidant enzymes that provide the first line of defense against reactive oxygen species and used as a biomarker of oxidative stress (Van der Oost *et al*., 2003). In the present study, CAT and GPx activities increased in the hepatopancreas of *P. clarkii* upon exposure to CuO NPs. Such increases in CAT activities may be explained as a response to the increased H_2O_2 levels and superoxide anions (John *et al.,* 2001). The increment of CAT and GPx activities may be an adaptive mechanism to prevent the accumulation of toxic reactive oxygen (Regoli*et al*., 2006). The elevated enzymatic activities might reflect the possibility of better protection against toxicity of metal-induced lipid peroxidation (Abdel-Tawwab and Wafeek, 2017). In the same trends, Mustafa *et al.* (2017) reported that GPx activity increased in liver of *Oreochromis niloticus* exposed to CuO NPs and attributed this increase to its protective role against damages induced by oxyradical.

Glutathione (GSH) reduces organic and hydrogen peroxides via a reaction catalysed by GSH-Px. It serves as a scavenger of O_2 and OH* (Fang *et al*., 2002; Hamre, 2011). In this study, the non-enzymatic antioxidant, GSH activities were significantly decreased in hepatopancreas exposed to CuO NPs. This decrease may be due to the intensification of turnover between reduced and oxidized glutathione under stress conditions and as a result caused an increase in the consumption of some peptides for the synthesis of heavy metal-binding proteins like metallothioneins (Radwan *et al.,* 2010). In this regard, Barim (2018) found that GSH was significantly decreased in the hepatopancreas of freshwater crayfish (*Astacus leptodactylus*) upon starvation. They attributed this decrease in GSH content during exposure to starvation to an increased utilization of GSH, which can be converted into oxidized glutathione, and inefficient GSH regeneration.

Metallothioneins (MTs) are other non-enzymatic antioxidants that play a vital role in the detoxification and metabolism of toxic metals (Kelly *et al.,* 1998). The present studyshowed that MTs levels were increased in crayfish treated with CuO NPs. Similarly, Martin-Diaz *et al.* (2006) reported an increase in the metallothioneins level in the hepatopancreas of crayfish, *P. clarkii* exposed to cadmium. Moreover, Cu exposure induces MTs production in white shrimp, *Litopenaeus vannamei* (Hui *et al.*, 2017) and in in signal crayfish, *Pacifastacus leniusculus* (Gunderson *et al.,* 2018). The increments of MTs in the investigated species upon exposure to nanometals explains the essential role of metallothioneins in the defense against toxic effects of nanoparticles and support the evidence that it is a suitable biomarkers in biomonitoring programs.

Histological and ultrastructural alterations are important biomarkers in toxicological studies. They are often observed in hepatopancreatic tissue of crayfish due to their role in detoxification (Jaiswal and Sanojini, 1990). It was found to be the major site of metal accumulation, and it is the organ in which cellular alterations are most evident after exposure to organic and inorganic pollutants (Zarai*et al.,* 2011).

**Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii*.
A combined histopathological, Biochemical and genotoxicological study**

The present study showed many histological alterations in hepatopancreatic cells of *P. clarkii* after being exposed to different concentrations of nano copper oxide .Low concentrations caused mild alterations as lumen dilatation, vacuolation and presence of number of yellowish brown granules in vacuole of secretory cells. On the other hand, higher concentrations of CuO NPs resulted in severe tubular disruption including haemocytes infiltration and cellular destruction. Similar histopathological alterations were also reported in the hepatopancreatic tubules of *P. clarkii* upon exposure to Malathion (Hamdi, 2001) ethion (Desouky *et al.,* 2013) and TiO$_2$ NPs (Abdel-Atti*et al.,* 2019). This finding is in agreement with Teresa *et al*. (2018) who found that Cu NPs caused many histological abnormalities in liver of rainbow trout (*Oncorhynchus mykiss*). These histological changes may be attributed to direct toxic effects of toxicants on hepatopancreatic cells because it is the main site of detoxification. The present electron microscopic studies have been supported the results obtained from the previous foundations. The obtained results showed that CuO NPs produce severe degeneration of cellular organelles including fragmented microvilli, swollen mitochondria, lytic and vacuolated cytoplasm, fragmented endoplasmic reticulum and presence of dense granules. Lysis of cytoplasm indicates that nanoparticles may interact with hepatic enzymes and other proteins which results in oxidative stress and reactive oxygen species formation which may cause apoptosis and necrosis of cells (Choi *et al*.,2010). Mitochondrial swelling might be due to pollutant-induced inhibition of Na$^+$/H$^+$ transporter and impairment of the overall osmoregulatory process of the cell (Vilella*et al.,* 1991). The disintegration of RER might be a result of final hyperactivity before cell necrosis (Roncero *et al.,* 1992).

Comet assay combines the simplicity of biochemical techniques for detecting DNA single strand breaks (Singh *et al.,* 1988). The present study showed that CuONPscaused DNA damages in hepatopancreatic nuclei of *P. clarkii* at high concentrations.Many studies have shown that these nanoparticles promote mitochondrial damage, DNA damage and oxidative DNA damage (Zhang *et al.,* 2014). DNA damage may be due to excessive generation of ROS which may cause deteriorations to DNA (Ozkan *et al.,* 2012).

In conclusion, CuO NPs were found to cause a moderate toxicity to the crayfish, *P. clarkii*. After exposure to sub-lethal concentrations of these nanoparticles, they were accumulated progressively inside the body organs over exposure period (28 days) and resulted in significant biochemical, histopathological and genetic alterations. These changes which may represent adaptive mechanisms to this stressful situation may be potentially disruptive for the survivability of this crustacean in contaminated aquatic ecosystems. Moreover, the present study revealed the utility of red swamp crayfish, *P. clarkii* to be used as a bioindicators for CuO NPs toxicity.

REFERANCES

Abd El-Atti, M. S. (2002).Effect of Water Quality and some Parasites on some Biological Aspects of Freshwater Crayfish *Procambarus clarkii* at Sharkia Governorate. Ph.D. Thesis, Fac. Sci., Zagazig Univ., Egypt. 152 Pp.

Abd El-Atti, M; Desouky, M; Mohamadien, A. and Said, R. (2019). Effects of titanium dioxide nanoparticles on red swamp crayfish, *Procambarus clarkii*: Bioaccumulation, oxidative stress and histopathological biomarkers. Egypt. J. Aquat. Res.,45: 11-18.

Abdel-Tawwab, M. (2016). Effect of feed availability on susceptibility of Nile tilapia, *Oreochromis niloticus* (L.) to environmental zinc toxicity: growth performance, biochemical response, and zinc bioaccumulation. Aquacult., 464: 309 -315.

Abdel-Tawwab, M. and Wafeek, M. (2017). Fluctuations in water temperature affected waterborne cadmium toxicity: hematology, anaerobic glucose pathway and oxidative stress status of Nile tilapia, *Oreochromis niloticus* (L.). Aquacul., 477: 106-111.

Alam, M.G.M; Tanaka, A; Allinson, G; Laurenson, L.J.B. andStagnitt, S. (2002).A comparison of trace element concentrations in cultured and wild carp (*Cyprinus carpio*) of Lake Kasumigaura, Japan. Ecotoxicol. Envir. Safe*.,* 53: 348-354.

Barim, O. (2018). The effects on some non-enzymatic antioxidants and oxidative stress of *Astacus leptodactylus* of starvation periods, Aquacul. Nut., 24: 492-503.

Boyd, C. E. (1984): Water quality in warm water fishponds. Auburn University Agriculture Experimental Station. Auburn, AL, USA.

Bradbury, S.P; Symonic, D.M; Costs, J.R. and Atchison, G.J. (1987). Toxicology of fenvalerate and its constituent isomers to the fathead minnow (*Pimephales promelos)* and blue gill (*Lepomis macrochirus*). Bull. Environ. Cont. Toxicol., 38: 727-735.

Choi, J.E; Kim, S; Ahn, J.H; Youn, P; Kang, J.S; Park, K; Yi, J. and Ryu, D.Y. (2010). Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat.Toxicol., 100:151-159.

Dalibor, K; Alzbeta, S; Eliska, Z; Antonin, K. and Josef, V.(2014). The effect of subchronicMetribuzin exposure to signal crayfish (*Pacifastacus leniusculus* (Dana1852). Neuroendocrinol.Lett., 35(2):51-56.

Derise, N.L. and Druilhet, R. (1988). Analysis of crawfish tail meat and fat. University of southwestern Louisiana, Lafayette, LA 70504.

Desouky, M.M.A; Abdel-Gawad, H. and Hegazi, B. (2013). Distribution, fate and histopathological effects of Ethion insecticide on selected organ of the crayfish, *Procambarus clarkii*. Food. Chem. Toxicol., 52: 42-52.

Dytham, C. (2011). Choosing and Using Statistics: a Biologist's Guide. Blackwell Science Ltd., London, UK.

Fang, T.Z; Yang, S. and Wu, G. (2002).Free radicals, antioxidants, and nutrition. Nutr., 18: 872-879.

Farlow, J. (2014). Chronic Toxicity of Nano Metallic on Red Swamp Crayfish (*Procambarus clarkii*) in Laboratory and Mesocosm Studies. Master's Thesis, Louisiana State University, Agricultural and Mechanical College, 89 Pp.

Griffitt, R.J; Weil, R; Hyndman, K.A; Denslow, N.D; Powers, K; Taylor, D. and Barber, S.D. (2007). Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Environ. Sci. Technol., 41: 8178-8186.

Gunderson, M.P; Nguyen, B.T; Cervantes Reyes, J.C; Holden, L.L; French, J.M.T; Smith, B.D. and Lineberger, C. (2018).Response of phase I and II detoxification enzymes, glutathione, metallothionein and acetylcholine esterase to mercury and dimethoate in signal crayfish (*Pacifastacus leniusculus*). Chemosph., 208:749-756.

Hagfeldt, A. and Graetzel, M. (1995): Light-induced redox reactions in nanocrystalline systems. Chem. Rev., 95(1): 49-68.

Hamdi, S.A.H. (2001). Some physiological changes in the haemolymph, hepatopancreas and muscle of the red swamp crayfish, *Procambarus clarkii* (Girard, 1852) exposed to Malathion. Egypt. Ph. D. Thesis, Fac. Sci., Cairo Univ., 150 Pps.

Hamre, K. (2011). Metabolism, interactions, requirements and functions of vitamin E in fish. Aquacult.Nut., 4: 492-503.

Huang, C.H; Chang, S.L. and Huang, W. (2003). Dietary vitamin E supplementation affects tissue lipid peroxidation of hybrid tilapia, *Oreochromis niloticus* x. *aureus*. Comp. Biochem. Phys. Part B: Biochem. Mol. Biol., 134: 265-270.

Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii*.
A combined histopathological, Biochemical and genotoxicological study

Hui, G; Kexu, L; Wei, W; Chenggui, W. and Yuchun, S. (2017). Effects of Copper on Hemocyte Apoptosis, ROS Production, and Gene Expression in White Shrimp *Litopenaeus vannamei*. Bio. Trace. Elem. Res., 179(2): 318-326.

Ibrahim, A.M; Khalil, M.T. and Mubarak, M.F. (1995). On the feeding behaviour of the exotic crayfish, *P. clarkii* in Egypt and its prospects in the biocontrol of local vector snails. J. Union. Arab. Biol., Cairo, 4: 321-340.

Isani, G; Falcioni, M.L; Barucca, G; Sekar, D; Andreani, G; Carpenè, E. and Falcioni, G. (2013):Comparative toxicity of CuO nanoparticles and $CuSO_4$ in rainbow trout. Ecotoxicol. Environ. Safe., 97, 40-46.

Jaiswal, K. and Sanojini, R. (1990).Histopathological lesion in the hepatopancreas of freshwater prawn *Macrobrachium kistnensis* induced by naphthalene poisoning. Environ. Pollut. Hlth. Hazards, Environmental series, III: 151-157.

Javed, M; Ahmad, I; Usmani, N.and Ahmad, M. (2016). Studies on biomarkers of oxidative stress and associated genotoxicity and histopathology in *Channa punctatus* from heavy metal polluted canal. Chemo., 151: 210-219.

Jitka, S; Karel, D; David, F; Natalia, L. and Pavel, V. (2017). Toxic and heavy metals as a cause of crayfish mass mortality from acidified headwater streams. Ecotoxico., 26(2):261-270.

John, S; Kale, M; Rathore, N. and Bhatnaga, D.(2001). Protective effect of vitamin E in Dimethoate and Malathion induced oxidative stress in rat erythrocytes. J. Nutr. Biochem., 12: 500-504.

Joh, D.Y; Kinder, J; Herman, L.H; Ju, S; Segal, M.A; Johnson, J.N; ChanGarnet, K.L. and Park, J.(2011). Single-walled carbon nanotubes as excitonic optical wires. Nat. Nanotechol., 6: 51-56.

Katwal, R; Kaur, H; Sharma, G; Naushad, M. and Pathania, D. (2015): Electrochemical synthesized copper oxide nanoparticles for enhanced photocatalytic and antimicrobial activity. J. Ind. Eng. Chem., 31, 173-184.

Kelly, S.A; Havrilla, C.H.M; Brady, T, C; Abramo, K.H.and Levin, E.D. (1998). Oxidative stress in toxicology: Established mammalian and emerging piscine model systems. Environ. Heal, Perspect., 106: 375-384.

Khashan, K.S; Sulaiman, G.M. and Abdulameer, F.A. (2016).Synthesis and antibacterial activity of CuO nanoparticles suspension induced by laser ablation in liquid. Arab. J. Sci. Eng., 41: 301-310.

Khatri, M; Dhimiter, B; Anoop, K; Joel, M.C; Susan, W; Thomas, G; Jiaqi, L April, Z; Philip, D.and Peter, G. (2013):Evaluation of cytotoxic, genotoxic and inflammatory responses of nanoparticles from photocopiers in three human cell lines. Partic, Fib.Toxicol., 10: 42.

Laurent, S; Forge, D; Port, M; Roch, A; Robic, C; van der Elst, L. and Muller, R.N. (2008). Magnetic iron oxide nanoparticles: synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications. Chem. Rev., 10: 2064 -2110.

Lazary, A; Irina, W; Vatine, J.J. and Ohana, N. (2014):Reduction of healthcare-associated infections in a long-term care brain injury ward by replacing regular linens with biocidal copper oxide impregnated linens. Inter, J. Infect. Dise., 24: 23-29.

Lushchak, V.I; Bagnyukova, T.V; Husak, V.V; Luzhna, L.I; Lushchak, O.V. and Storey, K.B. (2005). Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. Int. J. Biochem. Cell. Biol., 37: 1670-1680.

Martin-Diaz, M.L; Tuberty, S. R; McKenney, J.R; Blasco, J; Sarasquete, C. and DelValls, T.A. (2006). The use of bioaccumulation, biomarkers and histopathology diseases in shape *Procambarus clarkii* to establish bioavailability of Cd and Zn after a mining spill. Envir.Monit.Assesst., 116:169.

Muralisankar, T; Saravana, B. P; Radhakrishnan, S; Seenivasan, C. and Srinivasan, V. (2015).The effect of copper nanoparticles supplementation on freshwater prawn *Macrobrachium rosenbergii* post larvae. J. Trace. Elem. Med. Biol., 34: 39-49.

Mustafa, T; Servet, D; Özcan, A; Bedii, C. and Cahit, E. (2017). Effects of copper oxide nanoparticles on antioxidant enzyme activities and on tissue accumulation of *Oreochromis niloticus*. Env.Contam.Toxicol., 99: 360–364

Nations, S; Long, M; Wages, M; Maul, J.D; Theodorakis, C.W. and Cobb, G.P. (2015). Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on *Xeno puslaevis*. Chem., 135: 166-174.

Perreault, F; Melegari, S.P; Da Costa, C.H; Rossetto, A.L.; Popovic, R. and Matias, W.G. (2012). Genotoxic effects of copper oxide nanoparticles in Neuro 2A cell cultures. Sci. Total. Environ., 441: 117-124.

Prasath, P.M.D. and Arivoli, S. (2008). Biochemical study of freshwater fish *Catla catla* with reference to mercury chloride. Iran J. Environ. Health. Sci. Eng., 3:109-116.

OberdÖrster, G; Oberdörster, E. and Oberdörster, J. (2005):Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health.Persp., 113(7):823-839.

Oliveira-Filho, E.C; Muniz, D.H.F.and Ferreira, M.F.N.(2010).Evaluation of acute toxicity, cytotoxicity and genotoxicity of a nickel mining waste to *Oreochromisniloticus*. Bull. Envi.Contam.Toxico., 85:467-471.

Ostaszewska, T; Chojnacki, M; Kamaszewski, M. and Sawosz-Chwalibóg, E. (2015): Histopathological effects of silver and copper nanoparticles on the epidermis, gills, and liver of *Siberian sturgeon*. Environ. Sci. Pollut. Res., 23: 1621-1633.

Ozkan, F; Gündüz, S. G; Berkóz, M; Hunt, A. O. andYalin, S. (2012). The   protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*. Fish. Phys. Biochem., 38: 635-643.

Radwan, M.A; El-Gendy, K.S. and Gad, A.F. (2010): Oxidative stress biomarkers in the digestive gland of *Thebapisana* exposed to heavy metals. Arch. Environ. Contam.Toxicol., 58: 828-835.

Regoli, F; Gorbi, S; Fattorini, D; Tedesco, S; Notti, A; Machella, N; Bocchetti, R; Benedetti, M. and Piva, F. (2006). Use of the land snail *Helix aspersa* as sentinel organism for monitoring ecotoxicologic effects of urban pollution: an integrated approach. Environ. Heal. Perspe., 114: 63-69.

Roncero, V; Duran, E; Soler, F; Masot, J. and Gomez, L. (1992). Morphometric structural and ultrastructural studies of tench (*Tinca tinca* L.) hepatocytes after copper sulphate administration. Environ. Res., 57:45-58.

Ruas, B; CarvalhoCdos, H.S; de Araujo, E.L. and Espindola, M.N. (2008). Oxidative stress biomarkers of exposure in the blood of cichlid species from a metal-contaminated river. Ecotoxic. Environ. Safe., 71: 86-93.

Sahooli, M; Sabbaghi, S. and Saboori, R. (2012). Synthesis and characterization of mono sized CuO nanoparticles. Mater.Lett., 81, 169-172.

Schreck, C.B. and Moyle, P.M. (1990). Methods for Fish Biology. American Fishers Society Bathesda: Maryland. 115-119.

Serrano, A.A; Angulo, E; Ferrero, G. and Rallo, A. (2000). The use of two species of crayfish as environmental quality sentinels: the relationship between heavy metal content, cell

Impact of copper oxide nanoparticles on freshwater crayfish, *Procambarus clarkii.*
A combined histopathological, Biochemical and genotoxicological study

and tissue biomarkers and physico-chemical characteristics of the environment. Sci. Environ., 247 (2-3): 239-251.

Shaw, B.J; Al-Bairuty, G. and Handy, R.D. (2012). Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation. Aquat.Toxicol., 116–117:90–101

Singh, N.P; McCoy, M.T; Tice, R.R.and Schneider, E.L. (1988): A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell. Res., 175: 184-191.

Slaninova, A; Smutna, M; Modra, H. and Svobodova, Z. (2009). Oxidative stress in fish induced by pesticides. Neuroendocrinol.Lett., 30:2-12.

Smedes, F. and Thomasen, T.K. (1996): Evaluation of the Bligh and dyer lipid determination method. Mar. Pollut. Bull., 32: 681-688.

Stara, A; Machova, J. and Velisek, J. (2014). Effect of chronic exposure to prometryne on oxidative stress and antioxidant response on early life stages of common carp (*Cyprinus carpio* L.). Neuroendocrinol. Lett., 33(3): 130-135.

Stara, A; Steinbach, C; Wlasow, T; Gomulka, P; Ziemok, E; Machova, J. and Velisek, J. (2013): Effect of zeta-cypermethrin on common carp (*Cyprinus carpio* L.). Neuroendocrinol. Lett., 34(2): 37–42.

Sun, Y; Zhang, G; He, Z; Wang, Y; Cui, J. and Li, Y. (2015). Effects of copper oxide nanoparticles on developing zebrafish embryos and larvae. Int. J. Nanomed., 11: 905-918.

Tang, F; Li, L. and Chen, D. (2012). Mesoporous silica nanoparticles: Synthesis, biocompatibility and drug delivery. Adv. Mater., 24: 1504-1534.

Teresa , O ; Jerzy , S ; Maciej , K; Paweł , S. and Maciej ,C. (2018). Cytotoxicity of silver and copper nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Environ, Sci, Pollut, and Res., 25 (1):908-915.

Turner, C.D. and Bargnara, J.T. (1976): Endocrine mechanism in the invertebrates In: General Endocrinology Saunders, W. B. Co., Philadelphia, London, Toronnto., 545-558.

Usui, H; Matsui, H; Tanabe, N. and Yanagida, S. (2004).Improved dye-sensitized solar cells using ionic nanocomposite gel electrolytes. J. Photochem. Photobiol A: Chem., 164:97-101.

Valavanidis, A; Vlahogianni, T; Dassenakis, M. and Scoullos, M. (2006).Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Safe., 64: 178-189.

Van der Oost, R; Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharm., 13: 57-149.

Venancio, L.P.R; Silva, M.I.A. and da Silva T.L. et al. (2013): Pollution-induced metabolic responses in hypoxia-tolerant freshwater turtles. Ecotoxico. Environ. Safe., 97: 1-9.

Vilella, S ;Zonno, V; Cassano, G ; Maffia, M. and Storelli, C. (1991). Na^+/H^+ exchange in the kidney of eels *Anguilla anguilla* adapted to sea water or to freshwater environments, studies with brush border membrane vesicles. Comp. Biochem. Physiol., 102: 445-560.

Zarai, Z; Boulais, N; Karray, A; Misery, L; Bezzine, S; Rebai, T; Gargouri, Y. and Mejdoub, H. (2011):Immunohistochemical localization of hepatopancreatic phospholipase A2 in *Hexaplex trunculus* digestive cells. Lip. Heal. Dis., 1(1): 10-91.

Mahmoud Abd El-Atti *et al.*

Zhang, Q; Zhang, K; Xu, D; Yang, G; Huang, H; Nie, F; Liu, C. and Yang, S. (2014).CuO nanostructures: Synthesis, characterization, growth mechanisms, fundamental properties, and applications. Prog.Mater. Sci., 60: 208-337.

تأثير جزيئات أكسيد النحاس النانوية على استاكوزا المياه العذبة بروكمبلرس كلاركي :
دراسة نسيجية وبيوكيميائية وجينية

محمود عبدالعاطي ــ محمود دسوقي ــ آمال محمدين ــ رضوى سعيد
قسم علم الحيوان كلية العلوم جامعة الزقازيق

المستخلص

تستخدم جزيئات أكسيد النحاس النانوية (CuO NPs) بشكل شائع في العديد من التطبيقات الصناعية والطبية. وقد يؤدي الاستخدام المفرط لهذه المنتجات إلى تصريفها إلى النظم الإيكولوجية المائية، مما قد يسبب الكثير من الأضرار لتلك البيئة وأحيائها. تم إجراء هذه الدراسة لتقييم تأثير جزيئات أكسيد النحاس النانوية على معدل النفوق ،و التراكم الحيوي لتلك المادة ، والإجهاد التأكسدي ، والتغيرات المرضية النسيجية للغدة الهاضمة،   بالإضافة إلى دراسة تلف جزيئات   الحمض النووي في استاكوزا المياه العذبة بروكمبلوس كلاركي أحد كائنات المياه العذبة التي تستخدم بكثرة في مثل تلك الدراسات . ويتمثل الغرض من هذه الدراسة في محاولة تقييم أثر جزيئات النحاس النانوية، و فهم آلية سميتها، بالإضافة إلى تقييم إمكانية استخدام التأثيرات المحتملة لتلك المادة على الكائن محل الدراسة كمؤشرات حيوية للتلوث. وخلال الدراسة تم تعريض استاكوزا المياه العذبة بروكمبلوس كلاركيإلى تركيزات: 25 و 125 و 250 ملغم / لتر من CuO NPs لمدة 28 يومًا. وكانت نسبة الوفيات جراء التعرض لتلك التركيزات هي: صفر ٪ ، 6.7 ٪ و 36.7 ٪ ، على التوالي. وكشف تحليل التراكم الحيوي للجزيئات المدروسة أن أعلى مستويات تراكم للنحاس قد سجلت في الخياشيم، تليها الغدة الهاضمة، بينما تم تسجيل أدنى مستوياتها في العضلات. كما أظهر التحليل الكيميائي الحيوي أن تعرض   استاكوزا المياه العذبة بروكامبرس كلاركي للتركيزات المذكورة أعلاه من   CuO NPs   تسبب في زيادة ملحوظة في أنشطة انزيمات: Lipid peroxidase ، Glutathione-S-transferase Catalase وزيادة في معدل افراز بروتين الميتالوثيونين (Metallothioneins, MTs) في الغدة الهاضمة وكلها مركبات يتم افرازها للتعامل مع تلك الملوثات وتقليل آثارها الضارة ومحاولة التخلص منها. وعلى الجانب الآخر ، فقد انخفضت مستويات الجلوتاثيون (GSH) ، والدهون الكلية (TL) والبروتينات الكلية (TP) بشكل كبير. وقد كشفت الفحوصات المجهرية للغدة الهاضمة المعرضة لجزيئات النحاس النانوية وذلك باستخدام المجهر الضوئي عن توسع في تجاويف الأنيبيبات الهضمية والكثير من التغيرات المرضية للخلايا المبطنة لتلك الأنيبيبات مثل زيادة إفراز حبيبات بنية مصفرة داخل السيتوبلازم، وظهور فراغات سيتوبلازمية، وتحلل خلوي يشمل كل من الستوبلازم والنواة. بينما بينت صور المجهر الإلكتروني النافذ للغدة الهاضمة للحيوان محل الدراسة وجود الكثير من التأثيرات الضارة لجزيئات النحاس النانوية تمثلت في: ظهور فراغات سيتوبلازمية مع وجود حبيبات إفرازازية كثيفة ، وتمزق الزوائد الدقيقة للخلايا الممتصة ، وتحلل للشبكة الإندوبلازمية الخشنة RER ، وتشوه للميتوكوندريا. وأخيرا فقد أظهر تحليل   Comet Assay الخاص بالحمض النووي أن ال تركيزات العالية من هذه الجسيمات النانوية تسببت في تلف شديد لجزيء الحمض النووي. وقد خلصت الدراسة إلى أن التعرض لجزيئات النحاس النانوية قد أدى الى تراكمها داخل أنسجة الكائن محل الدراسة مسببة الكثير من الأضرار مثل زيادة معدل النفوق بالإضافة الى الكثير من التغيرات البيوكيميائية والمرضية داخل أنسجة هذا الكائن. وقد كانت تلك التأثيراتوالتغيرات واضحة وسهلة الرصد بما فيه الكفاية لإستخدامها كمؤشرات حيوية لتلوث كائنات المياه العذبة بالمركبات النانوية.