Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 - 6131 Vol. 22(2): 51- 60 (2018)
ejabf.journals.ekb.eg

Oxidative stress and histomorphological markers in the offspring of *Poecilia reticulata* maternally exposed to metallic and nanoformulated copper

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ARTICLE INFO

Article History:
Received:April 9, 2018
Accepted:May 25, 2018
Available online:June 2018

Keywords:
Maternal transfer
copper oxide nanoparticles
copper sulfate
Poecilia reticulate
oxidative stress
histopathology

ABSTRACT

The goal of present study is to determine whether metals or nanomaterials are transferred from exposed pregnant females of viviparous fishes to their developing offspring and the subsequent potential effects on the oxidative stress and histomorphological indicators. Gravid females of guppies (*Poecilia reticulata*) exposed to 1 mg/l copper sulfate ($CuSO_4$) or copper oxide nanoparticles (CuO-NPs) for 17 to 25 days depending on giving birth. Once a female gave birth, the female and her newborn offspring were analyzed for copper concentrations while some oxidative stress parameters and histopathology were performed for the newborn offspring. There were no malformations or histopathological lesions observed in the newborn offspring of guppies exposed for either $CuSO_4$ or CuO-NPs. Also, there was no maternal metal transfer for either $CuSO_4$ or CuO-NPs while females exposed to copper sulfate only showed significantly higher concentrations of Cu compared to control group. In addition, there were non-significant changes in catalase compared to control group. While, glutathione decreased significantly in offspring their mothers exposed to CuO-NPs only compared to control group. In addition, superoxide radical and lipid peroxidation increased significantly in offspring their mothers exposed to $CuSO_4$only compared to control group.

The findings of the present study indicated that the offspring were more sensitive to $CuSO_4$ than CuO-NPs. Although absence of metal Cu in the tissues of offspring as well as lack of morphological abnormalities and histopathology, maternal transfer of Cu metabolites may be suggested to be the causative factor for some oxidative stress.

INTRODUCTION

Maternal transfer of metals or nanomaterials had been studied scarcely in viviparous fish (Goodhead, 2011; Cazan and Klerks, 2014; Le Bourg *et al.*, 2014). In Poeciliidae, embryos develop within the follicle and are connected to their mother via a follicular pseudoplacenta (Wourms *et al.*, 1988), with the level of maternal provisioning changing among species (Greven, 2011). Guppy (*Poecilia reticulata*) is a species from the Poeciliidae family and broadly disseminated tropical fish in the world. They are used as a model organism in the field of ecology, evolution and behavioral studies (Moosavi and Shamushaki, 2015).

Copper compounds are widely used in aquaculture as biocides to control nuisance algae, macrophytes, freshwater snails, ectoparasites of fish and marine fouling organisms (Eisler, 1998). It should be considered that copper could be accumulated in water body and gradually increases the concentration will raise to lethal concentration for fishes (Reddy *et al.,* 2006). Recently, copper oxide nanoparticles (CuO-NPs) have found a wide spectrum of applications such as gas sensors, catalytic processes, solar cells and lithium batteries as well as face masks, wound dressings and socks (Mansouri *et al.*, 2015). There is a growing concern that these products and their byproducts may discharge hazardous biochemical particles into aquatic habitats which in turn can affect their biota (Mansouri *et al.,* 2015). Several studies have been conducted on the toxicity of copper and copper oxide nanoparticles to aquatic organisms, and these studies have shown that these substances can be toxic to aquatic organisms such as fish (Isani *et al*., 2013; Ates *et al*., 2014; Wang *et al.*, 2015; Soliman, 2015; Abdel-Khalek *et al*., 2016; Sun *et al.*, 2016). In the instance of copper and other metal oxide NPs, the dissolution of Cu and its speciation may be a key factor in their ecotoxicity, besides that related to the nanoparticle itself (Lagadic *et al.*, 2000).

A key mechanism thought to be responsible for oxidative stress exerted by nanomaterials, in particular by CuO NPs, involves an increased production of intracellular reactive oxygen species (ROS) (Di Bucchianico *et al*., 2013). Overproduction of ROS, to a point exceeding the antioxidant defense capacity of cells, causes oxidative stress which can directly modify membrane lipids, proteins and DNA (Winterbourn, 2008). The parameters of oxidative stress have a major role in ecotoxicological risk assessment in the aquatic environment and mentoring the health status of fish (Pritsos *et al.,* 2017). Moreover, histopathological evaluation of the affected target organ provides solid based evidence on the peroxidative nature of oxidant species which implicated in the cytotoxicity and associated with functional burdens (Sumi and Chitra, 2017). Therefore, the goal of present study was to investigate the maternally transfer of copper oxide nanoparticles and copper sulfate as well as determine oxidative stress and histopathology in the newborn offspring of guppy (*Poecilia reticulata*).

MATERIALS AND METHODS

Materials:

The Copper (II) oxide nanoparticles (CuO) were purchased from sigma-Aldrich, UK. The physical characteristics of the particles according to the manufactures data were; size (<50nm), purity (99.5%), trace metal basis, surface area (5.0 m2/g), density (10.49g/cc). The copper (II) oxide nanoparticles (CuO) were weighed and suspended in deionized water to prepare the required stock solution (1000 mgl^{-1}) and then dispersed by ultrasonic vibrations (100 w. 30khz) for 30 min. The stock suspension of copper (II) oxide nanoparticles (CuO) was re-sonicated for 3 to 5 min prior to tests, and then it was diluted with water to make final test concentrations according to the experimental design. Moreover, the copper ions used in the form of cupric sulfate ($CuSO_4.5H_2O$) were produced by BDH Chemical Ltd Poole, UK.

Female exposure and offspring collection:

Pregnant females of guppy (*Poecilia reticulata*) at first days of pregnancy depending on fish breeder experience (appearance of gravid spot) were obtained from a local aquaculture shop in Sohag province, Egypt. Prior to the beginning of experiments, pregnant females of guppy were acclimatized in1 L beakers supplied with continuously aerated tap water (22-27 ℃) under 12-hour light/12-hour dark cycles. Fish were fed on commercially available food at a rate of 2% body weight per day. Eighteen pregnant females were divided into three groups. One group served as a control, and the other two groups were treated with 1mg/l of CuO-NPs or $CuSO_4.5H_2O$ according to Soliman (2014) for 17 to 25 days depending on giving birth. To minimize degradation of CuO-NPs and $CuSO_4.5H_2O$ concentrations, the water in the aquariums was renewed every day. During the exposure, the beakers were aerated to prevent the tendency of aggregation. Once a female gave birth, the female and her newborn offspring were analyzed for copper concentrations, while some oxidative stress parameters and histopathology were performed for the newborn offspring.

Incidence of abnormalities:

Incidence of abnormalities of offspring were observed immediately and recorded after female gave birth according to the method of Mahmoud *et al.* (2009).

Histopathology:

Whole larvae were rinsed and fixed in 10 % neutral buffered formalin for at least 48 h, dehydrated in graded ethanol, and cleared in xylene prior to embedding in paraffin blocks. Dewaxed sections (5µm) were stained with Harris's hematoxylin and eosin stain (H & E), mounted with DPX and examined by light microscop.

Whole body copper analysis:

Whole body copper concentrations either of females or off springs were analyzed according to Soliman (2015). Briefly, whole fish was placed in 5 ml of concentrated nitric acid at 70 Cº for 24h. Each sample was analyzed for Cu by flame atomic absorption spectrophotometer (model 2380, Perkin Elmer). Analytical grade standards were used throughout, and the acidity and matrix of the standards were matched to the samples. Samples of water and food for metal analysis were taken immediately after each solution renewal, and acidified in 1% nitric acid before metal analysis by flame atomic absorption spectrophotometer (model 2380, Perkin Elmer) depending on the concentrations.

Oxidative stress:

The newborn offspring were homogenized in phosphate buffer (pH = 7.4), then the homogenates were centrifuged at 5000 rpm for 15 min to obtain the supernatant which stored at -20 °C for the estimation of oxidative stress markers. The total protein content was determined by the method of Lowry *et al.* (1951). Lipid peroxidation (LPO) products as thiobarbaturic acid reactive substance (TBARS) were determined according to the method of Ohkawa *et al.* (1979). Glutathione (GSH) content was determined using the method of Beutler *et al*. (1963). The superoxide radical (O^{-2}) was determined according to Prodczasy and Wei (1988). Catalase (CAT) activity was determined according to the procedure of Aebi (1984).

Statistical analysis:

All data were expressed as the mean ± SEM. Statistical comparisons were performed using Student's t-test (*P* values < 0.05 were considered statistically significant).

RESULTS

Characterization of copper oxide nanoparticles:

Transmission electron microscopy showed that the copper oxide nanoparticles were spherical in shape and not strongly aggregated, with mean diameter 22.23± 8.6 nm (Fig. 1).

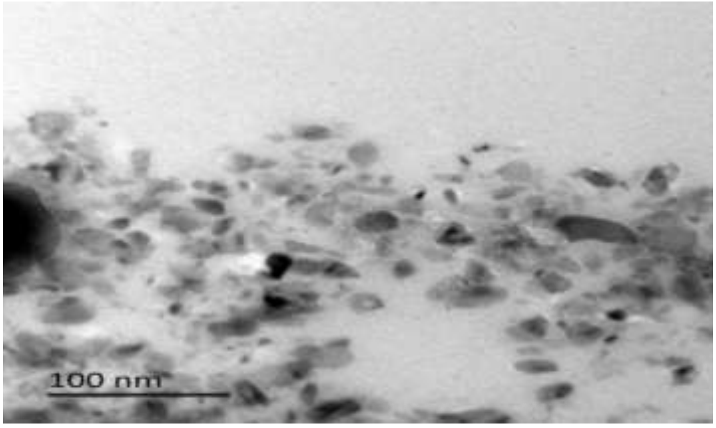

Fig.1. Transmission electron microscopy (TEM) image of copper oxide nanoparticles.

Incidence of morphological abnormalities and histopathological alterations:

There were no morphological abnormalities (notochordal curvature and finfold defects) in newborn offspring of exposed females compared to the control (Fig. 2). In addition, there were no histopathological changes in the brain and intestinal regions of the newborn offspring from the exposed females compared to the control (Figs. 3 & 4).

Fig. 2: Morphology of guppy larvae (*Poecilia reticulata*) showing (a) control, (b) after mother exposure to copper sulfate(c) after mother exposure to copper oxide nanoparticles.

Fig. 3: Histology of brain region of guppy larvae (*Poecilia reticulata*) showing (a) control, (b) after mother exposure to copper sulfate(c) after mother exposure to copper oxide nanoparticles. Brain (B), gills (G).

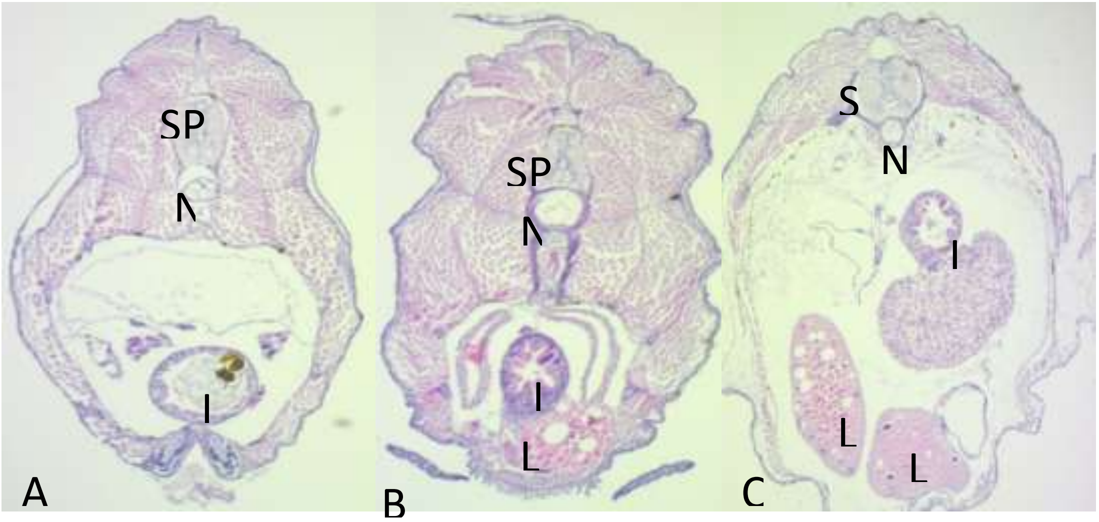

Fig. 4: Histology of intestinal region of guppy larvae (*Poecilia reticulata*) showing (a) control, (b) after mother exposure to copper sulfate (c) after mother exposure to copper oxide nanoparticles. Spinal cord (SP), notochord (N), intestine (I), liver (L).

Copper concentration in water:

The estimated Cu concentration of water media for $CuSO_4$ group and CuO NPs group was significantly higher than Cu concentration of water media in control (Table 1).

Table 1: Copper concentrations (mg/l) in water media and food pellet.

	Control	$CuSO_4$	CuO-NPs
Water media	**0.01±0.00**	**0.36±0.01***	**0.10±0.02***
Food pellet	**0.01±0.006**		

* Statistically significant difference at $P< 0.05$ (t test).

Whole body copper concentration:

Females exposed to copper sulfate only showed significantly higher concentrations of Cu compared to control group. On the other hand, the concentration of Cu in offspring their mothers exposed to both forms of copper (CuO NPs and soluble Cu) were under detectable levels (Table 2).

Table 2: Copper bioaccumulation concentration (mg/ g wet weight) in mothers and offspring of guppy (*Poecilia reticulata*) exposed to 1mg/l of CuO-NPs or $CuSO_4.5H_2O$.

	Control	$CuSO_4$	CuO-NPs
Mothers	**2.19±0.36**	**4.6±0.52***	**3.87±1.14**
Offspring	**ND**	**ND**	**ND**

* Statistically significant difference at $P< 0.05$ (t test).

Oxidative stress:

Offspring their mothers exposed to copper sulfate were characterized by higher levels of lipid peroxidation (LPO) and superoxide radical (O^{-2}) in comparison with the control group. While, offspring their mothers exposed to copper oxide nanoparticles were characterized by lower level of glutathione (GSH) in comparison with the control group. However, catalase (CAT) did not show any significant differences between the different experimental groups (Table 3).

Table 3: The antioxidant enzymes activities in offspring of guppy (*Poecilia reticulata*) exposed to 1mg/l of CuO-NPs or $CuSO_4.5H_2O$.

	Control	$CuSO_4$	CuO-NPs
Catalase (U/mg protein)	**0.95±0.21**	**1.69±0.81**	**1.75±0.71**
Glutathione (μg/mg protein)	**125.52±23.59**	**88.10±7.22**	**50.51±12.65***
Superoxide radical (nmol/min./mg protein)	**0.16±0.03**	**0.25±0.03***	**0.11±0.014**
TBARS (nmol/mg protein)	**0.76±0.14**	**1.46±0.26***	**0.62±0.11**

* Statistically significant difference at $P< 0.05$ (t test).

DISCUSSION

Maternal transfer is regarded as a potentially important route for exposure of embryos and early life stage animals to nanoparticles in live bearing animals (Goodhead, 2011).

In present study, there were no abnormalities (notochordal curvature and finfold defects) evident in offspring from control or exposed females as well as histopathological changes. The same results, the offspring of guppies (*Poecilia reticulata*) exposed to octylphenol or 17b-estradiol via the mother did not show any abnormalities (no significant effects on sex ratio, the growth and the gonopodium indices of the offspring). The absence of adverse effects on the offspring despite distinct effects on the parent generation raises the question whether 17b-estradiol and octylphenol reach the embryos in the ovary (Kinnberg *et al*., 2003). Also, there was no impact of silver on larval survival, birth weights and body condition indices in guppies (*Poecilia reticulata*) exposed to silver nanoparticles and bulk sized particles (Goodhead, 2011). Furthermore, there were no discernible effects on morphological endpoints in the exposed female fish or on somatic growth, gonadal development or sex partitioning in their developing offspring in *Xenotoca eiseni* exposed to 17α-ethinyloestradiol (Tinguely, 2015). In other hand, Morthorst *et al.* (2014) stated that maternal exposure to 17b-estradiol during early pregnancy causes severe malformations in fry of the viviparous eelpout. Furthermore, offspring of *Heterandria formosa* and *Gambusia affinis* exposed to copper and cadmium via females had a reduced size at birth and developmental abnormalities (Cazan and Klerks, 2015).

In current study, females exposed to copper sulphate only showed significantly higher concentrations of copper compared to controls. In other hand, the concentrations of copper in offspring exposed to both forms of copper (CuO NPs and soluble Cu) were under detectable levels. These differences probably reflect the fact that copper is efficiently acquired at low levels and regulated at low and intermediate levels (Kanakaraju *et al.,* 2009). The lower metal levels in offspring born after the exposure reflect changes in maternal transfer or are a direct reflection of lower metal levels in the females (Cazan and Klerks, 2014). Also, the guppy has macrolecital eggs, which are believed to contain enough yolk to meet the nutritive needs of the developing embryo until birth. So, maternal-embryonic transfer of nutrients seems to be negligible (Schindler and Hamlett, 1993). In other hand, maternal transfer of silver to the larvae was significantly higher for the nanoparticulate treatment compared with the bulk and larval burden was significantly higher compared with the maternal sires in guppies (*Poecilia reticulata*) exposed to silver nanoparticles and bulk sized particles, dosed via the diet for a full gestation cycle (Goodhead, 2011). Moreover, Cazan and Klerk (2014) observed maternal metal transfer of copper and cadmium in the western mosquitofish and least-killifish.

In current study, the marked increase in lipid peroxidation (LPO) and superoxide radical (O^{-2}) in the offspring of $CuSO_4$ exposed mothers and reduced GSH in the offspring of Cu NPS exposed mothers indicates possible damage of the cellular structures. These findings are corresponding with the ability of Cu to hasten free radicals generation accompanied with depletion of SOD and the components of glutathione redox cycle (Stohs and Bagchi, 1995). Also, offspring of *Gambusia affinis* exposed to cadmium via females had elevated levels of lipid peroxidation (Cazan and Klerks, 2015). Furthermore, exposing guppies (*Poecilia reticulata*) to UV-B increased catalase activity and glutathione concentrations, and reduced ROS-induced damage to membranes and proteins. There was no effect of parental exposure to UV-B on offspring superoxide dismutase activity (Kazerouni *et al.*, 2017).

CONCLUSION

Although there was no evidence about maternal transfer for metallic and nanoformulated Cu to offspring of *Poecilia reticulata*, a well marked increase in the lipid production and superoxide anion were observed in the offspring of $CuSO_4$ exposed mother and reduced GSH in the offspring CuO NPS exposed mother. These findings raise a question whether the undetectable levels of both forms of Cu or their metabolites still cause oxidant effects, and direct our attention towards increasing the exposure duration using a window of several time points and sampling a variety of developmental stages of offspring.

Acknowledgement

The authors are grateful to Prof. Dr. Hossam El-Din Omar. Prof. of Physiology. Zoology Department, Faculty of Science, Assiut University for manuscript revision.

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ARABIC SUMMARY

الإجهاد التأكسدي والعلامات الهيستومورفولوجية في نسل *Poecilia reticulata* أمومياً تعرضن للنحاس المعدني و النانومترى

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الهدف من الدراسة الحالية هو تحديد ما إذا كانت المعادن أو المواد النانومترية يتم نقلها من الإناث الحوامل للأسماك الولودة إلى نسلها النامى والتأثيرات اللاحقة المحتملة على الإجهاد التأكسدي والمؤشرات الهيستومورفولوجية.

تعرضت الإناث الحوامل من أسماك الجوبى (*Poecilia reticulata*) ل١ مجم / لتر من كبريتات النحاس ($CuSO_4$) أو جسيمات أكسيد النحاس النانومترية (CuO-NPs) لمدة تتراوح من ١٧ إلى ٢٥ يومًا تبعاً للولادة. وبمجرد أن أنجبت ألأناث ، تم تحليل الأناث ونسلها الوليد لتركيزات النحاس في حين تم قياس بعض معايير الإجهاد التأكسدي و الهستوباثولوجى للنسل حديث الولادة.

اظهرت النتائج عدم وجود تشوهات مورفولوجية أو نسيجية مرضية في نسل أسماك الجوبى التى تعرضت امهاتهم لكبريتات النحاس أو جسيمات أكسيد النحاس النانومترية . أيضا ، لم يكن هناك نقل للمعادن من الأمهات إلى نسلها سواء فى حالة كبريتات النحاس أو جسيمات أكسيد النحاس النانومترية بينما أظهرت الإناث المعرضة لكبريتات النحاس فقط تركيزات أعلى بشكل معنوى من النحاس مقارنة بالمجموعة الضابطة. بالإضافة إلى ذلك ، كانت هناك تغييرات غير معنوية في الكاتلاز مقارنة بالمجموعة الضابطة. في حين انخفض الجلوتاثيون بشكل معنوى في نسل الأمهات التى تعرضن إلى جسيمات أكسيد النحاس النانومترية فقط بالمقارنة مع المجموعة الضابطة. بالإضافة إلى ذلك ، زاد شق الأكسيد الفائق و بيروكسيد الدهون بشكل كبير في نسل أمهاتهم تعرضن لكبريتات النحاس مقارنة بالمجموعة الضابطة. أشارت نتائج الدراسة الحالية إلى أن النسل كان أكثر حساسية لكبريتات النحاس من جسيمات أكسيد النحاس النانومترية. على الرغم من عدم وجود معدن النحاس في أنسجة النسل فضلا عن عدم وجود تشوهات هيستومورفولوجية, من المحتمل ان النقل الأمومي لنواتج ايض النحاس هوالعامل المسبب لبعض الإجهاد التأكسدي فى النسل.