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Comparative toxicity of copper oxide bulk and nano particles in Nile Tilapia; *Oreochromis niloticus*: Biochemical and oxidative stress

Amr A. Abdel-Khalek *, Mohamed A.M. Kadry, Shereen R. Badran, Mohamed-Assem S. Marie

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

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Abstract Nile Tilapia; *Oreochromis niloticus* are commonly used in the assessment of aquatic environment quality and also considered as useful bio-indicators during environmental pollution monitoring. The LC_{50}/96 h of copper oxide (bulk & nano) particles [CuO (BPs & NPs)] were 2205 & 150 mg/l, respectively. Two tested concentrations of CuO (BPs & NPs) were selected: the first concentration was equivalent to ($^1/_{10}$) (220.5 & 15 mg/l), and the second was equivalent to ($^1/_{20}$) (110.25 & 7.5 mg/l) LC_{50}/96 h·CuO (BPs & NPs), respectively. While serum glucose, liver function tests (AST, ALT and ALP) and kidney function tests (creatinine and uric acid) showed a significant increase, serum total proteins, albumin, globulin and total lipids showed a significant decrease. Both liver and gill tissues of the studied fish showed a reduction in GSH content and an elevation in MDA and GPx activities. The present study also showed an elevation in liver CAT & SOD activities when exposed to both concentrations of CuO BPs and in the case of gills when exposed to both concentrations of CuO (BPs & NPs), although activity of these enzymes showed an inhibition in the liver when exposed to both concentrations of CuO NPs. The present study investigated whether CuO NPs are more toxic than CuO BPs.

Introduction

The excessive contamination of aquatic ecosystems has evoked major environmental and health concerns worldwide (McNeil and Fredberg, 2011). The pollutants could increase the level of metals in natural water and seriously affect both fresh and marine habitats (Muhammad et al., 2011; Yu et al., 2011; El Nemr, 2012; El Nemr et al., 2012). The devastating effects of heavy metals are mainly due to the dispersal performance and bio-magnification of metals into aquatic food chains in addition to their toxicity and accumulative behavior in the biological tissues (Matta et al., 1999; Islam and Tanaka, 2004; Yi et al., 2011). Copper is highly toxic to aquatic organisms and may cause irreversible harm at concentrations just over that required for growth and reproduction (Baldwin et al., 2003). Although copper is considered an essential element, its high

* Corresponding author.

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concentrations in water are toxic to freshwater organisms. Hardman (2006) reported that, the aquatic system which is an essential part of the environment is particularly at risk of exposure to engineered nanoparticles (ENPs). Chen et al. (2012) stated that ''Nanotechnology is concerned with materials and systems whose structures and components exhibit novel and significantly improved physical, chemical and biological properties, phenomena and processes due to their nano scale size''. As a result of the growing nanotechnology applications, many nano-metals are discharged into the aquatic habitats that affect its biota (Isani et al., 2013). The CuO NPs have been used in industrial applications, in medicine and as pesticides (Kiaune and Singhasemanon, 2011) and used as antifouling agents in paints (Perreault et al., 2012). Therefore, ENPs need to be evaluated in terms of their potential to pose risks to human health and environment (Handy et al., 2008; Nowack, 2009). Metal oxide NPs have been specifically studied because of their potential toxicity and common occurrence in consumer products and industrial pollutants (Melegaria et al., 2013). So, comparative studies of nanoscale and microscale materials are important because the intrinsic characteristic of NPs which may be directly related to their toxicity, and comprehensive characterization of suspensions of these particles is necessary (Ribeiro et al., 2013).

Fish are widely used to evaluate the health of aquatic ecosystems, because pollutants are building up in the food chain (Farkas et al., 2002). So, determination of metal levels in fish is tremendously important for the health of human beings (Uysal et al., 2008). Nile Tilapia; *Oreochromis niloticus*, is considered as one of the most common freshwater fish that is used in toxicological studies (Figueiredo-Fernandes et al., 2006a,b; Garcia-Santos et al., 2006). This species displays many characteristics making it an appropriate model to be used as an indicator species in bio-monitoring programs (Gadagbui et al., 1996), because of its high growth rate, significant tolerance to environmental stress, ease of reproduction, and high market demand (El-Sayed, 2006).

Analysis of biochemical parameters could help to identify the target organs of toxicity as well as the general health status of animals and it may also provide an early warning signal in stressed organism (David et al., 2010; Prashanth, 2012; Dube et al., 2014).

Oxidative Stress is induced by an increase in reactive oxygen species (ROS), an impairment of anti-oxidant defense systems, or incapacity to repair oxidative damage (Dorval and Hontela, 2003). The Cu can induce oxidative stress, because it catalyzes the formation of ROS via a Fenton-like reaction (Prousek, 2007). The positive Cu^{2+} ion can also act directly through binding to negatively charged protein-SH groups and denaturation of enzymes, or indirectly via generation of ROS resulting in oxidative stress (Ahmad et al., 2005; Bopp et al., 2008). The CuO NPs themselves may generate additional ROS and the investigation of the relationship between the cellular responses to sub-toxic concentrations of CuO NPs and the oxidative stress endpoints has been proposed (Fahmy and Cormier, 2009). Gomes et al. (2011) reported that CuO NPs cause oxidative stress in the mussel; *Mytilus galloprovincialis*.

The present study aims to provide a comparative study between CuO (BPs & NPs) at $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h concentrations to declare their deleterious effects on biochemical profiles and oxidative biomarkers of fresh water Nile Tilapia; *O. niloticus*.

Materials and methods

Characterization of CuO (BPs & NPs)

The CuO (BPs) were purchased from El-Nasr pharmaceutical chemicals Co. Egypt, while CuO (NPs) were purchased from Sigma–Aldrich, St. Louis, MO, USA, with an average size < 50 nm and 99% purity level. Structural studies of both CuO (BPs & NPs) were done by Field Emission Transmission Electron Microscopy (FETEM, JEM-2100F, JEOL Inc., Japan) at an accelerating voltage of 200 kV. The average hydrodynamic size of CuO (NPs & BPs) in water was determined by Dynamic Light Scattering (DLS) (Nano-zetasizer-HT, Malvern Instrument, UK). Both BP & NP suspensions were sonicated using a sonicator bath at room temperature for 15 min (40 W & 40 kHz) and the DLS experiments were performed as described by Murdock et al. (2008). Determinations of zeta potential were done in particle suspensions in deionized water, using a Malvern Zetasizer Nano ZS instrument.

Experimental fish

Experimental fish in the present study were Nile Tilapia; *O. niloticus*. They were purchased from unpolluted fish farm located in El-Ismailia governorate, Egypt. The initial body length and weight of fish were (13.5–17 cm) and (40–75 g), respectively. All Nile Tilapia were transported in plastic containers with continuous aeration to the lab. All fish (8 fish/aquarium) were maintained for two weeks in glass aquaria with 50 L aerated, dechlorinated tap water. Water temperature was maintained at 25 ± 1 °C, while dissolved oxygen and pH were 6.5–7.8 mg/l, and 7.1–7.3, respectively. Photoperiod was 12 h light:12 h dark. During the acclimatization period, fish were fed once daily with commercial pellet food (20% crude protein, 4% crude fat, 5% crude fiber, 12% crude ash and 10% crude moisture). Water was changed daily and dead fish as well as any fish showing any unusual performances were excluded.

Determination of CuO (BPs & NPs) LC50 values

After acclimatization period (2 weeks), groups) each four fish) were transferred to small glass aquaria for LC_{50} determination. Nominal concentrations used for BPs were (0, 1000, 2000, 3000, 4000 and 5000 mg/l) and for NPs were (0, 200, 400, 600, 800, 1000 mg/l). The exposure period was 96 h; with the same temperature, dissolved oxygen and pH as in the acclimatization period. The dead fish was recorded in each concentration to estimate the LC_{50} value via probit analysis according to Finney (1952), using statistical program (SPSS software, version 16.0, IBM, Chicago, IL, USA).

Preparation of CuO (BPs & NPs) suspensions

The two suspension concentrations of both bulk and nano CuO forms ($^{1}/_{10}$ and $^{1}/_{20}$ LC_{50}/96 h) were prepared by weighing dry CuO powder into the dechlorinated water (pH 7.4),

then ultrasonicated (100 W, 40 kHz) for 1 h to increase their dispersion.

Experimental design (30 days)

The fish were randomly allocated in glass aquaria (40 × 70 × 26 cm) in triplicate groups (each 8 fish/aquarium). Fish were then exposed to $^{1}/_{10}$ and $^{1}/_{20}$ of 96 h LC_{50} CuO (BPs & NPs) for 30 days. A control group was handled identically but without exposure to CuO particles. The conditions of the experiments were as those of acclimatization period and water was constantly (every day) checked for pH, temperature and dissolved oxygen. Water was changed every 2 days, and fish were fed 40 min before water change.

Fish sampling

After 30 days, blood sampling was withdrawn from the caudal vein of the control groups and the fish groups exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of CuO (BPs & NPs) using heparin as anticoagulant, then the vital organs (liver and gills) were isolated and stored frozen for further investigation.

Biochemical analyses

Blood samples were centrifuged to get the sera for biochemical analyses using enzymatic-colorimetric methods by means of commercial Biodiagnostic kits (Biodiagnostic, Dokki, Giza, Egypt). The serum glucose was determined according to the method described by Trinder (1969). Total lipid was determined according to Zollner and Kirsch (1962). In addition, serum total protein measurement was according to the biuret method (Gornal et al., 1949). While, serum albumin concentration was measured according to Doumas et al. (1971) globulin concentration was calculated as the difference between total protein and albumin according to the method described by Coles (1986). Serum AST and ALT activities were assessed according to Reitmans and Frankel (1957) while, the ALP activity was estimated according to Belfield and Goldberg (1971). Serum creatinine was measured using the colorimetric method described by Bartles et al. (1972), while uric acid was measured using enzymatic reaction according to Barham and Trinder (1972).

Determination of antioxidant biomarkers

For evaluation of oxidative damage, liver and gills were homogenized in 5 ml cold buffer (pH 7.4) per gram tissue using a homogenizer. Then the homogenates were centrifuged at 4000 r.p.m. for 15 min and the supernatants were stored at −80 °C until used. Oxidative stress was detected in supernatant of the tissue homogenate using Biodiagnostic kits (Biodiagnostic Dokki, Giza, Egypt).

Lipid peroxide

Malondialdehyde (MDA) concentration was used as the index of lipid peroxidation (LPO) as described by Ohkawa et al. (1979). MDA was determined by measuring the thiobarbituric acid reactive species. The absorbance of the resultant pink product was measured at 534 nm.

Glutathione reduced (GSH)

Glutathione reduced (GSH) levels depend on the reduction of 5,5′-dithiobis 2-nitrobenzoic acid with glutathione producing a yellow color whose absorbance is measured at 405 nm according to Beutler et al. (1963).

Glutathione peroxidase (GPx)

The assay is an indirect measure of the activity of glutathione peroxidase (Gpx) which reduced organic peroxide to oxidized glutathione (GSSG) which was recycled to its reduced state by glutathione reductase (GR). The oxidation of NADPH to $NADP^{+}$ is accompanied by a decrease in absorbance at 340 nm (A_{340}) providing a spectrophotometric means for monitoring the GPX enzyme activity as described by Paglia and valentine (1967).

Superoxide dismutase (SOD)

This assay relies on the ability of the enzyme to inhibit the phenazine methosulfate mediated reduction of nitro blue tetrazolium dye (Nishikimi et al., 1972) and the change in absorbance at 560 nm over 5 min was measured.

Catalase assay (CAT)

Catalase (CAT) reacts with a known quantity of H_2O_2 and the reaction is stopped after 1 min with catalase inhibitor. In the presence of peroxidase, the remaining H_2O_2 reacts with 3,5-Dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore with a color intensity inversely proportional to the amount of catalase in the sample. The absorbance was measured at 510 nm as described by Aebi (1984).

Statistical analyses

The results were expressed as mean ± SE. Data were statistically analyzed using *t*-test, analyses of variance (*F*-test) and Duncan's multiple range test to determine the difference in means using Statistical processor Systems support "SPSS" for windows software. A value of ($P < 0.05$) was considered significant.

Results

Fig. 1A and B shows (TEM) images of CuO BPs & NPs, respectively. According to TEM images, the CuO BPs size was (142–159 nm) greater than that of the CuO NPs size which was (35–37 nm). The average hydrodynamic size (Fig. 1C and D) and zeta potential (Fig. 1E and F) in water were (954 nm, 11.5 mV) for CuO BPs, and (588 nm, −5.32 mV) for CuO NPs, respectively. The LC_{50} of CuO (BPs & NPs) for Nile Tilapia; *O. niloticus* after 96 h exposure was 2205 and 150 mg/l, respectively. We selected $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h (220.5 & 110.25 mg/l) for CuO (BPs) and (15 & 7.5 mg/l) for CuO (NPs) in the present study for 30 days.

An analysis of variance (*F*-values) between control and fish exposed to both concentrations of CuO (BPs & NPs) showed a significant difference in all studied serum constituents, except albumin when fish groups were exposed to CuO BPs and uric acid when fish groups were exposed to CuO NPs. After 30 days, exposure of Nile Tilapia fish; *O. niloticus* to both

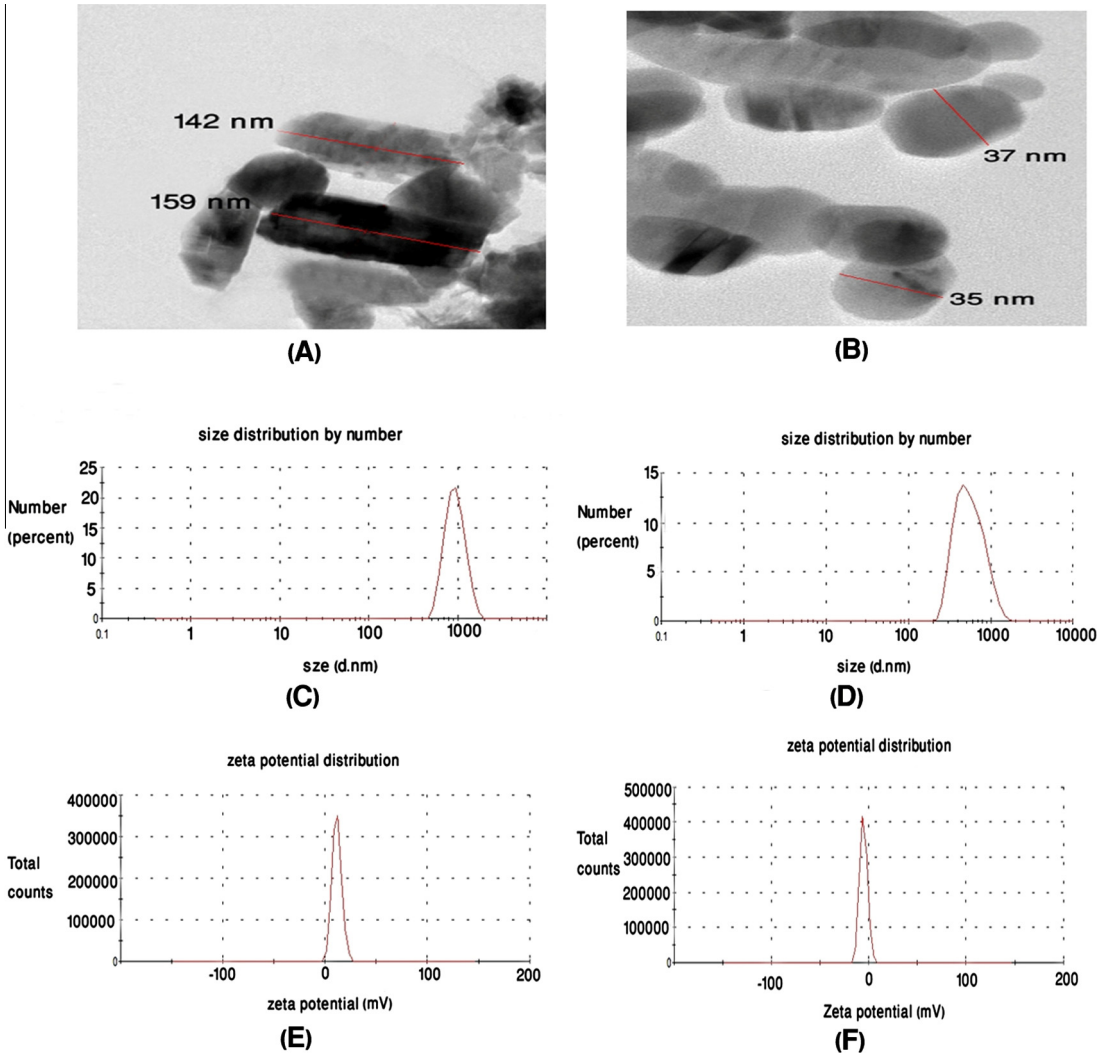

Figure 1 (A & B) shows TEM image of CuO (BPs & NPs); (C & D) shows size distribution of CuO (BPs & NPs) and (E & F) zeta potential distribution of CuO (BPs & NPs), respectively.

the studied concentrations of CuO (BPs & NPs) resulted in a significant increase in serum glucose, indicating that ($^1/_{20}$) LC_{50}/96 h of CuO NPs had more toxic effects than CuO (BPs) (Fig. 2A) while serum total lipids showed a significant decrease when compared to control groups when fish were exposed to ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h of CuO (BPs) and ($^1/_{10}$) of CuO (NPs), indicating that CuO BPs had more toxic effects than CuO (NPs) (Fig. 2B).

Regarding the effect of both the studied concentrations of CuO (BPs & NPs) on serum total protein, albumin and globulin, the obtained data showed a significant decrease in serum total protein, albumin and globulin compared with their matched control groups, except albumin when exposed to both concentrations of CuO (BPs), indicating that CuO NPs had higher effects at both concentrations than CuO (BPs) (Fig. 2C–E).

Serum AST, ALT, and ALP activities showed a generally highly significant increase in fish after exposure to ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h of (BPs & NPs) CuO in comparison to the control groups, indicating that CuO NPs at both concentrations are more toxic than CuO (BPs) (Fig. 3A–C).

While serum creatinine showed a significant increase in fish after exposure to both concentrations of CuO (BPs & NPs), without any variation between (BPs & NPs), uric acid showed a significant increase after exposure to ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h of CuO (BPs) without any change after exposure to ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h of CuO (NPs) (Fig. 3D and E).

An analysis of variance (*F*-values) between control and fish groups exposed to both concentrations CuO (BPs & NPs) showed a significant difference in all studied oxidative biomarkers in liver tissues.

After 30 days, exposure of Nile Tilapia fish; *O. niloticus* to both the studied concentrations of CuO (BPs & NPs) showed a significant increase in MDA levels in liver tissues, indicating that $^1/_{20}$ LC_{50}/96 h of CuO NPs had more toxic effects than $^1/_{20}$ LC_{50}/96 h of CuO (BPs) (Fig. 4A).

Concerning GSH content, it showed a significant decrease at ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h of CuO (BPs & NPs) when compared with control group, indicating that ($^1/_{20}$) LC_{50}/96 h·CuO NPs had more toxic effects than ($^1/_{20}$) LC_{50}/96 h·CuO (BPs) (Fig. 4B). Regarding the GPx activity the results showed a significant increase at ($^1/_{20}$) LC_{50}/96 h of CuO BPs and at both

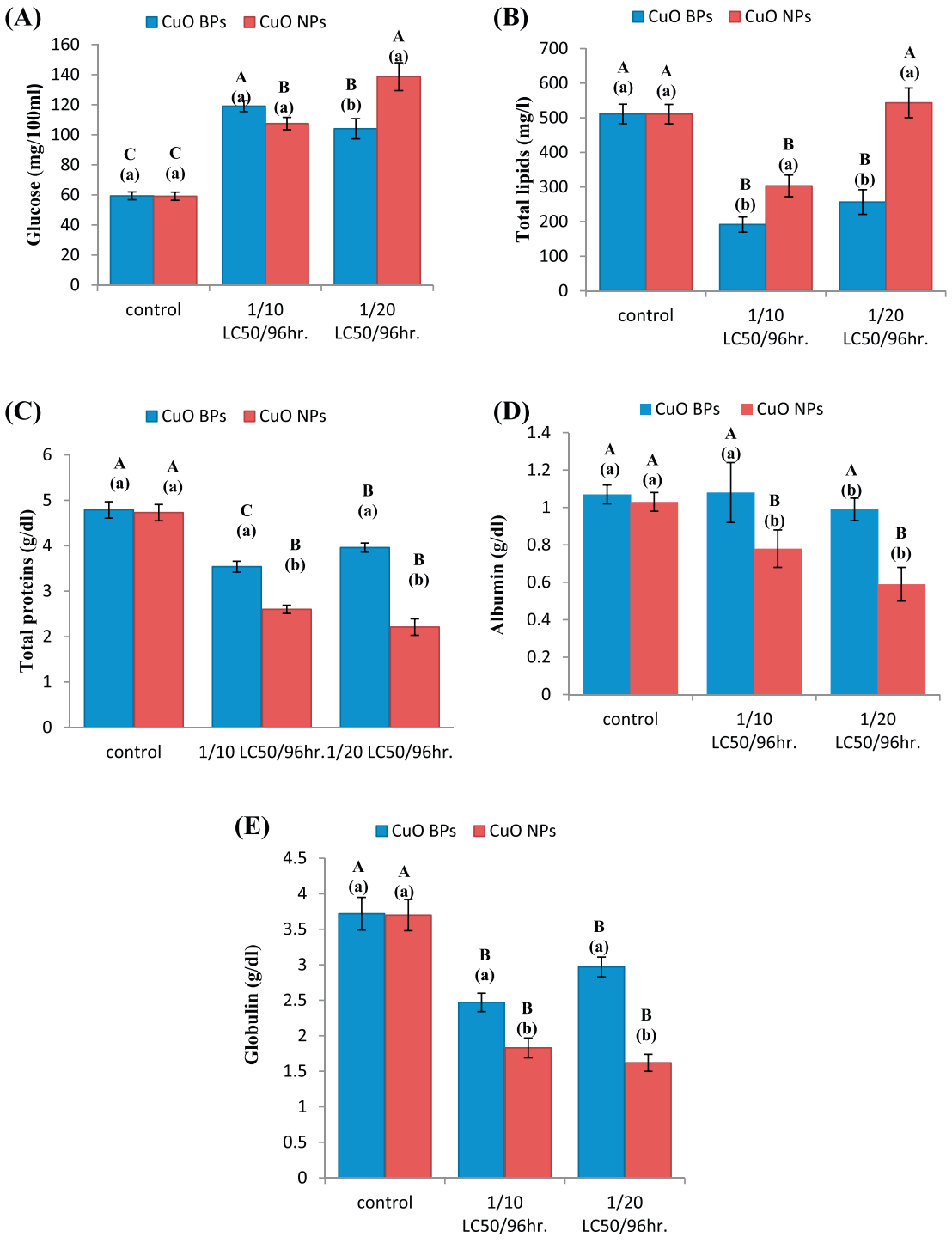

Figure 2 Effect of CuO (BPs & NPs) at different concentrations on serum glucose (A), total lipid (B), total protein (C), albumin (D) and globulin (E) of Nile Tilapia; *Oreochromis nilotcus* after 30 days. Mean ± SE with the same small letter on the different colored column for each parameter is not significantly different. Mean ± SE with the same capital letter on the same colored column for each parameter is not significantly different.

concentrations of CuO NPs, indicating that CuO NPs had higher effects at both concentrations than CuO (BPs) (Fig. 4C).

While, the exposure to ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h·CuO (BPs) caused a general significant increase in the activities of both CAT and SOD in the liver of fish when compared to control groups, the effects of ($^1/_{10}$ & $^1/_{20}$) LC_{50}/96 h·CuO (NPs) on both the studied enzymes recorded a significant inhibition in the liver of the exposed fish (Fig. 4D and E).

An analysis of variance (*F*-values) between control and fish groups exposed to both concentrations of CuO (BPs & NPs) showed a significant difference in all studied oxidative

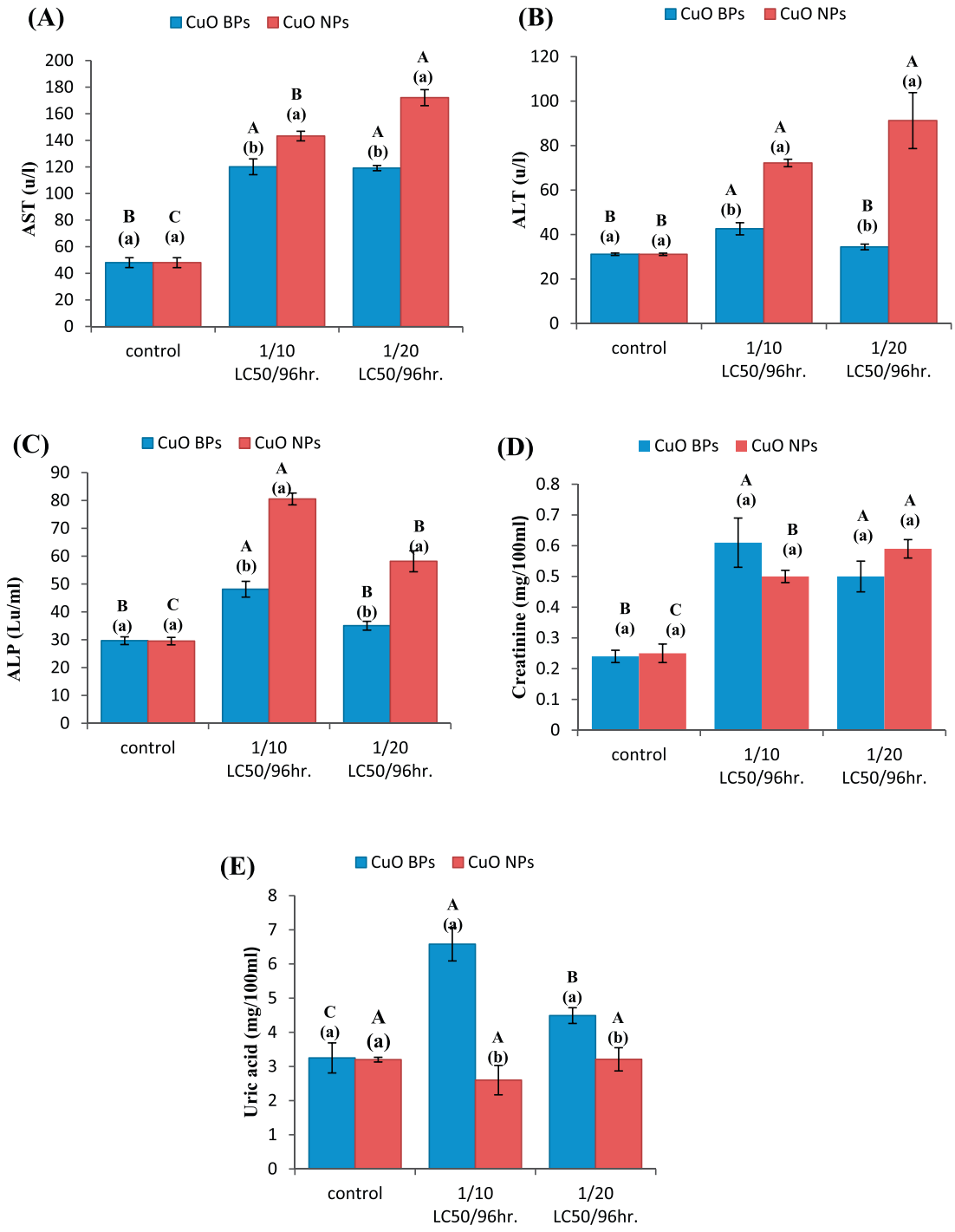

Figure 3 Effect of CuO (BPs & NPs) at different concentrations on serum AST (A), ALT (B), ALP (C), Creatinine (D) and uric acid (E) of Nile Tilapia; *Oreochromis nilotcus* after 30 days. Mean ± SE with the same small letter on the different colored column for each parameter is not significantly different. Mean ± SE with the same capital letter on the same colored column for each parameter is not significantly different.

biomarkers in gill tissues, except GSH when fish groups were exposed to CuO BPs and SOD when fish groups were exposed to CuO NPs.

After 30 days, exposure of Nile Tilapia fish; *O. niloticus* to the both studied concentrations of CuO (BPs & NPs) showed a significant increase in MDA levels in gills, indicating that ($^1/_{20}$) LC_{50}/96 h·CuO NPs had higher effect than ($^1/_{20}$) LC_{50}/96 h·CuO (BPs) in contrast when exposed to ($^1/_{20}$) LC_{50}/96 h·CuO (BPs) had a higher effect. (Fig. 5A).

According GSH content, it showed a significant decrease in gills at ($^1/_{20}$) LC_{50}/96 h of CuO (NPs) when compared with control group without any change when fish were exposed to the chosen concentrations of CuO (BPs) (Fig. 5B).

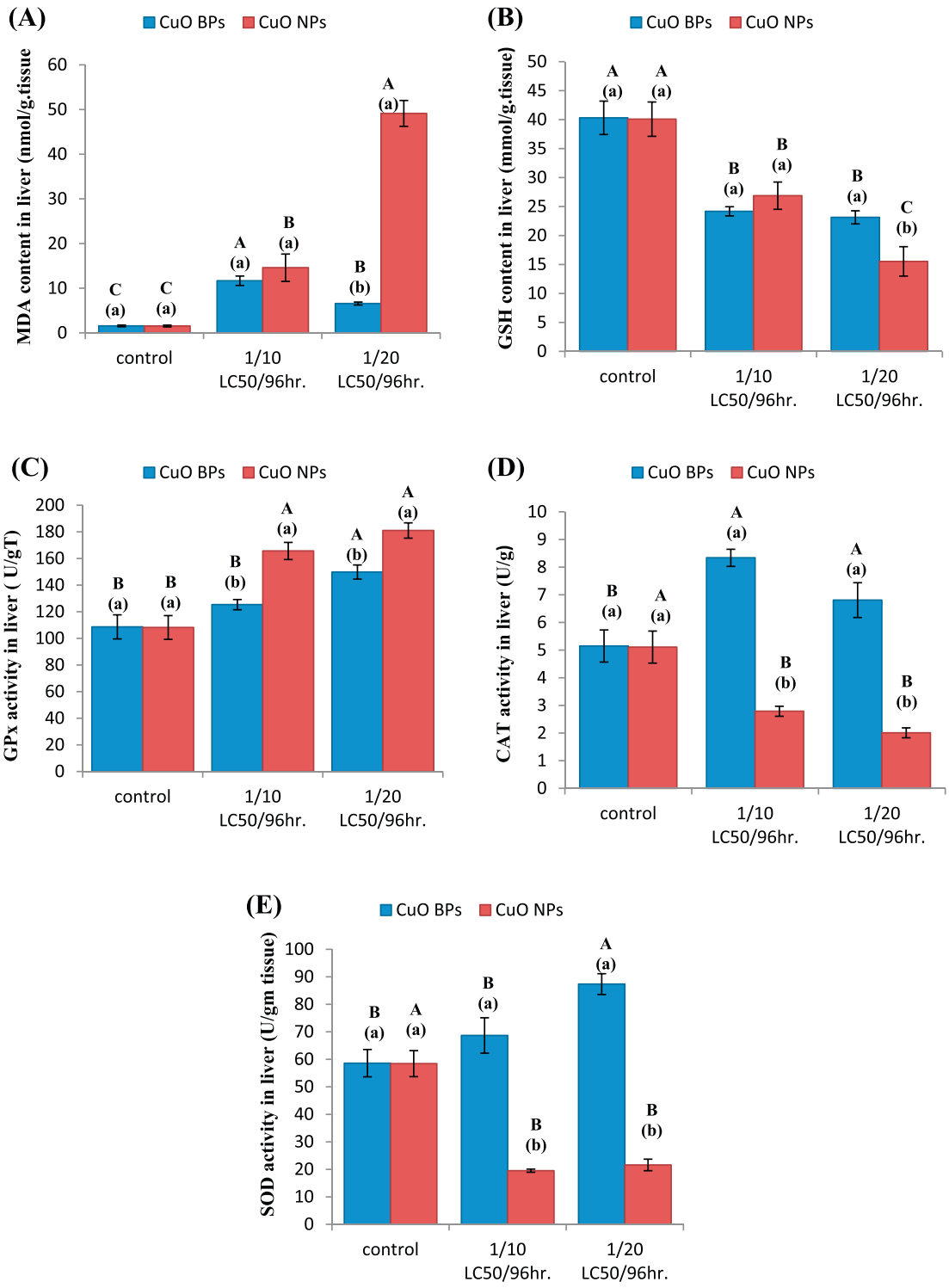

Figure 4 Effect of $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs & NPs) on MDA (A), GSH (B), GPx (C), CAT (D) and SOD (E) in the liver of Nile Tilapia; *Oreochromis nilotcus* after 30 days. Mean ± SE with the same small letter on the different colored column for each parameter is not significantly different. Mean ± SE with the same capital letter on the same colored column for each parameter is not significantly different.

Concerning the effect of ($^{1}/_{10}$ & $^{1}/_{20}$) LC_{50}/96 h of CuO (BPs & NPs) on GPx, CAT and SOD activities, a significant increase was recorded except SOD activity when exposed to two different concentrations of CuO (NPs), all showing that CuO NPs had higher effects at both concentrations than CuO (BPs).(Fig. 4C–E).

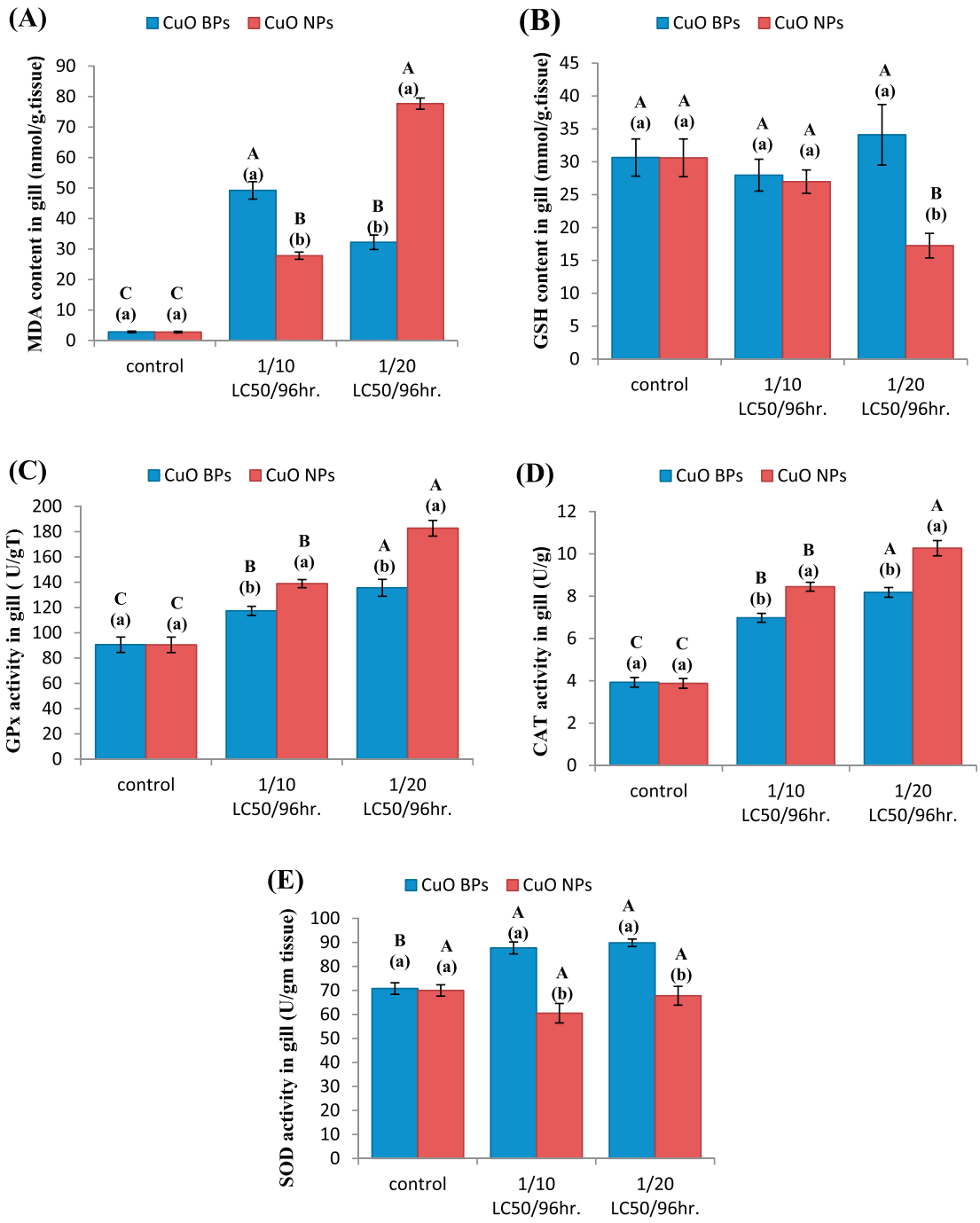

Figure 5 Effect of $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs & NPs) on MDA (A), GSH (B), GPx (C), CAT (D) and SOD (E) in gills of Nile Tilapia; *Oreochromis nilotcus* after 30 days. Mean ± SE with the same small letter on the different colored column for each parameter is not significantly different. Mean ± SE with the same capital letter on the same colored column for each parameter is not significantly different.

Discussion

The contamination of an aquatic resource with a wide range of pollutants has become a matter of concern over the past few decades (Vutukuru, 2005; Yousafzai and Shakoori, 2006; Narayanan and Vinodhini, 2008; Abdel-Khalek, 2015a). Production and use of engineered nanoparticles likely result in their release into aquatic environments and lead to unexpected hazards on aquatic organisms (Peralta-Videa et al., 2011). Health and environmental concerns of ENPs have been highlighted in several reports (Royal Commission on Environmental Pollution, 2008; European Commission, 2009).

In comparison with macro-materials of the same chemical composition, nanomaterials often display unusual chemical

characteristics (Wigginton et al., 2007; Smith et al., 2008; Nel et al., 2009). The TEM imaging in the present study indicated that, the particle size of individual CuO BPs was larger than that of CuO NPs. Rossetto et al. (2014) suggested that, the size is one of the key factors influencing the toxic effects of NPs.

In accord with the findings of Zhao et al. (2011), the results of the present study showed that CuO (BPs & NPs) had an average hydrodynamic diameter of 954 & 588 nm, respectively, which is much larger than the size measured by TEM, and this may be due to CuO aggregation and hydration.

NPs have unique physicochemical properties that are different from their bulk counterparts (Harman et al., 2002; McDonald et al., 2005). So, NPs have a special tendency to exhibit toxic effects and those supported by the studies of Kasemets et al. (2009) who reported that, NPs are more toxic than BPs. The NPs tend to aggregate as the surface charge approaches neutral (Ghosh et al., 2008). The zeta potential of CuO (NPs) is near to zero so, it is more unstable and shows more aggregation than bulk, which is similar to the result of Zhao et al. (2011) and Rossetto et al. (2014). Zhao et al. (2011) reported that, aeration which is used in the toxicity tests, might lead to a decrease in aggregation and sedimentation of NPs to a certain extent.

The obtained LC_{50} values are highly useful in the evaluation of safe levels or tolerance levels of a pollutant (Prentera et al., 2004). The present study showed that the recorded LC_{50}/96 h of the CuO BPs (2205 mg/l) was less toxic to Nile Tilapia; *O. niloticus* in comparison with the recorded LC_{50}/96 h (150 mg/l) of the CuO NPs. The CuO NPs were more toxic than BPs of the same composition, demonstrating that NP biological reactivity depended on both physical properties and chemical composition (Karlsson et al., 2009). This is in agreement with Zhao et al. (2011) who suggested that, CuO NPs had higher toxicity than CuO BPs in juvenile carp (*Cyprinus carpio*).

The evaluation of haematological and biochemical characteristics in fish has become an important health indicator (Saravanan et al., 2011). Blood glucose appeared to be a sensitive and reliable indicator of environmental stress in fish (Nemcsok and Boross, 1982). In the present study, results indicated a significant increase in serum glucose level, when Nile Tilapia; *O. niloticus* were exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of CuO (BPs & NPs) after 30 days. This is in accordance with Zaghloul et al. (2006) who studied the effect of copper toxicity on three fish species: *Clarias gariepinus, O. niloticus* and *Tilapia zillii*. They showed a significant increase in serum glucose in comparison to the control group. Pretto et al. (2014) observed also an increase in the plasma glucose levels after exposure of silver catfish to both Cu concentrations (16 and 29 μg/L), respectively. The alterations in the glucose level might be related to renal injury, liver damage, lack of nutrition and glycogenolysis and synthesis of glucose from extra hepatic tissue proteins and amino acids (Öner et al., 2008). Also, Haliwell (2007) and Wang et al. (2008) suggested that NP exposure may cause overproduction of ROS within the tissue, which can damage carbohydrates.

Lipids, as an important source of energy, play an important role in toelest fish (Shatunovsky, 1971; Harris, 1992; Haggag et al., 1993). The present study reported a significant decrease in serum total lipid when Nile Tilapia; *O. niloticus* were exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of CuO (BPs) and $^{1}/_{10}$ of CuO (NPs) when compared to control groups after 30 days. This is in agreement with Abu-El-Ella (1996), who reported a decrease in serum and muscle total lipids in grass carp; *Ctenopharyngodon idella* exposed to cadmium and attributed this decrease to the great demand of energy to confront this stress. This may also be due to the decrease in insulin levels because insulin has a greater effect on protogenic and lipogenic pathways (El-Naggar et al., 1998). Haliwell (2007) and Wang et al. (2008) suggested that NP exposure may cause overproduction of ROS within the tissue, which can damage lipids.

Measurement of total protein, albumin and globulin, in serum or plasma is of considerable diagnostic value in fish, as it relates to general nutritional status (Schäperclaus et al., 1992). In the present study, results indicated a significant decrease in total protein, albumin and globulin contents when Nile Tilapia; *O. niloticus* were exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of both CuO (BPs & NPs) without any significant difference in albumin in fish exposed to both concentration of CuO BPs after 30 days. These results were similar to those of Zaghloul et al. (2006) who studied the effect of copper toxicity on three fish species: *C. gariepinus, O. niloticus* and *T. zillii*. They showed a significant decrease in serum total proteins in comparison to the control group. Also Öner et al., 2008 found that, total protein concentration decreased significantly in Cu exposed *O. niloticus* fish and the greatest decrease was observed in 30 day Cu-exposed fish. And Pretto et al. (2014) found that, the decreases in the protein levels were by 33% and 43% in case of silver catfish exposed to both Cu concentrations (16 and 29 μg/L), respectively. The decrease in serum protein levels may be value for energy production during pollutant toxicity and/or due to other several pathological processes including renal damage and elimination in urine, decrease in liver protein synthesis, alteration in hepatic blood flow and/or plasma dissolution (Gluth and Hanke, 1985). The decrease in serum total protein may also be due to increased lipolysis (Ghosh and Chatterjee, 1989) and detoxification mechanism during stress (Neff, 1985). Haliwell (2007) and Wang et al. (2008) suggested that depletion in serum total protein after NP exposure may be due to overproduction of ROS within the tissue, which can damage proteins. Also NPs are coated with proteins, resulting in an NP-protein corona (Nel et al., 2009) and this may be the cause of depletion in serum total protein levels.

Serum enzymes such as AST, ALT and ALP could be used as sensitive biomarkers in ecotoxicology, because they provided an early warning of potentially hazardous alterations in contaminated aquatic organisms (Vaglio and Landriscina, 1999; Levesque et al., 2002; Nel et al., 2009). The results in the present study, indicated a significant increase in serum enzyme (AST, ALT and ALP) activities, when Nile Tilapia; *O. niloticus* were exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of CuO (BPs & NPs) after 30 days. These results were in agreement with Zaghloul et al. (2006) who studied the effect of copper toxicity on three fish species: *C. gariepinus, O. niloticus* and *T. zillii*. They showed a significant increase in serum enzyme (AST, ALT and ALP) activities in comparison to the control group. Öner et al., 2008 also reported that, continuous exposure of *O. niloticus* to sublethal (0.05 mg/l copper) concentration resulted in a significant elevated level of both AST and ALT activities. Again, Heydarnejad et al. (2013) reported increase in the ALP activity followed by Cu-exposure in 30 days. Wu et al. (2003) recorded an increase of AST and ALT activities in stressed juvenile areolate grouper

(*Epinephelus areolatus*) and this is maybe due to hepatic cell injury or increased synthesis of the enzymes by the liver. Changes in the ALP activity also could be due to the result of physiological and functional alterations in metal exposed fish (Jiraungkoorskul et al., 2003). Increase in AST, ALT and ALP activities in the present investigation could be due to a variety of conditions, including muscle damage, intestinal and hepato-pancreatic injury, and toxic hepatitis (Farkas et al., 2004). The significant increase in liver enzymes (AST, ALT & ALP) is confirmed by the histopathological examination of the liver in the present study (unpublished data), which showed clear damage in the liver tissue.

Serum creatinine and uric acid can be used as a rough index of the glomerular filtration rate and kidney dysfunction (Maita et al., 1984). In the present study, while serum creatinine showed a highly significant increase in Nile Tilapia; *O. niloticus* after exposure to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of CuO (BPs & NPs), uric acid showed a highly significant increase after exposure to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h of (BPs) only after 30 days. Zaghloul et al. (2006) studied the effect of copper toxicity on three fish species: *C. gariepinus, O. niloticus* and *T. zillii*. They showed a significant increase in levels of serum creatinine and uric acid in comparison to the control group. This elevation may relate to kidney dysfunction. The significant increase in levels of serum creatinine and uric acid is confirmed by the histopathological examination of kidney in the present study (unpublished data), which showed clear damage in the kidney tissue.

Oxidative stress is a convenient parameter to measure toxicity and ecotoxicity, because cells respond to oxidative stress by mounting a number of protective responses that can be easily measured as altered enzymatic or genetic expression (Kovochich et al., 2007; Abdel-Khalek, 2015b). It has been demonstrated that the presence of some metals (such as Cu and Fe) in biological systems can significantly increase the levels of oxidative stress (Pinto et al., 2003; Buzea et al., 2007). Also oxidative stress has been proposed as a common mechanism of cell damage induced by many types of NPs (Stone et al., 2007).

The measurement of MDA content (an index of LPO) provides a relative measure of the potential for pollutants to cause oxidative injury (Vlahogianni et al., 2007). LPO is one of the main processes induced by oxidative stress (Mela et al., 2013). It has been generally accepted that, active oxygen produced under stress is a detrimental factor, which causes lipid peroxidation and enzyme inactivation (Valko et al., 2004). In the present study, MDA contents in the liver and gill tissues of Nile Tilapia; *O. niloticus* exposed to both concentrations $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs & NPs) showed a significant increase when compared with control groups after 30 days. Metwally (1998) demonstrated that, MDA concentration in serum and tissues of Nile Tilapia and catfish increased with induction of heavy metal toxicity. Moreover, Roméo et al. (2000) reported that, cadmium and copper induced rises in MDA levels. LPO induced by CuO NPs was also reported in other biological models, indicating that oxidative stress is a common pathway for CuO NP toxicological effect (Barata et al., 2005; Wang et al., 2008; Fahmy and Cormier, 2009; Ahamed et al., 2010; Ghosh et al., 2010; Premanathan et al., 2011). The metabolism of heavy metals results in the formation of ROS which is known to extract hydrogen atom from unsaturated bonds thereby altering lipid structure or function (Grune et al., 2004). Also, Farombi et al. (2007) and Padmini and Rani (2009) concluded that, accumulation of heavy metals in high concentration in the liver and gills of fish induced lipid peroxidation. The increased MDA level suggests enhanced lipid peroxidation leading to tissue damage and the failure of antioxidant defense mechanisms to prevent formation of excessive free radicals (Kim et al., 2010).

GSH plays an important role in non-enzymatic antioxidant system, since it acts as a reductant in conjugation with xenobiotics (Kanak et al., 2014). In the present study, GSH contents in the liver and gill tissues of Nile Tilapia; *O. niloticus* exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs & NPs) showed a significant decrease, except those exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs) in the case of gill tissues, when compared with the control groups after 30 days. These results are similar to Xiong et al. (2011), who stated that ZnO NPs and a bulk ZnO suspension caused a decrease in GSH content in the liver tissue of zebra fish compared to controls. Redox active metal ions such as Cu (II) readily catalyze the oxidation of GSH giving rise to thiol and hydroxyl radicals (Stohs and Bagchi, 1995). This depletion can be a result of an increased binding of Cu (stabilization of Cu in oxidative state), an enhanced use of GSH's oxidizing ability (conversion into GSSG, the oxidized form of glutathione) or an ineffective GSH regeneration (Pandey et al., 2001; Parvez et al., 2003; Ahmad et al., 2005; Parvez and Raisuddin, 2006). This depletion of GSH level reduces the cellular availability to scavenge free radicals and can lead to more oxidative stress-related damage (Elia et al., 2003). GSH depletion can be associated with increased lipid peroxidation (Radu et al., 2010; Jozefczak et al., 2012). GSH depletion could probably be caused also by a significant dissolution of metal oxide NPs that released metal ions in the media (Jozefczak et al., 2012).

Aquatic organisms have developed defenses to protect against ROS-induced damage including antioxidant enzymes such as GPx, CAT and SOD (Dautremepuits et al., 2004; Eyckmans et al., 2011). GPx has a crucial role in intracellular protection against toxic compounds such as Cu and Zn (Anderson, 1997; Anderson and Luo, 1998; Mosleh et al., 2005). GPx is responsible for enzymatic defense against hydrogen peroxide (H_2O_2), and is strictly linked with the concentration of GSH, because it catalyzes the reaction between glutathione and (H_2O_2), resulting in the formation of glutathione disulfide (GSSG) (Paglia and Valentine, 1967; Alkaladi et al., 2013). In the present result, the GPx activity in the liver and gill tissues of Nile Tilapia; *O. niloticus* exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs & NPs) showed a significant increase when compared with control after 30 days. Orun et al. (2008) found a significant alteration in the GPx activity in the tissues of *Onchorhynchus mykiss* fish, after Cd and Cr exposures. Fahmy and Cormier (2009) reported that, CuO NPs were better able to increase the activity of GPx.

CAT and SOD have been classified as antioxidant systems of defense in various aquatic species (Almeida et al., 2007). In the present investigation, while CAT and SOD activities showed a significant increase in the case of liver tissues of Nile Tilapia; *O. niloticus* exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (BPs), they showed a significant increase in the case gill tissues, when fish were exposed to both concentrations of CuO (BPs & NPs) when compared with matched controls. Bainy et al. (1996) found an increase in the (SOD) activity after

exposure of Nile Tilapia to different pollutants. Basha and Rani (2003) stated that liver CAT and SOD activities increased in *Oreochromis mossambicus* after Cd exposures. An increase in the CAT activity was also recorded in different fish species after metal exposures (Dautremepuits et al., 2004; Sanchez et al., 2005; Atli et al., 2006). The liver CAT activity of piava (*Leporinus obtusidens*) exposed to 20 or 40 μg/L Cu (Gioda et al., 2007) and gills of common carp exposed to 60 μg/L Cu (Eyckmans et al., 2011) increased significantly.

In the present study, CAT and SOD activities recorded a significant inhibition in the case of liver tissues of the fish exposed to $^{1}/_{10}$ & $^{1}/_{20}$ LC_{50}/96 h·CuO (NPs). Pruell and Engelhardt (1980) reported that Cd-induced decrease in the CAT activity in the mangrove killifish (*Fundulus heteroclitus*). Cozzari et al. (2015) reported that Ag NP and bulk Ag particle exposure causes consistent decreases in both SOD and CAT activities particularly at the higher exposure concentrations. This inhibition may arise due to imbalance in ROS formation and the antioxidant defense system of the cells (Liu et al., 2012). Also, at higher concentrations, chemicals may directly inhibit the activity of enzymes, or indirectly reduce the concentration of the enzymes by damaging cell organs (Brown et al., 2004; Jemec et al., 2007). The reduction of the CAT activity may also result from the accumulation of H_2O_2 and other oxyradicals (Choi et al., 2010). Increased H_2O_2 levels resulting from CAT inhibition could ultimately further inhibit the SOD activity (Kono and Fridovich, 1982).

Results indicated that, CuO (NPs) have more toxic effect than CuO (BPs) in liver and gill tissues in most oxidative stress parameters. Hu et al. (2014) suggested that smaller-sized NPs caused more oxidative stress than larger particles of similar composition. They confirmed that, the NPs were internalized into the tissues of the blue mussel; *Mytilus edulis*. (NPs) can produce ROS as a consequence of their disproportionately large surface area compared to the bulk materials (Stoeger et al., 2006). The oxidative stress induced by metal oxide NPs was studied by exposing zebra fish and cells to NPs, the results showed that the quantities of ˙OH in metal oxide NPs' suspensions were much higher than in bulk formulations (Chang et al., 2012). Also, most of the results of biochemical determination in the present study showed CuO NPs had more toxic effect than BPs. The unique physical properties of NPs are mainly attributed to their high surface to volume ratio, with a large proportion of the atoms being exposed on the surface compared to the bulk material (Poole and Owens, 2003). While in bulk materials, the surface atoms constitute only a few percent of the total number of atoms, in NPs most of the atoms lay close to or at the surface (Casals et al., 2012). The toxicity of CuO NPs is likely explained by the combination of high surface reactivity and large surface area, thus constituting a ''double hazard'' as has been discussed regarding inflammatory potential (Duffin et al., 2007). The known effects of CuO NPs, in contrast to its bulk and ionic forms, are contradictory regarding the highest toxic form of Cu; nevertheless, evidence exists of different mechanisms of action dependent on the Cu form (Griffitt et al., 2007; Karlsson et al., 2008; Gomes et al., 2011). Therefore, CuO potential toxicity should not be ignored (Blinova et al., 2010; Saison et al., 2010; Buffet et al., 2011). Also the results indicate the difference in effects between the two selected concentrations ($^{1}/_{10}$ & $^{1}/_{20}$) of CuO (BPs & NPs) and these may be due to the aggregation of particles in water.

Conclusions

The results of this study represent the evaluation of CuO (BPs & NPs) toxicity to Nile Tilapia; *O. niloticus*. It can be concluded that: (1) the LC_{50}/96 h of CuO BPs was higher than that of nano indicating that CuO NPs are more toxic; (2) CuO NPs could cause more toxic effects and still smaller than CuO BPs despite that they formed aggregates in suspensions; (3) the present data also demonstrate that CuO (BPs & NPs) induce biochemical alterations and oxidative stress to *O. niloticus*, which may suggest ecological implications of CuO (BPs & NPs) release in aquatic ecosystems indicating that CuO NPs with different concentrations had more toxic effects in most of the determinations in this study. Also this study is helpful to understand and make comparisons between CuO BP and NP toxicity to aquatic organisms but those in laboratory, so further studies are required to assess the current environmental burden of NPs in aquatic ecosystems to determine, monitor and/or regulate the use and release of CuO NPs.

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