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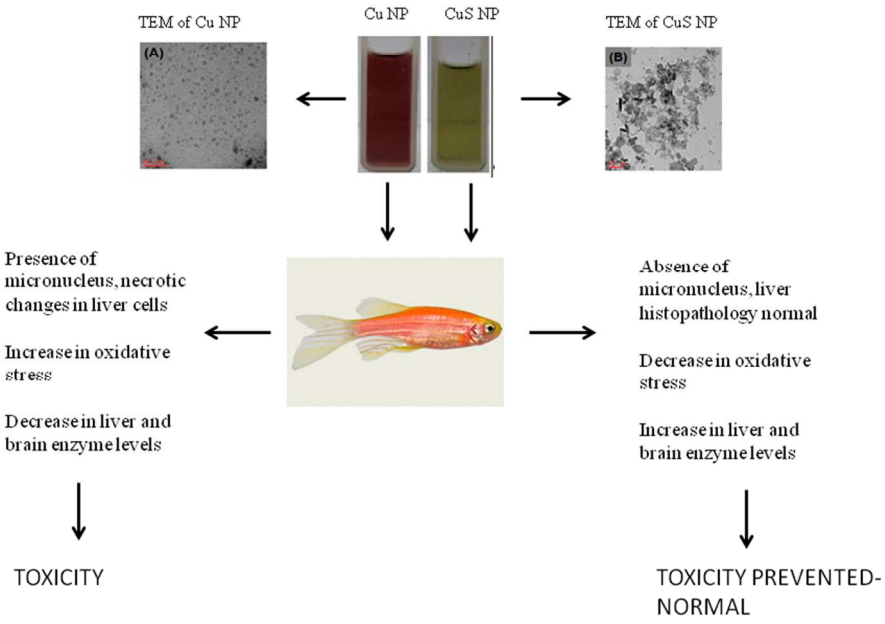

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Sulfidation Modulates Toxicity of Biogenic Copper Nanoparticles

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Abstract

Nanotoxicology, is an important field that has started to unravel the adverse effects of nanoparticles. Major emphasis thus is being laid on developing strategies that reduce toxicity of the nanomaterial. Chemical transformations of metal nanoparticles are suggested to be an important way to mitigate nanoparticle toxicity. In the present study, we investigated the toxicity of copper and copper sulfide nanoparticles in a zebrafish model by using a series of biomarkers of toxicity. Exposure of zebrafish to copper nanoparticles enhanced liver oxidative stress, altered detoxification enzymes and affected brain acetylcholinesterase activity. However, exposure of zebrafish to sulfidated copper nanoparticles rescued these parameters. Histopathological analyses of liver and metallothionein levels also support the significance of sulfidation as a potential mechanism for controlling copper nanoparticle toxicity. More importantly, micronucleus formation was shown to be highly reduced in liver of fish that were exposed to sulfidated copper nanoparticle when compared to non-sulfidated nanoparticle. The presented biochemical data provides strong evidence for the reduction in copper nanoparticles toxicity when it undergoes chemical transformation as copper sulfide.

Keywords: copper; nanoparticle; zebrafish; sulfidation; toxicity; pollution

1. Introduction

In the current scenario, nanoparticles are beginning to influence diverse fields including material science, catalysis, sensors, bioanalytics, medicine and so forth.[1-3] At the same time, - there is an extensive debate about the risks and benefits of the many manufactured nanomaterials .[4-5] The widespread use of engineered nanomaterials in consumer products could have serious consequences when it is released into the environment, where their fate and behavior are largely unknown.[6-7] Nanotoxicology, thus becomes an important area of research to evaluate the adverse effects of nanoparticles on the ecosystem and on human health.[8-9]

Among the coinage metal nanoparticles, copper nanoparticles (CuNPs) have attracted researchers due to their easy availability. CuNPs have wide applications in heat transfer systems,[10] electron conduction slurry,[11] sensors[12,13] and catalysts[14-16] and have also been suggested to be good replacements for the more expensive silver and gold nanoparticles in nanotechnology applications. Results from *in vitro* studies show that CuNPs exhibit good antibacterial activity and are suitable for biomedical applications.[17,18] At the same time, copper is a redox active essential trace element, whose transport into cells is tightly regulated. Recent studies have shown that CuNPs exhibit higher toxicity upon exposure to human lung cell line A549 and macrophage cell line THP-1, when compared to other metal nanoparticles.[19] Karlsson et al. have demonstrated that the CuNPs induce toxicity by damaging cell membranes.[20] Moreover, owing to their 'nanosize' NPs can be easily transported into cells, which may increase their intracellular concentration and toxicity.[21,22] Cells exposed to copper oxide NPs show signs of formation of intracellular reactive oxygen species and oxidative DNA lesions.[23-25] Therefore, we should exercise caution while utilizing NPs for biomedical applications. However, the widespread and unregulated use of CuNPs in other applications could create an immediate risk of

human exposure. In spite of the great developments in understanding the toxicity of metal-based nanomaterials, their general mechanism of toxicity, however, remains enigmatic.

Of late, green synthesis of metal NPs, that employs biomolecules and organisms, such as bacteria, fungi, proteins, biopolymers and plant extracts, has been receiving widespread attention in order to reduce their potential toxicity.[26-29] Nevertheless, both biogenic NPs and NPs prepared using synthetic materials exhibit good antibacterial activity. Any unwanted exposure to such an antibacterial agent, thus may produce adverse effect to non-target organisms. To the best of our knowledge, this is the first in vivo report to investigate the toxicity of biogenic CuNPs employing adult zebrafish. Zebrafish is an important model organism in biomedical research, which shares many common biological pathways with humans and is 80% genetically identical to humans. In this paper, we report the synthesis of biogenic CuNPs and copper sulfide (CuS) NPs using guar gum as capping agent and hydrazine as reducing agent. Guar gum is a biocompatible polysaccharide composed of galactomannan. Metal sulfidation has been suggested to be a natural method to minimize the toxicity of silver NPs.[30] To test this concept on CuNPs, we assessed the toxicity of CuNPs and CuS NPs in adult zebrafish employing various complementary biochemical assays that are routinely used in toxicological studies. Our results show that CuNPs enhances liver oxidative stress, alters liver detoxification enzymes, affects brain acetylcholinesterase activity and triggers micronucleus formation in liver; interestingly sulfidation of CuNPs resulted in significant rescue of all the biomarkers studied. In addition, histopathological analyses of liver and metallothionein levels also support the significance of sulfidation is an important strategy for reducing biological toxicity of nanomaterials.

2. RESULTS:

2.1. Preparation of copper and copper sulphide nanoparticles:

CuNPs were synthesized from copper chloride using guar gum as a stabilizer and hydrazine as a reducing agent. The color of the freshly prepared CuNPs was reddish brown – the characteristic color of CuNPs, which is consistent with previous reports.[31] The UV–Vis spectra of the CuNPs showed a characteristic surface plasmon resonance (SPR) band at 566 nm (Fig. 1). CuS NPs was synthesized from CuNPs using sodium sulphide as described in 'Materials and Methods'. The formation of CuS NPs was accompanied by color changes from reddish brown to green. The complete elimination of the CuNPs SPR peak at 566 nm clearly confirms the complete conversion of Cu to CuS NPs (Fig. 1). The absence of absorption peak at 800 nm clearly suggests that the formed CuNPs and CuS NPs are free from oxide form of copper.

The TEM images as shown in Fig. 2A, indicated that CuNPs are spherical in nature with a size of about 4 nm. However, upon sulfidation CuNPs undergo structural transformation and exhibit highly diversified shapes like spherical, rod-like, prism, triangular, pentagonal and hexagonal pattern (Fig. 2B). Presence of these features may be attributed to the formation of different aggregation states in stabilization of CuS NPs by guar gum. FTIR has been widely used to identify the possible interaction between the metal NPs and the stabilizing agent. The FTIR spectra of guar gum, guar gum stabilized CuNPs and guar gum stabilized CuS NPs are shown in Fig. 2C. The main characteristic peaks of guar gum at 1414 cm^{-1} (C-O stretch), (1643 cm^{-1} (C=O stretch), 2924 cm^{-1} (C-H stretch) and broad peak at 3436 cm^{-1} (O-H stretch) were observed. Both CuNPs and CuS NPs show all the peaks of guar gum, albeit significant shift was observed in the carbonyl stretching frequency region. The shifting of absorption frequencies clearly indicates the involvement of carbonyl group in stabilizing the nanostructures of CuNPs and CuS NPs. It is

also clear from HR-TEM images that the formed nanoparticles are isotropic (i.e., low aspect ratio) in shape. These results support the formation of NPs inside the nanoscopic polysaccharide templates. Thus, we propose that the electrostatic attractive forces between guar gum, hydroxyl and carbonyl group and copper ion in solution provide an effective driving force for the formation and stabilization of the CuNPs and CuS NPs.

2.2. Toxicity evaluation

The size of both CuNPs and CuS nanoparticles NPs were found to be about 4 nm (Fig. 2 a & b). For toxicity evaluation, the two types of nanoparticles were initially tested at three different concentrations - 1 ppm, 5 ppm, 10 ppm. However, exposure of fishes to all the three concentrations produced immediate mortality (data not shown); the fish were observed to breath with difficulty and sank down to the bottom of the tank, soon after introduction into the water containing CuNPs at these three concentrations. Subsequently, we selected lower concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 ppm and evaluated their toxicity. As shown in Table 1, CuNPs at 0.1 and 0.5 ppm produced mortality within a couple of hours after the start of the exposure, whereas in 0.05 ppm and 0.01 ppm the fish were able to survive for 1 day and 2 days, respectively. In the case of 0.005 ppm and 0.001 ppm the survival of fishes was better but still the fish were not able to survive beyond 7 days. Interestingly, sulfidation of Cu nanoparticles resulted in significant reduction in mortality for all the concentrations (Table 1). The best effect was seen with CuS NPs at 0.01 ppm, wherein, the fish were observed to survive for a maximum of 5 days when compared to 0.01 ppm CuNPs (48 hr), and hence we selected 0.01 ppm and 48 hr of exposure for both nanoparticles for all further analyses. We would like to mention that even in the case of sulfidation mortality was not completely reduced and even at the lowest concentration

tested (0.001 ppm) the fish were able to survive for just 8 days. However, these results suggest that sulfidation does significantly reduce toxicity of CuNPs, however the effect is not prolonged and this we speculate could be due to the extent of sulfidation. In addition we also exposed 10 fishes separately to guar gum (1 mg/ml in tap water) alone for ten days to check for its effect on fish survival. However, all ten fishes were alive without any observable changes (data not shown).

Histopathological observations (Figure 3) also supported our contention on the ability of sulfidation of CuNPs in mitigating its toxicity. Fish exposed to CuNPs (Figure 3b) showed extensive necrosis, degenerative changes and cell loss in liver, suggesting the toxicity of CuNPs. However, the liver from fish exposed to CuS NPs (Figure 3c) appeared normal and there were no adverse changes as observed upon CuNPs exposure. The histological feature of liver exposed to CuS NPs appeared similar to that of control fish (Figure 3a). These results show that sulfidation reduces CuNP toxicity in zebrafish.

2.3. Effect of CuNPs sulfidation on liver oxidative stress:

CuNPs was shown to be toxic to the zebrafish. To understand the mechanism of CuNPs toxicity we analyzed oxidative stress in the liver of zebrafish. As shown in Table 2, exposure of zebrafish to 0.01 ppm CuNPs for 48 h led to significant enhancement in nitric oxide generation in the liver of group II zebrafish (19.94±0.007, $p<0.05$), when compared to group I (14.87±0.006). This suggests that CuNPs increases oxidative stress by stimulating nitric oxide generation. However, when fish were exposed to sulphidated CuNPs, there was a significant reduction in nitric oxide generation (group III, 15.87±0.003, $p<0.05$) when compared to group II fishes.

The activities of liver antioxidants were also found to be decreased due to CuNPs toxicity. As can be seen from Table 2, liver SOD activity from group II fishes were found to be significantly reduced (0.742±0.02, $p<0.05$) due to exposure to CuNPs for 48 h. Similarly, the activity GSH (0.162±0.008, $p<0.05$) were also observed to be inhibited due to CuNPs exposure, when compared to control (group I) fishes. By contrast sulfidation of CuNPs resulted in a significant increase ($p<0.05$) in the activities of liver SOD (0.983±0.06), and GSH (0.253±0.006) and the values obtained were almost comparable with the respective controls.

Taken together, these results suggest that CuNPs exposure results in oxidative stress that could contribute to its toxicity. However, sulfidation of CuNPs significantly reduces its toxicity, as seen by reduction in tissue oxidative stress.

2.4. Effect of CuNPs sulfidation on liver carboxylesterases:

Since liver is considered to be the primary organ for xenobiotic detoxification, we analysed the effect of CuNPs exposure on liver carboxylesterases. As can be seen from Figure 4, exposure of fish to 0.01 ppm CuNPs for 48 hr resulted in a significant inhibition of α-carboxylesterases and not β-carboxylesterases. Though β-carboxylesterase activity showed a decrease in group II animals, the decrease was not significant. On the other hand α-carboxylesterase activity was significantly reduced in group II animals (49.14±2.86, $p<0.05$) that were exposed to CuNPs. However, liver α-carboxylesterase from group III fishes that were exposed to sulphidated CuNPs showed rescue in activity (65.72±5.99, $p<0.05$) when compared to group II animals . These results suggest that α-carboxylesterases are preferentially activated as a response to CuNPs toxicity and these enzymes could possibly play an important role in the detoxification response against CuNPs exposure.

2.5. Effect of CuNPs sulfidation on neuronal AChE:

In order to understand the effect of sulfidated CuNPs on neuronal functions, we tested brain AChE activity in fish exposed to CuNPs. As can be seen from the Figure 5, AChE activity in brain of CuNPs exposed fishes were significantly decreased (0.451±0.04, $p<0.05$) when compared to control (0.566±0.06). This suggests the ability of CuNPs in altering neuronal functions. However, when fish were exposed to sulfidated CuNPs, there was a small increase in brain AChE activity (0.478 ±0.07, $p<0.05$) when compared to group II fishes. These results show that sulfidation of CuNPs was able to protect zebrafish to some extent against neuronal toxicity due to CuNPs exposure.

2.6. Effect of CuNPs sulfidation on metallothionein levels:

Metallothioneins are metal binding proteins and their expression is induced by heavy metal exposure. In order to understand the role of metallothionein induction during CuNPs exposure, we analysed metallothionein in liver of zebrafish after 12 h and 24 h of CuNPs exposure. As can be seen from Figure 6, exposure of fish to CuNPs resulted in enhancement in metallothionein after 12 h (0.035±0.008) and 24 h (0.039±0.004, $p<0.05$). This suggests the ability of CuNPs in inducing metallothionein expression which could be a result of CuNPs toxicity. Interestingly, exposure of fish to sulfidated CuNPs resulted in significant reduction in metallothionein level after 12 h (0.031±0.003, $p<0.05$) and 24 h (0.036±0.008, $p<0.05$) of exposure. Though, the levels for 24 h were higher than the corresponding control, the values were nevertheless lower than CuNPs exposed fish. Taken together, these results suggest that metallothionein induction due to CuNPs exposure could be a response of fish to nanoparticle toxicity. Reduction in

metallothionein levels in group III fishes suggests the ability of sulfidation in reducing CuNPs toxicity.

2.7. Effect of CuNPs sulfidation on micronucleus:

Micronucleus formation is indicative of genotoxic stress that could be related to xenobiotic exposure. In the present study, liver samples from fish exposed to CuNPs showed extensive micronucleus formation (Figure 7b) as compared to normal cells observed in control fish. Increase in micronucleus formation in group II fishes suggests that CuNPs can easily reach nucleus of cells and cause damage to chromatin structure. This indicates onset of genotoxic stress suggesting the ability of CuNPs to cross into nucleus and affect chromatin structure. Micronucleus formation was completely absent from the liver of fish exposed to CuS NPs. This suggests that sulfidation of CuNPs (Figure 7c) probably protected liver cells against genotoxicity of CuNPs which resulted in normal appearance of liver of these fishes. These results strongly supports that sulfidation could play an important role in preventing nanoparticle induced chromatin damage in zebrafish.

3. Discussion

To check the role of sulfidation in reducing CuNPs toxicity, adult zebrafish were exposed to CuNPs and CuS NP at concentration ranging from 0.001 ppm to 10 ppm. Based on duration of exposure and mortality we found that sulfidation was able to reduce the toxicity of CuNPs by increasing the survival of zebrafish. Though the difference was just a few hours to days, for all the concentrations tested, these results clearly showed that sulfidation of CuNPs was able to significantly reduce its toxicity.[32] Histopathological analyses of liver samples from fish exposed to CuNP or CuS NPs support our initial toxicity findings on the ability of sulfidation of CuNPs in mitigating nanoparticle toxicity.

$CuCl_2$ and CuS are cytotoxic and this has been previously reported.[33,34] This toxicity is primarily attributed to free Cu ion that could be generated in vivo. However, Li et al.[33] have convincingly shown that when compared to CuCl2, CuS is less toxic. When concentration was increased to 1mM, there was a tremendous reduction in cell viability for CuCl2 compared to CuS at the same concentration. For CuCl2 at 1mM, the cell viability almost reached zero. This suggests that even though Cu and CuS are toxic, CuS is relatively less toxic and this toxicity is dependent on concentration. At lower concentration (0.001μM to 10μM) Cu and CuS show minimal toxicity.[33] On similar lines, the study by Lakshmanan et al.,[34] also show the toxicity of Au/CuS nanocomposites in control to be minimal when used for photothermal therapy (PTT). However, when PTT was used, there was significant toxicity, which could be attributed to the PTT activation of nanoparticles. Nevertheless, it should be noted that even though CuS exhibits some degree of toxicity, the concentration reached in vivo could prove to be important. Moreover, the above mentioned two studies also lend support to our study that sulphidation could reduce CuNP toxicity in biological systems.

Till now, most studies on nanotoxicology have laid emphasis on toxicity to ecosystem and its inhabitants. However, current focus is on understanding the possible transformations of nanomaterials in nature.[35] This is because in environment nanoparticles can be easily transformed by a variety of means that can have a profound impact on the basic characteristics of the nanomaterial as well as its toxicity. It is also important since such transformations can actually offer potential mechanisms for reducing nanoparticle toxicity.

In the present study we tested the effect of sulfidation of CuNPs on oxidative stress levels in the liver of zebrafish. Our results showed that exposure of zebrafish to 0.01 ppm CuNPs for 48 h resulted in enhanced oxidative stress[32,36-38] as observed with increase in nitric oxide and decrease in liver antioxidants such as SOD, and GSH. This shows that CuNPs stimulated oxidative stress is one of the major mechanisms of its toxicity.[32] It has been reported that in copper containing systems, free radicals can be produced from particle surfaces or by dissolved copper itself through mechanisms similar to Fenton chemistry.[32,36,38-40] This would be one of the direct ways by which CuNPs can produce biological toxicity. However, exposure of zebrafish to sulphidated CuNPs resulted in reduction in nitric oxide generation and enhanced activities of SOD, and GSH in the liver of zebrafish. This suggests that sulfidation of CuNPs probably reduces or masks CuNPs in biological systems that results in lowering of its toxicity.[32] This reduction in oxidative stress can also be advantageous in terms of increased survival seen with zebrafish exposed to CuS NPs. When compared to silver, CuNPs get oxidized more readily forming Cu(II) oxide.[41] Any transformation of CuNP either in the environment or biological system will be of the oxide forms of CuNP.[41,42] Nevertheless, dissolution of CuNP leading to the release of free copper could be a major mechanism of its toxicity.[42-44] Moreover, free Cu is highly redox active and is highly redox active and is quite capable of producing hydroxyl radical

through Fenton reaction involving hydrogen peroxide. Thus, it appears that in biological systems exposure to CuNP will result in oxidative stress by reaction of free Cu with biologically produced hydrogen peroxide.[32] In contrast the study by Fahmy and Cormier[45] has suggested that CuO by itself could have contributed to oxidative stress without the involvement of released Cu^{2+} ions. Some of the proposed mechanisms by which copper nanoparticles could generate oxidative stress include; generation of free radicals in CuO surface and diffusion across cell membrane,[46] oxidation of membrane lipids resulting in release of cytotoxic 8-isoprotane into the cytosol,[47] or induction of signaling network that can result in oxidative stress and apoptosis.[48] All these effects can result in loss of protective antioxidant response, proinflammatory changes (especially redox regulating signaling molecules and transcription factors) and widespread cellular damage. As far as nitric oxide generation is concerned, studies have shown that recruitment of inflammatory cells such as macrophage by nanoparticles to be the major source of nitric oxide.[49] This enhanced nitric oxide generation could be attributed to the activation of inducible nitric oxide synthase enzymes in these cells. Similar observation was also reported by Castranova et al.[50] and Carter and Driscoll.[51] Furthermore, Dyachenko et al.[52] have demonstrated that silica nanoparticles can stimulate endothelial nitric oxide generation by activating stretch-sensitive calcium channels. Thus, it appears that nanoparticles induced nitric oxide generation could be either due to proinflammatory changes or cellular responses to particle interaction.

Carboxylesterases are important components of the liver detoxification system and belong to the α/β-hydrolase family. These enzymes are activated as a response to xenobiotic stress[53] and are responsible for the cleavage of esters. In the present study exposure of zebrafish to CuNPs resulted in selective reduction in the activity of liver α-carboxylesterase but not β-carboxylesterase. This indicates that α-carboxylesterase are selectively inhibited due to CuNPs

exposure. In addition, it can also be speculated that reduction in liver α-carboxylesterase activity could represent turnover of the enzyme due to xenobiotic metabolism or this reduction indicates loss of enzyme into the extracellular fluid possibly due to liver damage induced by CuNPs exposure. Previous studies employing xenobiotics also have shown inhibition in carboxylesterase activity in zebrafish embryo.[54] In the present study, exposure of zebrafish to sulfidated CuNPs produced a slight increase in liver α-carboxylesterase activity, when compared to zebrafish exposed to CuNPs alone. This shows that sulfidation of CuNPs protected zebrafish from CuNPs toxicity, probably by delaying CuNPs release or exposure inside the zebrafish liver.

Similar to liver carboxylesterase, exposure of zebrafish to CuNPs resulted in neuronal toxicity, as shown by significant reduction in brain AChE activity. This suggests that CuNPs can cross blood-brain barrier and can affect brain functions too. Indeed, studies employing pesticides and insecticides have shown neurotoxicity in zebrafish with respect to decrease in brain AChE activity.[54-56] However, brain AChE activity was found to be rescued in the case of zebrafish exposed to sulfidated CuNPs. These results suggest that, CuNPs induced generation of free radical, and the resultant oxidative stress, can affect vital enzymes, viz., liver carboxylesterases and brain AChE. Inhibition of these can result in alterations in general physiological responses and thus increased lethality. On the other hand, sulfidation of CuNPs can reduce Cu exposure to biological systems, thus preventing or reducing oxidative stress, which can prove beneficial in terms of rescue of metabolic enzymes. We would like to point out that cholinesterase and carboxylesterases are well recognized biomarkers for assessing xenobiotic toxicity[52-54] and results of our study also suggests that these enzymes can also be used for assessing nanoparticle toxicity.

Metallothionein, metal-binding proteins, are well recognized biomarkers for heavy metal pollution affecting biological systems[57] and their levels are routinely monitored in toxicological studies.[58] In addition, studies employing xenobiotics, but not metals, too have analysed metallothionein expression.[59] This suggests that metallothionein are an important and critical indicator of environmental pollution. Metal based nanoparticles also have been shown to induce metallothionein activity and/or expression indicating the potential use of metallothionein as a biomarker for nanoparticle toxicity.[60] In the present study exposure of zebrafish to CuNPs resulted in significant increase in liver metallothionein expression after 12 h and 24 h of exposure. This suggests that metallothionein expression was enhanced as a host response to CuNPs toxicity, probably for inhibiting exposure of free Cu ions to tissues. On the other hand, liver metallothionein expression was found to be significantly reduced in zebrafish exposed to sulfidated CuNPs. This suggests that sulfidation of CuNPs could have resulted in a lower and/or reduced release of Cu in zebrafish, which could have resulted in lower CuNPs toxicity. Thus, sulfidation of CuNPs appears to be beneficial in terms of reduced metallothionein expression.

Micronucleus formation is indicative of genotoxicity[61] and this can have adverse consequences in terms of complete loss of cellular integrity and function. Micronucleus formation has been shown to be triggered by a variety of xenobiotics[62] including nanoparticles.[63] In this study exposure of fish to CuNPs resulted in prominent micronuclei in a number of hepatic cells when compared to control cells, indicating the genotoxic potential of CuNPs. However, exposure of fish to sulfidated CuNPs did not result in higher numbers of micronucleus formation in liver (only one micronuclei was seen in all the sections analysed), suggesting that sulfidation offers good protection against genotoxicity of CuNPs and could be a viable strategy for reducing metal nanoparticle toxicity in environment, if properly applied.

At this juncture we would like to point out that the study by Wang et al[32] has clearly shown the importance and relevance of sulfidation of copper oxide (Cu-O) nanoparticle in reducing its toxicity, by employing in vitro murine macrophages. In this, the authors have shown that sulfidation of Cu-O (mean size 10 nm) helps in reducing H_2O_2 generation and increasing cell viability. Our study differs from the earlier one in the choice and size of the nanoparticle and test system. In our study, we have selected CuNPs (mean size 4nm) and employed an in vivo model (zebrafish) to better understand the potential of sulfidation in reducing CuNPs toxicity in a biological system. It is important to note that the earlier study by Wang et al[32] demonstrated that sulfidation of Cu-O NP was not potent enough in completely reducing Cu-O toxicity. The authors showed that sulphides can be oxidized resulting in Cu release and toxicity. This is an important consequence since the amount of sulfidation of nanoparticle will determine the extent of nanoparticle toxicity.[32] This and our observations made in the present study suggest that sulfidation could be an important transformation resulting in lowering of toxicity of CuNPs in biological systems. This is due to the fact that sulfidation reduces the rate at which Cu ions are released in biological systems. In addition, it is also possible that decrease in the rate of Cu-ion release due to the sulfidation can prove advantageous under in vivo conditions in terms of a better ability of the system to mount an adaptive or protective response against CuNPs toxicity. This can positively influence the survival of the host by reducing lethality due to the CuNPs exposure. To our knowledge, this study is the first one to demonstrate the importance and relevance of transformation of CuNPs in reducing its toxicity under in vivo conditions.

Taken together, these results suggest that sulfidation results in lower bioavailability of copper metal,[32] however, this is true only if the sulfidation is very extensive. In addition, there is the

issue of oxidation reactions of nanoscale copper that can drastically affect the toxicity profile of copper-based nanoparticles.[32,64,65] A recent study by Rui et al.[66] has analysed extensively the properties of copper sulphide nanoparticles and shown the relevance of sulphidation for metal nanoparticles. However, we would like to point out that ours is the sulfidation of copper nanoparticles, whereas, the recent paper by Rui et al., reported the sulfidation of CuO nanoparticles and there is no report on toxicity. From the novelty perspective, we detailed the toxicity mechanism of copper and copper sulfide nanoparticles. The obtained result clearly showed that the toxicity of nano copper decreases significantly after sulfidation. However further study with aim of understanding the extent of sulfidation in modulating the toxicity of nano copper would be interesting.

In this connection, studies have indeed shown CuNPs induced toxicity in zebrafish.[67,68] However, to our knowledge, this study is the first one to show the protective role of sulfidation of CuNPs in adult zebrafish.

4. Materials and Methods:

4.1. Materials

Copper chloride ($CuCl_2$), sodium sulfide (Na_2S), hydrazine were obtained from Merck, India. 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), acetylcholine iodide, epinephrine, napthyl ethylenediamine hydrochloride, sulphanilamide were from Sigma Aldrich. All other chemicals and reagents were of the highest analytical grade and commercially available.

4.2. Synthesis of nanoparticles

Aqueous guar gum solution was prepared by dissolving 50 mg of guar gum in 50 ml of water. To the solution, $CuCl_2$ (1 mM, 8.5 mg, final concentration) were added slowly under vigorous stirring. About 200 µL of concentrated ammonia solution was added to the mixture and the reaction was allowed to proceed under gentle stirring at room temperature for 5 min. The color changes from greenish blue to blue due to the formation of copper-ammonia complex. After 5 min of gentle mixing, 400 µL of hydrazine hydrate was added as a mild reducing agent. After stirring the solution for 5 min, the reaction was allowed to proceed at room temperature for 3 h. The formation of copper nanoparticles was easily noticeable due to the change in the color of the solution to reddish brown – the characteristic color of the copper nanoparticles. Copper sulfide NPs (CuS NPs) were prepared by adding 4 mM sodium sulphide flakes to the preformed CuNPs. The color changes from reddish brown to olive green-indicating the formation of copper sulphide NPs.

Optical absorbance of synthesized CuNPs was monitored by UV-Vis spectrophotometer (Thermo Scientific Evolution 201) between the wavelengths of 400 to 900 nm at a resolution of 1 nm. Size and crystallinity of CuNPs was measured by using high-resolution transmission electron microscope (TEM). The FT-IR spectra were recorded using a PerkinElmer FT-IR

spectrometer with 1 cm^{-1} resolution. Guar gum capped CuNPs and CuS NPs was layered on a glass plate and dried in open air and scratched and grinded with KBr to obtain pellet for recording FT-IR analysis.

4.3. Animal acclimatization

Adult zebrafish (*Danio rerio*) irrespective of sex, measuring 4 to 5 cm in length, weighing approx. 300 mg, were purchased from local aquarium and used following standard experimental procedures. They were maintained in tap water (temperature: 25±2°C). Fish were was fed ad libitum and the tanks were cleaned, sterilized and water replaced periodically. Water quality was monitored regularly and water was used from the same source throughout the study after filtering. The fish were allowed to acclimatize for 1 week before initiation of nanoparticle exposure.

4.4. Nanoparticle exposure

To determine the optimum dosage for exposure, fish were first exposed to different concentrations of CuNPs and CuS NPs (0.001 ppm, 0.005 ppm, 0.01 ppm, 0.05 ppm, 0.1 ppm, 0.5ppm, 1 ppm, 5 ppm, 10 ppm) by directly mixing with the water. Ten fish were used for each concentration and the exposure period was for 10 days. Water and nanoparticles were renewed every day. Based on the mortality during the course of exposure and the number of fish alive at the end of 48 hours, CuNPs and CuS NPs at a concentration of 0.01 ppm was selected for all further analyses (Table 1). The study consisted of four groups: control, copper nanoparticle and copper sulphide nanoparticles, and guar gum and each group contained 20 fishes (4L/tank). The guar gum exposed fishes showed histopathological and biochemical features that were similar to control fishes and hence these results are not included in this study. Water parameters were: DO – 8.5±1.3 mg/L; pH - 7.58; Total Hardness (as $CaCO_3$) – 145±8.5 mg/L; chlorides - 73±3

mg/L; calcium 4.3±0.7 mg/L; magnesium 2.5±0.4 mg/L; Alkalinity - 352±9.6 mg/L; Total dissolved solid - 250±5 mg/L. Water temperature was 25±2°C. The water was always allowed to stand for 24 h before use.

4.5. Tissue preparation

At the end of exposure (48 h), fish were sacrificed (anesthetized by 150mM MS-222 and euthanized by decapitation), skin removed and the liver from two fish from the same group were pooled and homogenized in ice-cold buffer (Tris-HCl, 0.1M, pH 7.4). The homogenate was centrifuged (10,000 x g, 10 min, 4°C) and supernatant used for all analyses in duplicates. Brain was homogenized for acetylcholinesterase (AChE) assay. From the homogenate prepared from liver or brain pooled from two fishes, duplicates were derived for each assay.

4.6. Protein estimation

Protein was estimated by the method of Lowry et al.[69]

4.7. Estimation of metabolic enzymes

4.7.1. Carboxylesterase

Carboxylesterase activity was measured by the method specified by Argentine and James.[70]

500µl of the liver homogenate in 20mM sodium phosphate buffer (pH 7.0) was incubated with 2.5 mL of 250mM α-napthyl acetate or 2.5 mL of 250mM β-napthyl acetate for 30 min at RT. After incubation, 250µl of freshly prepared 0.3 % Fast blue B in 3.3% SDS was added to stop the enzymatic reaction. The mixture was incubated for 30 min at RT until a dark blue color for α-carboxylesterase and red for β-carboxylesterase was observed. The optical density of the samples was then measured at 430nm (α-carboxylesterase) or 588nm (β-carboxylesterase) against a blank

containing buffer instead of the homogenate. The amount of α and β-carboxylesterase was calculated using standard values and expressed as µM of α or β-napthol released/min.

4.7.2. Acetylcholinesterase (AChE)

AChE activity was measured by Edmann's degradation.[71] Briefly, 100µl of brain homogenate was added to 800 µL of 100mM sodium phosphate buffer (pH 7.5). To this mixture 50µl of 10mM DTNB solution was added and the enzymatic reaction was started by adding 50µl of 12.5mM acetylthiocholine iodide. The samples were incubated at RT for 5 min until the development of a yellow color. Optical density of the samples was then measured at 400nm against a blank containing buffer instead of sample. The activity was expressed as µM acetylthiochloine hydrolyzed/min.

4.8. Nitric oxide (NO)

NO was measured spectrophotometrically as described previously.[72] 200 µL of the liver homogenate was made up to 300 µL with Tris-HCl (pH 7.4). To this, 100 µL of 0.1% napthyl ethylenediamine hydrochloride and 100 µL of 1% sulfanilamide were added. The mixture was incubated for 10 min at RT and then centrifuged (12000 x g, 15 min, 4°C). Optical density of the samples was measured at 540nm against a blank containing buffer instead of the homogenate. Values were expresses as µM nitrite.

4.9. Superoxide dismutase (SOD)

SOD activity was determined as previously described.[73] To 100 µL of the liver homogenate, 750 µL of 100% ethanol, and 150 µl of chloroform (ice cold) were added and centrifuged (4000 x g, 5 min, 4◦C). To 500 µl of the supernatant, 500 µl of 0.6mM EDTA solution and 100 µL of bicarbonate buffer (0.1M, pH 10.2) were added. The reaction was

initiated by the addition of 500 µl of 1.3mM epinephrine and the increase in absorbance was read at 480nm.

4.10. Reduced glutathione (GSH)

GSH was analyzed as described previously.[73] 750 µl of the liver homogenate was mixed with 0.5 ml of 10% trichloroacetic acid and centrifuged (11,000 rpm, 15 min, 4◦C). The resulting protein-free supernatant was allowed to react with 250 µL of 0.2 M disodium phosphate (pH 8.0) and 1 ml of 0.6 mM DTNB. The absorbance of the resulting yellow color was read spectrophotometrically at 412 nm. GSH was expressed as µM/g wt.

4.11. Metallothionein

Metallothionein assay was carried out in 3 steps as follows:[74]

Step 1- tissue homogenization: Liver of adult zebrafish was homogenized in homogenization buffer (0.5 M sucrose, 20 mM Tris-HCl buffer (pH 8.6), 0.01% β-mercaptoethanol) and, distributed in aliquots (3 vol), which could be stored at -20◦C.

Step 2- concentration of metallothionein: The homogenates were then centrifuged at 30,000 x g for 20 min. The pellet was discarded after the first centrifugation and to every 1 ml of supernatant, 1.05 ml of ice cold absolute ethanol (-20◦C) and 80 µl of chloroform were added. The mixtures were again spun down at 6000 x g for 10 min at 4◦C. 3 volumes of ice cold ethanol was added to the supernatant & stored at -20◦C for 1 h.

Step 3- metallothionein purification and quantification: After an hour, the stored mixtures were again centrifuged at 6,000 x g for 10 min. Supernatant was discarded and the pellet was washed with ethanol: chloroform: homogenization buffer in the ratio 87:1:12. Mixture was centrifuged at 6,000 x g for 10 min. Supernatant was discarded and pellet was air dried. The dried pellets were resuspended in 300 µl of 5 mM Tris-HCl, 1 mM EDTA (pH 7.0). The resuspended

metallothionein fraction was then added to 4.2 ml of 0.43 mM DTNB in 0.2 M phosphate buffer (pH 8.0). The sample was finally incubated for 30 min at RT and, the concentration of reduced sulfhydryl was evaluated by measuring the absorbance at 412 nm.

4.12. Histopathology

For histopathological analyses a separate experiment containing all the exposure groups with 5 fishes each was performed. Fish were sacrificed. Scales were removed and the entire fish was washed in 0.9% ice cold saline and immediately fixed in buffered 10% formalin solution for 24 hours and embedded in a paraffin wax. Sections of 5μm thickness were cut and stained by both hematoxylin and eosin for histological examination. The histological observations were made under bright field using a Nikon Eclipse Ci (trinocular light microscope). Histopathological analyses of liver samples from exposed fish were done by an independent observer who was blind to treatment groups.

4.13. Micronucleus assay

For micronucleus assay, a separate experiment was performed, and viscera of five fish from control, CuNPs and CuS NPs exposure tanks were fixed in neutral buffered formalin and processed for paraffin sections (5 μm thickness) as mentioned above. Two sections from each specimen were then stained for 2 min with Wright's stain and then observed for micronucleus formation using a Labomed microscope under bright field illumination.

4.14. Statistical analyses

All assays were performed in duplicates, except for metallothionein which was run in triplicates. Results were expressed as mean±SD of 7 determinations using liver samples pooled from two

fishes of the same treatment group (total 14 fishes). One way analysis of variance ($p<0.05$) was followed by Tukey Post-hoc test to evaluate the significance between different treatment groups.

5. Conclusion

We report here a sulfidation strategy to combat metal nanoparticles, especially copper nanoparticle toxicity. Our observations revealed that sulfidation gives biological system time to mount protective responses against copper nanoparticle toxicity, thus increasing the ability of organisms to survive. By a series of experiments, we showed that the sufidation of copper nanoparticles rescue the various biochemical parameters of toxicity. Further studies aiming at analyzing the required extent of sulphidation and accumulated levels of NPs in various tissues would enable us to develop a potential strategy for combating environmental toxicity by various metal nanoparticles.

Conflict of interest: The authors declare no conflict of interest.

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Table 1: Effect of sulphidation of copper nanoparticle on zebrafish survivability

Groups	Concentration (ppm)	Day[@]									
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Control	**0**	10	10	10	10	10	10	10	10	10	10
CuNPs	**0.001**	10	10	10	10	9	5	3	3	1	1
	0.005	10	10	10	10	7	7	4	1	1	0
	0.01	10	8	5	0	0	0	0	0	0	0
	0.05	10	6	0	0	0	0	0	0	0	0
	0.1	10	0	0	0	0	0	0	0	0	0
	0.5	10	0	0	0	0	0	0	0	0	0
CuS NPs	**0.001**	10	10	10	10	10	9	9	7	5	3
	0.005	10	10	10	10	10	7	7	5	2	0
	0.01	10	10	10	8	8	7	4	4	3	0
	0.05	10	7	4	0	0	0	0	0	0	0
	0.1	10	0	0	0	0	0	0	0	0	0
	0.5	10	0	0	0	0	0	0	0	0	0

[@]Number of fish alive. A total of ten fish were used for each exposure. Cu-NP = copper nanoparticle; Cu-S_2 NP = copper sulphide nanoparticle

Table 2: Effect of sulphidation of copper nanoparticle on liver oxidative stress

Groups	Nitric oxide (μM nitrite)	SOD (OD@480 nm)	GSH (μM/g wt)
Control	14.8787 ± 0.006	1.198 ± 0.007	0.284 ± 0.009
CuNPs	19.94 ± 0.007[a]	0.742 ± 0.02[a]	0.162 ± 0.008[a]
CuS NPs	15.87 ± 0.003[b]	0.983 ± 0.06[b]	0.253 ± 0.006[b]

Values are expressed as mean±SD of 7 determinations using liver pooled from two fishes of the same treatment group. Concentration of nanoparticle used was 0.01 ppm and exposure was for 48 h. 'a' and 'b' indicates the difference observed between CuNPs and control and between CuS NPs and CuNPs are statistically significant at $p<0.05$. SOD – superoxide dismutase; GSH – reduced glutathione; CuNPs – copper nanoparticle; CuS NP – copper sulfide nanoparticle

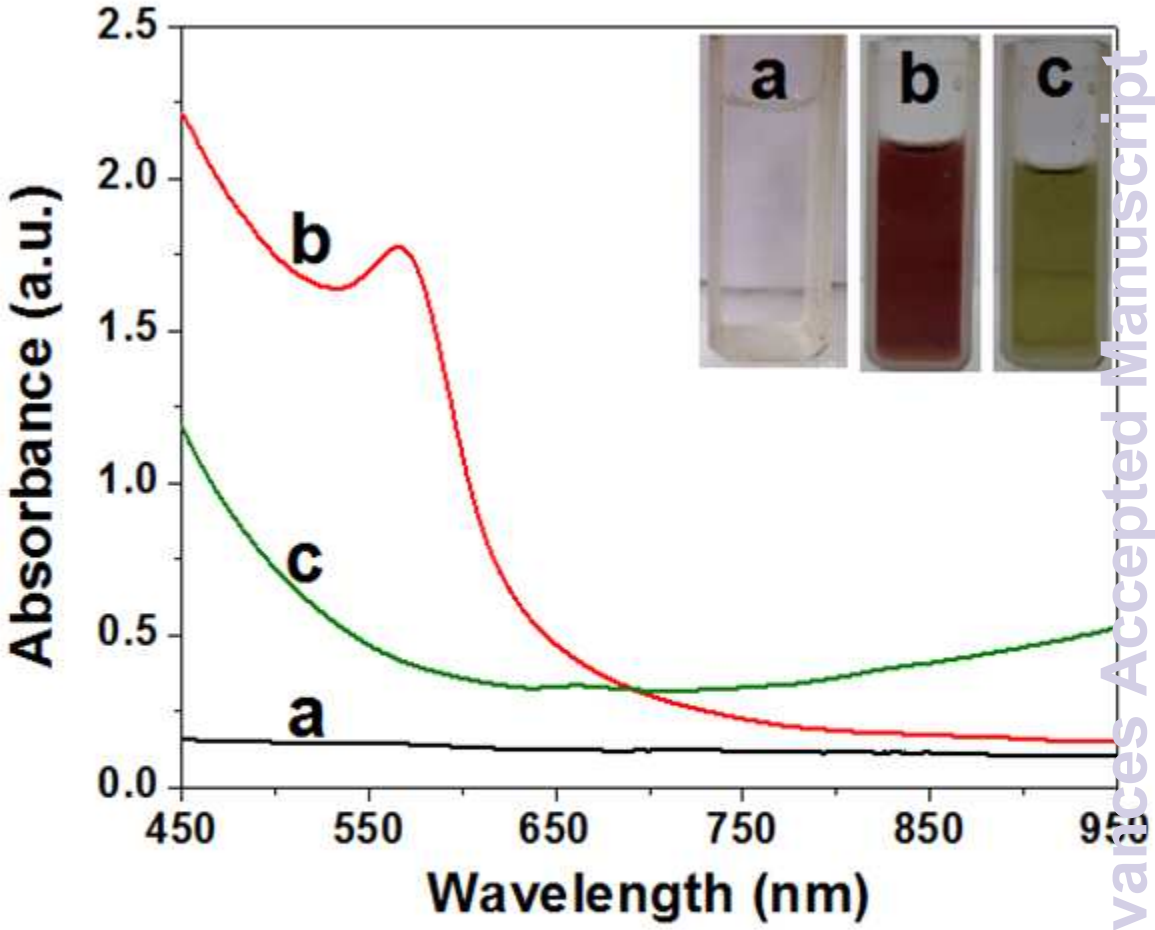

Figure 1

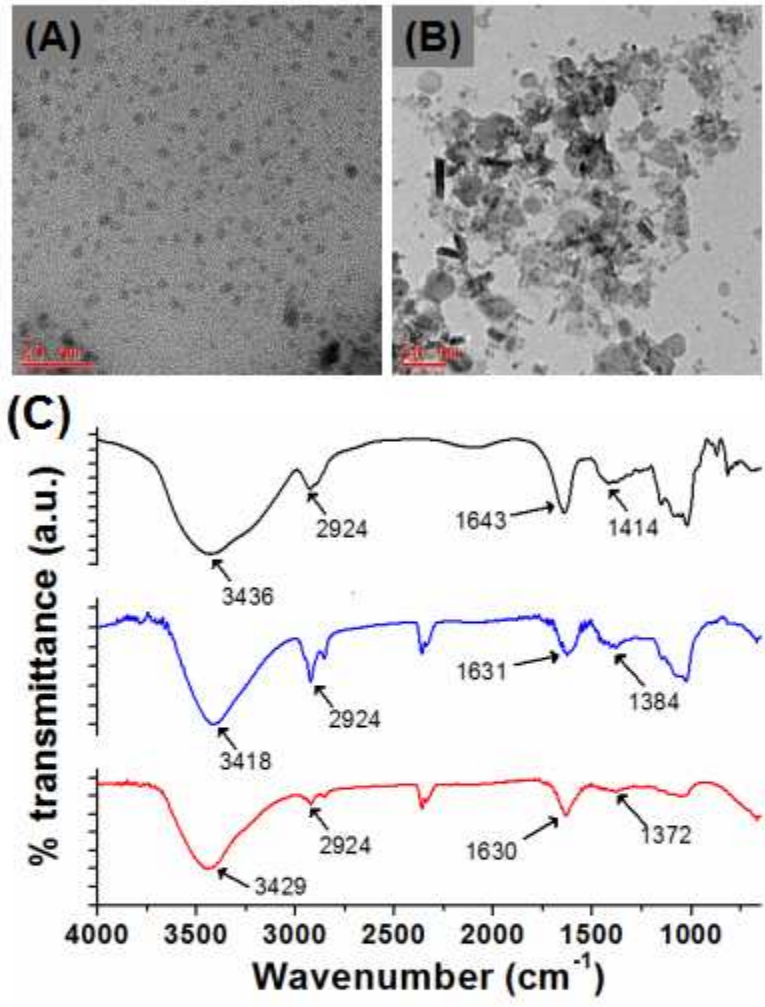

Figure 2

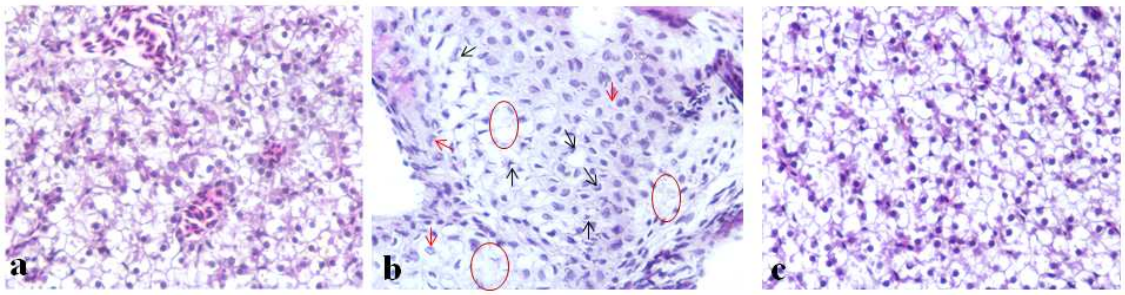

Figure 3

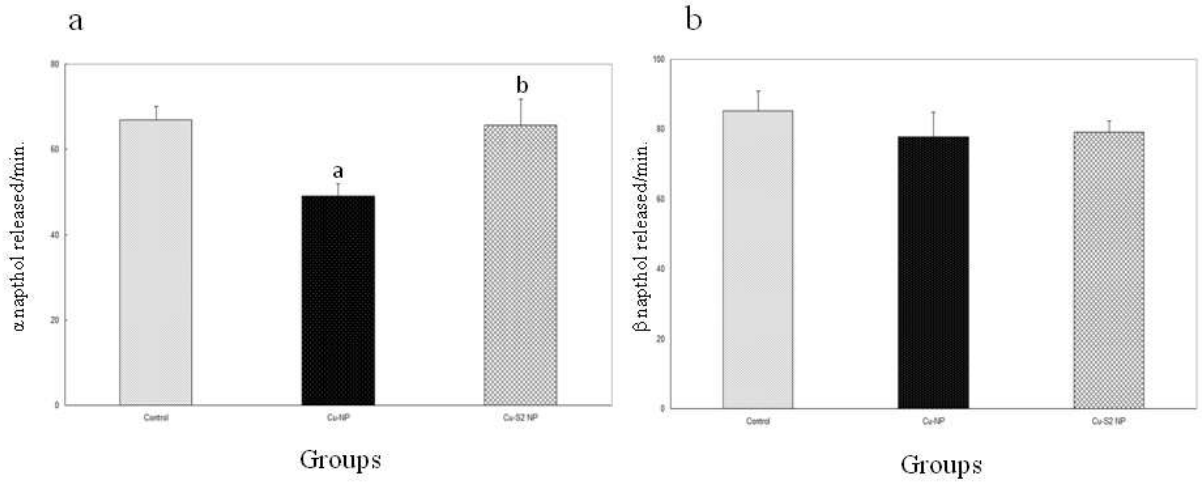

Figure 4

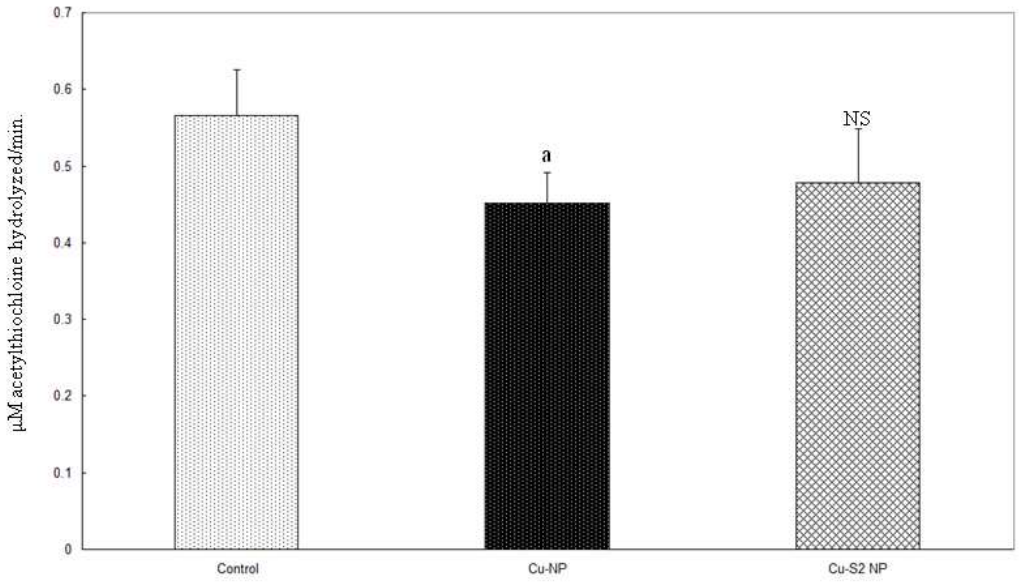

Figure 5

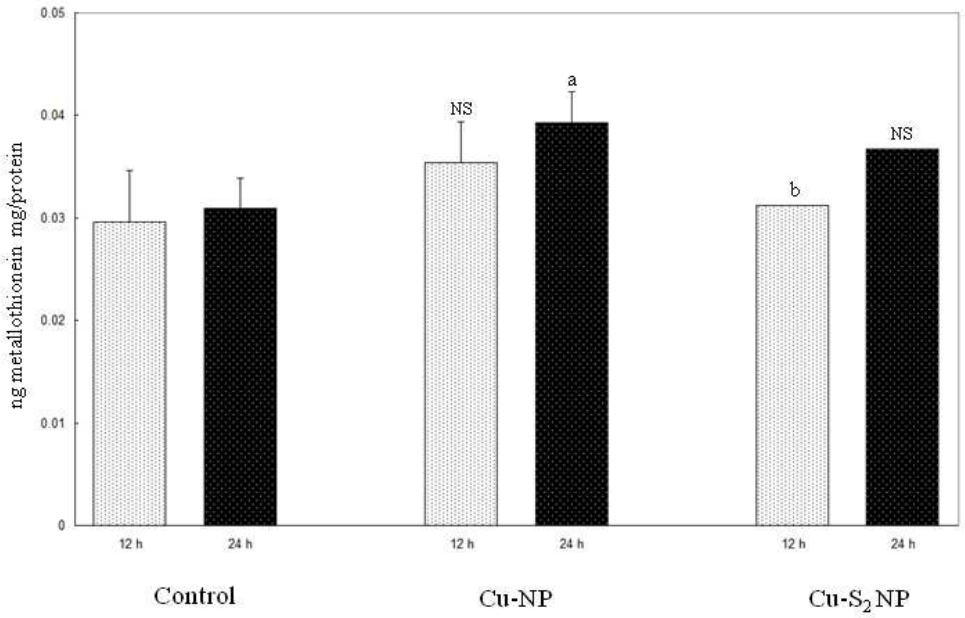

Figure 6

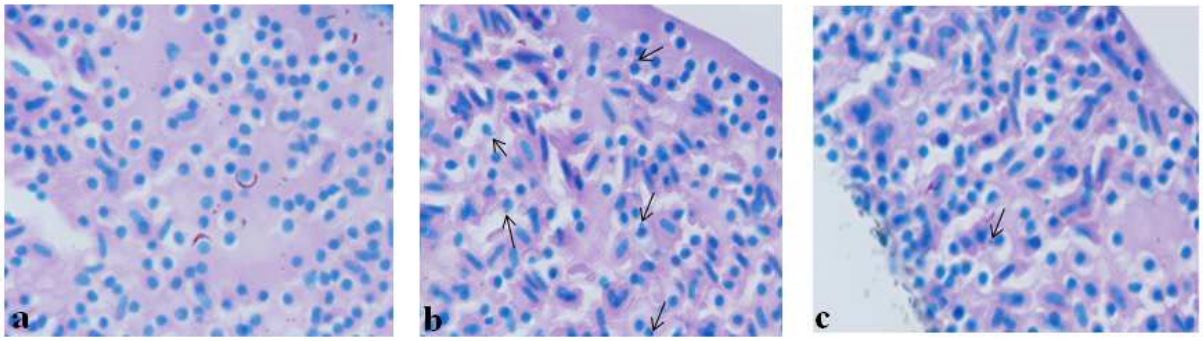

Figure 7

Figure Captions:

Figure 1: Absorption spectra of (a) guar gum, (b) CuNPs and (c) CuS NPs. *Inset* corresponds to the photographs of (a) guar gum, (b) CuNPs and (c) CuS NPs solutions synthesized as described in materials and methods.

Figure 2: TEM images of guar gum stabilized (A) CuNPs, (B) CuS NPs and (C) FTIR spectra of guar gum (black), CuNPs (blue) and CuS NPs (red).

Figure 3. Effect of sulfidation of copper nanoparticle on the histopathological changes in the liver of zebrafish. a) liver from control zebrafish, b) liver from zebrafish exposed to CuNPs. Note the extensive pathological changes due to CuNPs exposure. Black arrows indicate necrotic changes, red arrows show areas with degenerative changes in the liver while the encircled area represents areas with extensive cell loss. c) liver from zebrafish that was exposed to CuS NPs. Note the near normal appearance of the liver cells in general. The histological feature of liver from this group appeared similar to control liver. Similarly, liver from guar gum alone exposed fish was also analysed but there were no adverse changes (data not shown). Images are representative of five liver samples, isolated from each group, which were sectioned and processed for histopathology. Analyses were done by an independent observer who was blind to treatment groups.

Figure 4. Effect of sulfidation of copper nanoparticle on liver carboxylesterase activity in zebrafish. a) α-carboxylesterase and b) β-carboxylesterase. Values are expressed as mean±SD of 7 determinations using liver pooled from two fishes of the same treatment group. Concentration

of nanoparticle used was 0.01 ppm and exposure was for 48 h. ‘a’ and ‘b’ indicates the difference observed between CuNPs and control and between CuS NPs and CuNPs are statistically significant at $p<0.05$.

Figure 5. Effect of sulfidation of copper nanoparticle on brain acetylcholinesterase activity in zebrafish. Values are expressed as mean±SD of 7 determinations. Concentration of nanoparticle used was 0.01 ppm and exposure was for 48 h. ‘a’ indicates the difference observed between CuNPs and control is statistically significant at $p<0.05$. NS = not significant, the difference between CuS NPs and CuNPs.

Figure 6. Effect of sulfidation of copper nanoparticle on liver metallothionein in zebrafish. Values are expressed as mean±SD of 3 determinations using liver pooled from two fishes of the same treatment group. Concentration of nanoparticle used was 0.01 ppm and exposure was for 12 h and 24 h. ‘a’ and ‘b’ indicates the difference observed between CuNPs (24 h) and control (24 h) and between CuS NPs (12 h) and CuNPs (12 h) are statistically significant at $p<0.05$. NS – not significant, the difference between CuNPs after 12 h and control after 12 h and between CuS NP after 24 h and CuNPs after 24 h

Figure 7. Effect of sulfidation of copper nanoparticle on micronucleus formation in liver of zebrafish. a) liver from control fish, b) liver from fish expose to CuNPs. Note micronucleus are clearly visible in the liver next to nucleus of hepatocytes (black arrows). c) liver from fish exposed to CuS NPs. The liver section appears similar to control liver but with only one micronuclei. Images are representative of 5 liver samples, isolated from each group that were

sectioned and processed for Wright's staining. Analyses were done by an independent observer who was blind to treatment groups. Magnification 100X.