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Copper nanoparticles exert size and concentration dependent toxicity on somatosensory neurons of rat

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Abstract

Metal nanoparticles, due to their unique properties and important applications in optical, magnetic, thermal, electrical, sensor devices and cosmetics, are beginning to be widely manufactured and used. This new and rapidly growing field of technology warrants a thorough examination of the material's bio-compatibility and safety. Ultra-small particles may adversely affect living cells and organisms since they can easily penetrate the body through skin contact, inhalation and ingestion. Retrograde transport of copper nanoparticles from nerve endings on the skin can reach the somatosensory neurons in dorsal root ganglion (DRG). Since copper nanoparticles have industrial and healthcare applications, we determined the concentration and size-dependant effects of their exposure on survival of DRG neurons of rat in cell culture. The neurons were exposed to copper nanoparticles of increasing concentrations (10–100 μM) and sizes (40, 60 and 80 nm) for 24 h. Light microscopy, histochemical staining for copper, lactate dehydrogenase (LDH) assay for cell death, and MTS [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for cell viability were performed to measure the resultant toxicity and cell survival. DRG neurons exposed to copper nanoparticles displayed vacuoles and detachment of some neurons from the substratum. Neurons also exhibited disrupted neurite network. LDH and MTS assays revealed that exposure to copper nanoparticles had significant toxic effect with all the sizes tested when compared to unexposed control cultures. Further analysis of the results showed that copper nanoparticles of smaller size and higher concentration exerted the maximum toxic effects. Rubeanic acid staining showed intracellular deposition of copper. These results demonstrate that copper nanoparticles are toxic in a size- and concentration-dependent manner to DRG neurons.

Keywords

Copper nanoparticles; cytotoxicity; cell death; DRG neurons; histochemistry; neurotoxicity

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Introduction

When materials are engineered to nano-size (100 nanometers or less), they acquire unique physical and chemical properties. Hence these materials are increasingly being used in the commercial manufacture of fillers, opacifiers, catalysts, semiconductors, cosmetics, microelectronics, and drug carriers (Curtis et al. 2006). Just as the nano-scale fabrication has enabled several commercial applications, it can also lead to potential risks (Hussain et al. 2005; Nel et al. 2006). The many industrial uses as well as the direct contact of the nanoparticles to human skin increase the chances of nanoparticles entering the body easily through respiratory, gastrointestinal and dermal passages (Hoet et al. 2004; Oberdörster et al. 2005). Copper nanoparticles are now being widely manufactured and are available commercially to be used in applications such as facial spray, lubricants, anti oxidants and anode materials for lithium ion batteries (Gou et al. 2002; Liu et al. 2004; Yang et al. 2006). In the biological system, copper is an essential trace mineral critical for energy production in the cells. Copper is required for the formation of cupro proteins, like ceruloplasmin and for the activity of enzymes such as lisyl oxidase, cytochrome-c oxidase, superoxide dismutase, and tyrosinase (Evans 1973; Sternlieb 1980). The brain contains high levels of copper (Nalbandyan 1983) where it stimulates production of the neurotransmitters epinephrine and norepinephrine. In the human body, copper is maintained in homeostasis (Bertinato and L'Abbe 2004) since it becomes toxic when it is in excess and not properly bound. Under toxic conditions, its redox reactivity can lead to the formation of reactive oxygen species (ROS) such as, superoxide anion, hydrogen peroxide, and hydroxyl radical. Accumulation of ROS leads to cell damage through oxidative modifications of proteins, lipids, and nucleic acids, adversely affecting their structures and functions (Halliwell and Gutteridge 1990; Galhardi et al. 2004). In addition, copper can be toxic by directly binding to free thiols of cysteines and sulfhydryl groups in proteins, resulting in enzyme inactivation or altered protein conformation (Jeon et al. 2000; Cecconi et al. 2002).

Animal studies suggest that the toxicity of copper depends on the size of the ingested copper. As the size is reduced, the toxicity of copper increases sharply. According to Chen etal. (2006),the oral $LD_{50}$of copper was found to be 110 mg/kg for ionic copper < 413mg/kg for nano copper < 500 mg/kg for micro copper. Oral administration of copper nanoparticles heavily damaged the kidney, liver, spleen, and brain of experimental mice (Chen et al. 2006). Furthermore, they showed that nano-sized copper particles lead to the generation of highly toxic cupric ions suggesting copper nanoparticles might experience different metabolic processes *in vivo*, unlike other forms of copper (Chen et al. 2007; Meng et al. 2007a, 2007b). With an increase in the manufacture anduse,copper nanoparticles can gain easy entry into the body through the skin and respiratory system. They can then be retrogradely transported to the neurons innervating the skin or airways. Since copper nanoparticles can produce ROS, they can be even more toxic to neurons because neurons are very vulnerable to oxidative stress induced by ROS generation (Cadenas and Davies 2000; Brooking et al. 2001). Since the manufacturing of nanomaterial has recently started, there is no report yet on the effects of copper nanoparticles on neurons innervating the skin. Hence we decided to determine the effects of copper nanoparticles on sensory neurons that innervate the skin. Primary cultures of dorsal root ganglion (DRG) neurons is an ideal model in which to study the direct effect of copper nanoparticles on sensory neurons without the confounding effects of systemic administration. Therefore we cultured DRG neurons of neonatal rat pups and measured neuron survival and neurotoxicity in response to the exposure of copper nanoparticles of varying sizes and concentrations.

Materials and methods

Copper nanoparticles

Copper nanoparticles (40, 60 and 80 nm) were synthesized and generously received in powder form Dr Karl Martin of NovaCentrix, Austin (formerly Nanotechnologies, Inc.).

Characterization of copper nanoparticles

TEM characterization was performed to obtain the primary particle size and morphology of nanoparticles using a Hitachi H-7600 tungsten-tip instrument at an accelerating voltage of 100 kV. Nanoparticles were examined after dilution of nanoparticles stock solutions to 100 μg/ml suspensions in water and subsequent deposition of 5 μl onto formvar/carbon-coated copper TEM grids which were then dried. The AMT software for the digital TEM camera was calibrated for size measurement of the nanoparticles. The mean and standard deviation (SD) of particle sizes was calculated from measuring over 100 nanoparticles in random fields of view in addition to the images showing the general morphology of the nanoparticles. This has previously been described by Murdock et al. (2008).

Dynamic light scattering (DLS) and laser Doppler velocimetry (LDV), for characterization of size and zeta potential of the nanoparticles in solution, were performed on a Malvern Instruments Zetasizer Nano-ZS instrument. Samples were examined after dilution of nanoparticles stock solutions to 50 μg/ml suspensions in water and cell media; vortexed to provide a homogeneous solution, and then 1.5 ml was transferred to a 1 cm^2 cuvette for DLS measurements and 1 ml was transferred to a Malvern Clear Zeta Potential cell for DLS and LDV measurements, as previously described by Murdock et al. (2008).

Primary cultures of DRG neurons

All cell culture procedures were performed under sterile conditions in laminar flow hood. DRGs from one-day-old rat pups of Sprague Dawley strain were dissected and the connective tissue was digested by incubation for 20 min at 37°C in 0.5% collagenase (Invitrogen) followed by a second incubation in TrypLE (Invitrogen) for 15 min at 37°C. The ganglia were then washed with Dulbecco's modified essential medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, Invitrogen) and Deoxyriboneuclease (7.5 μg/ml DNase, Sigma) and resuspended in DMEM containing N2 supplement (Invitrogen) and nerve growth factor (50 ng/ml NGF, Invitrogen) and DNase. The ganglia were triturated through a 200 μl sterile pipette tip until the cell clumps disappeared. The cell suspension was then centrifuged for 3 min at 500 rpm at 4°C to gently pellet the cells. The cell pellet was resuspended in fresh DMEM containing 1 mM glutamax (GIBCO) and 1 mM glutamine (Invitrogen), antibiotic solution (100 U/ml Penicillin, 100 μg/ml Streptomycin, 250 ng/ml Amphotericin B, Sigma), uridine (70 μM/ml, Sigma), 5-fluoro-2′-deoxyuridine (30 μM, Sigma), NGF (50 ng/ml), and N2 supplement and pre-plated onto uncoated 100 mm culture dishes for a 30-min incubation at 37°C to allow the supporting cells to attach to the dish. The unattached neurons in the medium were collected by gently swirling the dish and pre-plated again two more times. The medium containing neurons in suspension was then collected, centrifuged to pellet the cells as before, and the pellet was resuspended in neurobasal™ medium (GIBCO) constituting the same mixture of supplements as above with B27 supplement (GIBCO) instead of N2 supplement. The cell density of the suspension was then determined, and cells were plated at a density of 3000 cells/each poly-d-lysine-coated 35 mm dishes for LDH assays and rubeanic acid staining and 1500 cells/each well of the 96-well plate for MTS assays. Pre-warmed neurobasal™ medium with appropriate supplements as mentioned before was added to the dishes and the wells. Cells were grown and maintained in a humidified incubator at 37°C in 5% CO_2 up to four days *in vitro*. The medium was changed the following day and every second day thereafter.

Exposure to copper nanoparticles

After four days of growth *in vitro*, DRG neurons were treated with different concentrations of copper nanoparticles ranging from 10–100 μM and sizes from 40–80 nm. Griffitt et al. (2007) reported that LC50 concentration of nanocopper that resulted in acute toxicity in zebra fish was 1.5 mg/l (23.6 uM); hence, we chose a range from 10–100 uM concentration of nanocopper for our experiments. The coppernanoparticles suspensions were prepared by making a 10 mM stock solution in phosphate buffered saline (PBS, 0.01 M, pH 7.4) and sonicated with a tip sonicator (Sonic Dismembrator Model 100, Fisher Scientific) at 4–6 W for a few seconds to get uniform distribution of the copper nanoparticles in the suspension. The desired concentration of the copper nanoparticles was obtained by diluting the stock solution with the neurobasal™ medium and added to DRG cultures for a 24-h treatment. At the same time, the control cultures were given a medium change. Toxicity experiments using DRG neurons are usually conducted with a 24 h exposure as it gives enough time to test for dose-dependent toxicity while exposure for a longer duration may mask such dose-dependent effects due to accumulated toxicity even for a low concentration over time. Hence 24 h exposure was chosen for all experiments reported here. After 24 h, the culture medium from both the experimental (copper nanoparticles exposed) and the control dishes was collected and the cells were fixed with 3% paraformaldehyde (Sigma) in PBS for 30 min and stained with rubeanicacid (Fluka) for copper (Thornburg et al. 1985). The culture medium samples collected above were centrifuged at 10,000 rpm for 10 min, and the supernatant was aliquoted and stored at −80°C for the LDH assay.

Rubeanic acid staining for copper

Rubeanic acid staining solution (0.005%) was prepared by diluting 0.1% stock solution prepared in absolute alcohol and diluted using 10% sodium acetate. The fixed cultures were washed three times and incubated in the staining solution overnight at 37°C. The dishes were washed and counter stained with 1% neutral red for 2 min followed by a rinse. The stained copper in neurons was observed under the Olympus IX71 inverted microscope and imaged using a Scion CCD camera.

Lactate dehydrogenase (LDH) assay for measuring cell death

LDH assay was performed in triplicate using the cytotoxicity detection kit (Roche) following the manufacturer's instructions for microplate assay using Bio-Rad microplate reader. Briefly, 100 μl of the previously collected culture medium supernatant and cytotoxic assay solution mixture was added to the microplate wells and incubated for 30 min at room temperature in the dark. Absorbance was measured at a wavelength of 490 nm using the Bio-Rad microplate reader. The LDH levels in experimental samples were calculated as a percentage of the LDH values in the control samples.

MTS assay for the determination of cell viability based on metabolic function

Cell viability was determined by performing MTS, a tetrazolium dye [(3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] based assay using the CellTiter 96 Aqueous One Solution (Promega) because unlike MTT, formazan crystals of MTS are soluble in culture medium and do not need solubilization. MTS is chemically reduced by reductase, enzymes present in mitochondria of metabolically active, viable, healthy cells into formazan, which is soluble in tissue culture medium. Thus the values from MTS assay indicate the mitochondrial metabolic activity and the healthy status or viability of the cells. Further, mitochondria are a major target of copper-induced cytotoxicity in Wilson disease (Sokol et al. 1994). Therefore, determining mitochondrial metabolic activity in neurons exposed to copper nanoparticles is appropriate. The loss of mitochondrial function leads to neurons death and hence MTS assay has been recognized as an appropriate determinant for

mitochondrial metabolic function as well as neurons viability (Aras et al. 2008). This assay was performed using live cells in culture. 20 μl of CellTiter 96 Aqueous One solution reagent was directly added to the control and experimental culture wells in the 96-well plate and cells were incubated in the humidified chamber at 37°C and 5% CO_2 for 3 h. Afterwards, the absorbance was measured at a wavelength of 490 nm using the Bio-Rad microplate reader. The cell viability of the experimental cultures was calculated as a percentage of cell viability in control cultures.

Statistical analysis

The differences between the values of experimental and control treatments were analyzed for statistical significance by analysis of variance (ANOVA) followed by Tukey's post hoc test for individual comparisons. A p value < 0.05 was considered significant. The means ± SD are from 6–9 independent experiments.

Results

Characterization of copper nanoparticles

The copper nanoparticles were examined under TEM to observe their morphology and obtain a size distribution for each sample. All three copper nanoparticles exhibited the same morphology of being in a highly aggregated state with non-definitive boundaries which made it difficult for individual particles to be determined (Figures 1a–c). Size distributions for the three copper samples were slightly different than the manufacturer labeled sizes, but still within the distribution found. Cu 40 nm particles were observed to have a size distribution of 55.2 ± 15.4 nm, while Cu 60 nm and Cu 80 nm particles were found to have size distributions of 69.0 ± 12.2 nm and 70.6 ± 20.0 nm, respectively (Figures 1d).

The copper nanoparticles were also observed after dispersion in deionized, Millipore-filtered water and neurobasal media. All three copper nanoparticles exhibited increased agglomeration in water and media, with a slight increase in agglomeration when dispersed in media over dispersion in water. Agglomeration sizes in water for Cu 40 nm, Cu 60 nm, and Cu 80 nm were 335 nm, 360 nm, and 365 nm, respectively (Figure 1d). When dispersed in neurobasal media, agglomerate sizes for Cu 40 nm, Cu 60 nm, and Cu 80 nm were measured at average sizes of 386 nm, 401 nm, and 409 nm, respectively (Figure 1d). This is a 5- to 6-fold increase in size over primary particle size once in water or media. For zeta potential readings, Cu 40 nm was highest with 30.9 mV, followed by Cu 80 nm at 23.5 mV and finally Cu 80 nm at 14.3 mV (Figure 1d).

Exposure to copper nanoparticles at higher concentrations disrupts morphology of DRG neurons

When the DRG neurons were maintained in cell culture in the presence of uridine and 5-fluoro 2′deoxyuridine, we obtained neuron-enriched cultures. When observed under inverted microscope (Olympus IX71) the neurons exhibited phase bright soma with intact membrane and long smooth neurites, profusely branched to form an extensive network among the neurons (Figure 2a). Exposure to copper nanoparticles at low concentrations (10 and 20 μM) for 24 h did not cause any morphological change in the DRG neurons. However, 24-h exposure to higher concentrations of copper nanoparticles (40–100 μM) resulted in significant changes in the morphology of DRG neurons. Some neurons displayed shrinkage and others vacuoles in their cytoplasm. Some cells detached partially from substratum and floated in the medium. Those cells that were attached showed the disrupted neuritic network (Figure 2b).

Exposure to copper nanoparticles of smaller size and higher concentration results in increased cell death

When LDH levels in the culture medium of experimental cultures were compared to that of controls, LDH release was found to be significantly increased in cultures exposed to copper nanoparticles at concentrations ranging from 40–100 μM indicating a loss of membrane integrity leading to increased release of LDH following exposure to copper nanoparticles at higher concentrations (Figure 3a). Statistical analyses of the results showed that the cell death was concentration-dependent. ANOVA showed that LDH levels of DRG cultures exposed to 40–100 μM were significantly different from those of control cultures ($p < 6.19$ E-08). Tukey's post hoc test confirmed that there was a dose-dependant increase in the LDH levels. While LDH levels of DRG cultures exposed to 10 and 20 μM concentrations of copper nanoparticles were not statistically different from those of control cultures, DRG neurons exposed to 40–100 μM copper nano particles showed a 22–31%, significant increase ($p < 0.05$) in LDH levels.

When we investigated whether there was a size-dependent neurotoxicity with regards to copper nanoparticles exposure, we found that the smaller sized nanoparticles caused significantly more LDH release than the larger sized nanoparticles. Exposure to 40 μM concentration of copper nanoparticles of all the sizes tested (40–80 nm) showed significant ($p < 0.005$) increase in LDH levels when compared to that of control DRG cultures. However, Tukey's post hoc test showed that the LDH release was significantly higher in cultures exposed to 40 and 60 nm and not in cultures exposed to 80 nm-sized copper nanoparticles, with exposure to 40 nm having the highest LDH release of 40% (Figure 3b).

Exposure to copper nanoparticles of smaller size and higher concentration results in reduced viability in DRG neurons

When MTS assay which indirectly reflects mitochondrial function and indirectly cell viability was performed, the results revealed that exposure to copper nanoparticles reduced cell viability. There was a 20% reduction in overall cell viability in cultures exposed to copper nanoparticles compared to that of control cultures. However, the reduction in cell viability in cultures exposed to 10 and 20 μM of copper nanoparticles was not statistically significant. On the other hand, 24-h exposure to copper nanoparticles at 40–100 μM concentration resulted in significantly reduced metabolic activity in mitochondria, indicating less DRG neuron viability ($p < 0.001$) when compared to that of the control DRG cultures (Figure 4a).

Furthermore, a size-dependent effect was also observed with regards to the reduction in cell viability. Thus exposure to copper nanoparticles of 40 and 60 nm sizes at 40 μM concentration showed significant ($p < 0.0001$) decrease in cell viability when compared to that of control DRG cultures. All the sizes tested appeared to affect cell viability more or less similarly because Tukey's post hoc test showed that the cell viability of neurons in the different experimental groups did not differ significantly from each other although they were significantly different from the controls. However, a trend showing that the smaller the size, the lesser was the cell viability was apparent as exposure to copper nanoparticles of 40 and 60 nm sizes leads to increased reduction in cell viability (23 and 30%, respectively) than exposure to copper nanoparticles of 80 nm size which had a 19% reduction in cell viability (Figure 4b).

Exposure to copper nanoparticles results in the presence of intraneuronal copper as revealed by rubeanic acid staining

The DRG neurons in control cultures which were stained with rubeanic acid and counter stained with neutral red showed pale red large spherical cell bodies with the absence of staining for copper in the neurons (Figure 5a). However, the neurons exposed to copper nanoparticles of 40 nm size at higher concentrations (60 and 80 μM) showed greenish black intracellular

deposition of copper (Figure 5b). Dark particles were also seen dispersed in the cytoplasm of some neurons (Figure 5c).

Discussion

In the present study, neurotoxicity resulting from exposure to copper nanoparticles was investigated by the morphological study and biochemical assays. Since nanomaterials from the periphery can be retrogradely transported into the nervous system (Praetorius et al. 2007), it is essential to know the effects of the exposure of neurons to nanoparticles. Sensory neurons innervating the skin are a potential target for such retrograde transport. Copper nanoparticles are now being widely manufactured because of the increasing demand due to their use in several applications. Although the toxicological effects of copper nanoparticles have been shown *in vivo* at organ levels in kidney, liver, and spleen (Chen et al. 2006), our study is the first to report that copper nanoparticles induced toxicity in sensory neurons. The need to characterize nanoparticles before assessing the *in vitro* toxicity is crucial. Particle size, morphology, surface area, surface chemistry and particle reactivity in solution are important factors which need to be defined to accurately assess nanoparticles toxicity. The primary sizes of the copper nanoparticles were fairly close to their manufacturer labeled sizes; however, once in neurobasal media, the nanoparticles aggregated to 350–400 nm sizes. Interestingly, all three copper nanoparticles aggregated to nearly the same average size in water and media, with only a minimal increase with increasing particle size. This is interesting in the fact that any differences in the observed effects of the particles would be attributed to other properties of the particles besides the primary particle size. For example, the particles may have different surface features or may behave differently once internalized by the cells. Also, the surface appears to be somewhat oxidized, even after only a few minutes of time in solution, suggesting that the particles are highly reactive. This would need to be verified by surface or elemental analysis of the particles, such as X-ray photoelectron spectroscopy or energy dispersive spectroscopy. However, it has been shown that the surface chemistry and characteristics of these copper nanoparticles began to show changes after one month in solution and they began to exhibit agglomeration in solution after five days and gradually increased their agglomeration by 34 days (Murdock et al. 2008). In our study, neurons were exposed to copper nanoparticles for only a short duration of 24 h. Since serum appeared to influence agglomeration, we used serum-free defined medium for our cell cultures.

At the end of the 24-h exposure to 40–100 μM concentration of copper nanoparticles, our inverted phase contrast microscopic observation of the DRG neurons showed that some neurons partially detached from the dish and floated in the medium. The detaching of cells from the substratum is an indication of the loss of membrane integrity of the neurons. In addition, many attached neurons showed vacuolation and neurite degeneration. Metal-induced neurotoxicity leading to neurite degeneration has been reported earlier. Methyl mercury has been shown to induce neurite degeneration in primary cultures of mouse dopaminergic mesencephalic cells (Gotz et al. 2002). Similarly, a 48-h exposure to aluminum chloride resulted in swollen nerve cell bodies in cultured primary cortical neurons from rats along with the presence of beaded and disrupted neurites (Munirathinam et al. 1996). The membrane compromised, dying neurons would have released the cytoplasmic content to the culture medium. As a result, the LDH values obtained from copper nanoparticles exposed cultures were significantly more compared to the control cultures. Copper nanomaterial appears to be toxic not only to DRG neurons in our study but also to glial cells. A recent report showed dose-dependent (10–100 μM) toxicity on human H4 neuroglioma cells exerted by cupric oxide nanoparticles (Li et al. 2007). In their study, an automated image analyzer system counted live and dead cells based on the differences in fluorescence between live and dead cells and revealed an increased cell death in H4 neuroglioma cells treated with 10 μM of cupric oxide nanoparticles. However, our results showed that at concentrations of 10 and 20 μM, copper

nanoparticles did not exert significant toxicity on DRG neurons. This could be due to differences in the properties of cupric oxide nanoparticles and pure copper nanoparticles and also could be due to the nature of the cells in the two studies and the duration of the nanoparticles exposure. While our cells were fully differentiated, sensory neurons from DRG and exposure duration was 24 h, Li et al. (2007) used an undifferentiated neuroglioma cell line with an exposure duration of 48 h.

The MTS assay is based on the reduction of the tetrazolium compound to soluble formazan by reductase mostly found in mitochondria, giving a measurement of mitochondrial function in the live cell. The results of the MTS assays showed that exposure to copper nanoparticles in concentrations of 40 μM or higher led to significantly reduced mitochondrial activity or reduced in DRG neurons indicating a reduction in metabolic activity or reduced cell viability. Thus this assay indicates that copper nanoparticles interfere with the reductase activity in mitochondria. This reduction in cell metabolism or viability could have resulted from an increase in oxidative stress. Several reports have shown that transition metals often result in cellular oxidative damage. The accumulation of transition metals in the cytosol may disrupt the intracellular redox status or alter protein conformation and inhibit protein function (Kawata and Suzuki 1983; Li et al. 1994). It has been reported that copper inhibits the mitochondrial dehyrdogenases both *in vitro* as well as *in vivo* and results in the generation of the reactive oxygen species (ROS) (Sheline and Choi 2004; Valko et al. 2005). When we stained the copper nanoparticles exposed neurons with rubeanic acid, intraneuronal presence of large deposits of copper was apparent. This copper inside neurons could have inhibited the mitochondrial dehydrogenases and resulted in ROS production. Various studies have shown the resulting cytotoxicity due to the initial induction of lipid peroxidation of the mitochondrial membrane by a metal which might cause decoupling of oxidative phosphorylation, disruption of electron transport, and a decrease in mitochondrial membrane potential (Freedman et al 1989; Saris and Skulskii 1991; Mattie and Freedman 2001). It has been suggested that neurodegenerative process associated with copper overload in Wilson's disease may be due to the mitochondrial damage, increased production of ROS, and failure of the antioxidant defense mechanisms (Gaetke and Chow 2003). Also, Pourahmad and O'Brien (2000) have shown that when isolated hepatocytes are incubated with copper, there is an immediate, rapid increase in ROS production. Thus, in our experiments, decreased neuronal viability following exposure to copper nanoparticles could also be due to an increase in the production of reactive oxygen species. Being a redox-active metal, capable of catalyzing the formation of hydroxyl radicals via a Haber-Weiss or Fenton-like reaction (Cadenas and Davies 2000; Valko et al. 2005), copper can also induce oxidative stress by depleting glutathione levels in neurons (Hultberg et al. 1997). Copper nanoparticles entering the cell therefore could target mitochondria and lead to an increase in oxidative stress.

A three-fold increase in the concentration of copper was seen in the mitoplasts of the SH-SY5Y neuroblastoma cells when exposed to 300 μM of copper sulphate (Arciello et al. 2005). Our results from rubeanic acid staining show the intracellular deposition of copper nanoparticles at 40–80 μM concentration. Nano-copper appears to enter neurons more readily and at lower concentrations than copper sulphate indicating that copper nanoparticles are capable of easily entering the cell similar to ionic copper.

The results from cell viability (MTS) and cell death (LDH) assays in our study were in accordance with each other. The cultures exposed to copper nanoparticles showed significantly less viability and more death of neurons when compared to those in unexposed control cultures. The higher the concentration and smaller the size, the greater was the neurotoxicity. The toxicity exerted by copper nanoparticles observed in our study was similar to other studies where micro/ionic copper was used (Freedman et al. 1989; Hultberg et al. 1997; Mattie and Freedman 2001; Arciello et al. 2005). It is interesting to note that when the sizes of particles

become small and eventually down to a nanoscale, copper becomes extremely reactive in a simulative intra body environment, and may even lead to changes in gene expression (Meng et al. 2007a, Wang et al., 2009).

In conclusion, our study demonstrated that exposure to copper nanoparticles resulted in significant toxicity to the cultured DRG neurons at concentrations of 40–100 μM but not at 10–20 μM. Although exposure to copper nanoparticles of sizes 40 nm, 60 nm, and 80 nm all had toxic effects on DRG neurons, copper nanoparticles of 40 nm and 60 nm sizes had higher toxic effect than the 80 nm-sized particles. Thus the toxic effect appears to be concentration and size-dependent. The mechanism underlying the toxicological effects observed with the exposure of DRG neurons to copper nanoparticles in the present study can be oxidative stress. Further studies identifying the subcellular location and dynamics of copper nanoparticles are needed to determine the specific mechanism of toxicity in DRG neurons.

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References

Aras MA, Hartnett KA, Aizenman E. Assessment of cell viability in primary neuronal cultures. Curr Protoc Neurosci 2008;44:7.18–7.18.15.

Arciello M, Rotilio G, Rossi L. Copper-dependent toxicity in SH-SY5Y neuroblastoma cells involves mitochondrial damage. Biochem Biophys Res Commun 2005;327:454–459. [PubMed: 15629136]

Bertinato J, L'Abbe MR. Maintaining copper homeostasis: Regulation of copper-trafficking proteins in response to copper deficiency or overload. J Nutr Biochem 2004;15:316–322. [PubMed: 15157936]

Brooking J, Davis SS, Illum L. Transport of nanoparticles across the rat nasal mucosa. J Drug Target 2001;9:267–279. [PubMed: 11697030]

Cadenas E, Davies KJ. Mitochondria free radical generation, oxidative stress and aging. Free Radic Biol Med 2000;29:222–230. [PubMed: 11035250]

Cecconi I, Scaloni A, Rastelli G, Moroni M, Vilardo PG, Costantino L, Cappiello M, Garland D, Carper D, Petrash JM, Del Corso A, Mura U. Oxidative modification of aldose reductase by copper ion. Definition of the metalprotein interaction mechanism. J Biol Chem 2002;277:42017–42027. [PubMed: 12183464]

Chen Z, Meng H, Xing G, Chen C, Zhao Y, Jia G, Wang T, Yuan H, Ye C, Zhao F, Chai Z, Zhu C, Fang X, Ma B, Wan L. Acute toxicological effects of copper nanoparticles in vivo. Toxicol Lett 2006;163:109–120. [PubMed: 16289865]

Chen Z, Meng H, Yuan H, Xing G, Chen C, Zhao F, Wang Y, Zhang C, Zhao Y. Identification of target of copper nanoparticles with ICP-MS technique. J Radioanalytical Nuclear Chem 2007;272:599–603.

Gaetke LM, Chow CK. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 2003;189:147–163. [PubMed: 12821289]

Curtis J, Greenberg M, Kester J, Phillips S, Krieger G. Nanotechnology and nanotoxicity: A primer for clinicians. Toxicol Rev 2006;25:245–260. [PubMed: 17288496]

Evans GW. Copper homeostasis in the mammalian system. Physiol Rev 1973;53:535–570. [PubMed: 4354642]

Freedman JH, Ciriola MR, Peisach J. The role of glutathione in copper metabolish and toxicity. J Biol Chem 1989;264:5598–5605. [PubMed: 2564391]

Galhardi CM, Diniz YS, Faine LA, Rodrigues HG, Burneiko RC, Ribas BO, Novelli EL. Toxicity of copper intake: Lipid profile, oxidative stress and susceptibility to renal dysfunction. Food Chem Toxicol 2004;42:2053–2060. [PubMed: 15500942]

Griffitt R, Weil R, Kelly H, Denslow ND, Powers K, Taylor D, Barber DS. Exposure to copper nanoparticles causes gill injury and acute lethality in Zebrafish (Danio rerio). Environ Sci Technol 2007;41:8178–8186. [PubMed: 18186356]

Gotz ME, Koutsilieri E, Riederer P, Ceccatelli S, Dare E. Methylmercury induces neurite degeneration in primary culture of mouse dopaminergic mesencephalic cells. J Neural Transm 2002;109:597–605. [PubMed: 12111452]

Guo K, Pand Q, Wang L, Fang S. Nano-scale copper-coated graphite as anode material for lithium-ion batteries. J Appl Electrochem 2002;32:679–685.

Halliwell B, Gutteridge JM. Role of free radicals and catalytic metals ions in human disease: An overview. Methods Enzymol 1990;186:1–85. [PubMed: 2172697]

Hoet PH, Bruske-Hohlfeld I, Salata OV. Nanoparticles-known and unknown health risks. J Nanobiotechnol 2004;2:12–27.

Hultberg B, Andersson A, Isaksson A. Copper ions differ from other thiol reactive metal ions in their effects on the concentration and redox status of thiols in HeLa cell cultures. Toxicology 1997;117:89–97. [PubMed: 9057888]

Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. Toxicology In Vitro 2005;19:975–983. [PubMed: 16125895]

Jeon KJ, Jeong JV, Jue DM. Thiol reactive metal compounds inhibit NFkB by blocking IkB kinase. J Immunol 2000;164:5981–5989. [PubMed: 10820281]

Kawata M, Suzuki KT. Relation between metal and glutathione concentrations in mouse liver after cadmium, zinc or copper loading. Toxicol Lett 1983;15:131–137. [PubMed: 6829037]

Li F, Zhou X, Zhu J, Ma J, Huang X, Wong ST. High content image analysis for human H4 neuroglioma cells exposed to CuO nanoparticles. BMC Biotechnol 2007;7:66–76. [PubMed: 17925027]

Li W, Kagan HM, Chou IN. Alterations in cytoskeletal organization and homeostasis of cellular thiols in cadmiumresistant cells. Toxicol Appl Pharmacol 1994;126:114–123. [PubMed: 8184421]

Liu G, Li X, Qin B, Xing D, Guo Y, Fan R. Investigation of the mending effect and mechanism of copper nanoparticles on a tribologically stressed surface. Tribol Lett 2004;17:961–966.

Mattie MD, Freedman JH. Protective effects of aspirin and vitamin E (alpha-tocopherol) against copper and cadmium-induced toxicity. Biochem Biophys Res Comm 2001;285:921–925. [PubMed: 11467839]

Meng H, Chen Z, Xing G, Yuan H, Chen C, Zhao F, Zhang C, Wang Y, Zhao Y. Ultra-high reactivity and grave nanotoxicity of copper nanoparticles. J Radioanalyt Nuclear Chem 2007a;271:595–598.

Meng H, Chen Z, Xing G, Yuan H, Chen C, Zhao F, Zhang C, Zhao Y. Ultra-high reactivity provokes nanotoxicity: Explanation of oral toxicity of nano-copper particles. Toxicol Lett 2007b;175:102–110. [PubMed: 18024012]

Munirathinam S, Lakshmana MK, Raju TR. (-) deprenyl attenuates aluminium induced neurotoxicity in primary cortical cultures. Neurodegeneration 1996;5:161–167. [PubMed: 8819137]

Murdock RC, Braydich-Stolle L, Schrand AM, Schlagger JJ, Hussain SM. Characterization of nanomaterials dispersion in solution prior to in vitro exposure using dynamic light scattering technique. Tox Sci 2008;101:239–253.

Nalbandyan RM. Copper in brain. Neurochem Res 1983;8:1211–1232. [PubMed: 6419137]

Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. Science 2006;311:622–627. [PubMed: 16456071]

Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 2005;113:823–839. [PubMed: 16002369]

Praetorius M, Brunner C, Lehnert B, Klingmann C, Schmidt H, Staecker H, Schick B. Transsynaptic delivery of nanoparticles to the central auditory nervous system. Acta Otolaryngol 2007;127:486–490. 2007. [PubMed: 17453474]

Pourahmad J, O'Brien PJ. A comparison of hepatocyte cytotoxic mechanisms for Cu2+ and Cd2+ Toxicology 2000;143:263–273. [PubMed: 10755712]

Saris NE, Skulskii IA. Interaction of copper with mitochondria. Acta Chem Scand 1991;45:1042–1046. [PubMed: 1662975]

Sheline CT, Choi DW. Cu2+ toxicity inhibition of mitochondrial dehydrogenases in vitro and in vivo. Ann Neurol 2004;55:645–653. [PubMed: 15122704]

Sokol RJ, Twedt D, McKim JM Jr, Deveraux MW, Karrer FM, Kam I, von Steigman G, Narkewicz MR, Bacon BR, Britton RS. Oxidant injury to hepatic mitochondria in patients with Wilson's disease and Bedlington terriers with copper toxicosis. Gastroenterology 1994;107:1788–1798. [PubMed: 7958693]

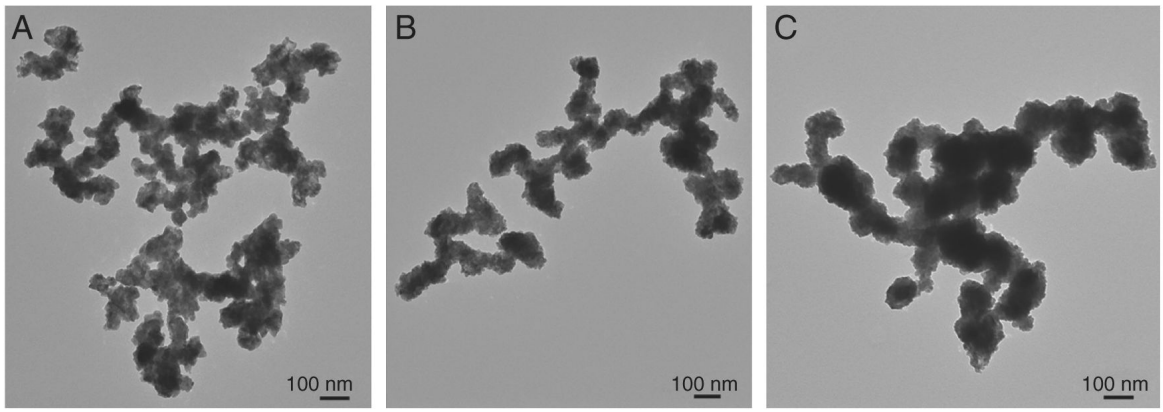
Sternlieb I. Copper and the liver. Gastroenterology 1980;78:1615–1628. [PubMed: 6245986]

Thornburg LP, Beissenherz M, Dolan M, Raisbeck MF. Histochemical demonstration of copper and copper-associated protein in the canine liver. Vet Pathol 1985;22:327–332. [PubMed: 2412331]

Wang J, Rahman MF, Duhart HM, Newport GD, Patterson TA, Murdock RC, Hussain SM, Schlager JJ, Ali SF. Expression changes of dopaminergic system related genes in PC 12 cells induced by manganese, silver and copper nanoparticles. NeuroTox. 2009 (in press).

Yang J, Okamoto T, Ichino R, Bessho T, Satake S, Okido M. A simple way for preparing antioxidation nano-copper powders. Chem Lett 2006;35:648–649.

Valko M, Morris H, Cronin MTD. Metals, toxicity and oxidative stress. Curr Med Chem 2005;12:1161–1208. [PubMed: 15892631]



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Particle	TEM	DLS		LDV		
	Size Distribution (nm)	Z-Average Diameter (nm)	PdI	Zeta Potential ζ (mV)	Electrophoretic Mobility U (μmcm/(Vs))	pH
Cu 40nm	55.2 ± 15.4					
DI H_2O		335	0.227	30.9	2.42	7.0
Neurobasal Media		386	0.322	* * *	* * *	7.58
Cu 60nm	69.0 ± 21.2					
DI H_2O		360	0.235	14.3	1.12	7.0
Neurobasal Media		401	0.217	* * *	* * *	7.58
Cu 80nm	70.6 ± 20.0					
DI H_2O		365	0.200	23.5	1.84	7.0
Neurobasal Media		409	0.208	* * *	* * *	7.58

Figure 1.

Characterization of copper nanoparticles. For TEM imaging (A–C), nanoparticles suspensions were dried on formvar/carbon film-coated Cu grids and all images taken at 100 kV accelerating voltage, as described in the *Materials and methods* section. 100 particles were measured per sample to obtain a size distribution. Distribution mean and standard deviation presented in associated table (D). For DLS and LDV, particles were dispersed in DI H_2O and neurobasal media. Mixing was done via vortexing, and samples were measured at a particle concentration of 50 μg/ml. (A) Cu 40 nm particles imaged at 25,000× magnification. (B) Cu 60 nm particles imaged at 25,000× magnification. (C) Cu 80 nm particles imaged at 30,000× magnification. (D) TEM size distribution, DLS, and LDV data for copper nanoparticles.

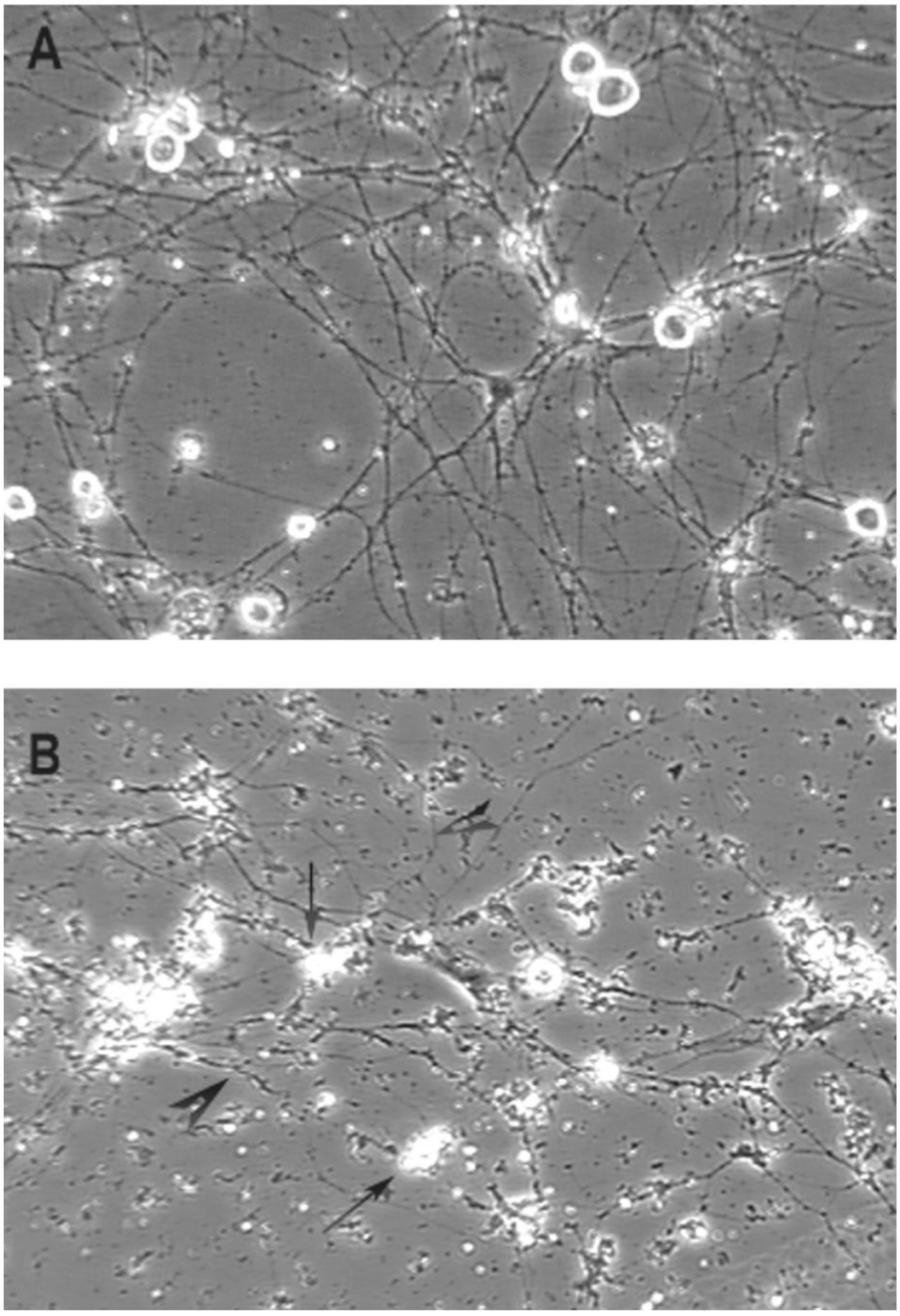

Figure 2.

Copper nanoparticles are toxic to sensory neurons as revealed by their morphology. Phase contrast images of DRG neurons in (A) show that in control conditions, neurons exhibit phase bright cell bodies, with long neurites indicating healthy neurons. In contrast, in (B) neurons which were exposed to copper nanoparticles (40 μM conc. and 40 nm in size) for 24 hours show morphological changes exhibiting bulged cell bodies of some neurons (arrows) along with disrupted neurites (arrow head) indicative of cell death.

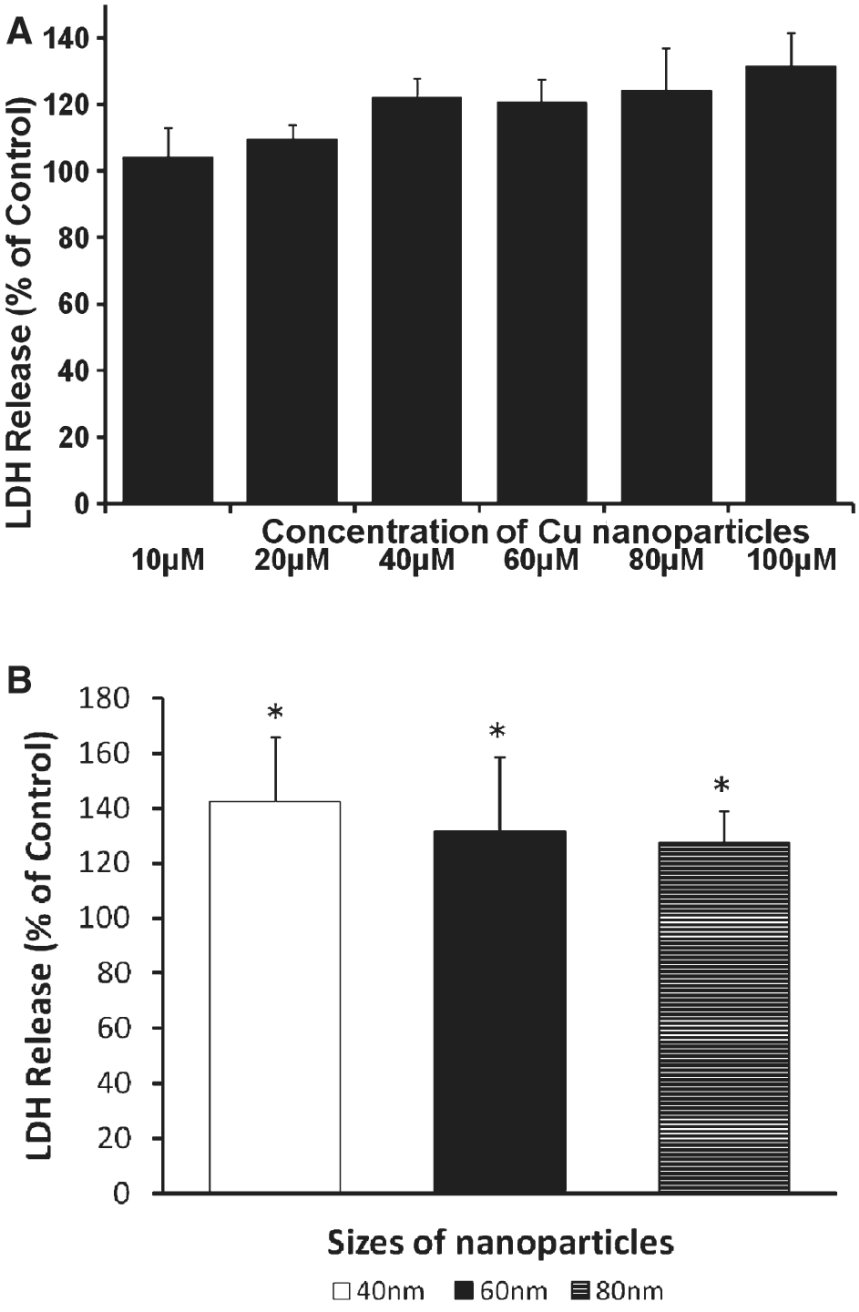

Figure 3.

Cell death resulting from exposure to copper nanoparticles is concentration- and size-dependent. In (A), levels of LDH released from DRG neurons exposed to different concentrations (10–100 μM) and in (B) exposure to different sizes (40–80 μM) of copper nanoparticles for 24 hours are shown. Data are displayed as percent of control concentrations and are mean ± SD. Data were analyzed by ANOVA with Tukey's post hoc test. In (A) LDH levels show that exposure to copper nanoparticles (80 nm size) at concentrations exceeding 20 μM resulted in significant neurotoxicity compared to unexposed neurons. [n = 6 independent experiments. F (6, 37) = 13.20, (**p < 6.19E-08)]. In (B), LDH levels were significantly high

in cultures exposed to copper nanoparticles (of 40 nM conc) when compared to control cultures for all the sizes tested [n = 10–12 independent experiments. F (3, 42) = 10.51, *p < 2.76E-05].

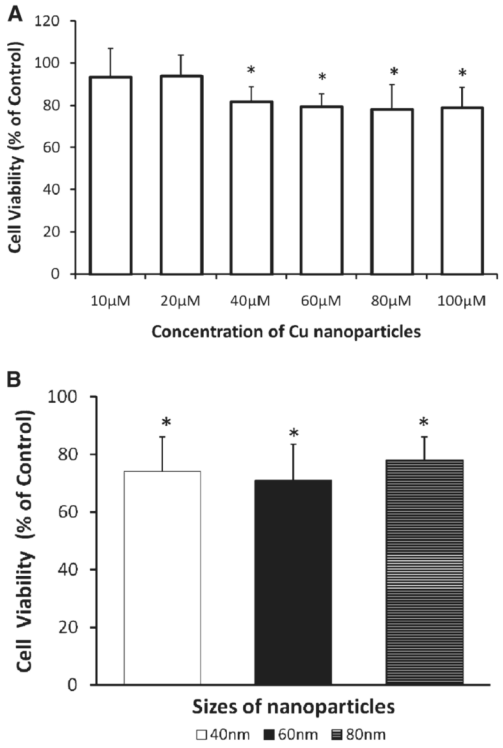

Figure 4.

When DRG neurons were exposed to copper nanoparticles, cell viability was reduced significantly in a concentration- and size-dependent manner as indicated by the results of the MTS (a tetrazolium dye-based) assay. Data are displayed as percentage of control concentrations and are mean ± SD. Data were analyzed by ANOVA with Tukey's post hoc test. (A) shows that at concentrations exceeding 20 μM, copper nanoparticles (of 80 nm size) caused a significant reduction in viability of DRG neurons when compared to that of unexposed, control neurons [n = 6–9 independent experiments. $F(6, 45) = 4.62$, (*$p < 0.001$)]. (B) shows that exposure to copper nanoparticles (40 μM concentration) of all the three sizes tested resulted in significantly reduced viability of the neurons when compared to that of unexposed control neurons,

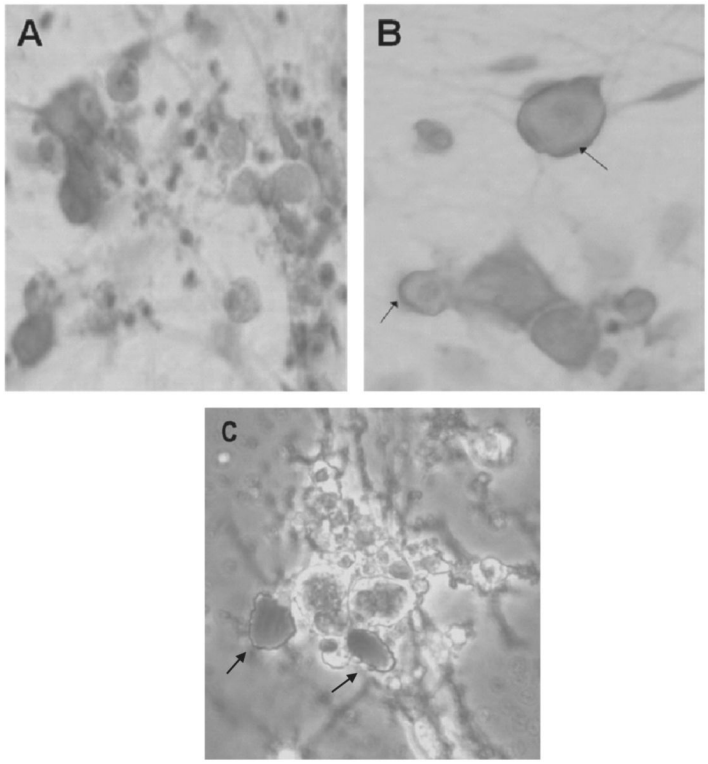

Figure 5.

Exposure to copper nanoparticles appears to result in the accumulation of copper in DRG neurons. DRG neurons unexposed (control) and exposed to copper nanoparticles, (experimental) were stained with rubeanic acid for copper and counter stained with neutral red. (A) shows control healthy neurons with cell bodies stained red exhibiting the presence of intact membrane and long neurites. (B) shows experimental neurons exposed to 40 μM of 40 nm size copper nanoparticles for 24 hours exhibiting rubeanic acid staining (greenish black) indicating deposition of copper along the cell membranes (arrows). (C) shows degenerating experimental neurons exposed to 80 μM of 40 nm size copper nanoparticles for 24 hours exhibiting increased rubeanic acid staining (greenish black) almost filling the entire cell indicating intercellular accumulation of copper nanoparticles (arrows) with disrupted neurites.