Cytotoxicity of Silver Nanoparticles (AgNPs) in Freshly-Prepared Isolated Rat Hepatocytes

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Abstract: Silver nanoparticles(AgNPs) are widely used in industrial, daily life, healthcare and medical products such as water purifiers, toys, sunscreen, wound dressings, urinary catheters and bone prostheses. So we need more studies about AgNPs adverse effect. In this study we discuss the effect of AgNPs (with mean particle size 20±1.2nm) at the cell level by using primary isolated rat hepatocyte. We highlight the AgNPs cytotoxicity by assessing cell viability through trypan blue exclusion test, determination of enzymatic leakage percent and mitochondrial damage through analysis of ALT and LDH enzymes release, assessment of oxidative stress by determination of reduced glutathione content and lipid peroxides formation, examination of histopathological changes under light and electron microscope. Results revealed a significant reduction in cell viability and glutathione level and significant increase in lipid peroxidation and enzymatic leakage levels in AgNPs- treated groups compared to control. Alteration in morphology and apoptosis were detected by light and electron microscopy in AgNPs- treated groups compared to control. In summary, these effects on the cell will help in understanding the mechanisms and risk assessment of AgNPs exposure in the future.
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1. Introduction

Nanoparticles (NPs) are materials manufactured within the nanoscale range of 1 to 100nm diameter. Since they possess novel physical and chemical functional properties, they have been used to create unique devices at the nanoscale level as well they have been introduced to many life and biomedical fields **(Colvin, 2003)**. In this respect, they have been used in gene therapy, drug design, modification of therapeutics, labeling of fluorescents, and tissue engineering **(Tian *et al.,* 2007; Yoon *et al.,* 2007; Kreuter and Gelperina, 2008; Su *et al.,* 2008; Hackenberg *et al.,* 2010)**. Among the nanoparticles, metal ones are widely used in different applications and the silver nanoparticles (AgNPs) have received the utmost attention for their excellent catalytic, optic, magnetic and antibacterial properties **(Kim *et al.,* 2006, Ng *et al.,* 2010)**. Silver nanoparticles have been used in the manufacture of disinfectants, shampoos, deodorants, humidifiers, wound dressings, textiles, paints, food packages, cosmetics, and medical devices products **(Johnston *et al.,* 2010; Zanette *et al.,* 2011)**; they have also been used as a coating for catheters, heart valves, and implants **(Chen and Schluesener, 2008; Chaloupka *et al.,* 2010)**.Despite their benefits, there has been serious concern about the possible side effects of AgNPs especially to workers at manufacturing plants as well to consumers of nanosilver-based products. These concerns have generated greater attention among public and scientists to evaluate the potential risks of AgNPs, especially their impacts on biological systems in order to facilitate the design of safer and more effective nanoparticles and nano-products in the future **(Zhang *et al.,* 2014)**.

Asharani *et al.* (2009 a,b) demonstrated that the uptake of AgNPs occurs by clathrin-mediated endocytosis and macropinocytosis. Once internalized intracellularly, AgNPs are degraded to release silver ions which interfere with normal mitochondrial functions and induce apoptotic cell death. Also, Silver nanoparticles stimulate stress pathways and result in free radical and cytokine production. Taking together, different studies suggest that AgNPs are cytotoxic and pro-inflammatory in nature **(Yang *et al.,* 2012; Jiang *et al.,* 2013)**.

The most common route of exposure among workers who handle and/or produce nanomaterials is thought to be inhalation **(Lee *et al.,* 2010)**. *In vivo* experiments in mice and rats have suggested that inhaled AgNPs are able to cross the blood–brain barrier as well reach several organs, including the lungs and liver **(Genter *et al.,* 2012)**. Previous studies have shown a high accumulation of silver NPs in the liver after injection **(Hirn *et al.,* 2011)**, while higher retention of particles in the liver was noticed after ingestion **(Schleh *et al.,* 2012)**. Generally, for any exposure route that involved translocation of nanoparticles to the bloodstream, liver is one of the most important targets, and appears to be the major

accumulation site of circulatory silver nanoparticles **(Takenaka *et al.*, 2001)**. Consequently, liver was selected in this study to be the target organ in order to identify adverse effects of silver nanoparticles on isolated rat hepatocytes.

The increased use of nanomaterials, and exposure of humans and the environment, necessitates the availability of reliable, cost- and time-effective screening mechanisms for NP toxicology.*In vivo* animal studies are expensive and time-consuming, plus there is continuous pressure from regulators and public to reduce their use in research. On the other hand, *in vitro* toxicity testing models accurately predicted *in vivo* toxicity and gained an advanced rank in the field of nanoparticles risk assessment **(Stone et al., 2009)**. *In vitro* nanotoxicity testing assays became important tools nowadays since they are fast, cheap and greatly control the experimental conditions. They imitate the *in vivo* realistic environment accurately; thereby reduce the number of laboratory animals required for testing **(Marquis *et al.*, 2009)**. Also, they predict to a large extent the possible starting doses for further *in vivo* acute toxicity testing as well reveal the mechanism of nanoparticles' action inside tested cells. For in vitro studies with NPs, cell-based assays are presently considered essential to toxicity testing **(Nel *et al.*, 2006; Lewinski *et al.*, 2008)**. Among cells, primary cell lines are representative of tissue and hence they are the most ideal for *in vitro* toxicity studies.

Up to these days, isolated and cultured hepatocytes represent the most commonly used *in vitro* model than precisely-cut liver slices and isolated perfused organ, since the liver is the gold-standard organ in biotransformation processes of different toxins. The freshly-prepared isolated liver cells suspension is much easier to prepare, after the vast modifications have been introduced to the technique **(El-Tawil and Abdel-Rahman 1997)**, than the hepatic slices and display a remarkable range of activities. Millions of isolated hepatocytes could be generated from a single liver, and then could be aliquoted and a significant combination of toxicity testing assays could be applied on the cells. In this study we aimed to determine the cytotoxicity of freshly-prepared isolated rat hepatocytes exposed to silver nanoparticles. Cytotoxicity end points were evaluated by Trypan-blue exclusion assay, cytosolic enzyme leakage percentage [Alanine aminotransferase (ALT), and Lactate dehydrogenase (LDH)], reduced glutathione (GSH) level and thiobarbituric acid reactive substances (TBARS) accumulation. Light and electron microscopical examination of AgNPs-treated cells was applied and results were compared to control group. Since scarce information of nanosilver toxicity is available, a simple *in vitro* toxicity testing model and general cytotoxicity parameters were chosen to help in understanding the mechanism of action after nanoparticles exposure. To our knowledge, there are no studies have been conducted on the cytotoxicity of AgNPs using isolated rat hepatocytes.

2. Materials and methods

2.1 Experimental animals

Adult male Sprague–Dawley rats, weighing 200-250 g, were used in this study; the animals were supplied from the animal house of department of Toxicology and Forensic Medicine, Faculty of Veterinary Medicine, Cairo University, Egypt. The animals were housed in stainless steel cages in a temperature- controlled (25±2 ◦C temp) environment and provided free access to pelleted food and purified drinking water. All animals received humane care in compliance with the regulations of Ethics of Animal Use in Research Committee (EAURC) in Egypt.

2.2. Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, USA), Aldrich (Germany) and El-Nasr Co. (Cairo).

2.3. Preparation of silver nanoparticles

Silver nanoparticles (AgNPs) were prepared from silver nitrate in form of nanospheres of mean size 20 ± 1.2 nm as revealed by high resolution TEM (JEOL, Japan). Upon preparation, the silver nanoparticles were re-suspended in distilled water in a concentration of 10µg/ml

2.4. Isolation of Hepatocytes

The hepatocytes were isolated by a collagenase two-step perfusion technique **(Berry and Friend, 1969)** with slight modifications as published by **El-Tawil and Abdel-Rahman (1997)**. The rat was anaesthetized with 100 mg ketamine/ kg, restrained, and an incision was made in the abdominal cavity to expose the portal vein. A polyethylene cannula was inserted into the portal vein and the liver was perfused *in situ* for 8 min with calcium-free Hank's bicarbonate buffer maintained at 37°C. The liver was then mechanically dislocated from the abdomen with the cannula in place and recirculated for 10 min in collagenase (0.67mg/ml) containing 5 mM calcium chloride. The isolated liver cells were filtered through four layers of cotton gauze and centrifuged for 2 min at 600 rpm. The cells were washed twice and suspended in HEPES-bicarbonate buffer (pH 7.4) containing 0.5% bovine albumin. The isolated hepatocytes were counted in a hemocytometer, while the viability of the cells was assessed by 0.4% Trypan Blue in Krebs Hanseliet buffer without albumin **(Baur *et al.*, 1985)**. Each freshly prepared cell suspension had 90% or greater viability prior to each experiment.

2.5. Incubation and treatment of hepatocytes

Freshly isolated hepatocytes ($5*10^6$cells/ml) were suspended in a HEPES-bicarbonate buffer (pH 7.4) and incubated 37°C in a shaking water bath at 30 oscillations per minute. Hepatocytes were incubated in plastic vials equipped with covers and were used for assessment of cytotoxicity of silver nanoparticles (at size range 20 ± 1.2nm) using two different concentration of AgNPs (1 µg and 2 µg/ml cell suspension). Six replicates were used for each group; each replicate contained 2ml of isolated rat hepatocytes ($1*10^7$ cells). Cytotoxicity of AgNPs was determined by assessing of cell viability using trypan blue exclusion method, cytosolic enzymes leakage percent [alanine aminotransferase (ALT) and lactate dehydrogenase (LDH)], reduced GSH content and thiobarbituric acid reactive substances (TBARS) accumulation. Cytopathological evaluation of isolated hepatocytes exposed to AgNPs was also evaluated by light and electron microscopes. Control replicates were carried out simultaneously under the same conditions and at the same time intervals (0 and 120 min).

2.6. Biochemical measurements

2.6.1 Trypan Blue Exclusion Test (Viability percent)

The viability percent of hepatocytes was assessed by the method of **Baur *et al.* (1985)**. After the incubation of isolated rat hepatocytes with AgNPs (1 µg and 2 µg/ml cell suspension), samples of cell suspension were collected at different time intervals (0 and 120 min). Aliquots (10µl) of hepatocytes were taken from each replicate and the same volume (10µl) of 0.5% trypan blue was added on the hepatocytes over a slide. Each sample was immediately mixed and covered by cover glass and examined under the light microscope. Three fields were counted from each slide for the viable and died cells. The viability percentage was calculated according to the following formula:

Viability % = (No of viable cells / No of total counted cells) ×100

2.6.2 Enzyme leakage percent

Enzymes activity (ALT) and (LDH) was monitored using Sigma–Aldrich ready-made kits (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) in an aliquot of cell-free medium then compared to the total activity achieved after lysis of the cells **(Moldeus *et al.*, 1978)**. The cell-free media were obtained by centrifugation of the aliquots at 2200 rpm for 15 minutes, while lysates were obtained by addition of 1% triton X-100 and shaking for 15 minutes followed by centrifugation at 2200 rpm. The leakage was expressed as percentage of total lysate activity at each time point. Determination of LDH activity was done using a test reagent kit according to the German Society for clinical chemistry **(Bergmeyer *et al.*, 1970)** while determination of ALT activity was done using a test reagent kit according to **Reitman and Frankel (1957)**.

2.6.3 Glutathione (GSH) assay

Reduced GSH levels were determined by measuring total soluble-reduced sulfhydryl content. Aliquots were collected at specified time points (0 min and 120 min) and centrifuged with phosphate buffer saline (PBS) at 3000 g for 5 minutes. The obtained precipitate was mixed with 0.7 ml of 0.2% triton X-100 and 2.5% sulfosalicylic acid and the solutions were centrifuged at 3000g for 5 minutes. A 0.5 ml aliquot of the acid-soluble supernatant medium was then added to 1ml of 0.3 M Na_2HPO_4 solution. Spectrophotometric determinations were performed at 412 nm after the addition of 0.125 ml of 5,5`-dithiobis-(2-nitrobenzoic acid) **(Beutler *et al.*, 1963)**.

2.6.4 Lipid peroxidation assay

Lipid peroxidation was assessed by determining thiobarbituric acid reactive substances (TBARS) in hepatocyte culture media by the method of **Uchiyama and Mihara (1978)**. Briefly, 2.5ml of TCA and 1ml of 0.6% thiobarbituric acid were added to 0.5ml sample or standard in a centrifuge tube. The mixture was heated for 20 minutes in a boiling water bath then cooled before 4 ml n-butanol were added and mixed vigorously. The upper n-butanol layer was separated by centrifugation at 2000 rpm for 10 minutes and the absorbance of the pink colored product was measured at 535nm against distilled water blank using spectrophotometer. The concentration of TBARS in the test samples was expressed as nmol/ml

2.6.5 Cytopathological evaluation of isolated hepatocytes

2.6.5.1 Light Microscopy

Ten µl of cell suspension from all replicates of each group at different time intervals (0 and 120 min) were placed on a glass slide and left to dry at room temperature. The cells were fixed by adding 10µl of absolute ethanol on the dried film and left to dry at room temperature. The slides were stained with haematoxylin and eosin according to **Kieman, 1999**.

2.6.5.2 Electron Microscopy

At the end of experiment after 120 minutes, cell suspensions from all replicates of each group, were centrifuged at 2000 rpm for 30 minutes to obtain cell pellets. They were fixed by adding 1% paraformaldehyde and 0.5% glutraldehyde in 0.1mol/l sodium cacodylate buffer (pH 7.4) for 24 hours at 4˚C. The cells were washed with the buffer and post fixed in 1% osmium tetroxide plus 1.5% potassium ferrocyanide overnight at 4˚C. The cells were then dehydrated with ascending grades of ethanol and then put in propylene oxide to prepare Epon-Araldit resin blocks. Ultrathin sections (70-90 nm) were cut and stained by uranyl acetate and lead citrate and

examined under transmission electron microscope according to **Glauret and Lewis, 1998**.

2.7. Statistical analyses

The obtained values were presented as means ± SE of the mean. Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by LSD comparisons test. The level of significance was set at P <0.05 using SPSS software (version 16, IBM Corp. NY, USA).

3. Results

3.1. Viability of isolated rat hepatocytes

Viability of hepatocytes was assessed by trypan blue exclusion assay after exposing the cells to different doses of silver nanoparticles (1 and 2 µg/ml of cell suspension). The viability of treated cells was significantly and progressively decreased in a time-dependent manner immediately after exposure to AgNPs compared to control cells (Fig.1), while the viability of exposed cells to higher concentration of AgNPs (2 µg/ml) was insignificantly different than that of the lower dose (1 µg/ml) at different time points (0 and 120 minutes).

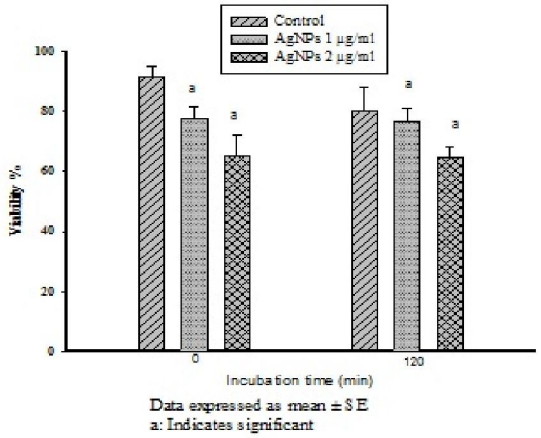
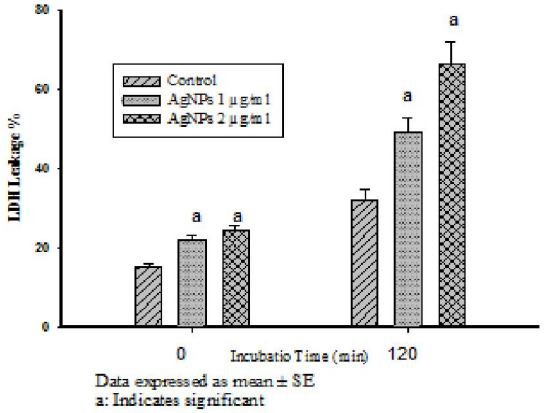


Figure 1: Trypan blue exclusion cell viability assay shows significant gradual drop in cells viability. They axis represents the viability percent of cells excluded trypan blue dye, while the x axis represents the time of incubation for different groups (0 and 120 minutes).The different patterns of the bars identify different groups; control group and treated groups with 1µg and 2µg AgNPs respectively. The values represent the mean ± standard error (SE) of three experiments; (a) small letter denotes P<0.05 level of significance between untreated and AgNPs treated groups.

3.2. Enzyme Leakage percent (LDH and ALT)

Leakage of hepatocytes enzymes may point to damage of their plasma membrane. This leakage was assessed by monitoring LDH and ALT enzymes leakage from isolated liver cells exposed to silver nanoparticles. Figure (2A) and (2B) illustrate the time- and dose-dependent leakage of LDH and ALT respectively in the media of AgNPs-treated hepatocytes compared to control group. Exposure of cells to 1 µg/ml of silver nanoparticles up to 120 minutes resulted in significant increase in the leakage of both enzymes into the culture medium, while higher dose of AgNPs (2µg/ml hepatocyte suspension) caused higher leakage of LDH and ALT enzymes than the lower dose of AgNPs as well the control groups.

A



B

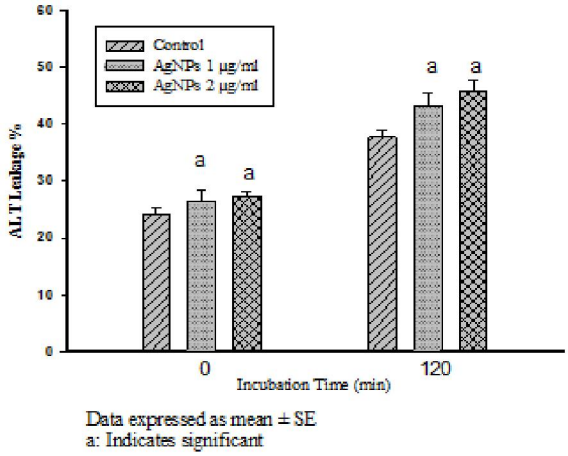


Figure 2: Enzymatic leakage assay of LDH (A) and ALT (B) shows significant gradual increase of hepatic membrane LDH and cytoplasmic ALT enzymes outside the cells in the media. The *y*axis represents the percent of cellular leakage, while the *x* axis represents the time of incubation for different groups (0 and 120 minutes).The different patterns of the bars identify different groups; control group and treated groups with 1µg and 2µg AgNPs respectively. The values represent the mean ± standard error (SE) of three experiments; (a) small letters denote$P$<0.05 level of significance between untreated and AgNPs treated groups.

3.3. Glutathione (GSH) depletion

Depletion of glutathione (GSH) is one of the parameters indicate onset of oxidative stress inside cells. In order to understand the mechanistic outlines of silver nanoparticles on isolated rat hepatocytes, cellular glutathione contents were determined in

AgNPs treated cells and compared to control. Figure (3A) demonstrates that exposure to silver nanoparticles started with marked decrease of glutathione levels and ended with significant depletion of GSH stores in isolated rat hepatocytes after 120 minutes incubation compared to control group.

3.4. Lipid peroxide contents

Lipid peroxidation is another parameter that indicates onset of oxidative stress inside cells and help too in understanding the mechanism of action of silver nanoparticles on isolated liver cells. Upon exposure of cells to AgNPs, increase in lipid peroxide formation was achieved as indicated by elevation of TBARS levels inside the cells, but this accumulation was not statistically significant when compared to control group (Fig. 3B).

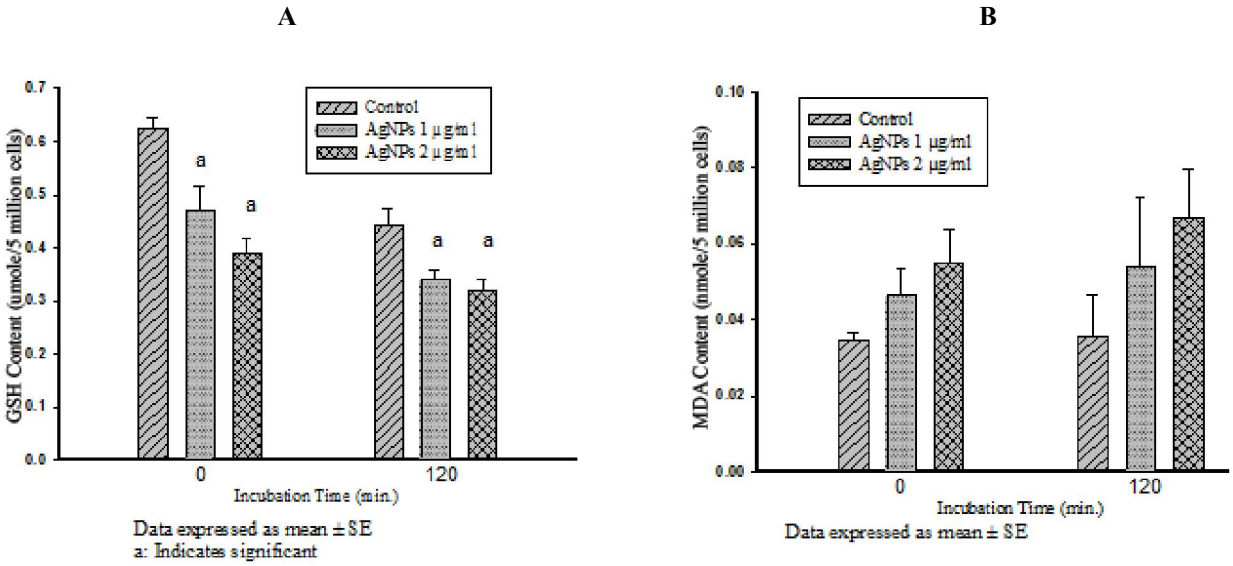


Figure 3: Oxidative stress results as revealed by quantification of cellular reduced glutathione GSH(A) and lipid peroxides formed as thiobarbituric acid reactive substances (TBARS)and measured as Malondialdehyde MDA(B). The results show significant depletion of GSH in AgNPs-treated cells compared to control group. In contrast, MDA was accumulated in treated cells but its levels were not significantly different compared to controls. The y axis represents the quantity of cellular GSH (A) and lipid peroxides (expressed as MDA content) (B), while the x axis represents the time of incubation for groups (0 and 120 minutes). The different patterns of the bars identify different groups; control group and treated groups with 1µg and 2µg AgNPs respectively. The values represent the mean ± standard error (SE) of three experiments; (a) small letters denote P <0.05 level of significance between untreated and AgNPs treated groups.

3.5. Cytopathological evaluation of isolated hepatocytes

3.5.1. Light microscopical evaluation of isolated hepatocytes

The histopathological examination of the control group revealed normal structure of hepatocytes. It appeared oval in shape with homogenous eosinophilic cytoplasm and basophilic nucleus; either single or binucleated cells (Figure 4A). The treated hepatocytes with silver nanoparticles revealed histopathological alterations varied in the degree of severity according to the concentration of AgNPs and incubation time; mostly vacuolar degeneration. The hepatocytes treated with 1µg/ml of silver nanoparticles revealed small vacuoles in the cytoplasm (Figure 4B) while the vacuoles increased in size, numbers and distribution in hepatocytes treated with2 µg/ml silver nanoparticles (Figure 4C)

3.5.2. Electron microscopical evaluation of isolated hepatocytes

Electron microscopical examination of hepatocytes after incubation of 120 minutes showed normal cellular structure of control group without any pathological changes (Figure 5A), while AgNPs-treated group showed different pathological alterations according to concentration and particles size of silver. Isolated hepatocytes exposed to 1 µg/ml AgNPs showed low numbers of cytoplasmic vacuoles, moderate mitochondrial degeneration and nuclearpyknosis (Figure 5B), while higher dose of AgNPs (2 µg/ml hepatocyte suspension) showed high numbers of cytoplasmic vacuoles, sever mitochondrial degeneration and nuclear karyorrhexis (Figure 5C).

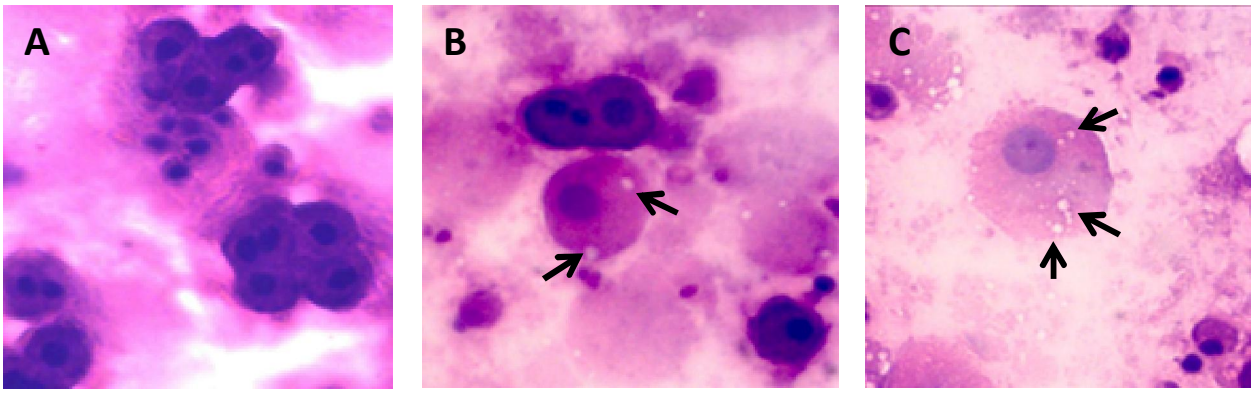


Figure 4: Light microscope images of cell smears after 120 minutes incubation. Untreated cells showed normal histological structure with no abnormalities (A), whereas cells treated with 1µg/mL AgNPs showed moderate number of vacuoles inside their cytoplasm (B). Higher dose of AgNPs (2µg/mL) showed increased number of vacuoles (C). All cells were stained with H&E and photographed at 400x

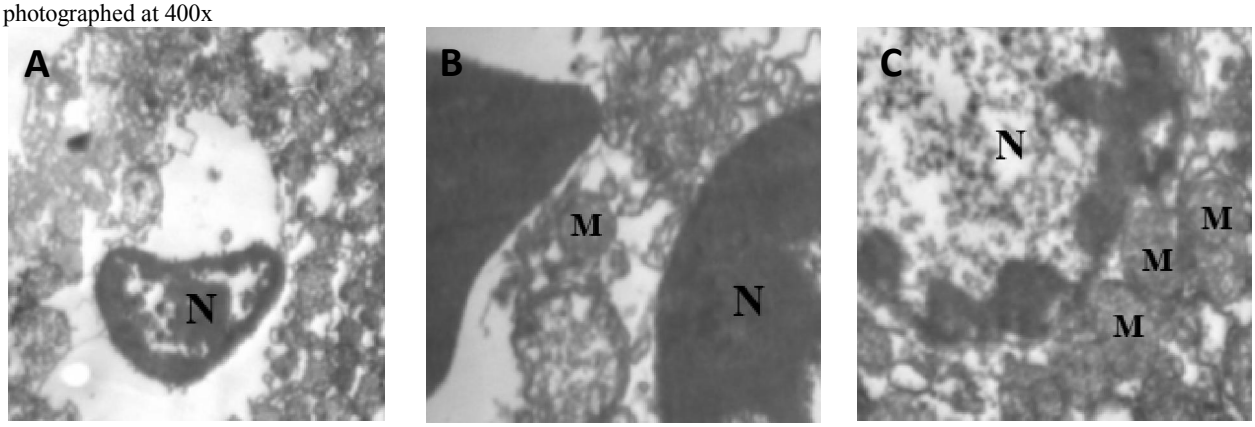


Figure 5: TEM images of ultrathin sections of cells. Untreated cells showed no abnormalities (5A), whereas cells treated with 1µg/mL AgNPs showed low numbers of cytoplasmic vacuoles, degeneration of some mitochondria (M) and nuclear **(**N**)** pyknosis (5B). Treated cells with higher doses of AgNPs showed multiple numbers of cytoplasmic vacuoles, degeneration of large number of mitochondria (M) and nuclear (N) karyorrhexis (5C)

4. Discussion:

Nanoparticles (NPs) are being used in diverse applications such as medicine, clothing, cosmetics and food. In order to promote the safe development of nanotechnology, it is essential to assess the potential adverse health consequences associated with human exposure. Silver nanoparticles (AgNPs) are presently the most commercialized of all nanomaterials and have a wide range of applications in electronics, paints, clothing, food, cosmetics, and medical devices **(Ng *et al.*, 2010)**. Nevertheless, biological and toxicological studies on AgNPs are still few, and the toxic properties of various forms of AgNPs have not been clearly defined yet **(Ahamed *et al.*, 2010; Johnston *et al.*, 2010).** Thus before incorporating AgNPs into our daily lives, it is necessary to determine its cytotoxic potentials on different organs and cells. Silver nanoparticles have been shown to be highly toxic not only to bacteria and fungi but also to a number of animal species and cultured cells **(Gaiser *et al.*, 2012).** Following the entry of silver nanoparticles into systemic circulation, they may migrate to liver, spleen, lungs, kidneys and brain and induce different toxicities **(Rahman *et al.*, 2009; Tang *et al.*, 2009)**. Other experimental studies showed that systemically administered AgNPs may translocate to various body compartments and induce different inflammatory and cytotoxic effects including hepatotoxicity **(Tiwari *et al.*, 2011)**. The liver is the target site for nanoparticles toxicity after ingestion, inhalation or absorptionand the hepatocyte bioassay has previously been shown to be capable of detecting toxic endpoints of single chemicals and environmental samples **(Tollefsen *et al.*, 2006)**. On the other hand, most studies were done using *in vitro* cultured hepatocytes or *in vivo* rodent models. In this study, all result data was generated using isolated rat hepatocytes as an important and informative *in vitro* model for assessing the impact of AgNPs on human and environmental health. It was hypothesized that AgNPs-induced cytotoxicity involves reactive oxygen species (ROS)-dependent pathway, cell cycle arrest

and intracellular liberation of Ag^+ ions. Some experimental studies demonstrated the discharge of Ag^+ ions from Ag NPs in simple salt solutions and culture medium **(Kittler *et al.*, 2010; Liu *et al.*, 2010; Miao *et al.*, 2010; Sotiriou and Pratsinis, 2010; George *et al.*, 2012)**. Additionally, intracellular AgNPs impaired cell membrane integrity and inflammatory responses **(Yang *et al.*, 2012; Jiang *et al.*, 2013)**. Depending on the size and surface modification of AgNPs internalized, silver nanoparticles can reach and enter the mitochondria as well the cellular nuclei**(AshaRani *et al.*, 2009b)**.

The purpose of the present study was to evaluate possible potential cytotoxicity and the general mechanism involved in silver nanoparticles toxicity. Since there are few studies investigating the in vitro toxicity of nanomaterials in primary isolated liver cells, we exposed our isolated rat hepatocytes to AgNPs in concentration of 1, and 2 µg/ml of freshly-prepared cell suspension. The results of**Asare *et al.* (2012)** suggested that silver nanoparticles were toxic and cytostatic as well caused apoptosis, necrosis and decreased proliferation of testicular cells. In this study, cytotoxicity of AgNPs was indicated by a significant decrease in the viability of hepatic cells, as well a significant increase in the leakage of intracellular enzymes (LDH, and ALT) into the incubation medium compared to control group. These data reflected that the cellular membrane integrity might be lost and consequent uptake of trypan blue dye inside the cytoplasm of affected cells as well outflow of their intracellular enzymes occurred. After 120 minutes of incubation, both concentration of silver nanoparticles caused clear reduction in viability of exposed hepatocytes and consequent frank induction of their LDH and ALT enzymatic leakages, compared to control group. On the other hand, comparing both concentration of AgNPs used in this study revealed that there was non-significant difference between both doses of silver nanoparticles treated groups in decreasing viability or increasing LDH and ALT enzymatic leakage. These results are in agreement with **Hussain *et al.*, 2005** who revealed that silver nanoparticles caused a significant increase in LDH leakage in rat hepatic cells exposed to silver nanoparticles *in vitro* compared to control group.

Further studies were conducted with silver nanoparticles in order to elucidate a general mechanism with reference to oxidative stress. In this study, exposure of primary isolated rat hepatocytes to AgNPs significantly depleted cellular GSH content in time- dependent but not in dose-dependent manner compared to control group. **Eom and Choi, 2010** concluded that internalization of NPs can induce stress responses due to stimulation of free radical production, which in turn, stimulates inflammatory signaling pathways. Hence, the production of reactive nitrogen species (RNS), ROS and cytokines following AgNPs exposure were directly correlated with cytotoxicity of AgNPs as reported by **Lu *et al.*, 2009**. Additionally, electron spin resonance has indicated that the active surface of AgNPs can directly induce the generation of original free radicals, and the dissolution of AgNPs into Ag^+ ions triggers the production of hydroxyl radicals in acidic endosome/lysosomes **(He *et al.*, 2012)**. On the other hand, our study revealed that both treatments of AgNPs increased lipid peroxides accumulation inside treated cells in dose- and time-dependent manner, but this accumulation was not statistically significant when compared neither to control nor to different doses of AgNPs used in this study.On the cytological level, treated groups with different concentrations of AgNPs induced morphological and pathological changes in cell structure in forms of vacuoles formation after 120 minutes. The number of vacuoles increased in dose-dependent manner by increasing concentration of silver nanoparticles. **Arora *et al.*, 2008** revealed that with high concentrations of AgNPs, the morphology of epithelial cells changedand became less polyhedral and more fusiform, shrunken, and rounded. The author stated that the reason for this was the higher levels of oxidative stress caused by AgNPs in forms of decreased glutathione (GSH) and superoxide dismutase (SOD) and increased lipid peroxidation, which finally leads to apoptosis by increasing caspase-3 activity and DNA fragmentation.

Also, cytopathological evaluation of isolated hepatocytes exposed to AgNPs was assessed by electron microscope. Exposure of isolated rat hepatocytes to silver nanoparticles for 120 minutes resulted in formation of cytoplasmic vacuoles, and mitochondrial degeneration. The severity of cytoplasmic vacuolation and degeneration of cellular mitochondria was dose-dependent where cells exposed to 2µg/ml AgNPs showed higher numbers of vacuole and degeneration of large numbers of mitochondria than cells exposed to 1µg/ml of AgNPs. Moreover, exposure to the lower dose of silver nanoparticles caused irreversible condensation of chromatin in the nucleus of cell which called karyopyknosis, while treated cells with the higher dose of AgNPs resulted in karyorrhexis; destructive fragmentation of the nucleus through the cytoplasm of dying cells. **Asharani and his colleagues (2009b)** treated human fibroblastsas wellglioblastoma cells (1.5x106) to Ag-starch nanoparticles (net concentration of 100µg/ml) for 48 hours. They detected a significant distribution of silver nanoparticles throughout the cytoplasm, inside lysosomes, and nucleus. In our study we used about 7 times more cells as well 50 times less concentration than used by **Asharani *et al.* (2009 b)**. This may

explain our inability to detect silver nanoparticles inside the isolated rat hepatocytes, but we detected signs of apoptosis and degeneration.

5. Conclusion

The present study concludes that silver nanoparticles are cytotoxic to isolated rat hepatocytes as revealed by cell viability, enzyme leakage, glutathione depletion and lipid peroxidation assays. Moreover, light and electron microscopy studies confirmed the same conclusion. These findings suggest and confirm that further studies must be conducted in this field to achieve the deeper understanding of silver nanoparticles toxicity.

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