Background: Arsenic, an environmental pollutant, is a carcinogenic metalloid and also an anticancer agent.

Objective: To evaluate the toxicity of arsenic nanoparticles in rat hepatocytes.

Methods: Freshly isolated rat hepatocytes were exposed to 0, 20, 40, and 100 µM of arsenic nanoparticles and its bulk counterpart. Their viability, reactive oxygen species level, glutathione depletion, mitochondrial and lysosomal damage, and apoptosis were evaluated.

Results: By all concentrations, lysosomal damage and apoptosis were clearly evident in hepatocytes exposed to arsenic nanoparticles. Evaluation of mitochondria and lysosomes revealed that lysosomes were highly damaged.

Conclusion: Exposure to arsenic nanoparticles causes apoptosis and organelle impairment. The nanoparticles have potentially higher toxicity than the bulk arsenic. Lysosomes are highly affected. It seems that, instead of mitochondria, lysosomes are the first target organelles involved in the toxicity induced by arsenic nanoparticles.

Keywords: Arsenic; Nanoparticles; Oxidative stress; Chemical and drug induced liver injury; Apoptosis

Introduction

As a major toxic metalloid, arsenic exists in air, water and soil. Exposure to higher-than-acceptable level of arsenic occurs either in workplace, eg, in smelting industries, coal-fired power plants, cosmetic industries, agriculture, etc, or through arsenic-contaminated food or drinking water.1,2 Arsenic is a carcinogen to both humans and animals and classified by the International Agency for Research on Cancer in group I of human carcinogens.3 Food and water contaminated with arsenic was shown to be linked to increased incidence of cancerous (eg, liver, skin, lung, urinary and bladder) and non-cancerous conditions (eg, diabetes mellitus, skin lesions, and peripheral vascular disease).4,5

Oxidative damage that has arisen from reactive oxygen species (ROS) is considered a central mechanism for arsenic pathogenesis.6 Intracellular accumula-

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tion of ROS leads to disruption of mitochondrial membrane potential, release of cytochrome c, activation of the caspase cascades, and ultimately, cell death. Moreover, during its cycles between different oxidative states, arsenic produces ROS and causes organ toxicity. Glutathione (GSH) is a ubiquitous essential tripeptide that protects cells against oxidants, electrophilic compounds and xenobiotics. GSH has been implicated in the protection of cells against cytotoxicity and in the metabolism of xenobiotics, including arsenic, through detoxification of reactive intermediates. GSH is produced in all organs, especially in the liver. Measurement of both reduced and oxidized glutathione (GSSG) has been considered an index for overall redox status and toxicity.

Insufficient maternal education and low socioeconomic condition have been linked to higher blood and urine arsenic levels in pregnant women living in gold mining areas in Tanzania. Epidemiologic studies on environmental and occupational exposure (metal smelting, wood treatment, pesticide application) to arsenic indicate a significant association between inorganic arsenic concentration in urine and proteinuria. Although arsenic can be toxic and arsenic exposure from industrial or natural sources can cause serious toxicities, it has been employed since more than 2400 years ago for therapeutic purposes. In treatment of cancers including esophageal cancer, chronic myeloid leukemia (CML), lymphoma, and particularly acute promyelocytic leukemia (APL), arsenic is likely to be effective through the induction of apoptosis.

In medicine, nanotechnology has considered a platform for delivering therapeutic agents in cancer imaging and treatment. Nanomaterials are engineered particles with dimensions <100 nm. These materials are increasingly being used for commercial purposes such as fillers, catalysts, opacifiers, semiconductors, microelectronics, cosmetics, and drug carriers. Nanomaterials have distinctive physicochemical properties such as large surface area to mass ratio, ultra-small size, and high reactivity. These properties can be used to overcome some of the restrictions exist in traditional therapeutic and diagnostic agents. Nanoparticles are designed to safely reach their targets, specifically release their cargo at the site of the disease, and finally promote the drug’s tissue bioavailability. The widespread use of nanomaterials in industrial and medical procedures created a number of toxic or adverse effects that are collectively discussed under the term “nanotoxicology.”

In this regard, materials like arsenic are more interesting due to their use in both industry and medicine. The nano form of these materials may induce relatively unidentified toxicities, even though they are provided with better physicochemical properties and kinetics. Arsenic nanoparticles have recently been reported to exert improved therapeutic activity. They are more effective than As (III) against visceral leishmaniasis; it is claimed that biocompatible arsenic nanoparticles along with reduced toxicity to normal cells, are as effective as bare As$_2$O$_3$ nanoparticles on prostate cancer cell lines.
this study to test if arsenic nanoparticles could damage hepatocytes more than that its bulk size does in freshly isolated rat hepatocytes.

Materials and Methods

Chemicals

Sodium arsenite (NaAsO₂) was purchased from Sigma Chemical Co. Collagenase (from Clostridium histolyticum) and HEPES were provided from Roche (Montreal, Canada). Rhodamine 123 was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Acridine orange and dichlorofluorescin diacetate were purchased from Molecular Probes (Eugene, Ore, USA). Glutathione (reduced), glutathione (oxidized), N-ethylmaleimide (NEM), O-phthalaldehyde, trypan blue, trichloroacetic acid (TCA) and heparin were obtained from Sigma (St. Louis, MO, USA). All chemicals used were of high analytical grade.

Animals

Male Wistar rats weighing 200–250 g were kept at a controlled condition of temperature (25±2 °C) with a 12:12 hrs light:dark cycle in polypropylene cages. They received standard rat chow and drinking water ad libitum. All experimental procedures were conducted according to the ethical standards and protocols approved by the University’s Ethical Committee. All efforts were made to minimize the number of animals and their suffering. Each experiment performed at least in triplicate.

Arsenic Nanoparticles (AsNPs) Preparation

Sodium arsenite powder was subjected to a high-energy planetary ball mill (Model PM 100) at a rotation speed of 300 rpm for 48 hrs to prepare the sodium arsenite nanoparticles. Ball milling process was implemented under atmospheric conditions (35 °C and 1 atm). Chamber and the balls were made of zirconium oxide. The mass ratio of ball to powder was chosen 30:1. Particle size analyzer (CILAS 920 [Madison, WI, USA]), equipped with a 280-µm aperture tube was used for analyzing particle size of the powder. The results revealed that the average particle size of the formed powder was <50 nm (Fig 1).

Isolation and Incubation of Hepatocytes

Hepatocytes were isolated by collagenase perfusion of the rat liver. Viabilities of >85% for hepatocytes (determined with trypan blue exclusion assay) were accepted. Cells were suspended (10⁶ cells/mL) in round bottom flasks rotating in the Krebs-Henseleit buffer (pH 7.4), supplemented with 12.5 mM HEPES under an atmosphere of carbogen gas (95% O₂ and 5% CO₂) in a 37 °C water bath; 10 mL of hepatocyte suspension in each flask was preincubated for 30 min prior to addition of As and AsNPs. Stock solutions of As and AsNPs (2, 4, and 10 mM) were prepared freshly prior to use. To incubate As and AsNPs with the required concentrations (20, 40, and 100 µM), we added 100 µL of concentrated stock solution (100×) to one rotating flask containing 10 mL hepatocyte
suspension and incubated with As and AsNPs at 37 °C for 1 h.

Cell Viability

The viability of isolated hepatocytes was assessed with trypan blue (0.2% [w/v]) exclusion test. Cell viability considered in this study was at least 85%–90%.

Determination of Reactive Oxygen Species (ROS)

The rate of hepatocyte ROS generation induced by As and AsNPs was determined by adding dichlorofluorescin diacetate (DCFH-DA) to the incubated hepatocytes. DCFH-DA penetrates hepatocytes and are hydrolyzed to form non-fluorescent dichlorofluorescin (DCF). DCF then reacts with ROS to form the highly fluorescent dichlorofluorescein that effluxes the cell. Aliquots of 1 mL of cell suspension (10^6 cells) were taken 1 h after incubation with As and AsNPs and centrifuged for 1 min at 1000 rpm. Cells were then resuspended in 1 mL of Krebs-Henseleit media containing 1.6 μM DCFD-DA and incubated at 37 °C for 10 min. The fluorescence intensity of dichlorofluorescein was measured using a Thermo Scientific fluorescence spectrophotometer at an excitation wavelength of 500 nm, and an emission wavelength of 520 nm.

GSH and GSSG Assay

Reduced (GSH) and oxidized glutathione (GSSG) levels were assayed by the method of Hissin and Hilf with minor modifications. Briefly, a 1-mL aliquot of the cell suspension (10^6 cells) incubated with As and AsNPs was taken and centrifuged at 1500 × g for 3 min. The supernatant was collected for determining of extracellular GSH and GSSG. The pellet was collected for measurement of intracellular GSH and GSSG. The supernatant was added to 500 μL of 10% TCA and centrifuged at 15,000 × g for 10 min at 4 °C; 700 μL of 10% TCA and 700 μL of water were added to the pellet and centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was collected for determining the intracellular GSH and GSSG levels. Fluorescence intensity was measured with a spectrofluorimeter (Thermo Scientific, USA) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. GSH and GSSG concentrations were measured using the calibration curve. Values were expressed as μmole/10^6 cells.

Mitochondrial Membrane Potential (ΔΨm) Assay

An aliquot of 0.5 mL of the cell suspension (10^6 cells/mL) was centrifuged for 1 min at 1000 rpm. Pellet was resuspended in 2 mL of fresh incubation medium containing 1.5 μM rhodamine 123, and incubated at 37 °C in a thermostatic bath for 10 min with
gentle shaking. Hepatocytes were removed by centrifugation at 3000 rpm for 1 min; the fluorescence of rhodamine 123 in the incubation medium was measured using a Thermo Scientific fluorescence spectrophotometer (490 nm excitation and 520 nm emission wavelengths). Altered fluorescence intensity among the control and treated cells indicated the capability of mitochondria to concentrate rhodamine 123 inside. The value was expressed as the percentage of the control.\(^{21}\)

**Lyosomal Membrane Stability Assay**

Hepatocyte lysosomal membrane stability was determined by redistribution of the fluorescent dye, acridine orange;\(^ {22,23}\) 0.5 mL aliquot of the cell suspension (\(10^6\) cells/mL) was centrifuged for 1 min at 1000 rpm. Pellet was resuspended in 2 mL of fresh Krebs-Henseleit media containing 5 μM acridine orange and incubated at 37 °C in a thermostatic bath for 10 min. Acridine orange redistribution was then measured fluorometrically using a fluorescence spectrophotometer (Thermo Scientific, USA) set at 495 nm excitation and 640 and 525 nm (integrity and damage, respectively) emission wavelengths.

**Neutral Comet Assay**

The apoptosis rate in hepatocyte was evaluated using neutral comet assay.\(^ {24}\) Hepatocytes at a concentration of \(10^6\) cell/mL were mixed with 1% low-melting-point agarose (LMPA) in PBS (phosphate buffered saline) at a ratio of 1:10 (v/v); 100 μL of this suspension was spread on a precoated slide with a supporting layer of 1% normal-melting-point (NMP) agarose in distilled water and covered with a coverslip. After gelling for 10 min at 0 °C, the coverslip was gently removed. Slides were placed in precooled lysis solution (2.5 mM NaCl, 100 mM EDTA, sodium lauroyl sarcosinate salt [SLS] 1%, 10 mM Tris base, 10% DMSO, and 1% Triton X-100) (the final pH of the lysis solution was adjusted to about 10) at 4 °C for 30 min and washed by TBE buffer three times (90 mM Tris base, 90 mM boric acid, 2.5 mM Na\(_2\)EDTA; all materials from Merck, Germany). Electrophoresis was performed for 20 min at 25 V and 300 mA.

Slides were stained with ethidium bromide solution (2 μg/mL) for 5 min and analyzed for the presence of apoptotic and non-apoptotic cells using a fluorescent microscope (Olympus BH-2) under a 10× objective lens. Each cell was characterized by its DNA fragmentation pattern as previously described.\(^ {25}\) Based on the head size and tail length, scores 0–3 were given to 50 nuclei from each slide. The apoptotic cells were categorized by scoring them as ‘2’ or ‘3.’

**Statistical Analysis**

SPSS\(^ \text{®}\) for Windows\(^ \text{®}\) ver 18 were used for data analysis. Assays were performed in triplicate. One-way ANOVA followed by Tukey’s HSD as the post hoc test, was used to compare means among three or more groups.

**Results**

Our study showed that As and AsNPs induced ROS production in a concentration-dependent manner. Both As and AsNPs significantly increased the ROS formation as compared with control cells (\(p=0.01\)). At concentration of 40 μM, AsNPs induced ROS formation more than As (\(p=0.016\)) (Fig 2). At higher concentrations, while the ROS produced by AsNPs was higher than As-treated cells, the difference was not significant.

Overall, AsNPs more robustly depleted GSH contents of hepatocytes in comparison with As. Significant level of ROS produced at 40-μM arsenic influenced the intracellular GSH and extracellular GSSG contents of hepatocytes (Fig 3). As and As-
NPs significantly depleted the intracellular GSH at 40 and 100 µM, compared with the controls. There was a significant increase in the extracellular GSH levels in both As and AsNPs at 20 µM, compared with the control group. Moreover, significant in-

*Figure 3:* The mean intracellular and extracellular levels of reduced and oxidized glutathione in isolated rat liver hepatocytes exposed to different concentrations of As and AsNPs. Error bars represent the SD.

*Significantly different from the control group (p<0.05).

*Significantly different from the group with the same corresponding concentration (p<0.05).
creases in the extracellular GSSG levels of hepatocytes exposed to AsNPs were noticed compared with As at 20 and 40 µM (Fig 3).

Both As and AsNPs, at all applied concentrations, significantly (p<0.001) decreased mitochondrial membrane potential (ΔΨm), compared with the control group. AsNPs disrupted mitochondrial membrane potential (ΔΨm) more than that As did alone, particularly at 20 µM (p=0.007) (Fig 4). Significant decrease in the membrane potential was observed at 40 µM or higher concentrations. It seems that AsNPs disturbed ΔΨm at 20 µM more prominently than that As did.

Both As and AsNPs, at all concentrations, significantly destructed lysosomal membrane integrity and damaged the lysosomal membrane (p<0.001). The extent of damage observed in the lysosomal membrane of hepatocytes exposed to AsNPs was significantly (p=0.03) more than that observed in As-exposed cells at all concentrations (Fig 5).

Lysosomal membrane disintegration and mitochondrial membrane potential collapse as well as their cross-talk can eventually activate the intrinsic pathway of apoptosis and lead to cell death. A significant increase in apoptosis was observed in As- and AsNPs-exposed hepatocytes at all applied concentrations (Fig 6); the intensity of apoptosis induced by As was lower than that induced after AsNPs exposure.

**Discussion**

ROS overproduction and mitochondrial dysfunction are among the most widely studied mechanisms for arsenic cytotoxicity. Collapse of ΔΨm, ROS generation and lipid peroxidation are considered major mechanisms for arsenic toxicity. Arsenic reportedly, activates the caspase cascades and induces apoptosis through opening of the mitochondrial permeability transition (MPT), modification of upstream signaling proteins (Bcl-2, Bax and cytochrome c), and activation of executive caspases.

Irregular arsenic exposure may possibly lead to serious toxicity, although it has been used therapeutically since more than 2400 years ago. As an anticancer agent, arsenic probably performs its cytotoxic action through the induction of apoptosis. Arsenic trioxide and cisplatin in nanoscale delivery systems showed synergistic anticancer effects. Arsenic nanoparticles were safer than arsenic trioxide with equivalent efficacy against breast cancer cell lines. It seems as if arsenic acts as a double-edged sword and, depending on the biological states of cell and arsenic concentration, diverse effects would be displayed. Therefore, we purposely selected arsenic and also considered a way to reduce its size (nanoparticles) to see if there...
would be any modification in its toxico-dynamic or mechanism of action. Despite the advantages of nano-structures, it has been reported that certain nanoparticles may lead to adverse effects due to their small sizes and unique properties.\textsuperscript{32-33}

ROS overproduction is one of the frequently reported responses to cellular damage and death.\textsuperscript{34} Arsenic throughout its metabolism, produces various types of ROS in cells.\textsuperscript{35} In our study, following the addition of arsenic to isolated rat hepatocytes, increased ROS formation occurred, which suggested the possible role for its major intracellular source, mitochondria. Previous studies suggest that complex I, II and III instabilities are the major sources for ROS generation throughout the respiratory chain activity.\textsuperscript{36,37} For this, the mitochondria supposed to be the main target of arsenic toxicity in cells, playing a pivotal role in arsenic-induced hepatotoxicity.\textsuperscript{38}

Here, arsenic significantly increased the mitochondrial membrane damage (Fig 4). The hepatotoxic effects of arsenic are associated with the development of oxidative stress and the consequent depletion of GSH. It has been reported that GSH plays an important role in detoxifying arsenic species as well as stimulating the excretion of methylated arsenic compounds.\textsuperscript{39} Depletion of hepatic GSH facilitates accumulation of arsenic in the liver and thus causes oxidative stress, particularly at higher doses.\textsuperscript{40} Exposure of liver mitochondria to oxidizing xenobiotics could decrease GSH levels with the concomitant increase in GSSG concentration.\textsuperscript{41}

It has been suggested that the loss of mitochondrial membrane potential and ROS overproduction (from the malfunctioned mitochondria) are the major mechanisms for arsenic toxicity and that mitochondria is supposed to be a specific target for its toxicity.\textsuperscript{37,42} Arsenic is metabolized (methylated) in rodents. Monomethylarsonous acid, one of the metabolites, which distrib-

\textbf{Figure 5:} The mean lysosomal membrane integrity and damage of isolated rat liver hepatocytes exposed to different concentrations of As and AsNPs. Error bars represent the SD.
ute irregularly in different tissues (RBC, liver, and kidney), is an ultimate toxicant to mitochondria. On the other hand, the pattern of metabolism and methylation, and accumulation and retaining of metabolites in tissues are not the same among distinct animals.

Thiol groups, residing in the inner mitochondrial membrane upon the oxidation, presumably lead to conformational changes in mitochondrial permeability transition pore (MPT) and collapse of ΔѰm, which are generally considered potential end-points in various insults associated with oxidative stress. According to these results, it seems that arsenic can induce the release of cytochrome c from mitochondria into the cytosol and trigger apoptosis pathway. Apoptosis was induced in a concentration-dependent manner at all concentrations and the amounts were prominently higher for AsNPs (Fig 6). The difference between arsenic and AsNPs with regard to mitochondrial damage, mitochondrial ROS production and GSH depletion was not as significant as that observed for lysosomal damage and apoptosis (Figs 2-6). Conversely, a considerable amount of lysosomal damage and apoptosis was comparably observed for AsNPs; this would create uncertainty about the first target of arsenic in the cell. It is more likely that the first target organelle by AsNPs is the lysosomes that in cooperation with mitochondria may finally initiate apoptosis.

Cross-talk between mitochondria and lysosomes has formerly been reported. Destabilization of lysosome results in the release of acidic lysosomal contents into the cytosol. Following arsenic exposure, cathepsins appear to pass through from lysosomes to the cytosol. Since arsenicals have been reported to accumulate in lysosomes, it is possible that arsenic-induced lysosomal destabilization involves a Fenton-type and Haber-Weiss reaction. These arsenic-induced reactions produce reactive hydroxyl radicals that cannot diffuse out of the lysosomes, resulting in further destabilizing of lysosomal membranes; hydrolytic enzymes are released and would eventually trigger the apoptosis.

Particle size reduction is believed to be the first and easiest way for increasing the drug dissolution rate. When particles become smaller, they further interact with the solvent; their solubility is improved. Theoretically, nano-materials, due to their higher surface reactivity, are estimated to be more toxic than their bulk equivalents. However, practically, they display unexpected behaviors that sometimes prevent them from being more toxic. Aggregation in the marine environment is an example for these behaviors. In our study, we were not seeking the behavior of arsenic nanoparticles in the medium or even in

Figure 6: The mean apoptosis proportion in isolated rat liver hepatocytes caused by different concentrations of As and AsNPs. Error bars represent the SD.
hepatocytes; instead, we wanted to know how cells behave in this condition, and what the toxic outcomes of this change on cells would be. Certainly, the study of particle dispersion, solubility, aggregation and other physicochemical properties of arsenic nanoparticles will be very interesting. However, in this study, we have just focused on their toxicity in rat hepatocytes to understand if there was any additional toxicity.

Our results indicated that a difference existed in the nature of adverse effects induced by nano-arsenic compared to its bulk counterpart; since this dissimilarity happened mainly in lysosomes, it suggested a higher concentration for nano-particle in the cell. Oxidative stress induced by nano-sized heavy metals was reported previously. The results of this study confirmed the previous results; they additionally indicated that lysosomal damage was rather involved in toxicity induced by the nano-particles, which led to higher death rate in the hepatocytes.

In conclusion, we have shown that nanoparticles of arsenic induced more prominent toxic effects in freshly isolated rat hepatocytes that seem to be mainly provoked by lysosomal damage. While these results indicated more toxic effects for nano-scale materials and raise more concerns about their safety, it is also revealed that changing the size may remarkably alter the mechanism of action and shows a way for future works to test whether these materials are effective against chronic states including cancer.

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