

Hepatotoxic Manifestations of Arsenic Trioxide Loaded Poly (Lactide-co-Glycolide) Nanoparticles in Wistar Rat

Yeshvandra Verma (✉ yeshvandra@gmail.com)

Chaudhary Charan Singh University <https://orcid.org/0000-0002-5994-7501>

Kavita Rana

Chaudhary Charan Singh University

Varsha Rani

Chaudhary Charan Singh University

Suresh VS Rana

Chaudhary Charan Singh University

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Abstract

Present study reports on the hepatotoxic manifestations of arsenic trioxide loaded poly lactide-co-glycolide nanoparticles (As₂O₃-PLGA- NPs) in rats. As₂O₃-PLGA- NPs enhances the activity of serum transaminases. As₂O₃-PLGA-NPs are potential inducer of lipid peroxidation in mitochondria as well as microsomes. Mitochondrial lipid peroxidation was higher than the microsomal lipid peroxidation. CYP₄₅₀ 2E1 was lower in the liver of As₂O₃-PLGA- NPs treated rats in comparison to arsenic trioxide treated rats. GSH showed lower values than control rats and arsenic trioxide treated rats. Glutathione-S-transferase inhibited by arsenic trioxide, non significant increase was recorded in the liver of As₂O₃-PLGA- NPs treated rats. As₂O₃-PLGA- NPs readily accumulates in liver and induces peculiar histopathological changes viz. intracytoplasmic/intranuclear inclusions and apoptosis. Since As₂O₃-PLGA- NPs are being considered as a suitable chemo-preventive agent against different types of cancer, its toxicological properties are of prime concern from bio-safety point of view. Thus, present observations seem to be important from human health risk assessment point of view.

Introduction

Arsenic is number one substance in the most recent comprehensive, environmental, response, compensation and liability act priority list of hazardous substances published by the Agency for Toxic Substances and Disease Registry (ATSDR 2007). It has been associated with several human diseases including cancer, diabetes, skin lesions, respiratory disorders and cardiovascular effects. Best studied mechanism of action (MOA) of arsenic toxicity known today is the formation of reactive oxygen species (ROS) and nitrogen species (NS) (Hughes et al 2006; Kitchen and Ahmad 2003; Kitchen and Conolly 2010). ROS formed by arsenic are involved in several of the proposed MOAs including genotoxicity, signal transduction, cell proliferation and inhibition of DNA repair (Hughes et al. 2010).

In recent years, several studies have appeared suggesting efficient use of arsenic trioxide in the treatment of promyelocytic leukemia (Evens et al. 2004) and a number of solid tumors including breast cancer (Ye et al. 2005; Chow et al. 2004; Plataniias 2009). Further, nanoparticles of arsenic trioxide has now been prepared and tested for their therapeutic effects on liver cancer cells (Wang et al., 2009), haematological malignancy (Ahn et al. 2013) and osteosarcoma (Li et al. 2007). Increasing experimental evidences suggest that special physiochemical properties of these nanoparticles might pose potential risk to human health (Nel et al. 2006). Therefore, considerable effort should be made to identify the potential toxicity of nanoparticles to cells and organisms. Nanoparticles of different sizes and composition do present toxicological problems. However, a comprehensive mechanism of nanoparticles cytotoxicity has not been investigated.

Recent advances in drug delivery have encouraged the development and use of nanoparticles as drug carrier. Several agents have been developed as drug carriers in the past, for example, encapsulation of drugs in poly lactide-co-glycolide (PLGA) nanoparticles is a safe system for human application (Zhao and

Zhang 2009). Ahn et al. (2013) demonstrated that the antitumor efficacy of arsenic trioxide loaded nanobins was superior to free arsenic trioxide. Li et al. (2007) investigated the therapeutic efficacy of the magnetic arsenic trioxide nanoparticles against osteosarcoma *in vivo* tumor models.

The objective of the present investigations was to study the toxicity of arsenic trioxide loaded PLGA nanoparticles (As_2O_3 -PLGA-NPs) in the liver of rat. As_2O_3 -PLGA-NPs toxicity is expected to be helpful in the management of cancer therapy. Further, biosafety issue of nanoarsenic is also addressed. Toxicological evaluation of As_2O_3 -PLGA-NPs is expected to add our knowledge on their therapeutic efficacy, if any.

Materials And Methods

Reagents /chemicals

Poly (lactide-co-glycolide), arsenic trioxide, thiobarbituric acid, 5'-5' dithiobis-2-nitrobenzoic acid, 1-chloro 2, 4-dinitrobenzene and bovine serum albumin were procured from Sigma Chemical Company (USA). Commercial kits for the determination of alanine transaminases, aspartate transaminase, and alkaline phosphatase were procured from Span Diagnostics, Surat (India). Other chemicals and reagents used in this study were of analytical grade and procured from E. Merck (India) and Glaxo (India).

Preparation of arsenic trioxide loaded PLGA nanoparticles

A polymer, PLGA was used to prepare arsenic trioxide nanoparticles with w/o/w double emulsion evaporation technique with minor modifications (Zhao and Zhang 2009). Briefly, the first emulsion was formed between an organic solution of the polymer (100 mg PLGA in 2ml methylene chloride) and an aqueous solution of arsenic trioxide. Then 12ml aqueous solution containing 2.25% of polyvinyl alcohol was added to this primary emulsion and sonicated (Sonics and Materials Inc. USA) to obtain the double emulsion. Afterwards the solvent evaporation was carried out by gentle magnetic stirring at room temperature. The suspension was washed with distilled water and centrifuged three times at 12,000 rpm. Subsequently, the sediments were freeze dried and sterilised. Finally arsenic trioxide PLGA nanoparticles were obtained.

Characterization of As_2O_3 -PLGA nanoparticles, animals and treatments

Physicochemical properties of As_2O_3 -PLGA nanoparticles were verified through transmission electron microscope, scanning electron microscope, Zetasizer and X-Ray diffraction. Due permission from institutional ethical committee was sought before making these experiments. Male albino rats of Wistar strain weighing $250\pm 50\text{g}$ were procured from the animal facility of Jamia Hamdard, New Delhi. They were housed in the animal house of Department of Zoology, Ch. Charan Singh University Meerut (India). All the rats were offered pelleted food (Golden Feeds, New Delhi) and tap water *ad libitum* and maintained under standard laboratory conditions (room temperature $25\pm 5^\circ\text{C}$ and relative humidity $50\pm 5\%$). After acclimatization to laboratory conditions for two weeks, the rats were divided into three

groups, each containing five rats. Rats of group A were administered a sublethal dose of As₂O₃-PLGA-NPs dissolved in saline (0.1 mg/100g body weight) by gavage on each alternate day for thirty days. Rats of group B were also given same dose of As₂O₃ whereas rats of group C were offered saline only to serve as controls.

Analyses

After scheduled treatments, the rats were starved overnight and sacrificed next morning by light ether anesthesia. Liver was carefully removed and weighed using electronic balance (Sartorius, Germany) and processed for further investigations.

Arsenic accumulation in liver

1g wet liver was digested in 10ml of concentrated nitric acid (A.R. grade) at 80°C for 16 hr. It was diluted to 100ml with double distilled water. 2ml aliquot of the digest was analysed for inorganic arsenic by hydride generation at pH 6.0 using sodium borohydride as the reducing agent. The analyses were performed using atomic absorption spectrophotometer (Electronic Corporation, India). Absorption was recorded at 193.7 nm, using a hollow cathode lamp for arsenic (Electronic Corporation, India).

Histopathological observations

Small pieces of liver were fixed in 10% neutral formalin at room temperature for 24 hours. They were washed in running tap water overnight. After dehydration, pieces were embedded in paraffin, sectioned (5-6 μ thick) on a rotary microtome and stained with hematoxylin and eosin. The lesions were observed under a light research microscope (Nikon, Japan).

Determination of serum enzymes

Blood was collected from anesthetized rats through cardiac puncture in sterilized tubes. It was allowed to clot at room temperature for about 1 hour. Thereafter, serum was separated by centrifugation and stored at 0°C for further analysis. Serum enzymes aspartate transaminase (AST) (E.C.2.6.1.1), alanine transaminase (ALT) (E.C.2.6.1.2) and alkaline phosphatase (ALP) (E.C.3.1.3.2) were determined according to the method of Rietman and Frankel (1957), using a commercial kit procured from Span Diagnostics (Surat, India).

Lipid peroxidation

Lipid peroxidation in the liver of experimental rats was determined by measuring mitochondrial and microsomal malondialdehyde following the method of Jordan and Schenkman (1982). Microsomes were separated using an ultracentrifuge (Sorvel, USA) following the method of Schenkman and Cinti (1978). Thiobarbituric acid reactive substances were measured at 532 nm using a spectrophotometer (Systronics, India). 1', 1', 3- tetramethoxypropane (Sigma, USA) was used as the standard.

Reduced glutathione

Glutathione (GSH) was determined in the liver of experimental rats using Ellman's reagent (5-5'-dithiobis (2-nitrobenzoic acid (Ellman 1959). Sulphosalicylic acid was used for protein precipitation. Absorbance was recorded at 412 nm using a spectrophotometer (Systronics, India).

Glutathione-S-transferase (E.C.2.5.1.8)

Glutathione-S-transferase was measured using 1-chloro -2, 4- dinitrobenzene as a substrate following the method of Habig et al. (1974). Enzyme activity was calculated as nm/NADPH/min/mg cytosolic protein. The absorbance was recorded at 340 nm.

CYP₄₅₀ 2E1. (EC 1.14.13)

Microsomes were separated using an ultracentrifuge (Sorvel,USA). Enzyme activity was estimated following the method of Koop (1986). In brief, the reaction mixture consisted of microsomal protein (0.2 mg/ml), 0.1 M potassium phosphate, pH 6.8 and 1 mM p-nitrophenol. Samples were incubated at 37°C for 3 min prior to the addition of NADPH to start the reaction. After 10 min, the reaction was stopped with 1.5N perchloric acid. Formation of p-nitrocatechol was measured at 510 nm.

Protein measurement

Protein content in the liver samples was measured applying the method of Lowry et al (1951). Bovine serum albumin (Sigma, USA) was used as standard.

Statistical analysis

The data was analysed using SPSS software version 20. P values of less than 0.05 were accepted to be significant.

Results

Fig.1A shows the representative TEM image of As₂O₃-PLGA- NPs (supplemental fig.1). The results shows an average particle diameter < 100nm. The SEM micrograph demonstrating the surface morphology and the formation of agglomerates (Fig.1B, supplemental fig.2). Zeta potential of As₂O₃-PLGA- NPs was found (- 2.93mV) (Fig.2, supplemental fig.3). Intensity-weighted particle sized distribution of As₂O₃-PLGA- NPs shows in fig. 3 (supplemental fig.4). Fig. 4 shows the XRD pattern of As₂O₃-PLGA- NPs depicted a cubic structure by comparing with the standard data (JSPDS: 01-071-0400) (supplemental fig.5).

Biological observations i.e. change in the body weight and liver/ body weight relationship apparently showed no sign of adverse effects of As₂O₃-PLGA- NPs in rat. As₂O₃-PLGA- NPs treatment helped the rats in gaining weight (fig. 5).

Hepatosomatic index was higher in As₂O₃-PLGA- NPs treated rats in comparison to control rats (table 1). Liver is a soft target for bioaccumulation of arsenic. Arsenic concentration in liver was found to increase in arsenic trioxide treated rats. Arsenic accumulated in liver of As₂O₃-PLGA- NPs treated rats however concentration was found to be low in comparison to arsenic trioxide treated rats (table 2). Observations on serum transaminases exhibited the state of liver function in As₂O₃-PLGA- NPs treated rats. Higher values were recorded for AST and ALT both in the serum of As₂O₃-PLGA NPs treated rats. Arsenic trioxide treatments also impaired liver function. However As₂O₃-PLGA- NPs were found to be more toxic. Arsenic trioxide inhibited ALP, however, As₂O₃-PLGA- NPs caused increased efflux of enzyme in the serum (table 1).

Hepatotoxicity of arsenic trioxide has been mainly attributed to reactive oxygen species thus generated and measured in terms of a phenomenon known lipid peroxidation. Our results showed that As₂O₃-PLGA- NPs are potential inducer of lipid peroxidation in mitochondria as well as microsomes. Mitochondrial lipid peroxidation was higher than the microsomal lipid peroxidation (table 3).

CYP₄₅₀ 2E1, was also estimated to study the metabolic fate of As₂O₃-PLGA- NPs. Values of this enzyme were lower in the liver of As₂O₃-PLGA- NPs treated rats in comparison to arsenic trioxide treated rats (table 3).

Oxidative stress caused by As₂O₃-PLGA- NPs, if any, was also studied. GSH thus estimated showed lower values than control rats and arsenic trioxide treated rats (table 2). Status of detoxication enzyme i.e. glutathione-S-transferase was also studied in the liver of As₂O₃-PLGA- NPs treated rats. Though it was inhibited by arsenic trioxide, non significant increase was recorded in the liver of As₂O₃-PLGA- NPs treated rats (table 2).

Finally, toxic impairment of liver caused by As₂O₃-PLGA- NPs was examined by histopathological observations. These observations showed peculiar and specific lesion in the form of nuclear and cytoplasmic changes. Several of the nuclei of hepatic parenchyma stained black and were filled with intranuclear inclusions. A few apoptotic bodies were also localized. Mild necrosis was recorded in the centrilobular region. Thus histopathological results suggested that As₂O₃-PLGA- NPs caused severe hepatotoxicity in rat. T.S. of liver of As₂O₃-PLGA-NPs treated rat shows the presence of several intracytoplasmic inclusions, apoptotic bodies and necrotic spaces (Fig.6A). These pathological changes are wanting in arsenic trioxide treated rats. However, arsenic trioxide treated rat shows perilobular necrosis in liver (fig.6B). Fig.6C shows the normal structure of liver of control rat with hepatocytes radiating from central vein.

Discussion

Cellular responses induced by nanomaterials or nanoparticles have been extensively studied in recent years by a number of laboratories using *in vitro* and /or *in vivo* systems. The nanomaterials have been

found to be potentially cytotoxic, therefore, their safety needs to be thoroughly assessed prior to the use in nano-based consumer products and technologies including nanomedicine (Sauer et al. 2014; Hussain et al. 2015).

Recent observations from a few workers suggesting that nano-arsenic can be applied to treat haematological malignancies, liver cancer and osteosarcoma have raised concerns for its toxicological evaluation in terms of hepatotoxicity, hematotoxicity and nephrotoxicity (Ahn et al. 2013; Li et al. 2007). Further, a comparison between the toxicity of bulk arsenic and nano-arsenic needs to be made to justify its application as a therapeutic agent.

Our results on initial characterization of nano-arsenic trioxide made through TEM showed that they were round / spherical in shape, measured less than 100 nanometer and formed agglomerates with PLGA. This information has been found to be critical in determining the biological response to nanoparticles (Richman and Hutchinson 2009; Sapsford et al. 2011). Oral administration of these particles for 30 days to rats caused a non significant increase in their body weight. While oral administration of arsenic trioxide inhibited the growth of rats. Hepatosomatic index increased in As₂O₃-PLGA- NPs treated rats as compared to arsenic trioxide treated rats. This observation associates As₂O₃-PLGA- NPs with the synthesis of major molecules viz. proteins, lipids, and carbohydrates. Further, accumulation of nanoparticles of arsenic trioxide decreased in liver as compared to the bulk metalloid. It has been observed that excretion of nanoparticles is quicker than the bulk particles. Opsonisation, the process that prepares foreign materials to be more efficiently engulfed by macrophages occur under certain conditions for nanoparticles depending on size and surface characteristics (Moghimi et al. 2005). Nanoparticles such as As₂O₃-PLGA- NPs can pass through the gastrointestinal tract and are rapidly eliminated through faeces and urine (Hagens et al. 2007). However, some nanoparticles accumulate in the liver during the first- pass metabolism (Oberdorster et al. 2005).

We observed that liver function as determined through activity of serum transaminases was also affected by As₂O₃-PLGA- NPs. The injury was higher in comparison to arsenic trioxide treated rats. Liver function disturbances caused by arsenic trioxide have been studied earlier in our laboratory (Singh and Rana 2009). However, present report confirms that liver is a target organ of nanoparticles. In general, nanoparticles stimulate macrophages via reactive oxygen species (ROS) and calcium signalling to make proinflammatory cytokines such as TNF α (Brown et al. 2004).

Thus hepatocytes function is inhibited by oxidative stress/ pro-inflammatory cytokines induced pathological changes in liver. The pathological changes caused by As₂O₃-PLGA- NPs in the liver included formation of intranuclear inclusion bodies, apoptosis and mild centrilobular necrosis. Arsenic trioxide has also been found to induce inflammatory effects in the liver (Singh and Rana 2009).

We could notice that As₂O₃-PLGA- NPs caused significant mitochondrial as well as microsomal lipid peroxidation in liver. These results are most important in delineating the MOA manifested by As₂O₃-PLGA- NPs and are in agreement with similar reports. In general, nanotoxicity has been attributed to the

generation of ROS (Fu et al. 2014). It has been hypothesized that cellular internalization of NPs activates immune cells including macrophages and neutrophils contributing to the generation of ROS/RNS (Risom et al. 2005; Knaapen et al. 2004). NPs with smaller size are reported to induce higher ROS owing to their unique characteristics such as high surface to volume ratio and high surface charge. It is the particle size that determines the reactive groups / sites on NP surface. Therefore, As₂O₃-PLGA- NPs induced higher generation of ROS as compared to arsenic trioxide. Nano-sized SiO₂ and TiO₂ and multiwalled carbon nanotubes have been reported to induce greater ROS as compared to their larger counterparts (Sohaebuddin et al. 2010). Arsenic trioxide induced lipid peroxidation has been reported earlier. Reactive species are formed *in vitro* and *in vivo* in the presence of arsenic and include superoxide anion, hydroxyl radical, hydrogen peroxide, RNS and arsenic centred arsenic peroxy radicals (Kitchen 2001; Shi et al. 2004).

An important paradigm for NPs-mediated toxicity is oxidative stress. Manke et al. (2013) reviewed different mechanisms of NPs induced oxidative stress and toxicity. It is known that metal based NPs induce oxidative damage to cellular macromolecules such as proteins, lipids and DNA via Fenton type and Haber-Weiss type reactions. The outcome of oxidative damage is membranous lipid peroxidation, protein denaturation and alteration of calcium homeostasis. As₂O₃-PLGA- NPs, as shown by present results on GSH caused hepatic parenchymal damage via oxidative stress.

In brief, it is concluded that arsenic with its carrier PLGA caused hepatotoxic effects through oxidative stress. Protective effects of antioxidants, yet to be made will confirm this hypothesis.

Declarations

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Authors contribution

YV and SVSR conceived and designed research. YV, KR, VR conducted experiments and analyzed data. YV and SVSR wrote the manuscript. All authors read, critically evaluated and approved the manuscript for publication. The authors declare that all data were generated in-house and that no paper mill was used.

Availability of data and materials

All data generated and analyzed during this study are included in this article.

Conflict of interest- Authors declare that they have no any conflict of interest.

Ethical approval- Permission from Institutional Animal Ethical Committee was received before making these experiments.

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table-1: Hepatosomatic index, aspartate amino transferase (AST), alanine transaminases (ALT) and alkaline phosphatase (ALK) in arsenic trioxide loaded PLGA nanoparticles induced rats

Groups	Hepatosomatic index	AST (IU/L)	ALT (IU/L)	ALK (KA units/dl)
Control	3.29 ± 0.38	89.13 ± 0.51	39.6 ± 1.01	11.62 ± 1.60
As ₂ O ₃	3.06 ± 0.12 ^{NS}	235.00 ± 4.5*	46.00 ± 1.41 ^{NS}	7.37 ± 0.94*
As ₂ O ₃ -PLGA-NPs	3.52 ± 0.15*	250 ± 0.12*	64.25 ± 0.04*	19.9 ± 0.12*

Results are expressed as mean ± S.E. (n=5), where n is number of observations

* Values are significant when compared with control group (p< 0.05).

^{NS} - Non significant values

Table-2: Reduced glutathione (GSH), Glutathione-S-transferase (GST) and arsenic concentration in liver of arsenic trioxide loaded PLGA nanoparticles induced rats

Groups	GSH ($\mu\text{g/g}$ wet liver)	GST (nmoles/NADPH/min/mg protein)	Arsenic concentration in liver ($\mu\text{g/g}$)
Control	0.88 ± 0.04	0.481 ± 0.39	0.011 ± 0.002
As_2O_3	$0.14 \pm 0.01^*$	$0.372 \pm 0.03^*$	$0.70 \pm 0.05^*$
As_2O_3 PLGA-NPs	$0.17 \pm 0.21^*$	$0.57 \pm 0.52^*$	$0.421 \pm 0.16^*$

Results are expressed as mean \pm S.E. (n=5), where n is number of observations

* Values are significant when compared with control group ($p < 0.05$).

Table-3: CYTP₄₅₀2E1, mitochondrial and microsomal malondialdehyde in liver of arsenic trioxide loaded PLGA nanoparticles induced rats

Groups	CYP ₄₅₀ 2E1 (µg/mg/microsomal protein)	Malondialdehyde (Mitochondrial) (n moles/mg/cytosolic protein)	Malondialdehyde (Microsomal) (nmoles/mg/microsomal protein)
Control	1.94 ± 0.16	0.18 ± 0.22	0.14 ± 0.008
As ₂ O ₃	3.01 ± 0.07*	0.23 ± 0.01 ^{NS}	0.31 ± 0.02 ^{NS}
As ₂ O ₃ -PLGA-NPs	1.10 ± 0.18 ^{NS}	1.54 ± 0.31*	0.36 ± 0.12*

Results are expressed as mean ± S.E. (n=5), where n is number of observations

* Values are significant when compared with control group (p< 0.05).

^{NS} - Non significant values

Figures

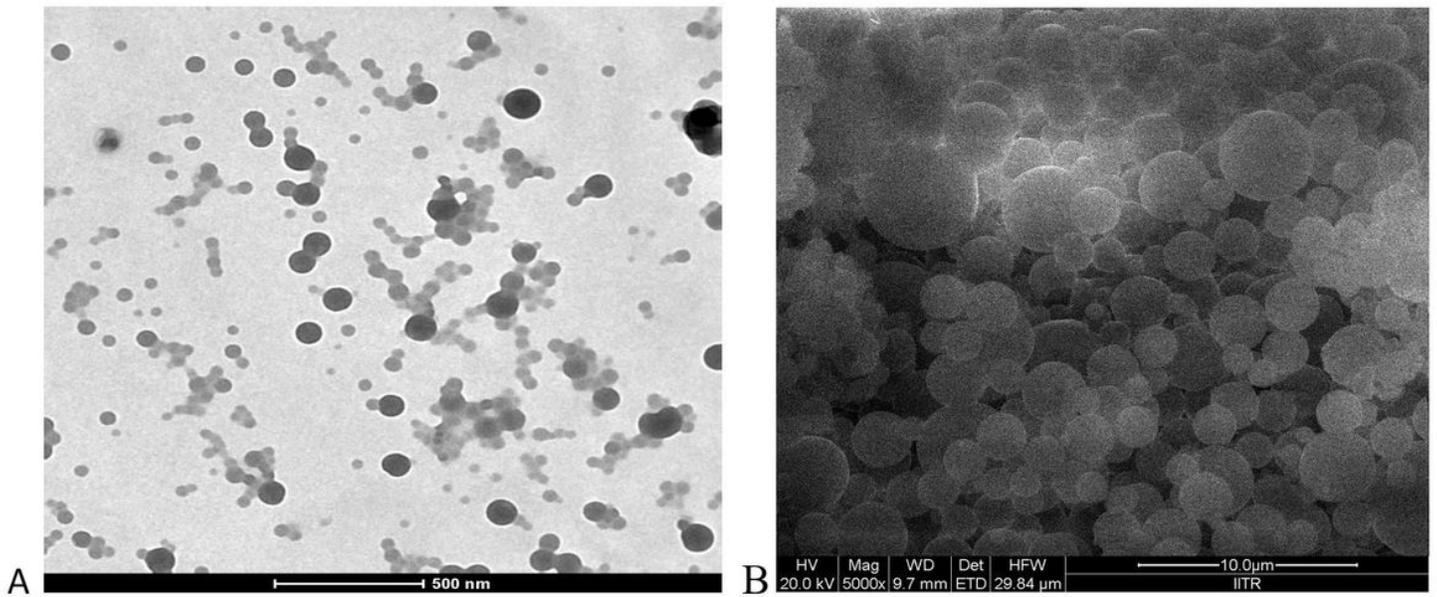


Figure 1

(A) Transmission electron microscopic image of As₂O₃ - PLGA NPs, (B) scanning electron microscopic image of As₂O₃ - PLGA NPs

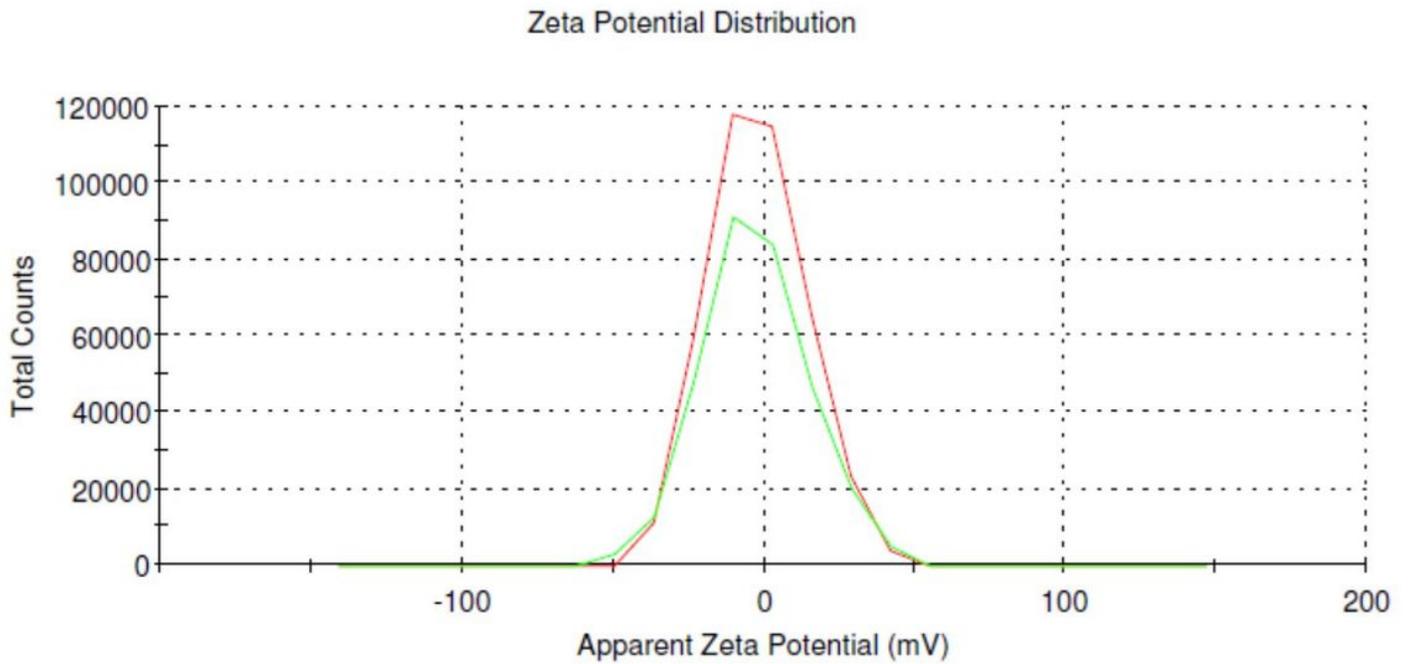


Figure 2

Measurement of Zeta potential of As₂O₃-PLGA-NPs

Size Distribution by Intensity

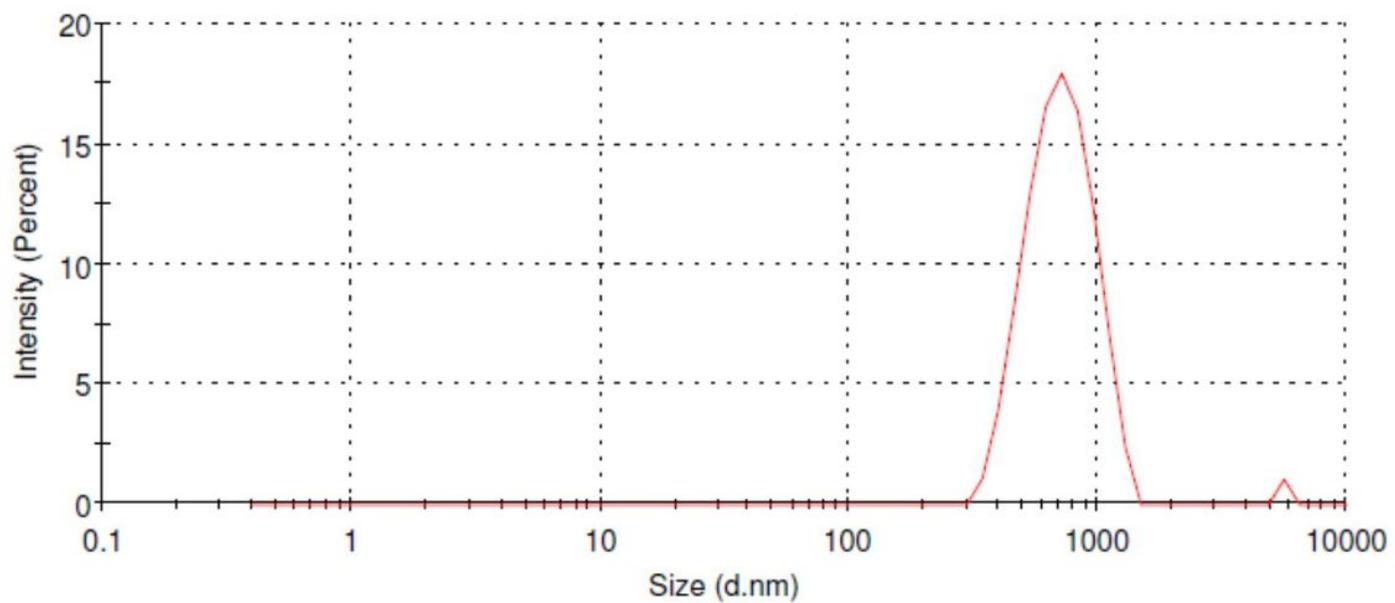


Figure 3

Size distribution (by intensity) analysis of As₂O₃-PLGA-NPs

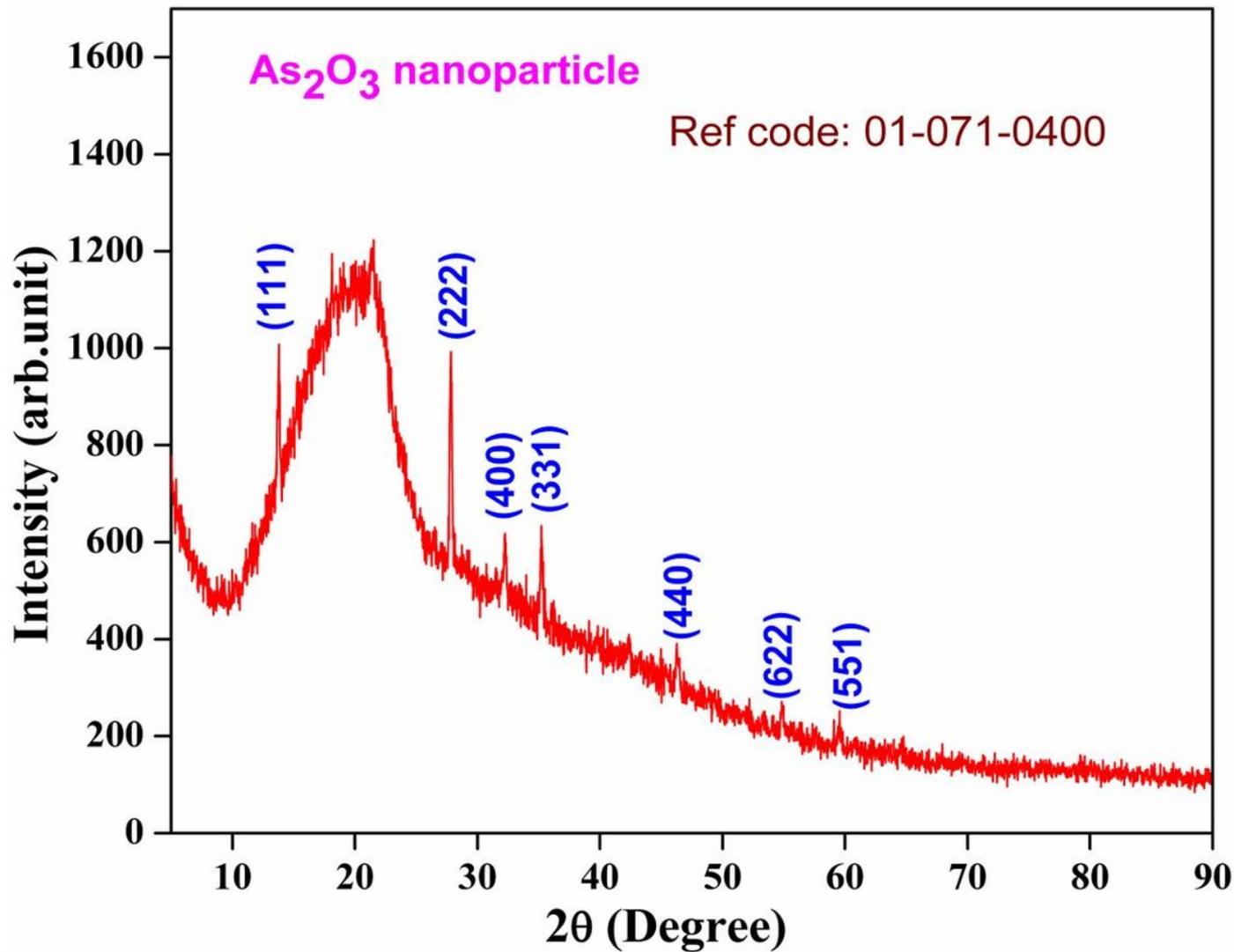


Figure 4

X-ray powder diffraction (XRD) spectra of As₂O₃ - PLGA NPs

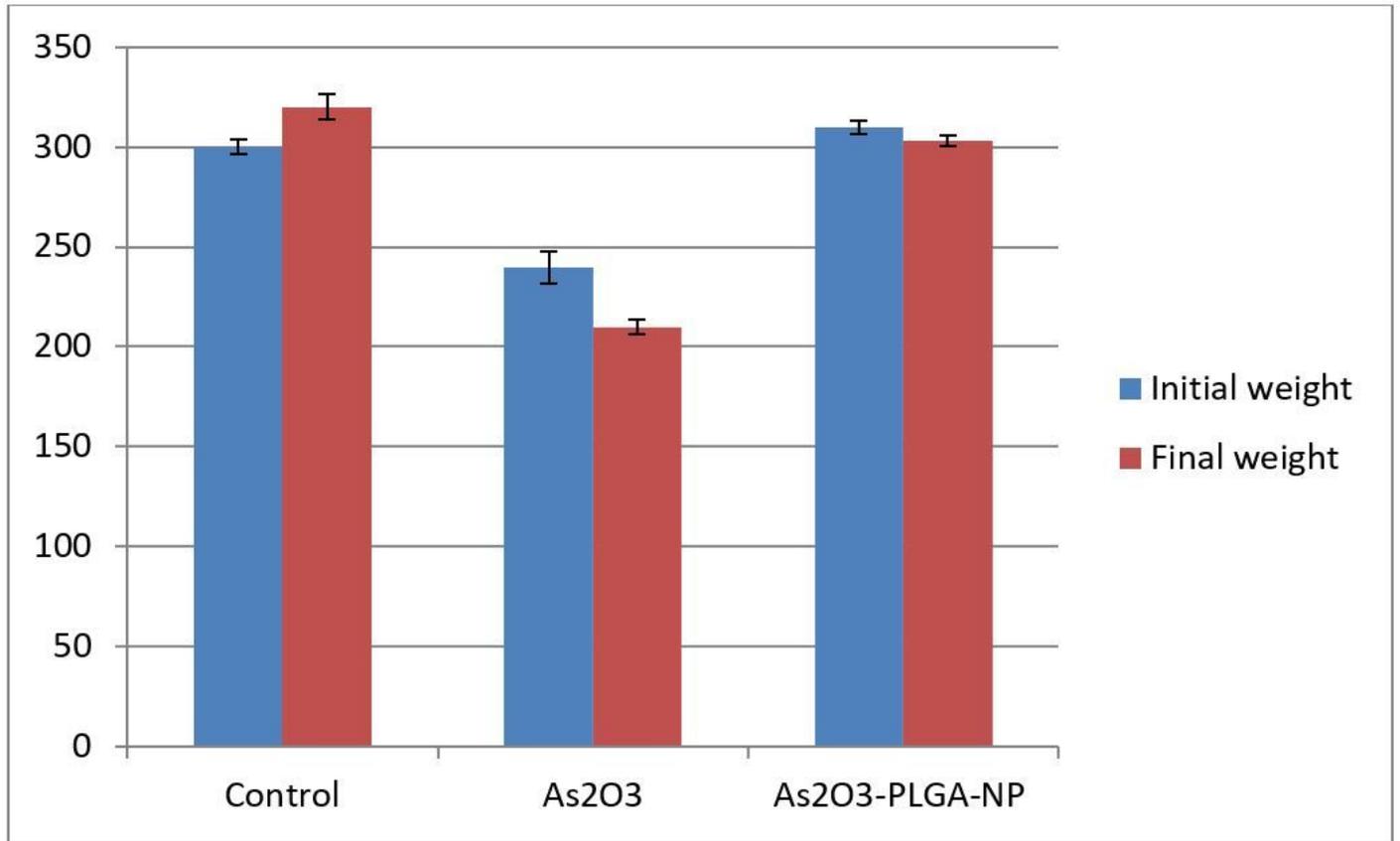


Figure 5

Body weight changes induced by arsenic trioxide and As₂O₃-PLGA-NPs in rats

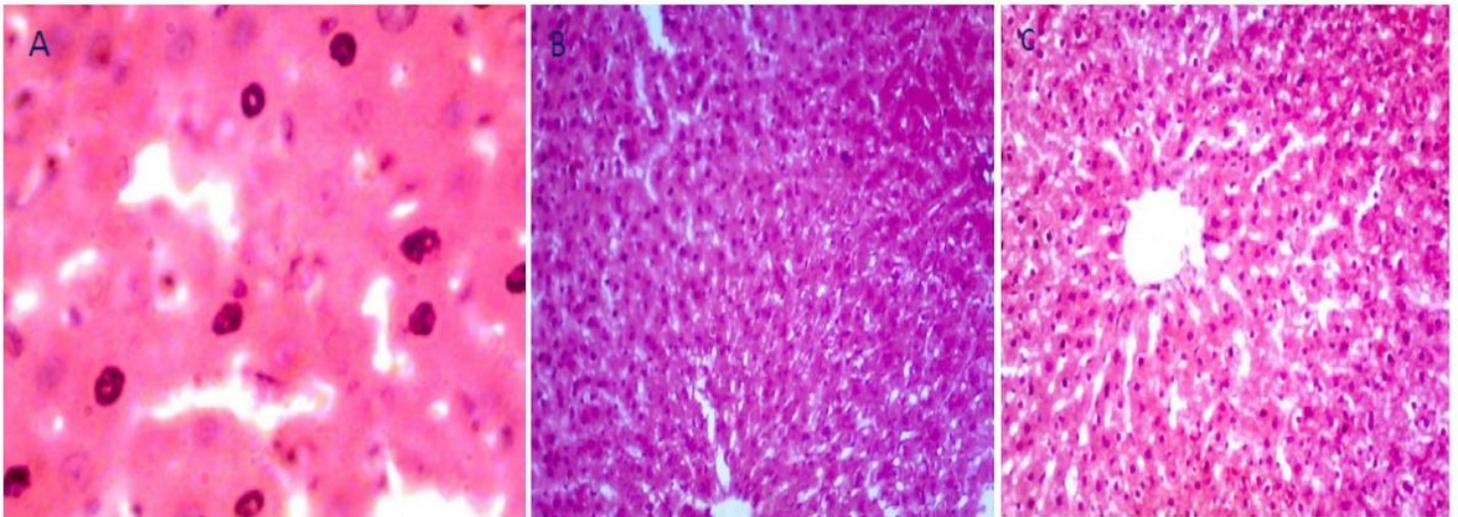


Figure 6

(A) T.S. of liver of an As₂O₃-PLGA-NPs treated rat shows the presence of several intracytoplasmic inclusions, apoptotic bodies and necrotic spaces (X400), (B) T.S. of liver of arsenic trioxide treated rat

shows perilobular necrosis (X200), (C) T.S. of liver of untreated control rat shows normal structure with hepatocytes radiating from central vein (X200)

Supplementary Files

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- [Supplementalmaterials.docx](#)