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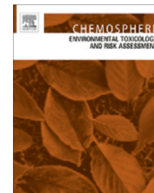

# Arsenic trioxide ($As_2O_3$) induces apoptosis and necrosis mediated cell death through mitochondrial membrane potential damage and elevated production of reactive oxygen species in PLHC-1 fish cell line

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## HIGHLIGHTS

- *In vitro* data's relevance to environmental arsenic exposure in whole fish is discussed.
- Mechanism of arsenic induced toxicity is similar to that observed in mammalian cell lines.
- This study represents a comprehensive approach, with several assays performed for the first time in PLHC-1 cells.
- Arsenic trioxide induced cell death is mediated by elevated production of ROS and mitochondrial damage.
- Arsenic trioxide induces both apoptotic and necrotic DNA damage as shown by comet assay.

## ARTICLE INFO

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## ABSTRACT

Several environmental pollutants, including metals can induce toxicological effect on aquatic animal species. Most studies to understand the toxicity of arsenic compounds were performed in mammalian cells; however, the study of the arsenic toxicity to the aquatic animals' species, including fish, is limited. So the objective of this study was first to investigate the effects of $As_2O_3$ induced toxicity particularly on apoptosis and necrosis mediated cell death in fish cell PLHC-1 as compared to the mechanism of toxicity from known mammalian cell lines, secondly to relate *in vitro* effects in fish to those demonstrated by *in vivo* systems. To conduct this study, PLHC-1 cells were exposed to various concentrations of $As_2O_3$ (0–100 μM) for 10, 20 and 40 h. The results indicate that $As_2O_3$ exposure promoted apoptotic and necrotic mediated cell death in a concentration and time dependent manner. Cell death (apoptotic and necrotic) induced by $As_2O_3$ was further confirmed by changes in various phases of cell cycle, DNA fragmentation (necro- comet and apo-comet) in the comet assay, alteration in mitochondrial membrane potential and formation of increased reactive oxygen species (ROS). Apoptotic mediated cell death was confirmed further by observing the increased caspase-3 activity and elevated expression of p53, cytochrome c and Bax proteins levels in the same experimental conditions. PLHC-1 cells were shown to be a good model for evaluating biochemical/cytotoxic effects following exposure to various reference chemicals and environmental contaminants. *In vitro* data obtained from this study provides a comprehensive approach for the elucidating the actual molecular mechanism for $As_2O_3$ induced toxicity particularly apoptosis and necrosis mediated cell death in PLHC-1 cell line.

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## 1. Introduction

Anthropogenic activities have increased the level of arsenic released into aquatic environment (Bhattacharya and Bhattacharya, 2007). Arsenic exists in both organic and inorganic forms, with the inorganic forms more toxic than organic (Gochfeld, 1995). In the aquatic environment, inorganic arsenic exists predominately in trivalent ($As^{3+}$) and pentavalent ($As^{5+}$) forms with the trivalent exhibiting more toxic effects than the pentavalent compound (Cervantes et al., 1994). Fish have been proven to be valuable test

*Abbreviations:* MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50, inhibitory concentration; FACS, fluorescence-activated cell sorter; FITC/PI, fluorescein isothiocyanate propidium iodide; JC-1 dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide; DEVD-AFC, 7-amido-4-methylcoumarine derivatives; GFP, green fluorescent filter; ECL solution, enhanced chemiluminescence; DMSO, dimethyl sulfoxide; μM, micromole; EBSS, Earle's balanced salt solutions; ANOVA, analysis of variation.

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species to study the effects of toxicant uptake and bioaccumulation on metabolic activities and other functions. The acute lethality test has been used to characterize the toxicity of arsenic in several fish species as $LC_{50}$, the dose of arsenic fatal to 50% of the individuals exposed (Buhl et al., 2002). The use of whole fish in metabolic studies is inconvenient, time consuming, expensive, and requires sacrificing organisms (Wang et al., 2004). Additionally, measurable endpoints in whole organism evaluation, such as condition indices, may be less sensitive than cellular changes.

*In vitro* assays have been developed to serve as an alternative or a supplementary bioassay for toxicity ranking of chemicals (Fent, 2001). The use of *in vitro* assays in ecotoxicological studies not only provides the opportunity for exploration from *in vitro* to *in vivo* systems, but also generates information on biological responses at a relatively high level of biological organization (Castano et al., 2003). Fish cell lines are appropriate for *in vitro* assays since they are believed to retain fish-specific traits in their metabolism of chemicals like arsenic. This paper reports for the first time the use of *Poeciliopsis lucida* hepato cellular cell line (PLHC-1) as test system to evaluate the cytotoxic effects of arsenic trioxide. PLHC-1 was selected for this study because (i) it retains the liver properties which is useful because liver is a major target organ of arsenic toxicity, (ii) it has metabolic activities, (iii) it is easy to propagate in culture at room temperature, and (iv) the PLHC-1 cell line has proven a versatile test system for evaluating cytotoxic effect of various compounds (Pichardo et al., 2005; Caminda et al., 2006).

Wang et al. (2004) compared toxicity of subacute and acute levels of $As_2O_3$ in the fish cell lines JP and T0-2 using colony forming assay, morphological changes, apoptotic mediated cell death confirmed by DNA fragmentation and increased cell cycle arrest at subG1 phase. The purpose of the present study was to further characterize the adverse effects of $As_2O_3$ induced toxicity in PLHC-1 cell line using specific bioassays to characterize the mechanism of toxicity and compare this mechanism with results described in mammalian cell lines. Several techniques were used including (i) Annexin V/PI staining to determine the amount of early apoptosis, late apoptosis and necrosis, (ii) Flow cytometry to evaluate progress of cell cycle, (iii) Comet assay to assess the pattern of DNA fragmentation, (iv) Expression of apoptosis associated regulatory proteins *by* immunoblotting, and (v) DEVD-AFC, DCDF-DA and JC-1 staining to determine caspase-3 activity, production of reactive oxygen species (ROS) and changes in mitochondrial membrane potential ($\Delta\psi_m$) respectively. A second objective was to determine the cytotoxic concentration ($IC_{50}$) of arsenic to relate *in vitro* adverse effect levels in the fish cell lines to those demonstrated by *in vivo* systems.

## 2. Materials and methods

### 2.1. Materials

$As_2O_3$ was purchased from Alfa Aesar (Ward Hill, MA); JC-1 Mitochondrial Membrane Potential Detection Kit from Cell Technology (Mountain View, CA); OxiSelect™ ROS Assay Kit from Cell Bio Labs, Inc. (San Diego, CA); Comet assay™ kit from Trevigen, Inc. (Gaithersburg, MD; USA); Caspase-3 Fluorometric Assay Kit from Bio-Vision Research Products (Mountain View, CA); Annexin V-FITC/PI Kit System from Beckman Coulter (Brea, CA,USA); Lonza PAGE* Gold precast Gels from (Lonza Group Ltd., Switzerland); ECL Western Blotting Detection Reagent & Membrane from GE* Health Care Amersham* (Piscataway, NJ); X-ray Film from (Thermo Scientific); All primary and secondary antibodies from Santa Cruz Biotechnology, Inc. (Delaware Avenue, CA); Positive Control Proteins, Biotinylated ladder and anti-biotined antibody from Cell Signaling Technology, Inc. (Danvers, MA); Pre-stained SDS-PAGE standards from Bio-Rad Life Science (Hercules, CA). All other tissue culture reagents and products were purchased from BD (Franklin Lakes, NJ).

### 2.2. Cell line

PLHC-1 cells (ATCC CRL-2406) were grown in 25 $cm^2$ cell culture flasks at 30 °C with 5% $CO_2$ in Minimum Essential Medium containing 2 mM $L^{-1}$- glutamine, 1% Pen/Strep (10,000 units' penicillin and 10 mg streptomycin $mL^{-1}$) and supplemented with 5% of fetal bovine serum.

### 2.3. Determination of cytotoxicity dose 50 ($IC_{50}$ of $As_2O_3$)

Concentrated stock of $As_2O_3$ (10 M) was prepared fresh each time in 0.1 N NaOH to minimize the oxidation of the chemical then serially diluted in PBS to desired concentrations and added to the growth media. The acute cytotoxicity dose ($IC_{50}$ of arsenic) was determined by MTT assay (Mosmann, 1983).

### 2.4. Propagation of PLHC-1 cells

PLHC-1 cells ($1 \times 10^5$ $mL^{-1}$) were grown in 24, 12 and 6 wells tissue culture plates for 2 or 3 d until 70–80% confluent. Once the desired cell density was reached, the culture medium was replaced with the fresh medium containing different doses of $As_2O_3$ (0–100 μM) for intervals of 10, 20 and 40 h. Negative control cells were treated with media and 100 μL PBS.

#### 2.4.1. Cell proliferation study

Cells were seeded in 12-well plates, grown to 80% confluence and treated with various concentration of $As_2O_3$ at various time points as mentioned in the experimental conditions. The cell proliferation was assessed based on MTT assay (Mosmann, 1983).

#### 2.4.2. Observation of morphological alterations of cells

Cells were seeded in 12-well plates, grown to 80% confluence and treated with $As_2O_3$ under the aforementioned experimental conditions. Cell morphology was observed (10×) and photographed using an inverted microscope (Olympus, Japan).

#### 2.4.3. LDH leakage assay

Lactate dehydrogenase (LDH) activity in the extracellular medium (an indicator of membrane leakage) was measured by ELISA plate reader using a LDH assay kit (Biovision Research Products, USA) following manufacture's specification.

#### 2.4.4. Induction of apoptosis and necrosis by $As_2O_3$

Detection of apoptosis and cell necrosis was performed with Annexin V-FITC and PI staining assay. Following treatment with $As_2O_3$, cells were harvested by trypsin, labeled with Annexin V-FITC and PI (Beckman Coulter, Fullerton CA, USA) and then examined by flow cytometry (Becton Dickinson FACSCalibur™) using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). A total of 10,000 cells were analyzed from each sample with the extent of early apoptosis, late apoptosis, and necrosis determined from the percentages of bound annexin $V^+/PI^-$, annexin $V^+/PI^+$, and annexin $V^-/PI^+$, respectively.

#### 2.4.5. Determination of cell-cycle progress by flow cytometry

To determine the progress of cell growth under treatment conditions, cultures were treated with $As_2O_3$ and their DNA content was estimated by the method of Wang et al. (2004) with minor modifications. Briefly, trypsinized cells were first washed twice with PBS containing 1% serum and then fixed overnight at 4 °C

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