University of Nevada, Reno

Differential Cytotoxicity and Reactive Oxygen Species Generation in Pulmonary and Aortic Cells Exposed to Inorganic Arsenic and Monomethylarsonous Acid

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MASTER OF SCIENCE

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Abstract

Chronic ingestion of arsenic (As), a common ground water contaminant, has demonstrated to result in numerous deleterious health outcomes including black foot disease, various cancers, and hyperpigmentation. Chronic arsenic ingestion has also been associated with the development of atherosclerosis and hypertension, though the cellular mechanisms have not been well elucidated. Recent studies have shown that an arsenic metabolite, monomethylarsonous acid (MMAs), causes a higher degree of toxicity than inorganic arsenic (iAs) and may be linked to arsenic-induced vascular diseases. This study explores possible reactive oxygen species that may contribute to atherosclerosis and examines the different cytotoxic effects of iAs and MMAs on thoracic aorta smooth muscle cells (A7r5) and rat pulmonary arterial smooth muscle cells (rPASMC) in culture to look for the generation of malondialdehyde, hydrogen peroxide, and superoxide. Cytotoxicity was determined by cell counts and Trypan Blue exclusion, MTT assay, and light microscopy to study altered smooth muscle cell morphology, cell viability and cytotoxicity.

Cells treated with various concentrations of either iAs and MMAs displayed cytotoxic effects and MMAs was significantly more toxic in both A7r5 and rPASMC. After a 24 hour exposure the LC_{50} in A7r5 treated with iAs was determined to be 11 µM, and 700 nM when treated with MMAs. In rPASMC treated with iAs the LC_{50} was determined to be 26.5 µM, and 4 µM with MMAs treatment showing higher toxicity in A7r5 cells than in rPASMC. No significant difference in MDA formation or superoxide production was observed with treatment of iAs and MMAs in both A7r5 and rPASMC. Hydrogen peroxide was measured in the extracellular medium of rPASMC and displayed

a 12% increase after a 2 hour treatment with 100 nM (p=0.006) iAs and 1 µM iAs (p=0.0002) when compared to untreated cells, a 7% increase when treated with 1 µM MMAs (p=0.03) and a 9% increase when treated with 10 µM MMAs (p=0.0003). In A7r5 cells there was a significant increase in the release of hydrogen peroxide with exposure to 1 µM MMAs displaying a 59% increase (p=0.0169) and 10 µM MMAs displaying a 70% increase (p=0.003) when compared to untreated cells.

These results suggest that the increased toxicity of MMAs could be due to increased hydrogen peroxide activity in A7r5 cells, but is not due solely due to oxidative stress when analyzed *in vitro*. Other mechanisms need to be further explored to understand the association between arsenic and cardiovascular disease.

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1. INTRODUCTION

The focus of this study was to examine the effects of inorganic arsenic (iAs) and the organic form, trivalent monomethylarsonous acid (MMAs), on rat thoracic aorta smooth muscle cells (A7r5) and pulmonary arterial smooth muscle cells (rPASMC). Chronic ingestion of arsenic-contaminated drinking water has been recognized as a public health concern. Arsenic can lead to several damaging health outcomes, but the presumed link of chronic arsenic ingestion and the development of cardiovascular disease has not been well studied (States et al., 2009). Therefore, aortic and pulmonary cell lines were used to model the *in vitro* toxicity of iAs and MMAs on vascular smooth muscle cells. The LC_{50} of iAs and MMAs exposure in both cell lines were determined to see whether there was a significant difference in toxicity between cell lines and between the organic and inorganic forms of arsenic.

Reactive Oxygen Species (ROS) are known to induce apoptosis and cell death in nearly all mammalian cells (Huang et al., 2002). ROS has also been demonstrated to cause deoxyribonucleic acid (DNA) and oxidative stress-induced damage (Ye et al., 2010). Although arsenic promotes free radical formation and generates bioactive molecules (Jomova et al., 2011), the generation of these molecules has not been demonstrated in vascular smooth muscle cells (VSMCs) after MMAs exposure. MMAs is not commercially available and was synthesized in our laboratory. Therefore, limited studies have examined the effects of MMAs, specifically generation of ROS molecules, to evaluate whether arsenic-induced cytotoxicity is due to oxidative stress in VSMCs.

Malondialdehyde (MDA) formation was measured to determine whether arsenic treatment promotes lipid peroxidation. MDA was specifically chosen for analysis because lipid peroxidation is a major risk factor for atherosclerosis, and MDA protein adducts have been found in atherosclerotic plaques of both animals and humans (Jurgens et al., 1993).

Extracellular hydrogen peroxide (H_20_2) was also measured in the cell culture medium after exposure to arsenic to determine whether this was a possible mechanism of cell death. Hydrogen peroxide has been known to cause cellular damage and is released by the mitochondria (Treberg et al., 2010). The mitochondria are known sites for ROS production and convert superoxide rapidly to hydrogen peroxide via superoxide dismutase (Perez-Vizcaino et al., 2010). Therefore, superoxide production was also be measured in this study.

Both iAs and MMAs were observed to be far more toxic in A7r5 cells than in rPASMC. Based on the experiments presented, it appears that cytotoxicity is not solely due to oxidative stress. The rPASMC showed significant increase in H_20_2 production when exposed to both iAs and MMAs. However, the A7r5 cells showed an increase in H_20_2 with MMAs exposure at 1 µM and 10 µM, but no significant increase with iAs exposure. This suggests that oxidative stress is not the main factor explaining cytotoxicity of arsenic in VSMCs.

2. LITERATURE REVIEW

2.1 Arsenic: A Public Health Concern

2.1.1 History of Arsenic

Arsenic is a naturally occurring metalloid found in the environment. Although arsenic displays properties of both metals and nonmetals it is frequently characterized as a heavy metal for purposes of explaining toxicological effects (Mandal and Suzuki, 2002). Arsenic has been used in industrial processes and found to be present in tobacco, but the greatest source of human exposure is the consumption of arsenic in contaminated ground water resulting in acute and chronic health outcomes (WHO factsheet no. 210, 2001). Arsenic is solubilized in ground water under proper conditions and with the world's increasing population the resources for clean water are dramatically decreasing (Smedley et al, 2002; Nordstrom, 2002). Exposure to arsenic occurs mainly through consumption of contaminated water, but can also occur through industrial processes and use of contaminated water for irrigation purposes and preparation of food crops (WHO factsheet no. 210, 2001).

2.1.2 Geographical Areas Affected

There has been an increased dependence on ground water around the world resulting from the lack of clean surface-water supplies. The demands for sanitary water have continued to rise with an increasing population (Nordstrom, 2002). Both natural and anthropogenic environmental conditions have been the cause of high arsenic concentrations in ground water. According to the World Health Organization (WHO), Bangladesh has a wide-spread occurrence of arsenic in well-water. Although the WHO

recommended limit for arsenic in drinking water is 10 µg/L, or 10 parts per billion (ppb), it is estimated that 20-45 million people are at risk of arsenic exposure greater than 50 ppb (WHO factsheet no. 210, 2001). Groundwater concentrations in Bangladesh and West Bengal have been found to range from 50-3200 ppb (Bhattacharya et al., 2003). Other areas affected by high concentrations of arsenic in ground water include locations in Asia, including West Bengal, Taiwan, Thailand, and Mainland China (Chowdhury et al., 2000). Locations in South America that are affected include Argentina and Chile. Many locations of elevated arsenic in groundwater exist in the United States, specifically the western states, where concentrations of arsenic in ground water have been found to be higher than the Environmental Protection Agency Standard (EPA) for public water supplies of 10 ppb (Smith et al., 2002).

2.1.3 Environmental Protection Agency Regulations

Arsenic was labeled as a cancer causing agent as early as 1879. In the United States, after a long national discussion, the EPA lowered the maximum contaminant level (MCL) of arsenic in public water supplies to the current 10 ppb. Before the regulations were set at 10 ppb, the standard was 50 ppb and set in 1942 (Smith et al., 2002). In 1962 health concerns were expressed by the U.S. Public Health Service (USPHS) and were advised that the standards should not exceed 10 ppb (USPHS, 1962). Almost 20 years later, in 1986, congress took action and directed the EPA to lower the standards to the previously advised 10 ppb. The lower standard was announced to take place in 2001 under the Clinton administration. Two months later, shortly after the Bush administration took office, this process was delayed due to the high costs and apparent concerns over the

science supporting the lower standards (Smith et al., 2002). During this delay the National Research Council (NRC) put out a report stating that the EPA had underestimated the cancer risks with standards at 50 ppb, and that they were in fact much higher than the EPA had previously reported (Smith et al., 1992). The change in regulations faced delays due to a lack of experimental studies with proper animal models causing uncertainties in data extrapolating from rodents to humans (Abernathy et al., 1999), and uncertainties with confounding factors in epidemiological studies (Smith, 1988). Finally, in 2006 the compliance date for the new standard of 10 ppb was implemented (Smith et al., 2002).

2.2 Arsenic Metabolism

2.2.1 Arsenic in the Environment

Arsenic exists in different forms and concentrations in the environment and comes from both natural and anthropogenic sources (Roy and Saha, 2002). Natural occurring arsenic is most commonly found in geological formations containing iron oxides. Iron oxides can adsorb to arsenic which can then cause high concentrations of arsenic to be released into the environment through an iron-reducing reaction (Welch et al., 2000; Dzombak and Morel, 1990). Anthropogenic sources of arsenic include industrial processes for wood preservation and glass production, and were widely used as a pesticide in the 1980s to 1990s prior to being banned for use in pesticides (Welch et al., 2000). There are two forms of inorganic arsenic (iAs) which consist of pentavalent arsenate (iAs^{5+}) and trivalent arsenite (iAs^{3+}) (Roy and Saha, 2002). Pentavalent arsenate is the main form found in surface water, and iAs^{3+} is the primarily form humans are

exposed to. It is found in the lower redox potential underground water reservoirs (Irgolic, 1994). The bioavailability of arsenic in the environment varies with specific factors such as pH, oxygen availability and whether or not it exists in an aquatic environment. There are four arsenate species that exist, depending on pH, and include H_3AsO_4, $H_2AsO_4^{-1}$, $HAsO_4^{-2}$ and AsO_4^{-3}. In conditions where arsenite is reduced the arsenous acid species consist of H_3AsO_3, of $H_2AsO_3^{1-}$, and $HAsO_3^{2-}$. The arsenous acids have been shown in experimental studies to be more toxic both *in vivo* and *in vitro* (Knowles et al., 1983, Cervantes et al., 1994). The figure below shows how numerous chemical and biological systems are involved in controlling the bioavailability of arsenic.

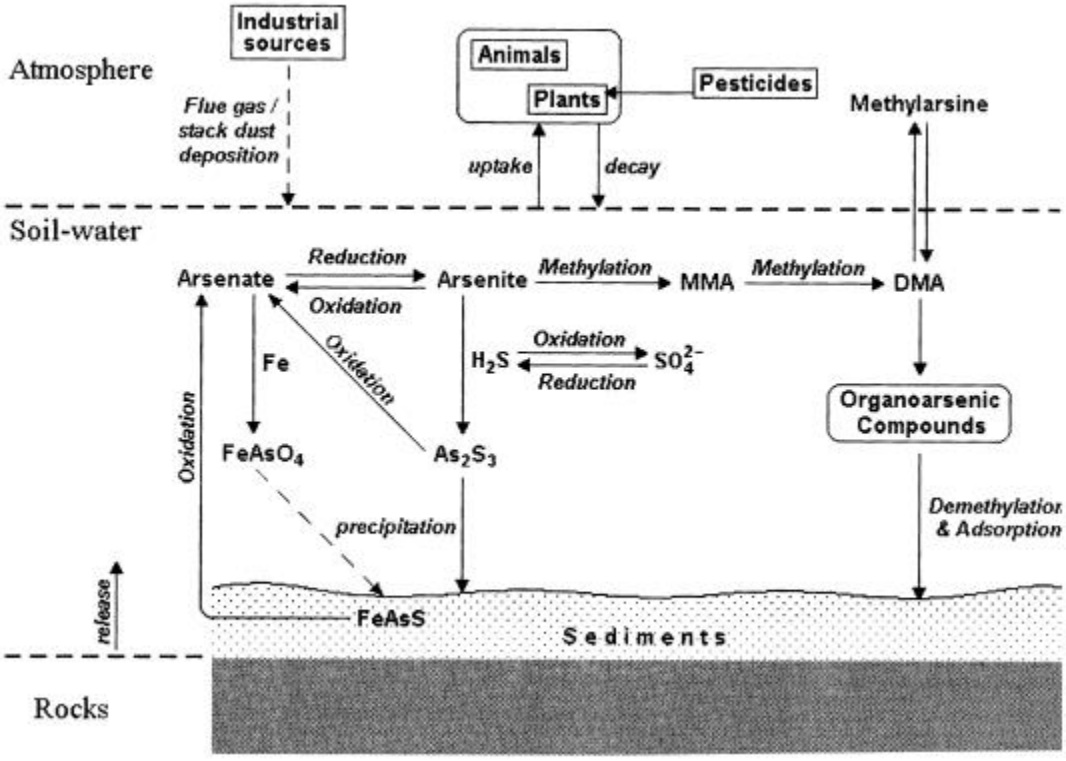


Figure 1. Various conditions that effect bioavailability of arsenic in the environment. Adapted from Roy and Saha, 2002.

2.2.2 Arsenic Metabolites

The toxicity of arsenic is largely dependent on its oxidation state. Trivalent inorganic arsenite has demonstrated to have higher toxicity than iAs^{5+} in experimental studies (Yamauchi and Fowler, 1994). In humans and a majority of mammals, iAs is metabolized by a two-electron reduction, facilitated by glutathione (GSH), which reduces iAs^{5+} to iAs^{3+}. It can then undergo methylation catalyzed by S-adenosylmethionine to form monomethylarsonic acid (MMA^{5+}) which will be reduced to monomethylarsonous acid (MMA^{3+}). Monomethylarsonous acid can undergo oxidative methylation enzymatically via arsenic (+3 oxidation state) methyltransferase (AS3MT) to form dimethylarsinic acid (DMA^{5+}) which can then be reduced to form dimethylarsinous acid (Hughes, 2002). In the figure below a proposed scheme of the metabolism of inorganic arsenic to organic arsenic is illustrated.

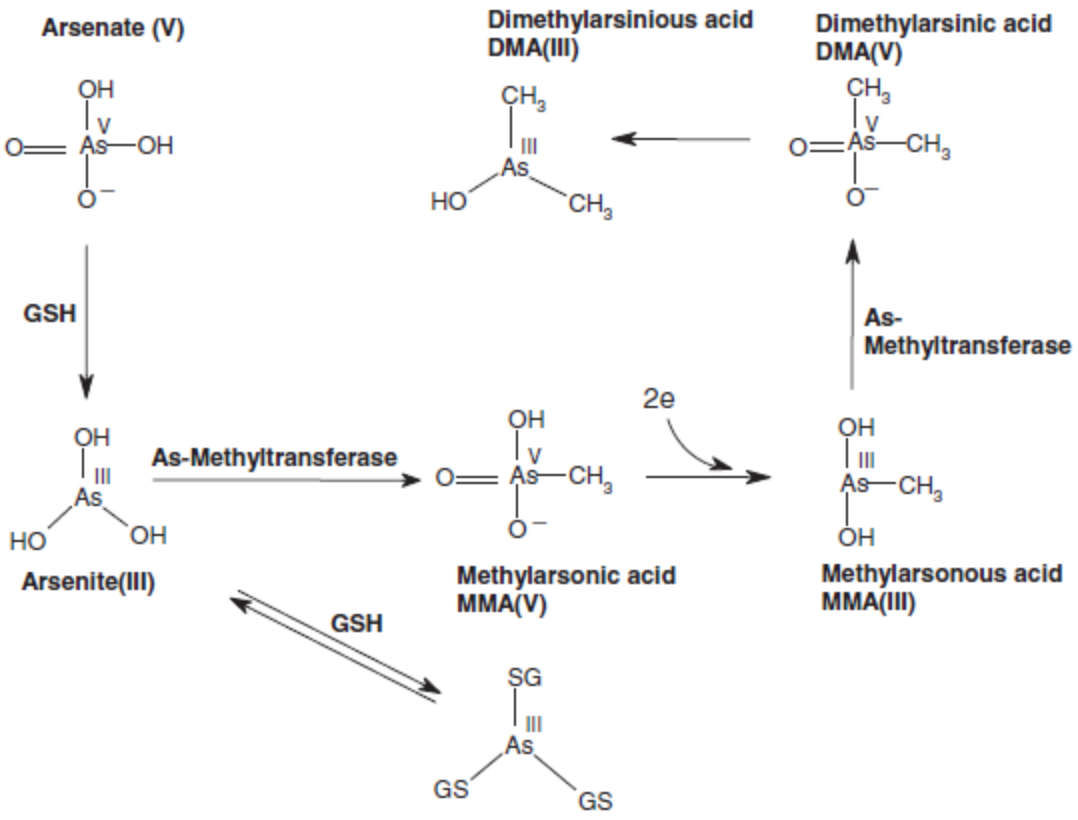


Figure 2. Inorganic arsenic metabolism. Adapted from Jomova et al., 2011.

Previous studies have reported that the biomethylation of inorganic arsenic is a detoxification process and the methylated intermediates are far less toxic than inorganic arsenic (Shi 2004, Valko, 2005). However, the trivalent metabolites were not previously studied as extensively as inorganic arsenic (Styblo et al., 2000). Recent studies have focused on the trivalent intermediates, MMA^{3+} and DMA^{3+}, which have been documented to be present in the urine of humans that are exposed to inorganic arsenic (Cohen et. al, 2006). It is now clear that the trivalent intermediates, specifically MMA^{3+}, are up to 20 times more toxic than trivalent iAs (Styblo et al., 2000). The trivalent, methylated metabolites, MMA^{3+} and DMA^{3+} have been reported to be the most toxic. This is possibly due to their ability to be taken up by the cell more efficiently than the

other metabolites (Dopp et al., 2010). Other organic forms of arsenic include arsenobetaine and arsenocholine. These forms are found to accumulate in fish, but have been reported to be nontoxic to humans (Hindmarsh, 2000).

2.3 Arsenic toxicity

2.3.1 Chronic arsenic exposure: Health Outcomes

Chronic inorganic arsenic exposure has shown to affect the majority of the organs in the body (Cohen et al., 2006). Cancers of the lung, skin, bladder, liver, kidney and prostate have been recognized as outcomes of chronic arsenic exposure (IARC, 2012; ROC, 2011). Arsenic enters the body primarily as the trivalent form via ingestion or inhalation (Cohen et al., 2006). Arsenic does not readily pass through the skin to enter the body, but is ingested when present in water, soil or food and it is inhaled with arsenic containing dust particles (Chen et al., 2006). The organic and inorganic forms of arsenic will be excreted through the urine. However, the inorganic form may remain in the body for a period of months (Aposhian et al., 2000). One positive health effect arsenic trioxide has had on health is with the treatment of acute promyelocytic leukemia (AML). The ability of arsenic to induce apoptosis in these malignant cells has been used as treatment for the past two and a half decades for relapsed patients. Studies have focused on understanding the mechanisms behind how it is able to target these cancer cells (Ferrara, 2010).

Arsenicosis is defined as the development of skin lesions, hyperkeratosis, and hyperpigmentation which are all associated with chronic arsenic exposure; these symptoms are early indicators for chronic arsenic poisoning (McCarty et al., 2007). A

study observing farmers in Taiwan showed that skin lesions had developed only with the individuals who were drinking arsenic-contaminated drinking water. It was also shown that the number of skin lesions developed was largely dependent on concentrations of arsenic in drinking water; with greater concentrations resulting in higher skin lesions (Tseng, 1977; Tseng et al., 1996).

Skin cancer is the primary form of cancer associated with arsenic exposure, but epidemiological evidence suggests that cancers of the lung, kidney, bladder and liver are also associated with arsenic exposure (Rossman, 2003). Inhalation of arsenic has been linked to lung cancer, as indicated in one study where an increased incidence of lung cancer was reported with workers exposed to dust containing arsenic trioxide (Wall, 1980; Welch et al., 1982). Workers who have been exposed to arsenate have displayed a higher incidence of lung cancer (Bulbulyan et al., 1996). The exact mechanism by which arsenic causes cancer is still not completely understood but accepted mechanisms include oxidative stress, induced cell proliferation and altered gene expression (Galanis et al., 2009).

Chronic arsenic exposure has demonstrated to result in hepatic injury. Consistent findings have shown an increase in liver damage and hepatic enzyme levels, including the antioxidant, glutathione (GSH), and antioxidant enzymes associated with lipid peroxidation (Liu et al., 2002; Mazumder et al., 2005). In one study, rats who received 3.33 mg of sodium arsenite per kg body weight per day displayed increased levels of GSH in the liver. Lipid peroxidation was enhanced in the kidneys and another antioxidant, superoxide dismutase, was decreased. This study suggests that the kidneys

are more sensitive to arsenic exposure than the liver, possibly due to the high concentrations of antioxidants present in the liver (Maiti and Chatterjee, 2000).

Acute exposure to high concentrations of inorganic arsenic, and chronic exposure to low concentrations of inorganic arsenic have shown to cause gastrointestinal disturbances including diarrhea, nausea, vomiting, and abdominal pain (Uede and Furukawa, 2003). When organic arsenicals were ingested the gastrointestinal tract seemed to also be affected by the same toxic effects as inorganic arsenic exposure, causing diarrhea and vomiting (Lee et al., 1995).

Neurotoxic effects have been observed with chronic arsenic exposure. Studies have demonstrated that ingestion of iAs can cause encephalopathy and symptoms of headache and mental confusion (Bartolome et al., 1999). When sodium arsenite was administered in animal studies neurological disorders were observed, and neurotransmitter levels displayed significant changes in central nervous system (Kannan et al., 2001). Neurobehavioral changes have been observed in children in areas of Taiwan and China where low levels of arsenic in drinking water were present. Children displayed a decreased attention span and ability to switch tasks (Tsai et al. 2003; Jomova et al., 2011).

A health outcome of arsenic that has been studied more recently is the development of type 2 diabetes. Epidemiological studies in Taiwan and Bangladesh have correlated chronic arsenic exposure to diabetes (Lai et al. 1994; Navas-Acien, 2008). A cross-sectional study conducted in the United States with adults 20 years of age or older who participated in the National Health and Nutrition Examination Survey (NHANES) had urine samples tested for arsenic. They found that those with type 2 diabetes had total

arsenic levels 26% higher than those without diabetes. This study demonstrates how low level arsenic exposure may be correlated to the prevalence of diabetes (Navas-Acien et al., 2008). The mechanism by which arsenic can induce type 2 diabetes is unclear but it has been indicated that symptoms of arsenic poisoning mimic those of diabetes with reduced secretion of insulin in populations exposed to high arsenic concentrations (Diaz-Villasenor et al., 2007). The study published by Navas-Acien et al. 2008, was faulted for the statistical methods used to analyze the correlation of arsenic exposure and type 2 diabetes. Several studies have re-examined the data with additional NHANES subjects demonstrating opposite results. The statistical modeling used when analyzing urinary creatinine and arsenobetaine in the urine may be why there are differences in the data obtained originally and the lack of reproducible data. It is important to include analysis of specific biomarkers for the development of diabetes for future studies (Chen et al., 2010).

In addition to these problematic health outcomes it has also been revealed that long-term exposure to arsenic has demonstrated to cause cardiovascular effects. Less attention has been paid to arsenic-induced cardiovascular disease than other health outcomes. However, several epidemiological studies have shown that arsenic exposure is associated with a higher risk of vascular diseases (States et al., 2009).

2.4 Arsenic and Cardiovascular Disease

2.4.1 Epidemiological Studies

Several epidemiological studies have researched the correlation between arsenic and cardiovascular diseases including hypertension (Chen et al., 1996), carotid atherosclerosis (Wang et al., 2002), ischemic heart disease (Hsueh et al., 1998), and

vascular disease mortality (Chen et al., 1996). The first documented case of arsenic-induced vascular disease was in 1969 when workers who were exposed to arsenic in a copper smelter showed a significant increase in mortality from ischemic heart disease (Lee and Fraumeni, 1969; Welch et al., 1982). In addition, epidemiological studies have also shown that arsenic causes "blackfoot" disease (BFD), which is the result of peripheral artery disease, eventually causing gangrene of the extremities (Tseng, 1989). Black foot disease is unique to Taiwan and not found in other places of the world. This may be due to malnutrition in the areas where BFD is prevalent. Other areas of the world affected by arsenic exposure have displayed less severe forms of peripheral vascular disease (WHO factsheet no. 210, 2001). Many epidemiological studies have been conducted in Taiwan, where populations are exposed to high levels of arsenic. One study showed that populations exposed to high levels of arsenic in well-water had increased mortality from cardiovascular and cerebrovascular diseases and also had BFD (Chen et al., 1988; Tseng et al., 2003). In Northeastern Taiwan researchers demonstrated a dose-dependent relationship between the prevalence of CVD and arsenic concentrations in well water after controlling for confounding factors such as smoking, alcohol consumption, age and gender (Wang et al., 2007). Furthermore, in Southwestern Taiwan there have been extensive studies focusing on the amputated extremities of those with BFD. In these cases there were typical pathological findings that involved the development of arteriosclerosis. This suggests that without the presence of any other coronary risk factors chronic arsenic exposure will lead to the development of arteriosclerosis (Rosenberg, 1974, Wang et al., 2007). One of the hallmarks for chronic arsenic exposure is carotid atherosclerosis, which has been used as a biomarker for

chronic arsenic exposure and poisoning. It is suggestive that chronic arsenic exposure can also lead to general atherosclerosis (Wang et al., 2007). Furthermore, in Taiwan the prevalence of carotid atherosclerosis and the concentrations of arsenic exposure from contaminated water show a dose-response relationship. After controlling for other risk factors it was determined that chronic arsenic exposure is independently a risk factor for atherosclerosis (Wang et al., 2002).

High pulse pressure is a risk factor for atherosclerosis and is an indicator for arterial stiffness (Safar et al., 2003). A study that took place in Bangladesh determined that there was a correlation between arsenic exposure via ingestion of drinking water and high pulse pressure. High pulse pressure was considered higher than 55 mmHg and was indicated to be 1.19-1.39 greater with the individuals who were exposed to higher levels of arsenic when compared to the control group of those exposed to low levels of less than 8 ppb. Nutrient intake was also taken into consideration during this study. The individuals who had a lower than average intake of B vitamins and folate, and were also exposed to high concentrations of arsenic, were 1.83-1.89 times more likely to have a high pulse pressure when compared to those exposed to low levels of arsenic. This study suggests that a significant association exists between the high pulse rate and chronic arsenic exposure, as well as with nutrient intake (Chen et al., 2007).

In the United States studies have been conducted to determine if high levels of arsenic in well water also correlate to an increased incidence of cardiovascular diseases. Areas where arsenic concentrations were greater than 20 ppb, an increase in deaths due to diseases related to arteriosclerosis and aneurysms was observed. However, limited knowledge exists on the correlation between low concentrations of arsenic ingestion via

drinking water and CVD, and in the US there have been inconsistent findings associating the two (Engel and Smith, 1994). Based on the accumulating epidemiological studies and data, chronic exposure to arsenic via water ingestion is recognized as an independent risk factor for CVD (Simeonova and Luster 2004).

2.4.2 Cellular Mechanisms

Many possible roles have been explored to better understand the association between arsenic and cardiovascular diseases, but the mechanisms are still not well known. Atherosclerosis progresses from vascular smooth muscle cell (VSMC) inflammation to the later stages of thrombotic and fibrotic destruction of the vessels. The endothelial cells facilitate transduction of physiological and chemical stimuli from the lumen of the blood vessel to the VSMC. They are also responsible for regulating vascular tone by maintaining a nitric oxide balance and providing cell signaling for a vast array of functions (Simeonova and Luster, 2004). When endothelial cells are disrupted or damaged by ROS generation inflammation cytotoxicity will occur, eventually leading to vasoconstriction. When dysfunctional endothelial cells display an altered cellular redox state apoptosis can occur along with altered anti-inflammatory properties that can eventually lead to atherosclerosis (Cai and Harrison, 2000).

The oxidation of low-density lipoproteins (LDL) has long been suspected as the primary mechanism behind the development of atherogenesis (Steinberg et al., 1989, Mertens and Holvoet, 2001). When an oxidized LDL particle is taken up by a macrophage a “foam cell” will develop. A “foam cell” is a macrophage overloaded with lipids and fatty substances that can be found within the blood vessels and can accumulate

causing inflammation of the artery walls, subsequently contributing to the development of an atheroma (Chen et al., 2003). Oxidized LDL has shown to be present in every stage of atherogenesis when studied in animal models (George et al., 1998). However, when human aortic endothelial cells have been exposed to arsenic *in vitro* no induction of oxidized LDL has been present (Simeonova et al., 2003).

Vascular adhesion molecules have been studied in the progression of atherosclerosis. Arsenic has shown to increase the activity of certain transcription factors including NF-kB and AP-1. Vascular adhesion molecules, (e.g., (VAM)-1), and proinflammatory molecules consisting of the chemokine, monocyte chemoattractant protein (MCP)-1), and cytokine, interleukin (IL)-8), result in inflammation when upregulated within the endothelium, and have been commonly identified in atherosclerotic lesions (Cushing et al., 1990; Takei et al., 2001). In double knock-out mice the proinflammatory molecules, MCP-1 and IL-6, were genetically deleted and the formation of atherosclerotic lesions were shown to decrease. This demonstrates the importance of these molecules in inflammation and the role they play in atherogenesis (Schieffer et al., 2004). Chronic inflammation is associated with development of atheroma and is important in the development of atherosclerotic plaques and the progression of atherosclerosis (Ross, 1999). Humans with two genotypic polymorphisms, ApoE (apolipoprotein E) and MCP1, have displayed greater than a 10 fold risk of developing carotid atherosclerosis if exposed to drinking water greater than 10 ppb of arsenic. ApoE functions as an LDL receptor and MCP-1 has been associated with the progression of atherosclerotic plaques; each aid in the activation of inflammatory molecules (Hsieh et al., 2008). This data continues to emphasize the importance of

arsenic-induced inflammation as an important risk factor for the development of atherosclerosis (Tsou et al., 2005).

Another hallmark of dysfunctional endothelial cells and development of atherosclerosis is an imbalance of nitric oxide (NO) (Rekka and Chrysselis, 2002). Nitric oxide plays many roles within the vascular wall and when functioning properly it's responsible for maintaining vasodilation and inactivating the molecule, NF-kB, which in turn inhibits platelets from becoming active and inhibits VSMC proliferation (Barchowsky et al., 1996; Thomas et al., 2003). When endothelial cells are exposed to sodium arsenite the integrity of the endothelium is compromised an increase in cytotoxicity occurrs. Vasorelaxation is affected because the bioavailability of NO is decreased with the inactivation of a protein kinase B/Akt and endothelial NO synthase (eNOS). Endothelial NO synthase is responsible for generating NO which in turn will inhibit the formation of platelets and vasorelaxation (Balakumar and Kaur, 2009). It has also been demonstrated that endothelial cell proliferation is disrupted with arsenic exposure (Chen et al., 1990).

Arsenic exposure has been associated with impaired NO activity. In a study performed on aorta rings *in vitro,* acetylcholine was used to induce relaxation of the rings. Guanosine 3,5-cyclic monophosphate (cGMP) was measured, in place of NO, after arsenic exposure. Following arsenic exposure, relaxation of the rings was inhibited and the levels of cGMP were decreased in a concentration dependent manner (Lee et al., 2003). Decreased bioavailability of NO due to arsenic exposure may be one mechanism in progression of atherosclerosis (Simeonova and Luster, 2004).

Hypertension has also been linked to chronic arsenic exposure (Yang et al., 2007). One mechanistic possibility of this effect is the activation of myosin light-chain by phosphorylation. A series of events can occur following phosphorylation which will lead to cell contraction, motility, proliferation and gene expression (Satoh et al., 2011). Phosphorylation of myosin light-chain will also increase calcium-sensitization within the blood vessels. The antioxidant defense system also plays an important role in hypertension. An increase in systolic blood pressure can occur when the defense system is impaired and there is a lack of antioxidant bioavailability. These are all possible mechanisms by which arsenic can contribute to the development of hypertension (Jomova et al., 2011).

Arsenic-induced oxidative stress has been suggested by several studies. In endothelial cells it was demonstrated that a variety of antioxidant enzymes were induced, including NADPH dehydrogenase and glutathione S-transferase (GST) (Hirano et al., 2003). Glutathione (GSH) is a thiol-based, tripeptide that conjugates with a number of different compounds and the conjugation with xenobiotics in the detoxification process is catalyzed by GST (Meister and Anderson, 1983). Porcine aortic endothelial cells (PAECs) exposed to arsenic trioxide have displayed an increase in intracellular GSH levels possibly in response to oxidative stress factors (Cheng, 2008). However, in the liver arsenic trioxide causes a depletion of GSH. Depletion of GSH can lead to levels of ROS being increased causing cytotoxic effects due to oxidative stress (Alarifi et al., 2013).

2.5 Reactive Oxygen Species

2.5.1 Oxidative Stress and ROS production

Substances that contain oxygen and react with biomolecules are called reactive oxygen species (ROS). ROS have been shown to cause tissue damage, lipid peroxidation, and protein and DNA damage. Oxidative stress is the term used to describe the toxic effects when ROS homeostasis is disrupted due to the decreased antioxidant levels (Perez-Vizcaino et al., 2010). Oxidative stress can be induced by arsenic, and the antioxidant response can form free radicals as a result of inflammation. When arsenic cycles between oxidation states oxidative stress may also be induced (Halliwell et al., 2004). Some important arsenic-induced ROS include hydrogen peroxide (H_20_2), hydroxyl radical ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot -}$) and peroxyl radicals ($ROO^{\cdot}$) (Flora, 2011). Cell signaling pathways are also disrupted due to ROS, primarily hydrogen peroxide and superoxide. ROS scavengers are antioxidants that used to prevent the damaging effects of ROS (Perez-Vizcaino et al., 2010). ROS have been demonstrated to display beneficial effects to a degree as well. Protective factors that are displayed with low concentrations of ROS generation include the elimination of cancerous cells via apoptosis and detoxification reactions utilizing the cytochrome P-450 complex (Salganik, 2001). Cytochrome P-450 is able to detoxify hydrophobic xenobiotics by generating superoxide which will in turn transform the molecule and metabolize it (Shenkman, 1993). A positive factor of ROS generation would be screening individuals with a high risk of developing diseases associated with an upregulation of ROS to determine if that individual can benefit in reducing their risk by applying antioxidant supplements (Salganik, 2001).

The mitochondrion is one of the primary sites of ROS production within the pulmonary vasculature. NADPH is a molecule in the mitochondria used as an electron donor for the electron transport chain to create ATP (Perez-Vizcaino et al., 2010). NADPH oxidase is the primary enzyme producing superoxide (Cai and Harrison, 2000), and an important enzyme involved in arsenic toxicity. Arsenite has demonstrated to upregulate NADPH causing DNA damage in vascular smooth muscle cells. This may be due to the production of superoxide and the ability of superoxide to cause DNA strand breaks (Lynn et al., 2000).

In cardiomyocytes ROS was demonstrated to cause the induction of caspase-3. The signaling of caspase-3 will lead to apoptosis (Hays et al., 2008). The same effect has been observed in human hepatocellular carcinoma cells. Arsenic trioxide displayed a dose-dependent increase in caspase-3 activity after 24 and 48 hour exposure (Alarfi et al., 2013).

The initiation of atherosclerosis induced through chronic arsenic exposure includes many mechanisms but the most widely accepted mechanism is arsenic-induced oxidative stress (Ercal et al., 2001). In summary, once arsenic induces oxidative stress and damage occurs to the vascular cells proinflammatory molecules are activated which will further activate platelets and monocytes to aggregate. When LDL is oxidized and taken up by macrophages “foam cells” may form and cause fatty streak lesions within the blood vessel. Inflammation will continue with the decreased NO availability causing vasoconstriction and inhibiting molecules, such as plasminogen activators, from breaking down the blood clots. This causes consistent plaque formation and atheroma development

will continue to worsen eventually leading to the development of atherosclerosis (Bunderson et al., 2004; Tsai et al., 2002; Jiang et al., 2002).

2.5.2 Malondialdehyde

Malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE) are the most abundant end products of LDL oxidation generated via ROS (Valko et al., 2005). They are also largely formed in polyunsaturated fatty acids due to their high sensitivity to free radical damage. MDA and HNE can be measured directly as indicators of oxidative stress (Wirtitsch et al., 2009). MDA induces lipid peroxidation and will form an adduct with DNA which has been found and isolated in human tissues (Niedernhofer et al., 2003).

In one study sodium arsenate was administered to rats for 1 hour. Following exposure, rats were sacrificed in order to analyze MDA formation and GSH activity. Analysis of GSH levels showed a significant decrease whereas an increase in lipid peroxidation was observed in the liver, kidney and heart (Ramos et al., 1995). Another study showed correlating results when human hepatocellular carcinoma cells were exposed to varying concentrations of sodium arsenite for 24 and 48 hours. An increase of MDA was observed and a decrease in GSH in a dose dependent manner (Alarifi et al., 2013). Typically GSH is used as a marker for oxidative stress, however, numerous studies have shown a decrease of GSH levels following arsenic exposure (Ramos et al., 1995; Alarifi et al., 2013). A possible explanation for this is that arsenite has a high affinity for GSH which in turn can cause the depletion (Jomova and Valko, 2011).

Oxidized LDL is a major risk factor for the development of atherosclerosis. It is the primary factor that stimulates oxidative injury resulting in the inflammatory response.

(Mitra et al., 2011). Lipid peroxidation has occurred in humans when exposed to arsenic. A study in China showed that residents who were using coal with high levels of arsenic had significantly higher MDA levels in their urine when compared to those in the same area without using arsenic-contaminated coal (Wang et al., 2009).

Arsenic-induced lipid peroxidation has shown a positive correlation between arsenic exposure and lipid peroxidation in plasma, urine, and tissue in a number of studies (Vizcaya-Ruiz et al., 2009; Wirtitsch et al., 2009).

2.5.3 Hydrogen Peroxide

Hydrogen peroxide is a non-radical ROS generated by arsenic exposure and is involved in cell signaling pathways (Yamanaka and Okada, 1994, Schroder and Eaton, 2008). Hydrogen peroxide is uncharged, and a less reactive molecule than superoxide; therefore it can diffuse across membranes easily (Schroder and Eaton, 2008). Arsenic has shown to induce superoxide which is dismutated quickly by superoxide dismutase (SOD) to hydrogen peroxide and oxygen (Figure 3). Hydrogen peroxide can induce cellular signaling in vascular smooth muscle cells and stimulate phosphorylation of kinases (Oeckler et al., 2005) which can cause both contraction and relaxant effects within the pulmonary arteries (Tang and Vanhoutte, 2009).

Although hydrogen peroxide is not a free radical, it can create a highly reactive hydroxyl radical when a high abundance of free iron is available in the body. Free iron decomposes hydrogen peroxide to a hydroxyl radical via the Fenton reaction. However, hydrogen peroxide can also be decomposed to water and oxygen with the antioxidant catalase (CAT) (Jomova et al., 2011).

2.5.4 Superoxide

The superoxide anion radical is formed with arsenic exposure and is generally considered to be the primary ROS molecule due to its ability to further interact with other molecules forming secondary ROS molecules. An example of a secondary ROS molecule is a hydroxyl radical generated by the Fenton reaction. Superoxide is extremely reactive and is able to interact with molecules directly or indirectly to generate additional ROS (Valko et al., 2005). Superoxide and hydrogen peroxide can form a hydroxyl radical which has shown to be important in the facilitation of the genotoxic effects of arsenic (Liu et al., 2001). Superoxide has the ability to interact with and suppress NO activity leading to impaired endothelial function. The cells will lose their ability to vasodilate, eventually leading to the progression of atherosclerosis as previously described (Napoli, 2002).

Superoxide and hydrogen peroxide are both involved in altering vascular tone. Superoxide is able to contract pulmonary arteries but can also be blocked by catalase because of the rapid conversion to hydrogen peroxide (Rhoades et al., 1990).

2.5.5 Antioxidants

Antioxidants provide cellular defense mechanisms providing protection from oxidative damage. The changes observed in antioxidant levels can be an indicator of oxidative damage. SOD and CAT are the first to respond to cellular exposure to oxygen and hydrogen peroxide. A superoxide anion will be dismutated by SOD to oxygen and hydrogen peroxide. CAT will catalyze the reaction of hydrogen peroxide to oxygen and water (Jomova et al., 2011). The enzyme activity of SOD and CAT are proportional to

the concentration of arsenic exposure (Flora, 2011). In one study, rats exposed to arsenic had decreased levels of SOD due to the increased activity of superoxide induced by arsenic exposure [specific tissue sites of expression unspecified by author] (Jomova et al., 2011). However, in another study, CAT and SOD were shown to increase with increasing concentrations of arsenic trioxide after a 24 and 48 hour exposure to human hepatocellular carcinoma cells (Alarifi et al., 2013). The thiol-based antioxidants also provide protection against free radicals. GSH is effective in maintaining cellular redox states and assisting in detoxification (Halliwell and Gutteridge, 2007). Depletion of GSH is a common phenomenon seen with arsenic exposure. GST has also been used as a biomarker for pulmonary changes following arsenic poisoning to determine the levels of GSH conjugation in xenobiotic detoxification (Lantz et al., 2007).

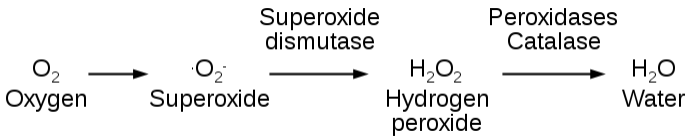


Figure 3. ROS detoxification pathway.

The purpose of this study was to evaluate the effects of inorganic arsenic and monomethylarsonous acid cytotoxicity and reactive oxygen species generation in rat thoracic aorta smooth muscle cells and rat pulmonary arterial cells. The preceding work has demonstrated an importance in better understanding the mechanisms of arsenic-induced vascular diseases.

3. MATERIALS AND METHODS

Reagents

Sodium arsenite, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoilium bromide (MTT), Catalase, Polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), Diethyldithiocarbamate (DDC), diethylenetriaminepenta-acetic acid (DTPA), Dimethyl Sulfoxide (DMSO), Trypan blue exclusion, 2-thiobarbituric acid, and RIPA Buffer were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium with high glucose, antibiotic-antimycotic, 0.05% Trypsin, Phosphate Buffered Serum, Amplex Red hydrogen peroxide assay kit were purchased from Invitrogen (Carlsbad, CA). Cyclic hydroxylamines 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine (TM-H), and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) were purchased from Enzo Life Sciences (San Diego, CA). Pierce BCA Protein Assay Kit was purchased from Thermo scientific (Pittsburg, PA). Methyldiiodoarsine was synthesized in Jeff Angermann's lab at the University of Nevada, Reno. Identity was established by comparison of capillary melting point, recrystallized product color and appearance, and product yield with published data (Millar et al., 1960).

3.1 Cell Culture

Thoracic aorta smooth muscle cells (A7r5) were a gift from Dean Burkin's pharmacology lab at the University of Nevada, Reno. Rat pulmonary arterial vascular smooth muscle cells (rPASMC) immortalized with telomerase were a gift from Cherie Singer's pharmacology laboratory at the University of Nevada, Reno. Both cell lines were subcultured to passages 15-20 and used to determine cell viability when exposed to

sodium arsenite and monomethylarsonous acid. Cells were cultured in Dulbecco's modified Eagle's medium with high glucose, sodium pyruvate and L-glutamine, supplemented with 10% Fetal Bovine Serum and 1% antibiotic-antimycotic. Cells were cultured at 37° C and 5% CO_2. Cells were passaged at 85-90% confluency with 0.05% Trypsin and subcultured in 75 cm^2 flasks, 96-well plates and 6-well plates for arsenic exposure.

3.2 Arsenic Exposure

Stock solutions of sodium arsenite and monomethylarsonous acid were made in ultra-pure DIH_20 at 20mM, 2mM, 200 µM, 20 µM, and 2 µM. Appropriate concentrations were added in cell culture medium for exposure experiments. Cells were exposed for 24 hours to various concentrations and harvested for experiments.

3.3 Cytotoxicity Determinations

Cell Morphology

Cell morphology of A7r5 and rPASMC were observed using a Zeiss Axiovision microscope following 24hr exposure to different concentrations of sodium arsenite and monomethylarsonous acid.

Cell Counts and Trypan Blue Exclusion

Cells were seeded in 6-well plates at $1x10^5$ and allowed to adhere to the surface for 24 hours. Afterwards, varying concentrations of sodium arsenite and monomethylarsonous acid (10 nM-1 mM) were added to the cell medium and incubated for 24 hours. Following treatment, cells were trypsinized and an aliquot of cell suspension was used for cell counts and Trypan Blue Exclusion was used to verify cell

counts. Lethal concentration at 50% (LC_{50}) was determined by graphing cell counts using the Origin software, version 6.0. Cell counts were determined with a Z1 Coulter Counter, and cells were manually counted using a 1:1 dilution of cell suspension and Trypan Blue Exclusion dye. Cell viability was calculated using the following formulas:

Trypan Blue:

$$\text{Cell Viability} = \frac{\text{unstained cells}}{\text{stained cells + unstained cells}} \quad (1)$$

Z1 Coulter Counter:

$$\%\ \text{Cell Viability} = \frac{\text{treated cell counts}}{\text{untreated cell counts}} \times 100 \quad (2)$$

Mitochondrial function

A7r5 and rPASMC were seeded in 96-well plates at $1x10^4$ cells per well and allowed to adhere to the surface for 24 hours. Different concentrations of sodium arsenite and monomethylarsonous acid (10nM-1mM) were exposed in triplicate for 24 hours. After 24 hours, MTT solution (5mg/mL) was added to each well and incubated for 3 hours resulting in formazan crystals formed by the mitochondria. Media containing MTT was removed and formazan crystals were dissolved with 100µL of DMSO. The 96-well plate was put on a plate shaker for 1 minute and absorbance was analyzed using a Tecan Ultra microplate reader at 560 nm. Signal from the untreated cells were used as controls. The percent viability was calculated with the following formula:

$$\%\ \text{Cell Viability} = \frac{OD_{560}\ \text{treated cells}}{OD_{560}\ \text{control}} \times 100 \quad (3)$$

3.4 Oxidative Stress biomarkers

Lipid Peroxidation

Lipid peroxidation within cell membranes was determined by measuring the formation of malondialdehyde using a method adapted from Ohkawa et al, 1979. Cells were extracted from 6-well plates using RIPA buffer after arsenic exposure. A mixture of 0.1 mL whole cell homogenate, 5% trichloroacetic acid, and 0.67% thiobarbituric acid was incubated at 90° C for one hour. After cooling, the mixture was centrifuged at 3000 rpm for 15 minutes. Absorbance of the supernatant was read at 530nm using a Tecan Ultra multiplate reader. Malondialdehyde standards were made to create a standard curve.

Hydrogen Peroxide

A7r5 and rPASMC were seeded in 6-well plates with $3x10^5$ cells per well and allowed to adhere for 24 hours. Media was replaced with fresh media containing a dilution of 1:1000 amplex red, 1:100 horseradish peroxidase (HRP) and different concentrations of sodium arsenite (100nM, 1uM, 10uM) and monomethylarsonous acid (1uM, 10uM). One milliliter of media was added to the wells in duplicate and incubated for two hours. Cell free media was also incubated to use as blanks for background. After two hours 0.1 mL of each well was measured in triplicate using the Tecan Ultra microplate reader at excitation 530 and emission 590. After subtracting the background from each sample the formula to calculate the percent increase of hydrogen peroxide in treated cells compared to untreated cells was as follows:

$$\% \text{ increase of } H_20_2 = 1 - \frac{530nm/590nm \text{ untreated cells}}{530nm/590nm \text{ treated cells}} \times 100 \quad (4)$$

Superoxide measurements

Stock solutions of cyclic hydroxylamine (10mM) were prepared in argon-purged 0.9% NaCl treated with 0.1 mM DTPA. Stock solutions were kept under argon on ice and prepared daily.

Superoxide radical was measured cell-free by EPR using cyclic hydroxylamine TM-H (0.5 mM) and the xanthine oxidase-superoxide-generating system containing xanthine oxidase 100 mU/ml, xanthine (100 mM), and DTPA (0.1 mM). A standard curve using xanthine ranging from 10 mM – 200 mM was also produced to check the sensitivity of the EPR.

Electron Paramagnetic Resonance (EPR) Spectroscopy

Continuous wave (CW) EPR spectra of the cyclic hydroxylamine 1-hydroxy-4-methoxy-2,2,6,6-tetramethylpiperidine (TM-H) were recorded at ~9.2-9.3 GHz on a commercial X-band nuclear magnetic resonance spectrometer with cavity, and liquid nitrogen-cooled gas flow system. The spectra were collected by use of the following operating conditions: 108 K, 1.0 G modulation amplitude at 100 kHz modulation, a microwave power of 2 mW, 128 ms time constant, and averaging five 150 G scans. The cyclic hydroxylamine concentration was calculated by double integration of the EPR signal and compared to the double integral for the signal from a tempol standard (10– 250 μM) recorded under the same conditions except for 0.5 G modulation amplitude.

All rPASMC intracellular superoxide measurements were performed in DMEM media containing 2% FBS, and 0.1 mM DTPA. Cells were seeded in 6-well plates at $3.5x10^3$ per well. Once cells were at 90% confluency they were washed with PBS and 0.1 mM DTPA and incubated for 1 hour at 37°C in 220 μL of DMEM with 2% FBS, 0.1 mM DTPA and 0.5 mM TM-H in the presence of absence of 1 μM sodium arsenite and 10

μM monomethylarsonous acid. The plates were kept on ice following treatment while the buffer and the cells harvested with a cell scraper were snap frozen in liquid nitrogen (100 μL each) and scanned as described for TM-H. Signal obtained in the buffer is used as blank and subtracted from the cell suspension signal. Samples were analyzed by Robert Usselman at the Magnetics Division at the National Institute of Standards and Technology (NIST) in Boulder, Colorado.

3.5 Statistical Analysis

Statistical analyses were conducted using the data analysis package in Microsoft Excel 2010 for Windows. To determine the statistical significance of treated cells to untreated cells in all experiments conducted Student t-test or ANOVA were used with a probability value of $p<0.05$. The following results are expressed as mean ± standard error of the mean (SEM). Lethal concentration at 50% (LC_{50}) was determined by graphing cell counts using the Origin software, version 6.0.

4. RESULTS

4.1 Cell Morphology and Cytotoxicity

A7r5 and rPASMC seeded at $1x10^5$ in 6-well plates were exposed to various concentrations of arsenic. After 24 hour exposure, A7r5 cells displayed cytotoxic effects and morphological changes of cell death and cell swelling. Cytotoxic effects were observed at 100 nM MMAs, 1 µM MMAs, and 10 µM MMAs,10 µM iAs and 100 µM iAs (Figure 4). In rPASMC cytotoxicity was observed at 10 µM and 100 µM iAs and MMAs. Cell counts were taken to determine the mortality rate and LC_{50} of each cell line. In A7r5 cells, the LC_{50} was determined to be 11 µM with cells exposed to iAs (figure 5), and 0.7 µM for cells exposed to MMAs (Figure 7). In rPASMC, the LC_{50} was 4 µM for MMAs and 26.5 µM for iAs (Figure 10, Figure 12). The MTT assay was utilized to determine cytotoxicity. Both A7r5 and rPASMC displayed a dose-dependent decrease in mitochondrial function when exposed to iAs and MMAs (Figure 6, Figure 8, Figure 11, Figure 13).

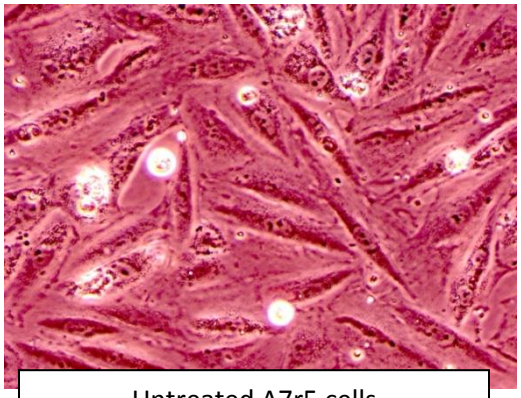
4.2 Generation of Reactive Oxygen Species

No statistically significance changes were observed in MDA production with cells exposed to iAs and MMAs when compared to untreated cells in both A7r5 and rPASMC (Table 1, Table 2).

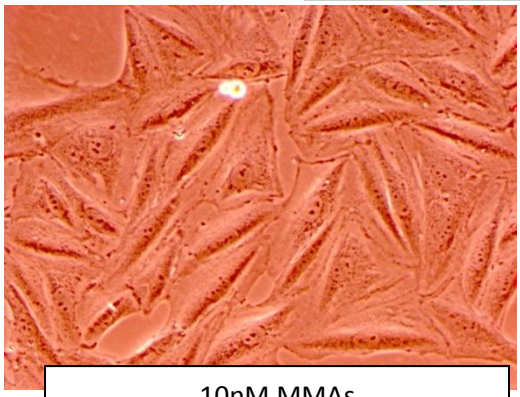
The generation of extracellular hydrogen peroxide was increased in rPASMC when exposed to 100 nM iAs, 1 µM iAs, and 10 µM MMAs. At 100 nM iAs there was a 12.2% increase in hydrogen peroxide. After 1 µM iAs exposure there was a 12.9% increase, after 1 µM MMAs there was a 7.0% 10 µM MMAs there was a 9.4% increase.

All other concentrations tested showed no significant difference when compared to an untreated control (table 3). In A7r5 cells there was no significant increase in hydrogen peroxide when exposed to 100 nM iAs, 1 µM iAs or 10 µM iAs. However, a significant increase in hydrogen peroxide was observed with 1 µM MMAs, and 10 µM MMAs. With 1 µM MMAs exposure there was a 59.1% increase and with 10 µM MMAs there was a 69.6% increase when compared to untreated cells. No significant difference was observed with exposure to 100 nM MMAs (table 4).

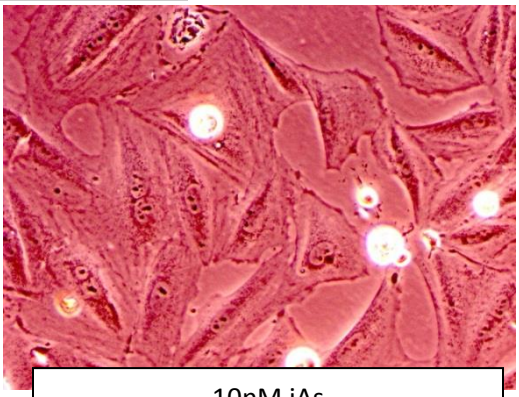
Superoxide radical was tested at 1 µM iAs and 10 µM MMAs in rPASMC. No statistically significant results were obtained (data not shown).



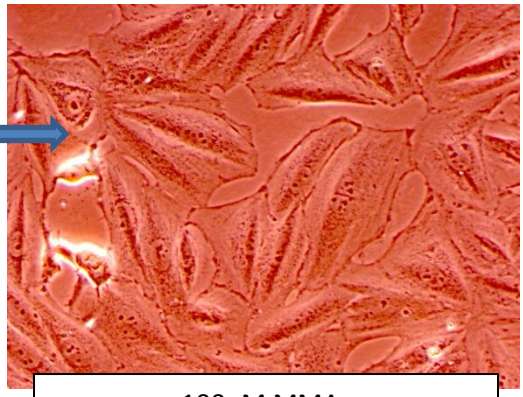
(a) Untreated A7r5 cells



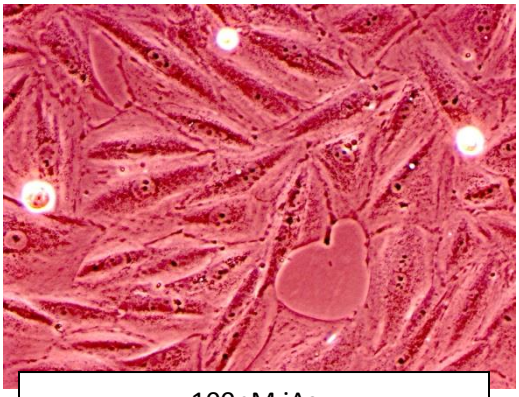
(b) 10nM MMAs



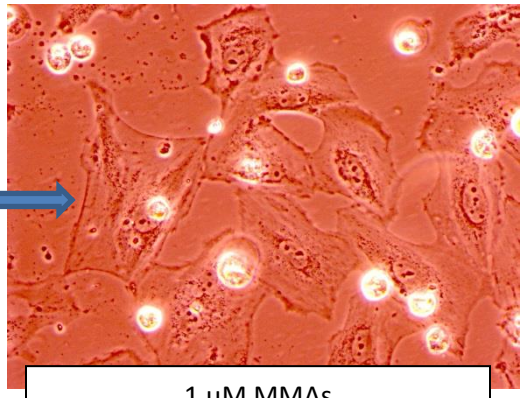
(c) 10nM iAs



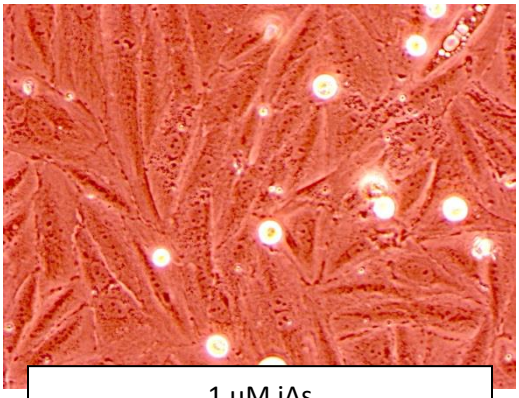
(d) 100nM MMAs



(e) 100nM iAs



(f) 1 µM MMAs



(g) 1 µM iAs

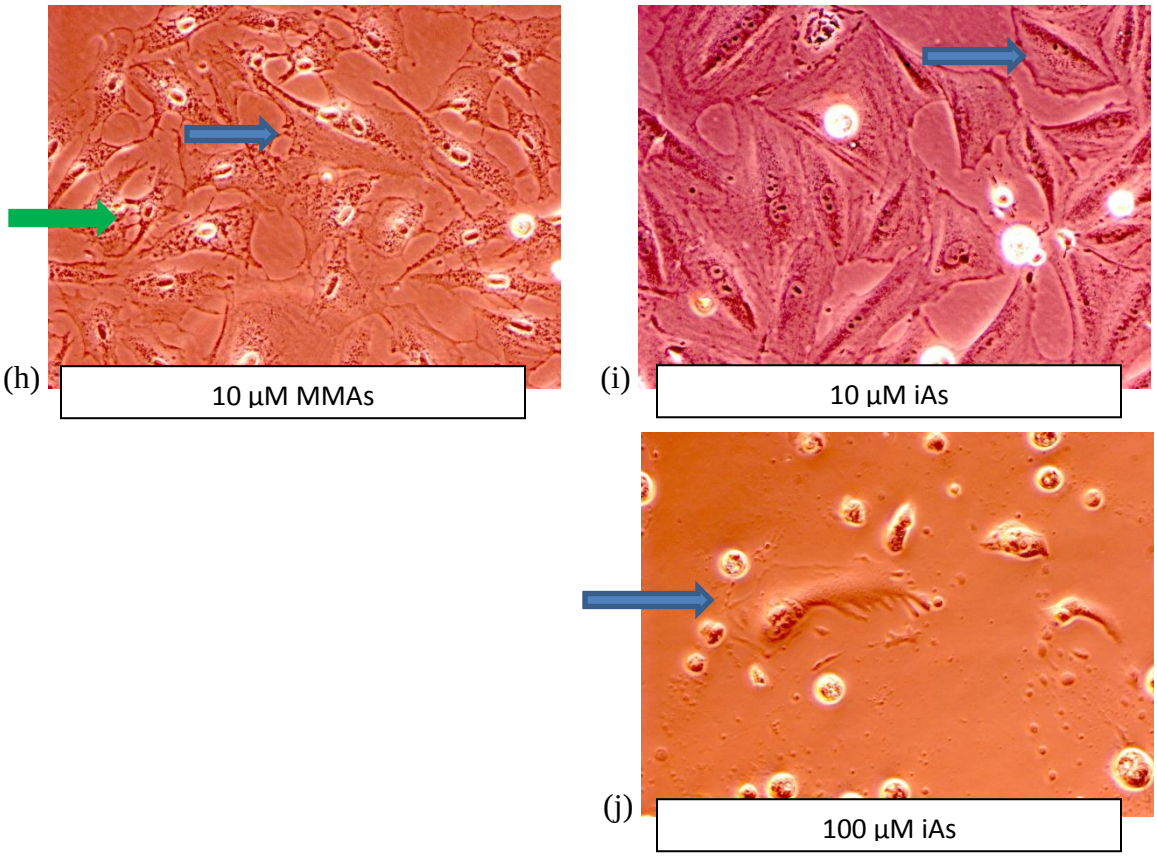


Figure 4. A7r5 cells exposed to different concentrations of arsenic for 24 hours and imaged at 20x magnification in phase contrast. (a) untreated, (b) 10 nM MMAs, (c) 10 nM iAs, (d) 100 nM MMAs (e) 100 nM iAs, (f) 1 µM MMAs, (g) 1 µM iAs, (h) 10 µM MMAs, (i) 10 µM iAs, and (j) 100 µM iAs.

Morphology change indicating cellular swelling.

Detachment of cells due to loss of viability

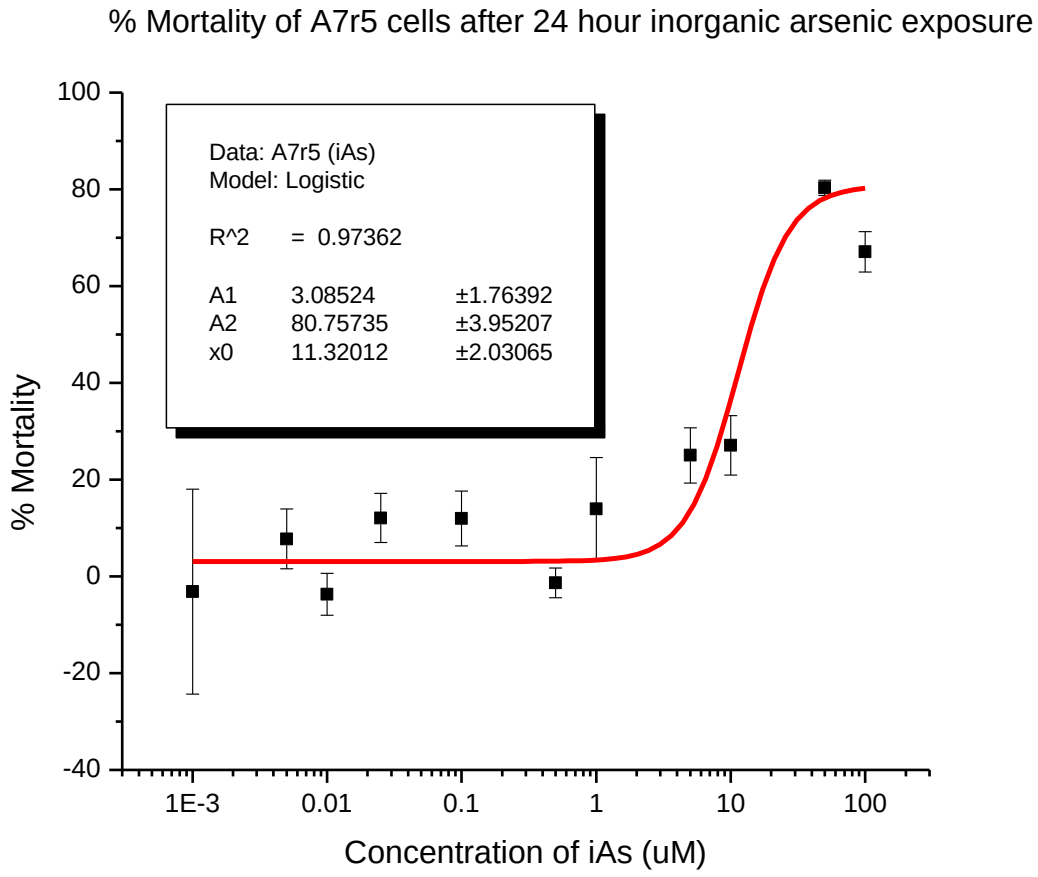


Figure 5. Percent mortality of A7r5 cells exposed to iAs for 24 hours. The LC_{50} was determined to be 11 µM by using cell counts and a logistic dose-response model:

$$y = \frac{A1 - A2}{1 + (x/x0)^p} + A2$$

(ANOVA analysis: $p<0.0001$)

A1: lower asymptote, A2: upper asymptote, x0: half maximal effective concentration (EC50) R^2: Statistical measure of linear regression.

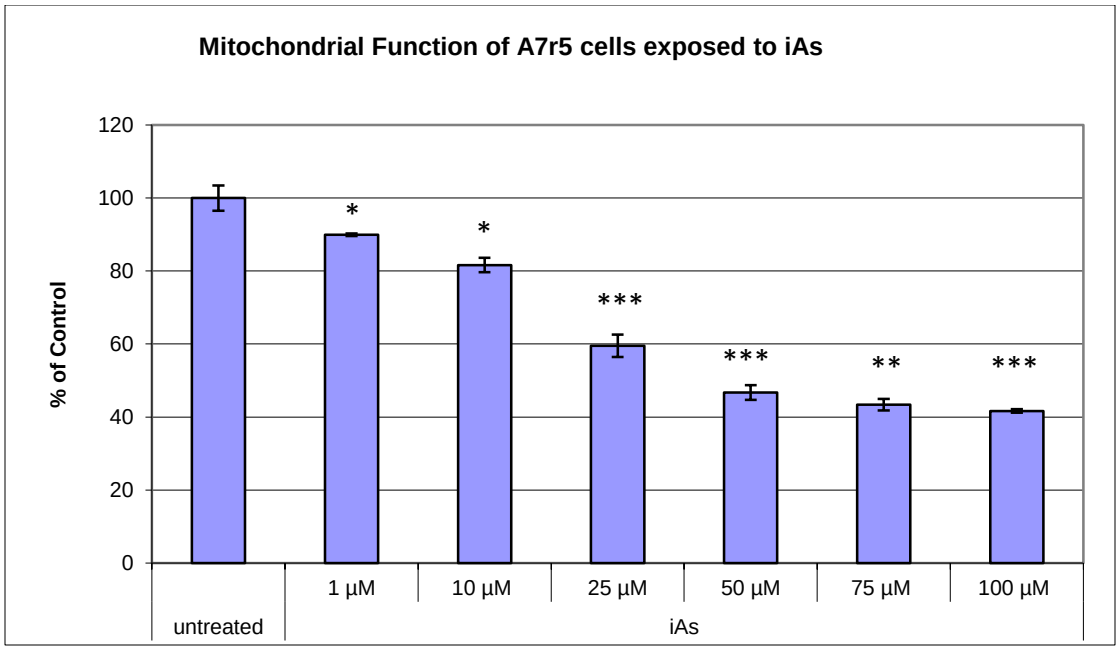


Figure 6. Mitochondrial function determined by MTT assay of A7r5 cells in seeded in a 96-well plate at $1x10^4$ cells per well and exposed to iAs for 24 hours. Mitochondrial function display a dose-dependent decrease in function.

*Statistically significant at $p<0.05$ **Statistically significant at $p<0.01$ ***Statistically significant at $p<0.001$

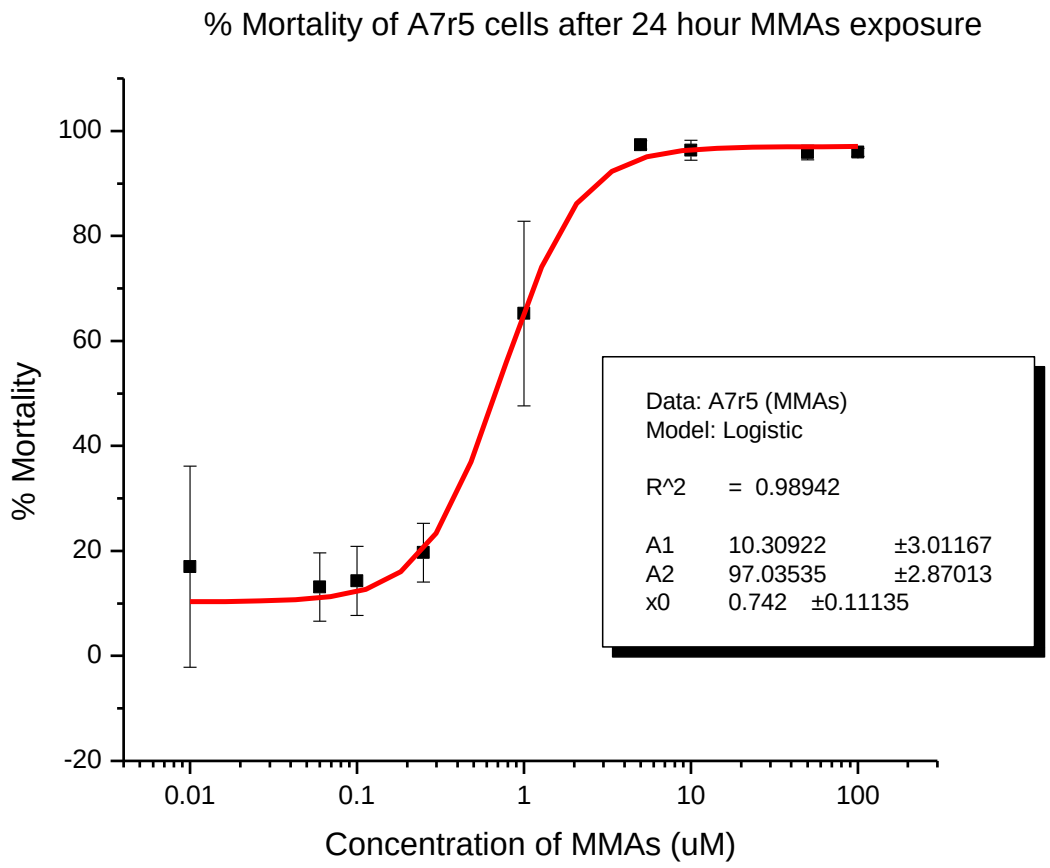


Figure 7. Percent mortality of A7r5 cells exposed to MMAs for 24 hours. The LC_{50} was determined by cell counts and shown to be 0.44 μM. (ANOVA analysis: $p<0.0001$) A1: lower asymptote, A2: upper asymptote, x0: half maximal effective concentration (EC50) R^2: Statistical measure of linear regression.

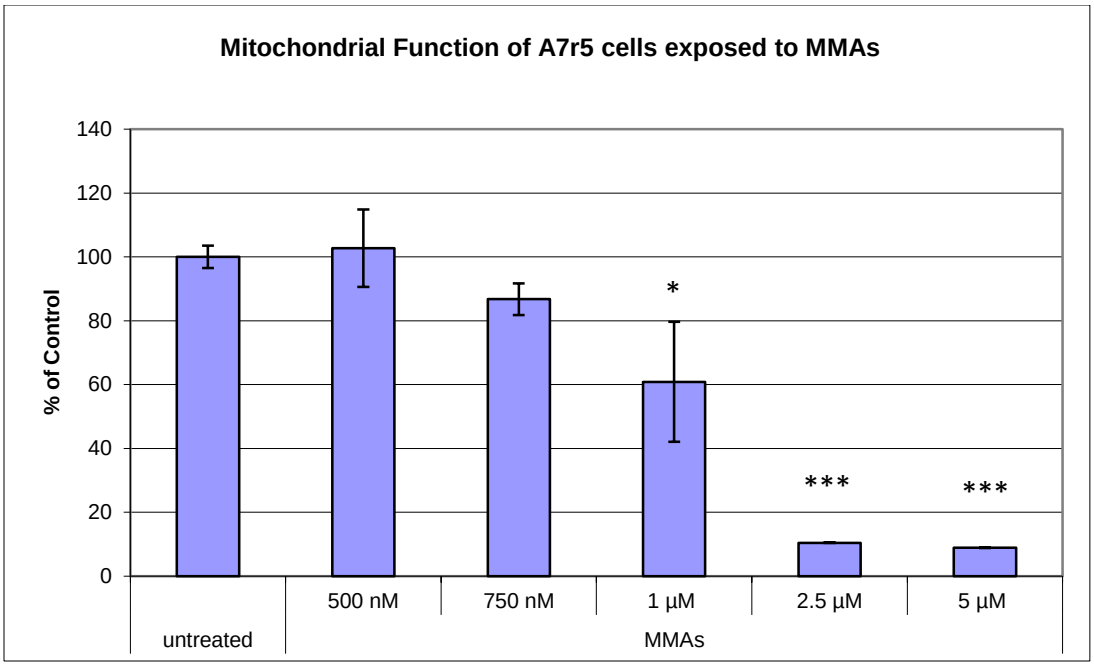
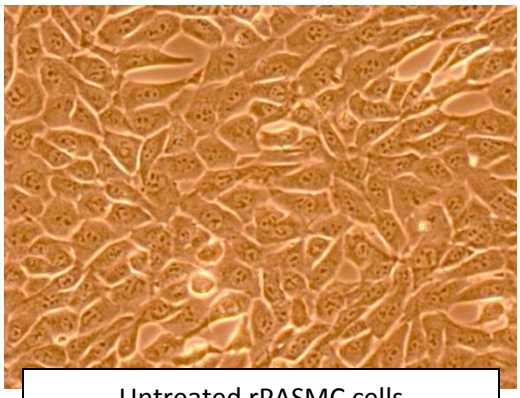
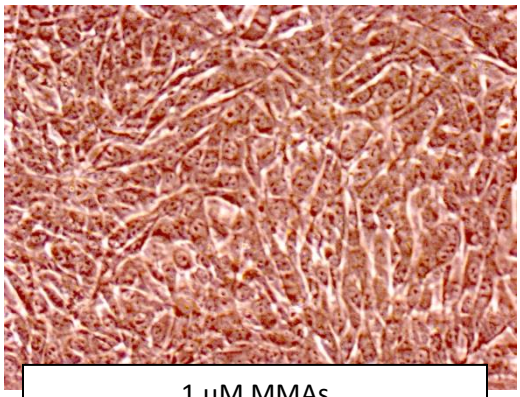


Figure 8. Mitochondrial function determined by MTT assay of A7r5 cells in seeded in a 96-well plate at $1x10^4$ cells per well and exposed to MMAs for 24 hours. Mitochondrial function diminishes in a dose dependent manner after 24 hours.

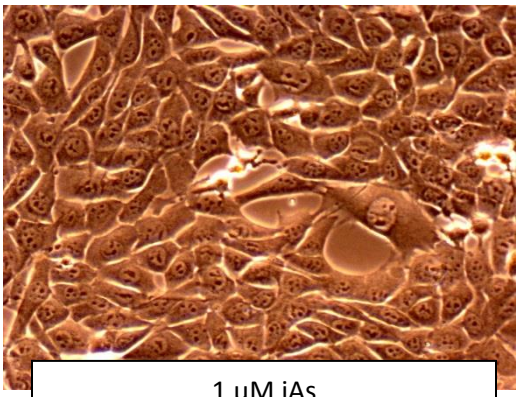
*Statistically significant at $p<0.05$ **Statistically significant at $p<0.01$ ***Statistically significant at $p<0.001$



(a) Untreated rPASMC cells



(b) 1 µM MMAs



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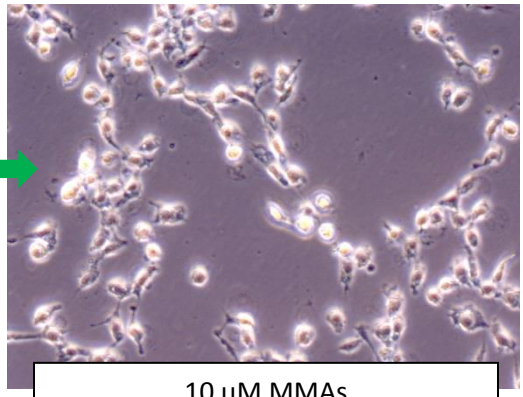
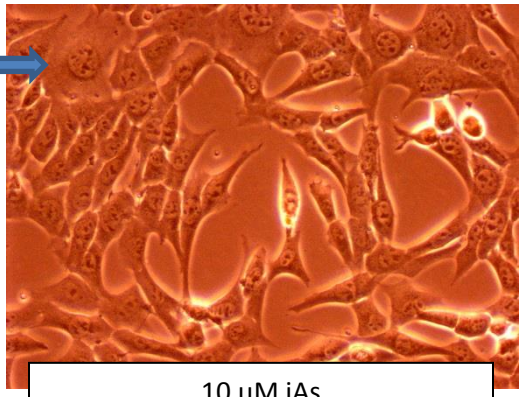
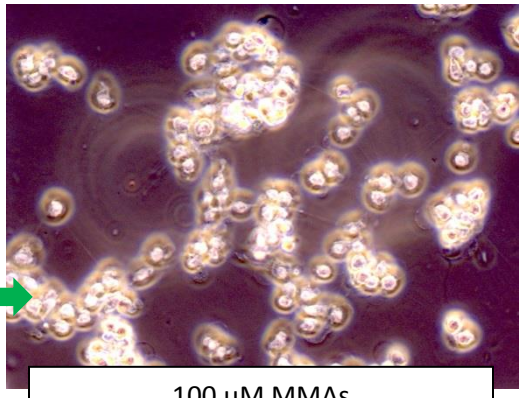


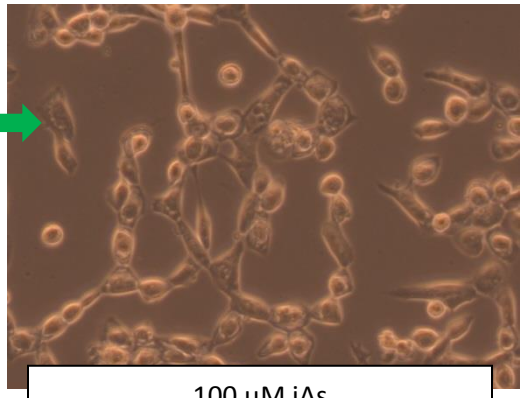
(d) 10 µM MMAs



(e) 10 µM iAs



(f) 100 µM MMAs



(g) 100 µM iAs

Figure 9. rPASMC cells exposed to different concentrations of arsenic for 24 hours and imaged at 20x magnification in phase contrast. (a) untreated (b) 1 µM MMAs, (c) 1 µM iAs, (d) 10 µM MMAs (e) 10 µM iAs, (f) 100 µM MMAs, (g) 100 µM iAs.

Morphology change indicating cellular swelling.

Detachment of cells due to loss of viability

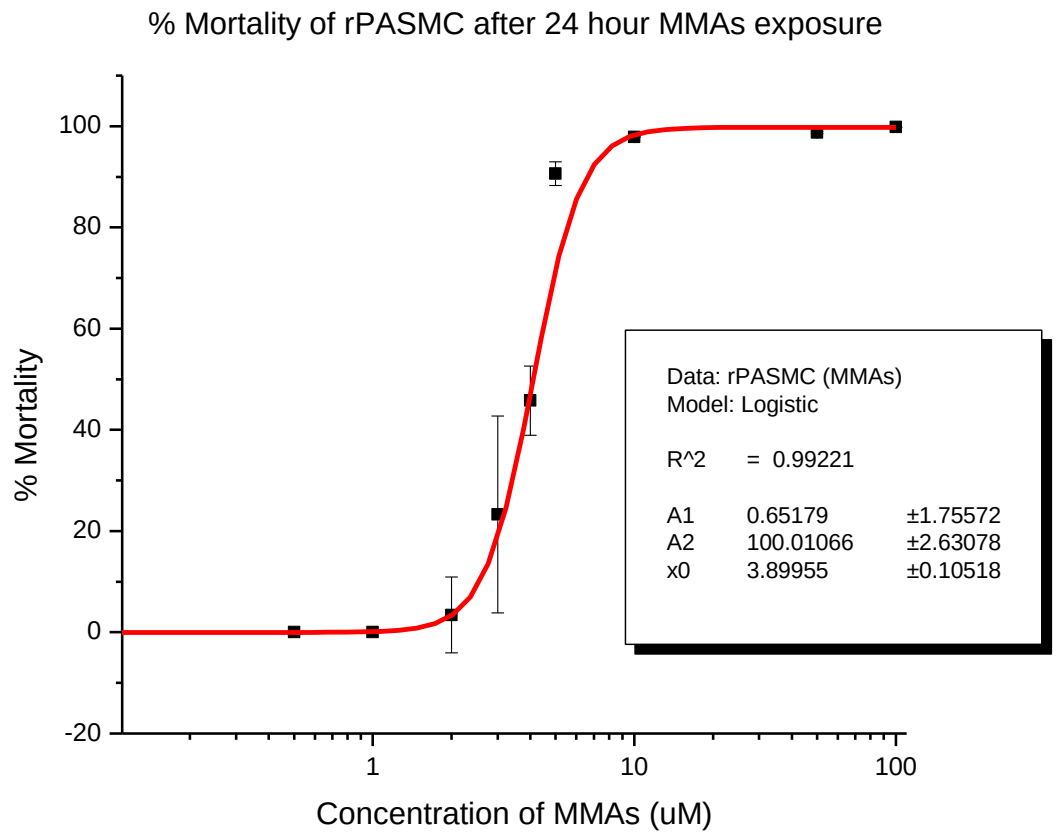


Figure 10. Percent mortality of rPASMC exposed to MMAs for 24 hours. The LC_{50} was determined by cell counts and shown to be 4 μM. (ANOVA analysis: $p<0.0001$)

A1: lower asymptote, A2: upper asymptote, x0: half maximal effective concentration (EC50) R^2: Statistical measure of linear regression.

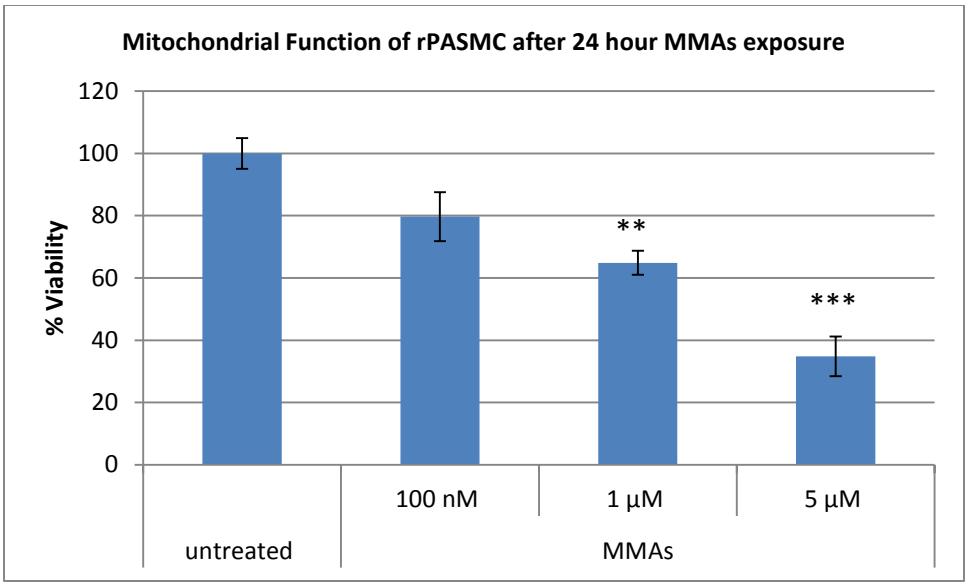


Figure 11. Mitochondrial function determined by MTT assay of rPASMC cells in seeded in a 96-well plate at $1x10^4$ cells per well and exposed to MMAs for 24 hours. Mitochondrial function diminishes in a dose dependent manner after 24 hours. *Statistically significant at $p<0.05$ **Statistically significant at $p<0.01$ ***Statistically significant at $p<0.001$

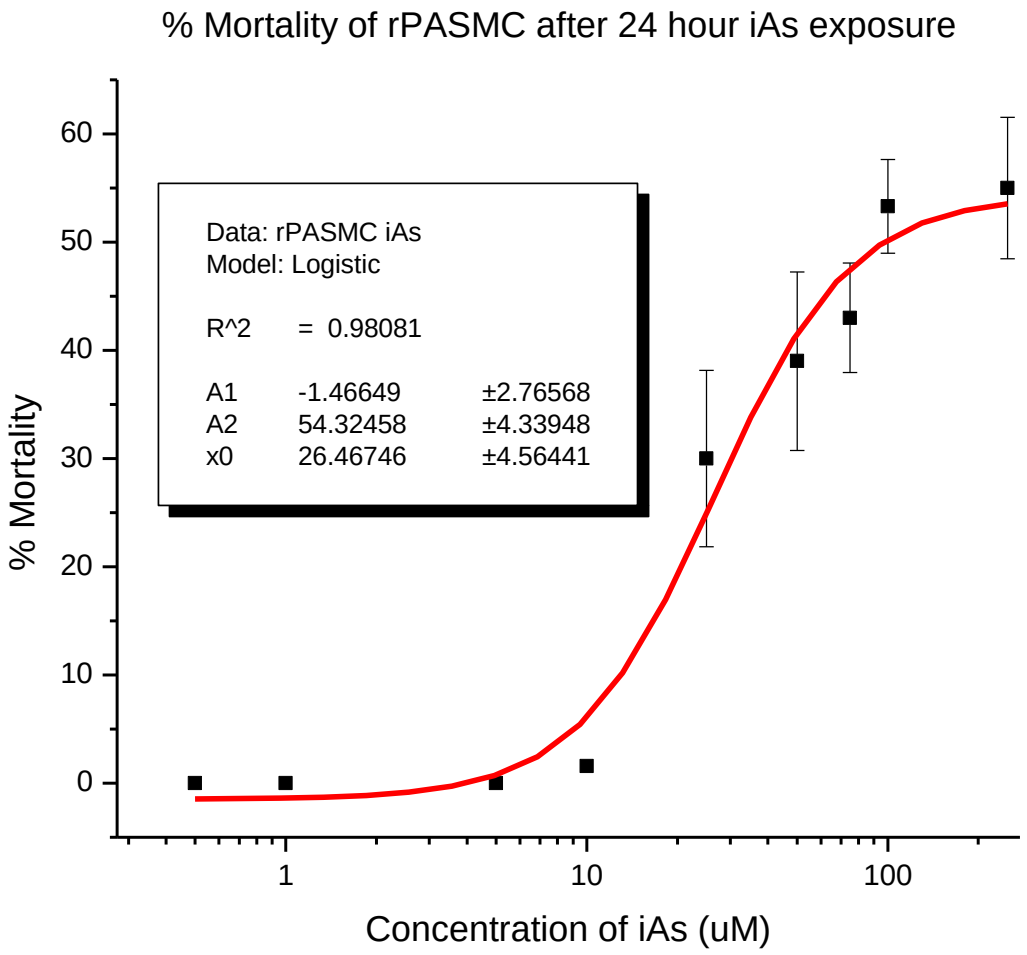


Figure 12. Percent mortality of rPASMC exposed to iAs for 24 hours. The LC_{50} was determined by cell counts and shown to be 26.5 μM. (ANOVA analysis: $p<0.0001$) A1: lower asymptote, A2: upper asymptote, x0: half maximal effective concentration (EC50) R^2: Statistical measure of linear regression.

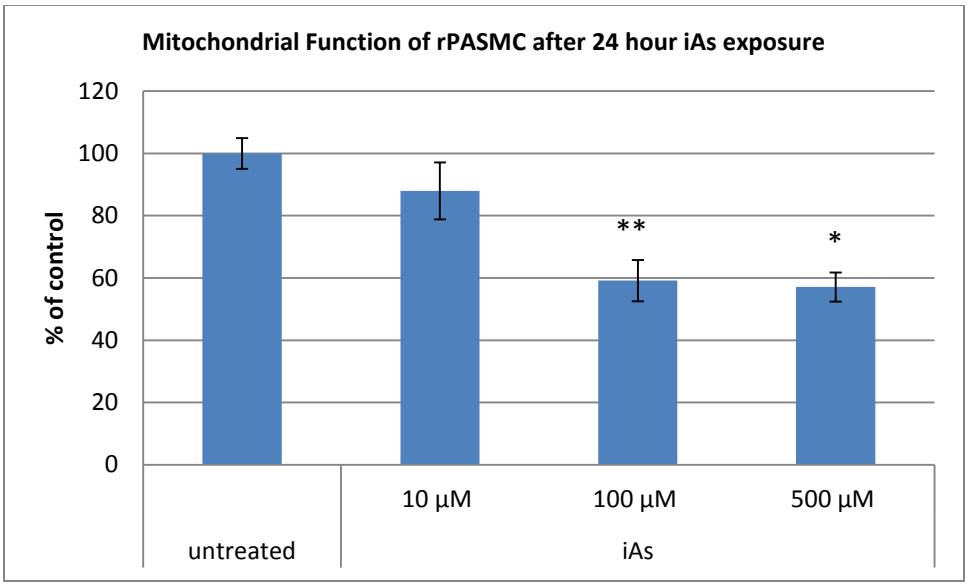
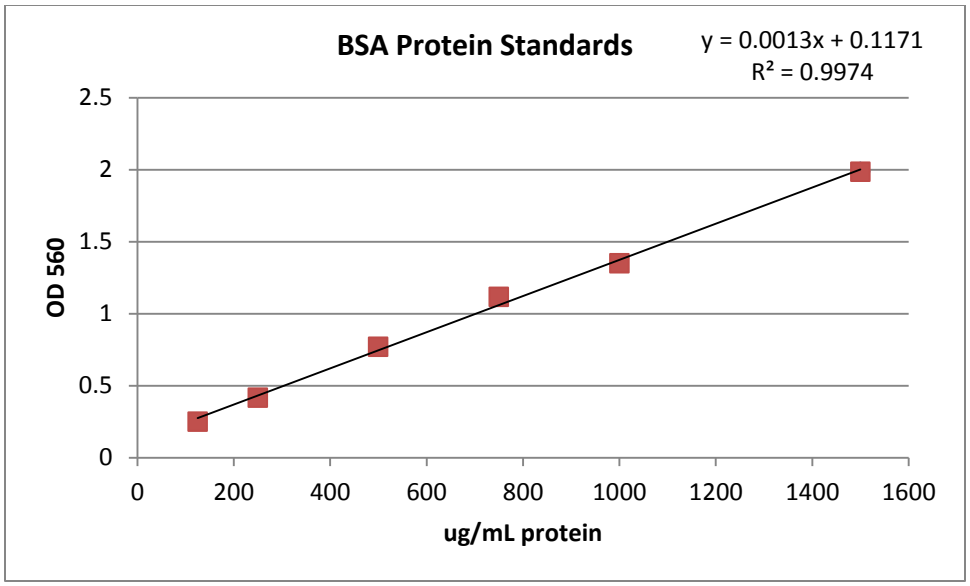


Figure 13. Mitochondrial function determined by MTT assay of rPASMC cells in seeded in a 96-well plate at $1x10^4$ cells per well and exposed to iAs for 24 hours. Mitochondrial function diminishes in a dose dependent manner after 24 hours.

*Statistically significant at $p<0.05$ **Statistically significant at $p<0.01$ ***Statistically significant at $p<0.001$

(a)



(b)

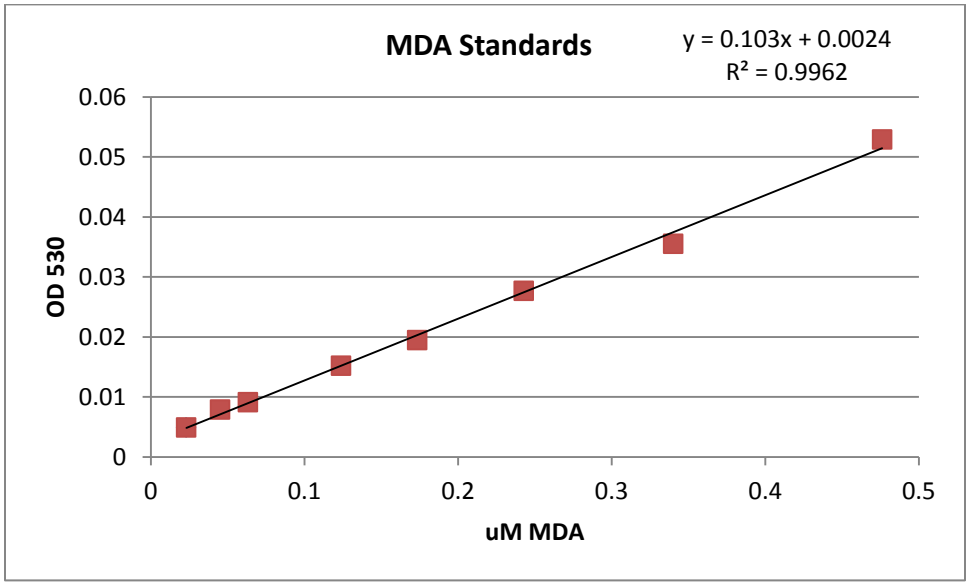


Figure 14. Standard curves of (a) BSA assay and (b) malondialdehyde assay.

Table 1. Total malondialdehyde concentrations in rPASMC exposed to arsenic (mean ± SEM).

rPASMC	nM MDA/mg/mL Protein	P-Value
untreated	$0.56 \pm 9x10^{-4}$	
1 µM iAs	0.60 ± 0.09	0.7597
10 µM iAs	0.63 ± 0.11	0.6434
100 nM MMAs	0.52 ± 0.12	0.7870
1 µM MMAs	0.59 ± 0.01	0.2403

N=3 with 2 replicates

No statistical significance at $P<0.05$ when compared to untreated cells

Table 2. Total malondialdehyde concentrations in A7r5 cells exposed to arsenic (mean ± SEM).

A7r5	nM MDA/mg/mL Protein	P-Value
untreated	1.2 ± 0.10	
1 µM iAs	3.9 ± 0.92	0.1875
10 µM iAs	2.0 ± 0.17	0.0562
100 nM MMA	2.2 ± 1.4	0.6352
1 µM MMA	-1.6 ± 2.6	0.4875

N=3 with 2 replicates

No statistical significance at $P<0.05$ when compared to untreated cells

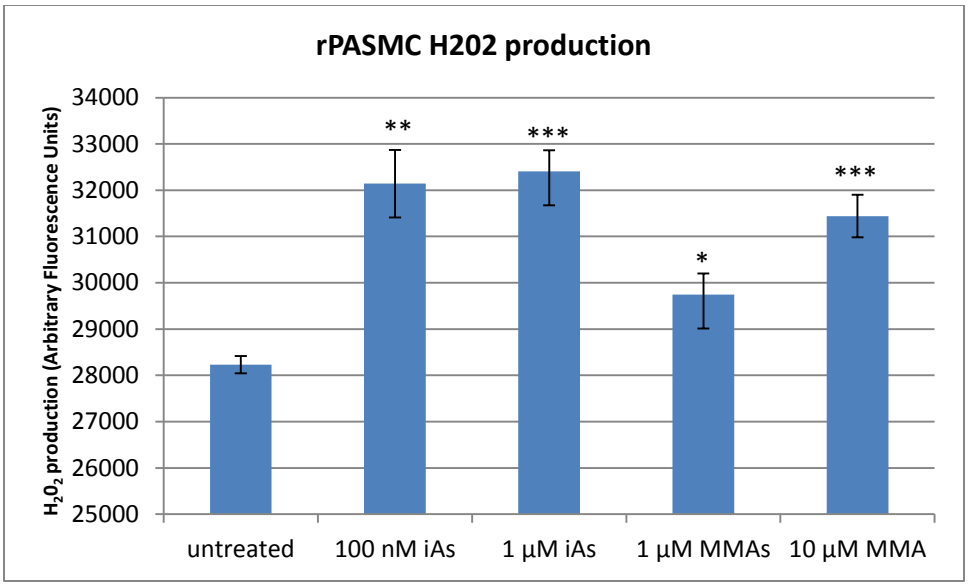


Figure 15. Hydrogen peroxide production in rPASMC after an acute, 2 hour exposure with arsenic.

*Statistically significant at $p<0.05$ **Statistically significant at $p<0.01$ ***Statistically significant at $p<0.001$

Table 3. Percent increase hydrogen peroxide production in rPASMC cells when exposed to arsenic (mean ± SEM).

rPASMC	% Increase in H202 production	p-value
100 nM iAs	12.2 ± 2.0	0.0062**
1 µM iAs	12.9 ± 1.3	0.0002***
1 µM MMA	7.0 ± 1.3	0.0332*
10 µM MMA	9.4 ± 0.78	0.0003***

N=2 with 3 replicates

*P<0.05, determined using student t-test when compared to untreated cells

**P<0.01, determined using student t-test when compared to untreated cells

***P<0.001, determined using student t-test when compared to untreated cells

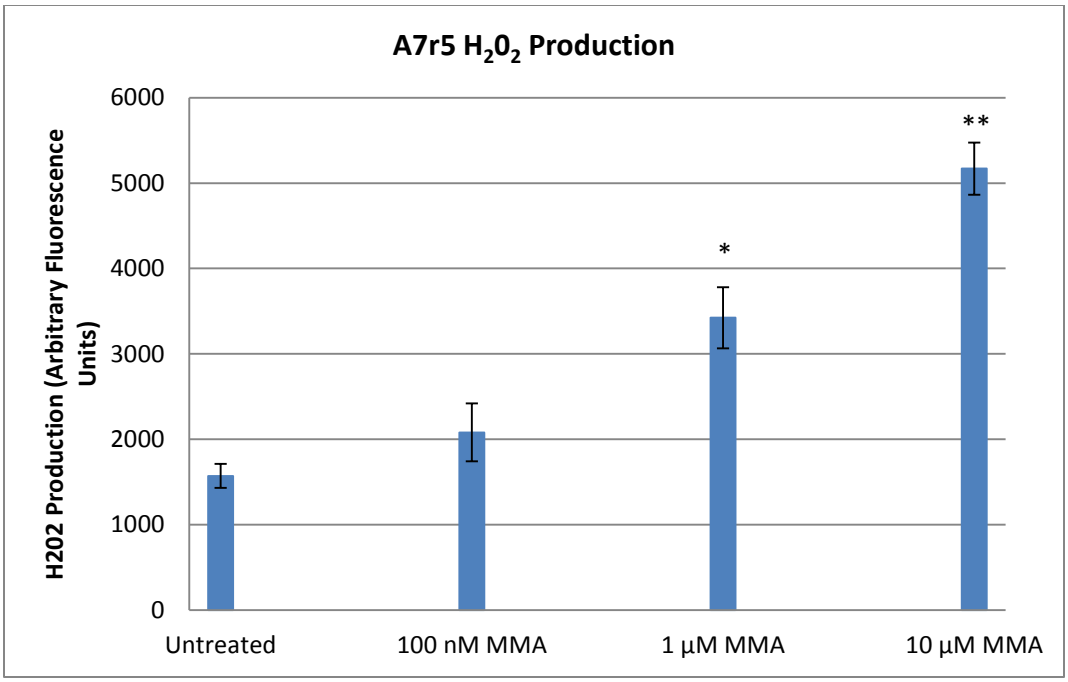


Figure 16. Hydrogen peroxide production in A7r5 cells after an acute, 2 hour exposure with arsenic.

*Statistically significant at $p<0.05$ **Statistically significant at $p<0.01$ ***Statistically significant at $p<0.001$

Table 4. Percent increase hydrogen peroxide production in A7r5 cells when exposed to arsenic (mean ± SEM).

A7r5	% Increase in H202 production	p-value
100 nM MMAs	24.5 ± 11.7	0.2478
1 µM MMAs	54.1 ± 4.7	0.0169*
10 µM MMAs	69.6 ± 1.8	0.0030**

N=2 with 3 replicates

*P<0.05, determined using student t-test when compared to untreated cells

**P<0.01, determined using student t-test when compared to untreated cells

***P<0.001, determined using student t-test when compared to untreated cells

5. DISCUSSION

5.1 Cytotoxicity

A7r5 and rPASMC are established cell lines that were treated identically during arsenic exposure. After 24 hour arsenic exposure MMAs was 15 times more toxic than iAs in A7r5 cells, and 7 times more toxic in rPASMC on a molar basis. Cell counts were used to determine the percent mortality in each cell line exposed to arsenic and an LC_{50} was determined for both iAs and MMAs. Once cell counts were obtained and graphed the LC_{50} was determined to be 11 µM for iAs and 0.7 µM for MMAs exposure in A7r5 cells. In rPASMC, the LC_{50} was 4 µM for MMAs and 26 µM for iAs. The A7r5 cells showed approximately 5 times higher toxicity with MMAs exposure and 2 times higher toxicity with iAs compared to rPASMC. The MTT assay displayed corresponding effects in mitochondrial activity. Diminished mitochondrial activity was observed in both rPASMC and A7r5 cells with iAs and MMAs exposure after 24 hours in a dose-dependent manner which explains the observed cytotoxicity.

A possibility for the increased cytotoxic effects of A7r5 cells could be due to the immortalization of the rPASMC. The rPASMC were immortalized using the enzyme telomerase. Telomerase inhibits telomere shortening and allows the cells to proliferate indefinitely. This is an important characteristic cancer cells possess, and the activation of telomerase limits events in the cell cycle such as apoptosis and cellular senescence (Dahse et al., 1997). Therefore, the rPASMC may be slightly more resistant to arsenic exposure than the A7r5 cells. Another possibility for higher cytotoxicity in A7r5 cells could be alterations in the differentiated state of the VSMC. In order to be sure both cell lines still retain their contractile functions the mRNA encoding for actin and myosin can

be analyzed via RT-PCR. This would verify that the natural characterizations of the cells have been maintained. Cell morphology also indicated possible mechanisms of toxicity. As concentrations of MMAs and iAs increased, cell morphology began to change. Cells transformed from an elongated shape to a shortened, swollen shape. Cell swelling is one characteristic for necrosis (Majno and Joris, 1995). The morphology change was observed at concentrations ranging from 100 nM to 10 µM MMAs in A7r5 cells and with 10 µM to 100 µM iAs exposure. In rPASMC morphology changes were observed also with iAs and MMAs exposure at 10 µM to 100 µM. Further experiments need to be performed to determine the susceptibility of arsenic in aorta and pulmonary cell lines that may account for differential toxicity.

5.2 ROS Generation

To investigate what mechanisms might be involved in the increased toxicity of MMAs in comparison to iAs in rPASMC and A7r5 cells, oxidative stress markers were studied. MDA is one of the major end products of lipid peroxidation and is an identified risk factor for atherosclerosis (Mitra et al., 2011). Therefore, MDA was one molecule analyzed after exposure of iAs and MMAs. After treatment with various concentrations of iAs and MMAs no significant increase in MDA was observed when compared to untreated cells. This indicates that no lipid peroxidation is occurring after 24 hour arsenic exposure in rPASMC and A7r5 cells. It also suggests that this may not be a mechanism in which arsenic causes cytotoxicity when studied *in vitro*. This may be due to the low sensitivity of the malondialdehyde assay. It may also be due to the low levels of lipids

available within the cell membrane to analyze. A large number of cells are necessary to obtain an accurate analysis of malondialdehyde when studied *in vitro*.

When extracellular hydrogen peroxide was analyzed, cells displayed a significant increase when compared to untreated cells. In rPASMC, 100 nM iAs showed a 12.2% increase in hydrogen peroxide, and a 12.9% increase with 1 µM iAs exposure. When exposed to MMAs there was a significant increase of 7% at 1 µM and 9.4% at 10 µM. The increase observed at 1 µM and 10 µM MMAs were still lower than both concentrations of iAs tested. With A7r5 cells no significant increase was observed with iAs exposure but with 1 µM MMAs there was a 59.1% increase in extracellular hydrogen peroxide, and a 69.6% increase with 10 µM MMAs. The increase in hydrogen peroxide was significantly higher with MMAs exposure in A7r5 cells than with rPASMC. This could be a possible mechanism that would explain why MMAs is more toxic in A7r5 cells than rPASMC, but does not explain why iAs is more toxic as well. Due to the lack of hydrogen peroxide activity observed in rPASMC this does not explain why MMAs is more toxic than iAs. There does not seem to be a similar trend occurring in the A7r5 cells and rPASMC when comparing the data for hydrogen peroxide. The A7r5 cells are more susceptible to cytotoxic effects caused by arsenic as indicated by the cell counts, mitochondrial activity, and the significantly higher levels of hydrogen peroxide produced by the cells when exposed to MMAs compared to rPASMC.

Superoxide is a free radical that is much more reactive than hydrogen peroxide and is able to interact with molecules to generate ROS (Valko et al., 2005). Superoxide is also quickly dismutated into hydrogen peroxide (Oeckler et al., 2005). Since significant increase in hydrogen peroxide levels were observed superoxide was tested to determine

whether high concentrations were present as well. No significant increase was observed in rPASMC at 1 µM iAs and 10 µM MMAs. This suggests that the release of hydrogen peroxide from the cells may not be due to dismutated superoxide, or that the superoxide is being dismutated too quickly to obtain an accurate analysis. Further studies will need to be conducted to determine if the same trend is observed with A7r5 cells.

5.3 Limitations

This study was limited primarily by the cell lines used to assess ROS production and cytotoxicity. The cell lines were used to model aortic and pulmonary effects after arsenic exposure to determine if specific ROS molecules that are known to contribute to the development of atherosclerosis were generated. If cells from primary culture were used results may have varied conflicting with the data obtained from established cell lines. Malondialdehyde was not observed in the cell lines used, but if tested with primary cells they may react differently and provide significant findings. Studies may also give conflicting results if performed *ex vivo* or *in vivo*. Another limitation of this study was determining the mechanism of cell death. Cell death was not determined to be due to apoptosis or necrosis. Cell morphology was assessed to determine changes resembling necrosis, but no specific assay or mechanism was studied to understand the mechanism of cell death. Superoxide was only tested in rPASMC at 1 µM iAs and 10 µM MMAs. In order to have additional concrete data superoxide should be tested at multiple concentrations in parallel with A7r5 cells.

6. Conclusions

These data indicate that within established rat aortic and pulmonary cell lines the organic form of arsenic, which was once thought to be a detoxification mechanism, displays higher cytotoxicity than the inorganic form. These cell lines were treated identically, but A7r5 cells displayed higher toxicity than the rPASMC. This suggests that different mechanisms may be responsible for the cytotoxicity of pulmonary cells versus the cytotoxicity of aortic cells. Out of the ROS molecules tested hydrogen peroxide was the only oxidant that displayed a significant increase in production when compared to untreated cells. Variations in results observing toxicity and ROS generation in rPASMC and A7r5 cells indicate that several mechanisms may be involved in arsenic-induced vascular diseases.

7. Suggestions for Future Studies

To better understand the cytotoxic effects of arsenic exposure to VSMC *in vitro* future studies to determine the mechanism of cell death need to be conducted. This study displayed morphological characteristics resembling necrosis, but studies comparing iAs and MMAs exposure in VSMC to determine whether apoptosis or necrosis is occurring would be useful. Caspase protease activation is unique to apoptosis and could be analyzed in cultured cells to determine if arsenic-induced cell death is due to necrosis or apoptosis.

Although in this study malondialdehyde was not shown to be significantly higher in cells treated with arsenic than untreated cells LDL oxidation should still be analyzed. A possible explanation of why there was no significant increase could be due to the limited amounts of lipids within the cell membrane. Future studies should supplement media with LDL and the addition of arsenic and compare malondialdehyde concentrations to untreated cells. Cytotoxicity can also be analyzed with the addition of LDL in treated cells to determine whether oxidation of LDL is occurring. Previous studies have shown that oxidized LDL displays higher toxicity than native LDL and is a major risk factor for the development of atherosclerosis (Mitra et al., 2011). If arsenic promotes oxidation of LDL further experiments can be conducted to look at which proteins that be upregulated with arsenic exposure leading to the oxidation of LDL. Studies have shown that the expression of a protein membrane receptor, LOX-1, has shown to be upregulated in endothelial cells. LOX-1 is a lectin-like oxidized low-density lipoprotein receptor-1, and is found in the membranes of various cells (Mitra et al., 2011).

Endothelial cells should also be studied for ROS generation as well as LDL oxidation after arsenic exposure. Endothelial cells should also be studied to determine if there is a difference between ROS generation when compared to VSMC and what affect that may have to arsenic-induced vascular disease. ROS generation can cause injury to endothelial cells. Disrupted endothelial cells can cause inflammation and display a vast array of cytotoxic effects which can lead to impaired vascular tone and nitric oxide balance (Simeonova and Luster, 2004). Future comparisons can be made with VSMC and endothelial cells to determine if arsenic promotes ROS generation in one cell line more than the other.

In this study hydrogen peroxide was increased with MMAs exposure in both rPASMC and A7r5 cells, but superoxide was not shown to be increased. Since superoxide is quickly dismutated into hydrogen peroxide it would be expected that superoxide would be increased. Only one concentration of iAs and one concentration of MMAs was tested for superoxide in rPASMC in this study. Future studies should test multiple concentrations of iAs and MMAs in both rPASMC and A7r5 cells to determine if the superoxide activity corresponds to the hydrogen peroxide activity observed.

A useful study would be to analyze the antioxidant concentrations of SOD and CAT expressed after arsenic exposure. This analysis would determine whether superoxide is being dismutated into hydrogen peroxide, and whether hydrogen peroxide is being detoxified by transformation to water and oxygen. If SOD is present then this would explain why superoxide is not being observed after arsenic treatment. The superoxide may be dismutated too quickly to be observed, or treatment time may have

not been long enough. A future study comparing treatment times of arsenic and superoxide generation should also be determined.

Cells used in this study were established cell lines that were immortalized. Future studies should analyze ROS generation in primary cells cultured and conduct cumulative population doubling studies. Low concentrations of iAs and MMAs exposed to primary cells would provide data on the number of population doublings cells exposed to arsenic experience in comparison to untreated cells.

This study determined ROS generation of cells *in vitro,* but future studies should examine the *ex vivo* effects of arsenic administered in rats and analyze the rat aorta and pulmonary. The abundance of cells and lipids within the tissue may provide a better determination for the analysis of malondialdehyde, superoxide and hydrogen peroxide. It would also be a useful comparison to determine whether differences exist with analyses of arsenic induced ROS generation *in vitro* and *ex vivo*. Future studies could also determine whether low concentrations of arsenic ingestion *in vivo* cause ROS generation with analysis of blood, plasma and urine.

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