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LOW-DOSE OF SODIUM ARSENITE CAUSES DELAYED DIFFERENTIATION IN C2C12 MOUSE MYOBLAST CELLS THROUGH THE REPRESSION OF THE TRANSCRIPTION FACTOR MYOGENIN

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LOW-DOSE OF SODIUM ARSENITE CAUSES DELAYED DIFFERENTIATION IN
C2C12 MOUSE MYOBLAST CELLS THROUGH THE REPRESSION OF THE
TRANSCRIPTION FACTOR MYOGENIN

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Amanda Ann Steffens
December 2009

Accepted by:
Dr. Lisa Bain, Committee Chair
Dr. William Baldwin
Dr. Richard Blob

Abstract

Arsenic is a contaminant of drinking water in many parts of the world. A number of epidemiological studies have correlated arsenic exposure with cancer, skin diseases, cardiovascular diseases, and adverse developmental outcomes such as stillbirths, spontaneous abortions, neonatal mortality, low birth weight, and delays in the use of musculature. The current study used C2C12 mouse myoblast cells to examine whether low concentrations of arsenic could alter their differentiation into myotubes, which would indicate that arsenic has the ability to act as a developmental toxicant. Myoblast cells were exposed to 20nM sodium arsenite and allowed to differentiate into myotubes and expression of the muscle-specific transcription factor myogenin, along with the expression of myosin light chain 2, and tropomyosin were investigated using real time PCR and immunofluorescence. Exposing C2C12 cells to 20nM sodium arsenite delayed the differentiation process, as evidenced by a significant reduction in the number of multinucleated myotubes. Additionally, arsenic exposure caused a time-dependant decrease in myogenin mRNA expression, as compared to the control cells, starting on day two of the differentiation process. Arsenic reduced myogenin mRNA levels by 1.4-fold on day two, 2.7-fold on day three, and 5.1-fold on day four of differentiation. This reduction in transcript number was confirmed by immunofluorescence, which also showed a decrease in the total number of nuclei expressing myogenin protein. Interestingly, myosin light chain 2 mRNA was significantly unregulated in the arsenic-exposed cells, although this did not translate into altered protein expression. This study

demonstrated that low concentrations of arsenic are able to disturb the differentiation process of myoblasts without causing overt toxicity.

Dedication

I would like to thank my parents, brother and aunt who have supported me through thick and thin. Without them life wouldn't be as much fun as it is. Thank you and I love you all.

Acknowledgments

I would like to thank Dr. Lisa Bain for allowing me to join her lab at Clemson and for helping me develop my scientific education and techniques. Thank you as well goes to my wonderful professors at UWRF: Dr. Daniel Marchand who inspired me to pursue toxicology, Dr. Jeff Rosenthal for giving me the chance to take part in research and allowing me to be part of the published work, and finally Dr. Kevin McLaughlin who gave science a wonderful twist with his amazing stories. I would also like to thank Dr. Paul Russo for giving me the chance to take part in summer research at LSU and Dr. Bhaskar Velamakanni and Paul Klaiber at 3M for giving me the courage to go for my masters degree. Thank you also goes to Dr. Raquel Contreras for helping me center myself through all my trials and tribulations. In addition, I want to thank Marilyn, Matt and Nikki for all their support though my undergrad and graduate career, their advice, encouragement and love is irreplaceable. Finally, thanks must go to my friends at Clemson who helped me get through these past two and a half years.

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Introduction

Arsenic is a ubiquitous element that can come in many oxidative states: -3, 0, +3 and +5. It ranks 12th in the body, 14th in seawater and 20th in the earth's crust and can be found in an inorganic or organic form (Mandal B.D., 2002). The trivalent inorganic form being the one humans are exposed to the most. This exposure can be from natural sources like soil, water, or food sources and from human sources like industrial pollution, mining (copper smelting) and medication.

Distribution

Today, a number of countries are affected by arsenic and its detrimental effects but Bangladesh and West Bengal have the largest affected areas. In these countries, the surface water is contaminated with pathogens, so tube wells were dug to reach the groundwater to use for drinking (Karn and Harada, 2001). Unfortunately, the well water contains high amounts of inorganic arsenic (Chowdhury *et al.*, 2000). When arsenic is found in high concentrations in the bedrock it is usually paired with sulfide minerals. The most common mineral forms are arsenian pyrite ($\text{Fe}(\text{S},\text{As})_2$) and arseno pyrite (FeAsS), and it can also come in the forms of realgar (AsS), orpiment (As_2S_3), or scorodite ($\text{FeAsO}_4 \cdot 2\text{H}_2\text{O}$). Large concentrations of arsenic in drinking water usually come from aquifers that are located in alluvial and delta planes. They create the best circumstances for the arsenic to separate from its mineral form through redox reactions that normally occur between a pH of 6.5-8.5. Hydrologically closed basin regions and a high pH of saline groundwater will also affect the adsorption of the arsenic (Welch A.H., 2000). The

poor adsorption of arsenic into evaporite minerals will only occur at a high salinity (> 9 molar) and as long as the redox stability of As(V) are at moderately reducing conditions and high pH values occur, it will decrease As affinity to the mineral surfaces (Levy, 1999). In southeastern Bangladesh alone, 90% of the boreholes have an arsenic concentration greater than 50 $\mu\text{g/L}$, which is due to the area being part of a large delta plane (Figure 1). The World Health Organization (WHO) has changed its limits of arsenic in the groundwater throughout the decades with the increasing concern for its adverse effects on human health.

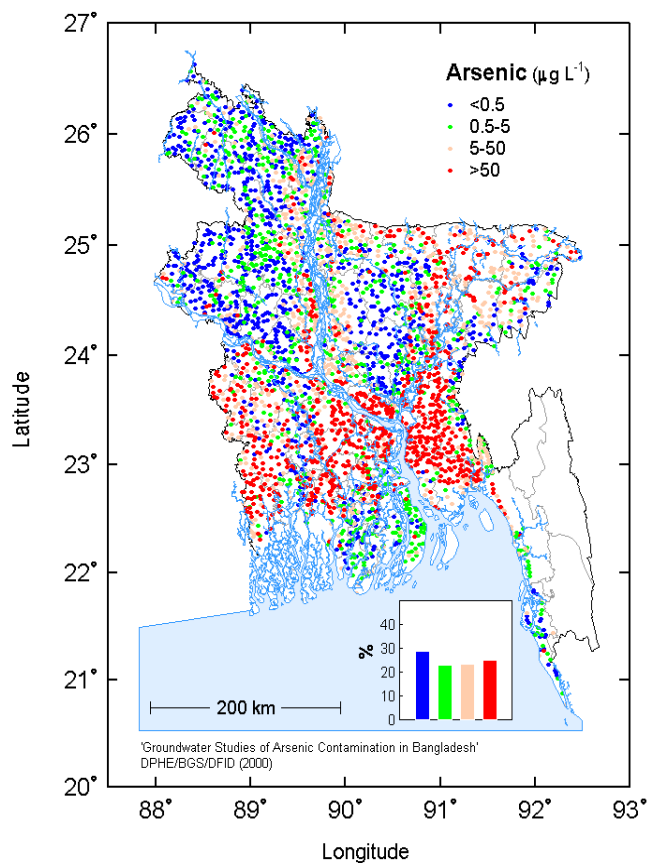


Figure 1. Arsenic concentrations in Bangladesh (DPHE/BGS/DFID, 2000)

In 1958, the regulation was 200 ppb, this amount was lowered to 50 ppb in 1963, and finally in 1993 was lowered to 10 ppb (WHO, 2003). The U.S. itself made the standard of 10 ppb effective on February 22, 2002, with water systems needing to be in compliance with the new standard by January 23, 2006. In the U.S., 31,350 public and private water supplies were tested for arsenic concentration from 1973 to 2001. A map that was developed from this data shows the different arsenic concentrations across the states (Figure 2). In certain areas like California (highest concentration was 2600ppb), and Nevada (highest concentration was 2200 ppb) (Ryker, 2001), the high concentrations are largely due to evaporation and concentration in shallow ground water.

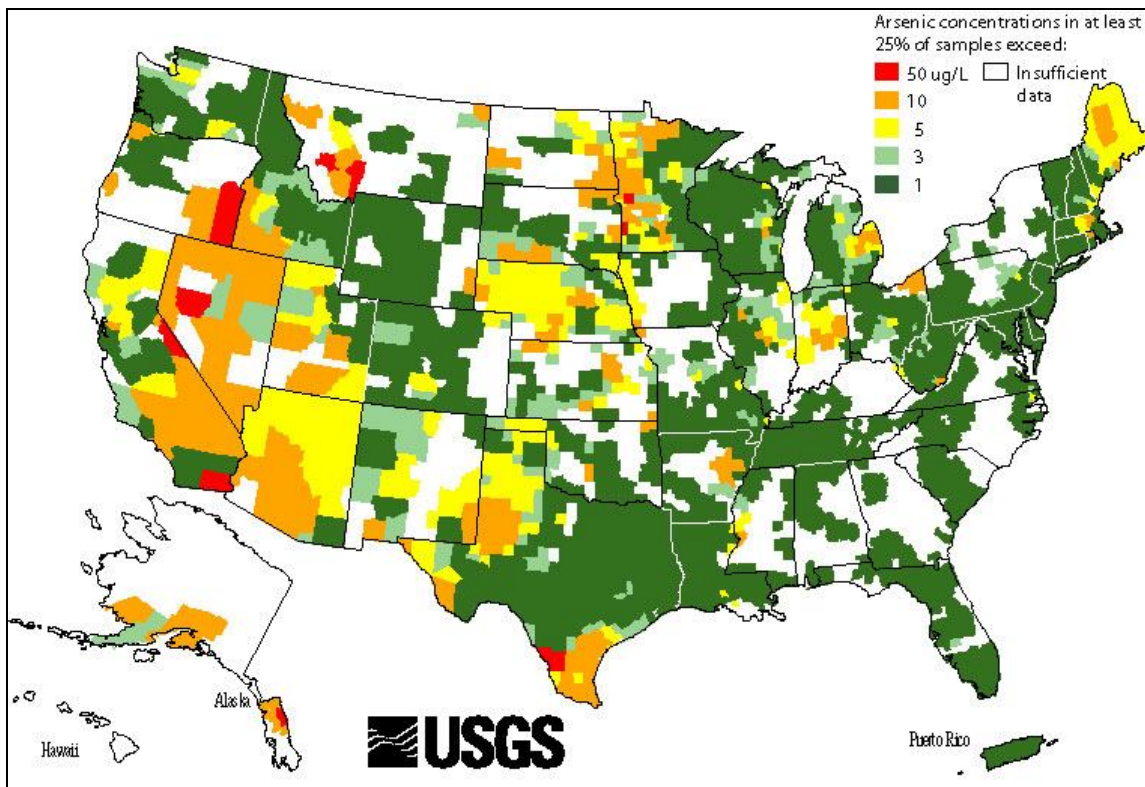


Figure 2. Concentrations of 31,350 ground-water arsenic samples collected during 1973 -2001 (Ryker, 2001)

Clinical Uses

Arsenic trioxide has been used as treatment for different types of leukemia, like multidrug-resistant human myeloid leukemia, acute promyelocytic leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, chronic lymphocytic leukemia, myelodysplastic syndrome, and multiple myeloma (Shen *et al.*, 1997; Li and Broome, 1999; Perkins *et al.*, 2000; Murgo, 2001; Chen *et al.*, 2002). The function of arsenic is to induce cell cycle arrest and apoptosis in the leukemia cells, but the same outcomes may also happen in noncancerous cells (Qian *et al.*, 2007).

In a study done by Perkins *et al.*, human promyelocytic leukemic cells (HL-60) and myelogenous leukemia cells (K562) that were incubated in arsenic trioxide saw a reduction in growth with a LC_{50} between 0.8 and 1.5 $\mu\text{mol/L}$. At 2 $\mu\text{mol/L}$ of arsenic trioxide, after 7 days of incubation, 30 to 50% of cells, in all cell lines, showed morphologic features of apoptosis, like blebbing and an expression of the cell-surface phospholipid phosphatidylserine, that is only released from the cell's cytosol to the cells surface during apoptosis (Perkins *et al.*, 2000). Another study showed that arsenic trioxide disrupted tubulin polymerization in K562 cells at concentrations ranging from 0.5–5 $\mu\text{mol/L}$ (Li and Broome, 1999).

Metabolism

When inorganic arsenic enters the body, the proposed metabolic pathway starts with the liver where it is metabolized through an oxidative process in which the trivalent inorganic arsenic will gain a methyl group that is donated from S-adenosylmethionine (SAM) (Fig. 3) (Aposhian, 1997). This process changes the oxidative state to a pentavalent form, creating methylarsenoic acid (MAs^V). From there, two electrons are removed from arsenic through a thiol oxidation process to reduce As(V) to As(III). Another oxidative methylation can take place creating dimethylarsinic acid (DMAs^V) (Thomas *et al.*, 2004). Normally the methylation and reduction process would be considered a detoxification step, with the arsenic metabolites exiting through the urine, but some studies have shown that the methylated species might also contribute to the effects seen with arsenic exposure (Kaise *et al.*, 1989, Styblo, 2000 #2750, Vega, 2001 #2748).

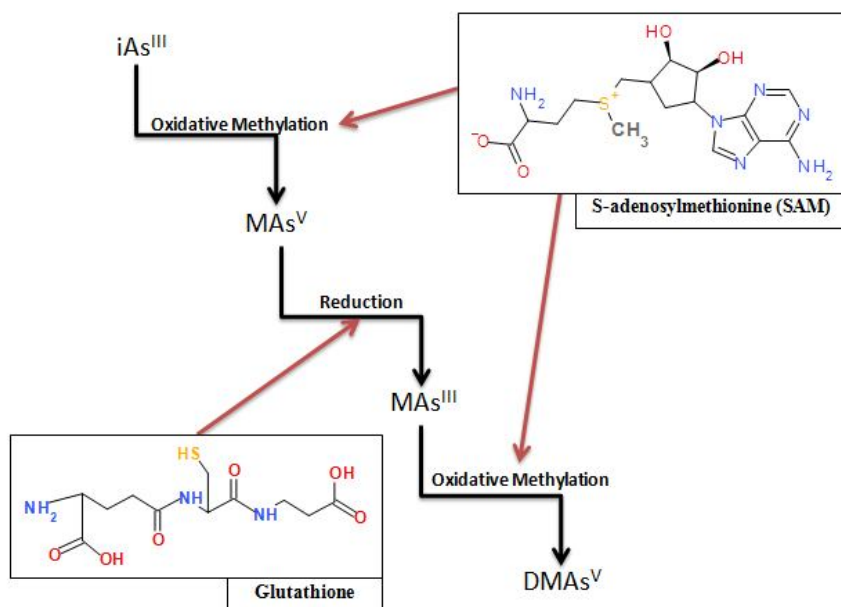


Figure. 3 Proposed methylation process with the methyl donor SAM and the reducing cofactor glutathione

An *in vivo* study examined the effects that methylarsonic acid, $\text{CH}_3\text{AsO}(\text{OH})_2$ (MAA), dimethylarsinic acid, $(\text{CH}_3)_2\text{AsO}(\text{OH})$ (DMAA), and trimethylarsine oxide had when orally administered to mice (Kaise *et al.*, 1989). DMAA had the highest toxicity of the methylated species with a LD_{50} of 1.2 g/kg, while MAA and TMAO had LD_{50} of 1.8 and 10.6 g/kg respectively. These concentrations are below arsenic trioxide's acute toxicity in which two mouse studies had an oral LD_{50} of 26-48 mg/kg (Kaise *et al.*, 1985) (Harrison *et al.*, 1958). Even though methylated species are not lethal it is important to consider their biological effects. The toxicity of inorganic arsenic, trivalent and pentavalent methylated arsenicals, was investigated in cultured human hepatocytes, bronchial cells, keratinocytes and urothelium cells, along with rat hepatocytes (Styblo *et al.*, 2000). Trivalent monomethylated arsenic was found to be less cytotoxic when compared to inorganic arsenic(III) in all cell types (Styblo *et al.*, 2000) The cell line with the greatest methylation capacity were the rat hepatocytes followed by the human hepatocytes, keratinocytes and bronchial cells. Another study also investigated the effects of exposed human epidermal keratinocytes to the different oxidative states of arsenic (Vega *et al.*, 2001). They observed that the lower the oxidative state, the more cytotoxic the arsenic was and within the pentavalent oxidative states the methylated versions had the higher cytotoxicity: iAsIII (arsenite) > MAsIIIIO (methylarsine oxide) > DMAsIIIIGS (dimethylarsinous acid with glutathione) > DMAsV (dimethylarsinic acid) > MAVs (methylarsonic acid) > iAsV (arsenate) (Vega *et al.*, 2001). This shows that the methylated species of arsenic may contribute to the adverse effects connected with exposure to arsenic.

Molecular events

Chronic arsenic exposure has multiple ailments connected to it but there is no specific order in which they appear, so diagnosis of arsenic exposure can be difficult. External characteristics can consist of dermal changes like lesions, pigmentation changes, increase of keratoses, or skin cancers (Tondel M., 1999; Mandal B.D., 2002). The internal ailments associated with exposure range from cardiovascular diseases, different forms of cancer, respiratory or gastrointestinal problems to developmental problems (Soffritti *et al.*, 2006) (Shi *et al.*, 2004). A clear mechanism or pathway for these ailments is still unknown but there have been various hypotheses like alteration of the DNA methylation pattern, impairment of DNA repair mechanisms, genetic damage or programmed cell death (Hughes, 2002).

For example, it is known that arsenic can modify DNA methylation patterns (Reichard *et al.*, 2007). Gene transcription can be regulated through methylation and an alteration to DNA methylation patterns can cause genes to be over- or under-expressed (Newell-Price *et al.*, 2000). A study showed that when the A549 human adenocarcinoma cell line was exposed to various concentrations of sodium arsenite, sodium arsenate, and DMAs^V, both sodium arsenite (0.08-2 μM) and sodium arsenate (30-300 μM) hypermethylated the promoter region of the tumor suppressor gene, p53 (Mass and Wang, 1997), which indicates that increasing arsenic concentrations likely suppressed p53 expression more efficiently, allowing for deregulation in the cell cycle process.

Arsenic exposure can both increase genetic damage as well as alter DNA repair mechanisms (Andrew *et al.*, 2006). There are three levels of genetic damage that can occur. The most basic is point mutation and frame shift mutation; the next level is chromosome or chromatid breaks and aneuploidy, otherwise known as a gain or loss of a whole chromosome. The final level of genetic damage is spindle disruption at metaphase and anaphase during the cell cycle and dicentric chromosome formation, where a chromosome contains two centromeres instead of one (Gonzalzo and Jones, 1997). A common test for mutation is the mouse lymphoma assay which looks at thymidine kinase (TK), an enzyme that takes part in the recycling of free thymidine which can get incorporated into DNA. A toxic analogue to thymidine is trifluorothymidine (TFT) and if thymidine kinase is active the TFT can disrupting DNA metabolism and cause cell death. The mouse lymphoma assay uses a cell line that only has one functioning TK gene and if the toxicant in question is mutagen the cells will be unaffected with addition of TFT, due to the loss of the TK gene. When mouse lymphoma cells were exposed to sodium arsenite, arsenic trioxide, monomethylarsonic acid and dimethylarsinic acid at 10 μ M , all were found to cause mutations at the TK gene (Soriano *et al.*, 2007).

The nucleotide excision repair pathway, which fixes nucleotide deletions caused by UV radiation, was studied to see if arsenic impaired it in a human lymphoblastoid cell line (TK6) (Danaee *et al.*, 2004). When 1 and 5 μ M sodium arsenite were combined with UV radiation, there was a synergistic effect on the number of mutations in the TK gene (Danaee *et al.*, 2004). To determine if the UV repair pathway was affected by arsenic, a comet assay was done within 15 minutes of exposure to UV and UV plus arsenite. It was

observed that the repair pathway was operational when the cells were exposed to just UV because of the low amount of comet scores. However, when the cells were exposed to both UV and arsenic, the comet scores were higher, implying that DNA repair mechanisms were impaired (Danaee *et al.*, 2004).

Finally, programmed cell death follows highly regulated pathways and can lead to either apoptotic or autophagic cell death (Burchell *et al.*, 1991). In apoptosis, the activation of various caspases lead to the formation of blebs, bulges that separate from the cell, taking a portion of cytoplasm with them containing the organelles of the cell so that other neighboring cells will not be affected. Autophagic cell death differs in that it forms vacuoles in which organelles of the cell are destroyed while maintaining membrane formation (Cuervo, 2004). A variety of studies have shown that arsenic is able to induce programmed cell death (Soignet *et al.*, 1998; Li and Broome, 1999; Kong *et al.*, 2005; Bashir *et al.*, 2006; Binet *et al.*, 2006; Cheung *et al.*, 2006; Qian *et al.*, 2007; Banerjee *et al.*, 2008; Leu and Mohassel, 2009). For example, when K529 myeloid leukemia cells were exposed to 2.5 μ M arsenic trioxide, only 36 \pm 5 % of the cells survived and those that did showed high levels of the apoptotic marker annexin V 12 hours post-exposure (Li and Broome, 1999). Using 4 μ M arsenic trioxide, another study not only reported apoptosis in two leukemia cell lines (Molt-4 and Mutz-1) but saw an increase in non-apoptotic cell death known as autophagy (Qian *et al.*, 2007). Additionally, male Wistar rats exposed to 2.5 and 5 mg/L sodium arsenite in their drinking water had an increase in apoptotic cells in their livers (Bashir *et al.*, 2006).

Developmental effects

Exposure to arsenic *in utero* or during infancy can be significant periods in which problems occurring with development may lead to diseases later in life (Daskeishi *et al.*, 2006). Arsenic has been shown to cross the placental barrier, causing numerous problems for the fetus, like stillbirth, spontaneous abortion, miscarriage, low birth weight, poor muscle movement or various forms of cancer (Ahmad *et al.*, 2001).

In Japan, back in 1955, around 13,000 infants were exposed to arsenic contaminated powdered milk. The company used low grade disodium hydrogen phosphate containing sodium arsenate in the production process and out of the initial count of exposed infants, 130 of them died (Daskeishi *et al.*, 2006). A year after their exposure the infants showed little dermal symptoms so doctors assumed the worst was over. About thirteen years after the incident, a group of public health and school nurses began to notice some health problems in the survivors, including dermatological problems like leukaderma, melanosis, and rashes, as well as neurological problems like epilepsy, retardation, cerebral palsy, and cardiovascular problems (Tsuchiya, 1977; Daskeishi *et al.*, 2006).

In utero arsenic exposure has been studied in various animal models, ranging from rodents to fish (Waalkes *et al.*, 2004a) (Gonzalez *et al.*, 2007) (Tilton and Tanguay, 2008). Exposures of 20ppm or greater caused a significant decrease in reproductive fecundity in mice which were caused by changes in the placenta, in which a defective vasculature network was observed (He *et al.*, 2007). This type of alteration could affect

the blood flow an embryo receives causing pregnancy complications like stillbirth, spontaneous abortion or miscarriage (He *et al.*, 2007).

The incidence of cancer also increases with arsenic exposure during gestation. When pregnant mice (C3H) were exposed to 42.5ppm or 85ppm sodium arsenite through their drinking water during gestation days 8 to 18 and offspring were grown to two years of age before sacrifice. The male offspring had an increased incidence of hepatocellular carcinoma and adrenal tumors, while females had increases in lung carcinomas, ovarian tumors, and uterine and oviduct preneoplasia compared to controls (Waalkes *et al.*, 2004b). In a previous study done in our laboratory, parent mummichogs (*Fundulus heteroclitus*) were exposed to 230 ppb arsenic for 10 days preceding spawning and their offspring were analyzed for physiological changes along with changes in differential gene expression. The offspring showed a 2.8 fold increase in the incidence of a curved or stunted tail. Muscle specific proteins, tropomyosin and myosin light chain 2 were induced by 3.1- and 4.2-fold, respectively (Gonzalez *et al.*, 2007). This curved tail phenomenon was also shown in zebrafish that were exposed to 2.0mM of sodium arsenite. Hatched embryos also had altered heart development, and aberrant somite and neuromuscular patterning that correlated to altered cell proliferation and genomic DNA methylation (Li *et al.*, 2009). With these occurrences of tail deformities and an increase in muscle specific proteins, arsenic might have a direct affect with muscle development.

Muscle effects

Arsenic exposure has been shown to effect muscle-related proteins and muscle function in smooth and cardiovascular muscles. Epidemiological studies in Bangladesh and Taiwan, two areas with exposure to high concentrations of arsenic, observed an increase in the development of hypertension leading them to believe that arsenic was a possible cause (Chen *et al.*, 1995; Rahman *et al.*, 1998). Hypertension can develop from a condition known as atherosclerosis, in which there is an accumulation of lipoprotein in the arteries. Arsenic has been shown to accelerate atherosclerosis in the offspring of ApoE-knockout mice, who were given drinking water that contained 85 mg/L, by reducing the arteries ability to relax (Srivastava *et al.*, 2007). Additionally, arsenic has been able to alter the arteriolar matrix in mice, exposed to 50 ppb sodium arsenite, by reducing collagen, elastin, and actin (Hays AM, 2008). During an *in utero* and postnatal exposure to 100 ppb sodium arsenite was able to increase the amount of smooth muscle actin in the lungs (Lantz *et al.*, 2008).

The functional contractile unit of muscle consists of myosin bound to actin. When adenosine triphosphate (ATP) binds to the myosin cross bridge (MCB) active site it causes myosin to dissociate from the actin filament (Geeves and Holmes, 1999). The MCB then orientates itself to a 90° angle from the actin during the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate. The products from hydrolysis are released and this causes the bound MCB to revert to its original angle of 45°, leading to a propelling action that moves the actin filament past the myosin filament (Geeves and Holmes, 1999) (Fig. 4). Other proteins, such as tropomyosin play a regulatory role.

Tropomyosin wraps itself around actin and hinders the attachment of the myosin cross bridge during low Ca^{+2} levels (Wolska and Wieczorek, 2003). As these levels rise, they activate tropomyosin which will in turn move to a non-blocking position to allow the myosin cross bridge to re-bind to actin during the contraction process.

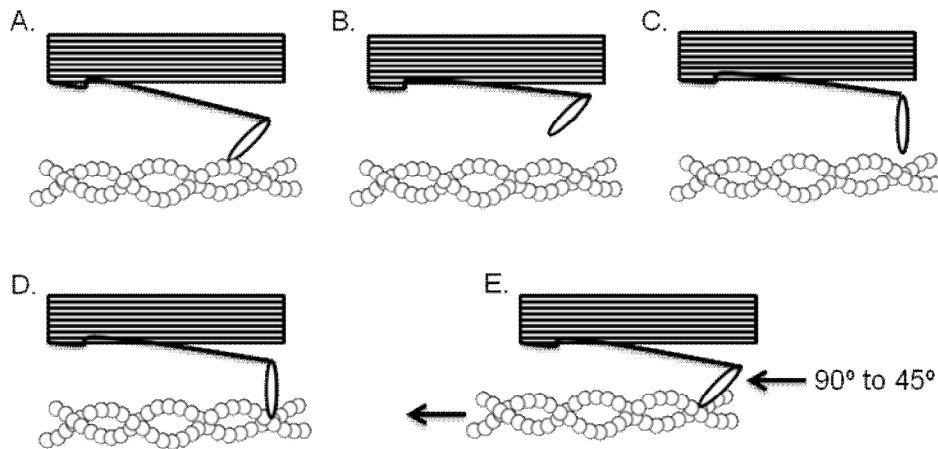


Figure 4. Mechanics for a muscle contraction

When smooth muscle, in the form of intact aortic rings, were exposed to $10\mu\text{M}$ monomethylarsonous acid (MMAIII), there was an irreversible relaxation of the blood vessel (Bae *et al.*, 2008). This failure to compress the blood vessels increases the risk for various cardiovascular diseases through the alteration of tonal regulation and blood circulation (Gutstein and Pérez, 2004).

Since arsenic has been shown to interact with various muscle types and our laboratory saw an increase in muscle-specific proteins after parental arsenic exposure to fish, we investigated the potential impacts of sodium arsenite during the differentiation C2C12 mouse skeletal muscle cells from myoblasts to myotubes (Fig. 5).

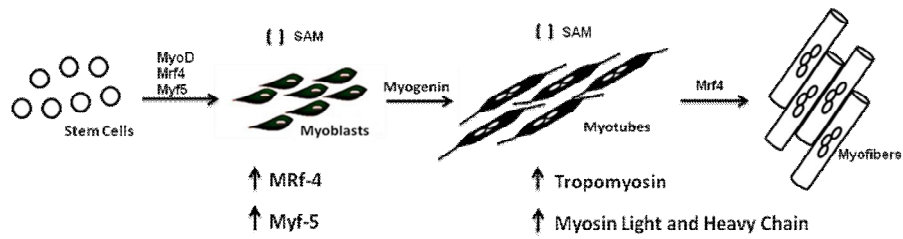


Figure 5. C2C12 mouse muscle cell cycle from stem cell to myofiber

Our hypothesis is that when the myoblast cells are incubated with arsenic, an inappropriate timing of transcription factor and muscle-specific protein expression may be in part responsible for some of the developmental abnormalities seen in our lab's previous study.

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Introduction

Arsenic is a known human toxicant, that causes diseases such as skin lesions, pigmentation changes, increase of keratoses, cardiovascular diseases, lung, bladder, and skin cancer, and developmental problems (DeSesso *et al.*, 1998) (Tondel M., 1999; Mandal and Suzuki, 2002; Shi *et al.*, 2004; Soffritti *et al.*, 2006; Hill *et al.*, 2008). Exposure can be from natural sources like soil, water, or food sources and from human sources like industrial pollution, mining and medication, but the most common exposure is through drinking water (Mandal and Suzuki, 2002). The World Health Organization (WHO) has changed its standard for arsenic concentrations in water throughout the decades with the increasing concern for its adverse effects on human health (WHO, 2003). Many of the epidemiologic studies are in areas of high arsenic concentrations which produce more consistent dose-response correlations. More recently, studies have begun to focus on exposure to lower arsenic concentrations (Cantor and Lubin, 2007; Reichard *et al.*, 2007) (Schoen *et al.*, 2004).

In utero and postnatal development are critical periods in which toxicants can disrupt gene expression and organ development which can result in problems later in life (Dolinoy *et al.*, 2007) (Morley, 2006) (Włodarczyk *et al.*, 1998) (Włodarczyk *et al.*, 2001). Arsenic has been shown to cross the placental barrier, and studies in rodent models have shown that dams exposed to arsenic in their drinking water had an increase in aborted fetuses, while their offspring had low birth weight and changes in locomotor

activity (Ahmad *et al.*, 2001) (Waalkes *et al.*, 2003; Waalkes *et al.*, 2004a; Jin *et al.*, 2005; He *et al.*, 2007). Additionally, arsenic exposure *in utero* increases cancer incidence in the offspring (Diwan *et al.*, 2000) (Waalkes *et al.*, 2007) (Waalkes *et al.*, 2008) (Ahlborna *et al.*, 2009).

In utero arsenic exposure also alters muscle function and muscle-related protein expression in the offspring. For example, mummichogs (*Fundulus heteroclitus*) that were parentally-exposed to arsenic had an up-regulation of tropomyosin and myosin light chain 2 (Gonzalez *et al.*, 2006). Zebrafish exposed during embryogenesis showed altered heart development, and somite and neuromuscular patterning that correlated to altered cell proliferation and genomic DNA methylation (Li *et al.*, 2009). *In utero* exposure accelerated atherosclerosis in the offspring while reducing the ability of their smooth muscle to properly relax (Srivastava *et al.*, 2007). An alteration to muscular regulatory factors during somitogenesis in zebrafish has resulted in impaired development of fast skeletal muscle (Tilton and Tanguay, 2008). Arsenic exposure *in utero* has resulted in changes in the vascular matrix by decreasing the amounts of collagen and elastin (Hays AM, 2008). *In utero* exposure can also lead to perturbation in placental vasculogenesis that can lead to spontaneous abortions (He *et al.*, 2007). It has also found that *in utero* or early postnatal arsenic exposure can lead to an irreversible change in airway smooth muscle by altering the expression of several genes that regulates the extracellular matrix (Lantz *et al.*, 2008). Additionally, arsenic exposure to frogs prior to metamorphosis resulted in reductions in swimming ability (Chen *et al.*, 2009).

Muscle development is regulated by several transcription factors that differentiate stem cells into smooth muscle, cardiac muscle, or skeletal muscle. In embryogenesis, progenitor cells originating in the somites develop into primary muscle fibers through a number of regulatory factors and the regulatory factor that initiates the conversion of a skeletal muscle myoblast to a myotube is myogenin (Buckingham *et al.*, 2003). This protein is essential for proper skeletal muscle development while the loss of Myf5 and MyoD will have little effect in its formation due to other family members that can act as substitutes (Braun *et al.*, 1992; Rudnicki *et al.*, 1992; Hasty *et al.*, 1993). The myogenin gene is activated by a histidine- and cysteine-rich region and helix III domains of MyoD but before that can take place the 5'-flanking region of the gene needs to be unmethylated (Sartorelli and Caretti, 2005) (Fuso *et al.*, 2001). In growth media with C2C12 cells, the 5'-flanking region of the myogenin gene will be completely methylated, which will result in a silent gene but in differentiation media (serum free) the region will be completely demethylated within 48 hours (Fuso *et al.*, 2001). When S-adenosylmethionine (SAM) is administered to C2C12 cells it can repress expression of myogenin by delaying the demethylation of the 5'-flanking region on the myogenin gene (Fuso *et al.*, 2001) (Lucarelli *et al.*, 2001). Once produced the myogenin protein is activated through phosphorylation by protein kinase C in its conserved DNA-binding domain (Li *et al.*, 1992). Demethylation of the myogenin promoter correlates with increased myogenin expression during muscle differentiation (Scarpa *et al.*, 1996) (Lucarelli *et al.*, 2001). This is likely due to a disruption of the methyl CpG-binding protein 2 (MBD2) complex which therefore releases myogenin transcription (Luo *et al.*, 2009).

Another function of methylation is to detoxify arsenic using SAM as a cofactor. Arsenic methyltransferase (As3MT) form tri- and pentavalent methylated species, such as methylarsonous acid [MAs(III)], methylarsonic acid [MAs(V)], dimethylarsinous acid [DMAs(III)], dimethylarsonic acid [DMAs(V)], trimethylarsine oxide [TMAs(V)O] and trimethylarsine [TMAs(III)] (Thomas *et al.*, 2001) (Thomas *et al.*, 2007). The use of SAM to detoxify arsenic likely depletes the same cofactor needed by DNA methyltransferase to methylate genes for silencing. This can lead to altered gene methylation and aberrant gene expression (Reichard *et al.*, 2007) (Poirier, 1994).

The present study examined muscle cell differentiation using low concentrations of arsenic, and determined that arsenic does indeed alter muscle cell differentiation and muscle-specific protein expression and patterning.

Methods and Materials

Cell Culture and Viability

Mouse muscle C2C12 cells from ATCC (#CRL-1772, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin (growth media) at 37° C in a humidified incubator containing 5% CO₂. To differentiate the cells, the medium was replaced with DMEM supplemented with 2% horse serum, 1% L-glutamine and 1% penicillin/streptomycin (differentiation media) (Kubo, 1991).

To determine the appropriate levels of arsenic exposure, C2C12 cells were seeded in quintuplet in a 96-well culture plate at a concentration of 2000 cells/well in growth media. Cells were cultured in increasing concentrations of sodium arsenite for three days (n=5 wells per concentration) before viability was assessed spectrophotometrically using a MTS/phenazine methosulfate assay (Cory *et al.*, 1991). From the assay, 20nM sodium arsenite was shown to be non-toxic, so this concentration was used for our experiments.

Quantification of Multinucleated Myocytes

To examine the rate of multinucleated myocyte formation, C2C12 cells were seeded at 8.6×10^2 cells/ well in 6 well plates, and cultured with or without 20nM sodium arsenite as described above. The cells were examined on differentiation days 0, 1, 2, 3, and 4 (n=6 per group per day). To determine the number of myotubes formed, cells were fixed in methanol, incubated in giemsa stain for 10 minutes, washed, and then incubated in 14%

giemsa stain in 100mM phosphate buffer for 20 minutes. Cells were considered to be multinucleated if they contained 3 or more nuclei (Duan and Gallagher, 2009). A multinucleation index was calculated by dividing the total number of cells by the number cells containing >3 nuclei. The average multinucleation index was calculated for each group and day, and statistical differences were determined by Student's t-test.

Arsenic Exposure and Real-time PCR

C2C12 cells were plated at 5000 cells/ 100mm dish (Corning, Corning, NY) in either growth medium or in growth medium containing 20nM sodium arsenite. On the fourth day, cells were washed twice with PBS and either differentiation medium alone or differentiation medium containing 20nM sodium arsenite. Cells were harvested on days 0, 1, 2, 3, and 4 (n=4 per group per day) by scraping into TriReagent (Sigma-Aldrich, St. Louis, MO) to extract total RNA. RNA concentrations were determined by spectrophotometry. 2µg RNA was incubated with 0.4mM deoxynucleotide mix, 2ng random hexamers, and 1000U MMLV reverse transcriptase (Promega, Madison, WI) for 1 hour at 37° C to prepare cDNA. The expression of myogenin, myosin light chain 2, and tropomyosin was quantified by real-time PCR (IQ5, Bio-Rad, Hercules, CA). Each reaction contained RT² SYBR® Green qPCR Master Mix (SABiosciences, Frederick, MD), 400nM primer, and 40ng cDNA, and was incubated at 95oC for 15 seconds followed by 60 seconds at the appropriate annealing temperature (Table 1). Samples were run in triplicate and the entire experiment was repeated at least twice. The cycle threshold values (Ct) were converted into number of molecules per 100ng cDNA using

known concentrations of the specific gene product. GAPDH was used for normalization, the fold change was calculated by dividing the treated value by the control value and statistical differences were determined using Student's t-test.

Cellular Expression and Localization of Myogenin, Myosin Light Chain 2, and Tropomyosin

C2C12 cells were seeded in Lab-tek II eight well coverglass slides (Nunc) at a concentration of 100 cells/well. Cells were cultured with or without 20nM sodium arsenite as described above and examined for specific protein expression on differentiation days 0, 1, 2, 3, and 4 (n=8 per protein per group per day). At the appropriate time point, the medium was removed and the cells were incubated in blocking buffer (1% bovine serum albumin, 0.1% Triton-X100 in 1X PBS) for 1 hour and washed. Cells were then incubated with the appropriate primary antibody for 1 hour (myogenin: 1:100 dilution, Imgenex # IMG-131; myosin light chain 2: 1:50 dilution, Santa Cruz #C-17; tropomyosin: 1:50 dilution, Santa Cruz # F-6). After washing, 1 μ g/ml secondary antibody conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, CA) was incubated with the cells for 1 hour and then the cells were incubated in 300mM DAPI (Invitrogen,) for 15 minutes. Cells were examined by conventional and confocal immunofluorescence on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY). Quantification of myogenin was done by dividing the total number of nuclei by the number of nuclei that were expressing myogenin. Quantification for myosin light chain 2, tropomyosin, and actin was done by outlining individual cells using Nikon NIS elements

software and acquiring the pixel intensity for Alexa Fluor 488. The mean pixel intensities for the individual cells were compared between controls and treatment with four fields per sample. These quantifications were done for each group and day, and statistical differences were determined by Student's t-test.

Cellular Concentration of S-Adenosyl Methionine

S-adenosyl methionine (SAM) concentrations were determined using the Bridge-It SAM Fluorescence Assay (Mediomics, St. Louis, MO). C2C12 cells were plated at 5000 cells/100mm dish. Culturing techniques were same as those done for Real-time PCR analysis. The cells were examined on differentiation days 0, 1, 2, 3, and 4 (n=4 per set per day). At the respective time points, cells were washed with PBS and all visible liquid was removed. Cells were scraped into microfuge tubes, homogenized using a 26 gauge needle, and analyzed by fluorimetry using 485nm for excitation and 665nm for emission. The relative fluorescence values were normalized to the amount of protein. Samples were normalized to the amount of protein (Bio-Rad's DC protein assay) and the average was determined for each group and time point. Statistical differences were determined using Student's t-test.

Results

Arsenic exposure and effects on C2C12 mouse muscle cells

Initial cell viability assays demonstrated that the LC50 of sodium arsenite in C2C12 cells was 5 μ M (data not shown), so concentrations lower than this were tested to determine whether they caused a delay in the differentiation of the cells. Cells were cultured in growth media for 3 days and then cultured in differentiation medium, either with or without 20nM sodium arsenite. Photographs were taken on the first day the differentiation media was added, and again over the next four days. In the 20nM arsenic exposed C2C12 cells on day 0 and day 1 of differentiation, there were no visible differences to that of control cells and both sets appeared predominantly as myoblasts (Figure 1 A and D). There was a noticeable decrease in the amount of myotube formation on day two in the 20nM arsenic exposed cells compared to the controls (Fig.1 B and E). By day two control cells had withdrawn from the cell cycle process and were already fusing together to form myotubes. The arsenic exposed cells were only at beginning stages of differentiation, in which they were elongate in shape, but only contained one nuclei. By day 3, the treated cells contained myotubes but not to the extent as the controls, yet by day 4 the arsenic exposed cells appeared the same as the controls (Fig.1C & F). C2C12 cells were also stained with giemsa during differentiation to determine the number of myotubes, which was considered to be those cells with 3 or more nuclei. There was a significant 17.4-fold increase in myotube formation in the controls on day two of differentiation as compared to the arsenic-exposed cells, but the number of myotubes

was equivalent by days three and four (Fig. 1 graph). This is in agreement with what we visually in the C2C12 cell differentiation.

Quantitative analysis of transcript levels from muscle transcription factor and muscle specific proteins

Gene expression was profiled using qPCR for myogenin, tropomyosin and myosin light chain 2. Myogenin is the transcription factor that initiates the differentiation of a skeletal muscle myoblast to a myotube. When C2C12 cells are cultured in growth medium, there was very low expression of myogenin in both unexposed and arsenic-exposed cells. However, by day 2 of differentiation, myogenin was significantly reduced by 1.4-fold in the arsenic-exposed cells (Figure 2A). Myogenin mRNA expression was down-regulated in the arsenic-exposed cells on days 3 (2.4-fold) and 4 (3.2-fold) of differentiation. The reduced expression of myogenin is likely causing the delay in differentiation in arsenic-exposed cells. The levels of tropomyosin and myosin light chain 2 were also examined since they are integral proteins in muscle contraction. Tropomyosin expression in treated cells showed no difference when compared to control cells (Figure 2B). Interestingly, the expression of myosin light chain 2 was up-regulated in the arsenic-exposed cells days 0, 3 and 4 by 2.6-, 2.2-, and 2.3-fold, respectively (Figure 2C).

Cellular Expression and Localization of Myogenin, Myosin Light Chain 2, and Tropomyosin Protein

Since changes were seen in transcript numbers for myogenin and myosin light chain 2 using real time PCR, the cells were investigated using immunofluorescence determine whether 20nM sodium arsenite would alter the expression and/or localization of myogenin, tropomyosin and myosin light chain 2. Actin was also investigated since it has been shown to go through filament reorganization when exposed to arsenic in endothelial cells (Qian *et al.*, 2004). Myogenin expression is restricted to the nuclei during myogenesis and in both controls (Figure 3 A-C) and treated cells (Figure 3 D-F). The number of nuclei expressing myogenin was significantly reduced in the arsenic-exposed C2C12 cells by 1.6-fold on day two and 1.7-fold on three of differentiation (Figure 5A). However, on day four the number of nuclei expression myogenin in the arsenic-exposed cells is significantly increased by 1.3-fold (Figure 5A). Tropomyosin appeared as filaments on day zero but this was replaced with an overall diffuse pattern of expression on the following days. There were no differences in localization or mean expression intensities between the control and treated cells (Figure 4 G-L and Figure 5 C). Myosin light chain 2 and actin both appear as individual filaments throughout the treatment period with no signs of disorganization (Figure 4 A-F and M-R). Likewise, there were no differences in mean intensities between treatment and control cells (Figure 5 B and D).

Quantification for S-adenosyl methionine

The cofactor S-adenosyl methionine (SAM) is normally used to methylate DNA to regulate gene expression and since there was a reduction in the expression of the

transcription factor myogenin, Sam was investigated to see if there was also a reduction with arsenic exposure. It was investigated using Mediomics Bridge-It® S-Adenosyl Methionine (SAM) Assay; however, there were no differences in S-adenosyl methionine (SAM) concentrations between control and arsenic-exposed cells (Figure 6).

Discussion

These results illustrate that 20 nM sodium arsenite can alter myoblast differentiation by reducing the expression of the transcription factor myogenin. Additionally, arsenic can alter the gene expression of the muscle-specific protein myosin light chain 2 during the differentiation process. To our knowledge, this is the first study that has examined the affects of sodium arsenite in the development of skeletal muscle cells while previous studies have looked at muscle development either *in vivo* or *in vitro* after arsenic exposure using smooth muscle (He *et al.*, 2007; Lantz *et al.*, 2008). When either 50ppb or 100ppb of arsenic were administered to female mice in their drinking water, their offspring had increases in the amount of smooth muscle mass and actin protein levels in the lung, especially in airways smaller than 100 μm in diameter (Lantz *et al.*, 2008). This demonstrates that arsenic can interfere in the development of the vascular system. When pregnant mice ingested drinking water containing 20ppm or higher of sodium arsenite, there was a decrease in their fecundity and a defective formation of blood vessels in the placenta (He *et al.*, 2007). This kind of dysfunction in vascular development was believed to contribute to spontaneous abortion seen in the study (He *et al.*, 2007).

In our study, a developmental delay was observed when the transcription factor myogenin was reduced in the arsenic-treated cells during days 2, 3 and 4. This coincides with the differentiation delay recorded by photography. We had hypothesized that the alterations in myogenin expression might be due to differences in methylation patterns in the promoter regions. Other investigators have seen altered methylation patterns with

arsenic exposure, like a hypermethylation in the promoter region of the tumor suppressor gene p53 in human lung adenocarcinoma A549 cells or a hypermethylation of p53 that has occurred in rat liver epithelial cell line when exposed to arsenic (Mass and Wang, 1997; Zhao *et al.*, 1997). This hypermethylation of the promoter region p53 and another tumor suppressor gene p16 has also been shown in blood samples obtained from people who have chronically exposed to arsenic (Chanda *et al.*, 2006). In one study, a decrease in SAM concentrations occurred during arsenic exposure of TRL 1215 rat liver cells (Zhao *et al.*, 1997). Human HaCaT keratinocytes exposed to 25 μ M arsenite also saw a decrease in SAM concentrations (Reichard *et al.*, 2007). Since SAM is used by both arsenic methyltransferase and DNA methyltransferase, a competition may occur causing a reduction in SAM levels to the point that the replenishment of SAM cannot keep up with the demand. It has even been shown that arsenic toxicity can be rescued by the administration of 170nM of SAM (Ramírez *et al.*, 2007). In HeLa cells that were exposed to 10 μ M sodium arsenite which can cause aneuploidy to occur, the addition of 170nM of SAM reduced the frequency of this chromosome abnormality (Ramirez *et al.*, 2003). As for our experiment, we found no changes in SAM levels between control and arsenic-exposed C2C12 cells. This might be due to the type of cell used, as some cells have are considered to be low methylators, such as urothelial cells and fibroblasts (Dopp *et al.*, 2009). If C2C12 cells are highly active in the methylation of arsenic, this might account for the SAM concentrations from C2C12 cells in the present study being extremely low, ranging from 5 μ M to 23 μ M. Rather than examining global changes in methylation

pools, studies are underway to investigate methylation patterns of the myogenin promoter.

As for the examination of muscle specific proteins, 20 nM sodium arsenite was also able to alter the mRNA levels of the muscle-specific protein myosin light chain 2, resulting in an average increase of 2.1 fold over controls. This is consistent with previous results in a model fish species termed mummichogs, in which the investigators also saw an increase in myosin light chain 2 (Gonzalez *et al.*, 2007). An increase in the amount of myosin light chain proteins could cause a dysfunction to the muscle's contractile force (Schiaffino and Reggiani, 1996). When myosin light chain 2 units were removed from myosin heavy chain, there was a reduction in the filament velocity (Lowey *et al.*, 1993). So an increase in myosin light chain 2 units might cause an over contraction by increasing the filament velocity. However, using immunofluorescence, there was no difference in myosin light chain 2, tropomyosin, or actin protein expression or localization in arsenic-exposed cells. In endothelial cells, an exposure of 10 μ M arsenic was needed to see a change in the organization of actin filaments (Qian *et al.*, 2004). This amount is 500 times higher than the concentration we used, which may be why we can detect differences in some of the transcript levels but not in protein expression.

This study shows that 20 nM of sodium arsenite was able to interfere with the differentiation of myoblasts to myotubes by reducing the production and expression of myogenin. This could translate into a loss of muscle mass or disorganization of muscle filaments, which may be one mechanism behind the developmental toxicity of arsenic.

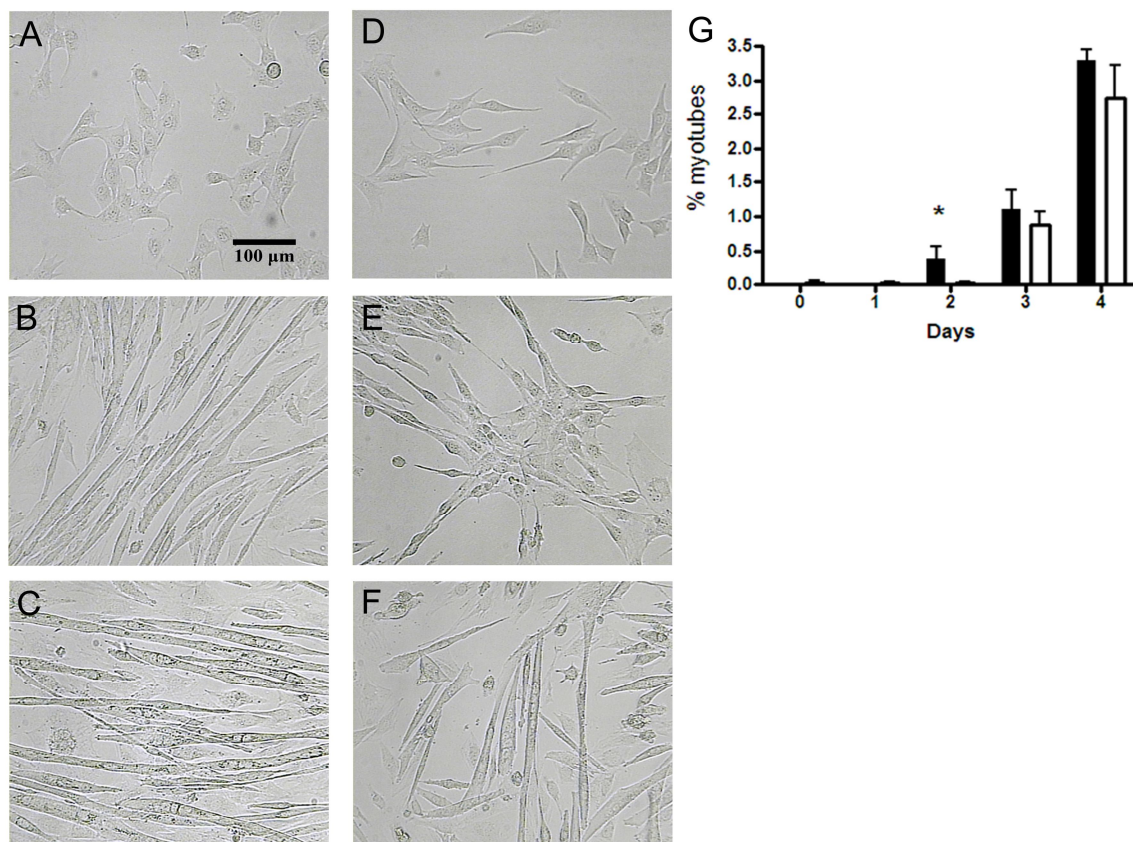


Figure 1. C2C12 cells differentiation from myoblast to myotube.

Both control (A-C) and exposed samples (D-F) had myoblast at day 0 after three days of growth media and zero days of differentiation media. Myotube formation is indicated by the white arrows and elongation is indicated by asterisks. Photographs are representative examples from 4 plates/day/group. Myotubes of 3 or more nuclei were counted for comparison (G) between controls (black bars) and treatment (white bars). Myotubes were counted from 4 random areas per plate (n=4 plates/time/group) and statistical differences were determined by student t-test ($p < 0.05$).

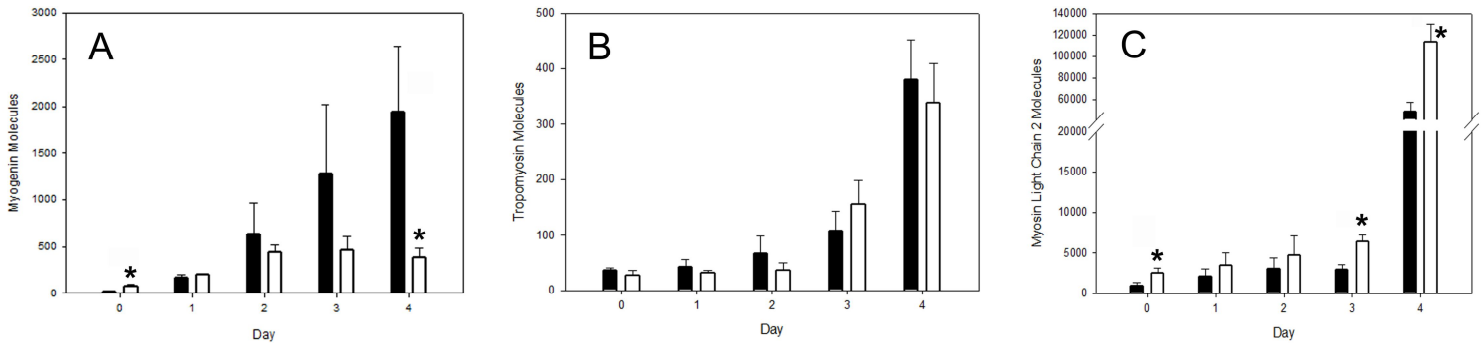


Figure 2. Real-time PCR for myogenin, myosin light chain 2 and tropomyosin

Alteration of myogenin (A), tropomyosin (B) and myosin light chain 2 (C) expression was determined by real-time PCR. Values were normalized against GAPDH as a housekeeping gene, with each sample run in triplicate (n=4 plates/day/group). Statistical differences were determined by Student's t-test ($p < 0.05$). Control samples are the black bars and treatment samples are the white bars.

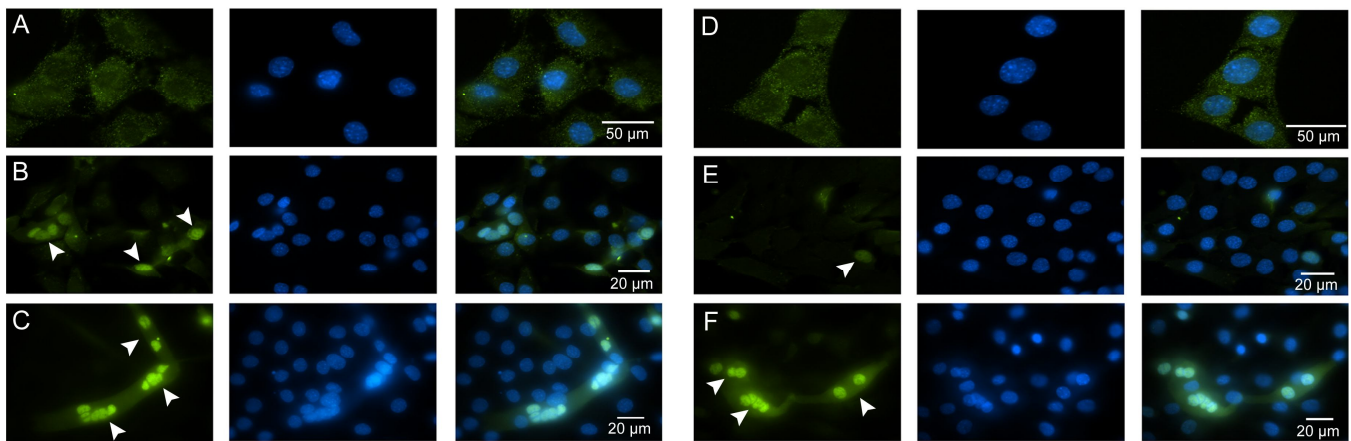


Figure 3. Localization and expression of myogenin through immunofluorescence

Immunofluorescence was used to examine myogenin localization and cells were counterstained with DAPI. Rows A, B, C are control cells for day 0, 2, and 4, respectively. Rows D, E, F are treated cells for day 0, 2, and 4. White arrows indicate nuclei that are expressing myogenin. Pictures are representative examples from 4 wells/time point/group.

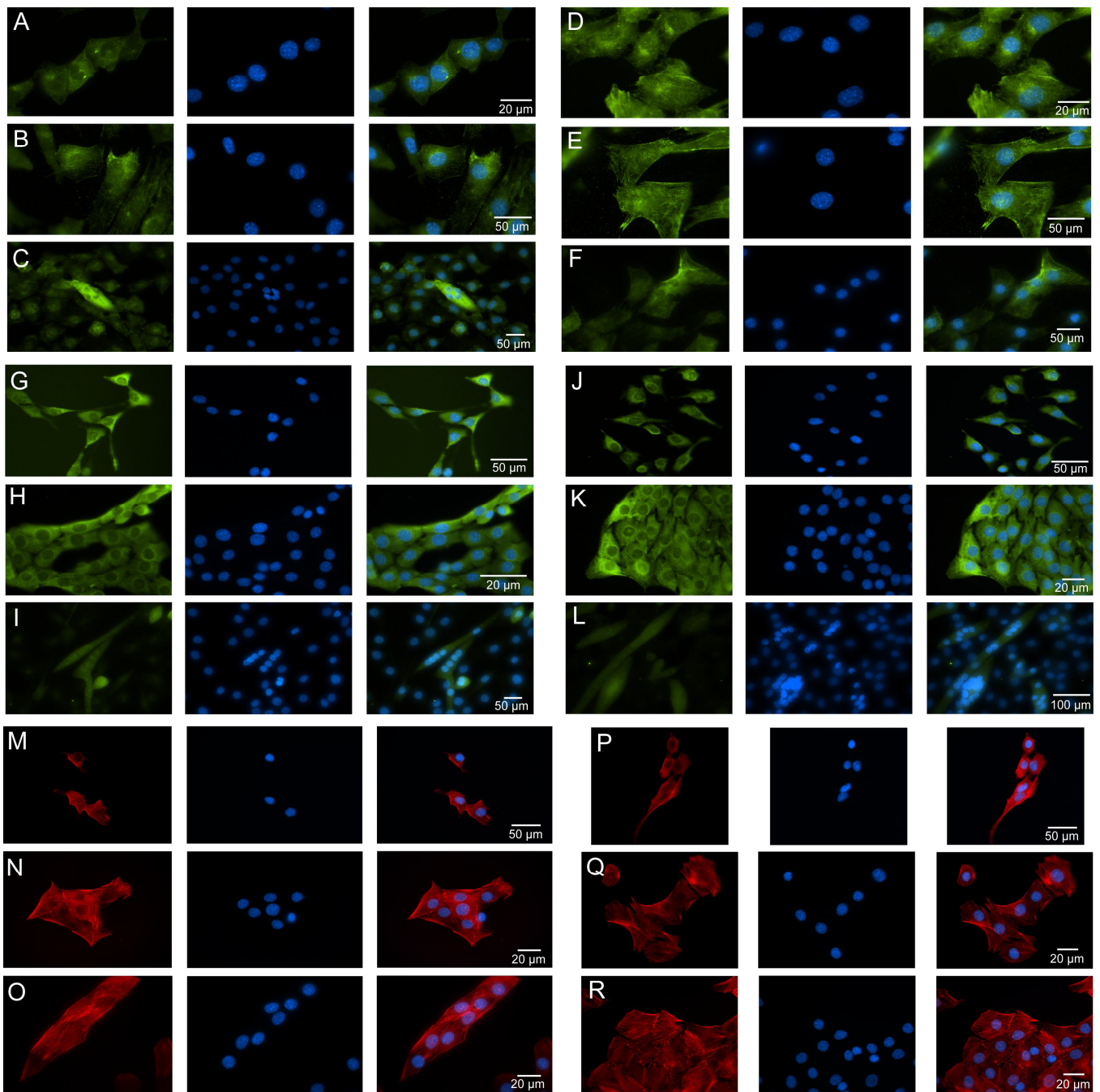


Figure 4. Immunofluorescence of myosin light chain 2, tropomyosin and actin

Immunofluorescence of muscle specific-proteins: myosin light chain 2 (rows A-F) - control cells in rows A-C and treated cells rows D-F; tropomyosin (rows G-L) - control cells in rows G-I and treated cells in rows J-L; actin (rows M-R) - control cells in rows M-O and treated cells in rows P-R. In each set the first row is day 0, second row is day 2 and third row is day 4. Pictures are representative examples from 4 wells/time point/group.

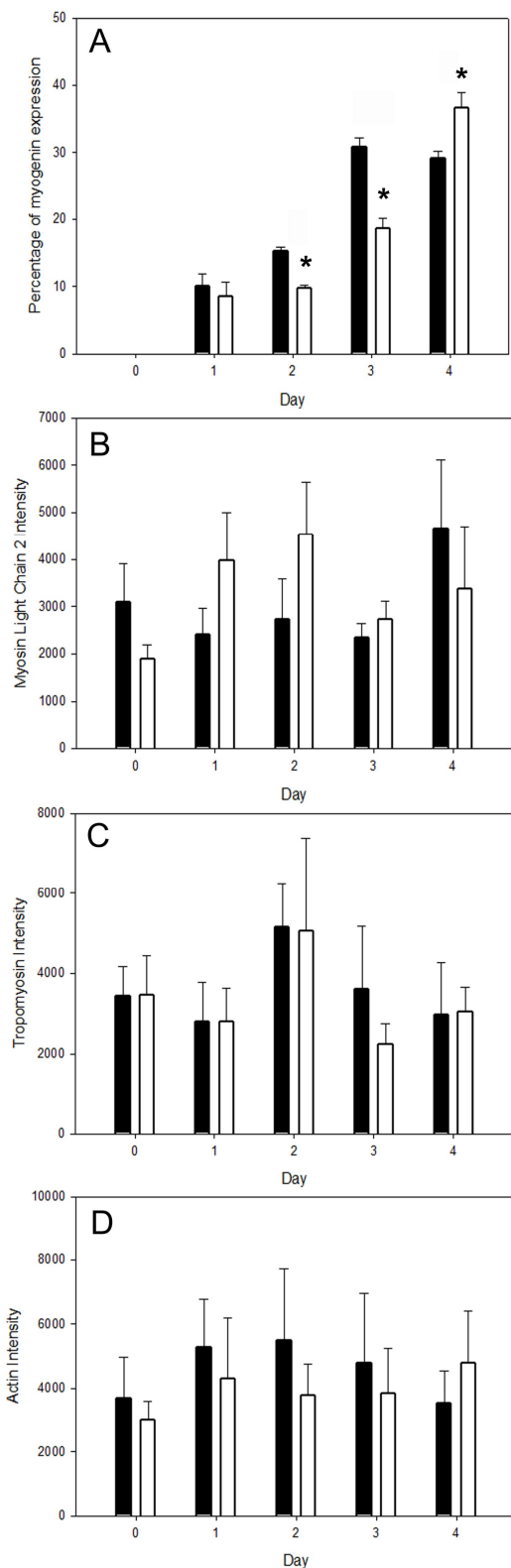


Figure 5. Percentage of expression for myogenin and expression intensities of myosin light chain 2, tropomyosin and actin

The percentage of nuclei expressing myogenin (A) was determined by comparing control cells (black bars) to treated cells (white bars), which was determined by counting the number of nuclei expressing myogenin per total cells in each photo taken ($n \geq 5$). For the mean intensities of myosin light chain 2 (B), tropomyosin (C) and actin (D) control cells (black bars) were compared to treated cells (white bars) cultures ($n=4$). NIS-elements software was used to outline individual cells to acquire their intensities and statistical differences were determined by student t-test ($p < 0.05$)

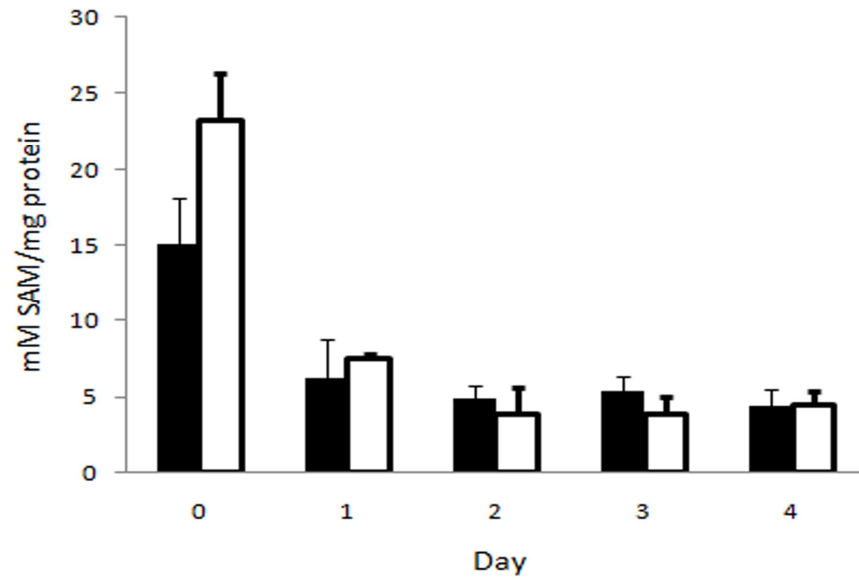


Figure 6. S-adenosyl methionine quantification

SAM levels were unaffected by the addition of 20nm sodium arsenite (white bars) compared to control values (black bars). Samples were done in triplicate on a 96-well plate and statistical differences were determined by student t-test ($p < 0.05$).

Conclusion

To reiterate the goal of this study, we wanted to see how a submicromolar concentration of sodium arsenite would affect the differentiation process of C2C12 mouse myoblast. What was observed was a delay in differentiation that began on day 2 after change from growth media to differentiation media. This was correlated to a decrease in myotube formation in the treated cells and a decrease in myogenin gene expression. This study provides evidence that 20nM sodium arsenite might have the ability to act as developmental toxicant.

Future studies examining the mechanism of myogenin reduction could investigate the methylation pattern of the myogenin promoter region. It has been shown that arsenic can cause a hypermethylation in the promoter regions of the tumor suppressor gene p53 and oncogene K-ras during low microcmolar concentration exposure which regulate cell cycle processes (Mass and Wang, 1997; Benbrahim-Tallaa *et al.*, 2005). A hypermethylation in the promoter region of myogenin could cause the repression that was seen in this experiment.

In addition, investigation of the genes that regulate the cell cycle, like proto-oncogenes and oncogenes, might be considered because an alteration could prevent a cell from exiting mitosis to begin differentiation. Some genes of interest could be cyclin-dependent kinase (cdk) inhibitor p21, c-myc or E2F-1, along with cdk2, cdk4, and cdk6. The cdk inhibitor p21 causes an irreversible cell cycle arrest with its induction in C2C12 cells, while cdk2, cdk4, and cdk6 decrease in expression upon cell differentiation (Walsh

and Perlman, 1997). If the cdk inhibitor p21 were to be down regulated with arsenic exposure while cdk2, cdk4, and cdk6 were up-regulated, this could delay the C2C12 cells' progression to differentiation. As for c-myc, an increase in its expression has been shown to inhibit a cell from exiting the cell cycle in epithelia and murine fibroblast (Freytag and Geddes, 1992). During the differentiation of murine preadipocyte fibroblasts into adipocytes *in vitro*, the cells were inhibited during short term exposure to concentrations $\geq 3\mu\text{M}$ sodium arsenite (Trouba *et al.*, 2000b). This concentration was able to keep the cells in a mitogenic stage even in conditions that should have induced differentiation. In a separate study published by this group, these fibroblasts had increased c-myc and E2F-1 levels after arsenic exposure, which may explain the inhibition of differentiation (Trouba *et al.*, 2000a).

The examination of the methylation pattern on the myogenin promoter region, possibly using a ChIP-on-chip assay, would determine if myogenin is being directly repressed by arsenic due to an altered methylation pattern. Determination of the transcript levels and protein expression for cdk inhibitor p21, c-myc, E2F-1, along with cdk2, cdk4, and cdk6 would be able to tell if the delay seen in the C2C12 cell differentiation was possible due to an inability to exit the cell cycle.

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