Mechanism of arsenic toxicity on vascular development

Wenjie He
The University of Montana

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Mechanism of Arsenic Toxicity on Vascular Development

by

Wenjie He

B.E China Pharmaceutical University, China, 1998

Presented in partial fulfillment of the requirement

for the degree of

Master of Sciences

The University of Montana

May 2004

Approved by:

Chairperson

Dean. Graduate School

Date
Epidemiological studies suggest that arsenic exposure is associated with various detrimental pregnancy outcomes. However, the mechanism for arsenic effects on pregnancy is unknown. We hypothesize that one of the reproductive outcomes, miscarriage, resulting from arsenic exposure may be due to placentation defects of pregnant mice, induced by endothelial vascular defects. The purpose of this study is to 1. Determine how arsenic exposure in drinking water affects fecundity, placentation and vascular morphogenesis in vivo. 2. Determine how arsenic exposure affects the balance between proliferation and apoptosis of bovine endothelial cells (BAE). The data show that the miscarriage rate is dramatically increased in pregnant mice exposed to arsenic in drinking water at concentration of 20 ppm (260 μM) (52.6%), 37.5 ppm (0.49 mM) (61.0%) and 75 ppm (0.98 mM) (69.7%) compared to control (28%). The miscarriage rate of 37.5 ppm arsenic exposure group increases from 43% at E7.5 to 60% at E12.5. The vascular abnormalities of placentas and embryos at E12.5 stage are examined with bandeiraea simplicifolia lectin I isolectin B4 (BSL-B4) staining. There is significant decrease in the vasculature of sodium arsenite (AsIII) treated placentas (52.0%). In vitro, we find a dose-dependent inhibition of cell growth and viability, and increased apoptosis by arsenic treatment for 24 hours on both confluent and non-confluent BAE cells. These data additionally suggest that confluent cells are more susceptible than proliferating cells to arsenic toxicity. However, proliferating cells are more susceptible in a loss of proliferation than confluent cells at doses of 5 μM and 10 μM arsenic. In conclusion, the data suggest that arsenic exposure through drinking water in mice does not substantially affect litter size or pup weight, but does cause spontaneous abortion of the entire litter between E7.5 and E12.5, which finally results in increased miscarriage rate of pregnant mice exposed to 37.5 ppm arsenic. The effects may be due in part to abnormal placenta neovascularization of pregnant mice after arsenic exposure.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>7</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>7</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>11</td>
</tr>
<tr>
<td>RESULTS</td>
<td>14</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>27</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>30</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>30</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>33</td>
</tr>
<tr>
<td>RESULTS</td>
<td>36</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>41</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>44</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>47</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>48</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

Table 1. Summary of arsenic effects on fecundity of pregnant mice  

Table 2. Miscarriage rate for birth  

Table 3. Effects of arsenic toxicity on E7.5 embryo development  

Table 4. Effects of arsenic toxicity on E12.5 embryo development
LIST OF FIGURES

Figure 1. Dose-response relationship for arsenic toxicity on fecundity of pregnant mice 19

Figure 2. Effect of arsenic toxicity on embryo development at E7.5 21

Figure 3. Effect of arsenic toxicity on embryo development at E12.5 23

Figure 4. Summary of arsenic toxicity on fecundity of pregnant mice and embryo development 24

Figure 5. Formation of vasculature of placentas and embryos at E12.5 25

Figure 6. Arsenic toxicity on cell viability of non-confluent and confluent BAE cells 38

Figure 7. Arsenic toxicity on cell proliferation of non-confluent and confluent BAE cells 39

Figure 8. Arsenic toxicity on cell apoptosis of non-confluent and confluent BAE cells 40
INTRODUCTION

Arsenic is an omnipresent metalloid element naturally occurring in the environment in the earth’s crust. It can be released into ground water and soil by both natural processes and human extractive activity. It also exists in pesticide and wood preservatives, pigments and glass (Waugh, 1982). Arsenic has organic and inorganic forms. There are two oxidation-states of inorganic arsenic: Arsenite (As^{III}) and arsenate (As^{V}). These two forms speciate in the environment depending upon the level of dissolved oxygen. However, arsenate can be converted to arsenite in vivo (Abernathy et al., 1999) and it can be methylated by microbes to MMA (monomethylarsonic acid) and DMA (dimethylarsinic acid) (Vahter et al., 2001). This process is traditionally regarded as detoxification pathway because of less acute toxicity of organic arsenic, but a recent study suggests this may not be the case (Tonner-Navarro, 1998). Sodium arsenite (As^{III}) was used throughout this project. The main source of human exposure to this toxicant is contaminated drinking water and some foods, such as rice, grains and fish (ACSH, 2002). Higher levels of arsenic contamination in drinking water from several hundred ppb to over 1 ppm have been observed in Argentina, Bangladesh, Chile, India, Mexico, Thailand and Taiwan (ACSH, 2002). In this country, some states, including Montana, New Mexico, Arizona, Nevada, Utah, Southern California, Idaho and Nebraska, have 50 ppb-100 ppb of arsenic in drinking water; higher than the currently approved 10 ppb MCL (maximum contaminant level) (ACSH, 2002).
Human epidemiological data suggest an association between arsenic exposure in drinking water and adverse reproductive outcomes. Basically, they can be concluded into four main aspects.

1. Spontaneous abortion. In 1989, it was reported that a 1.2-fold increased spontaneous abortion (95% confidence interval: 1.0-1.6) was found in a community of 286 women living with low levels of arsenic in drinking water (0.8-1.3 ppb) and a 1.7-fold increased spontaneous abortion (95% confidence interval: 0.7-4.2) was found in a community with high levels of arsenic (1.4-1.9 ppb) (Aschengrau et al., 1989). A study from Chile showed that higher incidence of late fetal mortality (rate ration (RR) = 1.7; 95% confidence interval (CI), 1.5-1.9), neonatal mortality (RR = 1.52; CI, 1.4-1.7) and postneonatal mortality (RR = 1.26; CI, 1.2-1.3) was associated with higher concentration of arsenic in drinking water (up to 860 ppb) compared to a control community (< 5ppb) (Hopenhany-Rich et al., 2000).

2. Stillbirth. Borzsonyi et al. (1992) reported the potential risk of stillbirth resulted from arsenic in drinking water in southern Hungary. Another study group in Bangladesh found the rate of stillbirth (p< 0.046), spontaneous abortion (p< 0.008) and preterm birth rate (p< 0.018) significantly increased in the arsenic exposed group in drinking water (>0.1 ppm) comparing with lower level arsenic exposure group (<0.02 ppm) (Ahmad et al., 2001).

3. Preterm birth rate. A report from Taiwan suggested that there was a higher preterm birth rate (3.74%) in a group with arsenic exposure in drinking water (from 0.9 ppb up to 3.59 ppm) vs 3.43% in a control group (<0.9 ppb) (with an odds ratio of 1.10). They also found that the babies’ birth weight in an exposed group was on average 30 g
(95 % CI= 13.55- 44.55; P=0.002) lighter than those in a control group (Yang et al., 2003).

4. Birth weight. An epidemiologic study in 2003 from Chile suggested an average reduction of average birth weight (-57 g; 95% confidence interval = -123 to 9) was associated with higher level of arsenic in drinking water (42 ppb) compared to control community (<1 ppb) (Hopenhayn et al., 2003).

These epidemiologic studies were limited by methodological concerns. Human data are too complicated for interpretation because there are multiple chemicals (copper, barium, fluoride etc) in drinking water and lifestyle factors could not be excluded. It is also difficult to determine chronic exposure levels in individuals to correlate the dose-response effects for arsenic exposure.

There are many experimental animal studies demonstrating arsenic as developmental toxicant, though the dosages of most of them are intravenous (i.v) or intraperitoneal (i.p) injection (Holson et al., 2000; Hood, 1978; Hood, 1972). These administration routes have made it difficult to assess the risk potential of arsenic on human health. Very few studies have examined the direct toxicity of arsenic exposure in drinking water on fecundity of pregnant mice, and none of them designed a dose-response study to determine the lowest concentration of As\textsuperscript{III} to cause toxicity in developing mice.

Although both these human and experimental studies suggested an association between arsenic exposures in drinking water with detrimental pregnancy outcomes, it is not clear why this happens. Both the nutrition and toxicant are transferred to the developing embryo through the abundant placental vasculature. Inorganic arsenic of both oxidation states can easily cross the placenta as a low weight molecule (Desesso et al., 1998;
Dencker 1983; Lindgren 1984). Eastman first discovered that arsenic is accumulated in human placenta (Eastman, 1931; Desesso et al., 1998). Concha et al. (1998) found that arsenic in exposed maternal placenta was 34 μg/kg, which was almost 5-fold higher than control women (7 μg/kg) and most of the arsenic was in DMA (dimethylarsinic acid) form in the blood plasma of both mother and newborns. In terms of the mechanisms for fetal and embryonic arsenic toxicity, Hopenhayn et al. (2003) suggested that arsenic might have vascular effects and cause placental abnormalities or decreased blood flow, which then retard fetal growth. Further research is needed to provide a better understanding of the mechanisms for arsenic-induced developmental anomalies.

Based on the current information, we hypothesize that arsenite toxicity impairs vascular development, causing abnormal placental neovascularization of pregnant mice that subsequently causes miscarriage. One purpose of this study is to determine how arsenic exposure affects fecundity, placentation and vascular morphogenesis in pregnant mice.

Endothelial cells are the innermost lining of blood vessels that form the vascular network. They are the major places where nutrients and toxicants enter the blood vessel. They are also the frontier targets of toxicants on vascular networks, and the function of endothelial cells is the foundation of a functional and homeostatic vasculature. The balance between endothelial cell proliferation and apoptosis, resulting in the viability of endothelial cells, directly controls vascular differentiation and angiogenesis. We hypothesize that arsenite toxicity impairs vascular development causing an inadequate perfusion of tissues that subsequently causes miscarriage. To better understand the toxicity of arsenic on the vasculature, it is very important to study the effect of arsenic on endothelial cells. In embryonic life, endothelial cells actively proliferate and differentiate.
The apoptosis of endothelial cells can severely destroy vasculogenesis in embryo development and placenta and cord development, leading to serious hemorrhage, finally causing embryo death (Dimmeler et al., 2000). In contrast, in adult life endothelial cells are typically quiescent and turn over slowly, similar to the situation for confluent cells in culture. The apoptosis of endothelial cells in adult neovasculature can regulate the adult angiogenesis to avoid excess angiogenesis (Dimmeler et al., 2000). The differences among cultured confluent and non-confluent bovine aortic endothelial (BAE) cells may reflect how cells behave in vivo. A study from Brooks et al. (1994) using a monoclonal antibody antagonist of integrin αvβ3 selectively induced apoptosis in the proliferating vascular cells but not in quiescent endothelial cells, which suggested a difference in apoptosis susceptibility between quiescent and proliferating endothelial cells (Ranta et al., 1998). There is a need for a better understanding of the different sensitivity between confluent (quiescent) and non-confluent (replicating) endothelial cells in response to arsenic. The second specific aim of this study was to determine the different physiology of confluent and non-confluent cells altered by arsenic toxicity. To our knowledge, it’s the first study on difference of proliferating and quiescent endothelial cells in response to arsenic.

The vascular system develops through two different processes: vasculogenesis and angiogenesis. Vasculogenesis is the formation of new blood vessels de novo from precursor cells, which mainly occurs during embryo development and placenta creation (Poole et al., 1989). Angiogenesis is the sprouting of new capillaries from pre-existing blood vessel networks (Coffin et al., 1988). In the adult body, one characteristic of the vascular network is stability, and angiogenesis is normally absent (Charnock-Jones et al.,
2004). However, angiogenesis could be activated and occur in certain circumstances, such as placentation (Gordon et al., 1995). The placenta is very crucial to pregnancy, providing adequate nourishment and oxygen to the fetus and exchanging wastes from the fetus. In humans, placental vasculature develops from gestational day 21 to delivery (Zygmunt et al., 2003) involving both vasculogenesis and angiogenesis processes. The angiogenesis in human placental vasculature is a complex and 3-dimensional process that involves sprouting, proliferation and migration of endothelial cells (Charnock-Jones et al., 2004). In this study, we investigated arsenic toxicity on endothelial cell physiology that underlies arsenic toxicity on placental angiogenesis.

Knowledge of the cellular and molecular mechanism of arsenic toxicity on neovascularization in pregnancy is very important for better understanding human reproductive outcomes during arsenic exposure. Inhibition of endothelial cell apoptosis and increase of endothelial cell survival in placenta may improve the angiogenesis, thus helping with vascular development in embryo development.
INTRODUCTION

Arsenic is an omnipresent metalloid element naturally occurring in the environment in the earth’s crust. It can be released into ground water and soil by both natural processes and human extractive activity. It also exists in pesticide and wood preservatives, pigments and glass (Waugh, 1982). Arsenic has organic and inorganic forms. There are two oxidation-states of inorganic arsenic: Arsenite (As^{III}) and arsenate (As^{V}). These two forms speciate in the environment depending upon the level of dissolved oxygen. However, arsenate can be converted to arsenite in vivo (Abernathy et al., 1999) and it can be methylated by microbes to MMA (monomethylarsonic acid) and DMA (dimethylarsinic acid) (Vahter et al., 2001). This process is traditionally regarded as detoxification pathway because of less acute toxicity of organic arsenic, but a recent study suggests this may not be the case (Tonner-Navarro, 1998). Sodium arsenite (As^{III}) was used throughout this project. The main source of human exposure to this toxicant is contaminated drinking water and some foods, such as rice, grains and fish (ACSH, 2002). Higher levels of arsenic contamination in drinking water from several hundred ppb to over 1 ppm have been observed in Argentina, Bangladesh, Chile, India, Mexico, Thailand and Taiwan (ACSH, 2002). In this country, some states, including Montana, New Mexico, Arizona, Nevada, Utah, Southern California, Idaho and Nebraska, have 50 ppb-
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Based on the current information, we hypothesize that arsenite toxicity impairs vascular development, causing abnormal placental neovascularization of pregnant mice that subsequently causes miscarriage. One purpose of this study is to determine how arsenic exposure affects fecundity, placentation and vascular morphogenesis in pregnant mice.
MATERIALS AND METHODS

Chemicals  Sodium arsenite (Formula: NaAsO₂, MW: 129.91) was purchased from J. T Baker (Phillipsburg, NJ), and diluted with deionized distilled water (ddH₂O) to yield 0 ppm (0 µM), 10 ppm (130 µM), 20 ppm (260 µM), 37.5 ppm (0.49 mM), 75 ppm (0.98 mM) and 150 ppm (1.9 mM) concentrations of drinking water for mice. It is changed twice weekly to maintain the desired oxidation states.

Animals  FVB/NJ strain mice (7-10 weeks age) were bred from our internal stocks. All mice were fed a diet of rodent chow (PMI, Brentwood, MO) and tap water ad lib. They were kept on a 12:12 h light: dark cycle at an environmental temperature of 22 ± 1.5°C, with a minimum relative humidity of 40 %. For mating, two virgin females were placed with one stud male and fed with deionized distilled water (ddH₂O). The females were examined daily early morning for semen plug. Detection of a semen plug was designated day 0.5 (E₀.5) of gestation. Pregnant females were placed in separate cages and exposed to As₃(III) (Sodium Arsenite) through drinking water on a continuous basis throughout gestational and weaning stage. The control group was kept on ddH₂O.

Data Collection  The number of newborn pups was counted in both the arsenic-treated group and the control group. The pups were weighed weekly until weaning, when they were sacrificed. The frequency of delivery after plug was determined by number of plugged females produced babies divided by total number of plugged females. Prenatal viability was determined by number of plugged females with embryos alive divided by
total number of plugged females. Miscarriage rate was determined by number of plugged females not producing babies divided by total number of plugged females. Miscarriage rate at E7.5 and E12.5 was determined by number of plugged females not having embryos divided by total number of plugged females.

Embryo examination At 7.5 and 12.5 days of gestation (E7.5 and E12.5), pregnant females were euthanized with carbon dioxide. The uterus was carefully transferred into sterile PBS and the number of live embryos or plaque resorptions was counted (data not shown). Live embryos were removed from their surrounding membranes and weighed. Due to the tiny weight of each embryo, all the live embryos of one litter weighed together. The mean embryo weight then generated by total weight divided by embryo numbers each litter. Embryos were stored in 4 % formalin/PBS at room temperature.

BSL-B4 labeling of mouse tissue After mounting the 4 % formalin-PBS fixed placentas and embryos in frozen tissue matrix (VWR, Bristol, CT), 10 micron sections were cut on a Cryostat (England). (Mouse embryos were cut longitudinally). To stain the endothelial cells in placenta and embryo, fix tissues were soaked in CMPBS (PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂) 3 times, 5 minutes each. The tissues were then permeabilized with 100 % methanol for 20 minutes and rehydrated in ethanol series (95 % - 70 % - 50 %) for 2 minutes each. After rinsing in CMPBS two times, Bandeiraea simplicifolia lectin I isolectin B4 (BSL-B4) (Vector Laboratories, Burlingame, CA) (staining (1, 3)-Gal of endothelial cells) was applied (1 μg/mL in CMPBS) for 30 minutes at room temperature. After a rinse with CMPBS, Texas Red
(546) (Molecular Probes, Eugene, OR) was applied at 1:500 for 30 minutes. Following a final rinse in PBS, the slides were coverslipped with Fluorsave reagent (Calbiochem, San Diego, CA) and examined with a fluorescence microscope (Nikon Eclipse E600, Japan). The immunofluorescence staining was analyzed with a Laser scanning cytometer (LSC) (CompuCyte Corporation, Cambridge, MA). Each datum is an average of three repeated sections on each slide and 5 different embryo litters (one litter on one slide) in each group.

**Statistical analysis.** Results are expressed as the mean ± standard deviation. Comparisons of means were analyzed by one-way analysis of variance (ANOVA) to determine intergroup differences. If the results of the ANOVA were significant (p< 0.05), Tukey’s honestly significant difference (HSD) and Dunnett 2-sided test were applied to the data to compare the treated group with control groups. Frequency of delivery after plug and prenatal vibility were analyzed by one-way analysis of Chi-square test to determine at what percentage of confidence the difference between groups were caused by arsenic exposure in drinking water.
RESULTS

Dose-response study

To determine how arsenic exposure affected the fecundity of pregnant females, a preliminary dose-response study was done. Time-pregnant females were exposed to 0, 1, 10, 20, 37.5, 75 or 150 ppm arsenic in drinking water on a continuous basis of gestational and weaning stages. The number of pups from each mother and average pup weight per litter at postnatal week 1, 2 and 3 was calculated (Table 1). There were strikingly increased miscarriage rates at 20 ppm (52.6 %), 37.5 ppm (61 %) and 75 ppm (69.7 %) vs control (28 %) (Table 2, Figure 1A). P value (<0.01) (Table 2) showed there is absolute dependence between the changing in miscarriage rate and the changing in arsenic exposure for these plugged mice. Although the pup number per litter in the arsenic treated group tended to be lower with increasing arsenic concentrations (from 1 ppm to 37.5 ppm) compared to controls (Table 1, Figure 1B), this finding was not statistically significant. We didn’t see any significant difference in mean pup weight per litter in each arsenic treated group compared to control group either (Table 1, Figure 1C). To minimize the effects of maternal toxicity (The group treated with 150 ppm arsenic died 2-4 weeks after exposure.), the concentration of arsenic in drinking water used in subsequent experiments was focused on 20 and 37.5 ppm.

Embryogenesis

Effects of arsenic on developmental competence of embryos were examined at E7.5 and E12.5 stages. The miscarriage rate for 20 ppm stayed the same at E7.5 and E12.5.
However, the miscarriage rate in 37.5 ppm arsenic group dramatically increased from 43% at E7.5 to 60% at E12.5 (Table 3, 4, Figure 3A, Figure 4A). There was a clear dose-response decrease of prenatal viability at E12.5 (Table 4, Figure 3A). However, the P-value from chi-square test showed that miscarriage rates in 10 ppm, 20 ppm and 37.5 ppm groups at E12.5 stage are independent of arsenic exposure in drinking water (Table 4). The same results were obtained at E7.5 stage (Table 3). Embryo number for each litter at E12.5 had a dose-related decrease (Table 4, Figure 3B), although the difference was not statistically significant. In contrast, we did not observe any decrease of embryo number of each litter mentioned above at E7.5 stage (Table 3, Figure 2B). We didn’t observe any significant difference of mean pup weight in the various arsenic concentration groups comparing to control group at E7.5 or E12.5 (Table 3, Table 4, Figure 2C, Figure 3C). Figure 4 shows a summary of arsenic toxicity and its effects on pregnancy and embryonic development. The data show trend toward smaller litters at both the E12.5 and birth stages. The data also show trend toward lower birth weight with increasing arsenic toxicity at E12.5. The most significant result is loss of the entire litter (P < 0.01), i.e. complete miscarriage, resulting from arsenic toxicity.

**Vasculature**

Placentas and embryos were examined for morphological variance and for vascular abnormalities. The insert micrograph (Figure 5E) shows three representative placentas from As$^{III}$ treated (top) and control (bottom) E12.5 females. The placentas of 37.5 ppm arsenic treated group were darker than those of control group may suggest a hemorrhage
and oxidation of hemoglobin. The enlargement of arsenic treated placentas may suggest a hypertrophy due to abnormal vasculogenesis (Figure 5E).

Fixed placentas and embryos were stained with Bandeiraea simplicifolia lectin I isolectin B4 (BSL-B4) (staining (1, 3)-Gal of endothelial cells) and fluorescence was quantified with the laser scanning cytometer (LSC). There were numerous large cavities in the tissues and more loosening of cell connections in arsenic treated placenta (Figure 5B) and embryo (Figure 5D) compared to control placenta (Figure 5A) and embryo (Figure 5C), which showed dramatic tissue damage. Quantification of fluorescence showed significantly loss of BSL-B4 positive staining (p<0.05) in placenta from 37.5 ppm As$_{III}^+$ treated group (51.99 %) at E12.5 versus the control group (100 %) (Figure 5E). The embryonic vasculature of this group decreased to about 65.69 % compared to control (100 %), though not statistically significant (Figure 5E). In contrast, the BSL-B4 labeling in 20 ppm arsenic treated placenta (122.09 %) didn’t have any statistically significant difference compared to control placenta (100 %), nor did the BSL-B4 labeling in 20 ppm arsenic treated embryos (65.55 %) compared to control embryos (Figure 5E).
Table 1. Summary of arsenic effects on fecundity of pregnant mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1ppm</th>
<th>10ppm</th>
<th>20ppm</th>
<th>37.5ppm</th>
<th>75ppm</th>
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<tbody>
<tr>
<td>Numbers of plugged females with pups (n)</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Pups per litter (n) (mean ± SD)</td>
<td>9 ± 4</td>
<td>7.91 ± 2.98</td>
<td>7.44 ± 3.13</td>
<td>6.89 ± 2.47</td>
<td>5.55 ± 2.66</td>
<td>7.1 ± 1.73</td>
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<tr>
<td>Mean pup Weight (g) Week 1 (Mean ± SD)</td>
<td>4.15 ± 0.62</td>
<td>4.60 ± 0.68</td>
<td>4.78 ± 0.85</td>
<td>4.73 ± 1.31</td>
<td>4.29 ± 0.56</td>
<td>3.65 ± 0.5</td>
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<td>Week 2</td>
<td>6.6 ± 1.50</td>
<td>7.25 ± 1.41</td>
<td>7.94 ± 1.55</td>
<td>7.35 ± 1.58</td>
<td>7.78 ± 1.32</td>
<td>5.43 ± 0.7</td>
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Table 2. Miscarriage rate for birth

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<tr>
<td>Number of plugged females without pups (n)</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Frequency of delivery after plug</td>
<td>72%</td>
<td>73%</td>
<td>81.8%</td>
<td>47.4%</td>
<td>39%</td>
<td>30.3%</td>
</tr>
<tr>
<td>Miscarriage rate</td>
<td>28%</td>
<td>27%</td>
<td>18.2%</td>
<td>52.6%</td>
<td>61%</td>
<td>69.7%</td>
</tr>
<tr>
<td>P-value (Chi-square test)</td>
<td>P&lt; 0.01</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 1. Dose-response relationship for arsenic toxicity on fecundity of pregnant mice. Plugged mice were exposed to various concentration of arsenic throughout all the gestational and weaning stages. Litters were counted and pups were weighted weekly. A. Calculated frequency of delivery after plug. B. Number of pups per litter. C. Mean pup weight per litter at week 1, 2 and 3 postnatal. Error bars represent the mean +/- SD for n≥9 each group.
Table 3. Effects of arsenic toxicity on E7.5 embryo development

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>20ppm</th>
<th>37.5ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers of plugged females with embryos (n)</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Embryos per litter (n) (mean ± SEM)</td>
<td>7.9 ± 2.92</td>
<td>8.8 ± 1.99</td>
<td>8.38 ± 3.81</td>
</tr>
<tr>
<td>Mean embryo weight (g) (mean ± SEM)</td>
<td>0.0141 ± 0.0054</td>
<td>0.0146 ± 0.0018</td>
<td>0.0139 ± 0.001</td>
</tr>
<tr>
<td>Numbers of plugged females without embryos (n)</td>
<td>5</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Prenatal viability</td>
<td>67%</td>
<td>53%</td>
<td>57%</td>
</tr>
<tr>
<td>Miscarriage rate (%)</td>
<td>33%</td>
<td>48%</td>
<td>43%</td>
</tr>
<tr>
<td>P-value (Chi-square test)</td>
<td>ns</td>
<td></td>
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</tr>
</tbody>
</table>

ns: not significant
Figure 2. Effect of arsenic toxicity on embryo development at E7.5. Plugged mice were exposed to various concentration of arsenic from E0.5 to E7.5. Live embryos were counted and weighed. A. Prenatal viability at E7.5. B. Numbers of embryo per litter at E7.5. C. Mean embryo weight per litter at E7.5. Error bars represent the mean +/- SD for n≥8 each group.
Table 4. Effects of arsenic toxicity on E12.5 embryo development

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10ppm</th>
<th>20ppm</th>
<th>37.5ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers of plugged females with embryos (n)</td>
<td>10</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Embryos per litter (n) (mean ± SEM)</td>
<td>8.9 ± 2.85</td>
<td>8.86 ± 2.97</td>
<td>8.38 ± 2.77</td>
<td>6.83 ± 2.5</td>
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<tr>
<td>Mean embryo weight (g) (mean ± SEM)</td>
<td>0.0965 ± 0.040</td>
<td>0.0779 ± 0.016</td>
<td>0.0828 ± 0.020</td>
<td>0.076 ± 0.</td>
</tr>
<tr>
<td>Numbers of plugged females without embryos (n)</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Prenatal viability (%)</td>
<td>83%</td>
<td>77%</td>
<td>53%</td>
<td>40%</td>
</tr>
<tr>
<td>Miscarriage rate (%)</td>
<td>18%</td>
<td>23%</td>
<td>50%</td>
<td>60%</td>
</tr>
<tr>
<td>P-value (Chi-square test)</td>
<td>ns</td>
<td></td>
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</tbody>
</table>

ns: not significant
Figure 3. Effect of arsenic toxicity on embryo development at E12.5. Plugged mice were exposed to various concentrations of arsenic from E0.5 to E12.5. Live embryos were counted and weighed. A. Prenatal viability at E12.5. B. Numbers of embryo per litter at E12.5. C. Mean embryo weight per litter at E12.5. Error bars represent the mean +/- SD for n≥ 6 each group.
Figure 4. Summary of arsenic toxicity on fecundity of pregnant mice and embryo development at E7.5 and E12.5 stages. Error bars represent the mean +/- SD for n≥ 6 each group.
Figure 5. Examination of vasculature of placenta and embryo at E12.5. Pregnant mice were exposed to 0, 20 and 37.5 ppm arsenic from E0.5 till E12.5. Placentas and embryos were stained with BSL-B4 and quantified with LSC. (A) Control placenta. (B) 37.5 ppm arsenic exposed placenta. (C) Control embryo. (D) 37.5 ppm arsenic exposed embryo. (A-D) 20x magnification. (E) Amount of endothelial cells in placentas and embryos at E12.5. The amount of endothelial cells is expressed as percentage of control. Error bars represent the mean +/- SD for n=5 each group. Inset shows photographs of placentas before staining (1x magnification). 37.5 ppm arsenic exposed placentas in upper level. Control placentas in lower level.
DISCUSSION

Arsenic exposure through drinking water from E0.5 through weaning causes defects in placentation and lower fecundity in pregnant females. Drinking water exposure to sodium arsenite did not cause a statistically significant difference in the litter size at any time point. Our study suggests that primary effect of arsenic on pregnant mice was a dose-dependent increase in conceptus mortality (miscarriage rate). Previous studies reported that embryonic malformations resulted from arsenic exposure by intraperitoneal (i.p.) or intravenous (i.v.) routes of pregnant mice (Hood, 1972; Morrissey, 1983). In this study, we didn’t observe any obvious offspring malformations, which is consistent with the research results of Stump et al. (1999) that oral administration of arsenic in rats by gavage only resulted in increased prenatal mortality. We also didn’t see any other adverse pregnancy outcomes, such as preterm delivery. All the pregnant females delivered at 19-21 gestational days without any observable increase in stillbirth. We did not observe any obvious increase of the resorptions plaques at E12.5 in 37.5 ppm arsenic treated group compared to control group. To our knowledge, this is the first study associating arsenic exposure in drinking water with lower fecundity of pregnant females. However, the results of this study should be considered within the context of limitations in the study design. In our study, we didn’t have exposure assessment and the consumption of arsenic water may vary between groups.

In this study, we examined the E7.5 and E12.5 stages to monitor vascular development from when it is first evident to functionally accomplished. Comparison of E7.5 to neonatal stage miscarriage rates shows that the spontaneous abortion mostly happened in
postimplantation stage between E7.5 to E12.5 stage. Examination of the placenta and embryonic vasculature showed arsenic toxicity impairs placental vascular development, which probably predisposes the embryo to miscarriage. In the 20 ppm arsenic treatment group, the miscarriage rate stayed almost the same from E7.5 (47 %), E12.5 (47 %) to birth (52 %). In contrast, the miscarriage rate for the 37.5 ppm arsenic treatment group increased from 43 % at E7.5 to 60 % at E12.5, which is within error, the same as the 61 % rate at birth. These data suggest that the mechanisms for miscarriage caused by arsenic exposure at 20 ppm may be different from the mechanisms at 37.5 ppm. However, the reason for this difference is not clear. The high miscarriage rates at E7.5 (47 %) and E12.5 (43 %) may suggest that the pre-implantation stage is also an important stage for arsenic toxicity. The data for 20 ppm may suggest that pre-implantation is a critical time for arsenic toxicity related spontaneous abortion; versus 37.5 ppm treatments where the mechanism of arsenic toxicity may have two stages: pre-implantation and placental neovascularization during post-implantation. In 2002, Krininger et al. demonstrated that arsenic could impair the pre-implantation development of bovine embryos. They showed that apoptosis and group 2 caspase activity were increased after exposure to arsenic at E5 (embryos ≥ 16 cells in number) and the cell number was reduced after exposure to arsenic at E1.5 (two-cell stage). They also found that the percentage of embryos that developed to the blastocyst stage was reduced by arsenic exposure at both E1.5 and E5 (Krininger et al., 2002). The magnitude of increasing miscarriage rate at post-implantation stage was less than that at pre-implantation stage, which is consistent with the report that developmental toxicity of inorganic arsenic was gestational age dependence (Tabacova et al., 1996). With increasing gestational age there was an
increasing resistance to arsenic-induced effects. In 1981, Harrison and Hood reported that exposure to arsenate by hamsters on E9 caused complete resorptions of all implantation sites while the same dose on E11 and E12 only resulted in weight reduction (Harrison and Hood, 1981; Tabacova et al., 1996). However, current study indicates that another possibility is maternal oxidative stress induced by arsenic followed by abnormal placental neovascularization that is likely to make embryo more susceptible to arsenic toxicity. We found that the placentas and embryos in the arsenic treated group contained vascular morphological anomalies. These results are consistent with reports of cavernous damage to both endodermal epithelium and mesodermal mesothelium in arsenic treated mouse embryo and yolk sac placenta (Li et al., 1997). Likewise, we found decreased quantities of endothelial cells in the E12.5 placenta of pregnant mice treated with 37.5 ppm (0.49 mM) sodium arsenic in drinking water; which is consistent with the finding of Soucy NV et al. (2003) that concentration of arsenic greater than 1 μM inhibits vessel growth in vivo in a chicken chorioallantoicmembrane (CAM) assay. They also found that the threshold \( \text{As}^{\text{III}} \) concentration for this transition from stimulation to inhibition of vessel density is 0.033 μM. In summary, our study shows the development of embryo highly dependent on a functional placenta and functional disturbance of placenta could heavily impair embryo development.

In this study, the different miscarriage rates for the control groups in the different experiments (Figure 4A) are likely to due to normal variance. Perhaps weighing the females before the experiments would have reduced the variance, though all of them were between 7 to 10 weeks age.
CHAPTER 2. A Comparison of Arsenic Toxicity on Viability, Proliferation and Apoptosis of Proliferating and Non-proliferating Bovine Aortic Endothelial Cells

INTRODUCTION

Endothelial cells are the innermost lining of blood vessels that form the vascular network. They are the major places where nutrients and toxicants enter the blood vessel. They are also the frontier targets of toxicants on vascular networks, and the function of endothelial cells is the foundation of a functional and homeostatic vasculature. The balance between endothelial cell proliferation and apoptosis, resulting in the viability of endothelial cells, directly controls vascular differentiation and angiogenesis. We hypothesize that arsenite toxicity impairs vascular development causing an inadequate perfusion of tissues that subsequently causes miscarriage. To better understand the toxicity of arsenic on the vasculature, it is very important to study the effect of arsenic on endothelial cells. In embryonic life, endothelial cells actively proliferate and differentiate. The apoptosis of endothelial cells can severely destroy vasculogenesis in embryo development and placenta and cord development, leading to serious hemorrhage, finally causing embryo death (Dimmeler et al., 2000). In contrast, in adult life endothelial cells are typically quiescent and turn over slowly, similar to the situation for confluent cells in culture. The apoptosis of endothelial cells in adult neovasculature can regulate the adult angiogenesis to avoid excess angiogenesis (Dimmeler et al., 2000). The differences among cultured confluent and non-confluent bovine aortic endothelial (BAE) cells may reflect how cells behave in vivo. A study from Brooks et al. (1994) using a monoclonal
antibody antagonist of integrin $\alpha_\beta_3$ selectively induced apoptosis in the proliferating vascular cells but not in quiescent endothelial cells, which suggested a difference in apoptosis susceptibility between quiescent and proliferating endothelial cells (Ranta et al., 1998). There is a need for a better understanding of the different sensitivity between confluent (quiescent) and non-confluent (replicating) endothelial cells in response to arsenic. The second specific aim of this study was to determine the different physiology of confluent and non-confluent cells altered by arsenic toxicity. To our knowledge, it’s the first study on difference of proliferating and quiescent endothelial cells in response to arsenic.

The vascular system develops through two different processes: vasculogenesis and angiogenesis. Vasculogenesis is the formation of new blood vessels de novo from precursor cells, which mainly occurs during embryo development and placenta creation (Poole et al., 1989). Angiogenesis is the sprouting of new capillaries from pre-existing blood vessel networks (Coffin et al., 1988). In the adult body, one characteristic of the vascular network is stability, and angiogenesis is normally absent (Charnock-Jones et al., 2004). However, angiogenesis could be activated and occur in certain circumstances, such as placentation (Gordon et al., 1995). The placenta is very crucial to pregnancy, providing adequate nourishment and oxygen to the fetus and exchanging wastes from the fetus. In humans, placental vasculature develops from gestational day 21 to delivery (Zygmunt et al., 2003) involving both vasculogenesis and angiogenesis processes. The angiogenesis in human placental vasculature is a complex and 3-dimentional process that involves sprouting, proliferation and migration of endothelial cells (Charnock-Jones et al.
al., 2004). In this study, we investigated arsenic toxicity on endothelial cell physiology that underlies arsenic toxicity on placental angiogenesis.
MATERIAL AND METHODS

**Chemicals** Sodium arsenite (Formula: NaAsO₂, MW: 129.91) was purchased from J. T Baker (Phillipsburg, NJ), and diluted with sterile PBS to yield arsenic concentrations of 0, 1, 5, 10, 20 and 50 μM to treat BAE cells.

**Cell Culture** Bovine aortic endothelial (BAE) cells were cultured in Dulbecco’s modification of eagle’s medium (DMEM) (Gibco, Grand Island, NY) supplemented with 1 % penicillin-streptomycin-glutamine (PSG) (Hyclone Laboratories, Logan, Utah) and 15 % fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. The cells were harvested with 0.05 % Trypsin-EDTA (Gibco, Grand Island, NY) and only passage between 2-6 were used. Cells reached confluence on 96 well plates and chamber slides two days after passage. One day post-confluent was used for all confluent cells. The culture medium for confluent cells was changed to fresh medium 18-24 hours before being treated with arsenic. Cells grown for one day (24 h) post-passage were considered non-confluent. Cell density was assayed with a Coulter Particle Counter (Beckman Coulter Corporation, Miami, FL).

**Cell viability assay** Cell viability was determined with the modified MTT assay (Bunderson, et. al., 2002). Cells were seeded in 96 well plates at 1.5 x 10⁵ cells/ml. Sodium arsenite solutions were applied for 24 hours. MTT (50 μg) (Sigma, St. Louis, MO) was added to each well and cells were incubated for four hours. Medium/MTT
solutions were removed and 100 μL DMSO was added to dissolve the MTT formazan crystals. Absorbance was measured with Spectra Max 340 (Molecular Device, Sunnyvale, CA) at 550 nm.

**Cell proliferation assay** BAE cells were seeded in 96 well plates at 1.5 x 10^5 cells/ml. After being treated with arsenic for 24 hours, a cell proliferation assay was performed with the use of BrdU (bromodeoxyuridine) Cell Proliferation Assay Kit (Oncogene, Cambridge, MA), according to the manufacturer’s instructions. Absorbance was determined with Spectra Max 340 at dual wavelengths of 450-540 nm.

**TUNEL assay** BAE cells were grown to confluence or non-confluence on 2% (w/v) gelatin (Sigma, St. Louis, MO) (diluted with PBS) coated Lab-Tek II Chamber slides (Nalge Nunc International, Naperville, IL) and incubated with arsenic concentrations of 0, 1, 5, 10, 20 or 50 μM for 24 hours. A TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay was performed with the use of ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. Propidium iodide used in this assay was purchased from Sigma (St. Louis, MO). Fluorescence was quantified with a Laser Scanning Cytometer (LSC; CompuCyte Corporation, Cambridge, MA).

**Statistical analysis.** Results are expressed as mean ± standard deviation. Comparisons of means were analyzed by one-way analysis of variance (ANOVA) to determine intergroup differences. If the results of the ANOVA were significant (p<0.05), Tukey’s honest
significant difference (HSD) and Dunnett 2-sided test were applied to the data to compare the treated and control groups.
RESULTS

Cell Viability assay

Confluent and non-confluent BAE cells were grown in 96-well plates (1.5 x 10^5 cells/ml) and a dose-response relationship to 24 hours of arsenic toxicity was determined with a MTT assay. There were dose-dependent decreases in viability after arsenic exposure in both confluent and non-confluent cells. In non-confluent cells, there was a significant decrease in viability from 5 μM to 50 μM compared to controls (Figure 6a), whereas confluent cells revealed a significant decrease in viability at arsenic concentration of 20 μM and 50 μM only (Figure 6b). However, at 50 μM arsenic exposure, the confluent cells had a significantly decreased cell viability compared to non-confluent cells (Figure 6c), which may suggest confluent BAE cells are more susceptible to arsenic toxicity.

Cell Proliferation assay

Confluent and non-confluent BAE cells were grown in 96-well plates and a dose-response study of cell proliferation was determined with the BrdU Proliferation Assay. Significant decreases in cell proliferation were found in proliferating (non-confluent) cells from 5 μM to 50 μM compared to control (Figure 7a); in contrast there was only a significant difference at highest concentration (50 μM) in confluent cells (Figure 7b). The non-confluent control BAE cells showed significantly more proliferation than confluent control cells. However, they only had significant difference at control and 1 μM point and lost this difference at more than 1 μM concentrations. This suggests that non-confluent BAE cells have more susceptibility to proliferation loss as a result of arsenic toxicity.
TUNEL assay for apoptosis

Confluent and non-confluent BAE cells were seeded in gelatin coated chamber slides and a dose-response study of cell apoptosis to 24 hours arsenic toxicity was conducted with a TUNEL assay. There was a significant increase in BAE apoptosis in both confluent and non-confluent cells at 1, 10, 20 and 50 μM sodium arsenite (Figure 8a, b). There was no significant difference between the two groups at any dose point (Figure 8c), though there was a trend of higher percentage of apoptosis cells in confluent BAE cells vs non-confluent cells.
Figure 6. Cell viability for confluent and non-confluent BAE cells treated with various concentration of sodium arsenite (As\textsuperscript{III}) for 24 h. Viability was measured using an adaptation of MTT colorimetric assay as described under Methods. Viability is expressed as percentage of control (untreated cells); Error bars represent mean +/- SD for n=4. There is significant difference at all concentration point between confluent and non-confluent cells (not marked). Significantly different from control p<0.05. The confluent control cells had higher viability (214 %) compared to non-confluent cells because they had 3 days longer to grow.
Figure 7. Cell proliferation for confluent and non-confluent BAE cells treated with various concentration of sodium arsenite ($\text{As}^{\text{III}}$) for 24h. Cell proliferation was measured using BrdU cell proliferation assay (Oncogene, Cambridge, MA). There is significant difference between confluent and non-confluent cells at control and 1 μM arsenic treatment. The non-confluent control BAE cells showed significantly more proliferation (127%) than confluent control cells (100%). Proliferation is expressed as percentage of control (untreated cells); error bars represent mean +/- SD for n=4. * Significantly different from control, ** Significantly different between two group, p<0.05.
Figure 8. Cell apoptosis for confluent and non-confluent BAE cells treated with various concentration of sodium arsenite (As$^{III}$) for 24h. Cell apoptosis was measured using ApoAlert DNA fragmentation assay (Clontech, Palo Alto, CA). Apoptosis is expressed as percentage of control (untreated cells); error bars represent mean +/- SD for n=4. Significantly different from control, p<0.05.
DISCUSSION

Our data showing a dose-dependent decrease of cell proliferation in proliferating (non-confluent) cells in response to arsenic treatment and a significant decrease starting from 5 µM are consistent with the decreased thymidine incorporation observed by others in exponentially growing porcine aortic endothelial cells (Barchowsky et al., 1996). This shows that the proliferation of non-confluent cells is not stimulated by arsenic at low concentration. In contrast, we observed a small trend of increasing cell proliferation from 1 µM to 5 µM sodium arsenite in confluent cells (though not significant), which is consistent with the previous report that low concentration of arsenic has a mitogenic effect on postconfluent endothelial cells (Barchowsky, 1996).

There was significant loss of cell viability of endothelial cells from 5 µM in both confluent and non-confluent BAE cells in this study. This is consistent with previous findings that concentrations greater than 5 µM are toxic to endothelial cells (Barchowsky, 1996; Deneke, 1992). From the data, we found proliferating cells (non-confluent) had higher cell viability after exposure to 50 µM arsenic for 24 than that of quiescent (confluent) cells, which may suggest a greater sensitivity of quiescent cells to arsenic toxicity.

From the TUNEL assay, we did not observe any significant difference of increased apoptosis in response to arsenic treatment between confluent and non-confluent situation. There was only a trend of more apoptosis in quiescent BAE cells than proliferating cells, which may suggest the more sensitivity of quiescent cells vs proliferating cells to arsenic.
toxicity which is consistent with the study from Queille et al. (2001) that growing cells had lower rates of apoptosis from UV damage than confluent cells.

However, we observed significant increase of cell apoptosis in both confluent and non-confluent situation compared to each control after 24 h treatment with 1 μM arsenic and lost this significant difference at 5 μM concentration followed by significant increase of apoptosis again. This is consistent with the study results from Barchowsky that there was no sign of cell death in primary porcine aortic endothelial cells exposure to 5 μM arsenite, even following exposure for 96 hours (Barchowsky et al., 1999). They also reported a significant cell loss after 48 h with concentrations greater than 10 μM, which agrees with the results showing here. In the other hand, low concentration of arsenic exposure (< 1 μM) stimulates angiogenesis in vitro (Kao et al., 2003). Another study from Barchowsky et al (1996) demonstrated that oxidant generation (dichlorofluorescin (DCF)) peaked at 5 μM sodium arsenite in porcine endothelial cells and decreased to basal line starting from 10 μM arsenite. They also suggested that this increased intracellular oxidant formation promote nuclear translocation of trans-acting factors (NF-κB) and therefore be mitogenic to endothelial cells growth. One implication from these literatures and the data of current study is that 1 μM – 5 μM – 10 μM may be a transit window of arsenic effects from stimulation angiogenesis to inhibition angiogenesis. The possible explanation for the phenomena we observed in TUNEL assay is that arsenic stimulates the apoptosis at 1 μM, which is then compensated by the mitogenic effect of arsenic at 5 μM and finally gets to inhibitory effect again at 10 μM.

At 50 μM arsenic concentration, we observed a significantly less viability of confluent cells compared to non-confluent cells. In contrast, we didn’t see any significant
difference of cell apoptosis or proliferation between confluent and non-confluent BAE cells. One of the reasons for that may be most of confluent cells are undergoing necrotic cell death instead of apoptosis. Previous research showed that bovine embryos can acquire increased resistance to certain cellular stress (cyanide toxicity) as they advance in development (Donnay et al., 2000). Like the actively proliferating cells in embryo, we can speculate that the surviving proliferating BAE cells may also gain a better resistance to exogenous toxicant (arsenic) than quiescent cells do.

In conclusion, there was a dose-dependent inhibition of both cell proliferation and viability, and an increase in apoptosis by arsenic treatment for 24 hours on both confluent and non-confluent BAE cells.
CONCLUSIONS

The major findings of this project were: Decreased fecundity in As\textsuperscript{III} exposed pregnant female mice due to vascular defects in the placenta (Chapter 1) and As\textsuperscript{III} mediated endothelial cell death (Chapter 2). Results from \textit{in vitro} arsenic exposure experiments with BAE cells suggest that the defects in vascular development could result from a loss of endothelial cell viability; particularly in the placenta. The decline in the viability could result from lower proliferation, increased apoptosis, or a combination of both factors (Chapter 2). In conclusion, this study suggests As\textsuperscript{III} induced apoptosis and/or inhibition of proliferation in the developing placental endothelium may be the cause of the increased miscarriages in As\textsuperscript{III} exposed pregnant female mice.

Future studies will address this hypothesis. Both the proliferation and apoptosis of endothelial cells will be assayed on placenta from As\textsuperscript{III} exposed mice. Currently, the mechanism of the regulation of angiogenesis in the placenta is not particularly well understood. Some endothelial growth factors, particularly vascular endothelial growth factor (VEGF), play very important roles in the process of vascular development. These growth factors may be the targets of an exogenous toxicant, resulting in vascular defects. It would also be interesting to study the differential expression of these proteins in placenta in response to arsenic toxicity in future research.

Arsenic readily reacts with thiol-containing molecules such as GSH and cysteine, which are critical in biochemical reactions, leading to toxicity (Scott \textit{et al.}, 1993). However, the mechanism of arsenic toxicity in molecular level is still not very clear. Some studies suggested arsenic genotoxicity, such as chromosomal aberrations, DNA-
protein crosslinks, and sister chromatid exchanges in hamster embryo cells after arsenic exposure (Rossman et al., 1980; Lee et al., 1985, Kochhar et al., 1996). Arsenic also alters DNA repair in human T-cell lymphoma cells by decreasing repair enzyme poly-(ADP-ribose) polymerase at 10 μM concentration, therefore causing decreased cell viability (Yager and Wiencke, 1997). Arsenic is also reported to cause some of the detrimental actions on cells by increasing superoxide anion generation (O2 ) and lipid peroxidation, which correlated with increased apoptosis of human gastric cancer cells (Chen et al., 2002). The reports referenced here and the results of this study clearly show that exposure to arsenic alters endothelial cell physiology. Does that alteration of endothelial cell physiology reach the threshold of pathological endothelial cell dysfunction? Collectively: The cardiovascular pathologies associated with arsenic toxicity in humans, *in vitro* studies exposing cultured endothelial cells to arsenic, and *in vivo* arsenic toxicity experiments such as this one all show that arsenic exposure causes endothelial dysfunction.

Lee et al. demonstrated that arsenic increased the susceptibility of platelets to aggregate, leading to the increased risk of arterial thrombosis, which is a causal factor in the development of cardiovascular disease (Lee et al., 2002). This study and the results presented here strongly suggest that arsenic related endothelial cell dysfunction is directly linked to cardiovascular disease and developmental disorders. The necessary arsenic exposure levels and the nature of the endothelial cells dysfunction are clearly different in a tissue specific manner. For example, arsenic exposure to atherosclerotic mice results in endothelial dysfunction that accelerates atherogenesis (Bunderson et al., 2002). Conversely, this report shows that arsenic exposure to the developing embryo results in
endothelial dysfunction manifest as placental insufficiency that result in miscarriage.

Future studies for this project will focus on endothelial cell dysfunction in the context of development. Particularly how arsenic mediated endothelial dysfunction affects angiogenesis and vasculogenesis, the two fundamental mechanisms for vascular development (Coffin and Poole, 1988).
ACKNOWLEDGMENTS

This work was supported by the University of Montana Center for Environmental Health Sciences under NIH grants: NIEHS # 1R21ES11749 and NCRR # 1P20RR17670.
BIBLIOGRAPHY


