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ARSENATE-INDUCED MATERNAL GLUCOSE INTOLERANCE AND NEURAL TUBE DEFECTS IN A MOUSE MODEL

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Abstract

Background—Epidemiological studies have linked environmental arsenic (As) exposure to increased type 2 diabetes risk. Periconceptional hyperglycemia is a significant risk factor for neural tube defects (NTDs), the second most common structural birth defect. A suspected teratogen, arsenic (As) induces NTDs in laboratory animals.

Objectives—We investigated whether maternal glucose homeostasis disruption was responsible for arsenate-induced NTDs in a well-established dosing regimen used in studies of arsenic's teratogenicity in early neurodevelopment.

Methods—We evaluated maternal intraperitoneal (I.P.) exposure to As 9.6 mg/kg (as sodium arsenate) in LM/Bc/Fnn mice for teratogenicity and disruption of maternal plasma glucose and insulin levels. Selected compounds (insulin pellet, sodium selenate (SS), N-acetyl cysteine (NAC), L-methionine (L-Met), N-tert-Butyl- α -phenylnitron (PBN)) were investigated for their potential to mitigate arsenate's effects.

Results—Arsenate caused significant glucose elevation during an I.P. glucose tolerance test (IPGTT). Insulin levels were not different between arsenate and control dams before (arsenate, 0.55 ng/dl; control, 0.48 ng/dl) or after glucose challenge (arsenate, 1.09 ng/dl; control, 0.81 ng/dl). HOMA-IR index was higher for arsenate (3.9) vs control (2.5) dams ($p=0.0260$). Arsenate caused NTDs (100%, $p<0.0001$). Insulin pellet and NAC were the most successful rescue agents, reducing NTD rates to 45% and 35%.

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Conclusions—IPGTT, insulin assay, and HOMA-IR results suggest a modest failure of glucose stimulated insulin secretion and insulin resistance characteristic of glucose intolerance. Insulin's success in preventing arsenate-induced NTDs provides evidence that these arsenate-induced NTDs are secondary to elevated maternal glucose. The NAC rescue, which did not restore maternal glucose or insulin levels, suggests oxidative disruption plays a role.

Keywords

arsenate; arsenic; glucose intolerance; insulin resistance; mouse; neural tube defect; oxidative stress; teratogen

Type 2 diabetes, characterized by hyperglycemia secondary to a relative lack of insulin, is a group of disorders whose multi-factorial etiology has both environmental and genetic components. While the cornerstone of type 2 diabetes prevention programs has focused on weight reduction and increased physical activity, established to reduce the incidence by up to 58% (Knowler *et al.*, 2002), its etiology remains to be elucidated, and the role of environmental pollutants in diabetogenesis has received little attention from the medical and research communities. While it has been known for nearly 30 years that exposure to a variety of environmental toxicants may contribute to diabetes risk (Franco *et al.*, 1978; Longnecker and Daniels, 2001), arsenic has only recently come under scrutiny for its contributing role.

While it is best known as a carcinogen, the link between arsenic exposure and type 2 diabetes was recognized in 1950 when a patient undergoing I.V. arsphenamine (Salvarsan) treatment was documented as developing type 2 diabetes (Stolzer *et al.*, 1950). Beginning in the 1990s, epidemiological studies in Bangladesh (Rahman *et al.*, 1998), Taiwan (Lai *et al.*, 1994), Mexico (Coronado-Gonzalez *et al.*, 2007) and Sweden (Rahman and Axelson, 1995; Rahman *et al.*, 1996) have consistently demonstrated a dose-response relationship between exposure to arsenic, (total arsenic or the inorganic arsenicals (Asi) arsenite and arsenate) from contaminated drinking water or in the workplace, and an increased risk of type 2 diabetes. Concerns over the relevance of these studies focused on the indirect measures of exposure, the extremely high-dose chronic exposure many individuals may have experienced in Taiwan and Bangladesh, and, to a lesser degree, the malnourishment affecting some of the individuals (Navas-Acien *et al.*, 2006). Most recently, research addressed these concerns and demonstrated a positive association between low-level chronic arsenic exposure via drinking water in the United States, as measured by urinary arsenical excretion, and an up to 3.6-fold increased risk of diabetes (Navas-Acien *et al.*, 2008).

As mentioned earlier, deliberate arsenic exposure alters glucose metabolism in humans, as well as in rodent and *in vitro* laboratory models. In fact, one of the most common side effects of arsenic trioxide treatment for cancer is hyperglycemia (Rust and Soignet, 2001). In rodent and β -cell models, both acute and sub-chronic exposure to arsenite has been shown to cause transient hyperglycemia or type 2 diabetes, respectively (Kawaguchi, 1981; Boquist *et al.*, 1988; Ortsater *et al.*, 2002; Izquierdo-Vega *et al.*, 2006).

Diabetes from environmental arsenic exposure is of interest to our laboratory because pre-gestational diabetes is associated with a two- to ten-fold increase in the risk of having an

NTD-affected pregnancy (McLeod and Ray, 2002). The second most common of all structural birth defects, NTDs affect approximately 2.6/1000 births worldwide, and 1/1000 births in the United States. NTDs represent a class of malformations wherein the embryo's neural tube fails to close, most commonly resulting in exencephaly or anencephaly, in which the majority of the brain and surrounding tissues are absent, or spina bifida, which often results in lower body paralysis and lack of bowel and bladder control (Mitchell, 2005). While a definitive association between arsenic exposure and an increased risk of NTDs in humans has not yet been established (Hill *et al.*, 2008), in laboratory animals, maternally administered inorganic arsenic crossed the placenta to result in embryonic or fetal exposure (Hanlon and Ferm, 1977; Lindgren *et al.*, 1984; Devesa *et al.*, 2006). Embryonic arsenic exposure via maternal intraperitoneal (I.P.) injection or oral exposure leads to a well-described pattern of fetal malformations, primarily characterized by NTDs, in a variety of animal models (Morrissey and Mottet, 1983; Hood *et al.*, 1987; Golub *et al.*, 1998; Wlodarczyk *et al.*, 2001; Paul *et al.*, 2007; Hill *et al.*, 2008). While oral maternal exposure results in an NTD phenotype identical to that caused by I.P. exposure, the penetrance of the phenotype is reduced to a degree that is impractical for use in birth defects research. While there was evidence of a significant positive dose-response relationship between oral maternal arsenate exposure and NTDs (exencephaly), the total percentage of affected embryos per treatment group was modest (water, 0.0%; As 4.8 mg/kg, 0.8%; As 9.6 mg/kg, 3.7%; As 14.4 mg/kg, 8.6%) (Hill *et al.*, 2008).

Maternal blood glucose equilibrates with the embryo, dictating embryonic glucose levels until onset of fetal pancreatic function, well after neurulation is complete. The teratogenicity of maternal hyperglycemia is well established in both human and laboratory animal models (Becerra *et al.*, 1990; Janssen *et al.*, 1996; Aberg *et al.*, 2001). As half of all pregnancies are unplanned, and women are typically past the critical time of neural tube closure when they initially detect a pregnancy, good prepregnancy glycemic control is essential in diabetic patients to reduce the risk of NTDs (Ray *et al.*, 2001; McLeod and Ray, 2002). Studies conducted using animal models provide further evidence that embryonic exposure to elevated glucose levels is mechanistically responsible for the increased risk of congenital malformation. Rodent models demonstrate that maternal hyperglycemia induced by direct injection of glucose or β -cell disruption from streptozotocin treatment results in exencephaly in the offspring, and explanted rodent embryos grown in high glucose media also exhibit a failure of neural tube closure (Loeken, 2005). Recent work shows that gene inactivation of the glucose transporter, *Glut2*, in embryonic mice protects them from maternal hyperglycemia-induced malformations (Li *et al.*, 2007).

In this study, we investigate arsenate-induced disruption of maternal glucose homeostasis and its role in the observed NTDs in a well-established dosing regimen used in studies of arsenic's teratogenicity in early neurodevelopment. We hypothesize that arsenic-induced disruption of maternal glucose homeostasis contributes to its observed induction of NTDs.

MATERIALS AND METHODS

Animals and Housing

The LM/Bc/Fnn inbred mouse strain has been maintained in the Finnell laboratory for several decades due to their low incidence of spontaneous malformations and resistance to most teratogens investigated in our laboratory, though they are especially sensitive to arsenate-induced malformations. The mice were housed in the Institute of Biosciences and Technology Vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (Harlan Teklad Rodent Diet #8606, Ralston Purina, St. Louis MO). The mice were maintained on a 12-h light/dark cycle. Nulligravid females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning, and the onset of gestation was considered to be 10 p.m. of the previous night, the midpoint of the dark cycle. All experiments were performed on gravid dams.

Test Agent and Treatment Regimen

Sodium arsenate (CAS# 10048-95-0, ACS Reagent, 98%) Sigma-Aldrich Chemicals, St. Louis, MO) was dissolved in sterile water (Sterile Water for Injection, USP, Abbott Laboratories Chicago IL) to prepare a 9.6 mg/kg body weight dose of arsenic in the form of sodium arsenate. This dose has been demonstrated to cause exencephaly in nearly all of the exposed offspring in this highly inbred mouse strain, without signs of embryotoxicity (i.e. increased resorption rates or decreased litter size). This enables us to make an unequivocal assessment of the efficacy of the rescue compounds. In addition, this is a commonly used dosing scheme in birth defects research, which facilitates comparison of results between this, and previous studies. Both As and water control treatments were administered by intraperitoneal (I.P.) injection at a dose volume of 10 μ L/g body weight. Treatments were administered on gestational day (E)7.5 and 8.5, immediately preceding, and at the onset of, neurulation, which generally occurs from E8.5–9.5.

I.P. Glucose Tolerance Test (IPGTT) with Insulin Assay

In order to establish the effects of this arsenate treatment model on maternal glucose metabolism, gravid dams were subjected to an IPGTT (n=6 for each group). Furthermore, to determine the relationship between circulating insulin and glucose, tailblood was concurrently collected for evaluation at selected timepoints.

Arsenate- and water-treated dams were fasted for six hours prior to sample collection (beginning at 3:00 PM through 9:00 PM) to allow for the 2-hour evaluation of glucose and insulin levels centered around 10:00 PM (E9.0), midpoint in neural tube closure. A basal blood sample (0 min) was collected by tailsnip, and maternal plasma glucose was evaluated using a glucometer (Bayer Ascencia Elite XL) (Bayer HealthCare, LLC, Tarrytown, NY). Dams were then injected IP with filtered D-glucose (2 mg/kg). Maternal glucose was evaluated again 15, 30, 60, 90, and 120 minutes later. Additional blood was taken at the peak glucose timepoint, 30 min, for evaluation of insulin.

An enzyme-linked immunosorbent assay (ELISA) using antibodies that recognize mouse and rat insulin was used following the manufacturer's protocol (Rat/Mouse Insulin ELISA Kit, LINCO Research, St. Charles, Mo.) to evaluate tailblood samples. The limit of sensitivity for this assay is 0.2 ng/ml insulin, with the appropriate range of the assay 0.2 ng/ml to 10 ng/ml (in a 10 µl sample size).

Ad Libitum Plasma Glucose

In order to confirm that ad libitum plasma glucose levels were different between the arsenate treated and water control dams, blood glucose was determined in a nonfasting state. Gravid dams were treated as described above with arsenate or water control, and ad libitum plasma glucose levels were determined at 8:00 PM, 2 hours after onset of the dark phase, just before E9.0 (n=10 for each group). A blood sample was collected by tailsnip, and maternal plasma glucose was evaluated using a glucometer.

Rescue Studies

Rescue Compounds—Five test compounds were selected to determine their ability to “rescue”, or restore, the normal phenotype of the arsenic-exposed embryos. These compounds are hypothesized to exert their effects through various mechanisms, and are therefore administered via routes and for durations anticipated to maximize their proposed rescue utility. Pregnant arsenic-treated dams were randomly selected to receive one of the rescue compounds. SS, (0.5 mg/kg) (CAS# 13410-01-0, Sigma Aldrich, SigmaUltra, 99%) is thought to exert its effects through possible multiple utilities: as part of selenocysteine, it is the active center of antioxidant selenoenzymes such as glutathione peroxidase, has been demonstrated to have diverse effects on glucose homeostasis, and has been shown *in vivo* to methylate arsenic without the use of a methyltransferase. L-Met (70 mg/kg) (CAS# 63-68-3, Sigma-Aldrich, BioChemika, 99.0%) was selected to provide additional methyl groups for arsenic's biotransformation and help speed its excretion from the body. NAC (200 mg/kg) (CAS# 616-91-1, Sigma-Aldrich, Puriss 99.0%) is an antioxidant that acts as a precursor of glutathione synthesis, a key substrate dismutating H₂O₂ to molecular oxygen and water. Sodium selenate, L-methionine, and N-acetyl cysteine were administered daily from E0.5–10.5 by oral gavage as a standardized solution in sterile water such that they receive 10 µL of fluid per gram of body weight. Each of the previous compounds may provide some antioxidant capacity through various mechanisms, potentially preventing maternal hyperglycemia thought to be due in part to mitochondrial H₂O₂ release in pancreatic βcells resulting in failure of ATP-dependent glucose stimulated insulin secretion (GSIS). The LinBit insulin pellet (LinShin Canada, Toronto, Canada) is implanted subcutaneously and dissolves over a period of weeks, releasing insulin to the dam to lower maternal circulating glucose levels. The LinBit pellet was implanted on E2.5 or 3.5, between onset of gestation and embryonic implantation. PBN (40 mg/kg) (CAS# 3376-24-7, Sigma Aldrich, SigmaUltra, 98%) is a spin-trap, a compound that traps and stabilizes oxidative radicals, preventing deleterious molecular interactions. In addition, the use of PBN would avoid the confounding factor of potential arsenic-thiol binding possible with SS, L-met, and NAC. PBN (Sigma-Aldrich) was injected I.P. on E7.5 and 8.5 as a standardized solution in sterile water such that the dams receive 10 µL of fluid per gram of body weight. The two

compounds most successful in rescuing the normal phenotype were characterized in further studies described below.

Fasting Plasma Glucose (FPG)—Fasting maternal plasma glucose was evaluated in tailsnip blood. Gravid dams were fasted for six hours prior to sample collection (beginning at 4:00 PM through 10:00 PM sample collection time) to allow for the evaluation of FPG levels at E:9.0, midpoint in neural tube closure. Experimental groups consisted of control, arsenate, LinBit+arsenate and NAC+arsenate treated dams.

Insulin ELISA—The amount of secreted insulin was measured to determine whether the most successful rescues modified maternal insulin levels. Tailblood samples (collected from gravid, fasting dams as described above for Fasting Blood Glucose) were evaluated for experimental groups consisted of control, arsenate, LinBit+arsenate, and NAC+arsenate treated dams using the Linco Kit described above.

Observations and Measurements

For the IPGTT with insulin assay, blood glucose and insulin values were collected. For all other studies (rescue studies), reproductive outcome was determined, and glucose and insulin values were reported when measured. To assess reproductive outcome, gravid dams were killed on E18.5, and litters were assessed by counting the number of implants, resorptions/dead, affected, and unaffected fetuses. A detailed gross external examination of each viable fetus was conducted.

Statistical Methods

Because the distribution of glucose and insulin in gravid mice is unknown, and could not be adequately assessed based on our study data (due to the relatively small number of treated mice), nonparametric tests were used exclusively. Maternal glucose levels and body weights were evaluated using a 2-sided Mann-Whitney. Maternal insulin levels were evaluated using Mann-Whitney for unpaired comparisons, and the Wilcoxon paired test for paired comparisons. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated for each dam using maternal glucose and insulin values obtained during the performance of the IPGTT using the following formula: $\text{fasting glucose (mg/dl)} \times \text{fasting insulin } (\mu\text{U/ml}) / 405$ and values for the two groups compared using a Mann-Whitney.

For all rescue studies, Fisher's exact test was used to evaluate differences between treatment groups for litter outcomes (resorption/dead, NTD). The Kruskal-Wallis test was used to evaluate the differences, between treatment groups, in the distribution of the number of implantations. If $p < 0.05$, a Dunn's post-test was performed. FPG and insulin levels were evaluating using 2-sided t-test.

All statistical analyses were conducted using GraphPad InStat (version 3.06; GraphPad Software, San Diego, CA, USA), and the results of all tests were considered to be statistical significant when the p-value (or adjusted p-value) was less than 0.05.

RESULTS

IPGTT and Insulin Assay

The plasma glucose (see Figure 1) was significantly higher in the arsenate treated dams when compared to water treated controls at the onset of the IPGTT (0 min, arsenate, 100 mg/dl; control, 72 mg/dl) ($p=0.0152$), and was significantly higher at all timepoints (15 min, arsenate, 293 mg/dl; control, 188 mg/dl; $p=0.0022$)(30 min, arsenate, 303 mg/dl; control, 211 mg/dl; $p=0.0087$)(60 min, arsenate, 208 mg/dl; control, 173 mg/dl; $p=0.0043$)(90 min, arsenate, 171 mg/dl; control, 145 mg/dl; $p=0.0249$)(120 min, arsenate, 144 mg/dl; control, 122 mg/dl; $p=0.0260$). Maternal weights were also compared due to their known effect on glucose homeostasis; there was no significant difference between the weights of the arsenate treated and water control groups (arsenate, 27.6 g; control, 26.3 g) ($p>0.05$).

The insulin levels (see Figure 2) were not significantly different between the arsenate and water treated dams at the initiation of the IPGTT (arsenate, 0.55 ng/dl; control, 0.48 ng/dl) ($p=0.0938$). At the time of highest circulating glucose, 30 minutes after the glucose challenge, while the insulin levels were slightly higher in the arsenate treated dams than controls, this difference did not reach statistical significance (arsenate, 1.09 ng/dl; control, 0.81 ng/dl) ($p=0.1563$). Importantly, in the arsenate treated dams, the circulating insulin did increase in response to the glucose challenge ($p=0.0313$), similar to the increase in insulin seen the water treated controls ($p=0.0313$).

The HOMA-IR index calculated for the arsenate treated dams (see Table 1) was significantly higher when compared to controls (arsenate, 3.9; control, 2.5) ($p=0.0260$), suggesting modest insulin resistance in the arsenate treated dams.

Ad Libitum Plasma Glucose

The ad libitum plasma glucose (see Figure 3) was significantly higher in the arsenate treated dams when compared to water treated controls (arsenate, 138 mg/dl; control, 113 mg/dl) ($p=0.0002$). Maternal weights were also compared due to their known effect on glucose homeostasis; there was no significant difference between the weights of the arsenate treated and water control groups (arsenate, 32.1 g; control, 30.8 g) ($p>0.05$).

Rescue Studies

Comparison of Experimental Treatments—The highly inbred LM/Bc mouse strain is known to have a very low rate of spontaneous NTDs, and no NTD-affected fetuses were observed in the absence of treatment with arsenate in these studies. Specifically, all test procedures (i.e. LinBit pellet placement, fasting, and additional maternal handling associated with blood collection for insulin testing) were evaluated in conjunction with a water control to determine whether they influenced the outcomes of interest (see Table 2 for all results). The LinBit pellet, which is placed subcutaneously to provide exogenous insulin, was also evaluated, because historically it had been suggested that insulin treatment was responsible for the increased risk of malformations observed in the offspring of diabetic women.

Water treated dams implanted with LinBit displayed no increase in NTDs or resorption (Group A3) in comparison to water treatment alone (Group A2), ($p>0.05$ for both comparisons). Further, fasted water-treated dams tested for FPG (Group C9) displayed no increases in NTDs or resorption when compared to water-treated dams that were not fasted (Group A2), indicating that the six hour fast did not disrupt embryonic neural tube closure ($p>0.05$ for all comparisons). Similarly, water treated dams fasted for blood collection for insulin for testing (Group D14) displayed no increases in NTDs or resorption when compared to water-treated dams that were not fasted (Group A2) ($p>0.05$ for all comparisons)

Arsenate Treatment—When treated with the arsenate regimen utilized in this study, nearly all of the exposed fetuses presented with NTDs. Arsenate treated dams had significantly more NTD-affected fetuses (Group A1, ~100%) than the water-treated controls (Group A2, 0%) ($p<0.0001$). However, the rates of resorptions were not significantly different in arsenate-treated dams when compared with water-treated dams ($p>0.05$).

Rescue of Arsenate-Induced NTDs—All compounds used to rescue the normal phenotype of embryos exposed to arsenate were successful. As previously described, arsenate treatment (Group A1) exhibited a ~100% rate of exencephaly. In the groups described above, PBN+As (Group B7) reduced the NTD rate to 77% ($p<0.0001$); SS+As (Group B8) reduced the NTD rate to 71% ($p<0.0001$); L-Met+As (Group B6) reduced the NTD rate to 71% ($p<0.0001$); LinBit+As (Group B5) reduced the NTD rate to 45% ($p<0.0001$) and NAC+As (Group B4) reduced the NTD rate to 35% ($p<0.0001$). With the one exception, the rescue treatments did not significantly altered the rate of resorptions in comparison to arsenate. The single exception, PBN, was associated with a significantly higher resorption rate of 28% ($p<0.0001$) as compared to arsenate (Group A1). As the NAC and LinBit treatments were the most effective, they were selected for further investigation into maternal FPG and insulin levels.

Maternal FPG and Associated Pregnancy Outcome—FPG testing was conducted in dams treated with water (Group C9), arsenate (Group C10), NAC+As (Group C12) and LinBit+As (Group C13) following a six hour fast. In all groups, tailblood was tested at the midpoint of neural tube closure. With one exception, fasted dams displayed no differences for NTD or resorption rates when compared to the similarly-treated dams that were not fasted (i.e. arsenate alone vs. fasting, (A1 vs. C10); water alone vs. fasting, (A2 vs. C9); LinBit alone vs. fasting, (A3 vs. C11); and NAC+As vs. fasting, (B4 vs. C12)). LinBit+As +fasted dams had a significantly higher rate of resorptions (Group C13, 19%) when compared to the LinBit+As dams that were not fasted (Group B5, 7%) ($p<0.0001$). It is notable that fasting did not diminish the effects of the NAC or LinBit on the rates of arsenate-induced NTDs (Table 1, Group C).

Consistent with the changes seen in the IPGTT, the mean FPG level of dams treated with arsenate (C10, 117 mg/dl) was significantly higher than dams treated with water (Group C9, 85 mg/dl) (two-tailed t-test, $p=0.0048$). Dams receiving LinBit in addition to arsenate (Group C13, 82 mg/dl) had mean FPG levels similar to dams receiving water controls (Group C9, FPG 85 mg/dl) ($p>0.05$). In contrast, in NAC treated dams (Group C12) FPG

levels (106 mg/dl) remained high, and were significantly different from water treated dams ($p=0.0065$). The LinBit+water (Group C11) was evaluated to confirm that LinBit treatment alone was sufficient to lower FPG in water-treated dams. LinBit+water treatment (Group C11) successfully lowered the FPG (60 mg/dl) below that associated with water treatment ($p=0.0019$). As indicated earlier, the low FPG associated with this treatment was not associated with an increase in NTD or resorption rates in comparison to water alone.

Maternal Insulin—Insulin testing was conducted in water+FPG+insulin (Group D14), arsenate+FPG+insulin (Group D15), NAC+As+FPG+insulin (Group D16) and LinBit+As+FPG+insulin (Group D17). Dams underwent a six hour fast in order to evaluate maternal blood glucose levels midpoint in neural tube closure, as well as additional handling to collect blood for an ELISA. Dams in these groups displayed no differences in NTD or resorption rates when compared with the similarly treated dams that were not fasted or tested for insulin levels, indicating that the additional handling and blood collection was neither teratogenic nor embryotoxic ($p>0.05$ for all comparisons).

All FPG levels associated with this insulin testing were similar to FPG levels in dams not tested for insulin, though they were slightly higher overall, possibly reflecting the stress of additional handling. Most importantly, arsenate treatment (Group D15) continued to be associated with a significantly higher FPG (124 mg/dl) in comparison to water treatment (Group D14) (FPG 98 mg/dl), and consistent with the values seen at the onset of the IPGTT. However, there was no difference between the insulin levels in these same groups, indicating that elevated levels of circulating glucose caused by arsenate failed to stimulate compensatory insulin secretion ($p>0.05$). Similarly, the FPG (122 mg/dl) associated with the NAC treatment (Group D16) was significantly higher than the water treatment (Group D14), with no difference in insulin levels. The LinBit treatment alone (Group D17) significantly lowered the FPG (59 mg/dl), and the exogenous insulin, as expected, elevated the insulin levels in comparison to water treatment (Group14).

DISCUSSION

While all selected rescue compounds served to restore the normal embryonic phenotype, insulin and NAC were the most effective. In particular, this study provides evidence that arsenate exposure causes glucose intolerance in this model, and suggests that some of the observed NTDs are secondary to this disrupted glucose homeostasis. Normally administered therapeutic doses of insulin are not observed to cross the placenta (Widness *et al.*, 1983), further supporting the conjecture that elevated maternal glucose may play a significant role in these NTDs. As demonstrated in the IPGTT, at the same maternal insulin levels, the plasma glucose is higher in the arsenate treated dams, indicating a degree of insulin resistance. However, the failure of additional compensatory insulin to be secreted in response to the higher maternal glucose in these arsenate treated dams indicates some degree of blunting of GSIS. A modest failure of GSIS and relative insulin deficiency are the hallmarks of impaired glucose tolerance, which is considered a transitional state from normoglycemia to frank diabetes type 2. The HOMA-IR index, which takes into account fasting insulin and blood glucose levels, was significantly higher in the arsenate treated dams, also indicating a degree of insulin resistance. The elevated FPG, blunted GSIS, and

modest resistance to insulin action observed during the IPGTT were consistently similarly altered throughout this study.

The normal FPG near 80 mg/dl observed in this strain is typical of many laboratory mouse strains, which display wide variability in normal FPG levels from 60mg/dl through a high of 145 mg/dl for the ICR strain, for example. It is difficult to compare the modest, but highly consistent, elevations observed in this model to conventional mouse diabetes models with dramatically elevated glucose levels frequently exceeding 300, and even 500, mg/dl. In our model, arsenate treatment resulted in average increase of 32 mg/dl (37%) in circulating glucose. The American Diabetes Association defines normal FPG in humans as below 100 mg/dl, 'impaired fasting glucose' as an FPG of 100 mg/dl to 125 mg/dl, with diabetic state beginning at 126 mg/dl (2007b). While the risks of periconceptional diabetic hyperglycemia are well established, median first-trimester FPG above 120 mg/dl (impaired fasting glucose) has been suggested by at least one study to confer an increased risk of abortion and major malformations (Rosenn *et al.*, 1994).

The oxidative stress component known to accompany both maternal diabetes and arsenic exposure may underlie the success of the NAC, PBN, and potentially SS, and L-Met, rescue agents in this study, though NAC was unable to rescue the maternal hyperglycemia thought to be potentially due in part to mitochondrial H₂O₂ release in pancreatic β cells resulting in failure of ATP-dependent GSIS (Nulton-Persson and Szweda, 2001; Pi *et al.*, 2007). NAC restored the normal embryonic phenotype without restoring normal maternal insulin or circulating glucose levels. NAC, which stimulates glutathione synthesis, has been shown to cross the placenta and to increase the fetal liver glutathione levels when given to pregnant women (Buhimschi *et al.*, 2003) and mice (Izzotti *et al.*, 2003). Neither PBN, SS, nor L-Met were selected for further investigation into maternal FPG and insulin levels due to their relatively low success rate. Nonetheless, the redox disruptive component of arsenate-induced NTDs is shown by the success of maternal administration of PBN, a radical trapping agent which inactivates toxic radical species, which was highly effective as rescue agent. However, it also significantly increased the number of resorptions and therefore was not further investigated. The success of SS and L-met may be due to diverse mechanisms, only a few of which are suggested here. SS may affect glucose homeostasis, and has been shown *in vivo* to methylate arsenic without the use of a methyltransferase. L-Met may provide additional methyl groups for arsenic's biotransformation and help speed its excretion from the body, as well as provide an antioxidant utility by lowering elevated maternal homocysteine levels due to arsenate treatment.

As expected, no single rescue agent was successful in restoring the normal phenotype to all arsenate-exposed fetuses. While we suggest that some of the NTDs observed in this model are due to elevated maternal glucose levels, arsenic is a complex toxicant whose teratogenicity may be mediated through several pathways. Our laboratory has previously performed a microarray study on anterior neural tube tissue collected from embryos exposed to arsenate at the onset of neurulation. While the results demonstrated that arsenate induced mitochondrial disruption and oxidative stress, this study also revealed many genes whose altered pattern of expression has previously been shown to induce birth defects in mouse

models. These genes included engrailed 1 (En-1), platelet derived growth factor receptor alpha (Pdgfra) and ephrinA7 (EphA7) (Wlodarczyk et al., 2006).

Diabetes has been described as a disease of reactive oxidative stress, which plays a role in both its etiology, as well as associated adverse health sequelae. Consistent with this observation, a growing body of evidence further suggests that maternal hyperglycemia induces teratogenic oxidative stress in the exposed embryo (Sakamaki *et al.*, 1999; Viana *et al.*, 2000; Cederberg *et al.*, 2001; Loeken, 2006). During neural tube closure, the concurrent onset of embryonic oxidative phosphorylation and cardiac function is a highly coordinated event involving maturation of the quiescent, delamellated, mitochondrial cristae and a related increased dependence on oxidative phosphorylation and decreased glucose utilization (Shepard *et al.*, 1997). It is suggested that excess glucose during this time may result in accumulation of associated excess toxic radical spillage from mitochondrial oxidative phosphorylation in an embryo still lacking competent vascular perfusion (Shepard *et al.*, 1998).

This link between oxidative stress and teratogenicity is not limited to diabetic disruption of neurulation, however. Disrupted oxidative radical homeostasis and oxidative signaling has been implicated in the teratogenic mechanism of several toxicants, including phenytoin and benzo[a]pyrene (Winn and Wells, 1997; Kasapinovic *et al.*, 2004), thalidomide (Parman *et al.*, 1999), and is proposed as general mechanism of teratogenicity associated with many toxicants (Wells *et al.*, 1997; Hansen, 2006). Arsenic is well known for its induction of oxidative stress, which is used both as a marker of exposure, as well as a therapeutic characteristic exploited during its use as the anticancer agent Trisenox.

Elucidation of the mechanisms that underlie the diabetogenic effects of arsenicals is an area of intense investigation. While epidemiological studies have been limited in their characterization of the arsenic species responsible for the observed increase in diabetes risk, both the trivalent and pentavalent forms, arsenite and arsenate, are found in drinking water (Magalhaes, 2002). While we have selected arsenate for our test agent because arsenic occurs in drinking water primarily as arsenate (2004), most studies use arsenite as their test agent. While administered arsenate converts to arsenite, and trivalent and pentavalent forms interconvert several times before excretion, the various arsenicals display both discrete, and overlapping, toxicities. The relationship between these two inorganic species and diabetes risk is not known.

Exposures to environmental toxicants are generally less conclusively associated with an increase diabetes and NTD risk. The first environmental contaminant to be associated with an increased diabetes risk was found in the 1970s, when carbon disulfide exposure's relationship to diabetes was reported (Franco *et al.*, 1978; 2007a). Interest in other environmental contaminants and their potential to increase diabetes risk is relatively recent, and has focused on arsenic, dioxin, and nitrates (Kostraba *et al.*, 1992; Rahman *et al.*, 1996; Rahman *et al.*, 1998; Longnecker and Michalek, 2000). For a comprehensive review of this subject, see work by Longnecker and Daniels (Longnecker and Daniels, 2001). Data concerning arsenic and TCDD were most suggestive of an association with diabetes, while nitrates, nitrites, and N-nitroso compounds had a weaker, but not null, association.

The experimental design limits the conclusions that may be drawn from this study. As the goal of this study was to determine whether a well-established arsenate dosing regimen was inducing NTDs via maternal hyperglycemia, a single dose of arsenate was explored. Further investigations should employ a dose-response design. Furthermore, future studies should be mindful of the strengthening case for arsenic as a risk factor for diabetes in humans, making oral exposure more interesting as an experimental design in spite of the inherent limitations already described. Use of genetically engineered knockout mice, such as the embryonic Glut2 $-/-$ model already described, may prove valuable in determining the role of embryonic glucose exposure in the observed NTDs. Finally, while maternally-administered insulin has not been demonstrated to reach the conceptus, it is unknown whether this is altered by maternal administration of arsenate.

In summary, we suggest that arsenate has direct effects on both the maternal and embryonic glucose levels that ultimately prove teratogenic in our model. Maternally administered arsenate causes teratogenic maternal glucose intolerance, as well as crossing the placenta to affect the embryo directly. The effectiveness of insulin, which does not cross the placenta, and the antioxidant NAC, which does, in protecting *in utero* arsenate-exposed embryos from NTDs suggests that elevated maternal blood glucose and embryonic oxidative stress are large contributors to the observed NTDs. We propose that the embryo is thus exposed to two separate, but related, oxidative insults: oxidative stress due to the maternally-controlled glycemic environment, as well as direct embryonic cellular damage due to arsenical exposure, which is well known for its mitochondriotoxicity and induction of H_2O_2 . As we continue to dissect the multifactorial causes of these complex diseases, diabetes and NTDs, exposures to environmental toxicants warrant further investigation, both for their relevance to human health, as well as their mechanisms of action.

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Abbreviations

As	arsenic
Asi	inorganic arsenic
FPG	fasting plasma glucose
GSIS	glucose stimulated insulin secretion
IP	intraperitoneal
L-Met	L-methionine
NAC	N-acetyl cysteine
NTD	neural tube defect
PBN	N-tert-Butyl- α -phenylnitrone

SS sodium selenate

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Figure 1.

Changes in maternal plasma glucose in arsenate and water treated dams induced by an I.P. glucose tolerance test. * $p < 0.05$, ** $p < 0.005$ when compared to control dams. Error bars indicate standard error.

Figure 2.

Maternal insulin values (ng/ml) for arsenate and water treated dams, both before (0 min) and 30 minutes after (30 min) glucose challenge during I.P. glucose tolerance test. *p<0.05 when compared to initial insulin values. Error bars indicate standard error.

Figure 3.

Maternal ad libitum plasma glucose values (mg/dl) for arsenate and water treated dams. ** $p < 0.005$ when compared to water treated dams. Error bars indicate standard error.

Table 1

HOMA-IR calculated from data collected at the onset of IPGTT.

Treatment Groups	FPG (mg/dl)	Fasting Insulin (μ U/ml)	HOMA-IR
Water Treated Controls (n=6)	71 $\pm$ 2.1	13.9 $\pm$ 0.4	2.5 $\pm$ 0.1
Arsenate Treated (n=6)	100 $\pm$ 3.8*	15.7 $\pm$ 0.5*	3.9 $\pm$ 0.2*

*
p<0.05 when compared to water treated dams, values are shown as mean $\pm$ SE.

Table 2

Summary of litter and maternal outcomes following treatment.

Group	Administered Compounds	No. of Dams	Implants per Litter Mean $\pm$ SE	No. of Resorptions (%)	No. of Fetuses with NTDs (%)	Maternal FPG (mg/dl) Mean $\pm$ SE	Maternal Insulin level (ng/ml)
A) Controls	1 Arsenate	10	8.3 $\pm$ 0.56	6(7.2)●	77(100.0)○	-	-
	2 Water	10	10.3 $\pm$ 0.37	3(2.9)	0(0.0)	-	-
	3 LinBit	9	9.8 $\pm$ 0.42	7(8.4)●	0(0.0)●	-	-
B) Rescue	4 NAC + As	11	9.7 $\pm$ 0.66	5(4.7)■	39(38.2)□	-	-
	5 LinBit + As	12	9.8 $\pm$ 0.42	8(6.8)■	51(46.4)□	-	-
	6 L-Met +As	10	8.2 $\pm$ 0.65	4 (4.9)■	53(67.9)□	-	-
	7 PBN +As	9	9.1 $\pm$ 0.7	23(28.0)□	43(74.1)□	-	-
	8 SS +As	11	8.9 $\pm$ 0.61	4(4.1)■	72(76.6)□	-	-
C) FPG	9 Water	9	9.2 $\pm$ 0.32	3(4.8)●	0(0.0)●	85 $\pm$ 6.3	-
	10 Arsenate	11	10.8 $\pm$ 0.63	2(2.2)■	91(100.0)■	117 $\pm$ 7.3 [†]	-
	11 LinBit	10	9.4 $\pm$ 0.27	3(3.2)□	0(0.0)□	60 $\pm$ 3.0 [†]	-
	12 NAC + As	13	8.3 $\pm$ 0.58	9(8.3)▲	40(40.4)▲	106 $\pm$ 3.8 [†]	-
	13 LinBit + As	11	7.7 $\pm$ 0.79	16(18.8)	29(42.0)▲	82 $\pm$ 9.8 [‡]	-
D) Insulin	14 Water	8	8.5 $\pm$ 0.68	0(0.0)● [‡]	0(0.0)● [‡]	98 $\pm$ 5.1	0.445 $\pm$ 0.043
	15 Arsenate	8	9.1 $\pm$ 0.72	10(13.7) [§]	63(100.0) [§]	124 $\pm$ 8.7	0.525 $\pm$ 0.064 [◆]
	16 NAC + As	9	7.9 $\pm$ 0.79	7 (9.9) ^{§§}	32(45.1) ^{§§}	122 $\pm$ 7.4	0.355 $\pm$ 0.028 [◆]
	17 LinBit +As	8	8.1 $\pm$ 0.77	9(13.8) ^{§§§}	28(43.1) ^{§§§}	59.1 $\pm$ 6.0	0.83 $\pm$ 0.075

□ p < 0.0001 compared to A1

■ p 0.05 compared to A1

○ p < 0.0001 compared to A2

● p 0.05 compared to A2

$\square$ p 0.05 compared to A3
 $\dagger$ p < 0.01 compared to C9
 $\ddagger$ p 0.05 compared to C9
 $\S$ p < 0.05 compared to D14
 $\P$ p 0.05 compared to D14
 $\blacktriangleleft$ p < 0.0001 compared to B5
 $\blacktriangleright$ p 0.05 compared to B5
 $\S$ p 0.05 compared to B4
 $\S$ p 0.05 compared to C10
 $\S\S$ p 0.05 compared to C12
 $\S\S\S$ p 0.05 compared to C13