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# Impact of Life Stage and Duration of Exposure on Arsenic-Induced Proliferative Lesions and Neoplasia in C3H Mice

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## Abstract

Epidemiological studies suggest that chronic exposure to inorganic arsenic is associated with cancer of the skin, urinary bladder and lung as well as the kidney and liver. Previous experimental studies have demonstrated increased incidence of liver, lung, ovary, and uterine tumors in mice exposed to 85 ppm (~8 mg/kg) inorganic arsenic during gestation. To further characterize age susceptibility to arsenic carcinogenesis we administered 85 ppm inorganic arsenic in drinking water to C3H mice during gestation, prior to pubescence and post-pubescence to compare proliferative lesion and tumor outcomes over a one-year exposure period. Inorganic arsenic significantly increased the incidence of hyperplasia in urinary bladder (48%) and oviduct (36%) in female mice exposed prior to pubescence (beginning on postnatal day 21 and extending through one year) compared to control mice (19 and 5%, respectively). Arsenic also increased the incidence of hyperplasia in urinary bladder (28%) of female mice continuously exposed to arsenic (beginning on gestation day 8 and extending though one year) compared to gestation only exposed mice (0%). In contrast, inorganic arsenic significantly decreased the incidence of tumors in liver (0%) and adrenal glands (0%) of male mice continuously exposed from gestation through one year, as compared to levels in control (30 and 65%, respectively) and gestation only (33 and 55%, respectively) exposed mice. Together, these results suggest that continuous inorganic arsenic exposure at 85 ppm from gestation through one year increases the incidence and severity of urogenital proliferative lesions in female mice and decreases the incidence of liver and adrenal

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tumors in male mice. The paradoxical nature of these effects may be related to altered lipid metabolism, the effective dose in each target organ, and/or the shorter one-year observational period.

**Keywords**

arsenic; carcinogenesis; life-stage; age susceptibility; urinary bladder; C3H mice

## Introduction

Significant drinking water exposure to inorganic arsenic may occur at any age in many human populations and has been associated with various cancers of the skin, lung, urinary bladder, liver and kidney (Bates et al., 1992; IARC 2004; NRC 1999; Pott et al., 2001; Kitchin 2001; Simeonova and Luster, 2000). Because the latency period of arsenic-related carcinogenesis in humans is considered to be in the range of 25-45 years, new epidemiological data on arsenic-induced cancers are currently becoming available from countries such as Taiwan, Chile, Argentina, and the USA, where human consumption of contaminated water has occurred for more than 50 years (Bates et al., 1995; Chiou et al., 1995; Hopenhayn-Rich et al., 1998; Ferreccio et al., 2000; Haque et al., 2003). Recent epidemiological studies in Chile suggest that arsenic exposure early in life predisposes adults to increased risks of lung and urinary bladder cancer (Marshall et al., 2007). Both *in utero* and early childhood exposures to arsenic in drinking water resulted in increased mortalities from lung cancer and bronchiectasis in an adult Chilean population (Smith et al., 2006). Consistent with these observations, both epidemiological and experimental data also demonstrate that arsenic crosses the placental barrier and that significant fetal exposure occurs when mothers are exposed to arsenic (Devesa et al., 2006; Hall et al., 2007; Fry et al., 2007).

Previous experimental studies in C3H and CD-1 mice have demonstrated increased tumor incidence after *in utero* exposure to inorganic arsenic between gestation days 8 and 18 (Waalkes et al., 2004a and 2006). Increased liver, lung, adrenal, ovarian and uterine tumors were found in adult mice two years after the 11-day gestational exposure. In contrast, induction of tumors in adult animals after sodium arsenite exposure has rarely been reported. The refractory nature of adult animals to arsenic-induced tumors may reflect specific aspects of arsenic metabolism in mature rodents (Hughes et al., 2005; Cohen et al., 2006). Most animal studies demonstrating carcinogenesis by inorganic arsenic or its metabolites have included the co-administration of another carcinogen or promoter in an adult animal model (Morikawa et al., 2000; Germolec et al., 1997; Rossman *et al.*, 2004; Mizoi *et al.*, 2005; Motiwale *et al.*, 2005; Uddin *et al.*, 2005). Induction of tumors by arsenic after gestational exposure may be mediated by an estrogenic mode of action based on estrogen receptor-α positive tumors and an increase in estrogen specific metabolizing enzyme transcripts (Waalkes et al., 2004b). Because other chemicals with estrogenic properties induce tumors in adult mice following neonatal exposure (Newbold et al., 1990), it was postulated that inorganic arsenic might also prove tumorigenic at other life stages, including the neonatal and prepubescent life stage.

To further characterize age susceptibility to arsenic carcinogenesis we have administered inorganic arsenic to C3H mice during gestation, prior to pubescence and post pubescence to compare proliferative lesion and tumor outcomes. This is the first investigation of the tumorigenic effects of inorganic arsenic at these different life stages using an experimental mouse model. A one-year exposure period was used in this study based on the high



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background rate of spontaneous hepatocellular tumors and mortality observed in this mouse strain after one year of age.

# Methods

## Chemicals

Sodium arsenite (99.9 % purity) was purchased from Sigma Aldrich (St. Louis, MO) and dissolved in tap water purified through a triple-carbon filtering device. Prior to filtering, there were no detectable levels of arsenicals in the water. Analytical grade chemicals were exclusively used throughout the study, including NaOH (EM Science, Gibbstown, NJ) and HCl (Fisher Chemicals, Fair Lawn, NJ).

## In Vivo Studies

Animal experiments were carried out at the United States Environmental Protection Agency, Research Triangle Park, NC in an AAALAC, International accredited facility, with all procedures involving the care and use of animals approved by the Institutional Animal Care and Use Committee. Timed-pregnant C3H dams were purchased from Charles River Laboratories (Raleigh, NC). Mice were housed in polycarbonate cages (one dam per cage) on Alpha Dry bedding with a 12 hour light/dark cycle. Room temperature was 70 +/- 2° F with a relative humidity of 50%. The basal diet was Ralston Purina 5001 (Ralston Purina Co., St. Louis, MO) with total arsenic levels listed as less than 0.22 ppm, of which over 90% of arsenicals present were in the form of arsenobetaine and arsenocholine. Water, with or without arsenic added, was provided ad libitum.

Preliminary testing regarding water consumption variables were evaluated for 12 weeks prior to the actual initiation of the trial. Deionized, distilled or carbon-purified water with and without sodium arsenite (85 ppm) was adjusted with 1M HCl or 1M NaOH to multiple pH values ranging from 9.2 to 3.0. Neither the water source nor pH affected water consumption in the dams from GD-8 through GD-19. However, F1 offspring showed significantly reduced water consumption in the deionized and distilled water at all pH ranges, with greater differences at neutral and higher pH values. Water consumption in mice administered sodium arsenite in carbon-purified water at pH 3.8-3.3 was slightly more depressed than their control counterparts, but these differences were not significant. After 60 days no differences in body weights were observed. As a result, carbon-purified water adjusted to pH 3.3 was considered to have fewer potentially confounding effects, and was chosen as the vehicle for administration of the control and sodium arsenite dosed water.

Timed-pregnant dams were allowed to acclimate for five days before sodium arsenite (85 ppm, ~8 mg/kg) was administered in the drinking water. Dosing solutions were made fresh and changed weekly. Pregnant dams and male and female offspring were randomly divided into 5 treatment groups (housed as littermates) : a water only control, *in utero* arsenic exposure only [gestational day (GD)-8 to GD-19], *in utero* through one year continuous arsenic exposure [GD-8 to postnatal day (PD)-365], pre-pubescence through one year chronic arsenic exposure [PD-21 to PD-365], and post-pubescence through one year chronic arsenic exposure [PD-56 to PD-365] (Figure 1). After 11 days of drinking water containing arsenic, up to five mice in each treatment group were euthanized with $CO_2$ asphyxiation; blood was collected via the right ventricle of the heart and placed in serum tubes. Tissue samples were harvested, flash frozen in liquid nitrogen and stored at -80°C and/or fixed for subsequent analysis. Remaining animals were euthanized at day 365 (Figure 1).



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## In-Life Data and Pathology

Individual animal weights were recorded weekly for the first 12 weeks and bimonthly thereafter. Water and feed consumptions of the dams and offspring were recorded bimonthly and 5 days prior to sacrifice. Animals were checked daily for signs of morbidity, and, if found, were further evaluated by the facility veterinarian.

A complete necropsy was performed on all animals following euthanasia. Tissues were taken and processed by standard techniques for histological analysis that included: adrenals, gonads (ovaries or testes), liver, lung, kidneys, urinary bladder, oviduct, pancreas, skin, uterus, and any grossly abnormal tissue. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological analysis. Pathological assessments were performed blind. Notably, the source of C3H mice for this study (Charles River Laboratories, Raleigh, NC) differed from the source used by Waalkes et al. (National Cancer Institute, Frederick, MD) in their studies of the transplacental carcinogenesis of inorganic arsenic.

## Immunohistochemistry

After the one year study period, liver sections from control and *in utero* only arsenic-treated mice were used to evaluate the immunohistochemical localization of estrogen receptor (ER-alpha). The sections were microwaved after deparaffinization for 10 min in citrate buffer. The immunohistochemical detection was performed with polyclonal rabbit anti-ER-alpha antibody (Santa Cruz Biotechnical, Santa Cruz, CA). The concentration of ER-alpha antibody used was 1:1500 (dilution). Reactions were visualized with an avidin-biotin-peroxidase Kit (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as a chromagen. To demonstrate the specificity of immunostaining, primary antibodies were omitted from each staining series.

## Total Arsenic Analysis

Freshly dissected liver samples were aliquoted into 2.0 ml cryotubes, snap frozen in liquid nitrogen and stored at -70°C until analyzed. Speciated arsenicals in liver tissue were determined by hydride generation- cryotrapping- gas chromatography-atomic absorption spectrometry (HG-CT-GC-AAS). Tissues were digested overnight in 2 M ultrapure phosphoric acid (90°C) (Hughes et al., 2005). The digestion converts all trivalent arsenicals to pentavalency. Arsines were generated at pH 1 in a reaction tube containing 1% antifoam B silicone emulsion (1 ml), 1M HCl (1 ml), deionized water (7 ml) and aliquot sample of the digested tissue (0.2 or 2 ml). Arsines were cryotrapped and separated by gas chromatography, as previously described (Devesa et al., 2005). The content of As in arsines was determined using a Perkin-Elmer model 5100 atomic absorption spectrometer equipped with a quartz multiatomizer. Under these conditions, this method routinely resolves arsines generated from iAs ($iAs^{III}$ + $iAs^{V}$), methylarsenic ($MAs^{III}$ + $MAs^{V}$), dimethylarsenic ($DMAs^{III}$ + $DMAs^{V}$), and trimethylarsenic ($TMAs^{III}$ + $TMAs^{V}O$) species. Five concentrations (0.25, 0.5, 1, 2 and 5 ng/ml) of each of these arsenicals were used to prepare calibration curves. Arsenicals in tissue samples were identified by spiking samples with appropriate As standards at several concentrations. The concentration of total speciated arsenic for each tissue sample was calculated as the sum of concentrations of iAs, MAs, DMAs, and TMAs.

## Clinical Chemistry Analysis

Clinical chemistry analyses were carried out using the Konelab 30 Analyzer (Thermo Electron Corp., Espoo, Finland). Serum creatinine (Kit #N221-30) and sorbitol dehydrogenase (Kit #N740-25) were measured using kits from Diagnostics Chemicals Ltd.

(Oxford, Conn). Alanine and aspartate aminotransferase (Kit #TR7001), blood urea nitrogen (Kit #TR1201), glucose (Kit #TR1501), lactate dehydrogenase (Kit #TR2001), total protein (Kit #TR34021), triglycerides (Kit #TR2221), total cholesterol (Kit #TR1351), high density lipoproteins (Kit #TR39601), and low density lipoproteins (Kit #TR53202), kits were obtained from Thermo Electron Corporation (Melbourne, Australia).

### Statistics

Treatment-related differences in food and water consumption, body and organ weights, and clinical chemistry parameters were determined using the student t-test ($p<0.05$). Significant differences in proliferative lesion and tumor incidence were determined using the one-sided Fisher exact test ($p<0.05$).

## Results

### Food and Water Consumption

Maternal water and food consumptions were not altered by the addition of arsenic in the drinking water. Control dams consumed 8.85 ± 0.36 ml daily per mouse (n = 6) compared to 8.52 ± 0.27 ml daily per mouse given 85 ppm (~8 mg/kg) sodium arsenite. Food consumption in the F1 offspring was not significantly reduced in male or female mice exposed to arsenic at any time throughout the 52-week exposure period. Water consumption was significantly reduced (40%) in *in utero*-exposed F1 male and female mice upon further exposure to sodium arsenite during acclimation to drinking water in the first week after weaning (PD32). By two months of age (PD67), and thereafter, there were no treatment-related differences from control in water consumption of male or female F1 offspring.

### Body Weight

Maternal body weight was not affected by arsenic exposure. At sacrifice, control dams weighed 42.5 ± 1.3 g, and treated dams weighed 41.7 ± 1.8 g. Although transplacental exposure to arsenic appeared to slightly depress body weights for offspring, reductions were not significant. Control male offspring taken from control dams at the GD19 sacrifice weighed 1.22 ± 0.013 g, while treated male offspring taken from treated dams weighed 1.14 ± 0.07 g. Control female offspring weighed 1.12 ± 0.06 g compared to treated female offspring that weighed 1.08 ± 0.09 g.

Male F1 body weights were significantly lower 11 days after weaning (PD32) in both the *in utero* continuously exposed (16.9 ± 2.02 g) and PD21+ treatment groups (20.2 ± 1.17 g) compared to controls (22.1 ± 0.54 g, n=5). Female F1 body weights 11 days after weaning (PD32) were significantly higher in the *in utero* only treatment group (19.2 ± 0.72) and significantly lower in the *in utero* continuously exposed group (15.6 ± 0.94) as compared to controls (17.4 ± 1.15, n=5).

Trends in reduced body weight gain continued for F1 male and female offspring exposed to arsenic chronically into adulthood and persisted until approximately six months of age (Figure 2). After six months of age, weight gain, although small, was similar among all treatment groups. Because the animals exposed to arsenic continued to gain weight at all time points and appeared healthy the dose was not lowered during the course of the study.

### Relative Organ Weights (% body weight)

There were no treatment-related differences in relative liver weights in male and female fetuses exposed to arsenic *in utero* (GD19). A small treatment-related increase in relative kidney weight (14%) was observed in male mice exposed continuously to arsenic for one year. No other treatment-related changes in relative organ weights were observed in male



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mice. Relative liver, lung, kidney and adrenal weights were statistically increased in female mice after the one-year exposure period in the continuous, PD21+ and PD56+ treatment groups. (Table 1). Relative lung and kidney weights were also increased 10-15% by arsenic in female mice sacrificed at postnatal day 67 (data not shown).

## Clinical Pathology

Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were significantly reduced (25-35%) in male mice following the one-year arsenic exposure period (Figure 3). There were no treatment-related increases in serum liver enzymes or changes in kidney function parameters associated with arsenic exposure in male mice. In contrast, serum blood urea nitrogen was significantly increased (25-30%) and creatinine decreased (20-25%) in female mice following the one-year arsenic exposure period. Serum alanine aminotransferase (ALT) levels were also elevated (20-35%) in female mice exposed to arsenic for one year. Cholesterol and triglyceride parameters were not measured in female mice due to an insufficient quantity of serum.

## Arsenic Levels

Total arsenic levels in male mouse livers were determined at postnatal day 32 and 67 interim sacrifices and at the end of the one-year exposure period (Figure 4). Hepatic levels from control and *in utero* only exposed mice ranged from 27-30 ng/gram liver while the levels in the chronically exposed mice (continuous, PD21+, and PD56+ exposure groups) ranged from 600-850 ng/gram liver at the postnatal day 32 and 67 interim sacrifices. Regardless of age, arsenic levels appeared to reach steady state after 11 days of exposure. The highest levels of hepatic arsenic, 1500-1800 ng/gram liver, were observed after the one-year exposure period in mice chronically exposed to arsenic in the PD21+ and PD56+ exposure groups. Arsenic levels in the chronically exposed continuous group (765 ng/gram liver) at one year were similar to levels observed at the interim sacrifices.

## Histopathology

The incidences of proliferative lesions and neoplasia in male and female mice after the one-year exposure period are presented in Table 2. Approximately 30% of control and *in utero* only exposed male mice developed liver tumors, while less than 2% of male mice chronically exposed to arsenic (continuous, PD21+, and PD56+ exposure groups) developed liver tumors ($p<0.002$). Although the incidence of carcinoma was observed to be much higher in the *in utero* only exposed animals compared to control animals (33% vs 5%), this finding was not statistically significant. Estrogen receptor-α (ER- α) was up-regulated in the liver of *in utero* only exposed male mice, as evidenced by widespread and intense nuclear staining, but not in the liver of control male mice (Figure 5).

In male mice a similar trend in reduced tumor incidence was observed for adrenal adenomas. Approximately 60% of control and *in utero* only exposed animals developed adrenal adenomas after one year while less than 2% of male mice chronically exposed to arsenic (continuous, PD21+, and PD56+ exposure groups) developed adrenal adenomas ($p<0.000001$).

A trend toward an increased incidence of urinary bladder hyperplasia was observed in male and female mice chronically exposed to arsenic (continuous, PD21+, and PD56+ exposure groups) compared to control mice (group 1). However, a statistical increase in urinary bladder hyperplasia was only observed in PD21+ female mice compared to their respective controls ($p<0.05$). Also when combining male and female data, a statistical increase was observed overall when comparing 8/41 control mice (20%) that developed urinary bladder hyperplasia with 41/117 chronically exposed mice (35%) that developed urinary bladder



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hyperplasia ($p<0.05$). One male mouse in the *in utero* only group developed severe urinary bladder hyperplasia.

In female mice, the incidence of mild uterine hyperplasia was observed in all arsenic treatment groups but was not statistically significant from control incidence. Moderate to severe uterine hyperplasia was only observed in mice chronically exposed to arsenic in the continuous and PD21+ treatment groups. A statistical increase in oviduct hyperplasia was observed in mice chronically exposed to arsenic in the PD21+ treatment group as compared to control mice ($p<0.02$). One female mouse in the continuous exposed group developed an ovarian tumor.

## Discussion

Several recent reviews have evaluated potential modes of action by which arsenic could act as a carcinogen (Kitchin, 2001; Rossman, 2003; Kligerman and Tennant, 2006). The goal of the present study was to evaluate the effect of life-stage on the incidence of arsenic-induced proliferative lesions and neoplasia in C3H mice. There is experimental evidence that early-life exposures (i.e. transplacental, neonatal, and pre-pubescent) to carcinogens with a mutagenic or an estrogenic mode of action may increase the incidence and multiplicity of tumor incidence compared to adult only exposures (Newbold et al., 1990 and Rice, 1976). Previous transplacental exposure studies have demonstrated an increase in arsenic-induced proliferative lesions and neoplasia in C3H and CD-1 mice (Waalkes, et al., 2003 and 2006). To further examine the impact of age of exposure on arsenic-induced tumorigenesis we report the comparative results of long, chronic arsenic exposure beginning at *in utero*, pre-pubescent and post-pubescent ages in C3H mice.

Similar to a previous report (Waalkes, et al., 2006), we found that arsenic increased the incidence of urogenital proliferative lesions in female mice exposed *in utero*. However, in our one-year exposure study the increased incidence was only observed in mice that received arsenic chronically following the *in utero* or pre-pubescent exposure. The lack of a statistical increase in the *in utero* only exposed group may, in part, be due to the reduced one-year observation period. A one-year exposure period was used in this study because of the high background rate of hepatocellular tumors and mortality observed in this mouse strain after one year of age. Nevertheless, chronic arsenic exposure increased the incidence of urogenital proliferative lesions (urinary bladder, uterine and oviduct hyperplasia) as compared to the control group and the *in utero* only treatment group (p  0.01 and $p<0.001$, respectively). This suggests that long term exposure to arsenic that includes *in utero* or pre-pubescent exposures can increase the risk for urogenital effects. This is also consistent with a previous study (Waalkes et al., 2006) in which arsenic administered *in utero*, and followed by neonatal exposure to the synthetic estrogen diethylstilbestrol (DES), resulted in increased urogenital proliferative lesions and neoplasia. Both our study and the Waalkes et al. 2006 study demonstrated more than double the incidence in urinary bladder lesions with subsequent arsenic or DES exposures after the initial *in utero* arsenic exposure. The increased incidence of urinary bladder hyperplasia reported here is also consistent with a previous epidemiology study done in Chile where human populations exposed to arsenic early in life through adulthood had a greater urinary bladder cancer mortality rate (Marshall et al., 2007) than those exposed as adults only.

Unlike previous reports with *in utero* arsenic exposure alone (Waalkes et al., 2003, 2004a and 2004b) the incidence of liver and adrenal adenomas was dramatically reduced in mice continuously exposed to arsenic following an *in utero* exposure. This apparent inhibition of tumor development was unexpected and may have been associated with altered fatty acid/cholesterol levels, reduced weight gain, and/or apoptosis of preneoplastic liver cells. Male

mice kept on arsenic after the *in utero* exposure weighed 20-25% less than control mice beginning on postnatal day 32 through postnatal day 365. However, food consumption was not significantly different between these groups during the course of the one-year study. Our companion study (in preparation) investigating hepatic gene expression patterns in these mice demonstrated a marked reduction in stearoyl-CoA desaturase-1 (*Scd1*) mRNA in mice continuously exposed to arsenic for one year compared to controls and the *in utero* only treatment group 2. *Scd1* is the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids and its down regulation is associated with reduced adiposity in mice (Ntambi et al., 2002). In addition to reduced weight gain, lower mRNA transcript levels of this gene are also associated with a reduction in spontaneous liver tumor formation (Ntambi et al., 2002 and Falvella et al., 2002). Serum cholesterol levels also influence hepatic *Scd* mRNA expression in mice (Ntambi 1999). Total cholesterol levels were lowered in male mice by more that 35% in our study and may account, in part, for the reduction in *Scd* mRNA and concomitant reduction in weight gain and lack of tumor response in male mice.

Increased apoptosis may also be involved in decreased liver tumor development observed in male mice chronically exposed to arsenic. In a previous mouse study (Liu et al., 2006), sodium arsenite at doses at or exceeding 1 mg/kg significantly reduced hepatocellular carcinoma cell growth after twenty days of i.p. treatment. However, doses below 1 mg/kg increased liver tumor growth. It is possible that a one year exposure to a lower concentration of arsenic (i.e. 5-10 ppm) in our study may not have caused such a dramatic decrease in liver tumor incidence, and perhaps may have caused an increase in liver tumor incidence in C3H mice. When apoptosis was assessed in these mouse tumors immunohistochemically, a dose-dependent increase in terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) was observed at doses of 1 mg/kg and higher, suggestive of an increased apoptotic frequency. Because doses used in our study were of a similar magnitude (~8 mg/kg) with an even longer duration compared to this previous report, a similar mechanism of increased apoptosis may be involved in reduction in liver tumor incidence observed in our study. Furthermore, our companion study (in preparation) investigating hepatic gene expression patterns in these mice demonstrated significant differences in apoptotic transcript levels in chronically exposed mice vs *in utero* only exposed or control mice. In summary, these one-year observational studies describe the impact of early life-stage and prolonged arsenic exposure on arsenic-induced proliferative lesions and neoplasia in mice. Continuous exposure to inorganic arsenic, extending from gestation through one year, increases the incidence and severity of urogenital proliferative lesions in female mice and decreases the incidence of liver and adrenal tumors in male mice. The paradoxical nature of these effects may be related to altered lipid metabolism, the effective dose in each target organ and/or the shorter one-year observational period for tumor incidence. Although drinking water exposures to inorganic arsenic may enable high levels of inorganic arsenic to reach the liver, higher levels of organic forms like dimethylarsinic acid (DMA(V)) may be important in urinary bladder toxicity and hyperplasia. Previous rat studies by multiple laboratories have demonstrated that dietary or drinking water administration of DMA(V) causes urinary bladder tumors following long term exposure (Wei et al., 1999 and 2002, Arnold et al., 2006). In contrast, high levels of sodium arsenite in the mouse liver may facilitate increased apoptosis of preneoplastic hepatocytes. Additional laboratory studies will need to be performed to substantiate these conclusions.

These studies provide additional evidence of differential age susceptibility to arsenic carcinogenesis. The duration of arsenic exposure was the major contributor to the differential tumor induction observed in male and female mice. Based on these findings, dose and length of exposure can significantly alter the incidence and severity of proliferative lesions and neoplasia following brief early-life arsenic exposures.



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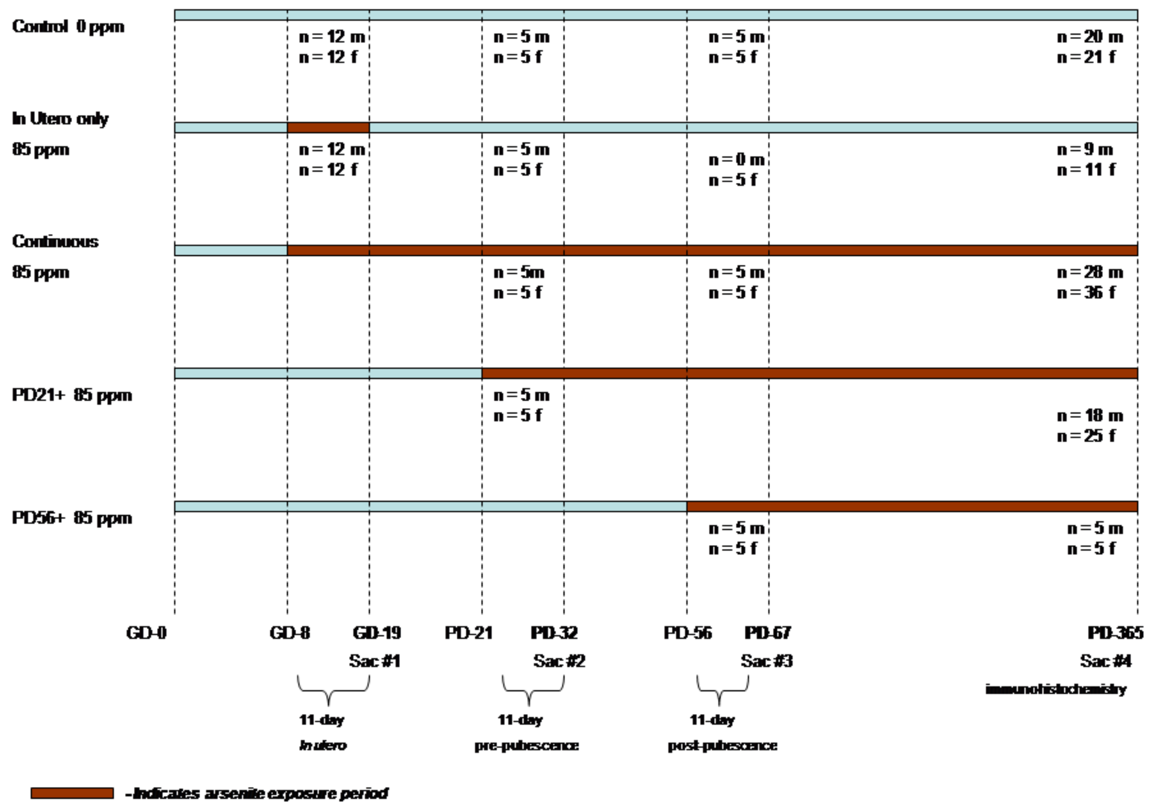


**Figure 1.**
Life-stage arsenic study design.

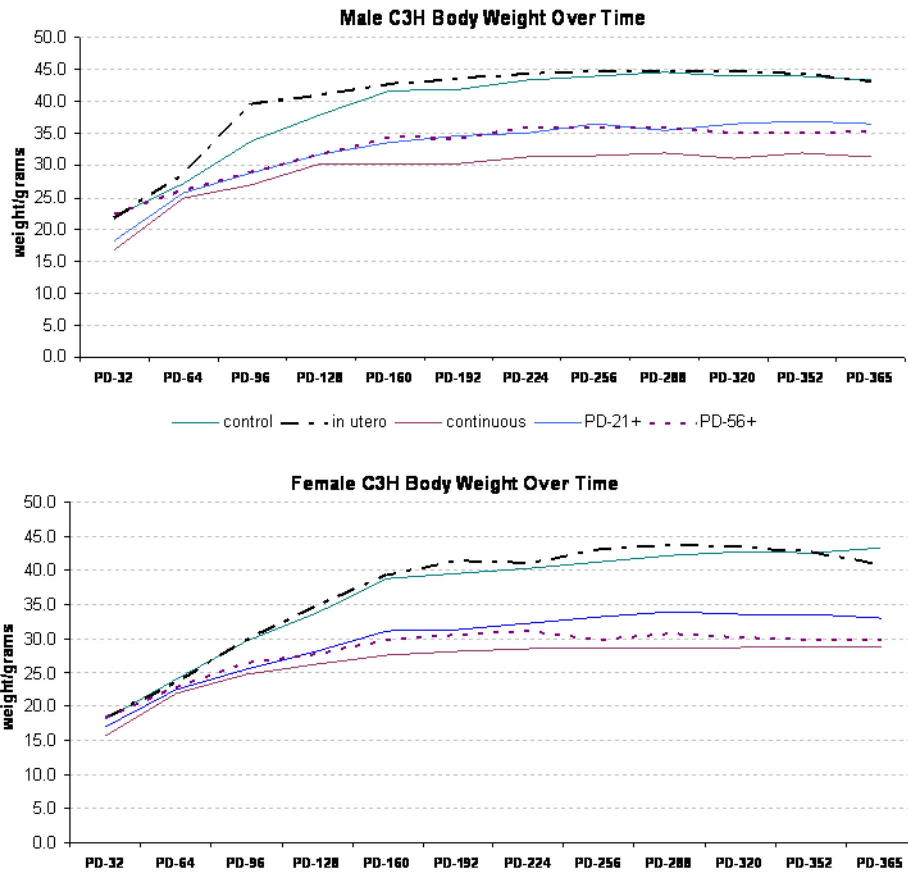


**Figure 2.**

Mean male (a) and female (b) body weights of each treatment group over the 52-week exposure period.



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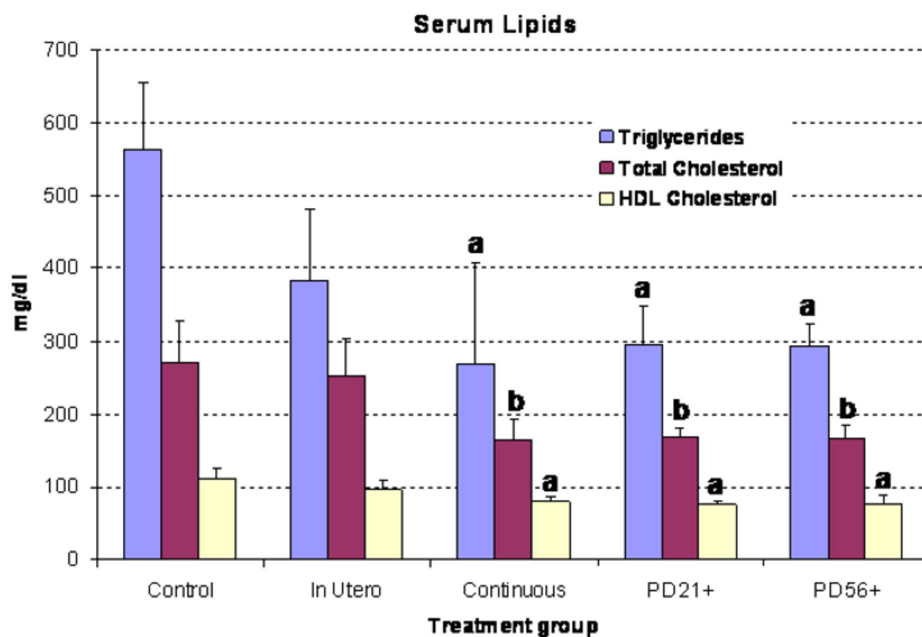


a – significantly different ($p<0.05$) from control group
b – significantly different ($p<0.05$) from both control and in utero groups

**Figure 3.**
Serum triglyceride, total cholesterol, and HDL cholesterol levels in male C3H mice (n = 3 to 6 mice per treatment group). Values are mean ± standard deviation.

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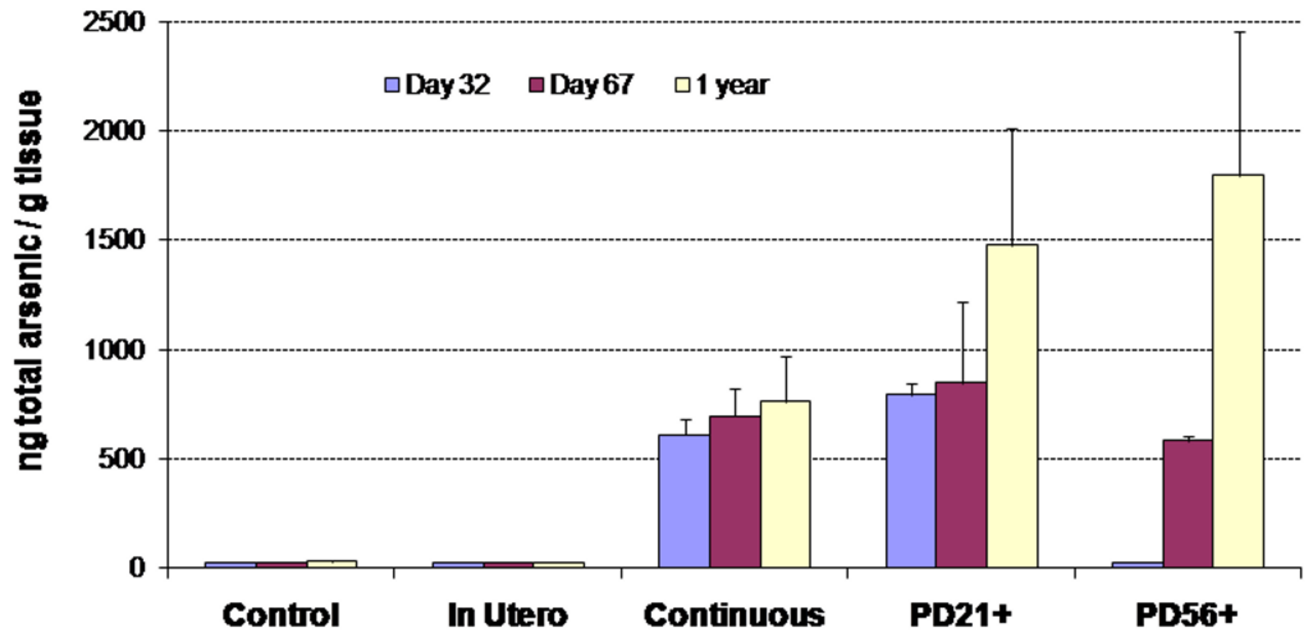


**Figure 4.**
Total levels of arsenic in the liver of C3H mice at 32, 67 days and one year. Values are mean ± standard deviation.

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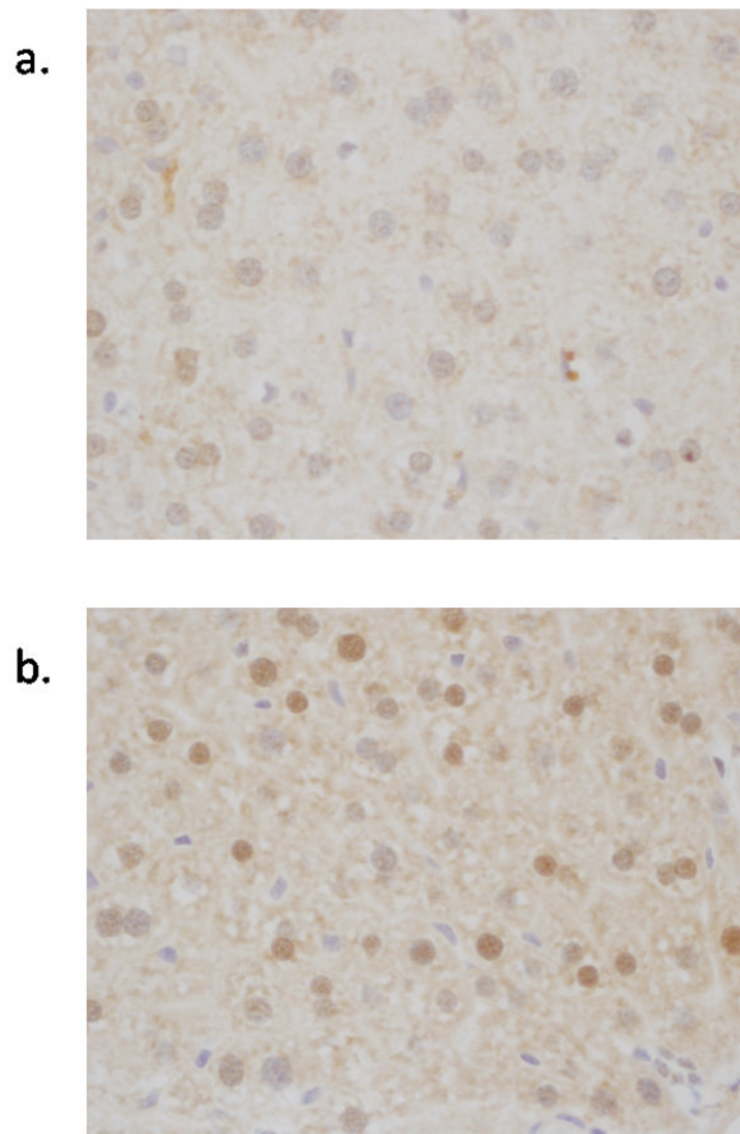


**Figure 5.**
Estrogen receptor-alpha (ER-α) staining of mouse liver from control (a) and *in utero* only exposed (b) male mouse.

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**Table 1**

**Final body and organ weights in male (a) and female (b) mice after the 52-week exposure period**

**a. Male**

| | Control | In Utero | Continuous | PD21+ | PD56+ |
|---|---|---|---|---|---|
| **No of animals** | **22** | **9** | **28** | **19** | **5** |
| **Body wt (g)** | 40.8 ± 7.6 | 43.1 ± 3.8 | 31.4 ± 2.9 * | 36.5 ± 3.0 | 35.3 ± 2.5 |
| **Liver** | | | | | |
| Weight (g) | 2.23 ± 0.43 | 2.44 ± 0.48 | 1.70 ± 0.14 | 1.90 ± 0.15 | 1.88 ± 0.13 |
| % Body weight | 5.55 ± 1.12 | 5.68 ± 1.13 | 5.41 ± 0.34 | 5.21 ± 0.35 | 5.33 ± 0.36 |
| **Kidney** | | | | | |
| Weight (g) | 0.41 ± 0.07 | 0.42 ± 0.06 | 0.36 ± 0.04 | 0.39 ± 0.04 | 0.38 ± 0.03 |
| % Body weight | 1.00 ± 0.19 | 0.96 ± 0.11 | 1.14 ± 0.12 * | 1.06 ± 0.13 | 1.07 ± 0.05 |
| **Adrenals** | | | | | |
| Weight (g) | 0.019 ± 0.014 | 0.014 ± 0.006 | 0.013 ± 0.011 | 0.019 ± 0.021 | 0.013 ± 0.003 |
| % Body weight | 0.046 ± .032 | 0.032 ± 0.013 | 0.040 ± 0.035 | 0.053 ± 0.061 | 0.036 ± 0.010 |
| **Testes** | | | | | |
| Weight (g) | 0.07 ± 0.02 | 0.08 ± 0.03 | 0.07 ± 0.01 | 0.07 ± 0.02 | 0.07 ± 0.01 |
| % Body weight | 0.18 ± 0.05 | 0.19 ± 0.06 | 0.21 ± .04 | 0.20 ± .06 | 0.21 ± .03 |
| **Lung** | | | | | |
| Weight (g) | 0.22 ± 0.05 | 0.22 ± 0.04 | 0.19 ± 0.01 | 0.19 ± 0.04 | 0.21 ± 0.03 |
| % Body weight | 0.54 ± 0.15 | 0.51 ± 0.09 | 0.60 ± 0.06 | 0.53 ± 0.10 | 0.59 ± 0.07 |

**b. Female**

| | Control | In Utero | Continuous | PD21+ | PD56+ |
|---|---|---|---|---|---|
| **No of animals** | **21** | **11** | **36** | **25** | **5** |
| **Body wt (g)** | 43.3 ± 4.3 | 40.9 ± 9.4 | 28.6 ± 2.4 * | 32.9 ± 4.3 * | 29.8 ± 2.6 * |
| **Liver** | | | | | |
| Weight (g) | 1.88 ± 0.25 | 1.94 ± 0.29 | 1.54 ± 0.13 | 1.68 ± 0.13 | 1.72 ± 0.25 |
| % Body weight | 4.34 ± 0.46 | 4.92 ± 1.19 | 5.40 ± 0.44 * | 5.18 ± 0.74 * | 5.78 ± 0.54 * |
| **Kidney** | | | | | |

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**a. Male**

| | Control | In Utero | Continuous | PD21+ | PD56+ |
|---|---|---|---|---|---|
| **No of animals** | **22** | **9** | **28** | **19** | **5** |
| Weight (g) | 0.24 ± 0.03 | 0.24 ± 0.02 | 0.22 ± 0.02 | 0.24 ± 0.02 | 0.25 ± 0.04 |
| % Body weight | 0.56 ± 0.04 | 0.62 ± 0.15 | 0.78 ± 0.07* | 0.73 ± 0.12* | 0.85 ± 0.11* |
| **Adrenals** | | | | | |
| Weight (g) | 0.013 ± 0.007 | 0.015 ± 0.008 | 0.012 ± 0.006 | 0.016 ± 0.009 | 0.011 ± 0.007 |
| % Body weight | 0.029 ± .015 | 0.038 ± 0.022 | 0.041 ± 0.022* | 0.049 ± 0.028* | 0.038 ± 0.022 |
| **Ovaries** | | | | | |
| Weight (g) | 0.023 ± 0.008 | 0.020 ± 0.008 | 0.027 ± 0.016 | 0.019 ± 0.010 | 0.015 ± 0.009 |
| % Body weight | 0.052 ± 0.014 | 0.051 ± 0.014 | 0.093 ± .026 | 0.061 ± .036 | 0.052 ± .028 |
| **Uterus (dry)** | | | | | |
| Weight (g) | 0.254 ± 0.109 | 0.230 ± 0.083 | 0.207 ± 0.079 | 0.181 ± 0.067 | 0.186 ± 0.030 |
| % Body weight | 0.598 ± 0.278 | 0.604 ± 0.307 | 0.713 ± 0.297 | 0.568 ± 0.264 | 0.626 ± 0.110 |
| **Lung** | | | | | |
| Weight (g) | 0.20 ± 0.02 | 0.21 ± 0.02 | 0.19 ± 0.02 | 0.20 ± 0.02 | 0.21 ± 0.02 |
| % Body weight | 0.46 ± 0.05 | 0.54 ± 0.13 | 0.66 ± 0.08* | 0.61 ± 0.10* | 0.69 ± 0.03* |

* denotes statistical difference ($p<0.05$) from control animals by the student t-test.

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**Table 2**

**Proliferative lesions and tumors in male (a) and female (b) mice following the 52 week exposure period**

**a. Male**

**Liver Adenomas and Carcinomas**

| Group | N | Incidence (%) | Adenoma | Carcinoma |
|---|---|---|---|---|
| control | 20 | 6 (30) | 6[(a)] | 1 |
| in utero | 9 | 3 (33) | 1 | 3[(b)] |
| continuous | 28 | 0 (0)* | 0 | 0 |
| PD21+ | 18 | 1 (6) | 1 | 0 |
| PD56+ | 5 | 0 (0) | 0 | 0 |

**Urinary Bladder Hyperplasia**

| Group | N | Incidence (%) | Mild | Moderate | Severe |
|---|---|---|---|---|---|
| control | 20 | 4 (20) | 3 | 1 | 0 |
| in utero | 9 | 3 (33) | 2 | 0 | 1 |
| continuous | 28 | 11 (39) | 9 | 2 | 0 |
| PD21+ | 18 | 4 (22) | 4 | 0 | 0 |
| PD56+ | 5 | 1 (20) | 0 | 1 | 0 |

**Adrenal Hyperplasia and Adenomas**

| Group | N | Incidence (%) | Hyperplasia | Adenoma |
|---|---|---|---|---|
| control | 20 | 13 (65) | 2 | 11 |
| in utero | 9 | 5 (55) | 1 | 4 |
| continuous | 28 | 0 (0)* | 0 | 0 |
| PD21+ | 18 | 1 (6)* | 0 | 1 |
| PD56+ | 5 | 0 (0)* | 0 | 0 |

**b. Female**

**Liver Adenomas**

| Group | N | Incidence (%) | Adenoma |
|---|---|---|---|
| control | 21 | 1 (5) | 1 |
| in utero | 11 | 1 (9) | 1 |
| continuous | 36 | 0 (0) | 0 |
| PD21+ | 25 | 0 (0) | 0 |

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| a. Male | | | | | |
|---|---|---|---|---|---|
| **Liver Adenomas and Carcinomas** | | | | | |
| **Group** | **N** | **Incidence (%)** | **Adenoma** | **Carcinoma** | |
| PD56+ | 5 | 0 (0) | 0 | | |
| **Urinary Bladder Hyperplasia** | | | | | |
| **Group** | **N** | **Incidence (%)** | **Mild** | **Moderate** | **Severe** |
| control | 21 | 4 (19) | 3 | 1 | 0 |
| in utero | 11 | 0 (0) | 0 | 0 | 0 |
| continuous | 36 | 10 (28) | 9 | 1 | 0 |
| PD21+ | 25 | 12 (48)* | 9 | 3 | 0 |
| PD56+ | 5 | 3 (60) | 2 | 1 | 0 |
| **Uterine Hyperplasia** | | | | | |
| **Group** | **N** | **Incidence (%)** | **Mild** | **Moderate** | **Severe** |
| control | 21 | 5 (24) | 5 | 0 | 0 |
| in utero | 11 | 1 (9) | 1 | 0 | 0 |
| continuous | 36 | 11 (31) | 6 | 3 | 2 |
| PD21+ | 25 | 8 (32) | 7 | 1 | 0 |
| PD56+ | 5 | 3 (60) | 3 | 0 | 0 |
| **Oviduct Mild Hyperplasia** | | | | | |
| **Group** | **N** | **Incidence (%)** | | | |
| control | 21 | 1 (5) | | | |
| in utero | 11 | 1 (9) | | | |
| continuous | 36 | 5 (14) | 1 ovarian tumor | | |
| PD21+ | 25 | 9 (36)* | | | |
| PD56+ | 5 | 2 (40) | | | |

(a) three mice had multiple tumors, two mice with two adenomas and one mouse with an adenoma and carcinoma.

(b) one mouse had an adenoma and carcinoma

* denotes statistical difference ($p<0.05$) by one sided Fisher exact test.