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Severe systemic toxicity and urinary bladder cytotoxicity and regenerative hyperplasia induced by arsenite in arsenic (+3 oxidation state) methyltransferase knockout mice. A preliminary report

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ABSTRACT

Arsenic (+3 oxidation state) methyltransferase (*As3mt*) catalyzes reactions which convert inorganic arsenic to methylated metabolites. This study determined whether the As3mt null genotype in the mouse modifies cytotoxic and proliferative effects seen in urinary bladders of wild type mice after exposure to inorganic arsenic. Female wild type C57BL/6 mice and *As3mt* KO mice were divided into 3 groups each ($n=8$) with free access to a diet containing 0, 100 or 150 ppm of arsenic as arsenite (As^{III}). During the first week of As^{III} exposure, *As3mt* KO mice exhibited severe and lethal systemic toxicity. At termination, urinary bladders of both *As3mt* KO and wild type mice showed hyperplasia by light microscopy. As expected, arsenic-containing granules were found in the superficial urothelial layer of wild type mice. In *As3mt* KO mice these granules were present in all layers of the bladder epithelium and were more abundant and larger than in wild type mice. Scanning electron microscopy of the bladder urothelium of *As3mt* KO mice treated with 100 ppm As^{III} showed extensive superficial necrosis and hyperplastic changes. In *As3mt* KO mice, livers showed severe acute inflammatory changes and spleen size and lymphoid areas were decreased compared with wild type mice. Thus, diminished arsenic methylation in *As3mt* KO mice exacerbates systemic toxicity and the effects of As^{III} on the bladder epithelium, showing that altered kinetic and dynamic behavior of arsenic can affect its toxicity.

Introduction

Inorganic arsenic is a known human carcinogen affecting urinary bladder, skin, and lung, with principle exposure from drinking water (National Research Council Subcommittee on Arsenic in Drinking Water, 1999, 2001). Inorganic arsenic exists mainly in the environment as trivalent arsenite (iAs^{III}) or pentavalent arsenate (iAs^{V}) (National Research Council Subcommittee on Arsenic in Drinking Water, 1999, 2001). Ingested inorganic arsenic undergoes reactions in which it is alternately reduced from pentavalency to trivalency, and trivalent arsenicals are oxidatively methylated to yield mono-, di-, and tri- methylated metabolites (Thomas et al., 2007). Although this methylation pathway has long been regarded as a detoxification process (Vahter, 1983), some intermediates formed during the methylation process (e.g.,. monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III})) are highly reactive and more cytotoxic *in vitro* than iAs^{III} (Petrick et al., 2000; Styblo et al., 2000; Cohen et al., 2002). Hence, the methylation pathway can also be described as an activation process that may be responsible for some of the toxic and carcinogenic effects commonly associated with exposure to inorganic As (Le et al., 2000; Thomas et al., 2001). In addition, metabolism of arsenic also involves conversion of methylated oxy-arsenicals to corresponding methylated thio-arsenicals. For example, dimethylmonothioarsinic acid ($DMMTA^{V}$) has been detected in urine of humans exposed to high levels of inorganic arsenic in the drinking water (Raml et al., 2007) and in urine of arsenite (As^{III})-treated rats in a study in our laboratory (Suzuki et al., 2010). Methylated thio-arsenicals that contain pentavalent arsenic may be more toxic than the corresponding methylated oxy-arsenicals that contain pentavalent arsenic. In our laboratory, *in vitro* studies found the LC_{50} for $DMMTA^{V}$ in MYP3 rat urothelial cells to be 1.34 μM which is closer to the toxicity of trivalent non-thiolated arsenicals

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such as arsenite (0.75 μM) and DMA^{III} (0.38 μM) than to pentavalent non-thiolated arsenicals such as monomethylarsonic acid (3.0 mM) and dimethylarsinic acid (0.66 mM). The unanticipated toxicity of methylated thio-arsenicals that contain pentavalent arsenic could depend on facile transport across cell membranes or on ease of conversion to methylated oxy-arsenicals that contain trivalent arsenic (Suzuki et al., 2010). Taken together, it is clear that the conversion of inorganic arsenic to methylated oxy-arsenicals and corresponding methylated thio-arsenicals is likely to be a critical determinant of toxic and carcinogenic effects associated with exposure to inorganic arsenic.

In short-term studies (1–10 weeks), we have recently found that treatment of mice with high concentrations of arsenic administered as sodium arsenite ($NaAs^{III}$) in the diet induced hyperplasia in the bladder epithelium (Lin et al., 2007; Suzuki et al., 2008b). We hypothesized that reactive methylated arsenicals produced during metabolism of inorganic arsenic bind to sulfhydryl groups of critical cellular proteins in the urinary bladder epithelium, leading to urothelial cytotoxicity and regenerative proliferation and, eventually, to tumors. Because the cytosolic enzyme, arsenic (+3 oxidation state) methyltransferase (*As3mt*) catalyzes reactions involved in the reduction and oxidative methylation of arsenicals (Thomas et al., 2007; 2010; Hester et al., 2009), it was of interest to determine the effects of inorganic arsenic exposure in a mouse with a null genotype for *As3mt*. Initial studies in an *As3mt* knockout (*As3mt* KO) mouse found that inhibition of arsenic methylation resulted in altered tissue distribution and clearance of arsenic (Drobna et al., 2009). In the current study, we examined the effect of iAs^{III} treatment on the urinary bladder epithelium in *As3mt* KO mice and in wild type mice in which the capacity of enzymatically catalyzed methylation of arsenic was intact. The *As3mt* null genotype was found to potentiate the effects of inorganic arsenic exposure on the urinary bladder epithelium, suggesting that the formation of methylated metabolites of inorganic arsenic was not required for cytotoxicity in this target tissue.

Materials and methods

Chemicals. $NaAs^{III}$ ($AsNaO_2$, anhydrous) was purchased from Sigma (St. Louis, MO) and was used as received without additional analysis. The purity stated by Sigma was 99%. $NaAs^{III}$ was stored desiccated at room temperature.

As3mt KO mice. Two male and four female mice homozygous for the disrupted *As3mt* gene (Drobna et al., 2009) were obtained from Dr. David Thomas (U.S. EPA, Research Triangle Park, NC). The homozygous *As3mt* KO mice were developed by deletion of exons 3 through 5 of the *As3mt* gene after homologous recombination. The altered gene was introduced and maintained in strain 129S6 mice and then introduced into strain C57BL/6 mice by marker-assisted accelerated backcrossing performed by Charles River Laboratories (Wilmington, MA) to produce homozygous $As3mt^{-/-}$ mice. The *As3mt* KO mice are fertile so brother/sister matings were used to maintain the homozygous *As3mt* knockouts.

Upon arrival, animals were placed in quarantine and housed on aspen wood shavings in polycarbonate cages with micro-isolator tops in a HEPA filtered, flexible film isolator in a quarantine facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) at the University of Nebraska Medical Center. The isolator was targeted at a temperature of 22 °C and relative humidity of 50% and on a 12 h light/dark schedule (0600/1800 CST). Mice were provided with diet (irradiated Teklad 2018S with Fenbendazole, Harlan Teklad, Madison WI) and hyperchlorinated (<10 ppm chlorine) reverse osmosis (RO) water *ad libitum*. After release from quarantine, mice were moved to the transgenic area of an AAALAC-accredited facility with the targeted environmental conditions listed above. Mice were provided autoclaved, pelleted Teklad 7012 diet (Harlan Teklad, Madison, WI) and housed on dry corncob bedding with Nestlets™ and mouse houses added for environmental enrichment. During mating, up to two females were housed with one male. Once pregnancy was noted, the female was separated from the male and singly housed. Pups were weaned 21 days or later following birth and group housed (5 or fewer animals/cage) based on dam and sex.

Animal experiment. Twenty-four female wild type C57BL/6 mice (Charles River Breeding Laboratories, Raleigh, NC) 7 weeks old at the time of arrival, were placed in an AAALAC-accredited facility and quarantined for 12 days prior to treatment. Twenty-four *As3mt* KO mice from the F2 generation, approximately 7 weeks old, were transferred from the breeding colony to the same room. The level of care provided to the animals met or exceeded the basic requirements outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication #86-23, revised 1986). All animals were housed in polycarbonate cages (4/cage) with micro-isolator tops and dry corncob bedding. Nestlets™ were added to the cage for environmental enrichment. The animal room was targeted at 22 °C and 50% humidity and on a 12 h light/dark schedule (0600/1800 CST). Mice were provided with pelleted Purina 5002 (Dyets Inc., Bethlehem, PA) and hyperchlorinated (<10 ppm chlorine) reverse osmosis (RO) water *ad libitum*.

At approximately 8 weeks of age, animals were randomized into 3 treatment groups per genotype (8/group) using a weight stratification method (Martin et al., 1986) and started on treatment diets. For each genotype, group 1 was treated with the basal diet, group 2 was treated with diet containing 173 ppm $NaAs^{III}$ (100 ppm As^{III}) and group 3 was treated with diet containing 260 ppm $NaAs^{III}$ (150 ppm As^{III}). Diets were mixed and pelleted by Dyets Inc. (Bethlehem, PA). Acute toxicity was observed in *As3mt* KO mice treated with 100 and 150 ppm As^{III}. Seven mice in the *As3mt* KO group treated with 150 ppm As^{III} died before termination and therefore, the remaining *As3mt* KO mouse and all wild type mice in the 150 ppm As^{III} groups were terminated on study day 7. Also on study day 7, the dose in the 100 ppm As^{III} *As3mt* KO and wild type groups was reduced to 50 ppm As^{III} (86.5 ppm $NaAs^{III}$) and the route of administration was changed to the drinking water. This necessitated a change from RO water to tap water for all remaining mice. Body weights of all animals were measured the day after arrival, prior to randomization, and on study days 0 and 7. Body weights were also measured on the majority of animals terminated on study day 9, and on surviving animals on study day 14.

Because of the perilous clinical status of As^{III}-treated *As3mt* KO mice, all remaining animals were terminated on study days 9 and 15. The animals terminated on the various study days are shown in Table 1. At termination, the urinary bladder was inflated *in situ* with Bouin's fixative, and after removal, the bladders were placed in Bouin's fixative. A small section of intestine was removed and placed in Bouin's with the bladder. Following fixation, the Bouin's-fixed bladders and intestinal tissue were rinsed in 70% ethanol, the bladders were weighed, and all tissues were observed macroscopically. The entire surface of one half of the bladder was examined by scanning electron microscopy (SEM) and classified in one of five categories as previously described (Cohen et al., 1990). Briefly, class 1 bladders have polygonal superficial urothelial cells; class 2 bladders have occasional small foci of superficial urothelial necrosis; class 3 bladders have numerous small foci of superficial urothelial necrosis; class 4 bladders have extensive superficial urothelial necrosis, especially in the dome of the bladder; and class 5 bladders have necrosis and piling up (hyperplasia) of rounded urothelial cells. Normal rodent urinary bladders are usually class 1 or 2, or occasionally class 3. The other half of the bladder was cut longitudinally into strips and with a slice of intestinal tissue was embedded in paraffin, stained with hemotoxylin

Table 1
Morbidity and mortality.

	Mouse		Animal fate[a]				
Group	Genotype	Treatment	d6[b]	d7	d8	d9	d15
1	*As3mt* KO	Control	–	–	–	4(T)	4(T)
2	*As3mt* KO	100/50 ppm As^{III}	–	–	1(D), 1(MT)	6(MT)	–
3	*As3mt* KO	150 ppm As^{III}	4(D)	3(D), 1(MT)	–	–	–
4	Wild type	Control	–	–	–	–	8(T)
5	Wild type	100/50 ppm As^{III}	–	–	–	8(T)	–
6	Wild type	150 ppm As^{III}	–	8(T)	–	–	–

[a] Number of animals (D, Dead; T, terminated; MT, moribund and terminated).
[b] Experimental day.

and eosin (H&E), and examined histopathologically (Cohen, 1983; Cohen et al., 1990; 2007). A diagnosis of mild simple hyperplasia was made when there were 4–5 cell layers in the bladder epithelium. The liver, spleen and kidneys were also removed and weighed, and the lungs were removed and inflated with formalin via the trachea. These organs were placed in formalin and embedded in paraffin, stained with H&E, and examined histopathologically.

Statistics. The group means for body weights, and tissue weights were evaluated using analysis of variance followed by the Duncan's multiple range test for group-wise comparisons. Histopathology was compared using the 2-tailed, Fisher's exact test. SEM data were analyzed using 1-way nonparametric procedures followed by a chi square test. *P* values less than 0.05 were considered significant. These statistical analyses were performed using SAS for Windows (Version 9.1).

Results

Morbidity and mortality

Four *As3mt* KO mice treated with 150 ppm As^{III} were found dead on study day 6 and an additional 3 mice in the same group were found dead on study day 7 (Table 1). The remaining animal in this group was terminated on study day 7 due to signs of severe acute toxicity. On study day 8, after reducing the dose in the 100 ppm As^{III} group to 50 ppm As^{III} on study day 7, one *As3mt* KO mouse was found dead and a second *As3mt* KO mouse was terminated due to extreme toxicity. On study day 9, the remaining 6 *As3mt* KO mice in the 100/50 ppm group were terminated due to severe acute toxicity. Because of autolysis, necropsies were not performed on animals found dead. Several of the *As3mt* KO mice treated with As^{III} showed symptoms of acute arsenic poisoning, including body tremors, decreased activity and an unsteady gait.

Body and tissue weights

Administration of As^{III} to *As3mt* KO mice (groups 2 and 3) caused severe depression in body weight gain by day 7 (Table 2). In the 150 ppm As^{III} *As3mt* KO group, 7 mice were found dead by day 7, but the single mouse that could be evaluated for body weight showed a dramatic loss (13.4 g vs a mean of 18.2 g in the *As3mt* KO control mice). Body weights in the *As3mt* KO 100/50 ppm As^{III} group on day 7 showed a significant decrease compared with the *As3mt* KO control group and the comparable wild type group. Body weights of wild type mice treated with 150 ppm As^{III} also decreased significantly on day 7 compared to wild type control mice indicating 150 ppm As^{III} (260 ppm $NaAs^{III}$) to be toxic even in wild type mice. However, none of these mice died or showed signs of acute poisoning. The organ weights were assessed, using relative weights for comparisons since the timing of the termination was not the same for all mice. Macroscopically, the size of the spleen in both *As3mt* KO and wild type mice treated with As^{III} was small, and the size in *As3mt* KO mice treated with As^{III} was much smaller than in wild type mice. Relative weights of spleens were decreased significantly in the *As3mt* KO 100/50 ppm As^{III} group compared with the wild type 100/50 ppm group by week 2 (Table 3). The weights of the liver, kidney and bladder showed no significant inter-group differences.

Histopathologically, simple hyperplasia of the bladder epithelium was detected in the single As3mt KO mouse treated with 150 ppm As^{III} that was necropsied, in 3 of 8 wild type mice treated with 150 ppm, and in 3 of 7 *As3mt* KO mice and 3 of 8 wild type mice in the 100/50 ppm treatment groups (Table 4). Numerous intracytoplasmic granules were present in the epithelial layer of the bladder in the *As3mt* KO and wild type As^{III}-treated mice. In wild type mice, the granules were present mostly in the superficial layer of the urothelium (Figs. 1E and F). However, in As3mt KO mice treated

Table 2
Body weights of the mice on day 0, 7 and 14.[a]

Group	Mouse Genotype	Treatment	d0[b]	d7	d14
1	*As3mt* KO	Control	18.0 ± 0.3(8)	18.2 ± 0.4(8)	17.9 ± 0.2(4)
2	*As3mt* KO	100/50 ppm As^{III}	17.4 ± 0.4(8)[c]	15.4 ± 0.4(8)[c,d]	–
3	*As3mt* KO	150 ppm As^{III}	17.6 ± 0.3(8)[e]	13.4(1)[f]	–
4	Wild type	Control	18.2 ± 0.3(8)	18.5 ± 0.3(8)	18.5 ± 0.3(8)
5	Wild type	100/50 ppm As^{III}	18.5 ± 0.2(8)	18.8 ± 0.2(8)	–
6	Wild type	150 ppm As^{III}	18.4 ± 0.2(8)	16.7 ± 0.1(8)[g]	–

[a] Values expressed as the mean ± S.E. (*n*) where appropriate.
[b] Experimental days.
[c] Significantly different compared to the wild type 100/50 ppm As^{III} group, $p<0.05$.
[d] Significantly different compared to *As3mt* KO control group, $p<0.05$.
[e] Significantly different compared to wild type 150 ppm As^{III} group, $p<0.05$.
[f] No statistical analysis of group performed.
[g] Significantly different compared to wild type control group, $p<0.05$.

Table 3
Relative weights of the organs in the mice.[a]

Week[b]	Group	Mouse		Body Weight	Bladder	Kidney	Liver	Spleen
		Genotype	Treatment	(g)	(mg/g BW)	(mg/g BW)	(mg/g BW)	(mg/g BW)
1	3	*As3mt* KO	150 ppm As^{III}	13.4(1)	2.48(1)	17.1(1)	50.0(1)	1.85(1)
1	6	Wild type	150 ppm As^{III}	16.7 ± 0.1(8)	1.65 ± 0.14(8)	13.4 ± 0.2(8)	44.0 ± 0.7(8)	2.49 ± 0.07(8)
2	2	*As3mt* KO	100/50 ppm As^{III}	14.0 ± 0.3(6)[c]	2.13 ± 0.27(6)	16.9 ± 0.2(6)[c]	51.3 ± 1.7(6)	1.72 ± 0.17(6)[c]
2	5	Wild type	100/50 ppm As^{III}	18.4 ± 0.3(8)	2.06 ± 0.15(8)	13.7 ± 0.3(8)	50.8 ± 1.9(8)	3.24 ± 0.12(8)
3	1	*As3mt* KO	Control	17.9 ± 0.2(4)	1.95 ± 0.21(4)	13.5 ± 0.4(4)	50.4 ± 1.7(4)	4.27 ± 0.13(4)
3	4	Wild type	Control	18.5 ± 0.3(8)	1.87 ± 0.12(8)	12.4 ± 0.3(8)	50.2 ± 1.7(8)	3.88 ± 0.14(8)

[a] Values expressed as the mean ± S.E. (n) where appropriate.
[b] The study week termination was performed.
[c] Significantly different compared to wild type 100/50 ppm As^{III} group, $p<0.05$, on week 2.

with As^{III}, granules were present in all layers of the urothelium, where they were plentiful, larger, and appeared to also involve the nucleus (Figs. 1B and C).

In the lung, liver, spleen, kidneys and intestines, no proliferative lesions were observed in any group. Livers from As^{III}-treated As3mt KO groups (Figs. 2B and C) showed severe acute inflammatory changes with inflammatory cell infiltration, increased apoptosis and cytoplasmic vacuolation. Livers from wild type mice treated with As^{III} (Fig. 2A), showed no histopathologic changes. The white pulp of the spleen was markedly decreased in the As^{III}-treated As3mt KO groups (Fig. 2E) compared with the As^{III}-treated wild type groups (Fig. 2D). The intestine, kidneys and lungs showed no remarkable changes in any group.

By SEM, the incidence of class 5 bladders was increased significantly in the *As3mt* KO mice treated with 100/50 ppm As^{III} compared with the *As3mt* KO control mice (Fig. 3A) and wild type mice treated with 100/50 ppm As^{III} (Table 4). The bladders of the *As3mt* KO mice treated with 100/50 ppm As^{III} showed cytotoxicity, necrosis (Fig. 3B) and pilling up of small round urothelial cells indicative of proliferation and hyperplasia (Fig. 3C).

Discussion

Characterization of arsenic distribution and retention in the *As3mt* KO mouse has shown that, following administration of a single oral dose of iAs^{V}, there is much diminished formation of methylated metabolites and that the rate of clearance of arsenic from tissues is much slower than in wild type mice (Drobna et al., 2009). The reduced rate of clearance of arsenic in *As3mt* KO mice demonstrates that the clearance of arsenic is facilitated by the formation of methylated metabolites. This finding is consistent with earlier work suggesting a critical role for methylation in the clearance of arsenic (Marafante and Vahter, 1984; Carlson-Lynch et al., 1994). Because of lower rates of whole body clearance of arsenic and of arsenic methylation, tissue concentrations of arsenic attained under the continuous exposure conditions used in this study would be expected to be much higher in *As3mt* KO mice than in wild type mice. As a consequence of the higher retention of arsenic in tissues of iAs^{III}-treated *As3mt* KO mice, there was histopathological evidence of injury in urinary bladder and liver which are target organs for cancer in humans chronically exposed to inorganic arsenic in drinking water.

In the present experiment, we demonstrated that this accumulation of arsenic following multiple doses rapidly leads to severe systemic arsenic toxicity. At a dose of 150 ppm arsenite in the diet, nearly all of the animals died within the first seven days of exposure. The animals showed clinical signs of severe toxicity including rapid and severe weight loss, trembling, decreased activity, and unstable movements, suggesting neurologic toxicity. Furthermore, histopathologically, there was severe damage to the liver, including necrosis and inflammation. There was also suppression of the lymphoid system, as evidenced by a marked decrease in splenic size, primarily due to a severe shrinkage of the white pulp (lymphocytes). In the liver, the histopathologic changes included hepatocellular balloon-like vacuolation, apoptosis and necrosis, and inflammatory cell infiltrate. In humans with acute arsenic poisoning, similar changes are seen in the liver, including the hepatocellular balloon-like vacuolation, apoptosis, necrosis, and an inflammatory infiltrate and fibrosis (Santra et al., 1999; Lu et al., 2001; Mazumder, 2005; Wu et al., 2008). In addition, there is also endothelial degeneration, and if the toxicity persists, the inflammation can lead to fibrosis and cirrhosis as well as increased incidence of hepatocellular carcinomas and liver hemangiosarcomas (Centeno et al., 2002).

Notably, we did not observe toxicity in either the lungs or the kidney, and there was no grossly observable toxicity in other tissues besides the liver and spleen. We did not examine the nervous system histopathologically. Thus, the histopathological evidence of adverse effects of arsenic exposure was confined to tissues which have been identified as sites of tumor initiation in arsenic-exposed humans.

Table 4
The histopathological findings and SEM classification analysis of the mice.

Week[a]	Group	Mouse genotype	Treatment	No. of mice examined	Histopathology		SEM classification				
					Normal	Simple hyperplasia	1	2	3	4	5
1	3	*As3mt* KO	150 ppm As^{III}	1	–	1	–	–	–	–	1
1	6	Wild type	150 ppm As^{III}	8	5	3	3	3	2	–	–
2	1	*As3mt* KO	Control	4	4	–	4	–	–	–	–
2	2	*As3mt* KO	100/50 ppm As^{III}	7	4	3	–	–	1	–	6[b,c]
2	5	Wild type	100/50 ppm As^{III}	8	5	3	8	–	–	–	–
3	1	*As3mt* KO	Control	4	4	–	3	–	–	–	–
3	4	Wild type	Control	8	7	1	4	4	–	–	–

[a] The study week termination was performed.
[b] SEM classification significantly different compared to the *As3mt*KO control group, $p<0.05$, on week 2.
[c] SEM classification significantly different compared to the wild type group treated with 100 ppm As^{III}, $p<0.05$, on week 2.

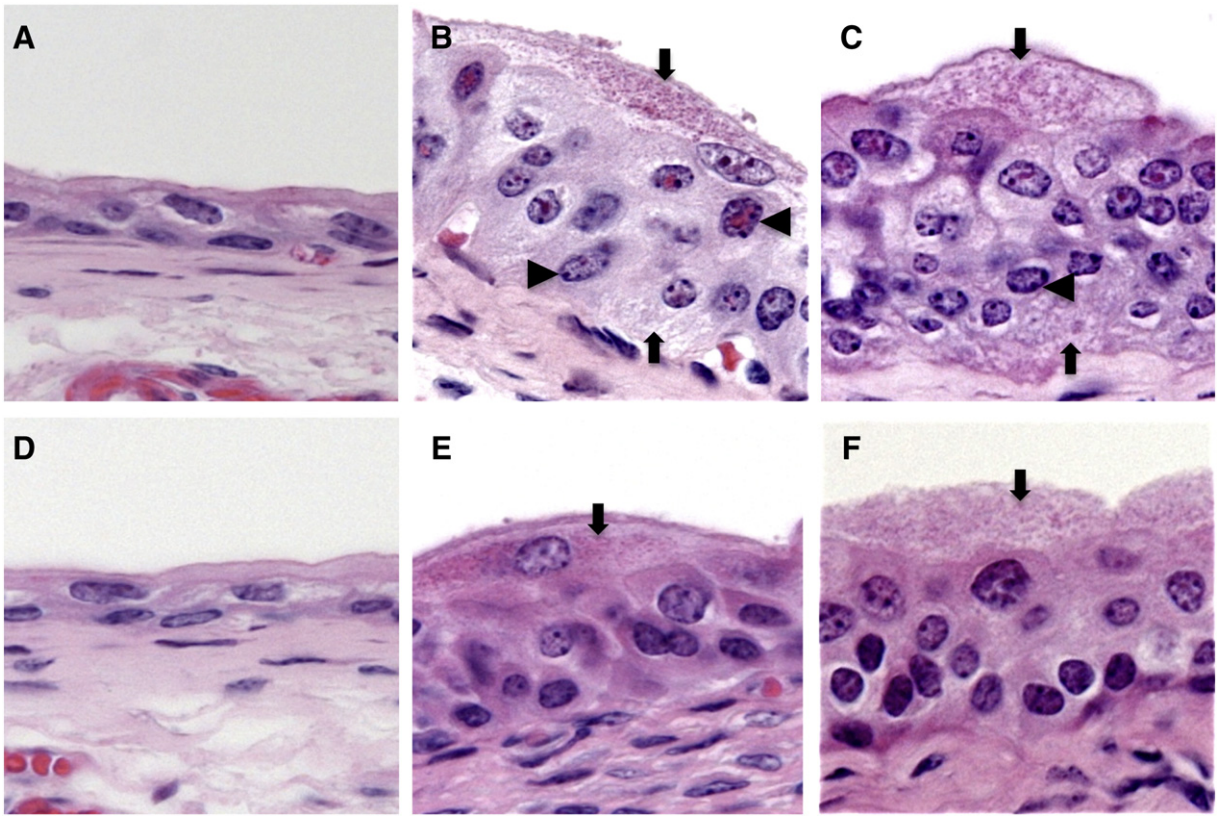

Fig. 1. Histopathological findings of the bladder epithelium. (A, *As3mt* KO control; B, *As3mt* KO 100/50 ppm As^{III}; C, *As3mt* KO 150 ppm As^{III}; D, wild type control; E, wild type 100/50 ppm As^{III}; F, wild type 150 ppm As^{III}). Granules in the epithelial layer of bladders with simple hyperplasia (B, C, E and F). In the As^{III}-treated *As3mt* KO mice, the granules occurred in all layers of the epithelium (arrows) and in both the cytoplasm and the nucleus (arrow heads) (B and C). Both *As3mt* KO and wild type control mice showed normal epithelium (A and D). Magnification ×400.

In previous work, we have focused on the generation of DMA^{III}, a highly reactive metabolite of inorganic arsenic, as a chemical species that produces cytotoxicity and consequent regenerative proliferation in the urinary bladder (Cohen et al., 2007; Suzuki et al., 2010). Thus, we hypothesized that in the *As3mt* KO mouse, without the formation of sufficient amounts of a reactive trivalent methylated arsenical (e.g., DMA^{III}), there would be no adverse effect on the urothelium. However, the current study found clear evidence of cytotoxicity and regenerative proliferation in the urothelium of *As3mt* KO and wild type mouse following treatment with either 150 or 100 ppm iAs^{III}. It is difficult to assess the findings in the *As3mt* KO mouse at 150 ppm since we were only able to evaluate 1 mouse bladder in this group because of the early deaths in the remaining animals. However, at the dose of 100 ppm, most *As3mt* KO mice were found to have evidence of severe cytotoxicity observed by scanning electron microscopy, frequently in association with evidence of increased proliferation with hyperplasia (piling up of small round cells). Clearly, in *As3mt* KO mice, cytotoxicity with regenerative proliferation of the urothelium following arsenite administration did not require methylation and the consequent formation of the highly reactive trivalent methylated arsenicals. Rather, uroepithelial injury could be the consequence of the retention of high levels of inorganic arsenic in this tissue in an animal which neither efficiently methylates nor clears arsenic. Effects on the urothelium in the *As3mt* KO mice and in the wild type mice are consistent with previous studies using human urothelial cells (UROtsa) *in vitro* which also do not have *As3mt* activity (Styblo et al., 2002; Sen et al., 2007).

We have previously reported the presence of numerous intracytoplasmic eosinophilic granules in the superficial cells of the urothelium of iAs^{III}-treated mice (Suzuki et al., 2008a), and have demonstrated that these granules are intramitochondrial and that they contain large amounts of arsenic in the form of arsenite (Arnold et al., 2006; Suzuki et al., 2008a; 2008b). In the current study, we found that these granules were even more pronounced in *As3mt* KO mice than in the wild type mice. Furthermore, in the *As3mt* KO mice, the granules were bigger, more plentiful, and appeared to involve all layers of the urothelium (not just the superficial cell layer). The granules appeared to also accumulate within the nuclei in addition to the cytoplasm in *As3mt* KO mice. The abundance and location of these granules in *As3mt* KO mice is likely due to the accumulation of very high concentrations of inorganic arsenic in urothelial cells of these animals.

The methylated trivalent arsenicals MMA^{III} and DMA^{III} are highly reactive and produce severe cytotoxicity of urothelial cells in vitro at concentrations that are below 1 μM (Nascimento et al., 2008; Suzuki et al., 2010). At slightly greater concentrations, approximately 0.8–1.5 μM, iAs^{III} is significantly cytotoxic. Hence, a relatively modest decrease in the rate of clearance of inorganic arsenic from urothelial cells in *As3mt* KO mouse could be sufficient to increase the concentration above the threshold for cytotoxicity. Thus, given the effects of the *As3mt* null genotype on the distribution and retention of inorganic arsenic, it is plausible that the genotype could render iAs^{III} a potent cytotoxin of urinary bladder cells. The finding of uroepithelial cell injury in *As3mt* KO mice is not inconsistent with earlier results suggesting that DMA^{III} is the arsenical responsible for the induction of cell injury. Rather, the results of the current study suggest that the combination of low rates of arsenic methylation and of tissue clearance of inorganic arsenic are sufficient to produce conditions in which inorganic arsenic can cause cytotoxicity in the urinary bladder.

Based on functions of LC_{50} concentrations rather than functions of specific concentrations, the effects of arsenic in vitro appear to be additive for the various trivalent arsenicals (Nascimento et al.,

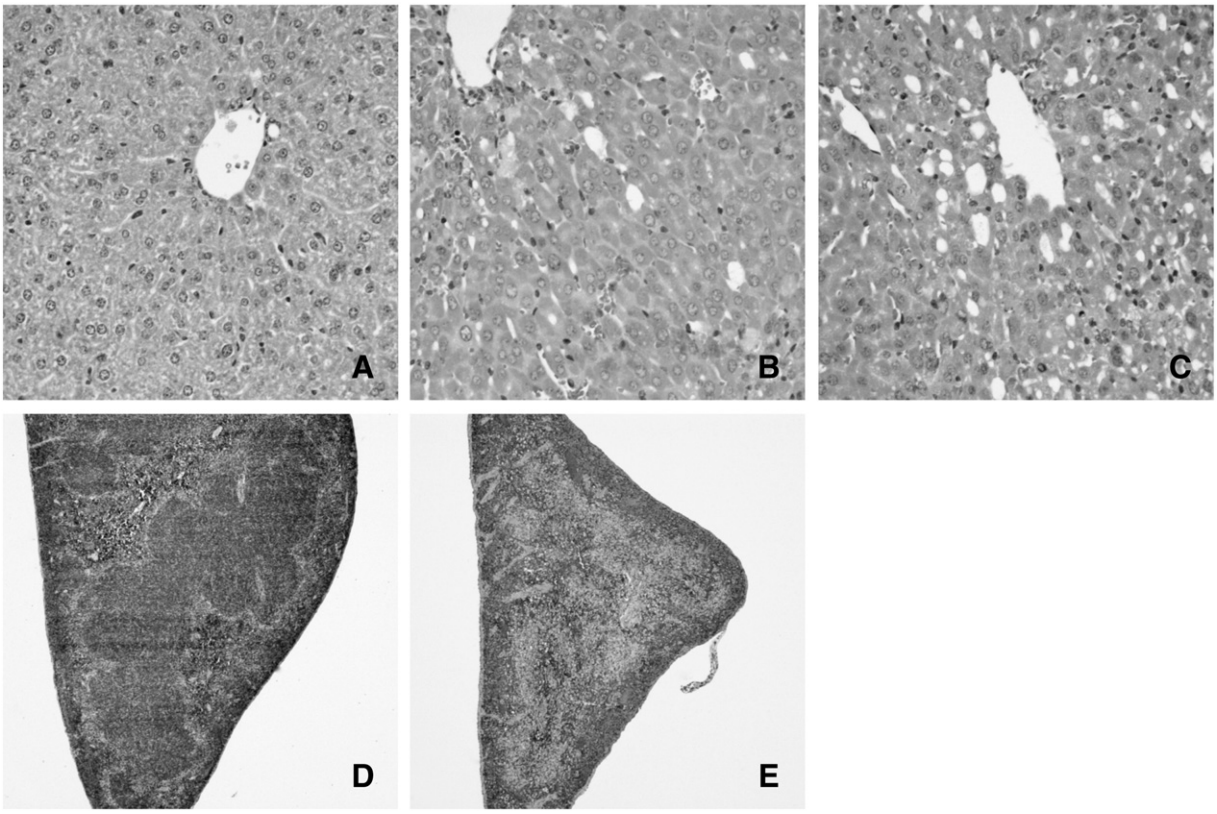

Fig. 2. Histopathological findings of the liver and spleen. The livers indicated severe acute inflammatory changes with inflammatory cell infiltration, increasing numbers of apoptotic cells and vacuolation of the cytoplasm were present in *As3mt* KO mice treated with 100/50 ppm (B) or 150 ppm As^{III} (C). The white pulp of the spleen was markedly decreased in the *As3mt* KO 100/50 ppm As^{III} group (E) compared with the wild type 100/50 ppm As^{III} group (D). Magnification, liver ×200; magnification, spleen ×40.

2008). We therefore hypothesize that the specific biologic effects of arsenic exposure depend on the toxicokinetic aspects of arsenic metabolism and distribution combined with the sensitivity of the targeted cellular constitutions (probably specific protein sulfhydryl groups) to the various trivalent forms of arsenic. This likely will vary between tissues and between species, as well as inter-individual variation and on conditions of exposure to factors that could influence the toxicokinetic and toxicodynamic aspects of arsenic. Thus, it is likely that the response to exposure to inorganic arsenic is dependent on the exposure of cells to the combination of trivalent arsenicals. Additional studies in *As3mt* KO mice of the disposition and retention of other intermediates in the pathway for arsenic methylation should help illuminate the relative potency of these arsenicals as cytotoxins.

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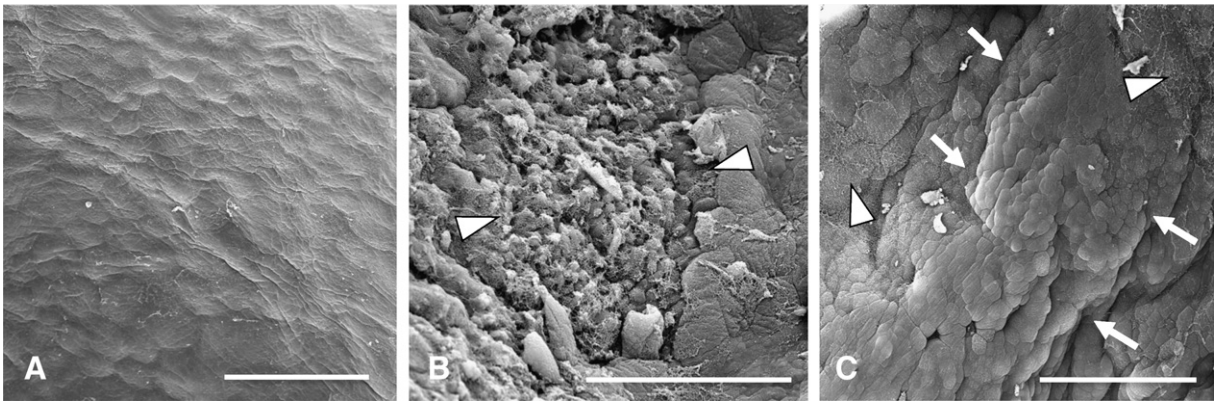

Fig. 3. SEM images of the surface of the bladder. (A) Surface of the bladder of an *As3mt* KO mouse fed control diet for 9 days showing the normal large superficial polygonal cells. Bar = 300 µm. (B) Surface of the bladder of an *As3mt* KO mouse treated with 100/50 ppm As^{III} for 9 days showing extensive necrosis (arrowhead). Bar = 200 µm. (C) Surface of the bladder of an *As3mt* KO mouse treated with 150 ppm As^{III} for 7 days showing piling up (hyperplasia) of rounded urothelial cells (arrows) and necrosis (arrowhead). Bar = 200 µm.

does mention of trade names or commercial products constitute endorsement or recommendation for use.

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