Arsenic in drinking water caused ultra-structural damage in urinary bladder but did not affect expression of DNA damage repair genes or repair of DNA damage in transitional cells

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Arsenic in drinking water caused ultra-structural damage in urinary bladder but did not affect expression of DNA damage repair genes or repair of DNA damage in transitional cells

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Abstract

Arsenic is a human carcinogen associated with urinary bladder transitional cell carcinoma and other cancers. Arsenic is also a strong comutagen and cocarcinogen. One possible mode of action for arsenic carcinogenesis/cocarcinogenesis is inhibition of DNA damage repair. In laboratory animals, urinary bladder transitional cell carcinoma has only been observed in dimethylarsinic acid [DMA(V)]-exposed F344 rats.

The goal of the present studies was to investigate inhibition of DNA repair as a mode of action for arsenic carcinogenesis/ cocarcinogenesis in the urinary bladder. Methods were first developed to harvest only transitional cells, the target cell type of arsenic carcinogenesis, suitable for RNA extraction or for DNA damage detection by Comet assay. Morphological studies established that DMA(V) in drinking water at 40 ppm was cytotoxic to the urothelium of Sprague-Dawley® and F344 rats, and mitochondria were targeted by DAM(V).

To investigate whether DMA(V) decreases the expression of DNA repair genes, mRNA levels of DNA repair genes in transitional cells were next measured in F344 rats exposed to up to 100 ppm DMA(V) in drinking water for 4 weeks. The mRNA levels of Ataxia Telangectasia mutant (ATM), X-ray repair cross-complementing group 1 (XRCC1), excision repair cross-complementing group 3/Xeroderma Pigmentosum B (ERCC3/XPB), and DNA polymerase β genes were not altered, as measured by real time RT PCR. These results suggested either that DMA(V) affects DNA repair without affecting the baseline expression of DNA repair genes or that DMA(V) does not affect DNA repair in the bladder.

Arsenic effects on DNA repair were further investigated in F344 rats given 100 ppm DMA(V) or arsenate in drinking water for 1 week. DNA damage levels in transitional cells and micronuclei frequency (MN) in bone marrow were measured. Dimethylarsinic acid did not affect *in vivo* cyclophosphamide-induced DNA damage, and neither DMA(V) nor arsenate inhibited *in vitro* repair of hydrogen peroxide- or formaldehyde-induced DNA damage, as measured by Comet assay. Neither DMA(V) nor arsenate increased MN or elevated *in vivo* cyclophosphamide-increased MN. These results suggest inhibition of DNA repair by arsenic, in the transitional epithelium, may not be a major mechanism responsible for carcinogensis/cocarcinogenesis in the bladder.

DEDICATION

I dedicate this dissertation to
Dr. Marc J. Mass (1954 -2001), who inspired me to study arsenic.

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β	regression-coefficient
γ GT	γ glutamyl transpeptidase
8-OHdG	8-Hydroxy-2'-deoxygaunosine, a form of oxidative DNA damage
ACE	accumulated arsenic exposure
Ach	acetylcholine
ADP	adenosine 5'-diphosphate
ALT	alanine aminotrnsferase
AP-1	activating protein-1
As^{III}, As(III)	arsenite
As^{V}, As(V)	arsenate
AST	aspartate aminotransferase
ATM	Ataxia Telangectasia mutant
ATP	adenosine triphosphate
AUM	asymmetric unit membrane, a structure of urinary bladder in the form of plaques
BBN	n-butyl-N-(4-hydroxybutyl)nitrosamine, a urinary bladder carcinogen
BrdU	bromodeoxyuridine
BUN	blood urine nitrogen
CA	chromosome aberrations
cAMP	cyclic adenosine monophosphate
cGMP	guanosine 3', 5'-cyclic monophosphate
CI	confidence interval
Cox-2	cyclooxygenase-2
CP	cyclophosphamide
DEN	diethylnitrosamine
DHPN	N-bis(2-hydroxypropyl)nitrosamine, a genotoxic carcinogen targeting the lung, liver, thyroid, and kidney
DMA^{III}, DMA(III)	dimethylarsinous acid
DMA(III)-GS	dimethylarsinous glutathione, a complex of dimethylarsinous acid with glutathione
DMA^{V}, DMA(V)	dimethylarsinic acid
DMBDD	diethylnitrosamine, N-methyl-N-nitrosourea, n-butyl-N-(4-hydroxybutyl)nitrosamine, 1,2-dimethylhydrazine and N-bis(2-hydroxypropyl)nitrosamine
DMH	1,2-dimethylhydrazine
DMI	dimethylarsinous iodide
DMSA	meso-2,3-dimercaptosuccinic acid, a chelator
EDTA	ethylenediaminetetraacetic acid
eNOS	nitric oxide synthase in endothelial cells
ERCC1	excision repair cross complementing group 1
ERCC3/XPB	excision repair cross-complementing group 3/xeroderma pigmentosum B

FBS	fetal bovine serum
FGF	fibroblast growth factor
Folbp	folate binding protein
Fpg	formamidopyrimidine-DNA glycosylase
FSH	follicle-stimulating hormone
GaAs	gallium arsenide
GC	guanylate cyclase
GD	gestational day
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	glutathione (reduced)
GSSH	glutathione
GST M1	glutathione S-transferase Mu 1-1
GST T1	glutathione S-transferase theta 1
GST	glutathione S-transferase
GST-P	glutathione S-transferase placental form positive
GTP	guanosine triphosphate
HE	hematoxylin-eosin
HSD	hydroxysteroid dehydrogenase
IARC	the International Agency for Research on Cancer
i.p.	intraperitoneal
IL-2	interleukin-2
ILK	integrin-linked kinase
LDH	lactate dehydrogenase
LH	leutinizing hormone
LI	labeling index; in nuclear immuno-enzymatic labeling, LI = (number of labeled cells) / (total number of cells); in cytoplasmic immuno-enzymatic labeling, LI = (area of labeled portions) / (total area of the structures)
MA(III)O	methylarsine oxide
MDR	P-glycoprotein/miltidrug resistance
MEM	minimal essential medium with Earles' salt
MI	mitotic index; MI = (number of cells in mitosis) / (number of all cells)
MiADMSA	monoisomayl dimercaptosuccinic acid, a chelator
MMA^{III}, MMA(III)	monomethylarsonous acid
MMA^{V}, MMA(V)	monomethylarsonic acid
MN	micronuclei or micronucleus
MPR	multidrug resistance protein
MT	metallothionein
MTF-1	metal- responsive transcription factor
MUN	N-methyl-N-nitrosourea
NAT	N-acetyltransferase
NBR	NCI (National Cancer Institute)-Black-Reiter, a strain of rats
NF-κB	nuclear factor-kappaB
NO	nitric oxide
NOAEL	no observed adverse effect level

NTD	neural tube defect, a form of developmental malformation/congenital malformation
ODC	ornithine decarboxylase
PAD	periodate-oxidized adenosine, an inhibitor of SAM-dependent methylation
PARP	poly(ADP-ribose)polymerase
PMI	primary arsenic methylation index; the ratios of MMA(V)/inorganic arsenic in urine
Polβ	DNA polymerase β
PTEN	phosphatase and tensin homolog
RFC	reduced folate carrier
RI	replication index
RT PCR	reverse transcription polymerase chain reaction
s.c.	subcutaneous
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCE	sister chromatid exchange
SD	Sprague-Dawley®, a rat strain
SE	standard error
SEM	scanning electron microscopy
SMI	secondary arsenic methylation index; the ratio of DMAV/MMAV in urine
SMR	standardized mortality ratio; SMR = (observed deaths) / (expected deaths)
SNP	sodium nitroprusside
TBP	TATA-box binding protein
TEM	transitional electron microscopy
TGFα	transforming growth factor-alpha
TMA	trimethyl-arsine
TMA^VO, TMA(V)O	trimethylarsine oxide
VEGF	vascular endothelial cell growth factor
VEGFR	vascular endothelial cell growth factor receptor
XPA	xeroderma pigmentosum group A
XRCC1	X-ray repair cross-complementing group 1

CHAPTER 1. LITERATURE REVIEW

1. TOXIC AND CARCINOGENIC EFFECTS OF ARSENIC IN HUMANS

Carcinogenic Effects

Since the late 19th century, when the carcinogenic effect of arsenic in skin was first noted by Hutchinson, cancers of skin, liver, urinary bladder, kidney, lung, breast, colon, stomach, parotid gland (a salivary gland), nasopharynx, larynx, buccal cavity, as well as leukemia and other hematopoietic cancers have been reported following chronic (years) arsenic exposure (National Research Council 1999). The International Agency for Research on Cancer (IARC) determined that arsenic compounds are dermal and pulmonary (via inhalation) carcinogens in humans in the 1980s (IARC 1980; IARC 1987a), and are human urinary bladder carcinogens in 2004 (IARC 2004).

Different types of cancer have been linked to exposure to inorganic arsenic via (1) drinking water, (2) occupational exposure (in copper smelters, glass manufacturing, or power generation by the burning of arsenic-contaminated coal) (National Research Council 1999; National Research Council 2001), (3) food (Gebel 2000; Sakurai and Fujiwara 2001), and (4) medical treatment (Fowler's solution and some anti-leukemia treatments) (Nevens *et al.* 1990). Epidemiological studies (see below) show that arsenic exposure increases mortality associated with bladder, kidney, lung, and skin cancers (Chiou *et al.* 2001; National Research Council 2001). Arsenic exposure is also associated with greater mortalities from neoplasms of the nasal cavity, bone, liver, larynx, colon, stomach, and from lymphoma (Tsai *et al.* 1999). A dose-response relationship was observed between arsenic levels in well water and the development of cancers of the urinary bladder, kidney, skin, and lung in both males and females, and cancers of

the prostate and liver in males (Wu *et al.* 1989). However, there was no discernible association between arsenic concentrations in well water and the development of cancers in nasopharynx, esophagus, stomach, colon, and uterine cervix, or of leukemia (Wu *et al.* 1989).

The effects of arsenic exposure on the development of several important organ-specific neoplasms are discussed more fully below.

Skin

Increased incidences of skin cancer in arsenic-exposed populations have been reported in epidemiological studies conducted in Taiwan, Mexico, Hungry, England, Japan, Argentina, and Bangladesh (Tseng *et al.* 1968; US EPA 1998; Smith *et al.* 2000). Arsenic exposure-related skin cancers include Bowen's disease (intraepithelial carcinoma, or carcinoma *in situ*), basal cell carcinoma, and squamous cell carcinoma (Guo *et al.* 2001; Centeno *et al.* 2002). Malignant melanoma is not associated with arsenic (Guo *et al.* 2001). The predominant types of arsenic-related skin cancer type vary among racial groups. For example, among Euro-Americans, basal cell carcinoma was the most frequent type of both arsenic-associated skin cancers and non-arsenic-associated skin cancers in this racial group. Among Asians, Bowen's disease was the most frequent type of arsenic-associated skin cancer, but not of skin cancers from other causes (Abernathy *et al.* 1999; Cabrera and Gomez 2003). The concurrent appearance of multiple skin lesions, particularly in areas of the skin that are not directly exposed to the sun (*i.e.*, trunk), is characteristic of arsenic-associated skin cancers in all races.

Lung

Inhaled inorganic arsenic is recognized as a cause of lung cancer (IARC 1980). An increased risk of lung cancer has been associated with cumulative exposure to arsenic-containing dust and

the duration of this exposure. Additionally, tobacco smoking further increases arsenic dust-associated lung cancer (Viren and Silvers 1999; Englyst *et al.* 2001; Chen and Chen 2002).

Similar to arsenic and skin cancer, arsenic-induced lung cancer appears to be cell-type specific. Squamous cell carcinoma and small cell carcinomas were related to arsenic ingestion, but adenocarcinoma was not (Guo *et al.* 2004). Exposure to arsenic in drinking water has been shown to increase lung cancers in Argentina, Chile, and Japan (Tsuda *et al.* 1995; Hopenhayn-Rich *et al.* 1998; Smith *et al.* 1998), as shown by increases of mortality rates, standardized mortality ratios, and odds ratios of lung cancer in arsenic-exposed populations compared to no-arsenic-exposed or low-arsenic-exposed populations.

Exposure to arsenic in drinking water increased lung cancer in both non-smokers and smokers (Ferreccio *et al.* 2000; Chen *et al.* 2004a). Furthermore, cigarette smoking and arsenic concentration had an additive or a greater than additive effect on lung cancer (Ferreccio *et al.* 2000; Chen *et al.* 2004a). Higher accumulative cigarette smoking exposure ($\geq$ 25 packs per year) increased relative risk of arsenic-associated lung cancer more than lower accumulative cigarette smoking exposure (< 25 packs per year) (Chen *et al.* 2004a).

Urinary Bladder

Several epidemiological studies have shown an association with an increased incidence and mortality due of transitional cell carcinoma of the urinary bladder and drinking arsenic-contaminated water. This has been demonstrated in Taiwan (Tsai et al. 1999; Chiou et al. 2001; Chen et al. 2003), Finland (Kurttio et al. 1999) Argentina (Steinmaus et al. 2006), Chile, and Japan (National Research Council 1999; Cohen et al. 2000) (Table 1). This association between urinary bladder cancer and arsenic exposure has also been documented in people using medicinal

Fowler's solution (potassium arsenite; once used to treat psoriasis) in the United Kingdom (National Research Council 1999).

Transitional cell carcinoma of the urinary bladder, the most common type of bladder cancer in developed countries, is associated with increased concentrations of arsenic in well water in a dose-response manner (Chiou *et al.* 2001). The incidences of transitional cell carcinoma were increased by high arsenic concentrations in the local well water in northeastern Taiwan (Chiou *et al.* 2001). Compared to the risk of developing transitional cell carcinoma when arsenic concentration at or less than 10.0 mg/liter, the risk of developing transitional cell carcinoma was 1.9 folds higher for arsenic concentrations of 10.1-50.0 mg/liter, and 8.2 folds higher for arsenic 50.1 to 100.0 mg/liter.

Kidney

An increase in the incidence of arsenic-associated kidney cancer and mortality due to these tumors has been reported in Taiwan, Argentina, and USA (Table 1). A positive correlation between the mortality rate of kidney cancer and arsenic concentration in the drinking water was reported in Taiwan (Wu *et al.* 1989).. The mortality rates from kidney cancer, were:

- 8.4 (men) and 3.4 (women) for average arsenic exposures <0.30 ppm,

- 18.9 (men) and 19.4 (women) for average arsenic exposures of 0.30-0.59 ppm, and

- 25.26 (men) and 57.98 (women) for average arsenic exposures of ≥0.60 ppm (Wu *et al.* 1989).

In the study conducted by Tsai and coworkers (1999), a median arsenic concentration in drinking water of 0.78 ppm was found and approximately 23 years of mortality data were analyzed from vital statistics published by the Taiwan government. A total of 222 deaths from kidney cancer were recorded, consisting of 94 males and 128 females. In people exposed to

excessive amounts of arsenic in drinking water, calculated mortality ratios for male kidney cancer were 6.76 (using the regional reference) and 6.80 (national reference), and for female kidney cancer were 8.89 (regional reference) and 10.49 (national reference). These data clearly indicate an increased risk of developing renal tumors following lengthy consumption of arsenic-contaminated drinking water.

Hopenhayn-Rich *et al.* (1998) analyzed mortality due to kidney and other cancers in the Cordoba region of Argentina between 1986 and 1991. The data were arbitrarily divided into high, medium and low arsenic exposure groups, using measured levels of arsenic in ground water throughout the country; individual exposure data were not available. When compared with reference statistics of the entire population of Argentina, standard mortality ratios for kidney cancer in Cordoba, from low to high arsenic exposure, were 0.87 (low level exposure), 1.33 (medium level exposure), 1.57 (high level exposure) (for men) and 1.00, 1.36, 1.81 (for women), respectively.

Kurttio and coworkers (1999) conducted an epidemiologic study of arsenic effect on the development of kidney cancer in Finland. They estimated arsenic exposure as arsenic concentration in the well, daily dose, and cumulative dose of arsenic. None of these exposure indicators showed a statistically significant increased risk for developing kidney cancer. This study did not assess the levels of arsenic in food, which may be an important source of arsenic in this population (Kurttio *et al.* 1999).

Liver

Several studies have shown that even a short period of exposure to Fowler's solution (potassium arsenite solution) can induce angiosarcoma of the liver, an uncommon hepatic neoplasm (Falk *et al.* 1981; Roat *et al.* 1982; Kadas *et al.* 1985). In several epidemiological

studies, arsenic exposure is associated with greater mortality due to liver cancer (Tsai *et al.* 1999; Chiu *et al.* 2004). A significant dose-response relationship was observed, correlating arsenic levels in well water and liver cancer in men (Wu *et al.* 1989).

The effects of arsenic exposure (from burning high arsenic-containing coal) on the development of liver lesions were studied in Guizhou, China (Liu *et al.* 2002). The estimated sources of total arsenic exposure in this area were arsenic-contaminated food (50-80%), air (10-20%), water (1-5%), and direct contact in coal mine workers (1%). The incidences of hepatomegaly, in areas where high-arsenic coal was burned, were 37% in 1992 (determined by physical examination) and 21% in 1998 (determined by ultrasound examination). The most serious outcomes of arsenic-associated liver injury were cirrhosis, ascites and hepatocellular carcinoma. However, the conclusion that arsenic was solely responsible for the observed liver lesions should be interpreted with caution. The coal, considered to be the primary source of arsenic, was contaminated with other heavy metals, such as lead. Likewise, effects of combustion products such as polycyclic aromatic hydrocarbons cannot be excluded in the pathogenesis of liver lesions.

Toxic Non-carcinogenic Effects

A distinction can be made between arsenic-induced non-carcinogenic acute and chronic effects. Acute arsenic exposure often causes immediate symptoms, which depend on the route and dose. Inhalation of the most toxic form of arsenic, AsH_3 (arsine gas), can cause death within 30 minutes at concentrations of 25 to 50 ppm, or immediate death at 100-150 ppm (Blackwell and Robbins 1979). Sub-lethal arsine poisonings may cause hemolysis/anemia and renal failure,

as well as gastrointestinal (vomiting, diarrhea, jaundice, and hepatomegaly) and central nervous system symptoms (headache, dizziness) (Ellenhorn 1988).

Acute massive inorganic arsenic ingestion can cause acute paralytic syndrome (cardiovascular collapse and central nervous system depression), and death within hours (Poklis and Saady 1990). After acute exposures to inorganic arsenic more than several mg per day, the most common symptons are gastrointestinal and severe overt cardiovascular symptoms followed by multiple organ failure such as delayed onset of central or peripheral nervous symptoms, renal failure, bone marrow suppression, and respiratory failure (Cullen *et al.* 1995; National Research Council 1999).

Chronic arsenic exposure causes characteristic cutaneous effects (hyperpigmentation and hyperkeratosis), peripheral vascular disease (Blackfoot disease), nonmalignant pulmonary disease, diabetes mellitus, and peripheral neuropathy. Overt gastrointestinal, cardiovascular, and central nervous symptoms are often absent (National Research Council 1999).

The following sections discuss arsenic non-carcinogenic effects by body system, with a focus on chronic arsenic exposure, especially through drinking water.

Cutaneous Effects

One of the early signs of chronic arsenic exposure is cutaneous lesions, which are relatively arsenic specific and easily identified (Smith *et al.* 2000). Furthermore, skin appears to be the most susceptible tissue to arsenic toxicity (Yoshida *et al.* 2004; ATSDR 2005), and the cutaneous lesions are reported in individuals chronically exposed to arsenic at 0.002 to 0.1 mg arsenic/kg body weight/day (ATSDR 2005). The pattern of arsenic-induced lesions changes over time, beginning with persistent erythematous flush, and then progressing to hyperpigmentation and hyperkeratosis (Schoolmeester and White 1980; Smith *et al.* 2000).

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Hyperpigmentation mainly appears on the eyelids, neck, upper chest, arms and legs (Graeme and Pollack 1998; Smith *et al.* 2000) in a "raindrop" pattern. This appears as numerous, rounded, hypopigmented maculae (2-4 nm in diameter), scattered on overall tan-to-brown hyperpigmented skin as if "raindrops on a dusty road" (Tay 1974; Gorby 1988; Wong *et al.* 1998). Other patterns (diffuse hyperpigmentation; localized or patchy pigmentation, particularly affecting skin folds), are less common (National Research Council 1999).

Hyperkeratosis predominately appears on the palms and soles (often with desquamation) (Graeme and Pollack 1998; McFall *et al.* 1998; Smith *et al.* 2000). Histologically, arsenic associated hyperkeratosis may have parakeratosis (persistence of nuclei of keratinocytes in the stratum corneum), acanthosis (increase in the thickness of the stratum spinousum), and enlargement of the rete ridges (National Research Council 1999; Centeno *et al.* 2002). Hair and nail signs include brittle nails, patchy alopecia, and Aldrich-Mees lines (transverse white lines across the nails) (Schoolmeester and White 1980; Graeme and Pollack 1998; McFall *et al.* 1998). Skin cancers, including Bowen's disease, basal cell carcinoma, and squamous carcinoma, may arise in the hyperkeratotic areas as well as nonkeratotic areas.

Gastrointestinal and Hepatic Effects

The most common manifestation of acute arsenic poisoning is acute overt gastrointestinal symptoms, which may include vomiting, abdominal cramping, profound watery or bloody diarrhea, and hemorrhagic gastroenteritis (Poklis and Saady 1990; Cullen *et al.* 1995; Graeme and Pollack 1998; National Research Council 1999; Ratnaike 2003). The consequent massive fluid loss may lead to reduced blood volume, circulatory collapse, hypovolumetric shock, and death (Graeme and Pollack 1998; Ratnaike 2003). These gastrointestinal symptoms result from intestinal injury caused by dilatation of splanchnic vessels leading to mucosal vesiculation. The

vesicles rupture, causing bleeding, diarrhea, and protein-wasting enteropathy (Schoolmeester and White 1980). Mild-to-moderate hepatocellular necrosis may occur (Cullen *et al.* 1995; National Research Council 1999).

Although chronic arsenic exposures usually do not present gastrointestinal symptoms, diarrhea, splenomegaly, and hepatomegaly have been reported in populations exposed to arsenic (Centeno *et al.* 2002; Liu *et al.* 2002; Guha Mazumder 2003). Chronic exposure to arsenic causes non-cirrhotic portal hypertension (Guha Mazumder 2005) with normal hepatic function. Non-cirrhotic portal hypertension, histologically, is characterized by thickening and sclerosis of the wall of large vein branches and periportal fibrosis (Centeno *et al.* 2002; Guha Mazumder 2005). Clinically, patients with arsenic-associated non-cirrhotic portal hypertension sometimes present enlarged spleen and liver, anemia, and gastrointestinal hemorrhage due to esophageal varices (Nevens *et al.* 1990; Centeno *et al.* 2002).

Cardiac and Vascular Effects

Acute arsenic poisonings cause serious overt cardiovascular symptoms, including hypertension, congestive heart failure, and tachycardia (National Research Council 1999; Stephanopoulos *et al.* 2002). Electrocardiographic changes are non-specific, and include QT prolongation, ST depression, or T wave inversion. Death may occur secondary to dysrhythmias, including torsades de pointes (polymorphic ventricular tachycardia) (Cullen *et al.* 1995; National Research Council 1999).

Chronic arsenic ingestion has been linked to increased mortalities due to cerebrovascular disease, ischemic heart disease, pulmonary-based heart disease, pericarditis, hypertension, and peripheral vascular diseases (Engel *et al.* 1994; Chen *et al.* 1995; Chiou *et al.* 1997b; Lewis *et al.* 1999; Tsai *et al.* 1999). Histological findings of arsenic-associated cardiovascular diseases

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included myocardial infarction, and subclinical markers of atherosclerosis (fibrous intimal thickening of arteries and carotid plaques) (Navas-Acien *et al.* 2005). Atherogenic pathway has been suggested to be involved in arsenic-associated ischemic heart disease and Blackfoot disease (Ross 1986). Possible mechanisms of atherogenicity associated with arsenic include increased oxidative stress, increased expression of inflammatory mediators and consequently inflammatory cell infiltration, endothelial cell damage, increased coagulability, smooth muscle cell proliferation, interference with functions of DNA, RNA and proteins, induction of apoptosis, interaction with other trace elements, and association with hypertension and diabetes mellitus (Tseng 2002; Simeonova and Luster 2004; Navas-Acien *et al.* 2005; Wu *et al.* 2006). Carotene and zinc deficiency, and/or immunological effects of arsenic may also be involved in arsenic vascular effects (Yu *et al.* 2002).

Blackfoot disease, a severe peripheral vascular insufficiency which causes gangrene of the extremities (particularly the feet), is a unique arsenic-induced peripheral vascular disease. It has been reported to occur in people living in southeastern Taiwan, where water was contaminated with inorganic arsenic (Tsai *et al.* 1999), and in German vintners who consumed wine substrate contaminated by arsenic-containing pesticides (Engel *et al.* 1994). Blackfoot disease initially appears as coldness or numbness in the extremities (usually the feet) and lameness, progressing to ulceration, gangrene, and spontaneous amputation (Tseng *et al.* 1961). Histologically, the affected limbs showed thromboangiitis obliterans (restricted or obstructed blood vessels of hands or feet, due to inflammation or clots) and arteriosclerosis obliterans (a vascular disease characterized by narrowing and hardening the arteries that supply the legs and feet), particularly affecting small vessels (Yu *et al.* 1984).

In contrast, Blackfoot disease was not reported in Mexico, Argentina, or in Inner Mongolia and Guizhou, China, in spite of comparable arsenic levels and the presence of other types of arsenic-induced skin lesions (National Research Council 1999; Gebel 2000; Tseng 2002). Possible explanations of this difference included shorter exposure in some areas, different nutritional status, genetic difference in the metabolism of arsenic, and potential co-contaminants (Tseng 2002). Nutritional zinc deficiency has been suggested to be a possible enhancer of or co-factor in the pathogenesis of arsenic-induced vascular disease. This deficiency could result from marginal malnutrition in Taiwan and excess alcohol consumption in the German vintners (Engel *et al.* 1994). However, the dietary zinc intake in Taiwan exceeded the WHO (World Health Organization) recommendation for zinc. Although without convincing evidence, co-contaminantion of humic acid in the arsenical well water in Taiwan and sulfuric acid (used to treat sugar cane) in the arsenic-contaminated wine in Germany as a cause of Blackfoot disease cannot be completely ruled out (Tseng 2002; Klatsky 2006).

It is worth noting that peripheral vascular syndromes similar to Blackfoot disease have also been reported in Antofagasta, Chile, Toruku, Japan, and West Bengal, India (Das *et al.* 1995; National Research Council 1999; Guha Mazumder 2003). They included Raynaud's syndrome (smallest arteries constrict and lead to loss of blood supply in fingers, toes and other areas), acrocyanosis (persistent blue color of the skin due to vasospasm of small vessels of the skin), or arterial spasms in the finger and toes; gangrene of fingers, toes, or distal extremities; ischemia of the tongue; mesenteric artery thrombosis; coronary arterial occlusions; arterial intimal thickening; and extensive coronary or cerebrovascular occlusion (Rosenberg 1974; Borgono *et al.* 1977; Zaldivar and Guillier 1977; Das *et al.* 1995; Guha Mazumder 2003). It is very

plausible that Blackfoot disease is actually more widely present in arsenic exposed populations than previously thought (only in Taiwan and German).

Renal Effects

Although kidney is not a major target of arsenic toxicity (Cullen *et al.* 1995; Guha Mazumder 2003), renal dysfunction has been reported in some acute arsenic poisoning cases and a few chronic exposure studies. Renal capillaries, tubules, and glomeruli are targets of arsenic toxicity (Schoolmeester and White 1980; Squibb and Fowler 1983). Hematuria, proteinuria, casts in the urine, oliguria and overt renal failure are seen in patients acutely exposed to arsenic orally (Gerhardt *et al.* 1978; Prasad and Rossi 1995; Stephanopoulos *et al.* 2002 ; Hantson *et al.* 2003). Chronic arsenic exposure is associated with increased standard mortality ratio of renal diseases, which includes nephritis, nephrotic syndrome, and nephrosis (Chiu and Yang 2005). The mechanism of arsenic-associated nephrotoxicity is much less understood than other heavy metal-induced nephrotoxicity (Fowler 1993). However arsine induced nephrotoxicity is probably, at least partially, due to hemolysis and the consequent renal tubular damage from red blood cell breakdown products (Squibb and Fowler 1983).

Hematological Effects

Hemolysis is the main toxicity from arsine gas, which targets erythrocytes and hemoglobin. The mechanism of arsine-induced hymolysis is not clear, and contradicting evidence has been reported regarding the involvement of GSH (Blair *et al.* 1990; Hatlelid and Carter 1997; Winski *et al.* 1997), Na^+, K^+-ATPase pump (Hatlelid *et al.* 1995), and binding between hemoglobin and erythrocyte membrane (Rael *et al.* 2006) in various animal (*in vivo* and *in vitro*) and human (*in vitro*) studies. Arsine did not alter ATP levels or inhibit ATPase in human erythrocytes (Winski *et al.* 1997), but it increased the release of heme from human oxyhemoglobin (Rael *et al.* 2006).

Acute and chronic arsenite or arsenate poisoning may result in anemia, granulocytopenia, and leukopenia (Feussner *et al.* 1979; Tchounwou *et al.* 1999), possibly due to bone marrow suppression (Feussner *et al.* 1979). The anemia can be aplastic (defects in blood forming organs, *i.e.* bone marrow) or megaloblastic (with megalobists in bone marrow and large erythrocytes in the blood) (Westhoff *et al.* 1975; Lisiewicz 1993). Based on an *in vitro* model, the anemia may be due to arsenate/arsenite-induced alteration of human erythrocyte membrane integrity by depleting ATP in erythrocytes (Winski and Carter 1998). Alterations in heme biosynthesis were reported in Mexicans who were chronically exposed to arsenic (mainly arsenite and arsenate) in drinking water (Hernandez-Zavala *et al.* 1999).

Respiratory Effects

In acute arsenic poisoning, patients developed pulmonary edema, respiratory failure secondary to muscle weakness, apnea secondary to phrenic nerve damage, or adult respiratory distress syndrome (extreme difficulty in breathing) (Bolliger *et al.* 1992; Graeme and Pollack 1998).

In chronic poisoning through consuming arsenic-contained water, increased prevalences of abnormal chest sounds (crepitation and/or rhonchi), shortness of breath, chronic cough and bronchitis have been reported in West Bengal, India and Antogafasta, Chile (National Research Council 1999; Guha Mazumder *et al.* 2000; Milton *et al.* 2003). Bronchiectasis (a chronic dilation of bronchi or bronchioles), shortness of breath, and cough were more severe or common in individuals with arsenic-associated skin lesions, as compared to those who were exposed to arsenic but did not develop skin lesions and to those who were not exposed to arsenic (Guha Mazumder 2003; Guha Mazumder *et al.* 2005; von Ehrenstein *et al.* 2005). Although there is a scarcity of studies in mechanisms of arsenic-associated non-malignant respiratory diseases in

humans, a recent study suggested arsenic-associated pulmonary function decline was due to inflammation, and not through direct toxicity (De *et al.* 2004).

Immunological Effects

Despite the high number of studies of the effects of *in vitro* arsenic exposure on human immune cells (mainly lymphocytes and monocytes) (McCabe *et al.* 1983; Vega *et al.* 2004; Sakurai *et al.* 2005) and genotoxicity/clastoxity of *in vivo* arsenic exposure on human lymphocytes (Gonsebatt *et al.* 1992; Gonsebatt *et al.* 1997; Maki-Paakkanen *et al.* 1998; Mahata *et al.* 2003; Basu *et al.* 2004; Basu *et al.* 2005; Ghosh *et al.* 2006), the immunological effects of *in vivo* arsenic exposure in human have only been reported in a few studies (Gonsebatt *et al.* 1994; Yu *et al.* 1998; Soto-Peña *et al.* 2006a).

Increased arsenic ingestion via drinking water is associated with decreased lymphocyte proliferation in response to *in vitro* phytohemaglutinin stimulation (Gonsebatt *et al.* 1994; Soto-Peña *et al.* 2006b). Both *in vivo* (Gonsebatt *et al.* 1994) and *in vitro* (Vega *et al.* 2004) arsenic-exposure-induced proliferation suppressions were more significant in lymphocytes from women than lymphocytes from men. Correlations between labeling index and mitotic index of lymphocytes from *in vivo* arsenic exposed individuals showed that progression from the initial S- to M-phase was altered by arsenic (Gonsebatt *et al.* 1994). Delay in cell cycle kinetics was also observed after *in vitro* exposure to arsenic (Gonsebatt *et al.* 1992).

Peripheral mononuclear cells from individuals who drank arsenic-contaminated water showed altered cytokine secretion, including decreased IL-2 (interleukin-2) secretion from lymphocytes, and increased GM-CSF (granulocyte-macrophage colony-stimulating factor) secretion from activated macrophage (Soto-Peña *et al.* 2006b).

Healthy individuals exposed to arsenic via drinking water displayed arsenic-associated trends toward decreased T helper cell number in peripheral blood and decreased T helper cell/cytotoxic T cell ratio (Soto-Peña *et al.* 2006b), while these effects became statistically significant in arsenic-associated Bowen's disease patients compared to controls (Yu *et al.* 1998) and in a study using *in vitro* arsenic exposure (Vega *et al.* 2004).

It is possible that arsenic simultaneously contributes to inflammation and immuno-suppression, which could explain arsenic-associated increases in the incidence of asthma, allergies (heightened immune response) and parasitic infection (suppressed immune response) (Soto-Peña *et al.* 2006a). The arsenic contribution to chronic inflammation is supported by increases in GM-CSF secretion by macrophages (Soto-Peña *et al.* 2006a). Arsenic association with immuno-suppression was supported by evidence of decreases in T helper cells (Yu *et al.* 1998; Soto-Peña *et al.* 2006a), in T helper cell/cytotoxic T cell ratio (Yu *et al.* 1998; Soto-Peña *et al.* 2006a), in lymphocyte proliferation (Gonsebatt *et al.* 1994; Soto-Peña *et al.* 2006a), and in lymphocyte activation via inhibition of IL-2 secretion (Soto-Peña *et al.* 2006a).

Neurological Effects

Both central nervous system and peripheral nervous system can be affected by arsenic (Rahman *et al.* 2001; Mukherjee *et al.* 2003), and symptoms vary among individuals (Rahman *et al.* 2001; Ratnaike 2003). In addition to neuropathy observed in arsenic-exposed children, adolescents and adults (Cullen *et al.* 1995; Greenberg 1996; McFall *et al.* 1998; National Research Council 1999; Mukherjee *et al.* 2003), reduced intellectual function was reported in children exposed to arsenic in drinking water (Wasserman *et al.* 2004). The recovery of arsenic-associated neurological effects can take years after the cessation of both acute and chronic

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arsenic exposure (Wax and Thornton 2000; Guha Mazumder *et al.* 2001; Ratnaike 2003; Hafeman *et al.* 2005).

In acute arsenic poisoning, the most common nervous system symptom is peripheral neuropathy (damage to the peripheral nervous system), and encephalopathy (any disease of the brain that alters brain function or structure) is also often observed (Ratnaike 2003). Peripheral nervous system symptoms include sensory dysesthesias (diminished senses), ascending weakness, and flaccid paralysis. Central nervous system symptoms include headache, mild confusion, altered mental status, encephalopathy, seizures, and coma (National Research Council 1999). The most common long-term neurological effect following acute arsenic exposure is polyneuropathy (a disease involving several nerves), resulting from direct arsenic toxicity to peripheral nerve cell bodies (Cullen *et al.* 1995; National Research Council 1999).

Sub-acute or chronic arsenic exposures cause from sub-clinical to overt peripheral neuropathy (National Research Council 1999; Hafeman *et al.* 2005). Demyelinating peripheral neuropathy is considered a classical hallmark of chronic arsenic exposure (Greenberg 1996; McFall *et al.* 1998). Main histological changes are demyelination and axonopathy (Rodriguez *et al.* 2003). Sensory polyneuropathy is common, but pure motor type is rare from chronic arsenic exposure through drinking water (Rahman *et al.* 2001; Mukherjee *et al.* 2003). Symptoms of chronic arsenic-associated neurological effects in general are more severe in the lower extremities than in upper extremities (Mukherjee *et al.* 2003; Hafeman *et al.* 2005), which is consistent with neurons with long axons being more sensitive to arsenic than neurons with short axons (Rodriguez *et al.* 2003). Neurological symptoms from sub-acute and chronic exposure may include tingling, decreased sensation or numbness or pain in the feet and later in hands, lose of ankle jerk reflex, pain in limbs (especially feet), general muscular weakness, tremor,

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decreased vision and hearing (not due to ophthalmologic or aural causes), mood change, headache, depression, and insomnia or somnolence (Rahman *et al.* 2001; Mukherjee *et al.* 2003).

Endocrinological Effects

The positive correlation between chronic arsenic ingestion and the occurrence of diabetes mellitus has been reported in Taiwan and Bangladesh (National Research Council 1999; Navas-Acien *et al.* 2006). Studies in Taiwan (Lai *et al.* 1994; Tseng *et al.* 2000) found the increased prevalence of non-insulin-dependent diabetes mellitus (defined by fasting blood glucose test) in Taiwanese ingesting inorganic arsenic in drinking water. Furthermore, dose-response relationships were found between arsenic exposure from well water and the prevalence ratio of diabetes mellitus in Bangladesh, whether the criteria of diabetes mellitus was glucosuria (Rahman *et al.* 1999), self-reported symptoms, glucosuria, or oral glucose tolerance test (Rahman *et al.* 1998). The correlation between exposures to low concentrations of arsenic and diabetes is unclear, as two studies in the United States of America, using death certificate or self-reported cases as diabetes criteria, did not show significant increased risk of diabetes with arsenic exposure (Lewis *et al.* 1999; Zierold *et al.* 2004).

Possible mechanisms of arsenic-associated diabetes have been recently reviewed (Tseng 2004). One of the possible mechanisms is via arsenic binding of vicinal dithiol groups in hormone receptors, particularly steroid receptors. In cultured cells, 100 µM As(III) showed inhibition on all steroid binding to glucocorticoid receptors, but not to androgen, estrogen, mineralocorticoid or progesterone receptors (Lopez *et al.* 1990). Oxidative stress and alterations in signal transduction related to insulin resistance and β cell dysfunction are also supported by epidemiological and experimental data (Tseng 2004). For example, arsenic-induced oxidative stress and activation of NFκB (nuclear factor kappa B) is associated with insulin resistance and β

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cell dysfunction. Chronic-arsenic-exposure-increased expression of TNFα (tumor necrosis factor α) and IL-6 (interleukin -6) can contribute to insulin resistance (Lagathu *et al.* 2003; Wu *et al.* 2003). Furthermore, the expression of PPARγ (peroxisome proliferators-activated receptor γ), a nuclear hormone receptor important for activating insulin action, is inhibited by arsenic (Wauson *et al.* 2002).

Reproductive and Developmental Effects

The evidence of arsenic-induced adverse reproductive and developmental effects is abundant in laboratory animals (for review, see (DeSesso *et al.* 1998; Wang *et al.* 2006) and Chapter 2 of this dissertation), but is very limited in human subjects.

Exposure to arsenic-contaminated drinking water was associated with (1) increased rates of spontaneous abortion in Hungary (Borzsonyi *et al.* 1992), Argentina (Hopenhayn-Rich *et al.* 2000), Bangladesh (Ahmad *et al.* 2001) and United States of America (Aschengrau *et al.* 1989), (2) increased rates of stillbirths in Hungary (Borzsonyi *et al.* 1992) and Bangladesh (Ahmad *et al.* 2001), (3) increased rates of preterm birth in Bangladesh (Ahmad *et al.* 2001), and (4) increased rates of lateterm fetal, neonatal, and post-natal mortality in Chile (Hopenhayn-Rich *et al.* 2000). However, some of these data are questionable. For example, the report of arsenic-induced high spontaneous abortion rates in Hungary (Borzsonyi *et al.* 1992) lacks details of arsenic concentrations and comparability of the study groups (DeSesso *et al.* 1998). In a study conducted in Massachusetts, the increase in the frequency of spontaneous abortion was associated not only with high levels of arsenic but also with detectable levels of mercury in the same water (Aschengrau *et al.* 1989).

Two reports showed arsenic-increased congenital cardiovascular anomalies in the United States of America (Zierler *et al.* 1988; Engel and Smith 1994). Zieler *et al.* reported (1998) that

the association was only found between arsenic exposure and coarctation of the aorta, a lesion not reported in arsenic-treated animals, and only in the subgroup analysis. Zierler *et al.* concluded that their study did not provide evidence of arsenic-induced cardiac teratogenicity (Zierler *et al.* 1988). Similarly, Engel and colleges (1994) reported a positive association between arsenic exposure and congenital anomalies of the heart and of the circulatory system, but did not conclude a causal relationship.

Summary

The carcinogenic effects of arsenic have been observed in lung, skin (Bowen's disease, basal cell carcinoma, and squamous cell carcinoma), urinary bladder (transitional cell carcinoma), as well as in liver (angiosarcoma), breast, colon, stomach, parotid gland, nasophynx, larynx, buccal cavity, leukemia and other hematopoietic cancers. The non-cancer effects of arsenic included cutaneous, gastrointestinal, cardiovascular, hematological, respiratory, immunological, neurological, endocrinological, reproductive and developmental effects. Most of the effects were observed using data from ecological and cohort studies of arsenic-exposed populations compared to control populations. Some of the effects were further studied *in vitro* using human cell culture or animal models.

2. TOXIC AND CARCINOGENIC EFFECTS OF ARSENIC IN RATS

Toxic effects of arsenic in rats

Rats have been widely used as a model to study the mechanisms of arsenic toxic effects observed in humans. The oral rat LD_{50} values are approximately 100 mg/kg for As(V) (Brown *et al.* 1997), 41 mg/kg for As(III) (Kitchin and Brown 1994), 961 mg/kg for MMA(V) (Gaines and

Linder 1986), and 644 mg/kg for DMA(V) (Gaines and Linder 1986). The following review of the toxic effects of arsenic is present by body system.

Renal effects

Both inorganic and organic arsenicals are nephrotoxic. In a 2-year bioassay study, female and male Sprague-Dawley (SD) rats that drank water containing As(III) showed dose-dependent renal pathological changes (Soffritti *et al.* 2006). The changes, most severe in the 200 mg/L group and less in the 100 and 50 mg/L groups, included inflammation, tubular enlargement with deposits of casts and fibrosis around glomeruli with distension of Bowman's space (Soffritti *et al.* 2006). In male SD rats which were exposed to 50 ppm As(V) via feed for 10 weeks, renal proximal tubular cells had moderate mitochondrial swelling by transmission electron microscopy (Mahaffey *et al.* 1981). Furthermore, As(V) increased urinary excretion of uroporphyrin and to a lesser extent coproporphyrin. Although the kidney weight and kidney weight/body weight ratios were not affected, total renal copper was twice that of control. The mechanism of As(V) or As(III)-induced copper accumulation in renal cortex and medulla remains unclear (Ademuyiwa *et al.* 1996; Ademuyiwa and Elsenhans 2000). Some researchers suggested diminished biliary arsenic excretion, which is secondary to arsenic-decreased hepatic copper from lowered food consumption, may be responsible (Yu and Beynen 2001). Others suggested arsenic and copper interaction in the kidney, rather than hepatic or biliary mechanisms, was the cause of arsenic-elevated renal copper levels (Ademuyiwa and Elsenhans 2000).

Arsine gas (AsH_3) is known to cause acute toxicity, including intravascular hemolysis with dark red urine (hematuria) and death by acute oliguric renal failure in humans. Nephrotoxicity of arsine was investigated by exposing cultured rat renal cells and *in situ* isolated kidney to arsine (Ayala-Fierro *et al.* 2000). The authors concluded that arsine nephrotoxicity was from the

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exposure to arsenic dissolved in plasma and then by hemolytic products from lysed erythrocytes. Damage was first seen in the endothelial cells from the glomerular capillaries and peritubular microvessels, which caused impeded filtration and edema. Hemolytic products reach the kidney and occluded the glomerulus, further compromising filtration.

Nephrotoxicity of DMA(V) was suspected as the main cause of death in male and female F344/DuCrj rats exposed to DMA(V) orally at levels of 57, 85, and 113 mg/kg for 4 weeks (Murai *et al.* 1993). The females were more sensitive to DMA(V) toxicity, based on the mortality rate and survival time. Both sexes showed dose-dependent decreases in body weight and survival rates. By light microscopy, renal proximal tubule degeneration and necrosis, hyperplasia of the epithelium covering the renal papillae, and papillary necrosis were seen. Because the extensive proximal tubular necrosis was only seen in rats that died before the end of the 4-week treatment, the authors concluded nephrotoxicity of DMA(V) was the main cause of death.

NCI-Black-Reiter rats, which cannot synthesize α_{2u}-globulin in the kidney, are similarly susceptible to the nephrotoxicity of DMA(V) as other stains of rats that synthesize α_{2u}-globulin (Vijayaraghavan *et al.* 2001). Aged NCI-Black-Reiter rats, 46 weeks old at the beginning of the experiments, were given 0, 5, 10, or 20 mg/kg BW of DMA(V) by gavage, once a day, 5 days a week, for 4 weeks. Although apoptotic index of the kidney and tubular necrosis in the renal cortex were not increased by DMA(V), other toxic signs (dilation of tubules, vacuolated degeneration in tubular cells, and congestion) were observed in 10 and 20 mg/kg groups, and these morphologic changes were more severe in the latter group. Also observed in the kidney were increased free radical-induced DNA damage [measured by 8-Hydroxy-2'-deoxygaunosine (8-OHdG) levels] at 10 and 20 mg/kg and increased cell proliferation [measured by proliferating

cell nuclear antigen (PCNA)-positive cells] at 5, 10 and 20 mg/kg. Although rats in the 5 mg/kg group showed an increase in cell proliferation but not in DNA damage, the authors stated the increased renal cell proliferation may be compensatory proliferation for lost cells. This study also showed no correlation between DNA damage and apoptosis in the tested conditions, and α_{2u}-globulin had no influence on the susceptibility of rats to DMA(V) renal toxicity.

Hepatic effects

Gallium arsenide (GaAs) is a group IIIa-Va intermetallic compound, and used commonly in the electronic and telecommunication industries. Occupational exposure to GaAs could occur in workers in these industries. Hepatic toxicity was observed in male Wistar albino rats, after exposures to 70 µmol/kg GaAs orally once a day, 5 days a week for 4 weeks (Flora *et al.* 1999). Serum aspartate aminotransferase (AST) activity was increased, as was the concentration of malondialdehyde in liver, indicating increased hepatic lipid peroxidation.   Serum alanine aminotransferase (ALT), hepatic ALT, hepatic AST and hepatic GSH were not affected. The toxicity of GaAs, in general, is due to the combined effects of gallium and arsenic (Carter *et al.* 2003).

Kitchin *et al.* (1992) developed a screen of four *in vivo* biochemical parameters to predict and mechanically classify chemical carcinogenicity. Hepatic DNA damage, assessed by alkaline elution, is a marker for possible initiation of carcinogeneis and for *in vivo* genotoxicity. Ornithine decarboxylase activity (ODC) and cytochome p450 content are markers for promotion of carcinogenesis, and increases in both suggests a Cell Proliferation Type (changes in ODC and p450) of non-genotoxic carcinogen. Hepatic ODC and serum ALT are markers for regenerative hyperplasia (cell toxicity and death), and induction of both suggests a Cell Toxicity Type (changes in ALT and ODC) of non-genotoxic carcinogen.

In a study using rats, the above four parameters plus hepatic reduced glutathione (GSH) and heme oxygenase were used to classify arsenic carcinogenicity (Brown and Kitchin 1996). The GSH and heme oxygenase measurements were added to the classification scheme because arsenic is known to induce heme oxygenase and to decrease GSH. The levels of GSH serve as an indicator of cellular protection against reactive stress. Female SD rats were given 1/25 oral LD_{50} (sodium arsenite 1.6 mg/kg), 1/5 oral LD_{50} (sodium arsenite 8.2 mg/kg), or 3/5 oral LD_{50} (sodium arsenite 24.6 mg/kg) in two doses at 21 and 4 hours prior to sacrifice via oral gavage (Brown and Kitchin 1996). Sodium arsenite increased hepatic ODC activity at 1.6 and 24.6 mg/kg and hepatic heme oxygenase activity at 8.2 and 24.6 mg/kg, but did not alter other parameters. Although the data could not classify As(III) as a rodent carcinogen or into either the Cell Proliferation Type or Cell Toxicity Type classifications (considering cytochrome P450 and serum ALT were not altered), the increase in hepatic ODC activity suggested that As(III) may be a promoter, not an initiator, of carcinogenesis.

In a later study utilizing the same protocol, sodium arsenate, MMA(V), and DMA(V) were used (Brown et $al.$ 1997). Female SD rats were given 1/5 or 3/5 LD_{50} by oral gavage divided in two doses at 21 and 4 hours prior to sacrifice (Brown et $al.$ 1997). For the 1/5 LD_{50} level, the doses were 20 mg/kg for As(V), 226 mg/kg for MMA(V), and 129 mg/kg for DMA(V). Arsenate exposure increased hepatic heme oxygenase concentrations at 60 mg/kg, but did not alter other parameters. At 1/5 LD_{50}, MMA(V) decreased serum ALT, and DMA(V) increased cytochrome P450. Both MMA(V) and DMA(V) at 3/5 LD_{50} (679 mg/kg and 387 mg/kg, respectively) decreased serum ALT, decreased hepatic GSH, and increased hepatic cytochrome P450. Heme oxygenase was increased significantly by DMA(V) and insignificantly by MMA(V). The same pattern of changes from MMA(V) and DMA(V) exposure suggested that

DMA(V) or even its metabolites (such as trimethylated arsenic species) may be the direct cause of MMA(V) effects. The MMA(V) and DMA(V)-induced *in vivo* decrease in serum ALT may result from direct inhibition, which is supported by *in vitro* data. The decreased hepatic GSH in both MMA(V) and DMA(V)-treated rats may be from the use of GSH for arsenic methylation and/or oxidative stress defense.

The effects of four major forms of arsenic [As(III), As(V), MMA(V), and DMA(V)] on six hepatic biochemical parameters in rats are summarized in **Table 2** [summarized from (Brown and Kitchin 1996; Brown *et al.* 1997)]. Inorganic arsenic and methylated arsenic investigated in this study showed distinct patterns. Inorganic As(III) and As(V) both significantly increased heme oxygenase concentration. MMA(V) and DMA(V) both significantly increased cytochrome p450 concentration, and decreased GSH and ALT concentrations, although heme oxygenase induction by MMA(V) was not significant.

The effects of sodium arsenate on the expression of cancer-related genes were studied in rat liver (Cui *et al.* 2004). Genes associated with both tumor progress and suppression showed changes. Male 7-week old SD rats were exposed to 0, 1, 10 or 100 ppm sodium arsenate (Na$_2$HAsO$_4$·7H$_2$O) in drinking water for either 1 or 4 months (Cui *et al.* 2004). Gene expression was measured at the mRNA level by quantitative real time RT PCR on 1-month and 4-month groups, using β-actin as an internal control (reference gene), and at the protein level by immunohistochemistry on 4-month group. Although arsenic concentrations in the liver were increased, there was no difference between the 4- and 1-month exposure groups. Histological examination was performed on livers in the 4-month exposure group, and showed dose-related disrupted hepatic cords, dilated sinusoids and fatty infiltration (steatosis). Arsenic-associated changes in the mRNA levels of cyclin D1, p27^{Kip1}, integrin-linked kinase (ILK, whose over-

expression facilitates anchorage-independent cell growth), phosphatase and tensin-homolog deleted on chromosome 10 (PTEN, inhibiting nuclear β-catenin accumulation and whose expression is often decreased in tumors), β-catenin (decreasing intercellular adhesion and whose expression is increased in tumors) are summarized in **Fig 1**. Protein level changes were generally the same as mRNA level changes. As(V) at low doses seems to simultaneously increase negative ($p27^{Kip1}$) and positive (cyclin D1, ILK) cell cycle regulators. Furthermore, both PTEN (considered to be a negative tumorigenic factor) and β-catenin (positive tumorigenetic factor) were suppressed by As(V). Cancer-related genes investigated in this study did not show a clear dose-or time-dependent change. The data suggested that As(V)-induced alteration of gene expression is a complicated process, and the authors speculate that As(V) affects gene expression through interfering with DNA methylation patterns and /or transcription factors like activation factor-1 and nuclear factor-kappaB (NF-κB).

Cardiovascular effects

Chronic exposure to As(V) in drinking water alters cardiovascular reactivity. Male SD rats were given 50 μg/ml of arsenic (as sodium arsenate) in drinking water for 320 days (Boscolo *et al.* 1982; Carmignani *et al.* 1983). Vascular reactivity to vascular beta-adrenoceptor stimulation was increased, while it was decreased to angiotensin I. Other parameters of cardiovascular function were unchanged. For example, systolic and diastolic blood pressure levels, electrocardiogram, cardiac inotropism and chronotropism, were normal. The cardiovascular reactivity to noradrenaline, acetylcholine (Ach), histamine, serotonin, bradykinin and angiotensin II was also unchanged. The authors concluded that chronic arsenic exposure did not affect the baroreflex sensitivity, but was able to induce sympathetic hyperactivity or hypersensitivity, or both, possibly through an antivagal action.

In another study, male Wistar rats were exposed to 50 μg/ml of sodium arsenite, As(III), or 50 μg/ml of sodium arsenate, As(V), for 18 months (Carmignani *et al.* 1985). Cardiovascular parameters and responses to substances that alter blood pressure were measured in anesthetized rats. Rats exposed to As(III) showed reduced stroke volume and cardiac output and increased vascular resistance, while rats receiving As(V) showed no cardiovascular change. On the other hand, As(V)-treated rats showed increased response in blood pressure to the following substance/procedure: clonidine, hexamethonium, isprenaline (all three decrease blood pressure), and vagotomy (increases blood pressure). The effects of tyramine, which increases blood pressure, was decreased in both As(V)- and As(III)-treated rats. As(III) administration increased vascular resistance, with consequent decreases of both stroke volume and cardiac output, and this might explain the reduced pressure responses to tyramine. The authors stated As(V) may increase the responses from the activation of vascular β-adrenoceptors, sympathetic nerve hyperactivity, and possibly vagal effects.

Because arsenic has been associated with the development of hypertension in human beings (Ng *et al.* 2003; Yoshida *et al.* 2004), arsenic effects in vasorelaxation have been investigated in rats. Arteriolotoxicity of inorganic arsenic was studied *ex vivo* in Wistar-Barby female rats, which were dosed orally with arsenic trioxide (As_2O_3), 5 days a week, by a stomach tube (Bekemeier and Hirschelmann 1989). The resting perfusion pressure (p_0), maximum perfusion pressure (p_{max}), and norepinephrine-induced maximum perfusion pressure increase [E_{Amax} = (p_{max}-p_0)] of the arteries of isolated, perfused hind leg were compared between treated and control rats. $E_{Amax}\%$ was designated as the maximum perfusion pressure upon infusing norepinephrine in arsenic-treated rats in relation to E_{Amax} of the respective control rats (=100%). While rats exposed to 15 mg/kg As_2O_3 up to 7 days showed no difference in p_0, E_{Amax}, or

$E_{Amax}\%$, 14- and 28-day exposure groups showed decreased E_{Amax} and $E_{Amax}\%$. The decrease in $E_{Amax}\%$ indicated the reduction of the ability of the blood vessel to constrict. Rats in 14-day exposure group also showed reduced mobility, shaggy coats, diarrhea and bloody stools. Although this study suggested that arsenic exposure reduced the ability of blood vessels to constrict, the high doses used (toxic to general health) may not be relevant to human environmental exposure.

Another study of arsenic effects on vasomotor tone (vasorelaxation) was conducted in isolated aortic rings from rats (Lee *et al.* 2003). The main pathways investigated are diagrammed in **Fig 2**. It is known that nitric oxide synthase in endothelial cells (eNOS) can produce nitric oxide (NO) (Moncada *et al.* 1991). Nitric oxide can activate guanylate cyclase within the smooth muscle cells, and cause vascular relaxation through the accumulation of guanosine 3', 5'-cyclic monophosphate (cGMP), which was synthesized by guanylate cyclase (Moncada *et al.* 1991). In the Lee *et al.* study (2003), aortic rings were collected from mature adult male SD rats and these isolated rings were exposed to arsenic in an organ-bath system for 14 hours. Acetylcholine (Ach)-induced relaxation was decreased by exposure to As(III) at 10 µM or higher, but other forms of arsenic [As(V), MMA(V), and DMA(V)] caused no change at 100 µM. To investigate the mechanism, only As(III) was used. As(III) decreased eNOS activity, basal and induced cGMP concentrations, SNP (sodium nitroprusside, an NO donor)-induced relaxation, as well as cGMP-induced relaxation. Although arsenic-induced superoxide production has been reported in cultured cells (Liu and Jan 2000; Liu *et al.* 2001b), it was not observed in this study. From the results in this study, it appears that superoxide, interacting with NO and blocking the NO pathway, does not play an important role in As(III)-decreased Ach-induced relaxation.

To confirm this *in vitro* observation, blood pressure was measured in conscious rats before

and after As(III) and Ach treatment (Lee *et al.* 2003) Basal blood pressure was not affected by

an intravenous bolus of As(III) at 1mg/kg, but the reduction of blood pressure induced by Ach

was suppressed by As(III). This was in agreement with *in vitro* As(III)-suppressed

vasorelaxation induced by Ach (Lee *et al.* 2003).

In summary, exposure to As(III) caused altered vascular tone by decreasing vasorelaxation.

The inhibitory effects associated with arsenic exposure may be due to suppression of NO

production medicated by eNOS inhibition in endothelial cells and interference with cGMP-

dependent relaxation machinery in arterial smooth muscle cells.

Hematological effects

Platelet aggregation is affected by arsenic, and both increased and decreased aggregation

have been reported by arsenic treatments (Kumar *et al.* 2001; Lee *et al.* 2002). Platelet

aggregation was greater in platelets collected from rats exposed to arsenic trioxide (As_2O_3) than

that from untreated rats. Adult male SD rats were given a subcutaneous injection of 0.66 mg

As_2O_3 /kg BW (one tenth of LD_{50}), and platelets were then collected and stimulated by

aggregation initiators. This *in vivo* arsenic treatment decreased platelet aggregation induced by

in vitro exposures to adenosine diphosphate (ADP), arachidonic acid, epinephrine, and collagen.

Furthermore, the cytosolic concentration of cyclic adenosine monophosphate (cAMP) in platelets

was also decreased. In contrast, platelet aggregation and thrombus formation were enhanced in

platelets collected from SD rats exposed to sodium arsenite, compared to platelets collected from

untreated rats (Lee *et al.* 2002). Rats were given sodium arsenite in drinking water, ranging from

2 to 25 ppm, for 4 weeks. Platelets collected from rats *in vivo* exposed to arsenic showed a dose-

dependent increase of *in vitro* thrombin-challenged platelet aggregation and P-selectin

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expression. Arterial thrombus formation was also increased in arsenic-exposed rats, as evidenced by shortened time to occlusion and increased incidence of irreversible occlusions measured in exposed carotid arteries in anesthetized rats (Lee *et al.* 2002).

In a chronic toxicity/carcinogenicity study of 2 year duration, with exposure up to 200 ppm of TMAO in water, there were no changes in hematological or serum biochemical findings in male F344/DuCrj rats (Shen *et al.* 2003b). Hematologic parameters measured were WBC and RBC, and the serum biochemistry parameters measured consisted of total protein, glutamic oxaloacetic transminase, glutamic-pyruvic transaminase (GTP), and blood urine nitrogen (BUN).

Pulmonary effects

In lung, DNA damage was observed after DMA(V) exposure (Yamanaka *et al.* 1989; Brown *et al.* 1997), but not after exposure to As(III) (Brown and Kitchin 1996). Young male Wistar rats were administered sodium dimethylarsinic acid, DMA(V), at 1950 mg/kg BW (Yamanaka *et al.* 1989). At 12 hours after administration of DMA(V), DNA single strand breaks were detected in lung. Observed DNA damage may have been induced by dimethylarsine, a gaseous metabolite of DMA(V), which was identified in the expired air of treated rats. This is supported by *in vitro* experiments in which DNA single-strand breaks were seen in dimethylarsine-treated, but not DMA(V)-treated, nuclei isolated from lung of mice (Yamanaka *et al.* 1989).

Pulmonary DNA damage, ODC activity, GSH concentration, cytochrome p450 concentration, and heme oxygenase activity were not altered in rats exposed to As(III) orally (Brown and Kitchin 1996). Ninety-day-old female SD rats were exposed to sodium arsenite at 1/25 oral LD_{50} (1.6 mg/kg), 1/5 oral LD_{50} (8.2 mg/kg), or 3/5 oral LD_{50} (24.6 mg/kg). For this study, sodium arsenite was dissolved in water and administered orally in two doses, 21 and 4 hours prior to sacrifice.

In another study, 90-day-old female SD rats were given DMA(V) at 1/5 or 3/5 rat LD_{50} (129 or 387 mg/kg, respectively) orally in two doses, 21 and 4 hours prior to sacrifice (Brown *et al.* 1997). In lung tissue, GSH was not altered. However, pulmonary DNA damage was increased at 387 mg/kg, and ODC activity was increased at both 129 and 387 mg/kg. Since there was no decrease of rat hepatic ODC caused by either As(V), MMA(V) or DMA(V) administration to rats, the *in vivo* ODC inhibition appears to be DMA(V)- and organ-specific. On the other hand, *in vitro* data showed lung and hepatic ODC inhibition by As(III), MMA(V) and, in particular, by DMA(V). A possible explanation of DMA(V)'s organ specific inhibition on pulmonary ODC activity offered by Brown *et al.* (1997) was that the lung generates different arsenic metabolites and free radicals that mediate both inhibition of rat lung ODC and pulmonary DNA damage. This spectrum of metabolites may not be generated as readily in rat livers as in rat lung, and hence neither DNA damage nor decreased ODC enzyme activity was observed in rat liver.

Immunological effects

Studies of immunotoxic effect of arsenic exposure in rodents have been very limited, and only inorganic arsenic has been examined.

Subchronic low-level exposure to arsenic can affect immune responses of rats (Schulz *et al.* 2002). Young male Wistar rats, 4 weeks old at the beginning of the treatment, were given 3.33, 6.66, or 26.6 mg $NaAsO_2$/kg/day by gavage,5 days per week, for 4 weeks. The humoral immune response was not significantly altered, even at the highest dose. There were significant decreases in the weights of thymus, spleen and adrenals, as well as the maximum delayed type hypersensitivity reaction, as measured by the footpad swelling, at the highest arsenic dose.

Altered immune function and structure of immune system tissue components was noted in rats exposed to GaAs (Flora *et al.* 1999). Gallium arsenide is a chemical compound composed of

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gallium and arsenic, and an important semiconductor. Gallium arsenide integrated circuits are commonly used in mobile phones, satellite communications, microwave point-to-point links, and some radar systems. Four weeks of oral exposure of 70 μmol/kg GaAs orally once a day, 5 days a week, caused decreases in spleen cellularity and primary IgM antibody formation cell response to challenge with sheep red blood cells in male Wistar albino rats. The delayed type hypersensitivity reaction, measured as foot pad swelling, was not altered by exposure to GaAs.

Neurological and behavioral effects

Inorganic arsenic in water increases lipid peroxidation in young growing rat brains at concentrations as low as 0.05 ppm (Chaudhuri *et al.* 1999). Lipid peroxidation in brain was of interest because arsenic-induced lipid peroxidation has been found in liver, kidney, and heart (Ramos *et al.* 1995), and brain is suspected to have higher sensitivity to lipid peroxidation than other organs. Brain is thought to be particularly vulnerable to oxidative damage because of its high rate of oxygen consumption, the high levels of polyunsaturated fatty acids, low levels of peroxisomal catalase and glutathione peroxidase activity, low levels of mitochondrial superoxide dismutase and catalase activities, and dependence of neurons on glial cells for synthesis of Cys from Cys-Cys and non regenerative nature of neuron (Chaudhuri *et al.* 1999). Furthermore, unlike lipid peroxidation in liver, which uses only NADPH as a reducing source and both Fe^{2+} and Fe^{3+} ion, lipid peroxidation in brain uses either NADPH or NADH as the reduction source and only Fe^{2+} ion. In Chaudhuri *et al.*'s study (1999), 4-week-old male SD rats were exposed to 0 (control), 0.05, 0.10, or 0.30 ppm Na_3AsO_4 in drinking water for 4 weeks. The brains of those young growing animals showed increased lipid peroxidation, decreased concentrations of GSH, and decreased activities of superoxide dismutase, catalase, and GSH reductase at all three tested concentrations. The activity of GSH peroxidase was not altered. These observations (decreased

GSH and inhibition of the defense enzymes) and/or generation of free radicals may be responsible for arsenic-induced lipid peroxidation in brain.

$NaAsO_2$ was given to male Wistar rats, 10 weeks old at the beginning of the treatment, for 4, 8, or 12 weeks by gavage on a 5 day per week schedule at 0, 6.6, 13.2, or 26.4 mg/kg/day (Schulz *et al.* 2002). Significant changes in behaviors (decreased horizontal ambulation in the open field and decrease in grooming activity) were only observed at the highest dose, equal to 15.4 mg arsenic/kg/day, at 4 weeks, but not at 8 or 12 weeks. No evidence of neurotoxicity, measured by electrophysiological parameters on anesthetized rats, was observed. The authors suggested the arsenic-induced hypoactivity may be from arsenic effects on dopaminergic neurotransmission.

In Rodriguez's study (2002), pregnant Sprague-Dawley rats were exposed to arsenite (36.60 mg arsenic/L in drinking water) from gestation day 15 or postnatal day 1, until pups were 13 weeks old (Rodriguez *et al.* 2002). Pups received the same concentration of arsenic in the drinking water as their mothers. When maternal behavior was tested on postnatal days 2, 6, and 10, there were no differences in exposed and controlled groups. Spontaneous locomotor activity was measured at the end of arsenic exposure (13 weeks old) and one month after the cessation of arsenic exposure (17 weeks old) in male, but not female, offspring. The numbers of errors in a delayed alternation task were increased in both groups with arsenic exposure started from gestation day 15 and from postnatal day 1, but an increase of rearing activity was only seen in groups exposed to arsenic from gestation day 15. The total arsenic content in brain was similar for both exposed groups and was significantly higher than that measured in the control group.

Reproductive and developmental effects

Both inorganic and organic forms of arsenic induce reproductive and developmental toxicity in rats, including malformations. The toxic effects depend on the form of arsenic, the dosing route, and the time (gestation date). Methylated or inorganic arsenic via drinking water or by repeated oral gavage induced male and female reproductive and developmental toxicities. Furthermore, at nonmaternally toxic levels, inorganic arsenic given to pregnant dams via drinking water affected fetal brain development and postnatal behaviors. However, arsenic given by repeated oral gavage to pregnant rats was not morphologically teratogenic. A detailed review is presented in Chapter 2.

Carcinogenic effects

Although epidemiological research showed strong evidence of arsenic carcinogenicity in humans, inorganic arsenic exposure studies in experimental animal carcinogenicity models have generally yielded negative results (National Research Council 1999; Kitchin 2001; Wang *et al.* 2002). With the use of inorganic arsenic metabolites (methylated arsenicals), researchers report independent studies in which various arsenicals behave as either promoters or complete carcinogens in rats at certain conditions, as detailed below.

Inorganic Arsenic

Inorganic arsenic as a promoter

Sodium arsenite promoted renal cancer in partially hepatectomized rats initiated by diethylnitrosamine (DEN) (Shirachi *et al.* 1983). In all four groups (control, DEN, arsenic, DEN+arsenic), male Wistar rats, weighing 70-100 g, underwent partial hepatectomy to stimulate hepatic regeneration. Eighteen to twenty four hours after the surgery, the DEN group and the

DEN+arsenic group were given DEN, an initiator carcinogen, by one-time i.p. injection at a dose of 30 mg/kg of body weight, while the control group received saline. Sodium arsenite was then given to the DEN+arsenic group and arsenic group in the drinking water at concentration of 160 ppm elemental arsenic for 175 days, which was equivalent to 8-9 mg/kg per rat per day, based on water consumption. After the 175-day exposure, all rats were sacrificed and major organs were weighed and examined by light microscopy. As(III) exposure decreased body weight in DEN+arsenic and arsenic groups, and this might be secondarily to decreased water consumption. Although this study was designed as an initiator-promoter model, utilizing partial hepatectomy to promote the development of hepatic tumors, there were no liver tumors in any group. Liver and kidney weights were not statistically different across groups. However, the DEN+arsenic group had a higher renal cancer frequency. More specifically, the renal tumor frequencies/n were 0/10, 1/7, 0/9, 7/10 in the control, DEN, arsenic, and DEN+arsenic groups, respectively. The pathological classification of renal tumors was not reported. This study suggested that As(III) increased the incidence of DEN-initiated renal tumors by acting as a promoter, but it is unclear if partial hepatectomy is a cofactor in this promoting effect.

Inorganic arsenic as a complete carcinogen

Inorganic arsenic, As(III) or As(V), given through diet or drinking water, for from 24 months to lifetime, traditionally did not act as a complete carcinogen in rats in several independent studies (for review, see (Wang *et al.* 2002)). Those studies were conducted before 1970, and lacked detailed information. However, recently one study showed that As(III) alone in drinking water increased incidences of pulmonary, renal and urinary bladder tumors, although not statistically significant, in SD rats (Soffritti *et al.* 2006)

After 2 years of exposure to sodium arsenite (NaAsO$_2$) in drinking water, the total number of malignant tumors (per 100 animals) was significantly increased in male SD rats in the 100 ppm

group (Soffritti *et al.* 2006). Although not statistically significant, the incidences of pulmonary adenomas and carcinomas, renal adenomas/papillomas and carcinomas, and urinary bladder carcinomas were increased in female and male rats exposed to up to 200 ppm As(III). These observations are important because these types of tumors are extremely rare (no more than 0.3%) in SD rats, and these types of tumors have been reported in humans exposed to inorganic arsenic in drinking water.

Adenocarcinomas were reported at the implantation sites in the stomach of rats given arsenic trioxide in a capsule by surgical implantation (Katsnelson *et al.* 1986). Male and female albino rats, weighing approximately 195 grams, were implanted with perforated polyethylene capsules, each containing 8 mg of arsenic trioxide in a fat-wax mixture as vehicle or control capsules containing the same mixture without arsenic. Out of 18 arsenic-exposed rats that survived, 17-24 months after implantation, two developed muconodular adenocarcinoma and one developed mucoid cystic adenocarcinoma in the stomach. No tumors were found in 9 rats implanted with control capsules.

Monomethylarsonic Acid [MMA(V)]
MMA(V) as a promotor

The promoting effect of MMA(V) on the development of liver tumors was shown by Nishikawa and colleagues using the Ito model (Ito *et al.* 1998) on male 10-week-old F344 rats (Nishikawa *et al.* 2000; Nishikawa *et al.* 2002). Two weeks prior to MMA(V) exposure, rats were initiated by an i.p. injection of DEN. MMA(V) was given at 100 ppm in the drinking water for 6 weeks. A week after the beginning of MMA(V) exposure, all rats underwent 2/3 hepatectomy. Glutathione-S-transferase placental form positive foci, preneoplastic lesions in rat

liver carcinogenesis, were increased by MMA(V) treatment. This study suggested that MMA(V) may act as a promoter in liver carcinogenesis.

MMA(V) as a complete carcinogen

Monomethylarsonic acid (MMA) alone did not induce tumors in rats in 2-year studies (Arnold *et al.* 2003; Shen *et al.* 2003a), with the exception of one report of thyroid cancer (National Research Council 1999).

Fischer F344 rats were treated with 50, 400, or 1300 ppm MMA in the diet (Arnold *et al.* 2003). The highest dose in the male and female rat groups was reduced to 1000 ppm during week 53 and then further reduced to 800 ppm during week 60 due to high mortality in the male rats. There were no increases of neoplasms in any organs. In another study, MMA(V) was given to 10-week-old male F344/DuCrj rats via drinking water at concentrations of 0, 50 and 200 ppm for 104 weeks (Shen *et al.* 2003a). Preneoplastic lesions were increased in both liver and urinary bladder, including increased glutathione S-transferase placental form positive (GST-P) foci in liver in 200 ppm group, and simple hyperplasia and PCNA-positive index in the urothelium. Nonetheless, there were no treatment-induced neoplasms in any organs.

Thyroid tumors were increased in male, but not in female, Sprague-Dawley rats fed 200 ppm MMA(V) in the diet for 2 years (National Research Council 1999). In another report, Fischer 334 (F344) rats fed MMA(V) for 2 years had an increased incidence of parathyroid gland tumors at high doses (400 and 1,300 ppm in males, and 1,300 ppm in females) when compared to control animals, but the statistical significance of incidence depended upon the statistical test used (National Research Council 1999).

Dimethylarsinic Acid [DMA(V)]

DMA(V) can act as a promoter, cocarcinogen, or complete carcinogen in laboratory animals in some circumstances. **Table 3** summarizes the results from a number of chronic exposure/carcinogenicity studies primarily, done using F344 or F344 crossbred rats, and varying levels of exposure to compound. Primary target organs are listed. Key studies are discussed in further detail following this table.

DMA(V) as a promoter

Yamamoto and colleagues conducted a multiorgan bioassay of arsenic carcinogenicity in male F344/DuCrj rats given various concentrations of DMA(V) in the drinking water for 24 weeks in a DMDBD model (using five mutagens as an initiator) (Yamamoto *et al.* 1995; Yamamoto *et al.* 1997). Animals in the initiated groups received five mutagens. They were DEN (i.p. at the beginning of the experiment), N-methyl-N-nitrosourea (MNU) (i.p. at days 5, 8, 11 and 14), and 1,2-dimethylhydrazine (DMH) (s.c. at days 18, 22, 26 and 30), n-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) (in the drinking water during weeks 1 and 2), and N-bis(2-hydroxypropyl)nitrosamine (DHPN) (in the drinking water during weeks 3 and 4). After a 2-week interval, rats were given 0, 50, 100, 200, and 400 ppm DMA(V) respectively. Uninitiated rats, which were not given DMDBD treatment, received 100 or 400 ppm DMA(V). DMA-increased tumor induction was observed in the urinary bladder, kidney, liver, and thyroid gland (**Table 4**). Uninitiated rats, that received only DMA(V), had no preneoplastic or neoplastic changes in any organs examined in this study. This study suggested that DMA(V) may act as a promoter.

The ability of DMA(V) to act as a promoter in BBN-induced urinary bladder tumors has been reported in three studies. In each study, the same protocol for initiation was used: rats were given 0.05% BBN in water for 4 weeks, followed by various concentrations of DMA(V) in

water. In the study by Wanibuchi et al. (1996), male F344 rats were given 0, 10, 25, and 100 ppm DMA(V) in water for 32 weeks initiation with BBN, a mutagen and bladder carcinogen (Wanibuchi *et al.* 1996). The incidences of BBN-induced urinary bladder hyperplasia (papillary or nodular), papilloma, and carcinoma were increased by DMA(V) at 25 ppm and higher. Furthermore, the multiplicity of tumors (number of bladder papillomas and carcinomas per rat) was increased starting at as low as 10 ppm. Rats exposed to 0 and 100 ppm DMA(V), but without BBN initiation, did not have preneoplastic lesions or tumors in urinary bladder.

Li and colleagues conducted a study similar to Wanibuchi et al.'s, but used male NCI-Black-Reiter rats, which lack α2u-globulin synthesizing ability (Li *et al.* 1998). α2u-globulin is a male rat specific protein and may affect susceptibility to carcinogens. Certain chemicals can bind to α2u-globulin and the consequent complex may cause cytotoxicity and regenerative proliferation (Cunningham 2002). Carcinogenicity in kidney that requires α2u-globulin is not a significant concern for humans (Alison *et al.* 1994). Following BBN initiation, NCI-Black-Reiter rats were given tap water or 100 ppm DMA(V) in drinking water for 32 weeks. The incidence and number of hyperplastic foci (papillary or nodular) in each rat, and the incidence of simple hyperplasia were significantly increased in the urinary bladders of DMA(V)-treated rats. Urinary bladder carcinomas were seen in 3 out of 8 DMA(V)-treated rats, but none were found in 8 controls. The numeric increase of urinary bladder carcinoma, however, was not statistically significant. BrdU labeling indexes for hyperplasia and normal epithelium of the urinary bladder in the DMA(V)-treated rats were significantly higher than the controls. The authors concluded that DMA(V) could function as a promoter in a BBN-initiated bladder carcinogenesis model, and this promotion is not associated with α2u-globulin.

Chen and colleagues used (Lewis x F344)F$_1$ rats to study DMA(V) promoting effects on the development of neoplasms (Chen *et al.* 1998; Chen *et al.* 1999). Rats underwent BBN initiation pretreatment, and then received no further treatment (group 1) or 0.01% DMA(V) in water (group 2) for another 36 weeks (Chen *et al.* 1998). The incidences of urinary bladder tumors (either papillomas and /or carcinomas) were increased by DMA(V) treatment (7% and 47% in groups 1 and 2 respectively). DMA(V)-treated rats also had a 5-times-higher average number of tumors per rat than controls (0.1 and 0.5 in groups 1 and 2, respectively) (Chen *et al.* 1998). Furthermore, loss of heterozygosity was detected in urinary bladder tumors in group 2 (2 out of 9, 22%) by polymerase chain reaction using 36 microsatellite markers (Chen *et al.* 1999). The authors concluded that DMA(V) enhanced urinary bladder carcinogenesis in LewisXF344 rats and this was associated with genetic alterations (losing heterozygosity) (Chen *et al.* 1999).

As discussed earlier, rats initiated by pretreatment with DMDBD, and then subsequently exposed to DMA(V) did not develop lung tumors (Yamamoto *et al.* 1995; Yamamoto *et al.* 1997). Likewise, no promoting effects of DMA(V) in drinking water on F344 rat lung cancer were seen in rats initiated by pretreatment with N-bis(2-hydroxypropyl)nitrosamine (DHPN) (Seike *et al.* 2002). In Seike's study (2002), eight week old male F344/DuCrj rats received DHPN in drinking water for 1 week, followed by 1 week of no treatment and then 30 weeks of DMA(V) in drinking water at 0 ppm (group 1), 100 ppm (group 2), 200 ppm (group 3), or 400 ppm (group 4). Group 5 received no DHPN or DMA(V), and group 6 received no DHPN but 400 ppm DMA(V). DHPN exposure induced alveolar epithelial hyperplasia, adenomas, and adenocarcinomas in groups 1 to 3, but no DMA(V) effect was seen on the incidence frequencies or multiplicities. Among 18 rats in group 2, a urinary bladder transitional carcinoma was found in one rat, and a thyroid adenoma was found in the thyroid gland of one rat. All rats that

received 400 ppm DMA(V) died by week 9. The authors reported no changes in BrdU labeling

index in the lung of rats exposed to 200 ppm DMA(V) alone for 30 weeks, compared to rats

received no treatment. There was also no change in 8-OHdG level in the lung of rats exposed to

525 ppm DMA(V) in the diet for 7 days and sacrificed after 3 days of no treatment, compared to

that of rats received no treatment. This study suggested that DMA(V) in drinking water does not

act as a promoter in lung carcinogenesis. Using the Ito model, DMA(V) acted as a promoter in

the liver of male F344 rats (Nishikawa *et al.* 2000; Nishikawa *et al.* 2002). Rats were initiated

by DEN and hepatectomy, and the development of Glutathione-S-transferase placental form

positive foci was increased by 6-week exposure to DMA(V) at 100 ppm in drinking water. This

promotion effect of DMA(V) was similar to that of MMA(V) discussed above and that of

TMA(V)O discussed below.

DMA(V) as a complete carcinogen

When male and female F344 rats were given DMA(V) in the diet for 104 weeks, urinary

bladder transitional cell neoplasms were increased (US EPA 1994). The concentrations of

DMA(V) in the diet were 0, 2, 10, 40, and 100 ppm. In males, transitional cell carcinomas and

papillomas were seen in all dose groups except the control group, although the numeric increase

in the incidences of neoplasms was not statistically significant. In females, the 100 ppm group

(8.0 mg/kg BW/day) had significantly increased incidences of papilloma (4 out of 58 rats) and

carcinoma (6/58), while no tumors were seen in control or lower concentration groups. The

increase of urinary bladder transitional cell tumors, combining papilloma and carcinoma, was

also statistically significant in females.

In contrast to the effects of DMA(V) in the diet, DMA(V) in the drinking water increased

incidences of urinary bladder tumors in male F344 rats after 2 years of dosing. Male F344 rats

(10 weeks old at the start of the experiment) received 0, 12.5, 50 or 200 ppm DMA in drinking

water for 104 weeks (Wei *et al.* 1999; Fukushima 2001). During weeks 97 to 194, urinary

bladder tumors were observed in 12 (2 papilloma and 10 carcinoma)/31, 8 (2 papilloma and 6

carcinoma)/31, 0/33, and 0/28 rats in 200, 50, 12.5, and 0 ppm treatment groups respectively

(Wei *et al.* 1999). BrdU labeling indices were also significantly increased in DMA-treated rats

over controls (Fukushima *et al.* 2000), indicating increased proliferation.

Trimethylarsine oxide [TMA(V)O]

TMA(V)O as a promoter

TMA(V)O, similar to MMA(V) and DMA(V) discussed above, was found to act as a

promoter in the livers of F344 rats, following initiation with DEN and hepatectomy (Ito model)

(Nishikawa *et al.* 2000; Nishikawa *et al.* 2002). Using the Ito model, 100 ppm TMA(V)O in

drinking water for 6 weeks increased glutathione-S-transferase placental form positive foci

(preneoplastic lesion) in the liver, at a similar potency to that of MMA(V) and DMA(V).

TMA(V)O as a complete carcinogen

TMA(V)O increased hepatocellular adenomas in male F344 rats in a 2 year carcinogenicity

study (Shen *et al.* 2003b). Rats were exposed to TMA(V)O in the drinking water at

concentrations of 0, 50, or 200 ppm. The incidences and multiplicities of hepatocellular

adenomas were significantly increased in the 200 ppm group, whereas increases seen in the 50

ppm group were not statistically significant. Moreover, in the normal appearing liver, the PCNA

index was significantly higher in the 200 ppm group than in the control. The amounts of 8-

OHdG were also increased in the liver of rats in the 200 ppm group. The authors suggested that

TMA(V)O may acts as a complete liver carcinogen by initiating cells through oxidative stress,

and by elevating cell proliferation in the promotion stage.

Summary

Arsenic, particularly DMA(V), has been shown to be carcinogenic in rats in multiple studies conducted in the past decade (**Table 5**), after a long history of using inorganic arsenic in various animal models that failed to induce cancer. Since exposure routes greatly affect the absorbance and metabolism of arsenic, the toxic and carcinogenic effects are expected to be influenced by exposure routes as well. Most arsenic carcinogenicity studies exposed rats to arsenic through water or diet, which correspond to the common human exposure routes. The concentrations of arsenic showing carcinogenic effects in rats, however, were much higher than the current US EPA drinking water standard, but are compatible to levels found in the water in Bangladesh and other areas.

Liver and urinary bladder appear to be target organs for arsenic carcinogenicity in both rats and humans, while thyroid carcinogenicity is only seen in rats, when the exposures were through diet or drinking water and no initiation took place. The review of the literature indicates that F344 or F344 hybrid rats may be the only proven animal model for arsenic-induced urinary bladder cancer. It is unclear if DMA(V) acts as a complete carcinogen in other strains of rats, since no report, negative or positive, is available.

3.   RISK FACTORS IN THE DEVELOPMENT OF URINARY BLADDER CANCER ASSOCIATED WITH ARSENIC EXPOSURE

In addition to the known risk factors for the development of urinary bladder cancer in general, such as cigarette smoking (La Vecchia and Airoldi 1999) and age, alcohol consumption may also be a risk factor in arsenic-induced urinary bladder cancer. Most data regarding the risk factors in urinary bladder carcinoma, regardless of etiology, were from studies of transitional cell

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carcinoma, the major form of bladder carcinoma (about 95% cases in Caucasians, 85% in Africa Americans, but less in Middle East populations), which is also the primary type of arsenic-induced bladder cancer. Other risk factors, including nutritional status and genetic polymorphism of enzymes associated with bladder carcinogens, were studied in relation to arsenic metabolism. In light of the scarcity of research on the risk factors in arsenic-induced bladder cancer, possible factors that have not been proven in epidemiological studies are also discussed.

Cigarette smoking

Cigarette smoking increases the risk of arsenic-induced urinary bladder cancer. In case-control studies conducted in southwestern Taiwan (Chen *et al.* 2003), New Hampshier, USA, (Karagas *et al.* 2004), and in Argentina and Chile (Moore *et al.* 2003), cigarette smoking significantly increased the odds ratio of bladder cancer in individuals exposed to arsenic through drinking water. Sometimes, no association between arsenic and bladder cancer risk was detected among non-smokers, while the association was significant in smokers (Karagas *et al.* 2004).

In addition to epidemiological studies, molecular studies also showed that cigarette smoking increases arsenic-induced adverse effects. The frequencies of mutation in p53 genes of arsenic-associated bladder cancer were higher in smokers than in non-smokers (Moore *et al.* 2003) In the buccal epithelial cells from people exposed to arsenic in drinking water, arsenic-induced DNA fragmentation, an indicator of apoptosis, was increased by cigarette smoking (Feng *et al.* 2001).

Genetic polymorphism of enzymes associated with urinary bladder cancer

N-acetyltransferase (NAT) is known to acetylate aromatic amines, which are urinary bladder carcinogens. NAT2 gene is reported to be more relevant to bladder neoplasm risk than NAT1,

and its polymorphism affects acetylation ability in individuals. Slow acetylators are known to have increased risk to bladder cancer induced by aromatic amines and cigarette smoking (Cohen *et al.* 2000). However, among people exposed to arsenic in southwestern Taiwan, NAT2-related slow acetylators did not show increased risk for bladder cancer, in contrast to the association seen in residents in another Taiwan area without arsenic contamination in water (Su *et al.* 1998). This suggested arsenic-assoicated bladder cancer is manifested by a mechanism different from aromatic amines.

Arsenic metabolism

The valence and methylation extent greatly affect the toxicity and carcinogenicity of arsenic (Mass *et al.* 2001; Wang *et al.* 2002; Cohen *et al.* 2006; Kligerman and Tennant 2006), so the ability to metabolize arsenic affects susceptibility to arsenic toxicity and carcinogenicity. For humans, arsenic methylation is usually studied by analyzing the species of arsenicals in urine. The ratios of MMA(V) and DMA(V) to total arsenic are commonly used indicators. A high MMA(V)/total arsenic ratio, accompanied with a low DMA(V)/total arsenic ratio, indicates low methylation ability. Since methylated arsenicals are more readily excreted in urine than inorganic arsenic, and the inhibition of methylation by chemicals increases arsenic concentrations in tissues, low methylation ability/efficiency indicates slower overall excretion.

On the other hand, a high methylation ability also increases the production of highly toxic trivalent methylated arsenicals, namely MMA(III) and DMA(III). These trivalent methylated arsenicals have been detected in human urine (Aposhian *et al.* 2000; Mandal *et al.* 2001). Although trivalent methylated arsenicals have very short half lives, they are more toxic than As(III) or pentavalent methylated arsenicals. For example, the acute toxicity of MMA(III) is about 4 times of As(III), since LD_{50}s in hamster were 29 and 112 μM/kg for MMA(III) and

As(III) respectively (Petrick *et al.* 2001). When cytotoxicities of various arsenicals were measured in primary cells from human and rat liver, human neonatal foreskin, human cervix, and a normal human urinary bladder-derived cell, MMA(III) was the most cytotoxic, DMA(III) was at least as toxic as As(III), and pentavalent arsenicals were less cytotoxic in all tested cell lines (Styblo *et al.* 1999a). High methylation ability facilitates the excretion of arsenic, but also increases the concentrations of trivalent methylated arsenicals.

The relationship of arsenic methylation and arsenic-induced urinary bladder risk was studied in southwestern Taiwan, where residents were exposed to arsenic via drinking water (Chen *et al.* 2003). The ability of arsenic methylation was defined as the ratios of MMA(V)/inorganic arsenic (primary arsenic methylation index, PMI) and DMA(V)/MMA(V) (secondary arsenic methylation index, SMI) in urine. Among individuals with high accumulated arsenic exposure (more than 12 mg/L-year), individuals with low SMI had nearly 3.8 times the risk of bladder cancer compared to those with high SMI, whereas individuals with high PMI have 1.1 times the risk of bladder cancer compared to those with low PMI. The estimation was adjusted for age, gender, body mass index, hair dye usage, cigarette smoking, and education. Authors concluded the results support the hypothesis that people with high PMI or low SMI could accumulate MMA(III), prolong the contact of MMA(III) with bladder epithelium, and therefore have increased risk of bladder cancer.

Recently, a case-control study of individuals without arsenic drinking water exposures showed that low SMI and high PMI increase urinary carcinoma risk, even at low arsenic exposure (Pu *et al.* 2006). In this study, none of 177 cases (patients with urothelium-derived carcinoma from renal pelvic, ureter, urether, and most commonly urinary bladder) and 313 controls were from areas with arsenic-contaminated water in Taiwan. Arsenicals were detected

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in their urine, and were at significantly lower concentrations than those from individuals drinking arsenic-contaminated water. The urinary arsenic profile of urinary carcinoma cases showed higher sums of arsenicals, higher MMA percentages, and lower DMA percentages than that of controls. Furthermore, cases also had lower SMI and higher PMI than controls. In short, unfavorable urinary arsenic profile (low SMI, high PMI) was associated with higher risk of urinary carcinoma in individuals with low arsenic exposure.

Factors affecting arsenic metabolism

Nutritional Status

In spite of a plethora of animal studies on nutritional status modulating arsenic toxicity, information in humans on this topic is not only rare, but also inconsistent. Although there are no data on the relation between nutritional status and arsenic-induced toxicity and carcinogenicity in urinary bladder, a brief review on how nutritional status may affect arsenic metabolism and susceptibility is provided.

Associations with poor nutritional status and increased susceptibility of arsenic-induced skin lesions and skin cancer have been reported in several countries. In West Bengal and surrounding areas, underweight individuals (weight less than 80% of age- and sex-adjusted standard body weight) had 2.1 (in females) and 1.5 (in males) times prevalence of keratosis (Guha Mazumder *et al.* 1998). Similarly, in Taiwan, individuals who consumed dried sweet potato as a staple food for more than 20 years, compared to less than 9 years, showed higher prevalence of arsenic-associated skin lesions and skin cancer (Hsueh *et al.* 1995). Before 1960, dehydrated sweet potato and /or rice were the staple food for Taiwanese, and dried sweet potato was consumed much more in the low socioeconomic group than in the high socioeconomic group. The high

consumption of dried sweet potato is associated with low intake of other nutrients and poor

nutritional status (Chen *et al.* 1988), so dried sweet potato consumption was used as an indicator

of nutritional status in Hsueh's study. A recent study in West Bengal, India showed that

individuals with low intakes of animal protein, calcium, fiber and folate were more likely to

develop arsenic-associated skin lesions (Mitra *et al.* 2004).

The intakes of protein, iron, zinc, and niacin (a B vitamin) were associated with arsenic

methylation in western USA (Steinmaus *et al.* 2005). People with low intake of protein, iron,

zinc, and niacin excreted higher percents of ingested arsenic as MMA and lower percents of

DMA in their urine (Steinmaus *et al.* 2005). Because high percents of MMA or MMA: DMA

ratios in urine have been reported to be associated with a higher risk of skin lesion, skin cancer,

and bladder cancer in Taiwan (Hsueh *et al.* 1997; Chen *et al.* 2003; Chen *et al.* 2005), USA, and

Argentina (Steinmaus *et al.* 2006), this study suggested people whose diets are deficient with

protein, iron, zinc and niacin may be more susceptible to arsenic toxicity.

Genetic polymorphism

Large inter-individual and inter-population variances of arsenic methylation efficiency have

been reported. Since the arsenic methylation efficiency of an individual over time is very stable

(Concha *et al.* 2002), the variance is suspected to come from genetic polymorphism. The

polymorphism of glutathione S-transferases (GST) has been investigated in relation to arsenic

methylation.

In addition to detoxifying various metals by conjugation, glutathione is necessary for arsenic

reduction and methylation. Glutathione S-transferases, which affect the concentrations of

glutathione, are logical candidates whose polymorphism may affect arsenic metabolism.

Glutathione S-transferase Mu 1-1 (GST M1) has two allelic variants, A and B. Individuals who

have none of the alleles are null genotype, and might have increased risk of developing cancers,

including bladder cancer (Cohen *et al.* 2000). Chiou *et al.* (1997) found that genotypes of GST M1 and GST theta 1 (GST T1) affected methylation and body retention of arsenic. Individuals with null genotype of GST M1 had a slightly higher percentage of inorganic arsenic in urine, whereas those with null genotype of GST T1 showed a higher percentage of DMA in urine. The polymorphisms of GST M1 and T1 did not affect arsenic concentrations in hair or toenail (Chiou *et al.* 1997a). In contrast, Kile *et al.* (2005) found that individuals with null genotype of GST T1 had higher levels of total arsenic in toenail than individuals with wild type of GST T1 (Kile *et al.* 2005).

Human MMA(V) reductase is a member of GST, and can directly affect arsenic metabolism by reducing As(V) to As(III), MMA(V) to MMA(III), and DMA(V) to DMA(III) (Zakharyan and Aposhian 1999). The rate of reduction of MMA(V) to MMA(III) by MMA(V) reductase is the rate-limiting step in the arsenic methylation pathway. Human MMA(V) reductase protein is encoded by the *hGSTO1* gene (Marnell *et al.* 2003). There are at lest 9 polymorphisms of the *hGSTO1* gene, and individuals heterozygous for E155del [a deletion of amino acid 155, glutamic acid (E)] or Glu208Lys [a substitution of amino acid 208 from glutamic acid (Glu) to lysine (Lys)] may have a different ability to reduce As(V) than individuals with other polymorphism (Marnell *et al.* 2003).

Age

Infants and children may be more rone to arsenic-induced adverse effects, at least partially due to their higher water consumption to body weight ratio. The roles of other factors, such as difference in methylation capacity, are less clear. For example, the ability of arsenic methylation was reported to vary by age, although different researchers showed higher and lower arsenic methylation in younger populations (Calderon *et al.* 1999; US EPA).

On a body weight basis, the water intake (and consequently the arsenic exposure) of infants (less than one year old) is estimated to be 3 to 4 times of the mean for the whole population (US EPA 2000), so infants may be more likely to show arsenic effects. The breast milk from mothers exposed to arsenic via drinking water is another possible source of arsenic (Watanabe *et al.* 2003), although the arsenic concentrations in break milk are usually very low, and infant arsenic consumptions through breast milk were estimated well within the safety limit (Concha *et al.* 1998b; Sternowsky *et al.* 2002). Total arsenic concentration in urine was greater in people 18 years old or younger, compared to the urinary concentration from people older than 18, and this difference may be from the discrepancy in water intake (Calderon *et al.* 1999).

Arsenic methylation, indicated by the percentage of DMA in urine, was reported higher in children than in adults in Taiwan (Hsueh *et al.* 1998) and northern Argentina (Concha *et al.* 1998b). On the other hand, studies in northern Taiwan (Hsu *et al.* 1997) and Finland (Kurttio *et al.* 1998) showed an increased DMA percentage in urine with age. There are also studies that showed no difference of arsenic methylation with age in Mexico (Del Razo *et al.* 1999), and under background concentration arsenic exposure (Buchet *et al.* 1980; Kalman *et al.* 1990). In short, the age effect on arsenic methylation is still unclear, as are the effects of methylation ability on susceptibility to arsenic adverse effects.

Age-related sensitivity to arsenic exposure was reported in male Wistar rats, in terms of body weight and organ weight (Schulz *et al.* 2002). Four- and 10-week-old rats were treated with 3.3 to 26.6 mg $NaAsO_2$/kg/day for 4 weeks by gavage 5 days per week. A significant decrease in body weight was seen in the 4-week-old rats (juvenile) at the highest dose from day 15 on, but not in 10-week-old rats (young adult). Furthermore, some organ weight (relative to body weight) changes were different, and some in opposite directions. After 4 weeks of treatment, the liver

relative weight was increased in juvenile rats, but decreased in young adult rats. The adrenal relative weight was increased in juvenile rats, but not altered by arsenic in young adult rats.

Summary

In epidemiological studies, cigarette smoking, high MMA(V)/inorganic arsenic ratios and low DMA(V)/MMA(V) ratios in urine (high PMI and low SMI) are associated with greater risk for urinary bladder cancer associated with arsenic exposure. Cigarette smoking is a risk factor in the development of bladder cancer, and it also increases odds ratio of arsenic-induced bladder cancer. Individuals with high PMI and low SMI are less efficient in adding a second methyl group on arsenic than methylating inorganic arsenic, and may therefore have increased urinary MMA(III), a highly toxic arsenic metabolite.

Malnutrition or undernourishment is associated with increased susceptibility to arsenic effects on skin lesions, but the association to bladder cancer has not been studied. In animal studies, antioxidants and trace metals also affect arsenic metabolism, which may influence susceptibility to arsenic effects. Other bladder cancer risk factors, such as genetic polymorphism of NAT2, did not show association with arsenic-induced bladder cancer. Finally, infants and children may be exposed to more arsenic on a body weight base because of their high water ingestion on a body weight base than adults in the same envionrment. More investigation is needed to clarify the importance of those plausible risk factors in urinary bladder cancer associated with arsenic exposure.

4.   METABOLISM OF ARSENIC IN HUMANS AND RATS

Metabolism of arsenic in humans and rats

Most mammals biomethylate inorganic arsenic. For methylation to occur, pentavalent

arsenate, As(V), is first reduced to trivalent arsenite, As(III), either non-enzymatically via

glutathione or enzymatically via As(V) reductase (Kitchin 2001). For most species, as much as

50-70% of absorbed As(V) is rapidly reduced to As(III) (Vahter 1999), and this reduction

happens primarily in the blood (Vahter and Marafante 1983; Marafante *et al.* 1985; Vahter and

Marafante 1985). As(III) then goes through both oxidation and methylation to form MMA(V),

which then can be reduced to genotoxic and unstable MMA(III). The technique to separate

trivalent forms of the methylated arsenic species from the pentavalent forms was developed

recently (Le *et al.* 2000; Sampayo-Reyes *et al.* 2000). Receiving a methyl group from S-

adenosyl methionine (SAM), MMA(III) is further methylated into DMA(V), whose intermediate,

DMA(III) is also highly genotoxic and cytotoxic (Styblo *et al.* 2000; Mass *et al.* 2001).

Methylation mainly occurs in the liver (Marafante *et al.* 1985; Styblo *et al.* 2000), although other

tissues (keratinocytes, bronchial cells) also have methylation capacity for inorganic arsenic

(Styblo *et al.* 2000). In mice, rats, hamster, and humans, a small percentage of orally

administered DMA is methylated to trimethylarsine oxide [TMA(V)O] (Marafante *et al.* 1987).

The postulated scheme is as follows: As(V) → As(III) → MMA(V) → MMA(III) → DMA(V)

→ DMA(III) → TMA(V)O (Yamauchi and Yamamura 1984; Cullen *et al.* 1989; Yoshida *et al.*

1997).

The methylation of inorganic arsenic is important for excretion. Among people exposed to

arsenic via drinking water, the ones with higher arsenic methylation efficiency have lower blood

arsenic (Concha *et al.* 1998b). When the methylation is inhibited, the excretion of DMA is

decreased, and the arsenic tissue concentrations are increased (Marafante and Vahter 1984). At the cellular level, cells with low methylation capacity retained more arsenic compared to cells with a high capacity to methylate As(III) or MMA(III) (Styblo et al. 2000). In most mammals, urine is the main excretion pathway, followed by feces (Marafante et al. 1987; Hughes and Kenyon 1998; Vahter 2000). In the urine of people and rats exposed to arsenic via drinking water, As(III), As(V), MMA(V), MMA(III), DMA(V), and DMA(III) are all detected (Mandal et al. 2001; Chen et al. 2002; Cohen et al. 2002). Most mammals excrete arsenic mainly as DMA in the urine. Humans, the most sensitive species so far identified to arsenic toxicity (Rossman et al. 1997; Wang et al. 2002), are the only species having a significant percentage of MMA in urine (Vahter 2000).

Methylation has traditionally been viewed as a primary detoxification pathway, because methylation increased excretion, and pentavalent methylated arsenicls [MMA(V) and DMA(V)] are less toxic than inorganic arsenic in terms of cytotoxicity, (Kaise et al. 1985; Kaise et al. 1989), genotoxicity, (Eguchi et al. 1997), and DNA clastogenicity, (Moore et al. 1997c)). However, new evidence shows that MMA(III) and DMA(III) are more toxic than inorganic arsenic, and the methylation may not protect cells.

MMA(III) has been reported being from 2- to 10-fold more cytotoxic than As(III) in human hepatic cell lines (Styblo et al. 1999b; Petrick et al. 2000). MMA(III) also shows more inhibition on the activities of enzymes than As(III), such as yeast glutathione reductase, a key enzyme of redox metabolism of GSH (Styblo et al. 1997), and mouse liver thioredoxen reductase (Lin et al. 1999).

The cytotoxicity of As(III), As(V), MMA(V), DMA(V), DMA(III) and three trivalent methylated arsenicals: methylarsine oxide, iododimethylarsine, and the complex of DMA(III)

with GSH were compared in various cell types (Styblo *et al.* 2000). All the trivalent methylated arsenicals were more toxic for normal human hepatocytes, epidermal keratinocytes, bronchial epithelial cells and urinary bladder cells than pentavalent arsenicals. Methylarsine oxide was more toxic than As(III). DMA(III) was at least as toxic as As(III) for most cell types studied. Moreover, the effects of trivalent arsenicals on cell viability were similar in cells with high, low or no methylation capacity (Styblo *et al.* 2000). These data suggest that high methylation capacity does not protect cells from the acute toxicity of trivalent arsenicals.

After being exposed to inorganic arsenic, TMA(V)O can be detected in the urine of rats, but not in the urine of humans. Although TMA(V)O is basically non-cytotoxic (Hirano *et al.* 2004), TMA(V)O has been reported to increase cell numbers, suggesting potential mitogenic activity, in cultured rat heart microvessel endothelial cells (Hirano *et al.* 2004). However, rats exposed to TAM(V)O did not show morphological changes in the urinary bladder transitional cells under scanning electron microscope, in contrast to rats exposed to DMA(V) (Shen *et al.* 2006).

Methylated trivalent arsenicals were much more potent in damaging DNA than any other arsenicals tested, as measured by a single-cell gel ("Comet") assay in human lymphocytes (Mass *et al.* 2001). Furthermore, only methylated trivalent arsenicals were observed to damage naked DNA without exogenous enzymatic or chemical activation systems (Mass *et al.* 2001). In other words, methylated trivalent arsenicals are direct-acting forms of arsenic that are genotoxic. This observation also provided the first data that arsenic is directly genotoxic, which significantly changes the risk assessment of arsenic carcinogenicity.

Summary

In summary, arsenic metabolism is consistent in most mammals: As(V) → As(III) → MMA(V) → MMA(III) → DMA(V) → DMA(III) → TMA(V)O → TMA(III) (Yamauchi and

Yamamura 1984; Cullen *et al.* 1989; Yoshida *et al.* 1997). The process of methylation of arsenic compounds can no longer be thought of only as a detoxification process but also as an activation process (Cullen *et al.* 1989; Styblo *et al.* 1999b; Aposhian *et al.* 2000; Petrick *et al.* 2000; Styblo *et al.* 2000).

5.   MECHANIMS OF ARSENIC-INDUCED TOXICITY AND CARCINOGENESIS IN URINARY BLADDER

Carcinogenic Modes of Action

Although there is a plethora of epidemiological data of arsenic as a bladder carcinogen, and there are successful animal models for studying arsenic-induced bladder cancer, the mechanism of action or mode of action of arsenic carcinogenesis on urinary bladder is not clear. Furthermore, there is no consensus on mechanisms of arsenic carcinogenicity, regardless of the target organs cited and studied. In any discussion of carcinogenesis, it is important to distinguish modes of action and mechanisms of action. The mode of action of carcinogenesis is the necessary (but not sufficient) steps whereby a particular agent causes tumor development. The mechanisms of action includes the necessary steps and their specific natures (e.g. the specific genes involved) whereby an agent induces a tumor. In this part of the literature review, some proposed modes of action of arsenic carcinogenicity are reviewed. Whenever possible, specific data on urinary bladder are provided.

1) Chromosomal Abnormalities

The results of many arsenic clastogenicity (the capability to damage chromosomes) studies are positive (reviewed in Rossman 1996 and Basu et al. 2001). As(III), As(III), MMA(V), and

DMA(V) all increase chromosome aberrations (CA), micronuclei (MN) formation and sister chromatid exchange (SCE) in various mammal cells. TMA(V)O also induces SCE in human lymphocytes *in vitro*.

Bladder-specific measurement of arsenic clastogenicity included the frequencies of MN in exfoliated epithelial cells in urine samples. Micronuclei are fragments of DNA or whole chromosomes that are left out of the main daughter nuclei during cell division, and are incorporated into much smaller nuclei in the cytoplasm (Smith *et al.* 1993; Moore *et al.* 1997b). Micronuclei can be generated by damage to DNA, chromosomal structure, or the mitotic spindle, and the frequencies of MN show positive correlation with other bladder cancer risk factors, including cigarette smoking, *Schistosoma haematobium* infection, occupational exposure to pesticides, and leather tanning. Not surprisingly, increased levels of MN in exfoliated bladder cells from people exposed to high concentrations of arsenic in drinking water have been reported in Chile (Biggs *et al.* 1997; Moore *et al.* 1997a; Moore *et al.* 1997b), Mexico (Gonsebatt *et al.* 1997), Nevada, USA (Moore *et al.* 1997a), and Inner Mongolia, China (Tian *et al.* 2001).

The immediate causes of MN can be classified into aneuploidogenic events and clastogenic events by examining the presence of centrometric probe within a MN. Micronuclei with a centrometric part are assumed to contain a whole chromosome and to have arisen from an aneuploidogenic event (e.g. spindle disturbances). In contrast, MNs without a centrometric part are thought to contain only acentric fragments of DNA and to have arisen via a clastogenic event (e.g. chromosome breakage). Moore *et al.* (1997) found that increases in the concentration of arsenic in water were accompanied by increased portions of MN without centrometric parts and decreased portions of MN with centrometric parts (Moore *et al.* 1997b). These data suggested

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that the mechanism of formation of arsenic-induced MN in urinary bladder cells is mainly by chromosome breakage.

In Gonsebatt's study (1997), individuals exposed to an average of 408.17 μg/L of arsenic in water, compared to individuals exposed to average 29.88 μg/L of arsenic, had a 2-fold increase in the frequency of chromatid and isochromatid deletion in lymphocytes and a 4-fold increase in the MN in exfoliated epithelial cells from urine. The difference in fold change between exfoliated cells from urine and lymphocytes could be that exfoliated cells from urine were directly exposed to arsenic and its metabolites in the urine, and the high concentrations of arsenicals in urine. Moore et al. (1997a, b) reported that the prevalence of MN in exfoliated epithelial cells from urine decreased after lowering the concentration of arsenic in drinking water After an 8-week exposure of 45μg/L arsenic in water, individuals who used to drink water with higher than 600 μg/L arsenic lowered the MN from 2.63 to 1.79 per 1000 exfoliated bladder cells. The decrease was more pronounced in the subgroups exposed to subcytotoxic concentrations of arsenic (600-700 μg/L) and smokers, compared to the whole group and non-smokers, respectively.

Chromosome number was also affected by *in vitro* exposure to arsenic compounds. Both aneuploid [by DMA(V) in murine bone marrow cells (Kashiwada *et al.* 1998), and by As(V) in cultured human lymphocytes (Ramirez *et al.* 1997)] and tetraploid [by DMA(V) in Chinese hamster V79 cells (Ueda *et al.* 1997)] have been reported. The potential of various arsenicals for induction of tetraploidy appears to be TAMO > DMA(V) > MMA(V) (Kenyon and Hughes 2001).

Coclastogenicity of arsenic has been invested *in vitro* by treating cells with arsenic compounds and UV radiation, DNA crossing agents, alkylation agents, or direct acting

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mutagens. Most results were additive or more than additive (Basu *et al.* 2001). Other arsenic-induced chromosomal aberrations reported include: a) As(III) induces intrachromosomal homologous recombination, but not non-homologous recombination, in the *hpry* gene of V79 Chinese hamster cells (Helleday *et al.* 2000), b) humans exposed to inorganic arsenic via drinking water have higher frequency of chromatid and isochromatid deletions in lymphocytes (Gonsebatt *et al.* 1997), c) As(III) produces endoreduplication of chromosomes in cultured Chinese hamster ovary cells (Kochhar *et al.* 1998), and d) As(V) produces chromosome gaps and chromatid type of aberrations (Kochhar *et al.* 1998).

Several mechanisms of arsenic-induced chromosomal aberrations were suggested, although very few studies have specifically examined these. Li and Rossman (1989b) have suggested that the clastogenicity of As(III) is due to the high affinity of arsenic for sulfhydryl groups. Errors in DNA replication may be responsible for arsenic-induced chromosomal changes since G0/G1 phase lymphocytes exposed to arsenic generate only chromatid-type aberrations, and not chromosomal types (US EPA 1997). This is consistent with the observation of arsenic-induced SCEs (Sahu *et al.* 1989; Mahata *et al.* 2003), which are also produced by errors in DNA replication. Disruption of microtubule assembly and spindle formation have been considered the most probable mechanism for inducing aneuploid cells (Ramirez *et al.* 1997). Inhibition the activity of protein phosphatase has been suggested to be a reason for endoreduplication observed in cultured human skin fibroblasts exposed to As(III) (Huang *et al.* 1995). The connection between arsenic clastogenicity and carcinogenicity is not clear (Kitchin 2001), and more research needs to be done before the relationship between arsenic-induced clastogenicity and cancers can be determined.

2) Oxidative Stress

Oxidative stress due to arsenic exposure has been demonstrated by the increase in free radical damage, arsenic-induced antioxidants in the body, direct free radical formation, and decrease in arsenic toxicity by free radical scavengers. Oxidative stress and heat shock responses of arsenic have been reviewed elsewhere (Bernstam and Nriagu 2000).

One of the biomarkers of oxidative stress to DNA is the presence of 8-OHdG. DMA(V) treatment increased 8-OHdG in murine urine (Yamanaka *et al.* 2001a) and rat liver (Wanibuchi *et al.* 1996). In human arsenic-related skin neoplasms, a higher percentage of samples were 8-OHdG positive (detected by immuno-histochemical stain) than non-arsenic related Bowen's disease, squamous cell carcinoma *in situ* (Matsui *et al.* 1999). Direct evidence of arsenic-induced free radical production was gained by electron spin resonance studies. Mice fed either As(V) or As(III) showed positive electron spin resonance signals in the liver (Liu *et al.* 2000a).

Arsenite in the body activates antioxidants, such as superoxide dismutase, catalase, and metallothionein, a metal binding protein (Kreppel *et al.* 1993). Arsenite administration also induces hepatic and renal heme oxygenase isoform I (an enzyme that catalyzes heme degradation and protects cells from oxidative injury) in rats (Kitchin *et al.* 1999). However, glucose-6-phosphate dehydrogenase, glutathione peroxidase and hepatic glutathione were significantly lower in mice drinking water contaminated with arsenic (3.2 mg/L) than those of the control group (Santra *et al.* 2000). Taking consideration of the presence of arsenic-induced oxidative stress biomarkers, arsenic-activated oxidative stress defense may not completely prevent/reverse the oxidative damage, and the defense may be overwhelmed over time.

Nitric oxide (NO) synthase inhibitors and calcium chelators decreased As(III)-induced MN formation (Gurr *et al.* 1998). Arsenic-induced DNA strand breaks in bovine aortic endothelial cells were decreased by NO scavengers (>75%), peroxynitrite ($ONOO^-$)/$^\bullet OH$ scavengers (50-

75% and >75%) (Liu and Jan 2000). Natural radical scavengers, *e.g.* mustard oil and garlic extract, also have shown protection against As(III) induced chromosomal aberrations *in vivo* (Choudhury *et al.* 1997a; Choudhury *et al.* 1997b).

Interestingly, the effect of As(III) on NO production seems to be cell type specific. Increased NO can lead to more oxidative stress by interacting with superoxide anions to generate highly reactive peroxnitrites (Liu *et al.* 2005), whereas decreased NO can lead to blood vessel endothelial cell dysfunction, which contributes to atherosclerosis (Simeonova and Luster 2004). Some cells [Chinese hamster ovary cell, C3H/10T1/2 cells (a fibroblast cell line isolated from a line of C3H mouse embryo cells) and bovine aortic endothelial cells] have increased NO production, and some (rat aortic smooth muscle cells, hepatocytes, and human liver cells) are not affected. Moreover, As(III) inhibits NO synthase gene expression in cytokine-stimulated human liver cells, rat pulmonary artery smooth muscle cells, and hepatocytes (Liu and Jan 2000). There is no clear trend on which cell types are prone to arsenic-induced NO production.

Oxidative damage due to arsenic exposure has been reported in organs that display arsenic-induced cancer (skin, liver, kidney, and lung) as well as tissues that are damaged by arsenic but without a carcinogenic response (aorta). Specific organelles or cellular functions that are affected by arsenic-induced oxidative damage need to be further investigated. Nonetheless, oxidative damage may well be an essential part of arsenic carcinogenicity.

3) Increased Cell Proliferation

Increased cell proliferation after arsenic exposure has been reported in various mammal cells both *in vivo* and *in vitro*. Arsenite exposure in rat liver, and DMA(V) exposure in rat liver and rat kidney increased ODC activity, a biomarker for cell proliferation (Kitchin 2001). In primary human kerotinocytes, As(III) increased cell proliferation as indicated by increases in cell

numbers, c-myc gene expression, and incorporation of 3H-thymidine into cellular DNA (Germolec *et al.* 1996). Moreover As(III), methylarsine oxide [MA(III)O], and complex of dimethylarsinous acid with glutathione [DMA(III)-GS], increased normal human epidermal keratinocyte proliferation at very low concentrations (0.001 to 0.01 μM) as measured by thymidine intake. Pentavalent arsenicals tested did not increase cell proliferation, and all tested arsenicals decrease cell proliferation at high concentrations (Kitchin 2001).

One unusual pattern of proliferation was observed in Swiss mice receiving As(V) injections. The uterus, testes, and liver showed proliferative lesions, and the author suggested a possible estrogenic mode of action (Waalkes *et al.* 2000).

Arsenic exposure induces cell proliferation and increased cellularity by several mechanisms in different organs. The urinary bladder epithelium of rats treated with DMA(V) showed cytotoxicity and necrosis prior to hyperplasia (Cohen *et al.* 2001), while arsenic-induced growth factors have been reported in skin (Germolec *et al.* 1997; Germolec *et al.* 1998; Simeonova *et al.* 2002). While arsenic-induced apoptosis is the basis for the use of arsenic to treat leukemia, this effect seems to be cell type- and p53-dependent (Bode and Dong 2002). Arsenic-associated inhibitions in cellular differentiation were reported in cultured epidermal keratinocytes (Perez *et al.* 2003).

3.1) Altered Growth Factors and Differentiation

In primary human epidermal keratinocytes, mRNA transcripts and secretion of keratinocyte growth factors were increased by *in vitro* exposure to As(III). Increased cell proliferation was also observed. Elevated GM-CSF levels were observed and were due to the increase in its transcription and its mRNA. The presence of elevated transforming growth factor-alpha (TGFα) levels is likely the result of mRNA stabilization (Germolec *et al.* 1996). Increases in GM-CSF

and TGFα mRNA transcripts were also found in the epidermis at clinically normal skin sites of arsenic-exposed Tg.AC mice, which carry the v-Ha-ras oncogene (Germolec *et al.* 1998).

In another human keratinocyte study, exposure to low doses of trivalent arsenicals, containing As(III), MA(III)O and DMA(III)GS, stimulated secretion of the growth-promoting cytokines (GM-CSF) and tumor necrosis factor-alpha. DMA(V) reduced cytokine secretion at lower concentrations than those at which proliferation increased (Vega *et al.* 2001). Growth factors changes induced by arsenic should be investigated in other organs/tissues, such as liver, lung and kidney, which are sensitive to arsenic carcinogenicity.

Inhibition of cell differentiation and maturation has been seen in cultured epidermal keratinocytes (Perez *et al.* 2003) and a myelomonocytic leukemia cell line (McCabe *et al.* 2000). It is noteworthy that the effects of arsenic on cell growth are concentration-dependent. For example, for leukemia cells, differentiation is impaired at low concentrations, cell cycle is affected at intermediate concentrations, and apoptosis is induced at high concentrations (McCabe *et al.* 2000).

3.2) Regeneration followed by cell death

After DMA(V) exposures, rat urinary bladders showed necrotic foci prior to proliferation (Cohen *et al.* 2001; Cohen *et al.* 2002). Based on the timeline of lesions, the authors suggested that DMA(V) resulted in cytotoxicity with necrosis, followed by regenerative hyperplasia of the bladder epithelium.

4) Promotion and /or Progression in Carcinogenesis

In laboratory animals, arsenic acts as a promoter more often than a complete carcinogen. The promoter function was seen in mouse skin and lung, and rat bladder, kidney, liver, and thyroid, while arsenic acts as a complete carcinogen only in mouse lung and rat bladder (Kitchin

2001). However, it must be considered that humans appear to be the most sensitive species to arsenic carcinogenicity, and seem to be the only species that develop skin, lung, liver, kidney and bladder tumors due to natural exposure.

5) Decreased DNA Repair

Trivalent arsenicals have high affinity towards sulfhydryl (SH-) groups, especially two nearby sulfhydryl groups (dithiols). Since zinc finger proteins, proteins with endogenous zinc, have dithiol groups, it is reasonable to predict that arsenic may interfere the function of zinc finger proteins. Zinc finger domains are mostly involved in DNA binding, and have been found in DNA binding enzymes, transcription factors and DNA repair proteins. Examples of zinc finger DNA repair proteins are UrvA protein (DNA damage recognition), formamidopyrimidine-DNA glycosylase (Fpg) protein (oxidative DNA base modification removal), mammalian xeroderma pigmentosum group A (XPA) protein (recognition of DNA damage in nucleotide excision repair), DNA ligase II, and poly(ADP-ribose)polymerase (PARP).

The fact that arsenic causes chromosomal damages, but acts as a very poor mutagen also suggests that arsenic promotes DNA damage by inhibiting DNA repair (Basu *et al.* 2001). A number of studies have shown arsenic inhibits DNA repair, including global genome repair and transcription-coupled repair (Hartwig *et al.* 1997). For example, As(III) and As(V) inhibit the excision of UV-induced thymine dimers (Okui and Fujiwara 1986). As(III) reduces the incision frequency at low concentrations and the ligation efficiency at higher concentrations (Hartwig *et al.* 1997). As(III) inhibits the nucleotide excision repair system (especially incision step) for UV-induced DNA photoproducts (Hartwig 1998). As(III) also inhibits DNA repair in cells treated with N-methyl-N-nitrosourea (MMU) (a mutagen, recognized animal carcinogen and possible

human carcinogen) (Li and Rossman 1989b), and methyl methanesulphonate (a possible human carcinogen) (Lee-Chen *et al.* 1993; Lee-Chen *et al.* 1994).

DNA repair processes have a common sequence. First, DNA damage is recognized, possibly by zinc finger proteins via DNA-protein interaction. Secondly, the incision/ excision or other repair occurs, displacing the damaged DNA. Finally the gap in DNA is filled by polymerization and ligation. If arsenic inhibits most of the repair enzymes, a co-genotoxic effect would be expected when cells are treated with a mutagen and arsenic (Lee-Chen *et al.* 1992). However, no co-clastogenic effect was found in stationary-phase mammalian cells treated with ethyl methanesulfonate (a human carcinogen) and As(III) (Huang *et al.* 1987), nor did post treatment with As(III) have any co-cytotoxic effects on UV-irradiated XPA cells (a human epidermal fibroblast cell line deficient in excision repair) (Okui and Fujiwara 1986), or bleomycin-treated Chinese hamster ovary cells, human skin fibroblasts, and HeLa cells (Jan *et al.* 1990). This verifies the need to look for other DNA repair steps affected by arsenic.

Arsenic-affected DNA repair pathways are mostly studied by treating cells with As(III). It has been suggested that As(III) does not alter DNA-protein interactions during DNA damage recognition (Hartwig *et al.* 1997); however, it inhibits the incision step at low concentrations of arsenic and the ligation step at higher/cytotoxic concentrations (Hartwig *et al.* 1997). Arsenic inhibits steps of DNA repair in various degrees. When cells are treated with methyl methanesulphate, As(III) inhibitory effects on DNA repair processes (from high to low) are ligation $\approx$ DNA polymerization $\geq$ DNA repair synthesis > excision (Lynn *et al.* 1997). In UV-irradiated Chinese hamster ovary K1 cells, the potency of As(III) inhibitory effects are ligation > post-replication repair >> incision > polymeration (Lee-Chen *et al.* 1992). As(III)-induced inhibition of post-replication repair was also reported in UV-irradiated *E. coli* (Fong *et al.* 1980).

The activities of DNA repair enzymes, especially of DNA ligases, have been reported to be significantly lower in As(III)-treated cells than in control cells. As(III) inhibited the activity of DNA ligase II in Chinese hamster V79 cells (Li and Rossman 1989a). Both constitutive and methylnitrosourea-inducible levels of DNA ligase II were inhibited by As(III) (Li and Rossman 1989a). DNA ligase I and II were inhibited by As(III) in methyl methanesulfonate-treated cells (Lee-Chen *et al.* 1993). The activity of poly(ADP-ribose) polymerase (PARP) was decreased to about half at 10 μM As(III) in Molt-3 cells (a human T-cell lymphoma-derived cell line) (Yager and Wiencke 1997).

Purified DNA repair enzymes appear to be very insensitive to arsenic. In spite of high affinity to dithiol groups, As(III) up to 1000 μM did not inhibit 2 isolated zinc finger DNA repair enzymes: bacterial Fpg and mammalian XPA proteins (Asmuss *et al.* 2000b). Additionally, low sensitivity to As(III) in purified DNA polymerase β, DNA ligase I, and DNA ligase III has been reported (Hu *et al.* 1998). These three enzymes were activated, rather than inhibited, by mM concentrations of As(III) or As(V). These data suggested that arsenic may not inhibit DNA repair by direct inhibition of one or more specific enzymes, but rather by indirect mechanisms, such as changes in gene expression, post-translational modification, or signal transduction (Hu *et al.* 1998; Asmuss *et al.* 2000b).

6) Altered Gene Expression

6.1) Altered p53 Gene Expression

p53 gene is a tumor-suppressor gene. P53 protein controls the G1 checkpoint of the cell cycle and contributes to the G2 arrest. P53 induces P21 protein, a cyclin-kinase inhibitor which inhibits all Cdk1-, Cdk2-, Cdk4-, and Cdk 6- complexes, and eventually arrests cells in G1 and G2. P21 protein in turn decreases p53 gene expression through a p53-p21 negative feedback loop. If the

p53 gene is not expressed, cells with DNA damage do not arrest. Without adequate time to repair DNA damage, cells can accumulate multiple mutations and may become cancer cells. More than 50% of human cancers (all types) show changes in p53 gene structure or activity.

Arsenic-induced changes in p53 gene expression have been reported. Both decreased and increased p53 gene expressions have been reported in arsenic-induced skin cancer and arsenic-treated cells (see below).

Arsenic-related skin basal cell carcinoma expressed p53 gene less often and at a lower intensity than sporadic basal cell carcinoma (Boonchai *et al.* 2000). The authors suggested the TP53 gene is down-regulated by methylation in arsenic-related basal cell carcinomas, or mutations in TP53, that stabilize the protein, are less common in arsenic-related basal cell carcinomas (Boonchai *et al.* 2000). *In vitro*, human keratinocytes treated with As(III) showed reduced p53 protein levels, while concomitantly showing increased mdm2 protein (murine double minute 2 proto-oncogene product) (which decreases p53 protein). The authors proposed the disruption of the p53-mdm2 loop regulating cell cycle arrest as a model for arsenic-related skin carcinogenesis (Hamadeh *et al.* 1999).

p53 over-expression was found in some arsenic-related skin cancers, and some tumors showed a high frequency of p53 mutations, which are different from mutations in UV-induced skin cancers (Hsu *et al.* 1999). P53 protein accumulation was reported in As(III)-treated human fibroblasts, along with DNA strand breaks, and increased p53 downstream proteins: p21 and human homologue of murine double minute-2 proteins (Yih and Lee 2000). Similarly, irradiated normal human fibroblasts showed As(III)-induced increases in p53 expression, increases in cyclin D1, and suppression of the radiation-induced increase in p21 (Vogt and Rossman 2001).

As(III) effects on p53 expression were studied in different cell lines: HeLa, C33A (a transformed human non-differentiated carcinoma cell line), Jurkat (a transformed human T-lymphocyte cell line), and LCL-EBV (a lymphoblast cell line transformed with Epstein-Barr virus) (Salazar *et al.* 1997). Increased p53 expression was seen in Jurket, HeLa, and LCL-EBV cells. When Jurket and human lymphocytes had a mutated p53 gene, they become more sensitive to arsenic cytotoxicity than those with wild-type p53 gene. Salazar's data showed that cells with a functional p53 gene cope with arsenic-induced damage better than cells with a mutated p53 gene (Salazar *et al.* 1997).

6.2) Altered DNA Methylation Patterns

The metabolism of arsenic requires methyl groups from the SAM pool (**Fig 3**), and continuous methyl depletion may induce hypomethylation. Arsenic-decreased expression of DNA methyltransferase genes also contributes to the arsenic-induced hypomethylation (Reichard *et al.* 2007). Hypomethylation has been linked to transformation (the change of normal cells into cancerous cells) (Zhao *et al.* 1997) and aberrant gene activation (Leder *et al.* 1986). A potential mode of action has been hypothesized: arsenic exposure →depleted methyl pool + decreased DNA methyltransferase activity → genomic DNA hypomethylation →aberrant gene expression → oncogene over-expression → → cancer (James *et al.* 2003; Benbrahim-Tallaa *et al.* 2005; Reichard *et al.* 2007).

A positive correlation between hypomethylation and transformation was seen in arsenic-treated cells. Genome-wide DNA hypomethylation occurred concurrently with As(III)-induced cell transformation and reduced SAM levels in TRL 1215 cells, a rat liver epithelial-cell line (Zhao *et al.* 1997; Chen *et al.* 2001). These arsenic-transformed cells induced malignant tumors in nude mice, and the rate of tumor formation was highly correlated with the extent of As(III)-induced genome-wide DNA hypomethylation (Zhao *et al.* 1997; Chen *et al.* 2001). Oncogene c-

myc was hopomethylated, and consequently (Leder *et al.* 1986) over-expressed in TRL 1215 cells after As(III) exposure (Chen *et al.* 2001). Furthermore, the expression level of oncogene c-myc was highly correlated with transformed cell-induced tumor formation in nude mice and was also highly correlated with genome-wide DNA hypomethylation.

Consistent with *in vitro* findings, hypomethylation was observed at genomic level and oncogene-specific level in the liver of mice exposed to As(III) (Okoji *et al.* 2002). C57BL/6J mice were given methyl-deficient diet and As(III) through drinking water. In the liver, genome-wide hypomethylation and hypomethylation in the promoter region of oncogne Ha-ras were observed (Okoji *et al.* 2002).

In contrast to the SAM pool depletion and suppression of DNA methyltransferase hypotheses, some studies showed hypermethylation in arsenic-treated cells. About 10% of human lung A549 cells exposed to As(III) or As(V), but not DMA(V), showed hypermethylation in a portion of the p53 promoter region (Mass and Wang 1997). There are two possible hypotheses for arsenic-induced hypermethylation. Because a hypermethylated p53 promoter may result in inactivation of p53, this may somehow allow selection for cells that are resistant to arsenic toxicity. Arsenic might act as a differentially specific inhibitor of the numerous methyltranferases that engage the SAM pool as the methyl donor (Goering *et al.* 1999). Recently, gene-specific DNA hypermethylation was observed in the lung tumors of mice exposed to As(V) for 18 months, compared to that in lung tumor from the control and to that in non-tumorous lung tissues from both control and exposed mice (Cui *et al.* 2006). The hypermethylation of two tumor suppressor genes, p16INK4a and RASSF1A was accompanied by decreased protein expression of these genes (Cui *et al.* 2006).

Both hypomethylation and hypermethylation on DNA coexist in As(III)-treated human lung A549 cells (Zhong and Mass 2001). In DNA obtained from blood of people exposed to arsenic, most samples showed hypomethylation in the promoter region of p53 genes, while a small subgroup of samples showed hypomethylation of p53 gene with high arsenic exposure (Chanda *et al.* 2006). Although all of these results support a unifying hypothesis that DNA methylation imbalance could conceivably disrupt appropriate gene expression in arsenic-exposed cells (Zhong and Mass 2001), the changes in methylation status may not be an early step in the mode of action of arsenic carcinogenicity (Mass 2001).

6.3) Transcription Factors

Urinary bladders of mice exposed to As(III) showed increased DNA-binding activity of activating protein-1 (AP-1), a functionally pleomorphic transcription factor regulating diverse gene activities (Simeonova *et al.* 2001). Arsenic-induced alterations in AP-1 activity were also seen in other *in vivo* and *in vitro* system (Parrish *et al.* 1999; Jessen *et al.* 2001; Liao *et al.* 2004). In the livers of mice exposed to As(III) or As(V), c-Jun/AP-1 transcription complex was activated after arsenic treatments (Liu *et al.* 2001a). The protein concentrations of metal-responsive transcription factor MTF-1, nuclear factor kappa B, and c- Jun/AP-1 were also increased (Liu *et al.* 2001a). Transcription factor Nrf was also increased by As(III) and As(V) (Aono *et al.* 2003).

7. Gene Amplification

Amplification of oncogenes has been observed in many human cancers including breast carcinoma, pancreatic ductal adenocarcinoma, gastric cancer, childhood adrenocortical tumors, prostate cancer, and neuroblastoma (Ruggeri *et al.* 1998; Raggi *et al.* 1999; Figueiredo *et al.* 2000; Miyoshi *et al.* 2000; Rennstam *et al.* 2001; Yokozaki *et al.* 2001). Amplification of

oncogenes results in overexpression of oncogenes, and is often associated with aggressive types of cancer.

In mouse methotrexate-resistant 3T6 cells, 2-10 fold duplication of the normal copy number of the dihydrofolate reductase gene was observed at 0.2-0.8 μM As(III) and 1-4 μM As(V) (Lee *et al.* 1988). Arsenic-induced dihydrofolate reductase gene amplifications were also found in SHE cells and in arsenic-resistance trypanosomes (Kitchin 2001). The amplification of chromosomal DNA into extrachromosomal circles has been suggested as the mechanism of arsenite resistance in *Leishmania mexicana amazonensis*, an arsenite-resistant variant of a trypanosomatid protozoan (Detke *et al.* 1989).

8. Summary

There may be several modes of action of arsenic carcinogenicity, and it is possible that different modes of action exist in different organs/tissues. The list is not exclusive, and the events discussed above often interact with each other. For example, altered methylation has effects on p53 gene expression, which in turn affects DNA repair due to inadequate time to repair DNA damage. Cells with deficient p53 enter S phase in spite of DNA damage, and replicate DNA with damage. Replication of DNA damage can lead to chromosome abnormalities and gene amplification (Alberts *et al.* 1994). Oxidative stress can cause chromosome abnormalities, as well as damage DNA repair enzymes.

From currently available data, chromosomal abnormalities, oxidative stress, cell proliferation by altered growth factors, and decrease in DNA repair are the most promising explanations of arsenic carcinogenicity. The oxidative stress theory works very well for lung and bladder, since lungs are exposed to high partial pressures of oxygen, and bladder stores high concentrations of DMA and MMA that can generate free radicals (Kitchin 2001). Effects on growth factors and

cell proliferation, on the other hand, have strong evidence both *in vivo* and *in vitro* in human skin. As Kitchin (2001) discussed, all the parts of cell proliferation theory of arsenic action have been demonstrated in one or more systems (Kitchin 2001). Variants of the DNA repair theory of arsenic carcinogenicity are relatively new and developing quickly, especially considering arsenic-induced action on DNA dithiol groups. Testing methylated arsenic, which is no longer a pure detoxification product, as well as different types of DNA damage, will shed more light on this theory.

Toxicity Modes of Action

The mechanism of arsenic toxicity depends on its chemical form and oxidation status. As(V) may substitute for phosphate and consequently affects the activities of enzymes that binds to As(V) instead of phosphate (Gresser 1981; Moore *et al.* 1983). As(III) may react with sulflhydryl groups and affect various enzyme activities (Delnomdedieu *et al.* 1995). Oxidative stress and alterations in gene expression, as discussed in Carcinogenic Modes of Action, are also likely to be responsible for arsenic toxicity.

As(V) can replace phosphate in many biochemical reactions, because of their similar structure and properties. In energy transfer phosphorylation reactions, As(V) inhibits adenosine triphosphate (ATP) production by forming adenosine diphosphate (ADP)-As(V), instead of ATP (Gresser 1981). The resulting ADP-As(V) is unstable and undergoes hydrolysis, effectively depleting cofactors and therefore uncoupling oxidative phosphorylation (Gresser 1981; Moore *et al.* 1983). Unlike As(V), As(III), MMA(V) and DMA(V) do not perturb phosphate metabolism, and are not phosphate analogs (Delnomdedieu *et al.* 1995).

As previously discussed, As(III) has high affinity to sulfhydryl groups, and readily interacts with thiol groups of proteins (Delnomdedieu *et al.* 1995). MMA(III) and DMA(III) bind to protein *in vitro* at a greater extent than MMA(V) and DMA(V) (Styblo *et al.* 1995; Vahter and Concha 2001), and As(V) does not react with sulfhydryl groups (Delnomdedieu *et al.* 1993). The binding between As(III) and thiol groups has been reported on GSH, cystein and dihydropoamide (Jin *et al.* 2004; Tseng 2004). The latter is a cofactor of pyruvate dehydrogenase, and arsenic inhibits the pyruvate dehydrogenase multienzyme complex and consequently the mitochondrial-based citric acid cycle and ATP production (Aposhian and Aposhian 1989). As(III) can also compete with zinc in metal binding proteins, and display vicinal dithiols contained in zinc fingers of DNA binding and repair proteins and transcription factors (Asmuss *et al.* 2000a; Asmuss *et al.* 2000b). However, this effect occurs at very high arsenic concentrations (Asmuss *et al.* 2000a; Asmuss *et al.* 2000b), and repair enzymes appear to be less sensitive to arsenic toxicity than whole cells. Although methylated arsenicals have been reported to be more potent in releasing zinc (Schwerdtle *et al.* 2003a), they have not been studied specifically on DNA repair enzymes.

Interference with gene expression by arsenic can have a wide range of effects. The mechanism of arsenic-induced diabetes mellitus may be arsenic-induced insulin resistance which develops through enhancement of the expression of NF-κB, TNFα, and IL-6 and by inhibition of the expression of PPRγ, a nuclear hormone receptor important for activating insulin action (Tseng 2004). Methylated arsenic metabolites, rather than consumed inorganic arsenic, may be directly involved in arsenic-induced diabetes mellitus.

Other mechanisms of arsenic toxicity include interacting with other metals and inducing apoptosis. Arsenic can enhance the toxicities of other metals, including lead and cadmium

(Mahaffey *et al.* 1981; Liu *et al.* 2000b). The ability of arsenic trioxide (As_2O_3) to induce apoptosis is associated with gene expression changes, oxidative stress and target cell cycle status (McCabe *et al.* 2000; Park *et al.* 2001; Huang *et al.* 2002; Miller 2002; Davison *et al.* 2003), and is used to treat acute promyelocytic leukemia (Miller 2002).

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Table 1. Epidemiological studies of renal and urinary bladder cancers in areas with arsenic-contaminated water.

Study	Location	Exposure	Endpoint	Number of cases		Study Outcome		Comments
Ecological studies								
(Chen *et al.* 1985)	SW Taiwan	Blackfoot endemic area				SMR (95% CI):		SMRs; age-specific Taiwan rates as standard
			Bladder cancer mortality	Male 167	Female 165	Male 11.00 (9.3-12.7)	Female 20.09 (17.0-23.2)	
						SMR:		
			Kidney cancer mortality	Male 42	Female 62	Male 7.72	Female 11.19	
(Wu *et al.* 1989)	SW Taiwan					Rate:		Mortality, 1973-1986 in 42 villages in Taiwan
		Average arsenic:		Male	Female	Male	Female	
		<0.30 ppm	Bladder cancer	23	30	22.6	25.6	
		0.30-0.59 ppm	mortality	36	36	61.0	57.0	
		≥ 0.60 ppm		26	30	92.7	111.3	
			Kidney cancer			Rate:		
		Average arsenic:	mortality	Male	Female	Male	Female	
		<0.30 ppm		9	4	8.42	3.42	
		0.30-0.59 ppm		11	13	18.90	19.42	
		≥ 0.60 ppm		6	16	25.26	57.98	

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome		Comments
(Chen and Wang 1990)	Taiwan	Data from 83, 656 wells, national survey	Bladder cancer mortality	National data	β (SE) from regression:		Moratality, 1972-1983 in 314 precincts and townships, regression-coefficient (β) estimates increase in age-adjusted mortality per 100,000 per increase in arsenic at 100 μg/L of water.
					Male	Female	
					3.9 (0.5)	4.2 (0.5)	
			Kidney cancer mortality		1.1 (0.2)	1.7 (0.2)	
(Guo *et al.* 1997)	Taiwan	Data from 83, 656 wells, national survey: <0.05 ppm 0.05-0.08 ppm 0.09-0.16 ppm 0.17-0.32 ppm 0.33-0.64 ppm >0.64 ppm	Bladder caner incidence (shown transitional cell carcinoma only)	National rates	β (SE) from regression: Mixed results for exposure levels: at >0.64 ppm, the β (SE) was		Incidence, 1980=1987; positive associations of high arsenic levels in drinking water with transitional cell carcinomas of the bladder, kidney, and ureter and all urethral cancers combined, also in adenocarcinomas of the bladder in males, but not in squamous cell carcinomas
					Male	Female	
					0.57 (0.07)	0.33 (0.04)	

Study	Location	Exposure	Endpoint	Number of cases		Study Outcome		Comments
			Kidney cancer incidence (shown renal-cell carcinoma only)			Male 0.03 (0.02)	Female 0.142 (0.013)	of the bladder or renal cell carcinomas or nephroblastomas of the kidney.
(Hopenhayn-Rich *et al.* 1998)	Cordoba province, Argentina	Low (few high concentration)	Bladder cancer mortality	Male 113	Female 39	SMR (95% CI): Male 0.80 (0.7-1.0)	Female 1.21 (0.9-1.6)	Mortality, 1986-1991; national rates for 1989 used as the standard for the SMR; SMR for chronic obstructive pulmonary disease below the expected level, indicating low smoking rates; high concentration was defined as arsenic 40 mg/L or higher in water
		Medium (scattered high concentration)		116	29	1.28 (1.1-1.5)	1.39 (0.9-2.0)	
		High (178 µg/L average)		131	27	2.14 (1.8-2.5)	1.82 (1.2-2.6)	
		Low (few high concentration)	Kidney cancer mortality	Male 66	Female 38	SMR: Male 0.87 (0.66-1.10)	Female 1.00 (0.71-1.37)	
		Medium (scattered high concentration)		66	34	1.33 (1.02-1.68)	1.36 (0.94-1.89)	

Study	Location	Exposure	Endpoint	Number of cases		Study Outcome		Comments
		High (178 µg/L average)		53	27	1.57 (1.17-2.05)	1.81 (1.19-2.64)	
(Smith *et al.* 1998)	Region II, Northern Chile	420 µg/L average; 5 year average ranged from below 100 µg/L after 1980 to 569 µg/L in 1955-1959; by city and 5 year period, range was 40-870 µg/L	Bladder cancer mortality	Male 93	Female 64	SMR: Male 6.0 (4.8-7.4)	Female 8.2 (6.3-10.5)	Mortality, 1989-1993; national rates for 1991 used as the standard for the SMR; arsenic concentration is population-weighted average for major cites or towns in Region II, 1950-1974

Study	Location	Exposure	Endpoint	Number of cases		Study Outcome	Comments
(Tsai et al. 1999)	Blackfoot endemic area of SW Taiwan			Male	Female	SMR: Male	SMRs calculated compared with mortality experience of the whole of Taiwan and
			Bladder cancers	312	295	8.92 (7.96-9.96) Regional 10.50 (9.37-11.73) National	compared with mortality in the two counties in SW Taiwan where the Blackfoot endemic area is located
						Female 14.07 (12.51-15.78) Regional 17.65 (5.70-19.79) National	
				Male	Female	Male	
			Kidney cancer	94	128	6.76 (5.46-8.27) Regional 6.80 (5.49-8.32) National	
						Female 8.89 (7.42-10.57) Regional 10.49 (8.75-12.47) National	

Cohort Studies

| (Cuzick et al. 1992) | United Kingdom | Total arsenic exposure: | Bladder cancer mortality | | | SMR | 478 patients treated with Fowler's solution (potassium |

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome	Comments
		224 mg		1	1.2 ((0.04-7.0)	arsenite) during the period
		504 mg		1		1945-1969 and followed until
		963 mg		1	5.00 (2.0-15)	1991
		1901 mg		1	(Exposure ≥ 500 mg)	
		3324 mg		1		
(Tsuda *et al.* 1995)	Niigata Prefecture, Japan	Arsenic in water <0.05 μg/L 0.05-0.99 μg/L ≥ 1.0 μg/L	Bladder cancer mortality	0 1 8	SMR 0.0 (0-12.5) 0.0 (0-47.1) 31.2 (8.6-91.8)	454 persons who drank arsenic-contaminated well water for approximately 5 years (1955-1959) were followed for 33 years - death certifications between 1959-1992 were examined; reference is mortality in Niigata Prefecture 1960-1989
(Chiou *et al.* 1995)	SW Taiwan	Average arsenic <0.05 mg/L 0.05-0.70 mg/L	Bladder cancer incidence	6 7	Relative risk: 1.0 1.8 (0.6-5.3)	263 patients with Blackfoot disease and 2293 healthy residents in the endemic area of arseniasis were recruited

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome		Comments
		≥0.71 mg/L		7	3.3 (1.0-11.1)		and followed for 7 years
		Cumulative arsenic					
		<0.1 mg/L x years		4	1.0		
		0.1-19.9 mg/L x years		7	2.1 (0.6-7.2)		
		≥20 mg/L x years		9	5.1 (1.5-17.3)		
(Lewis et al. 1999)	Utah, USA	Cumulative arsenic exposure during residence in study towns:	Kidney cancer mortality		SMR (CI) Male	Female	Retrospective cohort mortality study of 4,058 residents of Millard County, Utah, born between 1900 and
		<1,000 ppb-years			2.51	2.36	1945; vital status followed
		1,000-4,999 ppb-years			1.13	132	through 1996 SMRs used
		≥5,000 ppb-years			1.43	1.13	Utah state rates as the
		All			1.75 (0.80-3.32)	1.60 (0.44-4.11)	reference
		<1,000 ppb-years	Bladder and		0.36	1.18	

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome		Comments
		1,000-4,999 ppb-years	other urinary organ cancers		-	-	
		≥5,000 ppb-years	mortality		0.95	1.10	
		All			1.42 (0.08-1.22)	0.81 (0.10-2.93)	
(Chiou *et al.* 2001)	arseniasis-endemic area in NE Taiwan	Arsenic in water	Bladder and kidney cancer incidence	Incidence rate per 100,000 (no. of cases)	Relative risk (95% CI):		Prospective cohort study of 8,102 person; relative risks calculated using subjects with exposures ≤ 10 µg/L as a reference population, only relative risks in model one are shown.
		≤ 10 µg/L		37.6 (3)	1.0		
		10.1-50.0 µg/L		4.8 (3)	1.5 (0.3-8.0)		
		50.1-100 µg/L		66.4 (2)	2.2 (0.4-13.7)		
		>100 µg/L		134.1 (7)	4.8 (1.2-19.4)		
			Transitional cell carcinoma incidence	Incidence rate per 100,000 (no. of cases)	Relative risk:		
		≤ 10 µg/L		12.5 (1)	1.0		
		10.1-50.0 µg/L		14.9 (1)	1.9 (0.1-32.5)		
		50.1-100 µg/L		66.4 (2)	8.2 (0.7-99.1)		

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome	Comments
		>100 µg/L		114.9 (6)	15.3 (1.7-139.9)	

Case control studies

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome	Comments
(Bates *et al.* 1995)	Utah, USA	Cumulative lifetime exposure: <19 mg 19 to <33 mg 33 to <53 mg >53 mg	Bladder cancer incidence	117 cases, 266 pop7ulation-based controls	Odds ratios (95% CI) 1.0 1.56 (0.8-3.2) 0.95 (0.4-2.0) 1.41 (0.7-2.9)	Exposures estimated by linking residential-history information with water-sample data from public water supplies; 81 out of 88 towns <10 µg/L; 1 town >50 µg/L
(Kurttio *et al.* 1999)	Finland	Average arsenic exposure: <0.1 µg/L 0.1-0.5 µg/L ≥ 0.5 µg/L Average arsenic exposure:	Bladder cancer incidence, diagnosed in the third to the ninth years following exposure Kidney cancer incidence,	23 19 19	Relative risk (95% CI): 1 1.53 (0.75-3.09) 2.44 (1.11-5.37)	Population-based case-control study of individuals who drank water from their own drilled wells between 1967 and 1980; cases were diagnosed between 1981 and 1995; reference group of 275 age- and sex-matched persons form the same population;

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome	Comments
		<0.1 µg/L	diagnosed in the	23	1	cases with longer latency
		0.1-0.5 µg/L	third to the ninth	12	0.78 (0.37-1.66)	(diagnosed after 10 or more
		≥ 0.5 µg/L	years following	14	1.49 (0.67-3.31)	years after exposure) are not
			exposure			shown
(Steinmaus et al. 2003)	western Nevada and central California, USA	Accumulative arsenic exposure	Bladder cancer incidence, shown only cases with exposures 40 or more years ago	Number of cases (number of controls)	Odds ratios (95% CI)	All primary bladder cancer diagnosed from 1994 to 2000 were recruited; 181 cases and 328 controls. Overall, no increased risks were identified for arsenic intakes
		<6.4 mg		153 (282)	1.00	greater than 80 µg/day (odds
		6.4-82.8 mg		9 (13)	1.63 (0.64-4.13)	

Study	Location	Exposure	Endpoint	Number of cases	Study Outcome	Comments
		>82.8 mg		19 (33)	1.40 (0.73-2.70)	ratio = 0.94, 95% CI: 0.56- 1.57). When the analysis was focused on exposures 40 or more years ago, an odds ratio of 3.67 (95% CI: 1.43- 9.42) was identified for intakes greater than 80 µg/day (median intake, 177 µg/day) in smokers.

CI, confidence interval. SMR, standardized mortality rate.

Table was modified and updated from (National Research Council 1999; National Research Council 2001)

Table 2. The response of six hepatic biochemical parameters to As(III), As(V), MMA(V), and DMA(V) at up to 3/5 oral LD50 in female SD rats [summarized from (Brown and Kitchin 1996; Brown et al. 1997)]. ⇑ indicates increase, ⇓ indicates decrease, and – indicates no significant change.

	DNA damage level	ODC activity	Heme oxygenase concentration	GSH concentration	Cytochrome P450 concentration	Serum ALT concentration
As(V)	-	-	⇑	-	-	-
As(III)	-	⇑	⇑	-	-	-
MMA(V)	-	-	-	⇓	⇑	⇓
DMA(V)	-	-	⇑	⇓	⇑	⇓

Table 3. DMA(V) carcinogenesis in rats.

Organ	Animal	Description (lowest effective DMA(V) concentration)	Reference
Action: DMA(V) as a promoter			
Bladder, kidney, liver, and thyroid gland (but not lung)	Male F344/DuCrj rats	Initiated with 5 carcinogens (DMDBD treatment), then DMA(V) in drinking water for 24 weeks (50 ppm for bladder tumors, 200 ppm for kidney and liver tumors, 400 ppm for thyroid gland tumor)	(Yamamoto *et al.* 1995; Yamamoto *et al.* 1997)
Bladder	Male F344 rats	N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), then DMA(V) in drinking water for 32 weeks (10 ppm)	(Wanibuchi *et al.* 1996)
Bladder	Male Lewis x F344 rats	BBN, then DMA(V) in drinking water for 36 weeks (100 ppm)	(Chen *et al.* 1998; Chen *et al.* 1999)
Bladder	Male NCI-Black-Reiter rats	BBN, then 100 ppm DMA(V) in drinking water for 32 weeks	(Li *et al.* 1998)

Organ	Animal	Description (lowest effective DMA(V) concentration)	Reference
Liver	Male F344 rats	DEN, partial hepatectomy, then DMA(V) in drinking water for 6 weeks (100 pm).	(Nishikawa *et al.* 2000; Nishikawa *et al.* 2002)
Lung*	Male F344 rats	N-bis(2-hydroxypropyl)nitrosamine, and staring 1 week later DMA(V) in drinking water for 30 weeks at up to 400 ppm. No promoting effects.	(Seike *et al.* 2002)

Action: DMA(V) inducing preneoplastic lessons

Bladder	Male F344/DuCrj rats	0, 10 or 25 ppm DMA(V) in drinking water for 8 weeks (BrdU labeling increase at 10 ppm, but not at 25 ppm)	(Yamamoto *et al.* 1997)
Bladder	Female F344 rats	DMA(V) in diet for 10 weeks, simple hyperplasia under light microscope (100 ppm), necrosis and regeneration under SEM (40 ppm)	(Cohen *et al.* 1998)

Organ	Animal	Description (lowest effective DMA(V) concentration)	Reference
Bladder	Female and male F344 rats	Female rats: 0, 2, 10, 40 or 100 ppm DMA(V) in Purina diet for 10 weeks (BrdU labeling increase at 40 and 100 ppm, higher SEM classification and hyperplasia under light microscope at 100 ppm); 0 or 100 ppm DMA(V) in Altromin diet for 10 weeks (higher BrdU labeling index and SEM classification at 100 ppm) Male rats: 0 pr 100 ppm DMA(V) in Purina diet for 10 weeks (BrdU labeling index at 100 ppm)	(Arnold *et al.* 1999)
Bladder	Female F344 rats	100 ppm DMA(V) in diet for 6 hours to 10 weeks, changes under SEM (cell pitting, 6 hours; necrosis, 24 hours), labeling index (7 days), hyperplasia (10 weeks)	(Cohen *et al.* 2001)

Organ	Animal	Description (lowest effective DMA(V) concentration)	Reference
Action: DMA(V) as a complete carcinogen			
Bladder	Male F344/DuCrj rats	DMA(V) in drinking water for 104 weeks (50 ppm)	(Wei *et al.* 1999)
Bladder	Male F344 rats	200, 50, 12.5 or 0 ppm DMA(V) in drinking water for 104 weeks (50 ppm)	(Fukushima *et al.* 2000)
Bladder	Male F344 rats	DMA(V) for 2 years (100 ppm)	Unpublished data, mentioned in (Chen *et al.* 1998)
Bladder	Female, but not male, F344 rats	Transitional cell neoplasms (papillomas and /or carcinomas) of the urinary bladder after DMA(V) in the diet for 104 weeks (100 ppm).	(US EPA 1994)

* indicates negative results.

Table 4. The lowest effective concentrations of DMA (ppm) found to increase preneoplastic lesions, tumors, and the number of tumor-bearing animals after DMDBD initiation.

	Urinary bladder	Kidney	Liver	Thyroid	Skin	Lung
Preneoplastic lesions [a]	50	200	100	200	X	X
Tumors [b]	50	200	200	400	X	X
Number of tumor-bearing animals	50	200	200	400	X	X

X indicated no significant increase at the highest concentration tested. [a] Preneoplastic lesions are hyperplasia in the urinary bladder; atypical tubules, which have a basophilic cytoplasm and occasionally contain granules, in the kidney; and glutathione S-transferase placental form-positive foci in the liver. [b] Diagnosis was totals of transitional cell papilloma and carcinoma in urinary bladder; adenoma, adenocarcinoma, and nephroblastoma in kidney; hepatocellular carcinoma, cholangioma, and hemangioma in the liver; adenoma and adenocarcinoma in thyroid; sebaceous cancer in skin; and adenocarcinoma and squamous cell carcinoma in lung.

Table 5. Arsenic carcinogenicity in rats.

	Arsenic acted as a promoter (initiation treatment)	Arsenic acted as a complete carcinogen
As(III)	Kidney: rats (DEN)	Stomach: rats (arsenic implant)
As(V)	N/A	N/A
MMA(V)	Liver: F344 male rats (diethylnitrosamine and partial hepatectomy)	Thyroid: Sprague-Dawley male, but not in female, rats
DMA(V)	Liver: F344 male rats (diethylnitrosamine and partial hepatectomy) Bladder, kidney, liver, and thyroid gland: F344/DuCrj male rats (DMBDD) Bladder: F344 male rats, Lewis x F344 male rats, male NBR rats (BBN)	Bladder: F344/DuCrj male rats, F344 rats (one report in female but not male rats, two reports in males)
TMA(V)O	Liver: F344 male rats (diethylnitrosamine and partial hepatectomy).	Liver: F344 male rats

N/A: not available.
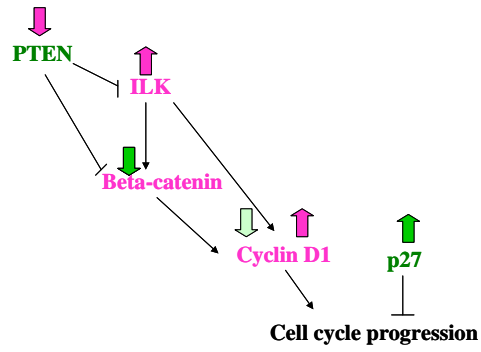
Figure 1. Simplified schematic representation of cancer-related gene expression by As(V) in rat liver. Arrow ($\rightarrow$) indicates an increase or facilitation. Blunt head arrow ($\perp$) indicates a decrease or hindering. Upward arrow ($\Uparrow$) indicate increased expression. Downward arrow ($\Downarrow$) indicates decreased expression.
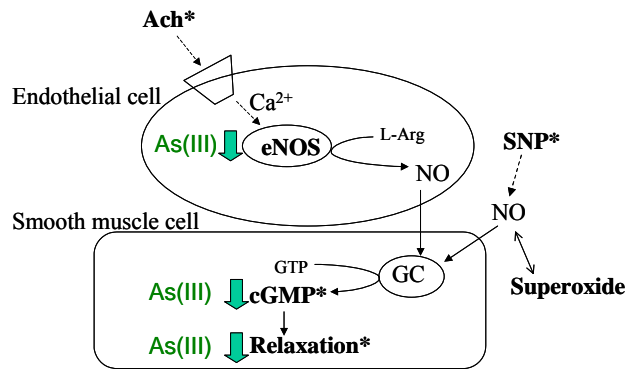
Figure 2. The pathways of vasorelaxation regulation and As(III) inhibitory mechanism on vasorelaxation. Components in bold were investigated (Lee et al. 2003). As(III) suppressed vasorelaxation induced by Ach, SNP, cGMP analog (marked by *). The suppression could be due to the decrease in NO production mediated by eNOS inhibition [As(III) decreased eNOS activity] in endothelial cells and interference of cGMP-dependent relaxation machinery [As(III) decreased cGMP concentration] in smooth muscle. Superoxide concentration, which may interact with NO and block NO pathway, was not affected by As(III). Abbreviations: Ach, acetylcholine; GC, guanylate cyclase; GTP, guanosine triphosphate; SNP, sodium nitroprusside.
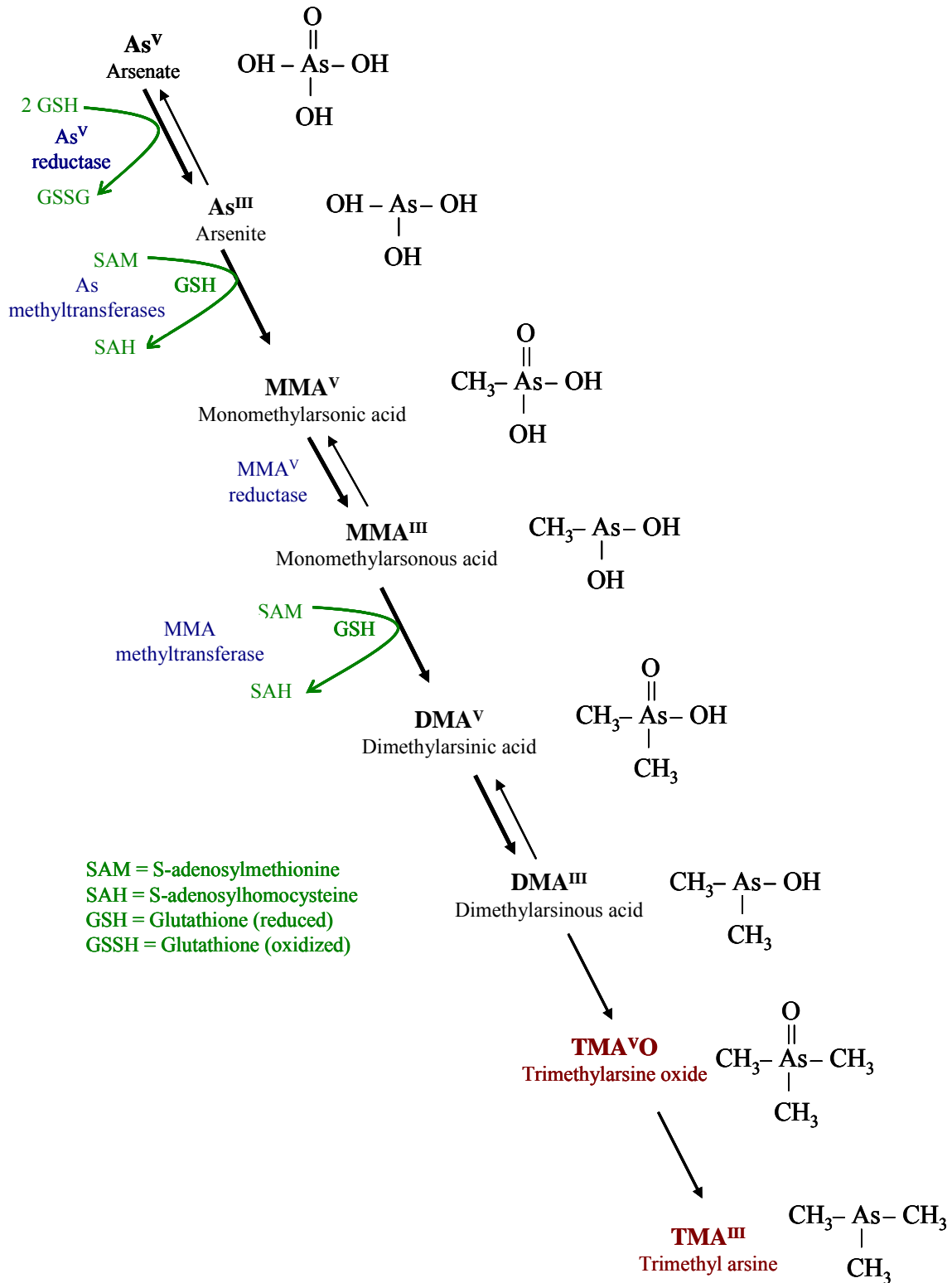
Figure 3. Metabolism of arsenic

CHAPTER 2   REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF ARSENIC IN RODENTS: A REVIEW

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ARSENIC REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

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ABSTRACT

Arsenic is a recognized reproductive toxicant in humans and induces malformations, especially neural tube defects, in laboratory animals. Early studies showed that murine malformations occurred only when a high dose of inorganic arsenic was given by intravenous or intraperitoneal injection in early gestation. Oral gavage of inorganic arsenic at maternally toxic doses caused reduced fetal body weight and increased resorptions. Recently, arsenic reproductive and developmental toxicity has been studied in situations more similar to human exposures and using broader endpoints, such as behavioral changes and gene expression. For the general population, exposure to arsenic is mostly oral, particularly via drinking water, repeated and prolonged over time. In mice and rats, methylated or inorganic arsenic via drinking water or by repeated oral gavage induced male and female reproductive and developmental toxicity. Furthermore, at non-maternally toxic levels, inorganic arsenic given to pregnant dams via drinking water affected fetal brain development and postnatal behaviors. However, arsenic given by repeated oral gavage to pregnant mice and rats was not morphologically teratogenic. In this review of arsenic reproductive and developmental toxicity in rats and mice, we summarize recent *in vivo* studies and discuss possible underlying mechanisms. The influences of folate, selenium, zinc and arsenic methylation on arsenic reproductive and developmental toxicity are also discussed.

Key words: arsenic, *in vivo*, fetus, folate, methylation.

INTRODUCTION

Arsenic is a major global health concern due to its wide distribution and adverse health effects. Arsenic naturally occurs in the Earth's crust, and can contaminate drinking water sources through leaching, erosion, and mining. High concentrations of arsenic in water have been reported in Asia (Bangladesh, China, India, Inner Mongolia and Taiwan), Europe (Hungary) and the Americas (Argentina, Chile, Mexico, and the northeast and western United States of America) (National Research Council 1999; National Research Council 2001; Karagas *et al.* 2002; Ayotte *et al.* 2003; Akter *et al.* 2005). Grains and vegetables grown in arsenic-contaminated soil or irrigated with arsenic contaminated water incorporate arsenic in their tissue. Arsenic is also used in the manufacture of wood preservatives, glass, pesticides and herbicides, semiconductors and pharmaceuticals. Therefore, ingestion of arsenic-containing food, inhalation of arsine in industrial settings, and exposure to arsenical herbicides and pesticides can also contribute to arsenic intake (Akter *et al.* 2005). For the general population, arsenic in drinking water is the main exposure source (National Research Council 2001), and more harmful than arsenic in food, since the bio-availability (actual amount absorbed into the bloodstream) of arsenic from water is greater than that from grains or vegetables (Akter *et al.* 2005). Furthermore, arsenic in drinking water is mainly inorganic arsenic. Arsenic found in seafood is predominately organic forms, such as arsenobetaine. The organic forms tend to be less potent toxicants than inorganic arsenic (De Gieter *et al.* 2002; Guillamet *et al.* 2004; Sakurai *et al.* 2004).

Developmental effects, cancer, and cardiovascular disease have all been associated with long-term exposure to arsenic in humans. There is a paucity of epidemiological studies of arsenic reproductive toxicity, and there are only a few of arsenic developmental toxicity

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(DeSesso *et al.* 1998; Tchounwou *et al.* 2003). Epidemiological studies have reported that arsenic exposure *in utero* increased spontaneous abortion and stillbirth and decreased birth-weight (DeSesso *et al.* 1998; Ihrig *et al.* 1998; Ahmad *et al.* 2001; Milton *et al.* 2005). However, these studies lack detailed information on confounders (exposure to other metals, smoking, maternal age, *etc*.) and accurate maternal arsenic exposure. The use of animal models therefore is essential for investigating arsenic developmental and reproductive toxicity, because it allows greater experimental controls, including the confounders mentioned above, and direct observations of developmental changes before birth. Furthermore, it provides opportunities to investigate the effects of interventions that have not been approved for humans.

In early studies, arsenic induced fetal malformations in rats and mice after an intraperitoneal (i.p.) or intravenous (i.v.) injection in early gestation (DeSesso *et al.* 1998; Hood 1998; Stump *et al.* 1999; Holson *et al.* 2000a; DeSesso 2001). Both inorganic and methylated arsenic injections caused developmental toxicity at maternally toxic or near lethal doses. In contrast, single or repeated oral exposure to inorganic arsenic did not induce fetal gross malformation in mice or rats (Stump *et al.* 1998a; Stump *et al.* 1999; Holson *et al.* 2000b). At maternally toxic doses, repeated oral exposures to dimethylarsinic acid increased resorptions and decreased fetal weight in mice and rats (Rogers *et al.* 1981). While no gross abnormalities were reported in these rats, an increase in incidences of cleft palate was observed in mice by Roger *et al.* (1981). Inhalation of inorganic arsenic or arsine did not cause developmental toxicity (Morrissey *et al.* 1990; Stump *et al.* 1998b). In summary, the above studies detected arsenic developmental toxicity only at maternally toxic doses.

In the past five years, arsenic reproductive and developmental toxicity studies have expanded to include more human-related exposure conditions and diverse endpoints. In

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particular, following United States of America Food and Drug Administration (US FDA) Guidelines for Developmental Toxicity Studies (US FDA 2000), recent arsenic studies used repeated or prolonged oral exposures (via drinking water or gavage). Reproductive and non-gross developmental endpoints (molecular events, brain development, and behaviors) were also investigated. These studies revealed that prolonged exposure to arsenic could cause developmental toxicity at maternally non-toxic levels. For example, maternal exposures to inorganic arsenic in drinking water throughout gestation affected fetal brain development and newborn behaviors in rats (Chattopadhyay *et al.* 2002; Rodriguez *et al.* 2002).

Arsenic metabolism

Inorganic arsenic is the most common form of arsenic in the environment. Inorganic arsenic is readily absorbed through the gastrointestinal tract, and bio-transformed in the liver and other tissues. Most mammals, including mice, rats, and humans, bio-transform inorganic arsenic by alternating reduction and oxidative methylation (Fig 1) (Vahter 2002; Carter *et al.* 2003). The classical arsenic transformation is as follows: from inorganic pentavalent arsenate (As^V) to inorganic trivalent arsenite (As^{III}), to monomethylarsonic acid (MMA^V), to monomethylarsonous acid (MMA^{III}), to dimethylarsinic acid (DMA^V), to dimethylarsinous acid (DMA^{III}), and in some animals, including rats (Lu *et al.* 2003), to trimethylarsine oxide (TMA^VO). The reduction is facilitated by reductases and the reduced form of glutathione (GSH) along with possibly other thiols as electron donors. Oxidative methylation is carried out by methyltransferases, and S-adenosylmethionine (SAM) serves as the main methyl donor. Both mice and rats are more effective in arsenic methylation than humans.

Inorganic and methylated arsenicals are excreted mainly in the urine and to a lesser degree in the bile. More importantly, arsenic can pass through the placenta to the developing fetus. When

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pregnant mice inhaled arsine gas, fetal brain and liver had higher arsenic concentrations than placenta and maternal liver (Miyazaki *et al.* 2005). Inorganic arsenic in the drinking water of pregnant rats can also result in arsenic accumulation in fetuses, including the fetal brain (Rodriguez *et al.* 2002). Inorganic, monomethyl and dimethyl arsenicals all have been detected in the fetuses of pregnant mice exposed to As^{III} or As^{V} through i.p. injection or oral gavage (Hood *et al.* 1987; Hood *et al.* 1988). However, placenta and maternal blood, urine, liver, and kidneys were only analyzed for total arsenic in Hood's studies (1987, 1988). It is not clear what form(s) of arsenic transfer across the placenta or if fetuses methylate arsenic. Because the exposure to As^{III} or As^{V} results in exposure to its methylated metabolites, these studies cannot tell the active form of arsenic that is directly responsible for developmental toxicity. More information is therefore needed to identify the importance of arsenical species and methylation in placental transfer and arsenic developmental toxicity in mice and rats. In humans, DMA^{V} accounts for nearly 90 % of all detected arsenic in the blood plasma of both the newborns and their mothers (Concha *et al.* 1998). This form of arsenic accounts for 60-70% of the total detected arsenic in urine of the general population, while urine from pregnant women contained more than 90% arsenic as DMA^{V}. This suggested that arsenic methylation may be increased during pregnancy and that DMA^{V} is the major form of arsenic transferred to the fetus.

Rats are known to retain arsenic, as a consequence of their red blood cells having a high affinity for arsenic, particularly DMA^{III} (Lu *et al.* 2004). The whole body retention of inorganic arsenic in rats was twenty times higher than that in similarly exposed mice (Vahter 1981). In spite of the arsenic accumulation in rat erythrocytes, arsenic distributes to other tissues including the fetus as well. Although not an ideal test species for arsenic kinetic studies, rats are an appropriate animal model for arsenic reproductive and developmental toxicity studies.

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Reproductive effects of arsenic in mice and rats

1) Male reproductive toxicity

Arsenite exposure causes male reproductive toxicity when given through drinking water (Pant *et al.* 2001; Chinoy *et al.* 2004; Pant *et al.* 2004) or by i.p. injection (Sarkar *et al.* 2003). As[III] interferes with spermatogenesis (Pant *et al.* 2001; Sarkar *et al.* 2003; Pant *et al.* 2004) and alters activities of spermatogenetic enzymes (Pant *et al.* 2001; Chinoy *et al.* 2004; Pant *et al.* 2004). Furthermore, As[III] lowers levels of testosterone and gonadotrophin (Sarkar *et al.* 2003; Chinoy *et al.* 2004)(Fig. 2). These results suggest that arsenic may act on the brain or pituitary as well as directly on the germ cells (Sarkar *et al.* 2003).

Male mice exposed to sodium arsenite in drinking water showed reproductive toxicity without clinical effects (Pant *et al.* 2001). Sodium arsenite was given to mice via drinking water at up to 533.90 μmole/L for 35 days. As[III]-treated mice did not show changes in body weight, testes weight, or accessory sex organ weights. However, at 533.90 μmole/L, the activity of 17 β hydroxysteroid dehydrogenase (HSD) was decreased. Conversely, the activities of lactate dehydrogenase (LDH) and γ glutamyl transpeptidase (γ GT) were increased in the testes. LDH was used as a marker of Leydig cell function, and γ GT as a marker of Sertoli cell function. In addition to amino acid transport across the plasma membrane, γ GT regulates GSH levels and contributes to protection against oxidative stress (Meroni *et al.* 2000). Because arsenic lowers follicle-stimulating hormone (FSH), which in turn may decrease the activity of γ GT (Schteingart *et al.* 2002), the observed increases in the activity of γ GT could result from arsenic-induced oxidative stress. As[III]-treated mice also showed decreases in sperm count and motility along with an increase in abnormal sperm. One possible cause of arsenic-decreased sperm motility may be arsenic binding to thiols (Uckun *et al.* 2002). Sperm nuclear chromatins have large amounts of

thiol-rich protamines, and the sperm flagellum is rich in thiols. In addition, arsenic might affect

sperms through lowering levels of gonadotrophin and testosteronen (see below).

Spermatogenesis and plasma levels of gonadotrophin and testosterone were affected by As^{III}

in rats (Sarkar *et al.* 2003). Sodium arsenite was given to Wistar rats via i.p. injections at 4, 5, or

6 mg/kg/day for 26 days. At 5 and 6 mg/kg/day, relative testicular weight, accessory sex organ

weights and epididymal sperm counts were decreased. The same was true for plasma

concentrations of luteinizing hormone (LH), FSH and testosterone. Since late stages of

spermatogenesis were especially sensitive to testosterone, quantitative analysis of

spermatogenesis was carried out by counting the relative number of each variety of germ cells at

stage VII of the seminiferous epithelium cycle, as defined by Leblold and Clermont (1952).

Massive degeneration of all the germ cells at stage VII was observed at 5 and 6 mg/kg/day.

These authors suggested the observed arsenic-induced low levels of LH and FSH might be the

trigger of suppressed testosterone synthesis. Low testosterone consequently increased spermatid

degeneration. While As^{III} may act on the brain or pituitary to suppress LH and FSH levels, direct

inhibition on germ cells by binding to thiol cannot be ruled out. Another possible cause of the

reduction in serum LH, FSH and testosterone levels could be high serum corticosterone levels.

High corticosterone can reduce serum gonadotrophin and testosterone levels (Vreeburg *et al.*

1988; Hardy *et al.* 2005), and has been reported in As^{III} treated rats (Biswas *et al.* 1994).

In mice, in addition to spermatogenesis, cholesterol metabolism and testicular testosterone

level were affected by As^{III} (Chinoy *et al.* 2004). Male Swiss mice were given arsenic trioxide

(As$_2$O$_3$) orally at 0.5 mg/kg for 30 days.   Treated mice showed increased cholesterol levels and

decreased protein levels in the testes. Testicular structural damage observed included

degeneration of tubules and denudation of germinal epithelial cells. There was also a lack of

sperm in the lumen of seminiferous tubules. In addition, testicular activities of 3β-HSD and

17β-HSD, and testosterone levels in the serum were decreased. In the testis, cholesterol in the

interstitial tissue is used for testosterone synthesis (Kabbaj *et al.* 2003). 17β-HSD converts

androstenedione into testosterone. In the seminiferous tubes of the testis, cholesterol in the

membrane of developing cells influences the gamete's fertility (Kabbaj *et al.* 2003). These data

suggest that low serum testosterone after As[III] exposure was due to low enzymatic conversion

(17β-HSD), rather than a lack of the synthetic precursor (cholesterol). Chinoy et al. (2004) also

tested the effects of co-exposures to arsenic and fluoride (NaF), and found that the recovery from

arsenic and fluoride-induced effects can be facilitated by ascorbic acid, calcium and vitamin E.

These results suggest arsenic and fluoride-induced reproductive toxicity was at least in part

mediated by oxidative stress.

Male reproductive effects of long-term exposure to As[III] via drinking water were investigated

in mice by Pant et al. (2004). Swiss albino mice were given sodium arsenite (NaAsO$_2$ at 53.39

μmole/L, equivalent to 4 ppm arsenic) via drinking water for 365 days. The mice showed

decreases in absolute and relative testicular weights, but no change in epididymal or accessory

sex organ weights. Sperm count and sperm motility were decreased, and the percentage of

abnormal sperm was increased. Additionally, the activities of marker testicular enzymes were

altered. For example, enzymes associated with postmeiotic spermatogenic cells showed changes

in both directions. The activities of sorbitol dehydrogenase and acid phosphatase were

decreased, and the activity of LDH was increased. The changes in biochemical activities of

these testicular enzymes, which are associated with specific types of germ cells, suggested

damage to germ cells. The testicular activity of γ-GT, associated with Sertoli cells, was

increased. Meanwhile, activity of 17β-HSD, which converts androstenedione to testosterone in

Leydig cells, was decreased. These authors suggested the decreased 17β-HSD activity might be due to low levels of plasma gonadotrophins, which has been reported in rats i.p. injected with sodium arsenite (Sarkar *et al.* 2003).

2) Female reproductive toxicity

In female mice and rats, inorganic arsenic suppresses ovarian steroidogenesis, prolongs diestrus, and degenerates ovarian follicular and uterine cells (Zhang *et al.* 2000; Chattopadhyay *et al.* 2001; Chattopadhyay *et al.* 2003; Navarro *et al.* 2004). It also increases meiotic aberrations in oocytes, and decreases cleavage and pre-implantation development (Navarro *et al.* 2004).

Arsenic can induce ovarian and uterine toxicity, and influence neuroendocrine regulation of female sex hormones (Chattopadhyay *et al.* 2001). In female Wistar rats gavaged with 10 ml of 0.4 ppm sodium arsenite daily for 28 days, a consistent diestrous stage was observed. There were also decreases in relative ovarian and uterine weights, activities of Δ^5-3β-HSD and 17β-HSD in ovary, and the activities of peroxidase in the ovary and uterus. Moreover, levels of LH, FSH and estradiol in the plasma, and norepinephrine levels in midbrain and diencephalon were decreased, while serotonin levels in midbrain and diencephalon were increased. The primary cause of the observed As[III] toxicity in the female reproductive system could be arsenic-induced changes in the levels of catecholamines in the brain. The elevation in serotonin and decrease in norepinephrine in the midbrain and diencephalon could lower gonadotrophin synthesis and secretion. Low gonadotrophin levels could in turn decrease activities of ovarian Δ^5-3β-HSD and 17β-HSD, two important regulatory enzymes for steroidogenesis (Ghersevich *et al.* 1994a; Ghersevich *et al.* 1994b; Miro *et al.* 1995; Kaminski *et al.* 1997). These observations suggest that low plasma levels of estradiol could be the cause of consistent diestrous. These arsenic-

induced ovarian and uterine toxicities and steroidogenic dysfunction were decreased by co-administrations of ascorbic acid orally. Possible mechanisms of ascorbic acid protection included its antioxidant property, facilitating the elimination of arsenic, and influences on hormones. Regarding hormonal influences, ascorbic acid can enhance endogenous norepinephrine secretion and consequently stimulate gonadotrophin releasing hormone release (Miller and Cicero 1987). Ascorbic acid also facilitates the synthesis and secretion of gonadotrophins from the anterior pituitary (Wun *et al.* 1994), and is a stimulator of gonadal steroidogenesis (Murray *et al.* 2001).

Using the same strain of rat and As^{III} treatment, Chattopadhyay *et al.* later observed ovarian follicular and uterine cell degeneration (Chattopadhyay *et al.* 2003). This was accompanied by increases in dopamine levels in the midbrain and diencephalon, as well as arsenic levels in the ovary, uterus and plasma (Chattopadhyay *et al.* 2003). Similarly to norepinephrine, low dopamine levels could decrease gonadotrophin synthesis and secretion. The observed low FSH level may contribute to the observed decreased number of healthy follicles and increased number of apoptotic follicles. The authors suggested that uterine cell degeneration may be due to low ovarian estradiol and /or increased production of reactive oxygen species (ROS) after arsenic treatment. The As^{III} toxicity in the female reproductive system was decreased by co-administrations of sodium selenite orally. Conversely, this selenite supplement did not reduce As^{III}-increased activities of renal and hepatic enzymes. The causes of selenite selective protection against arsenic toxicity are not clear. Other studies showed that arsenic and selenium decrease the tissue concentrations of each other by increasing mutual excretions (Berry and Galle 1994; Csanaky and Gregus 2003; Zeng *et al.* 2005). Gastrointestinal and biliary excretion was increased via the formation of seleno-bis(S-glutathionyl) arsinium ion (Csanaky and Gregus

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2003; Zeng *et al.* 2005). Arsenic and selenium also precipitate each other by forming insoluble selenide (As_2Se), which were seen in lysosomes of renal cells and in dense deposits in the urinary lumen (Berry and Galle 1994; Zeng *et al.* 2005). These lysosomes and their precipitate were consequently excreted in the urine. Furthermore, selenite inhibits As^{III}- and As^{V}-induced activation of C-Jun N-terminal kinase (JNK), activator protein (AP-1), and nuclear factor-κB (NFκB) signaling. Consequently, selenite inhibits arsenic-induced apoptosis and necrosis.

Oocyte meiotic abnormalities and compromised pre-implantation development were observed in As^{III}-treated mice (Navarro *et al.* 2004). Female CD-1 mice were i.p. injected with 0, 8, or 16 mg/kg sodium arsenite every 2 days for a total of 7 injections over 14 days. Super-ovulation was induced by injections of equine and human chorionic gonadotrophins overlapping the end of As^{III} treatment. Metaphase II oocytes from these As^{III}-treated mice had increased meiotic aberrations, characterized by spindle disruption and chromosomal misalignment. Additionally, zygotes from As^{III}-treated mice showed lower rates of cleavage, decreased morula formation, and decreased development to blastocysts. More apoptotic nuclei were seen in the blastocysts of As^{III}-treated mice. The authors suggested that arsenic-induced meiotic aberrations could subsequently compromise oocyte fertilization, pre-implantation development and embryo viability. Some of these arsenic effects on oocytes were observed at 8 mg/kg, a previously established maternal non-observed-adverse-effect-level (NOAEL).

Developmental effects of arsenic in mice and rats

Developmental toxicity is the adverse effects of an agent on the developing organism. Developmental toxicity can be manifested as death, structural anomaly, altered or retarded growth, and functional deficiency (US FDA 2000). The last includes biological dysfunctions and behavioral deficits that become evident as the animal grows.

Developmental toxicity observed without maternal toxicity is a clear indication of selective toxicity to the embryo/fetus. However, maternal toxicity does not preclude the possibility that an agent is also a developmental toxicant. US FDA 2000 Redbook, Guidelines for Developmental Toxicity Studies (US FDA 2000) stated that "developmental effects that occur in the presence of minimal maternal toxicity are considered to be evidence of developmental toxicity, unless it can be established that the developmental effects are unquestionably secondary to the maternal effects. In situations where developmental effects are observed only at doses where there is a substantial amount of maternal toxicity, then the possible relationship between maternal toxicity and the developmental effects should be evaluated". Maternal toxicity can be measured as changes in body weight and adjusted body weight, and feed and fluid consumption. Daily clinical observations and necropsy data, such as organ weights, are also maternal toxicity parameters.

In 2001, the US EPA announced a new maximum contaminant level for arsenic in drinking water of 10 μg/L (10 ppb). Several excellent reviews of arsenic developmental toxicity had been published prior to the year 2001 (DeSesso *et al.* 1998; US EPA 1998; Holson *et al.* 2000a). In the present review, new findings of arsenic developmental toxicity are emphasized. *In vivo* studies and those following FDA guidelines (US FDA 2000) provide the most relevant information to humans. Additionally, studies on the influences of folate and methylation on arsenic developmental toxicity may help in identifying susceptible populations. All gestation days (GDs) in this review have been adjusted such that gestation day (GD) 0 was recorded upon demonstration of vaginal sperm plug in mice or sperm in the vaginal smear in rats.

Inorganic arsenicals, As^{III} and As^V, are more toxic than organic arsenicals to embryos/fetuses (National Research Council 1999). Similar to other systemic toxicity, the teratogenic potential is

greater from As^{III} than As^V (Hunter 2000; Lammon *et al.* 2003). Few studies have examined developmental effects of arsine gas (Morrissey *et al.* 1990) or organic arsenic (Rogers *et al.* 1981; Chernoff *et al.* 1990); nor have trivalent organic arsenicals been studied in this respect. Inorganic arsenic given to pregnant dams induces slow development, behavior changes, and malformations in the fetus (DeSesso *et al.* 1998; National Research Council 1999; Stump *et al.* 1999; Holson *et al.* 2000b; DeSesso 2001; Chattopadhyay *et al.* 2002). Arsenic-induced malformations have been reported in the neural tube, skull and skeleton, eye, and urogenital system.

1) Arsine gas

Arsine gas (AsH_3) was not fetotoxic or teratogenic in rats or mice (Morrissey *et al.* 1990). Pregnant F344 rats and CD-1 mice were exposed to up to 2.5 ppm (8 mg/m^3) arsine by inhalation for 6 hours/day during GDs 6-15. No developmental or reproductive toxicity was observed, although maternal splenomegaly and evidence of hemolysis occurred in the 2.5 ppm group (Morrissey *et al.* 1990). When pregnant rats were exposed to up to 5 ppm arsine during GDs 6-17, the arsenic concentrations in both maternal blood and fetal liver were increased in a dose-dependent manner. This indicated that the lack of arsine fetotoxicity/teratogenicity was not due to the lack of embryonic arsenic exposure. The arsine NOAEL for maternal toxicity was 0.5 ppm (increased spleen weight in rats and mice). This was also the arsine NOAEL for developmental effects (increase in average fetal body weight per litter in rats).

2) Methylated arsenic

Dimethylarsinic acid given to pregnant mice and rats by oral gavage caused developmental toxicity (Rogers *et al.* 1981; Chernoff *et al.* 1990). CD-1 mice were orally gavaged with DMA^V at 200, 400 or 600 mg/kg/day during GDs 7-16 (Rogers *et al.* 1981). These mice showed lower

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maternal weight gain and fetal weight at 200 mg/kg/day, and a higher incidence of cleft palate at 400 mg/kg/day. Similarly, during GDs 7-16, CD rats were orally gavaged with DMA^V at 7.5 to 60 mg/kg/day (Rogers *et al.* 1981). Maternal weight gain and fetal weight were decreased at 40 mg/kg/day and higher. Fetal mortality was increased at 50 or 60 mg/kg/day. However, no fetal gross malformations were seen in these rats. In a later study, DMA^V was given to pregnant Sprague Dawley (SD) rats at 40 mg/kg/day by oral gavage during GDs 6-15. This treatment did not induce maternal weight reduction or maternal lethality, but decreased fetal weight (Chernoff *et al.* 1990).

3) Inorganic arsenic

Fetal malformations were only reported when pregnant rats and mice were i.v or i.p. injected with inorganic arsenic at early gestation (Stump *et al.* 1999; DeSesso 2001). Maternal inhalation or oral ingestion of inorganic arsenic affected fetal development and behavior, but did not cause malformations (Holson *et al.* 1999; Stump *et al.* 1999; Holson *et al.* 2000b; DeSesso 2001; Chattopadhyay *et al.* 2002). The importance of administration routes in determining adverse developmental effects of inorganic arsenic can be explained by toxicokinetic differences. The toxicokinetic differences among administration routes include maternal circulation levels of arsenicals as well as rates and pathways of biotransformation and excretion (DeSesso 2001). The maternal circulation levels of arsenicals are influenced by absorption rates. In oral exposure, arsenic is absorbed into the blood from the intestines. It is then transported to the liver and may undergo first-pass metabolism prior to being delivered to the uterus (Stump *et al.* 1999; DeSesso 2001). Additionally, first-order elimination was observed for maternal arsenic in mice given inorganic arsenic (Hood *et al.* 1987; Hood *et al.* 1988). Intraperitoneal injections, on the other hand, allow arsenic to be taken up by blood vessels directly. Some arsenic may bypass first-pass

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metabolism by going into vessels that line the inner surface of peritoneal cavity (Stump *et al.* 1999; DeSesso 2001). Oral administration of arsenic at a dose twice that used in i.p. injection resulted in a peak maternal arterial blood arsenic concentration that was roughly only 30% of the i.p. injection (Hood *et al.* 1987; DeSesso *et al.* 1998). Furthermore, the uterus may be directly exposed to arsenic in the peritoneal cavity, which may have higher concentrations than blood. As a result, the arsenic concentration differences in embryos from mothers exposed to arsenic from i.p. injection and oral gavage are even greater than those in maternal blood. A maximal embryonic inorganic arsenic concentration after maternal oral exposure to a dose twice that used in maternal i.p. injection was only 22% of that caused by the i.p. injection (Hood *et al.* 1987). When the same dose of arsenic was given to pregnant rats, the embryonic total arsenic concentration from i.p. injected mothers was more than 10 times higher than that from the orally exposed mother (Holson *et al.* 2000a). Inhalation is the least effective means of increasing maternal or embryonic arsenic concentrations, compared to i.p. and i.v. injections and oral exposure (Holson *et al.* 2000a).

3.1 Intraperitoneal injection

Consistent with earlier studies, sodium arsenate given by a single i.p. injection induced fetal malformations in Swiss mice (Fascineli *et al.* 2002). Without affecting maternal weights, an i.p. injection of 45 mg/kg sodium arsenate on GD 8 decreased placental weight and increased fetal malformations. The increased malformations included external (exencephaly and eye abnormalities), visceral (hydrocephalus and hydronephrosis) and skeletal malformations.

3.2 Oral gavages

In a multiple administration study (Holson *et al.* 2000b), arsenic trioxide via oral gavage did not cause neural tube defects, even at maternally toxic dose levels. Female Crl:CD®(SD)BR rats

were gavaged with arsenic trioxide from 14 days prior to mating through GD 19. At the highest

dose tested (10 mg/kg/day), fetal weights were decreased. There were no arsenic-induced

changes in mating index, fertility index, implantation, or fetal malformation. It is worth noting

that maternal toxicity[1] was observed at 10 mg/kg/day and the maternal NOAEL was 2.5

mg/kg/day due to transient decreases in food consumption at 5 mg/kg/day.

3.3 In drinking water

When As^{III} was given to pregnant rats in the drinking water throughout gestation, fetal

behavior and brain development were affected (Chattopadhyay *et al.* 2002). Sodium arsenite

was administered to pregnant rats in drinking water at 0.03, 0.3, and 3 ppm. While rats exposed

to up to 0.3 ppm As^{III} could complete gestation and parturition on schedule, an exposure of 3

ppm caused 25% neonatal death. At 0.3 and 3 ppm, both post gestational mothers and 1 day old

neonatal pups showed decreased spontaneous behavior, with this effect being more dramatic in

neonatal pups. The spontaneous behavior was measured as the changes of weight of an animal

when placed on a single pan balance. It included, and was not limited to, movement, shaking,

tremors, and grooming. Neonatal rat brain cells derived from pups of 0.3 ppm arsenic treated

mothers had increased cell membrane damage (as measured by Trypan Blue dye exclusion test).

They also showed increased intracellular generation of ROS and nitric oxide (NO), and

decreased DNA and protein synthesis (measured by ^{3}H-thymidine and ^{14}C-leucine incorporation,

respectively). These data demonstrated that the developing brain can be affected by *in utero*

exposure to non-maternal-lethal levels of As^{III} in drinking water.

Postnatal developmental changes were observed when As^{III} was given in the drinking water

to pregnant and lactating rats and continually to the newborns (Rodriguez *et al.* 2002). Sodium

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[1] The maternal toxicity in the 10 mg/kg/day group was evidenced by decreased food consumption, decreased body
weigh gain during gestation, increased liver and kidney weights, and stomach adhesions and erosions.

arsenite at 36.7 mg/L was administered to SD rats from GD 15 or postnatal day 1, until newborns were approximately 4 months old (Rodriguez *et al.* 2002). Weaned pups received the same As^{III} treatments in water as mothers. While both female and male pups were assessed for developmental indices, only male pups were subjected to behavioral tests. The behavioral tests measured spontaneous locomotor activity in a chamber and motor coordination on a rotating cylinder. Two learning tasks, spontaneous and delayed alternation tests, were also included in the behavioral tests. Maternal behaviors (retrieval of pups, cleaning and sniffing pups, nest-building and self-grooming) and body weights were unaffected by either arsenic treatment. In behavioral tests, the pups in the group exposed from GD 15 showed increased spontaneous locomotor activities, and pups in both exposed groups showed increased numbers of errors in a delayed alternation task in comparison to the pups in the untreated control group. Since performing a delayed alternation task requires sensory information on the body in space, the increased errors in arsenic-exposed pups suggested that the striatum, hippocampus and prefrontal cortex, along with neurotransmitter metabolism may be affected by arsenic. Among the developmental indices, the group exposed from GD 15 had more litters showing full pinna detachment on postnatal day 12. Conversely, more litters showed low ratings on eye opening on postnatal day 14, compared to the untreated controls. However, there was no difference in these developmental indices on postnatal day 16. These data showed that arsenic induced an asynchrony of the maturation processes during postnatal development. Furthermore, arsenic caused behavioral changes, including deficits in spontaneous locomotor activity and more errors in completing a spatial learning task.

3.4 Influence of selenium

In utero exposures to As^{III} decreased the activity of thioredoxin reductase (an antioxidant enzyme) in the brain (Miyazaki *et al.* 2005). Furthermore, combination of As^{III} and a selenium deficient diet decreased the activity of type II iodothyronine deiodinase (a selenoenzyme important for brain development) (Miyazaki *et al.* 2005). Pregnant ICR mice were fed a selenium-sufficient or selenium-deficient diet from GDs 0 to 16. From GDs 7 to 16, half of the rats were orally gavaged with 58 μmol/kg/day sodium arsenite. On GD 17, maternal and fetal tissues were harvested and analyzed. None of the treatments caused changes in maternal weight, litter size, mortality or fetal body weight. The selenium-deficient diet increased arsenic concentrations in maternal liver and fetal brain in arsenic treated groups. Arsenic decreased thioredoxin reductase activities in the fetal brain in both diet groups, and in the fetal liver in the selenium-deficient group. This decrease of antioxidant selenoenzyme activity could enhance oxidative stress and damage. Furthermore, with mothers on a selenium-deficient diet (but not selenium-sufficient diet), fetal brains showed arsenic-increased activity of type II iodothyronine deiodinase. Among four types of iodothyronine deiodinases, types II and III are expressed in the brain (Kodding *et al.* 1986; Polk 1995). Type II iodothyronine deiodinases transform inactive thyroxine (T4) to receptor-active triiodothyronine (T3) by outer ring deiodination. Meanwhile, type III iodothyronine deiodinases transform inactive T4 to inactive reverse T3 by inner ring deiodination. The balance of these activities is important in determining brain T3 level, which affects brain development. Miyazak (2005) therefore suggested that *in utero* exposure to As^{III} in conjunction with selenium deficiency might disturb fetal thyroid hormone balance in the brain, and potentially brain development.

3.5 Influence of zinc

Zinc did not lessen arsenic fetotoxicity in mice (Fascineli *et al.* 2002). Both arsenic and zinc are known to induce expression of metallothionein (Liu *et al.* 2001), a protective protein that binds heavy metals. Metallothionein isotype 1 may be involved in developmental processes during gestation (Nordberg and Nordberg 2000). Zinc also induced arsenic tolerance in mice in a metallothionein independent manner (Kreppel *et al.* 1994). Zinc, however, did not ameliorate arsenic teratogenicity, when it was given either prior to arsenic or simultaneously with arsenic (Fascineli *et al.* 2002). For zinc pretreatment, Swiss mice were gavaged with zinc on GDs 7 and 8, and i.p. injected with As^V on GD 8. For simultaneous treatment, mice were i.p. injected with As^V plus zinc on GD 8. The dose of sodium arsenate ($NaHAsO_4.7H_2O$) was 45 mg/kg. Zinc sulfate ($ZnSO_4.7H_2O$) was given by oral gavage at 40 or 20 mg/kg or i.p. injection at 10 or 5 mg/kg. Controls received no treatment, As^V alone or zinc alone. When dams and fetuses were examined on GD 17, As^V alone decreased fetal and placental weights and increased fetal malformations. Zinc alone caused delayed fetal development, but not malformations, with the exception that an i.p. injection of 10 mg/kg zinc caused exencephaly. Excenphaly is a condition in which the brain is located outside the skull due to defects in neutral tube closure. Exposures to zinc plus As^V (in sequence and simultaneously) decreased maternal weight gain, fetal weight and placental weight, and delayed fetal ossification. Moreover, zinc did not decrease As^V-induced malformations. In fact, vertebrate skeletal anomalies were more frequent in the zinc plus As^V group than As^V alone group. This report demonstrated that neither pretreatment nor simultaneous treatment of zinc prevented arsenic-induced teratogenicity in mice. The fetotoxicity from arsenic and zinc exposure may be a combination of their effects on the fetus, placenta, and mother.

3.5 Influence of folate

Folate can affect arsenic methylation, and both folate deficiency and arsenic can induce malformations, including neural tube defects (NTDs). Using transgenic mice deficient in folate transport, the relationship between folate and arsenic developmental toxicity has been intensively studied in the past 5 years. Arsenic methylation is catalyzed by methyltransferases and requires S-adenosylmethionine (SAM) as the methyl donor. SAM is eventually regenerated through the homocysteine remethylation cycle, a process that requires 5-methyl-tetrahydrofolate as a cofactor (Fig 3). Thus, inorganic arsenic metabolism is dependent on the folate supply (Spiegelstein *et al.* 2003). Both folate deficiency and arsenic exposure have been reported to increase congenital malformations, including NTDs, in humans and rodents. Folate enters cells through folate binding proteins (Folbp) in conjunction with reduced folate carriers (RFC). Mouse Folbp1-/- embryos have multiple structural malformations, including NTDs, and die before GD 10.5. Similarly, RFC-/- embryos die immaturely *in utero*. On the other hand, Folbp1+/-, Folbp2-/-, and RFC+/- embryos develop normally with no apparent congenital abnormalities (Wlodarczyk *et al.* 2001; Spiegelstein *et al.* 2005b).

Mice lacking a functional *Folbp 2* gene (Folbp2-/-) were more sensitive to *in utero* arsenic exposure than wild type mice, and a folate deficient diet further increased arsenic-induced teratogenicity (Wlodarczyk *et al.* 2001; Spiegelstein *et al.* 2005b). In a study of embryonic genotype effects on arsenic teratogenicity, female Folbp2-/- and Folbp2+/+ mice were mated with males of the same genotype, and were i.p. injected with 40 mg/kg sodium arsenate on GDs 7.5 and 8.5, the critical period of neural tube closure (Wlodarczyk *et al.* 2001). This As^V treatment increased resorption rates and NTDs in the surviving fetuses from both strains. After subtracting spontaneous NTDs in untreated controls of the same genotype, the increase of As^V-induced NTDs was bigger in Folbp2-/- embryos than in Folbp2+/+. A folate-deficient diet

further increased the NTD frequency in Folbp2-/- embryos, but not in Folbp2+/+ embryos (Wlodarczyk *et al.* 2001). In addition, maternal genotype affected the sensitivity to *in utero* As^V exposure of Folbp2+/- embryos. Folbp2+/- embryos were from Folbp2-/- females mated with Folbp+/+ males or from Folbp2+/+ females mated with Folbp-/- males. The resorption rates of embryos from the Folbp+/+ maternal group were higher than that from the Folbp-/- maternal groups after corresponding As^V 30 and 40 mg/kg single i.p. doses, but there were no differences in excencephaly rates between maternal genotype groups. Conversely, no difference was found in the 24-hr urinary arsenical profiles of Folbp2-/- and Folbp+/+ female mice i.p. injected with 30 mg/kg sodium arsenate (Wlodarczyk *et al.* 2001). In a later study (Spiegelstein *et al.* 2005b), arsenicals were measured in the 24-hr urine of wild type and Folbp2-/- male mice after a single i.p. injection of 1 mg/kg sodium arsenate. In spite of the decreased plasma folate and SAM levels in Folbp2-/- mice compared to the wild type, there were no differences due to genotype in urinary arsenical profiles of male mice. Overall, impairment of Folbp2-mediated folate transportation due to inactive *Folbp2* gene increased developmental defects induced by *in utero* exposure to As^V without affecting arsenic metabolism/excretion.

In contrast to the elevated sensitivity to As^V teratogenicity seen in Folbp-/- mice, no RFC or Folbp1 genotype-related differences in embryonic susceptibility to As^V exposure were observed (Spiegelstein *et al.* 2005a). Due to the embryonic lethality in Folbp-/- and RFC-/- mice, Folbp+/- and RFC+/- mice were used by these authors (Spiegelstein *et al.* 2005a). In a study of *Folbp1* effects, Folbp1+/- female mice were mated with Folbp1+/+ males, after which pregnant mice received an i.p. injection of sodium arsenate at a dose of 30, 35, or 40 mg/kg on both GDs 7.5 and 8.5. All three doses increased resorption rates and NTD rates with no embryonic genotype (Folbp+/+ or Folbp1+/-) difference. Similarly, in a study of *RFC* effects, RFC+/-

female mice were mated with RFC+/+ males, and RFC+/+ females were mated RFC+/- males; pregnant mice received an i.p. injection of 40 mg/kg sodium arsenate on GDs 7.5 and 8.5. Arsenate treatment increased NTD rates, but no differences were observed by RFC geneotypes or mating strategy. Regarding *RFC* effects on arsenic metabolism, RFC+/+ and RFC+/- mice were i.p. injected with 1 mg/kg sodium arsenate after receiving a normal or folate deficient diet. With a normal diet, RFC+/- mice had the same plasma folate levels, plasma SAM levels, and urinary arsenical profiles as the wild type (RFC+/+). With a folate deficient diet, both RFC+/+ and RFC+/- mice had lower plasma folate and normal plasma SAM levels, and lower total arsenic in the urine. Thus, there were no RFC-genotype- or Folbp-genotype-related differences in embryonic susceptibility to *in utero* As^V exposure-induced NTDs, and arsenic metabolism appeared unaltered in RFC+/- mice.

Folate supplements failed to protect mice from arsenic teratogenicity (Gefrides *et al.* 2002). Arsenic acid (Na$_2$SeO$_4$) given at 40 mg/kg (i.p.) once each day on GD 7.5 and 8.5 increased NTDs and resorptions in LM/Bc, SWV, and CXL-Splotch mice. LM/Bc embryos were highly susceptible to arsenic developmental toxicity, while SWV embryos were relatively resistant. CXL-Splotch mice carry a mutation in the transcription factor *Pax3*, and heterozygous litters have high incidences of spontaneous NTDs. Arsenic increased NTDs in both CXL wild-type (+/+ x +/+) and heterozygous (+/Sp x +/Sp) litters. Neither folic acid nor folate[2] given to pregnant mice at 25 mg/kg (i.p.) once daily from GDs 6.5 to 10.5 provided protection against arsenic-induced resorptions or NTDs in CXL wild-type and heterozygous embryos, LM/Bc, or SWV. Folic acid and folate also did not decrease spontaneous NTDs in Splotch heterozygous

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[2] While folate and folic acid are often used as interchangeable terms, folic acid is the synthetic form of folate, which is a B vitamin found naturally in some foods Kurtzweil, P. (1999, February 1999). "How Folate Can Help Prevent Birth Defects." Retrieved August 30, 2005, from http://www.cfsan.fda.gov/~dms/fdafolic.html..

embryos. Unexpectedly, folate given to arsenic treated pregnant mice caused maternal deaths in all three strains.

Studies of folate effects on arsenic developmental toxicity can be summarized as follows. Folbp2-/- mice, but not Folbp1+/- or RFC+/- mice, had increased sensitivity to *in-utero*-arsenate-exposure-induced teratogenicity. There was no apparent RFC or Folbp2 genotype-related difference in arsenic metabolism based on urine arsenicals, and therefore the increased sensitivity to arsenate teratogenicity in Folbp2-/- mice was unlikely due to changes in arsenic metabolism. Furthermore, folate and folic acid supplements did not protect mice from arsenic-induced resorptions or malformations.

3.6 Influence of methylation

Inhibition of arsenic methylation, by either a methylation inhibitor or a protein deficient diet, increased As^{III} and As^{V} developmental toxicities (Lammon *et al.* 2003; Lammon and Hood 2004). Periodate-oxidized adenosine (PAD) inhibits SAM-dependent methylation, and therefore inhibits arsenic methylation. In mice treated with PAD and then either As^{III} or As^{V}, inhibition of arsenic methylation was evidenced in the urinary arsenic profile (Lammon *et al.* 2003). Those mice had increased inorganic arsenic and decreased DMA^{V} in the urine, as compared to mice treated with As^{III} or As^{V} alone. This PAD pre-treatment enhanced As^{III} and As^{V} developmental toxicities, including resorptions and fetal malformations (Lammon *et al.* 2003). CD-1 mice were given PAD (i.p.) followed by 7.5 mg/kg sodium arsenite or 17.9 mg/kg sodium arsenate (i.p.) on GD 8. Controls received As^{III}, As^{V}, PAD alone, or were untreated. Mothers were sacrificed on GD 17, and litters were examined. Compared to untreated mice, PAD-alone-treated mice had increased prenatal mortality and percentages of litters with grossly malformed fetuses. Similarly, mice treated with As^{III} alone had increased prenatal mortality. As^{V}-alone-treated mice had

increased incidences of ablepharia (a partial or total absence of the eyelids). Compared to either arsenical alone, PAD pre-treatment resulted in higher maternal morbidity and mortality, more complete resorptions and lower fetal weight. Furthermore, PAD pre-treatment increased fetal malformations, possibly by PAD-induced maternal toxicity or PAD-enhanced arsenic toxicity. Increased incidences of exencephaly, ablepharia, misshapen vertebral centra, and abnormalities of the ribs and sternebrae were observed. In summary, both As^{III} and As^{V} developmental toxicity can be enhanced by PAD, possibly by inhibition of arsenic methylation.

A low protein diet also enhanced both As^{III} and As^{V} developmental toxicity (Lammon and Hood 2004). Female CD-1 mice were given diets with 20% (protein sufficient), 10% (moderate protein deficiency), or 5 % (severe deficiency) protein. A single i.p. injection of As^{III} (7.5 mg/kg $NaAsO_2$) or As^{V} (17.9 mg/kg Na_2HAsO_4) was given on GD 8. These treatments were expected to induce low levels of malformations; fetuses were examined on GD 17. While 5% protein diet alone decreased maternal weight, protein deficiency alone did not cause developmental toxicity. With a protein-sufficient diet, increases in malformations were observed in either As^{III} or As^{V} treatment. The As^{III} or As^{V} alone increased incidences of exencephaly, ablepharia, rudimentary ribs, and sternebrae abnormalities. Arsenic plus protein deficiency decreased maternal weight gain, and increased the incidences of exencephaly, ablepharia and skeletal defects. The observed skeletal defects included malformed vertebral centra, fused ribs and abnormal sternebrae (bipartite, rudimentary or unossified). These data showed that as the dietary protein content decreases, the incidence of fetal malformations of the offspring of arsenic-treated pregnant mice increases. Since the protein deficiency alone did not cause developmental toxicity in this study, the authors suggested that protein deficiency probably enhanced arsenic developmental toxicity by impairing arsenic methylation.

CONCLUSION

The forms of arsenic and administration routes greatly affect the severities and types of arsenic-induced reproductive and developmental toxicity. Inorganic arsenic caused reproductive and developmental toxicity, as demonstrated in *in vivo* studies using rats and mice. DMA^V caused developmental toxicity, while arsine did not. Gross structural malformations were only induced by i.p. or i.v. injections of inorganic arsenic. Other reproductive and developmental toxicity, however, was seen after maternal oral inorganic arsenic exposure. Inorganic arsenic exposure, including via drinking water, affects hormonal regulation and functions of both the male and female reproductive systems. Newborn behaviors and fetal brain development were also affected by As^{III} in the drinking water.

Selenium, but not zinc or folate, supplement was protective against arsenic-induced reproductive and developmental toxicity. Selenite supplementation decreased arsenic-induced female reproductive toxicity. A selenium-deficient diet increased arsenic-induced changes in selenoenzymes, important for brain development. Folate deficiency is associated with neural tube defects, and may interfere with arsenic methylation (Fig 3). Folbp2-/- mice, but not Folbp1+/- or RFC+/- mice, had increased sensitivity to *in utero*-arsenate-exposure-induced teratogenicity. However, folate and folic acid supplements did not protect mice from arsenic-induced resorptions or structural malformations. Inhibition of arsenic methylation also increased As^{III} and As^V developmental toxicity.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST STATEMENT
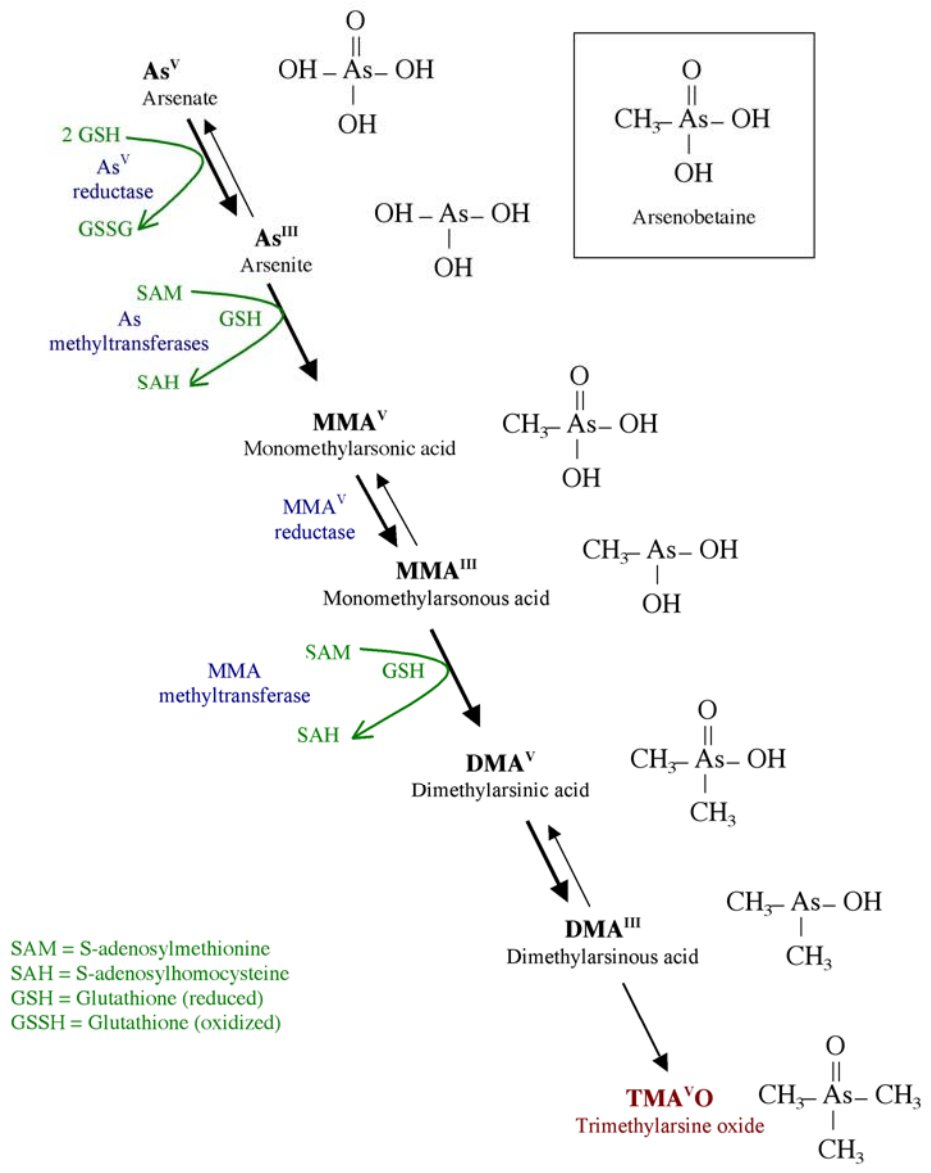
FIGURE 1. Classical arsenic metabolism pathway includes reduction and oxidative methylation.
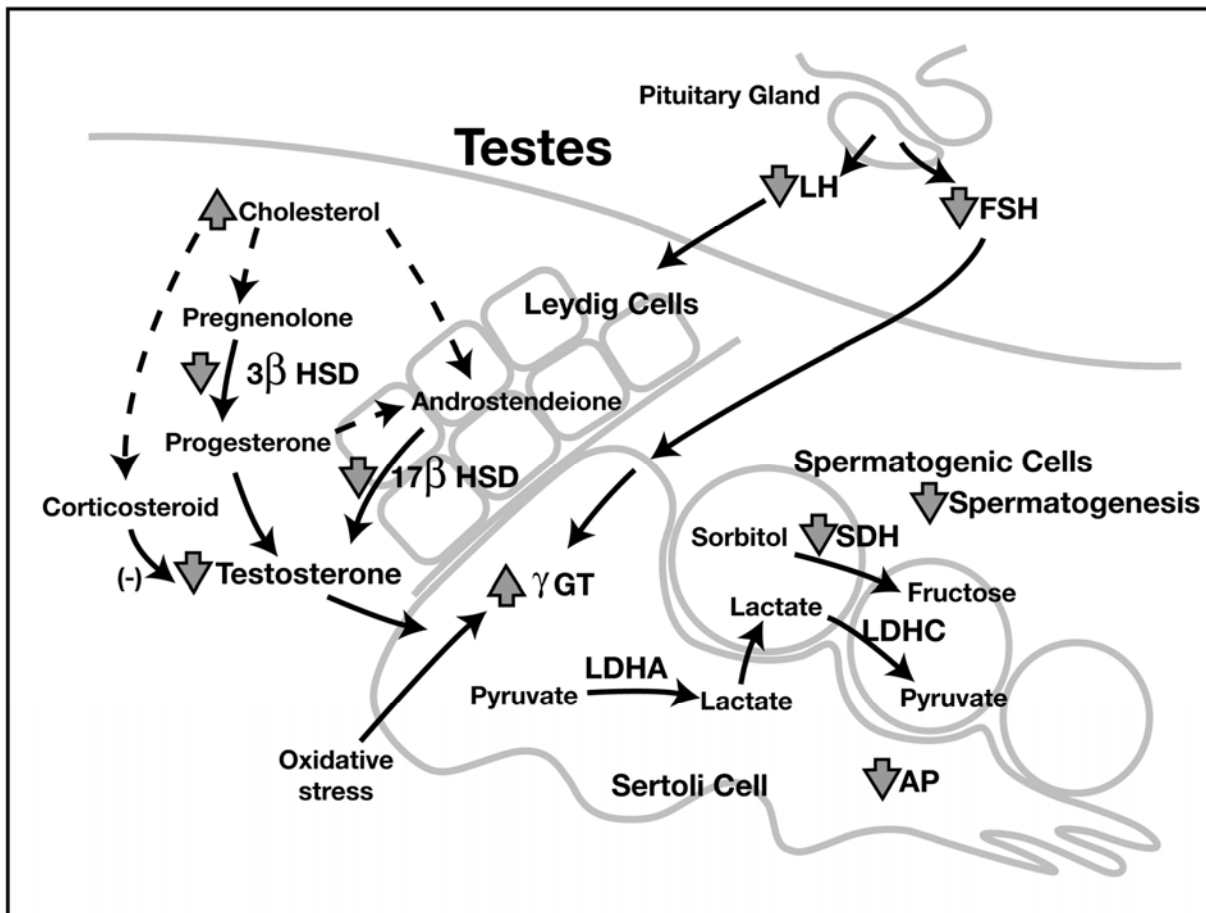
FIGURE 2. Arsenic affects male reproductive system at hormonal control and enzymatic activities. The upward arrow heads (⇧) indicate increases that have been demonstrated in the review studies after arsenic exposure. The downward arrow heads (⇩) indicate decreases after arsenic exposure. Low LH and FSH concentrations could be the leading cause of observed arsenic effects, including low testosterone concentration. Increased γ GT activity could be from arsenic-induced oxidative stress. LDH and SDH, enzymes involved in providing energy substrate for germ cells, are also affected. Overall LDH activity was increased by arsenic, while SDH activity was decreased. Spermatogenesis is also inhibited by arsenic. AP = acid phosphatase. LDHA = lactate dehydrogenase A. LDHC= lactate dehydroganse C. SDH = sorbitol dehydrogenase.
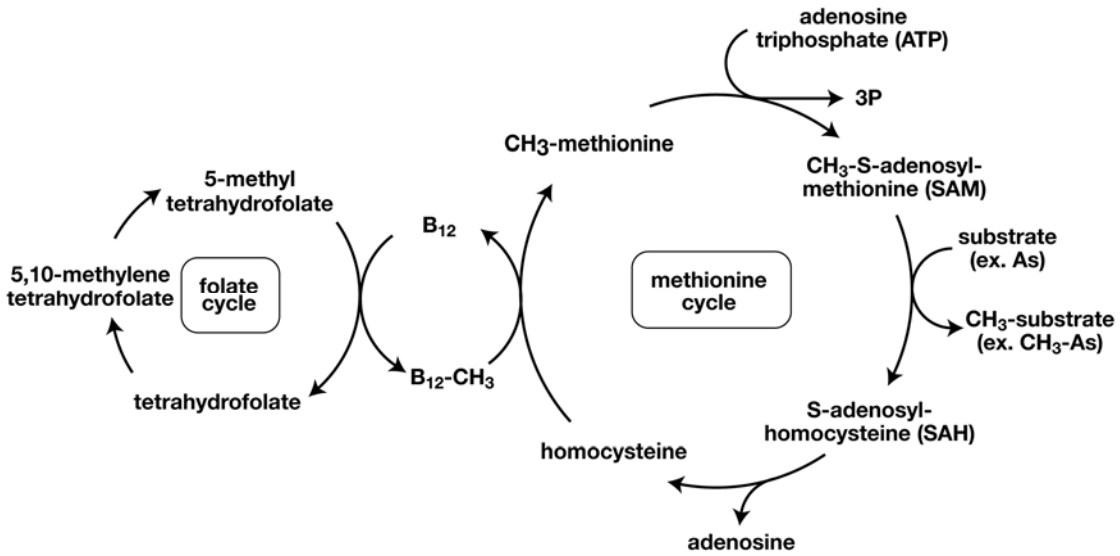
FIGURE 3. Folate influences arsenic methylation by affecting regeneration of SAM, the methyl donor in arsenic methylation. Adapted from E. Somundsun (2004) and C. Bolander-Gouaille (2004).

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CHAPTER 3   SUBACUTE TOXICITY OF DIMETHYLARSINIC ACID IN DRINKING WATER IN SPRAGUE-DAWLEY RATS: A PRELIMINARY EVALUATION OF GENERAL TOXICITY, ULTRASTRUCTURAL CHANGES IN THE URINARY BLADDER AND TRANSITIONAL CELL COLLECTION TECHNIQUES

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ABSTRACT

Arsenic-associated urinary bladder cancer is primarily transitional cell carcinoma. While animal models for arsenic carcinogenesis studies exist, commonly used analyses of whole bladders may lack the sensitivity and specificity for detecting key events of arsenic carcinogenicity within target transitional epithelial cells. In this methods-development study, Sprague-Dawley® rats received 0, 4, or 40 ppm dimethylarsinic acid [DMA(V)] via drinking water for 4 weeks. No overt toxic responses including changes in body weight or consumption of food or water were observed. Scanning and transition electron microscopy were used to detect arsenic-related morphologic damage in transitional cells. Results suggested that a four-week exposure of 40 ppm DMA(V) in drinking water is cytotoxic to the urinary bladder. Methods for selectively collecting transitional cells that might allow focused gene expression and DNA damage detection were then evaluated. Neither enzymatic (collagenase plus Dispase) nor mechanical scraping of bladder yielded sufficient live transitional cells for Comet assay, to detect DNA damage by Comet assay. However, each of two Trizol® striping techniques resulted in pure transitional cells, and in sufficient numbers for mRNA extraction. These techniques should therefore allow target cell-focused gene expression studies in the arsenic-exposed rats, something previously not available.

INTRODUCTION

Arsenic is a natural, ubiquitous element in the Earth's crust and is a human carcinogen. Exposure to arsenic induces neoplasms in the skin, lung, and urinary bladder (Ng *et al.* 2003; Tchounwou *et al.* 2003). A primary modality of arsenic exposure for the general population is through drinking water. The high cost and technical expertise associated with removing arsenic from water make it difficult to routinely remediate. Subsequently, high concentrations of arsenic in drinking water are present not only in Argentina, Bangladesh, Chile, China, Japan, and Taiwan, but also in western and northeastern states of the United Sates of America (National Research Council 1999; Ayotte *et al.* 2003; Steinmaus *et al.* 2003). The International Agency for Research on Cancer (IARC) determined that arsenic compounds are dermal and pulmonary (via inhalation) carcinogens in 1980 (IARC 1980; IARC 1987), and are human urinary bladder carcinogens in 2004 (IARC 2004).

Epidemiological studies revealed that arsenic-induced bladder cancer arises from transitional cells (Guo *et al.* 1997; Chiou *et al.* 2001), and may be fundamentally different from non-arsenic-associated bladder cancer (Moore *et al.* 2002).

The mechanisms underlying the development of arsenic-induced bladder cancer have been studied in a variety of laboratory animal models; however, only F344 rats showed increased bladder cancer after exposures to arsenic, specifically to DMA(V) (Wang *et al.* 2002). Other combinations of rat and mouse strains and species of arsenic did not yield bladder cancer, although lung, skin and other types of cancer were increased in some studies (reviewed by (Wang *et al.* 2002). When exposed to 100 ppm DMA(V) via the diet for 2 years, female F344 rats had a 17% incidence of urinary bladder transitional cell tumors (6 carcinoma and 4 papillomas in 58 rats), while control female rats displayed no bladder tumors (US EPA 1994). In

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the same study, male rats showed bladder hyperplasia and a non-significant increase in bladder

tumors (US EPA 1994). When exposed to DMA(V) via drinking water for 97-104 weeks, male

F344 rats showed increases in urinary bladder tumors at 50 ppm and higher concentrations of

DMA(V). Urinary bladder tumors were seen in 39% of the animals (2 papillomas and 10

transitional cell carcinomas in 31 rats) in the 200 ppm group, in 26% of the animals (2

papillomas and 6 carcinomas in 31 rats) in the 50 ppm, and in 0% of the animals in the 12.5 and

0 ppm groups (Wei *et al.* 1999).

The effects of arsenic exposure on urinary bladder morphology, DNA, and protein expression

have been studied by several investigators, using F344 rats and DMA(V). Cohen and colleagues

fed rats DMA(V)-containing diets, and observed DMA(V)-induced hyperplasia in the

transitional epithelium, based on light and scanning electron microscopy and bromodeoxyuridine

(BrdU) labeling index. The observed hyperplasia was suggested to be from regeneration of

bladder cells after arsenic-induced necrosis (Cohen *et al.* 1998; Arnold *et al.* 1999; Cohen *et al.*

2001). Fukushima and colleagues exposed rats to DMA(V) via drinking water, and detected a

low rate of H-*ras* mutations (but not *p53*, K-*ras* oncogene or β-catenin, which is associated with

cell-cell adhesion) (Clairotte *et al.* 2006) and unaltered microsatellite instability in DMA(V)-

induced bladder tumors. An increased BrdU labeling in normal-appearing bladders in the 50 and

200 ppm groups indicated an increase in cellular proliferation of bladder epithelium.   They also

found altered protein expression of p27, cyclinD1 and Cyclooxygenase-2 (COX-2, an important

enzyme for bladder cancer development) by immunohistochemistry; and formation of 8-

hydroxy-2'-deoxyguanosine (8-OHdG, an oxidative DNA change) in control and DMA(V)-

treated bladders (Wei *et al.* 1999; Fukushima *et al.* 2000; Wei *et al.* 2002). Fukushima and

colleagues' study showed that DMA(V) is a bladder carcinogen in rats, and various gene alterations and oxidative stress may be involved in its carcinogenesis.

There is a need for further understanding of mechanisms by which arsenic causes carcinogenesis in the urinary bladder. While arsenic cytotoxicity and subsequent regeneration have been proposed to be the underlying cause of DMA(V) induced bladder cancer in F344 rats, other modes of action (e.g., inhibition of DNA damage repair, oxidative stress, altered growth factors) cannot be ruled out. Furthermore, arsenic administration via diet or oral gavage may generate different responses compared to exposure to arsenic through drinking water, the main human exposure route. Perhaps of greatest importance, analyzing the whole urinary bladder may interfere with or dilute arsenic-induced changes in the transitional epithelium, the target of arsenic carcinogenicity.

In this preliminary study female Sprague-Dawley® (SD) rats were exposed to arsenic, in the form of DMA(V), in drinking water for four weeks. SD rats were used because they are a common strain not yet evaluated for arsenic transitional cell effects, and are considerably less expensive than F344 rats. Electron microscopy was used to examine arsenic-related morphologic lesions to the transitional epithelium. Methods were then developed and tested for selectively collecting transitional cells. Such collection will be necessary to support focused mRNA isolation and subsequent gene expression analysis by real time reverse transcription polymerase chain reaction (RT PCR), and for measuring DNA damage by Comet or other assay.

MATERIAL AND METHODS

Materials

Dimethylarsinic acid, sodium cacodylate trihydrate, Azure II, and uranyl acetate were purchased from Electron Microscopy Science, Fort Washington, Pennsylvania. The chemical form of DMA(V) was sodium cacodylate-trihydrate [$(CH_3)_2AsO_2Na \cdot 3H_2O$], with a purity of 99.52%. Propylene oxide, methylene blue, basic fuschin, and sodium citrate were obtained from Fisher, Fairlawn, New Jersey. Osmium tetraoxide was from Stevens Metallurgical Corp, New York, New York. Poly/Bed 812 was from Polysciences, Warrington, Pennsylvania. Lead nitrate was from Malinckrodt, Paris, Kentucky. Collagenase-Dispase was supplied by Boehringer Mannheim, Mannheim, Germany. TRIZOL® Reagent was from Invitrogen, Carlsbad, California.

Animals and Animal Care

All procedures for the described experiments were reviewed and approved by the Virginia Tech Institutional Animal Care and Use Committee for the humane treatment of laboratory animals, prior to initiation of experiments.

Thirty-two female albino SD rats were purchased at 8 weeks of age from a commercial supplier of laboratory rodents (Harlan, Madison, Wisconsin, USA). On receipt, rats were placed in quarantine for one week. Thereafter, the rats were transferred to standard housing. Rats were uniquely identified by ear tags. The rats were single-housed in polycarbonate shoebox-style cages with long fiber paper bedding, and the order of cages on the rack was assigned by complete randomization. The temperature and humidity of the animal room were monitored continuously and a 12/12-hour light/dark cycle was maintained.

Standard rat chow (Teklad 2018 SC diet, from Harlan Teklad,. Madison, Wisconsin) and water in plastic water bottles with stainless steel sipper tubes with stoppers were available *ad*

libitum. Both the rat chow and tap water were assayed for the presence of contaminant arsenic, and arsenic levels were below detection limits (diet <0.10 ppm, water <0.002 ppm). Selenium, an antagonist of arsenic-induced toxicity (Zeng *et al.* 2005; Wang *et al.* 2006) was also assayed and found to be present at levels of 0.16 ppm in diet and <0.01 ppm in water.

Dosing and Measurements of Body Weight, Food and Water Consumption

After 2 weeks of acclimation rats were assigned randomly into one of three groups, which received DMA(V) at concentrations of 0 (11 rats), 4 (10 rats), or 40 (11 rats) ppm in the drinking water. Rats within these exposure levels were then randomly divided into one of four groups: morphology (2 rats), DNA damage (3 rats, with the exception of 2 rats in 4 ppm group), Trizol-original (3 rats), or Trizol-modified (3 rats). Group sizes were based on experience of the investigators with the endpoints of interest. The intent was to provide needed preliminary data using fewer animals, to support future studies in our and other laboratories, rather than attempting more detailed statistical comparisons.

Dosing began on experimental day 0, when the rats were approximately 11 weeks of age and continued for 28 days. DMA(V) solution was prepared with tap water once a week, and stored at room temperature. Water, with or without DMA(V), was changed three times a week. Food consumption and water consumption were measured daily, and body weight was measured weekly when rats were transferred to clean cages with fresh bedding.

Terminal Necropsy

All rats survived the 4-week treatment without signs of overt toxicity. Rats in all three groups received tap water from experimental day 28 until sacrifice beginning on experimental day 30.   Necropsies took place over the next three days, with about 8 rats per day selected in random order, until all rats were sacrificed. This number of rats was the maximum that could be

comfortably evaluated per day. Rats were euthanized by CO_2 asphyxia. Urinary bladders were removed within 2 minutes of death, and either: (1) fixed immediately for morphology studies; (2) stripped using two Trizol® techniques to determine suitability of these collection methods for RT PCR; or (3) enzymatically stripped (collagenase plus Dispase) or mechanically scrapped to determine if sufficient viable transitional cells could be collected by either method for measuring DNA damage by Comet assay. **Table 1** shows the distribution of rats to each treatment group and the collection method of urinary bladder.

Ultrastructural Examination

Selected bladders were examined (see **Table 1**) for morphological evidence of transitional cell damage, using scanning and transitional electron microscopy (SEM and TEM, respectively).

Bladders for ultrastructural studies (both SEM and TEM) were fixed in General Fixative (4.4% formaldehyde, 5% glutaraldehyde, and 2.75% picric acid in a 0.05M sodium cacodylate buffer at pH 7.4) for at least 24 hours, washed in 0.1M sodium cacodylate, post-fixed in 0.1M sodium cacodylate containing 1% osmium tetroxide (OsO_4), and washed in 0.1M sodium cacodylate. Dehydration was performed by placing samples in graded ethanol solutions increasing in concentration as follows: 15%, 30%, 50%, 70%, 95%, 100% for 10 minutes each, followed by submersion in propylene oxide for 15 minutes.

For TEM, samples were embedded in a 50:50 solution of propylene oxide: Poly/Bed 812. To determine the areas for thin sectioning, thick sections (1.0 μm) were cut and stained with three dyes (Methylene blue stain in sodium borate, Azure II stain in water, and basic fuschin in sodium borate) (Humphrey *et al.* 1974). The thick sections were observed under a light microscope, and areas with transitional epithelium were identified and cut into thin sections (60 nm, 600Å), which were stained with uranyl acetate and lead citrate, which was generated from lead nitrate and

sodium citrate. The thin sections were examined with a Zeiss 10CA transmission electron

microscope (Zeiss, West Germany), and images were recorded digitally.

For SEM, samples were dried using a Ladd Critical Point Dryer model 28000 (Ladd

Research Industries, Burlington, Vermont), mounted onto SEM specimen stubs, and sputter-

coated with gold in SPI-Module™ Sputter Coater (SPI Supplies, West Chester, Pennsylvania).

The specimens were examined in a Philips Scanning Electron Microscope 505 (Philips,

Eindhoven, Netherlands). Tissue samples evaluated by SEM were classified using previously-

described semi-subjective methods (Cohen *et al.* 1990; Cohen *et al.* 2001). Briefly, using this

classification system, class I bladders show flat, polygonal superficial urothelial cells without

necrosis, with only single or paired necrotic foci, or with loss of single or paired superficial cells.

Class II bladders show occasional small foci of superficial urothelial necrosis or superficial cell

loss (2 to 4 cells in diameter). Class III bladders show numerous small foci of superficial

urothelial necrosis or superficial cell loss. Class IV bladders show extensive superficial

urothelial necrosis or superficial cell loss. Class V bladder show necrosis or superficial cell loss

and piling up of rounded urothelial cells. Normal bladders are class I or II.

Trizol® Stripping for RNA Extraction

Two Trizol® stripping techniques were assessed to determine if urinary bladder transitional

cell lysate could be selectively collected in amounts suitable for gene expression analysis by real

time RT-PCR. A previously-published Trizol method (Original Trizol; Wolf and Hester, 2002)

was first evaluated, which utilized injection of 1 ml of cold Trizol® solution into tied urinary

bladders, followed by a 10-minute incubation for Trizol®-mediated lysing of transitional cells.

The cell lysate was aspirated with a syringe, placed in a cryo-preservation tube, and immediately

flash frozen in liquid nitrogen prior to storage at -70°C. A modified method (Modified Trizol)

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was also evaluated. The Modified Trizol method was similar in most respects to the Original Trizol method, except that urine, if any, was withdrawn first, and then the bladders were rinsed using 1 ml of cold RNAse free phosphate buffered saline (PBS) (injected and withdrawn through the wall of the tied bladder) prior to the injection of Trizol® solution. These modifications were performed to remove exfoliated and potentially degenerated cells in the urine and on bladder surface, since those cells cannot undergo tumorigenesis. It was hypothesized that gene expression of these damaged and potentially degenerated cells might interfere with analysis and interpretation of changes in gene expression related to arsenic carcinogenesis. All stripped bladders were fixed in formalin for visual confirmation of the completeness and specificity (exclusion of other cell layers) of transitional cell layer stripping under light microscope.

Collection of Transitional Epithelial Cells Suitable for Comet Assay

Potential ability to utilize Comet assay for detecting arsenic-induced DNA damage in urinary bladder transitional cells is of interest, because Comet assay measures the levels of DNA damage in individual cells and requires limited numbers of cells per sample (Tice *et al.* 2000; Hartmann *et al.* 2003). For Comet assay, briefly, cells are suspended in agarose and layered on a glass slide. After lysis (to break cells and release DNA) and alkaline treatment (to unwind double stranded DNA into single stranded DNA), samples undergo electrophoresis. Shorter strands of DNA (such as those generated from strand breaks) migrate faster than intact DNA strands and generate the "tail" of the Comet, while long DNA strands form the head. In order to avoid detecting DNA damage in dying or dead cells, specimens used for data collection must have at least 75% viable cells (Henderson *et al.* 1998). There are limited data published on the collection of viable urinary bladder transitional cells for Comet assay, including ability to precede through the above assay steps with this cell type.

Our first attempted collection of urinary bladder transitional cells suitable for Comet assay used enzymatic digestion with collagenase and Dispase, a method modified from a published procedure for collecting astrocytes (Allen *et al.* 2000). The excised and tied urinary bladder was rinsed with PBS, injected with 1 ml dissociation medium [minimal essential medium with Earles' salts (MEM) with 10% v/v heat-inactivated horse serum, 3.2 unit/ml Dispase, and 0.4 unit/ml collagenase], and allowed to sit for 10 minutes. The dissociation medium was aspirated, divided into two tubes each containing 0.5 ml ice-cold freezing medium (MEM with 10% v/v heat-inactivated horse serum), and kept on ice. The cell count and viability were determined by Trypan blue exclusion assay. The enzymatically stripped urinary bladder was fixed in formalin to confirm that only epithelium was removed under light microscope.

The scraping method for transitional epithelium collection was a modification of the technique of Morimoto *et al.* (1987; 1989). In initial attempts, excised bladders were inverted, but not inflated, and the epithelium was scrapped off with a rectangular glass coverslip into 3 ml cold PBS (see below). A Trypan blue exclusion assay was then performed to evaluate viability. Some bladders were scrapped with round coverslips (no sharp corners) into MEM, to determine if viable cell recovery could be enhanced by possibly gentler scraping and more supportive medium. For both attempted techniques, the scrapped remnant urinary bladder was fixed in formalin for light microscopic examination to confirm that only epithelium was removed by the mechanical scraping method.

Statistical Analysis

For health monitoring data (water consumption, food consumption and body weight gain), observed values were adjusted by body weight and expressed as percentage of body weight. The effects of arsenic concentration, time, and interaction of arsenic concentration and time on group

means of body weight, body weight gain, food consumption and water consumption were evaluated by repeated measure analysis of variance and Wilks' Lambda approach to test if the means of groups are different in a multi-variance test. P values less than 0.05 were considered significant. All statistical analyses were performed using a SAS computer program version 8.02 (SAS Institute Inc., Cary, NC).

RESULTS

General Health

All animals survived the treatments, and showed no gross sign of intoxication (such as ruffled coat). DMA(V) did not affect mean body weight (**Fig. 1**) or body weight gain (**Fig. 2**). The water and food consumptions based on body weight were not affected by DMA(V) concentration (**Figs. 3 and 4**). The body weight, body weight gain, food consumption, and water consumption all changed over time, which were expected as rats grew larger over the study period. Furthermore, statistical analysis revealed these parameters were also not affected by the interaction of DMA(V) and time.

Ultrastructural Changes of Transitional Epithelium

Scanning electron microscopy (SEM)

All bladders examined under SEM were considered normal, with the exception of one bladder that was not classified because only the outside of the bladder was apparent in this particular SEM specimen. Sections from two control bladders were evaluated by SEM. One bladder showed typical large polygonal superficial cells (**Fig. 5a**) and dense microridges, or beaded necklace-like short microvilli under high magnification (above 4000x) (**Fig. 5b**), and was classified as class I. The second control bladder showed mild hyperplasia, suggested by the

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microvilli of intermediate cells on the surface, and was classified as class II (not shown).

Normal epithelium was also seen in the bladders of rats that received 4 ppm DMA(V) in drinking

water for 28 days (N=2) (not shown). Two rats that received 40 ppm DMA(V) in drinking water

were similarly examined. The urinary bladder of one 40 ppm rat showed normal polygonal

epithelial cells (**Fig. 5c**) and was classified as class II. Under high magnification, the surface of

the epithelium was covered by short microvilli (**Fig. 5d**). The urinary bladder collected from the

second 40 ppm rat showed both cobblestone-like epithelium and granular areas, but was not

classified due to tissue folding that reduced confidence in ability to reliably classify the sections

(not shown).

Transmission electron microscopy (TEM)

The ultrastructural (TEM) appearance of the normal transitional epithelium of the rat urinary

bladder consists of a layer of superficial cells facing the lumen, 1-3 layers of intermediate cells,

and a layer of basal cells (Pauli *et al.* 1983). The urothelium from control rats (N=2) was normal

(**Fig. 6a**) and showed a scalloped luminal surface with asymmetric unit membrane (AUM)

plaques, a unique characteristic of mature mammalian transitional cells. The fusiform vesicles,

formed by AUM plaques and identified by their thick walls, were also seen. Vacuoles appeared

only in the cytoplasm of superficial cells, while lysosomes appeared in superficial and

intermediate cells. Similar to SEM results, rats (N=2) exposed to 4 ppm DMA(V) via drinking

water for 28 days also showed normal transitional epithelium under TEM.

Changes were seen in tissue specimens of one of two rats in the 40 ppm group under TEM.

Tissue preparations from the first 40 ppm rat lacked sufficient transitional epithelium for

adequate evaluation. Samples from the second 40 ppm rat showed ultrastructural changes that

included isolated local areas of superficial cells undergoing necrosis and vacuoles in intermediate

cells (**Fig. 6b**). Some intermediate cells were exposed to the lumen. Necrotic superficial cells were identified by the presence of pyknosis (condense of chromatin in the nucleus) and increased cytoplasmic vacuoles, which do not have thick and rigid AUM plaques as walls (**Fig. 6c**).

Collection of Transitional Epithelial Cells Suitable for Comet Assay

1. Enzymatic digestion. Collection of transitional cells using collagenase plus Dispase digestion yielded extremely low cell count ($<1x~10^5$ cells/bladder) and poor viability ($< 10\%$) of cells from urinary bladders, suggesting this technique was incompatible with Comet assay. Therefore, this procedure using collagenase plus Dispase was not further investigated for transitional cell collection.

2. Mechanical scraping. The two scrapping techniques yielded adequate cell numbers to support a Comet assay. With rectangular cover slips and PBS, approximately 3×10^6 total cells/bladder were obtained; while with round cover slips and MEM, approximately 15×10^6 total cells/bladder were obtained. In both cases, however, Trypan blue assay revealed viability of only about 10%. These results again indicated that other epithelium collection techniques must be developed before the Comet assay can be applied to transitional cells.

Trizol® Stripping for RNA Extraction.

Trizol® solution lyses cells, and was intended to lyse the superficial transitional cells lining the inner surface of the urinary bladder, to generate a lysate from which mRNA could be collected. Light microscopic examination of formalin-fixed bladders after Trizol® stripping verified the specificity of the transitional layer stripping, i.e., deeper cell layers were not reached (data not shown). Subsequently, the original Trizol stripping method produced sufficient mRNA

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from transitional cells for successful gene microarray studies (Hester et al., unpublished data) in F344 rats.

DISCUSSION

The purpose of the present investigations was development of methods for studying the effects of arsenic on rat urinary bladder transitional cells, a target cell type of arsenic carcinogenicity. We tested modified collection methods for suitability to harvest highly pure transitional cells, which might be used for real time RT PCR, microarray and Comet assay. The development and refinement of such new methods will be a necessity for target cell-specific mRNA collection, as well as focused detection of DNA damage that might be associated with arsenic exposure.

Dosing was via drinking water to mimic the principal route of human exposure, and at levels intended to affect the sensitive urinary bladder target cells while producing limited overt toxicity. Statistical analysis of several health parameters verified the lack of overt toxic effects, including altered water consumption. These results are in contrast to increased water consumption in F344 rats exposed to DMA(V) at 40 ppm and higher (Arnold *et al.* 1999; Nishikawa *et al.* 2002; Wei *et al.* 2002), and suggest the SD rat may be less sensitive to DMA(V) as compared to the F344 rat.

Ultrastructural evaluation of transitional epithelium by SEM and TEM suggested TEM may be the more sensitive of these techniques for detecting arsenic-induced changes, in spite of smaller areas that can be examined by TEM. Increases of vacuoles and alterations of mitochondria in the cytoplasm occur before more severe changes (e.g. exfoliation and hyperplasia), and only TEM allows observation of these organelles in the cytoplasm. Present morphologic results are clearly preliminary, however one 40 ppm DMA(V) bladders evaluated

under TEM showed focal necrosis, including increased pyknosis and cytoplasmic vacuoles, changes consistent with arsenic-induced cytotoxicity.

The Comet assay requires at least 75% viable cells for reliable results (Henderson *et al.* 1998). Previously, Morimoto *et al.* (1987, 1989) examined rat urinary bladder transitional epithelial cells for DNA damage using an alkaline elution method, which utilizes similar principles as Comet assay but measures the DNA damage levels at the whole sample level (not individual cells). These authors reported 70% viability in the assayed cells measured by Trypan Blue exclusion assay, insufficient to support the Comet assay. Rather than improving transitional cell collection, the present enzymatic digestion (collagenase plus Dispase) and mechanical scraping techniques resulted in both fewer and less viable cells, thus could not support continued DNA damage assays.

Wolf *et al.* (2000) previously harvested bladder transitional cell lysate for RNA extraction using a Trizol stripping technique. However, the reported technique also harvests cells in the urine, which may include exfoliated urinary bladder transitional cells, exfoliated epithelial cells from kidney, lymphocytes, and other cell types. Although the percentage of these cells in urine compared to target urinary bladder transitional cells (live cells that are lining the urinary bladder) is generally very small, DMA(V) treatment may induce urothelium necrosis (Arnold *et al.* 1999) and therefore increase exfoliated, dead transitional cells in the urine. The present modified Trizol method excludes cells in the urine, and harvests RNA sufficient for real time RT PCR and microarray.

In conclusion, SD rats exposed to up to 40 ppm DMA(V) in drinking water for 4 weeks did not show overt intoxication signs or changes in the body weight gain, food consumption or water consumption. Morphological changes were detected by TEM in the urothelium of one rat

exposed to 40 ppm arsenic in the drinking water. Enzymatic (collagenase plus Dispase) and mechanical collection techniques would not permit proceeding to a Comet assay with urinary bladder transitional cells. Finally, both Original Trizol and Modified Trizol methods were suitable for harvesting appropriate number and quality transitional cells for mRNA isolation, and should permit focused gene expression studies in these urinary bladder arsenic target cells.

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Table 1. Distribution of SD rats in DMA(V) treatmentand tests on urinary bladders.

Endpoints intended	DMA(V) treatment (number of rats)
Urinary bladder collection method	
DNA Damage	
Collagenase and Dispase	0 ppm (3), 40 ppm (1)
Scraping (MEM)	4 ppm (1), 40 ppm (1)
Scraping (PBS)	4 ppm (1), 40 ppm (1)
Morphology	
Fixation	0 ppm (2), 4 ppm (2), 40 ppm (2)
mRNA levels	
Modified Trizol	0 ppm (3), 4 ppm (3), 40 ppm (3)
Original Trizol	0 ppm (3), 4 ppm (3), 40 ppm (3)
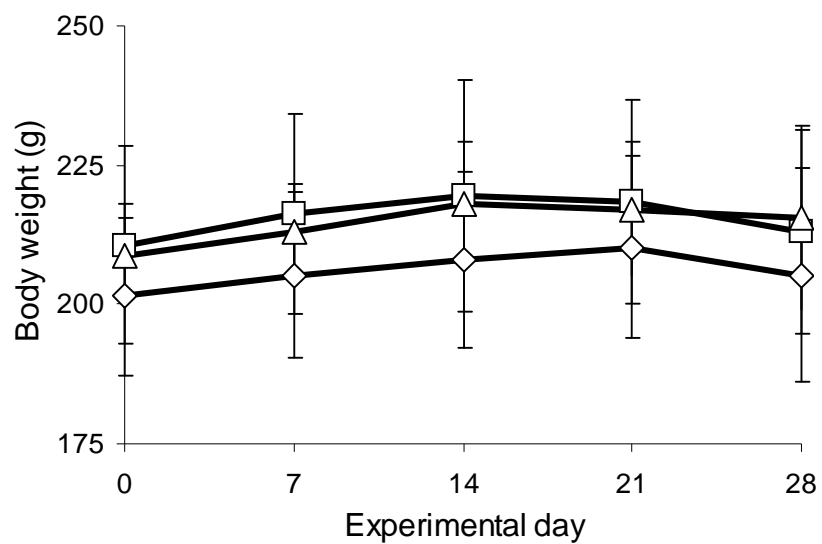
Figure 1. Body weights of SD rats were not affected by 4-week DMA(V) treatments up to 40 ppm. Y error bars indicate standard deviations. ◊ = 0 ppm, □ = 4 ppm, Δ = 40 ppm DMA(V).
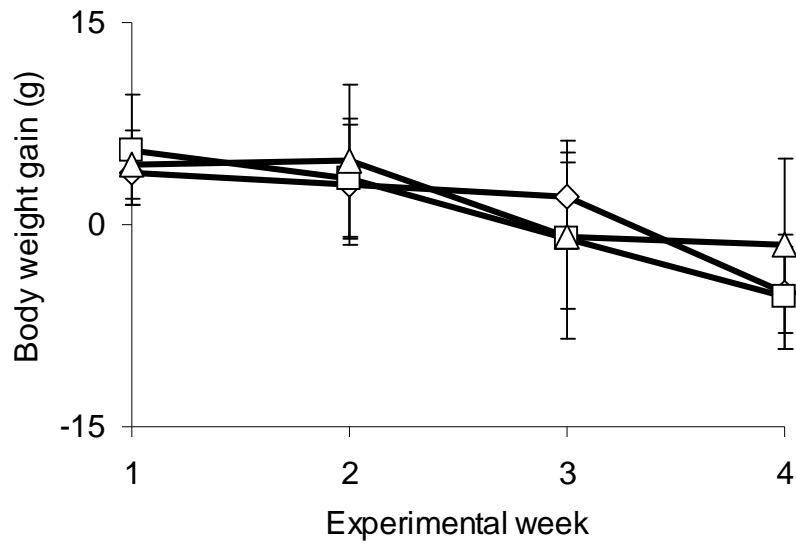
Figure 2. Body weight gains, adjusted by body weight, were not affected by 4-week DMA(V) treatments up to 40 ppm. Y error bars indicate standard deviations. . ◊ = 0 ppm, □ = 4 ppm, Δ = 40 ppm DMA(V).
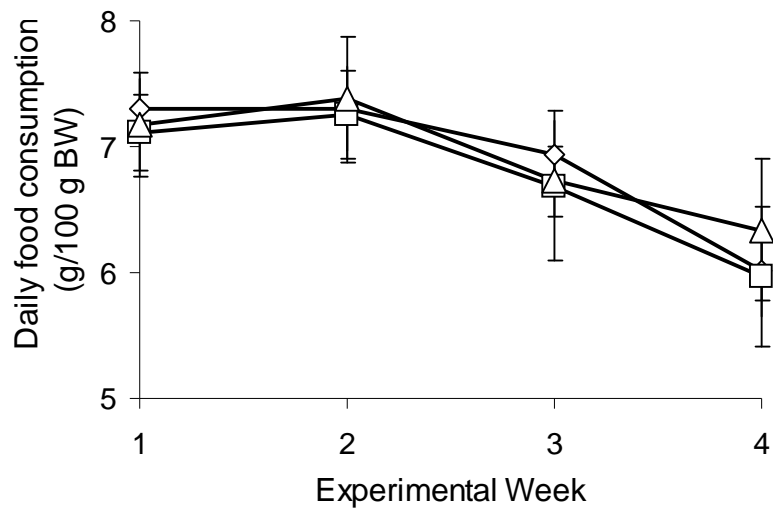
Figure 3. Food consumption of SD rats were not affected by 4-week DMA(V) treatments up to 40 ppm. Y error bars indicate standard deviations. ◊ = 0 ppm, □ = 4 ppm, Δ = 40 ppm DMA(V).
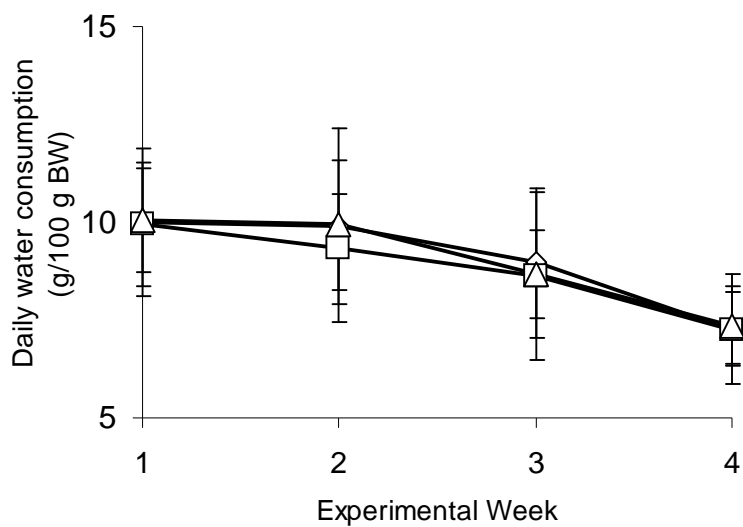
Figure 4. Water consumption of SD rats were not affected by 4-week DMA(V) treatments up to 40 ppm. Y error bars indicate standard deviations. ◊ = 0 ppm, □ = 4 ppm, Δ = 40 ppm DMA(V).
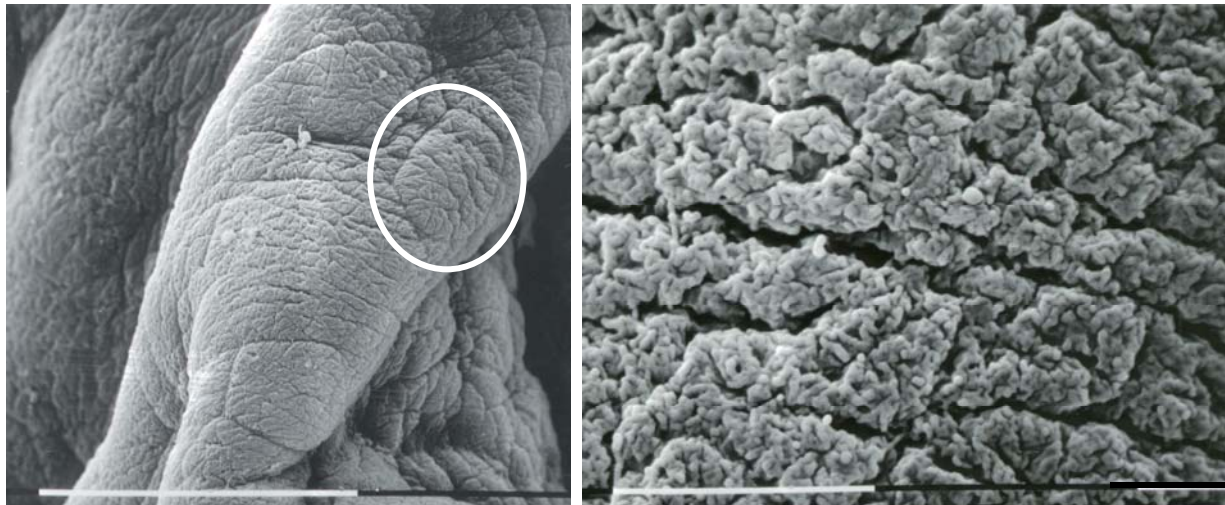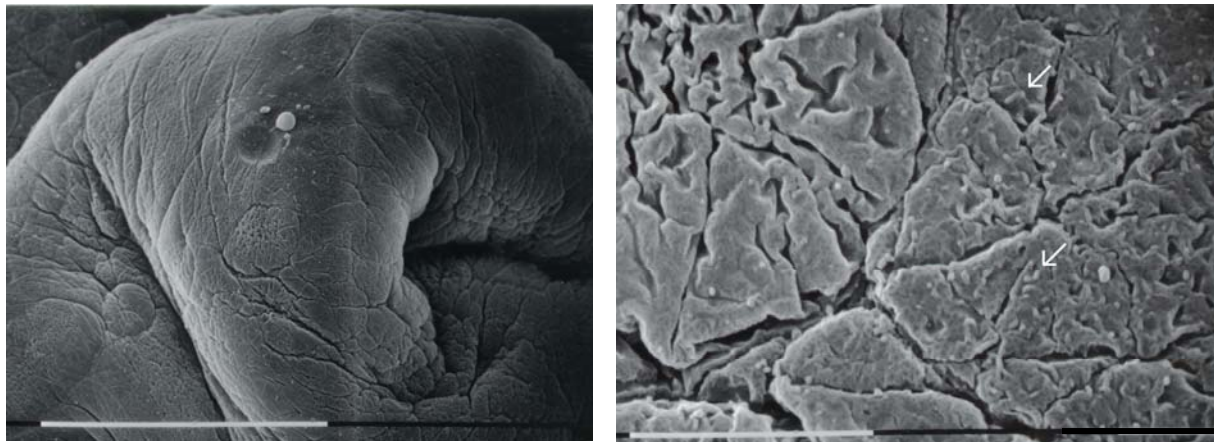
Figure 5. Bladder epithelium under SEM. (a) Normal bladder epithelium from a control SD rat (Class I) showed polygonal epithelial cells (circle). White bar = 0.1mm. (b) Normal bladder epithelium from a control SD rat showing dense microridges. White bar = 10 μm. (c) Class II urinary bladder epithelium from a female SD rat exposed to 40 ppm DMA(V) in drinking water for 4 weeks and then tap water for 2 days. White bar = 0.1 mm. (d) Same bladder as in (c) showed microvilli under high magnification. White bar = 10 μm.
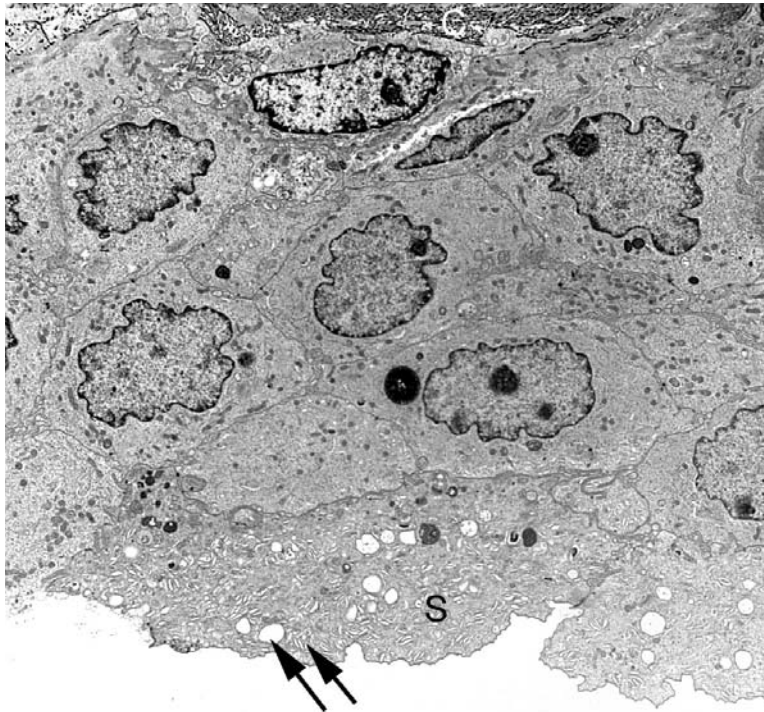
(a)
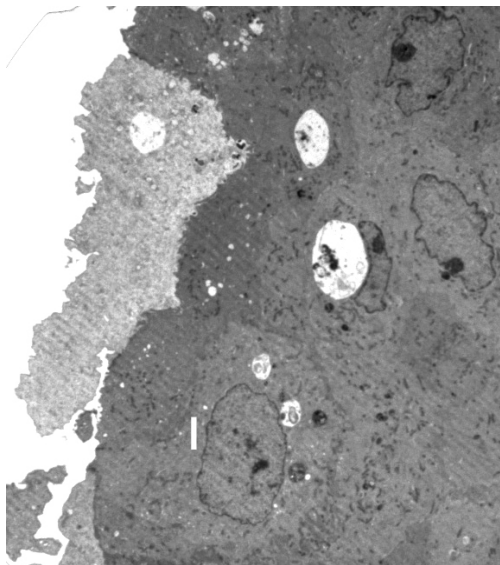
(b)
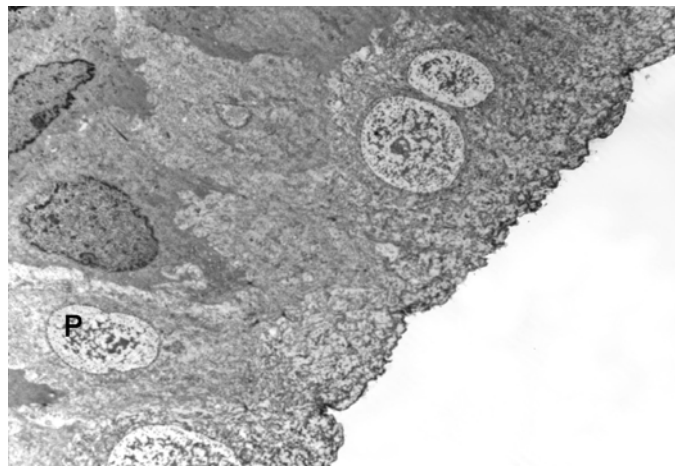
(c)

Figure 6. Urinary bladder epithelium under TEM. (a) Normal urinary bladder epithelium under

TEM from a control rat. The superficial cells (S) facing the lumen at the bottom of the figure

have characteristic asymmetric unit membrane plaques, which forms scalloped outlines of the

cellular membrane and fusiform vesicles (arrows) in the cytoplasm. At the top, collagen fibrils (C) are visible outside the basal membrane. Original magnification: 2500 X. (b and c) Necrosis and exfoliation of superficial cells in the urothelium from a rat exposed to 40 ppm DMA(V) for 4 weeks. Although most transitional cells appeared normal, lesions that were not in control or 4 ppm group were seen in isolated regions. (b) Exfoliation of superficial cells and exposure of intermediate cells. Intermediate cells (I), exposed to lumen and underneath the superficial cells, had vacuoles. Original magnification: 1250 X. (c) Necrotic superficial cells were observed in isolated regions. Pyknosis (P) and numerous vacuoles, which do not have thick walls as fusiform vesicles, were observed. Original magnification: 1600 X.

Chapter 4   Measurement of DNA damage in rat urinary bladder transitional cells: improved selective harvest of transitional cells and detailed Comet assay protocols

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ABSTRACT

Urinary bladder transitional epithelium is the main site of bladder cancer, and the use of transitional cells to study carcinogenesis/genotoxicity is recommended over the use of whole bladders. Because the transitional epithelium is only a small fraction of the whole bladder, the alkaline single cell gel electrophoresis assay (Comet assay), which requires only a small number of cells per sample, is especially suitable for measuring DNA damage in transitional cells. However, existed procedures of cell collection did not yield transitional cells with a high purity, and pooling of samples was needed for Comet assay. The goal of this study was to develop an optimized protocol to evaluate DNA damage in the urinary bladder transitional epithelium. This was achieved by an enzymatic stripping method (trypsin-EDTA incubation plus gentle scraping) to selectively harvest transitional cells from rat bladders, and the use of the alkaline Comet assay to detect DNA strand breaks, alkaline-labile sites, and DNA-protein crosslinks. Step by step procedures are reported here. Cells collected from a single rat bladder were sufficient for multiple Comet assays. With this new protocol, increases in DNA damage were detected in transitional cells after *in vitro* exposure to the positive control agents, hydrogen peroxide or formaldehyde. Repair of the induced DNA damage occurred within 4 hours. This indicated the capacity for DNA repair was maintained in the harvested cells. The new protocol provides a simple and inexpensive method to detect various types of DNA damage and to measure DNA damage repair in urinary bladder transitional cells.

Keywords: Comet assay; urinary bladder; transitional epithelium

1. INTRODUCTION

   Urinary bladder cancer is one of the five most common cancers in Europe and the United

States of America [1], and more than 90% of malignant bladder cancer is transitional cell

carcinoma [2]. The urinary bladder consists of 3 layers of tissues: inner epithelium (urothelium),

submucosa (connective tissue), and smooth muscle. The urothelium consists of transitional cells

which are constantly exposed to metabolic wastes and other chemicals through contact with the

urine [3]. Laboratory research addressing the mechanisms of urinary bladder carcinogenesis

should ideally be conducted using pure transitional cells instead of a mixture of separate cell

types from whole bladders [4,5] to avoid dilution or misleading responses [5]. Because

transitional urothelium comprises only a small fraction of total bladder tissue, assays utilizing

transitional cells must make use of very small tissue samples or sample pooling may be needed.

   Genotoxicity is important for characterizing the mode of action for carcinogens, including

bladder carcinogens [4]. An assay for genotoxicity that is gaining widespread use is the single

cell gel electrophoresis assay, commonly called the Comet assay. This assay has the advantages

of requiring a small number of cells per sample (as few as 10^5), being amenable to almost all cell

types whether actively dividing or quiescent, and being relatively low cost requiring only basic

equipment [6-8]. In the Comet assay, cells are embedded in agarose on a glass slide, lysed to

break cell and nuclear membranes, and unwound in an alkaline solution to yield single stranded

DNA from liberated double-stranded DNA. Following electrophoresis, the sample is stained

with a DNA-binding dye and viewed under a microscope. Short strands of DNA generated from

DNA strand breaks and/or alkaline labile sites (depending on pH of the electrophoresis solution)

migrate further than intact DNA during electrophoresis and form the "tail" of the "Comet". In

the presence of DNA-protein crosslinks or rare DNA-DNA crosslinks, DNA migration is

inhibited by crosslinks. The degree of DNA migration is usually presented as percentages of

DNA in the tail (by image analysis software) or morphological classes of Comets measured

visually [9]. When compared to control samples, a significant increase in DNA migration

indicates the presence of increased levels of strand breaks, alkali-labile sites, or incomplete

excision repair sites, whereas a significant decrease indicates the presence of DNA-protein or

DNA-DNA crosslinks [6-8].

   The ability to conclude that altered DNA migration is due to genotoxicity generally requires

samples with a minimum of 70 to 75 % viability as measured by the trypan blue exclusion assay

[10,11]. Concurrent measurements of cell viability/cytotoxicity in samples subjected to the

Comet assay are important because cytotoxicity can cause artifactual changes in DNA migration

[12]. For example, apoptotic and necrotic cells that contain highly degraded DNA can increase

mean DNA migration of the samples [6]. On the other hand, at cytotoxic doses loss of heavily

damaged or dying cells during sample processing and electrophoresis can decrease the recorded

mean DNA migration [12].

   While the Comet assay has already been recommended [13] and used on urinary bladder cells

[14-18], previously published studies using the Comet assay have several drawbacks, mainly

regarding collection of target cells. These drawbacks include (1) use of the nucleus instead of

whole cells from scraped bladder mucosa without information on cell viability [14], (2) use of

minced bladder tissue containing a mixture of different cell types with low cell viability [15], and

(3) difficulty in collecting viable cells through scraping bladder mucosa and resuspending the

cells in Merchant's solution[3] [16]. Robbiano *et al.* (2002) reported a selective harvest of bladder

transitional cells with 95-98% viability by scraping trypsin and ethylenediaminetetraacetic acid

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[3] Merchant's solution is 0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.53 mM Na_2EDTA, pH
7.4.

(EDTA)-treated bladders that is based on a method originally developed to establish a primary cell line [17]. However, each sample for the Comet assay required pooling cells collected from three bladders [18], and the pooling greatly increased the number of animals needed for *in vivo* studies.

Here we present a method, modified from the previously published method using trypsin and EDTA [17,18], to selectively harvest urinary bladder transitional cells suitable for the Comet assay. This new method requires no pooling of samples. Sufficient cells for six samples were collected from each bladder, which can simultaneously be used for performing Comet assays with and without enzymes, or studying DNA repair over time while still allowing sufficient samples for positive and negative experimental controls. Detailed Comet protocols for the alkaline Comet assay and the detection of crosslinks in rat bladder transitional epithelial cells are presented below.

2. MATERIALS AND METHODS

2.1 Chemicals

Isoflurane (CAS no. 26675-46-7) was purchased from Abbott Animal Health, North Chicago, Illinois. Beuthanasia [390 mg pentobarbital sodium (barbituric acid derivative) and 50 mg phenytoin sodium per ml of Beuthanasia] (CAS no. 8024-20-2) was from Schering-Plough Animal Health Corporation, Union, New Jersey. Fetal bovine serum (FBS) was from Cambrex Bio Science Inc., Walkersville, Maryland and Atlanta Biologicals, Lawrenceville, Georgia. L-glutamine (CAS no. 56-85-9, Gibco), Proteinase K (Catalog no. 25530-049) and SYBR gold (Molecular Probe) were from Invitrogen, Grand Island, New York. CometAssay[TM] LMAgarose (low melting temperature agarose) and CometAssay[TM] Lysis Solution were from Trevigen,

Gaithersburg, Maryland. Williams' medium E, trypsin, ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA) (CAS no. 6381-92-6), DMSO (CAS no. 67-68-5), sodium hydroxide (NaOH) (CAS no. 1310-73-2), and Trizma base (CAS no. 77-86-1) were from Sigma, St. Louis, Missouri. Ethanol (200 proof ethyl alcohol) was from AAPER, Shelbyville, Kentucky.

2.2 Rats

Animal use and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee for Animals Used in Research and Testing. Female F344 rats were purchased from Harlan Teklad (Madison, Wisconsin). All rats were quarantined and acclimated for at least 2 weeks. The rats were single-housed in polycarbonate shoebox-style cages with Alpha Dry bedding (Shepherd Specialty Papers, Watertown, Tennessee) for at least 5 weeks prior to sacrifice. The temperature and humidity were monitored continuously, and a 12-hour light/dark cycle was maintained. Teklad 2018 SC diet (Harlan Teklad, Madison, Wisconsin) and tap water in plastic water bottles with stainless steel sipper tubes with stoppers were available *ad libitum*. At the time of sacrifice, rats were 13 to 15 weeks old.

2.3 Harvest of urinary bladder transitional cells

To prevent ultraviolet light-induced DNA damage, all procedures (except after dehydration by ethanol and before staining by SYBR gold) were done under safety yellow light and green LED (*l*ight-*e*mitting *d*iode) light, neither of which emits ultraviolet light.

Rats were anesthetized by isoflurane inhalation, shaved, and their bladders were surgically exposed. After the connective tissues and fat on the bladder were trimmed off, the bladder was tied by a surgical suture (SOFSILK, 3.5 metric coated braided silk) (Synture, Norwalk,

Connecticut) near the urethra. A catheter (Angiocath Catheter, 24G x 3/4 inch) (BD Medical, Sandy, Utah) was inserted into the bladder at the trigon area, between the tips of a pair of forceps which was used to secure the catheter position [**Fig. 1 (a)**]. The inner needle of the catheter was removed [**Fig. 1 (b)**], allowing attachment of syringes. Urine, if any, was withdrawn through the catheter to an empty syringe [**Fig. 1 (c)**] to remove exfoliated bladder cells. The bladder was then rinsed by injecting and withdrawing 1 ml PBS [**Fig. 1 (c)**] to remove any residual dead cells in the urine or bladder surface. These steps to remove dead or exfoliated cells, which may be from kidney, bladder or other origins, were used to increase the purity of the transitional cells to be collected. The bladder was then inflated with 1 ml trypsin and EDTA (0.25 % trypsin and 0.05% Na_2EDTA) [**Fig. 1 (c)**]. An additional knot was tied with a surgical suture at the opposite side of the catheter insertion location [**Fig. 1 (d)**], the catheter was removed [**Fig. 1 (e)**], and the knots were pulled tighter to prevent leakage [**Fig. 1 (f)**]. The inflated bladder was separated from the rat [**Fig. 1 (f)**] and placed in a covered Petri dish, followed by euthanasia of the rat by an intra-cardiac injection of Beuthanasia. The bladder was incubated in a humidified incubator at 37 ºC with 5 % CO_2 for 30 min, which yielded higher percentages of live cells than a 45 min incubation (data not shown). After incubation, the Petri dish was placed on ice and filled with 10 ml ice cold Williams' E medium supplemented with 10% FBS (W+S). The bladder was nicked at the dome, and then turned inside out. The transitional cells were gently scraped off using a pair of curved forceps. The resultant cell suspension was transferred into a centrifuge tube and immediately centrifuged at 250 x g for 5 min at 10 ºC. The cell pellet was resuspended in 1 ml cold W+S. Cell density and viability were measured by the trypan blue exclusion assay. Cells were diluted in W+S at 10^5 cells/ml.

2.4 Measurement of DNA damage by the alkaline Comet assay

Briefly, cells in PBS were resuspended with LMAgarose and loaded onto slides. Cells then underwent lysis, unwinding, and electrophoresis [6]. After being stained, Comets were scored using fluorescence microscopy as described below.

Cell treatment: For the positive controls, cells were treated with 100 μM H_2O_2 for 10 min on ice by adding 10 μl of 10 mM H_2O_2 into a 1 ml cell suspension. PBS served as the negative control. The trypan blue exclusion assay was performed on all samples after the 10 min treatments to ensure that cell viabilities remained not much below 70%, by which samples were deemed acceptable. For DNA repair studies, approximately 50% of the cells were resuspended in cold PBS at 10^6 cells/ml (0 hour recovery), and the remaining cells were resuspended in W+S and incubated in a humidified 37 ºC incubator for 4 hours (4 hour recovery). At the end of recovery, cells were centrifuged at 720 x g for 5 min and resuspended in cold PBS at 10^6 cells/ml.

Sample loading: Samples were loaded onto FLARE Slides (Trevigen), each having 3 ready-to-use sample loading circular areas pre-coated with normal melting temperature agarose. Samples were loaded onto a minimum of 2 circles on different slides for each Comet assay test condition. To reach a final concentration of 5 x 10^4 cells/ml upon loading, 10 μl of cell suspension in PBS (10^6 cells/ml) were added to 190 μl melted 37 ºC LMAgarose for every two circles. Cell suspensions in LMAgarose were immediately pipetted onto circles (75 μl/circle) and spread over the whole circles with the pipette tip. The slides were placed in a metal tray horizontally at 4 ºC for 30 min for the agarose to solidify.

Lysis of cells: The slides were then immersed in a 4 ºC Lysis Solution with 10% DMSO for 1 hour. The addition of DMSO helps to prevent oxidative DNA damage from radicals generated

by the Fenton reaction from the iron released from erythrocytes in the sample during lysis [6].

Even with the addition of DMSO, it is critical to separate samples treated with H_2O_2 from other

samples by placing them in a separate container for the lysis step [19]. Otherwise, untreated

negative control samples could show increased DNA damage.

Unwinding of DNA: After a rinse in deionized water, slides were immersed in a 4 ºC alkaline

solution (300 mM NaOH, 1 mM Na_2EDTA, pH >13) for 30 min to unwind DNA.

Electrophoresis: Electrophoresis was carried out at a constant voltage of 40 V (1 V/cm

distance between electrodes) beginning with 300 mA for 30 min at 4 ºC. The edges (2 slides

wide, approximately 25% of total width) of the electrophoresis apparatus were not used for slides

loaded with samples, and all empty spaces (at the edge and middle areas) were filled with blank

slides to establish nearly identical alkaline solution levels regardless of the number of sample

slides.

Neutralization and drying: After electrophoresis, slides were immersed in neutralization

buffer (0.4 M Tris-HCl, pH 7.5) at 4 ºC for 15 minutes, rinsed in deionized water, and

dehydrated in 70% ethanol at room temperature for 5 minutes. Neutralization and dehydration in

ethanol decreases background stain in the later staining step. The slides were then air dried.

Comet scoring: All slides were coded, randomized (for scoring sequence), and scored by the

same individual without knowledge of the treatment. Immediately before being viewed under

the microscope, each circle was stained with 20 µl diluted SYBR gold [5 µl 10,000 x concentrate

in 1 ml TE buffer (10 mM Tris, 1mM Na_2EDTA), pH 7.4]. No cover slips were used. Slides

were scored using a Nikon Microphot-FXA microscope (Nikon, Tokyo, Japan) equipped with a

Nikon mercury lamp. Live images were captured with a Hamamatsu ORCA-AG digital camera

(model C4742-80-12AG) (Hamamatsu, Japan) connected to the microscope and a personal

computer. The Comets were scored using image analysis software, Komet 5.5. (Kinetic Imaging Ltd, Liverpool, UK). Only Comets inside a concentric circular area with a diameter that was half the diameter of the gel loading circle were scored. No Comets near edges were included because they would be vulnerable to agarose drying prior to electrophoresis. A total of 100 Comets was scored from each sample, consisting of 50 arbitrarily chosen Comets per circle. The average percentage of DNA in the tail was calculated for each sample as the primary indicator of DNA migration and DNA damage.

2.5 Measurement of DNA-protein crosslinks by the alkaline Comet assay

The procedures were the same as those in the alkaline Comet assay described above, except for the following steps.

Cell treatment: For the positive control for DNA-protein crosslinks, cells were treated with 5 mM formaldehyde for 10 min on ice by adding 10 µl of 0.5 M formaldehye into a 1 ml cell suspension. Each sample was loaded onto 4 circles on 4 different slides (2 circles for Proteinase K treatment, and 2 circles without Proteinase K treatment).

Lysis of cells: The slides of formaldehyde-treated samples and untreated samples were in the same containers in the lysis step, which lasted overnight. All slides were maintained in a horizontal position for the overnight lysis to prevent solidified agarose from sliding off the slides.

Proteinase K treatment: After lysis, slides were rinsed in deionized water and immersed in TE buffer (pH 8.0) for 5 minutes at room temperature. After slides were air dried to the point where no liquid was visible on the non-loading area, freshly made Proteinase K (1 mg/ml in TE buffer, pH 8.0) or TE buffer was added to samples at 100 µl per circle. The slides were then

placed in a wet-Kimwipe® lined plastic box and incubated in a humidified 37 ºC incubator for 2 hours.

Unwinding of DNA and electrophoresis: The slides were rinsed and unwound in the alkaline solution for 20 minutes after which they underwent electrophoresis at a consistent 32 V (0.8 V/cm), initially at 300 mA, for 30 minutes at 4 ºC. This electrophoresis condition was optimized to yield 5-10 % DNA in the tail in untreated control samples when no Proteinase K was used.

Comet scoring: A total of 200 Comets were scored from each sample, consisting of 50 arbitrarily chosen Comets per circle. The average percentage of DNA in the tail was calculated separately for samples treated with and without Proteinase K.

2.6 Statistics

Data are presented as the mean ± standard deviation. For DNA migration, Student's t-test (paired) was used for statistical analysis, and data from the same rat were treated as pairs. A two-tailed t-test was used for the alkaline Comet assay results because the percentage of DNA in the tail could be increased or decreased by various types of DNA damage. Mixed model analysis of variance (ANOVA) and *post hoc* Tukey comparison were used for the results from Comet assay with and without Proteinase K. A p value of < 0.05 was considered to be statistically significant.

3. RESULTS

3.1 Only the transitional epithelium was removed from the bladder

That only transitional epithelium was harvested was confirmed by examining the scraped bladders under a light microscope. While intact urinary bladders showed purple-stained

transitional cells facing the lumen of the bladder [**Fig 2 (a)**], the scraped bladders showed no

transitional cells, and yet the basal membrane and underneath tissue remained intact [**Fig 2 (b)**].

This demonstrated that the transitional epithelium was selectively harvested for the Comet assay.

3.2 Collected cells had more than 70% viability and were sufficient for multiple Comet assay tests

All urinary bladder cell samples collected from the enzymatic stripping method (trypsin and

EDTA) showed typical large and round transitional cell morphology using light microscopy.

Occasionally, binuclear transitional cells were seen. Red blood cells, very small and round, were

present but not counted toward cell number or viability. Muscle cells and squamous epithelial

cells, which are as large as transitional cells but with pointed outlines, were not seen. This

indicated that the final Comet cell preparations were almost all transitional cells.

Viabilities were greater than 70% in all urinary bladder cell samples (range: 79% to 91%) as

measured by trypan blue exclusion assay. The total numbers of cells collected from each bladder

were greater than 2×10^5 (range: 2.2×10^5 cells/bladder to 3.7×10^5 cells/bladder) for 6 out of 7

bladders, and 1 bladder yielded 1.2×10^5 cells. These data showed that cells collected from each

bladder were sufficient for the Comet assay without using pooled samples.

After being treated with hydrogen peroxide, formaldehyde, or PBS for 10 minutes, the vast

majority (19 of 22) of the transitional cell preparations maintained higher than 70% viabilities

(range: 63% to 100%). After the 4 hour recovery period, all of the cell viabilities were at least

67% (range: 67% to 100%). These data showed that the hydrogen peroxide and formaldehyde

treatments had a minimal cytotoxic effect.

3.3 DNA damage was detected after in vitro treatments and repaired within 4 hours

Hydrogen peroxide significantly increased DNA migration as detected in the alkaline Comet assay, indicating increased DNA damage (**Fig. 3**). After 4 hours of recovery, both untreated samples and hydrogen peroxide-treated samples had the same levels of DNA migration as untreated samples at 0 hour. These data indicated that hydrogen peroxide-induced DNA damage in bladder transitional cells was repaired within 4 hours.

Formaldehyde significantly decreased DNA migration after both 0 and 4 hour recovery when no Proteinase K was used [**Fig 4(a)**]. This indicated the presence of DNA-protein and/or DNA-DNA crosslinks. There was a significant decrease of DNA migration in untreated cells after 4 hour recovery, but not in formaldehyde-treated cells [**Fig 4(a)**]. This suggested repair of DNA strand breaks and alkaline-liable sites in the untreated cells. Such repair in protein-crosslinked DNA in the formaldehyde-treated cells could not be shown in this assay without Proteinase K. The DNA damage observed at 0 hr recovery may be endogenous or from the cell collection process.

When Proteinase K was used, the DNA migration levels were the same between samples treated with and without formaldehyde both at 0 hour recovery and at 4 hour recovery [**Fig 4 (b)**]. This indicates that the majority of damage induced by formaldehyde was DNA-protein crosslinks. The DNA migration was significantly decreased after 4 hours of recovery at both untreated and formaldehyde-treated cells indicating the repair of DNA damage.

4. DISCUSSION

This study demonstrated that a trypsin and EDTA stripping method can be used to selectively harvest urinary bladder transitional cells for the Comet assay. Transitional cells collected from

one rat bladder were sufficient for at least 6 Comet assay tests, and transitional cells maintained their DNA repair capacities.

Several methods to harvest viable transitional cells from the urinary bladders have been reported [16,18], and here we presented a method that is easy to perform and yields cells sufficient for multiple Comet assays. We initially attempted an enzymatic stripping using collagenase plus Dispase digestion, representing a method modified from a published procedure for collecting astrocytes [20]. However, transitional cell numbers obtained were extremely low ($<1x\ 10^5$ cells/bladder) as were viabilities (< 10%). We next tried scraping untreated bladder mucosa with a cover slip and resuspending cells in PBS, minimal essential medium with Earles' salts, or Merchant's solution [16]. Harvested cells consistently had unacceptably low viabilities (approximately 10%). Morimoto's method (scraping and suspending in Merchant's solution) has been reported to yield cells with more than 80% viabilities [16]; however, we were unable to duplicate these results. Finally, gentle scraping of trypsin and EDTA treated-bladders was used, and this method yielded transitional cells in sufficient numbers and viabilities for the Comet assay.

Transitional cells collected maintained their DNA repair capacities, as evidenced by decreased DNA migration after 4 hours of recovery. Hydrogen peroxide-induced DNA damage was completely repaired within 4 hours. Formaldehyde-induced DNA-protein crosslinks were inferred by comparing DNA migration after incubations with and without Proteinase K. It is possible that DNA-protein crosslinks were partially repaired in 4 hours in rat urinary bladder transitional cells, because cells from rat (a tracheal epithelial cell line) showed partial repair of formaldehyde-induced DNA-protein crosslinks after 4 hours of recovery, and complete repair after 16 hours [21]. It has been reported that formaldehyde-induced DNA-protein crosslinks

have shorter half-lives (as short as 2-5 hours in human cell lines) than other chemical-induced DNA-protein crosslinks [22,23]. While collected rat urinary bladder transitional cells demonstrated their DNA repair capacity, it cannot be ruled out that the collection processes altered the levels of repair activity. Because all samples were collected the same way, the alteration, if any, is expected to be the same among samples. These data indicated that cells from a single rat bladder were sufficient to serve as both positive and negative controls in studying repair of total DNA damage and of a specific type of DNA damage.

In the present method urine was removed, and the bladders were rinsed prior to trypsin and EDTA incubation, allowing a focused study of only cells with a potential to develop into transitional cell carcinomas. Cells in the urine are primarily exfoliated transitional cells but also may include lymphocytes and exfoliated cells from the kidney or ureters, backflow from the urethra, vaginal epithelium in females, and sperm and prostatic epithelium in males. When the cells in the urine and rinsing solution were collected, the numbers of cells were very low, with almost all of the cells being dead (data not shown). However, other studies of cells collected by similar bladder washing showed 60% viable cells from dogs [24] and humans [25]. These different results may be due to non-transitional cells in the samples. The presence of non-transitional cells in the bladder wash was confirmed by immuno-precipitation in Gontijo's study [25] and constituted approximately half of the total cells. This shows the importance of removing the urine and rinsing the bladders to maximize the purity of urinary bladder transitional cells.

Hydrogen peroxide treatment is a convenient way to induce DNA damage as a positive control; however, the necessity of placing samples treated with hydrogen peroxide in a separate container from other samples in the lysis step has not been previously published. Even in the

presence of DMSO in the Lysis Solution, negative control samples showed increased DNA damage levels after incubation with hydrogen peroxide-treated samples. This increase was seen about 50% of the time. Possibly, reactive oxygen species diffused from the hydrogen peroxide-treated cells into the Lysis Solution when the positive control slides were lysed, and subsequently affected the negative controls in that solution.

It is possible to collect bladders from euthanized rats, instead of anesthetized rats, and still achieve desired cell viability from scraped bladders [18]. When male rats are used, excessive stress to the animal during the induction stage of euthanasia or anesthesia should be especially avoided. Strong animal movement may cause sperm migration into the urinary bladder (retrograde ejaculation). Sperm cells sometimes could be seen among collected bladder cells during the trypan blue exclusion assay (unpublished observation), and sperm heads could show up as small Comets after electrophoresis due to their 1n DNA content, compared to bladder cells with 2n DNA content [26]. Such sperm contamination can be prevented by ensuring a smooth induction of anesthesia or euthanasia (such as having CO_2 build up in the chamber before rats are placed in the chamber), and removal of urine and rinsing the bladder prior to trypsin and EDTA incubation.

In summary, an easy and efficient enzymatic stripping method to selectively harvest urinary bladder transitional cells is presented here with detailed protocols for Comet assays to detect DNA damage in transitional cells. The removal of urine, the rinsing of the bladder, trypsin and EDTA incubation, and gentle scrapping yielded more than 2×10^5 transitional cells per rat bladder. Transitional cells collected in this manner had high viabilities and were able to repair *in vitro* induced DNA damage.

ACKNOWLEDGMENTS

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(a)

(b)

(e)

(f)

(e)

(f)
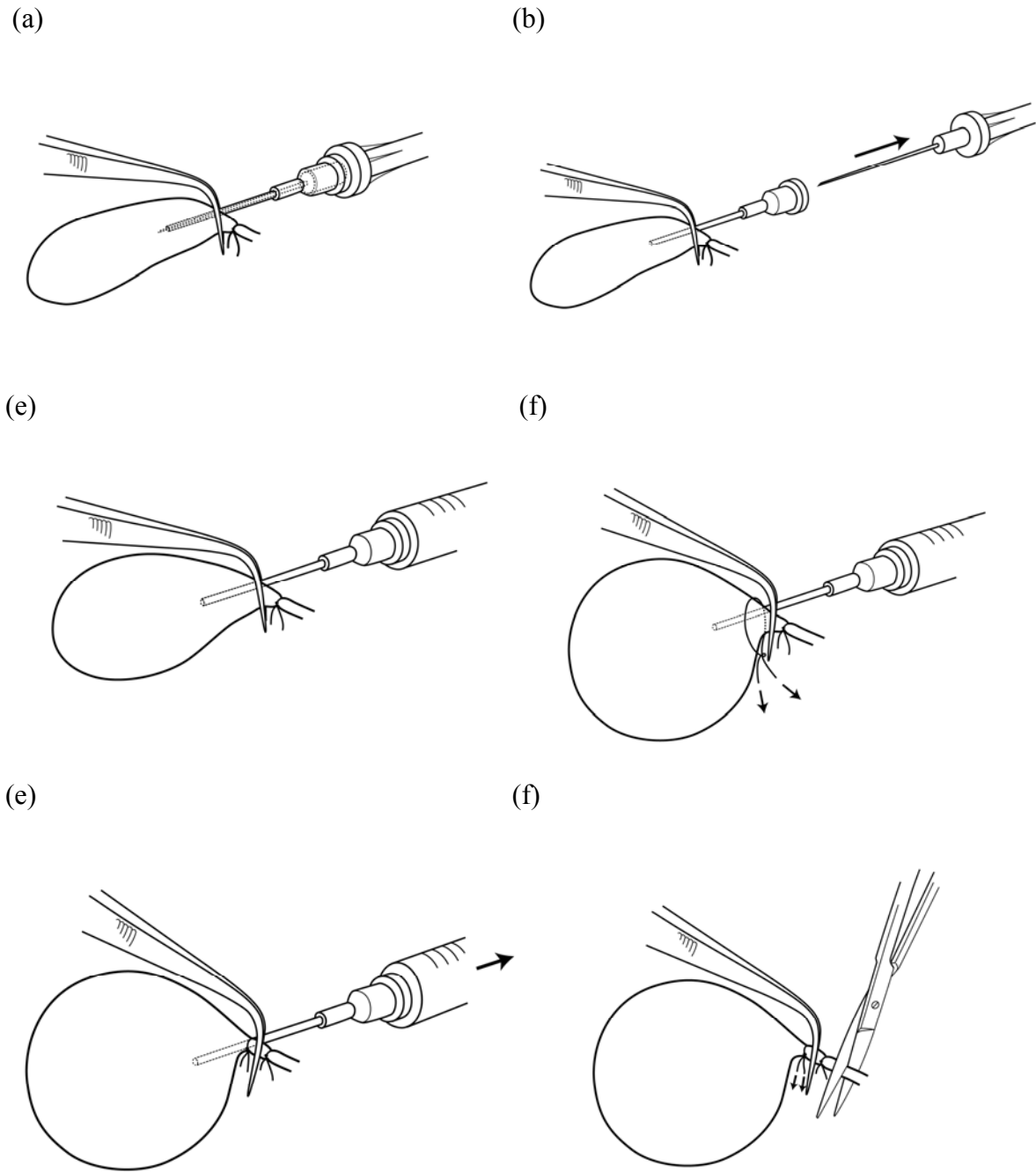
Figure 1. Inflation of a rat urinary bladder with trypsin and EDTA. The bladder was tied near

the urethra and held by a pair of curved forceps at the trigon area. (a) A catheter was inserted

into the bladder lumen between the tips of the forceps. (b) The inner metal needle of the catheter

was removed while the forceps kept the catheter in place. (c) The urine was removed through

the catheter. The bladder was rinsed with PBS and inflated with trypsin and EDTA. (d) A

second knot was tied. After the removal of the catheter (e), the second knot was further

tightened (f) to create a sealed bladder, and the bladder was separated from the animal.

(a)
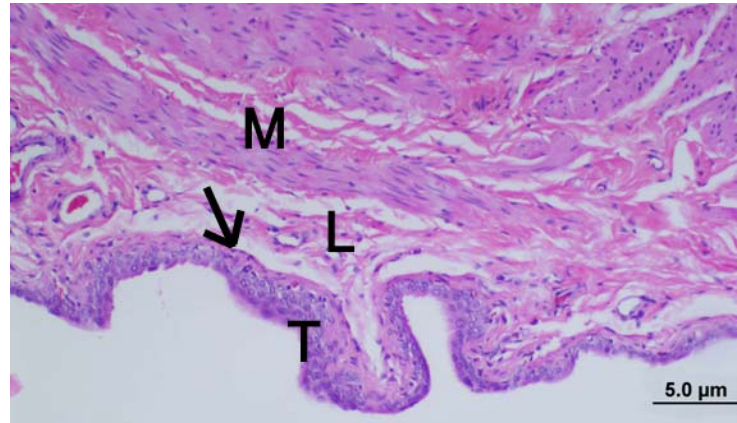
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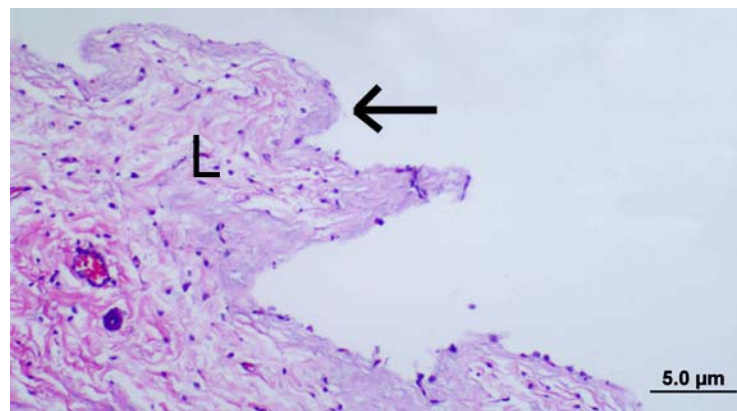
Figure 2. Selective harvest of the urinary bladder transitional epithelium by enzymatic stripping (trypsin and EDTA incubation and scraping).   **(a)** The wall of an intact bladder contains smooth muscle (M), submucosa [lamina propria (L)], basal membrane (arrow) of the epithelium, and transitional epithelium (T). **(b)** After enzymatic stripping, the transitional epithelium was collected from the bladder. The basal membrane (arrow) and tissue underneath it (L) were left on the bladder. Specimens were stained with hemotoxylin-eosin.
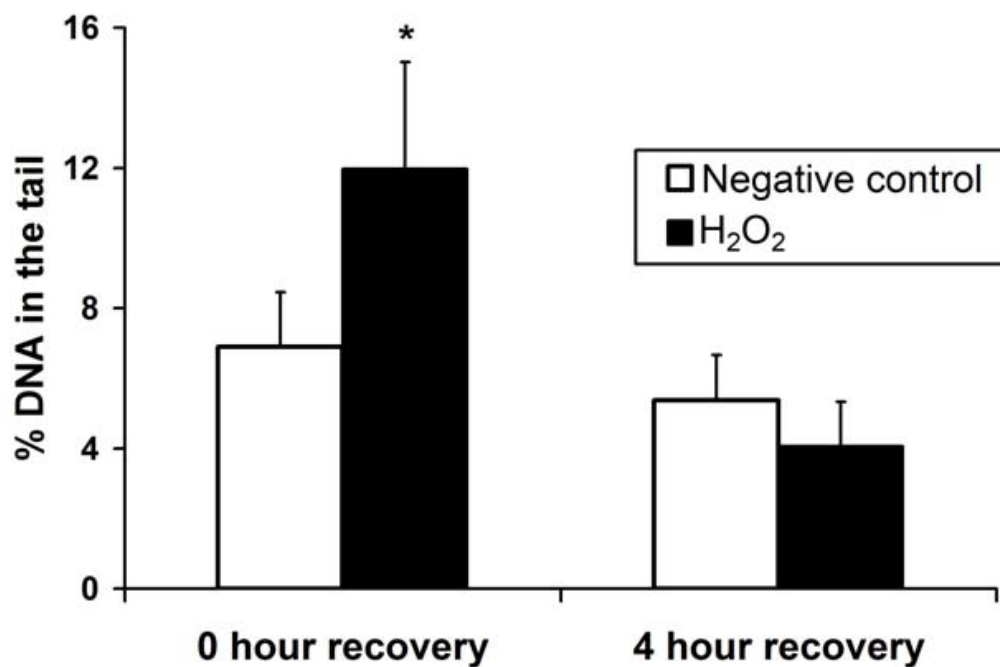
Figure 3. DNA damage and repair in rat transitional cells treated with hydrogen peroxide. Hydrogen peroxide significantly increased DNA damage, and the damage was repaired within 4 hours. Each bar is the mean from 6 rats. Y error bars indicate standard deviations. * indicates a significant difference compared with negative control at 0 hour ($p < 0.05$).

(a)
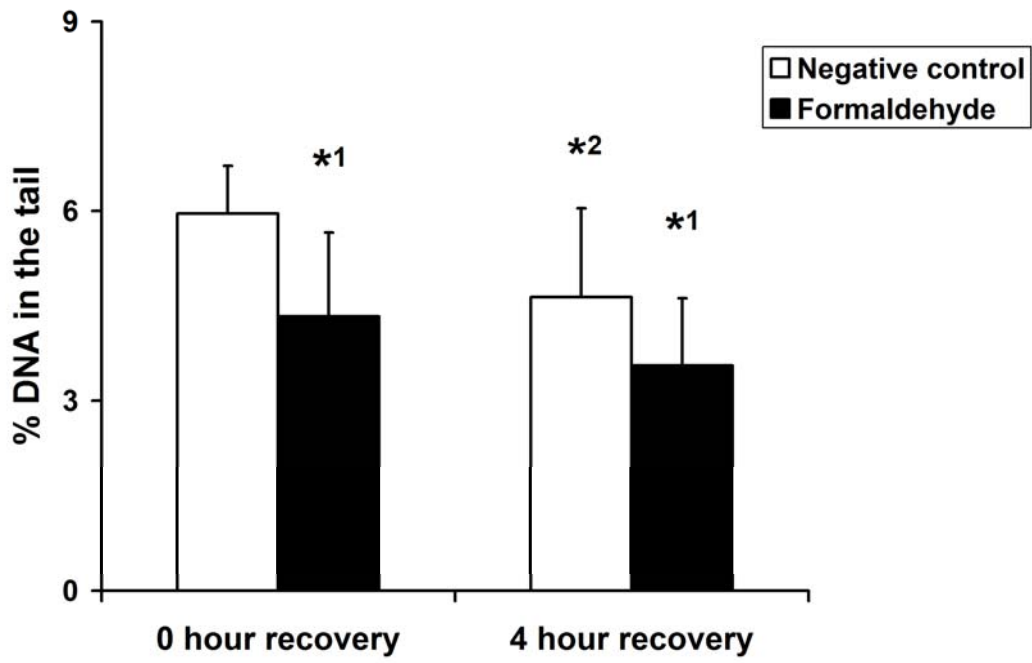
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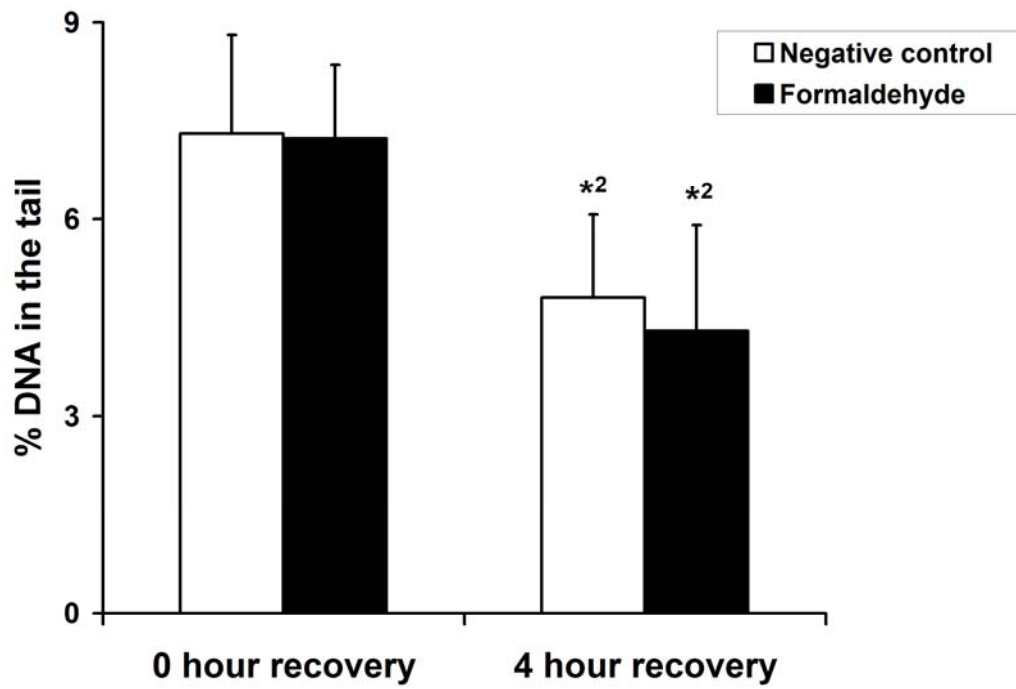
Figure 4. DNA damage and repair in rat transitional cells treated with formaldehyde. Cells were treated with formaldehyde in PBS (Formaldehyde) or PBS (negative control). (a) When no Proteinase K was used, formaldehyde-treated cells had significantly less DNA in the tail than untreated cells at 0 and 4 hour recovery time. When cells were allowed to recover for 4 hours at 37^0 C, the percentage of DNA in the tail was decreased compared to cells at 0 hr recovery time.. (b) When Proteinase K was used, formaldehyde-treated cells showed the same amount of DNA in the tail as untreated cells. DNA migration was decreased after 4 hours of recovery in both formaldehyde-treated and negative control cells. The means of 5 rats are shown. *1 indicates a significant difference compared with negative control samples at the same time point ($p<0.05$). *2 indicates a significant difference compared with the same samples at 0 hour recovery ($p<0.05$).

Chapter 5   Dimethylarsinic acid changed the morphology but not the expression of DNA repair genes of urinary bladder transitional epithelium in F344 rats

(preparing for submission; presently being reviewed by co-authors)

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Short title: DMA(V) affects urinary bladder

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ABSTRACT

Inorganic arsenic increases urinary bladder transitional cell carcinoma in humans, whereas dimethylarsinic acid [DMA(V)], a major arsenic metabolite in the urine of inorganic arsenic-exposed people, increases transitional cell carcinoma in F344 rats. A potentially important critical effect necessary for arsenic carcinogenesis is inhibition of DNA damage repair, which would increase the chance of DNA errors and contribute to carcinogenesis or cocarcinogenesis. Arsenic-induced inhibition of DNA repair has been observed in various cultured cell lines, in mice, and in the lymphocytes of arsenic-exposed people, but has not been previously studied in urinary bladder. We investigated morphological changes of the urinary bladder transitional epithelium of F344 rats exposed to DMA(V) through drinking water, and the expression of DNA repair genes in the transitional cells. Mitochondria were found to be very sensitive to DMA(V), and swollen mitochondria appeared to be the main source of vacuoles observed in the transitional epithelium. Real time RT PCR results showed the mRNA levels of tested DNA repair genes [Ataxia Telangectasia mutant (ATM), X-ray repair cross-complementing group 1 (XRCC1), excision repair cross-complementing group 3/xeroderma pigmentosum B (ERCC3/XPB), and DNA polymerase β (Polβ)] were not altered in the transitional cells by DMA(V). These data suggested that either DMA(V) does not affect DNA repair in the bladder or DMA(V) affects DNA repair without affecting baseline mRNA levels of repair genes. The possibility remains that DMA(V) may lower damage-induced increases in repair gene expression or cause post-translational modification of repair enzymes.

Key Words: arsenic; urothelium; ultrastructure; DNA damage repair; gene expression.

INTRODUCTION

People exposed to arsenic in drinking water have a higher risk of urinary bladder transitional cell carcinoma, particularly smokers (Karagas *et al.* 2004; Steinmaus *et al.* 2003). While inorganic arsenite [As(III)] and arsenate [As(V)] are the main forms of arsenic in drinking water, humans are also exposed to methylated arsenic generated through metabolism of inorganic arsenic in the body, such as monomethylarsonic acid [MMA(V)], monomethylarsonous acid [MMA(III)], dimethylarsinic acid [DMA(V)], and dimethylarsinous acid [DMA(III)]. Additionally, the use of herbicides containing DMA(V) and the consumption of seafood containing DMA(V) or arsenosugars that can be metabolized to DMA(V) are also potential sources of human exposure to DMA(V) (Adair *et al.* 2007). Arsenic is mainly excreted through urine, with DMA(V) being a major metabolite detected in the urine of humans drinking arsenic-contaminated water.

The only laboratory animal model in which arsenic acts as a complete urinary bladder carcinogen is F344 rats exposed to DMA(V) (Cohen *et al.* 2006; Wang *et al.* 2002). Studies using this model showed that the mode of action for arsenic-induced urinary bladder transitional cell carcinoma involves regenerative proliferation of transitional cells following DMA(III)-induced cell death in the urothelium (Cohen *et al.* 2006). Other factors that increase the chance of stable genetic errors in normal proliferating cells may be involved as well. For example, DMA(V)-induced oxidative stress could damage DNA in the bladder and increase the chance of mutation and cancer in the long term. Inhibition of DNA damage repair, similarly, could contribute to genomic instability and carcinogenesis.

Arsenic-induced decreases in DNA repair and in the expression of DNA repair genes have been reported in cultured cells, mouse skin, and lymphocytes collected from arsenic-exposed

humans (Andrew *et al.* 2003; Hartwig *et al.* 2003; Wu *et al.* 2005). However, whether arsenic affects DNA repair in the urinary bladder is not clear, because arsenic effects on DNA damage and repair appear to be cell-type specific (Evans *et al.* 2004; Fischer *et al.* 2005) and arsenic effects on DNA repair have not been previously studied in an animal model that develops bladder cancer after arsenic exposure. *In vitro* studies showed that base excision repair, nucleotide excision repair, and the repair of DNA double strand breaks were all affected by arsenic (Hartwig *et al.* 1997; Lynn *et al.* 1997; Maier *et al.* 2002), and several mechanisms of arsenic-induced inhibition on DNA repair have been proposed. Possible mechanisms include direct inhibition of DNA repair enzymes by trivalent methylated arsenic (Schwerdtle *et al.* 2003; Walter *et al.* 2007), indirect inhibition of DNA repair enzymes through reactive oxygen or nitrogen species (Bau *et al.* 2001; Chien *et al.* 2004), and alternations in the expression of DNA repair genes (Andrew *et al.* 2006; Hamadeh *et al.* 2002). Alterations in signal pathways (Vogt and Rossman 2001) and post-translational modification on proteins involved in DNA repair (Yuan *et al.* 2002) also have been reported.

The first objective in the present study was to determine whether DMA(V) in drinking water causes ultra-structural changes in the urothelium of F344 rats. Previously published studies showed hyperplasia, necrosis, and exfoliation in the urothelium of rats exposed to DMA(V) in the feed (Cohen *et al.* 1998; Cohen *et al.* 2002), but morphological changes were not evaluated at the organelle level. Recently, mitochondria have been shown to play an important role in arsenic-induced toxicity, including oxidative stress, apoptosis, and genotoxicity (Liu *et al.* 2005; Pourahmad *et al.* 2003; Santra *et al.* 2007). With a focus on the mitochondria, the present study investigated DMA(V) effects on the organelles in the urothelium, using transmission electron

microscopy, in addition to light microscopy and scanning electron microscopy. The ultra-structural changes of the urothelium are provided in detail for the first time.

The second objective was to test the hypothesis that DMA(V) affects the expression of DNA repair genes in rat urinary bladder transitional cells. We harvested mRNA from urinary bladder transitional cells, the target cell of DMA(V) carcinogenesis in the rat and measured the expression of DNA repair genes by real-time RT PCR. Ataxia Telangectasia mutant (ATM), X-ray repair cross-complementing group 1 (XRCC1), and excision repair cross-complementing group 3/xeroderma pigmentosum B (ERCC3/XPB) were tested in this study because their expressions were affected by inorganic arsenic exposure in other cell types (Andrew *et al.* 2003; Bae *et al.* 2002). DNA polymerase β (Polβ) was tested because the ligation step in DNA repair was inhibited by inorganic arsenic, but isolated Polβ protein was insensitive to arsenic (Hu *et al.* 1998). The present study adds understanding of DMA(V) effects in the urinary bladder, particularly on DNA damage repair inhibition as a critical feature of carcinogenesis.

MATERIALS AND METHODS

Chemicals. Dimethylarsinic acid, in the form of sodium cacodylate-trihydrate [(CH3)2AsO2Na·3H2O], (purity of 99.52%, with inorganic arsenic 0.0030%) was purchased from Electron Microscopy Science, Fort Washington, Pennsylvania. Trizol® solution was from Invitrogen, Carlsbad, California.

Animals and treatments. Animal use and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee for Animals Used in Research and Testing, prior to the initiation of the study. Female Fisher 344® rats were purchased from Harlan, Madison, Wisconsin, USA at the age of 6 weeks. Female rats were used because they are more sensitive to

DMA(V)-induced urothelium toxicity than the males (Arnold *et al.* 2006; Cohen *et al.* 2002; Cohen *et al.* 2001; Shen *et al.* 2006). Rats were uniquely identified by ear tags after 1 week of quarantine. The rats were single-housed in polycarbonate shoebox-style cages with long fiber paper bedding. The temperature and humidity were monitored continuously and a 12-hour light-dark cycle was maintained. Teklad 2018 SC diet and water in plastic water bottles with stainless steel sipper tubes with stoppers was available *ad libitum*. Since chlorination byproducts have been suspected as a human carcinogen for the urinary bladder, chlorinated tap water, instead of deionized or distilled water, was used to simulate human exposure (Huff *et al.* 1998). Both the diet and tap water contained below-detection-limit levels of arsenic (diet <0.10 ppm, water <0.002 ppm). Selenium, an antagonist to arsenic toxicity, was 0.16 ppm in diet and below detection level in water (<0.01 ppm).

After 1 week of acclimation, rats were assigned into experiments by body weight stratification. Twenty rats were assigned to the morphology study, and 30 rats were assigned to the study of expression of DNA repair genes. Within each experiment, rats were randomly divided into five treatment concentrations: 0, 1, 4, 40 or 100 ppm of DMA(V).

Rats were given DMA(V) in drinking water for 4 weeks. DMA(V) solution was prepared with tap water once a week and stored at room temperature. Fresh water with or without DMA(V) was provided three times a week, and water consumption was measured three times a week at each water change. Food consumption and body weight were measured weekly while rats were transferred to clean cages with fresh bedding.

Terminal necropsy. All rats survived the 4-week treatment without signs of overt toxicity. Rats were euthanized by carbon dioxide asphyxia. The bladders were removed immediately

(within 2 minutes after death), and stripped by Trizol® solution for mRNA extraction. Bladders were fixed for morphology studies within 4 minutes after death.

Morphological examination for urinary bladder. Each bladder was cut into approximate halves longitudinally, bisecting the trigone, at the time of necropsy. One half (for light microscopy) was fixed in 10% neutral buffered formalin, and the other half (for TEM and SEM) was dissected and fixed in cold general fixative for electron microscopy samples (4.4% formaldehyde/5% glutaraldehyde/2.75% picric acid, in a 0.05M sodium cacodylate buffer at pH 7.4).

Each half bladder fixed in 10% neutral buffered formalin was trimmed and submitted to Carilion Histology Laboratory (Roanoke, Virginia) to be processed into slides for routine histology. Briefly, the samples were dehydrated through a graded series of alcohols, infiltrated with paraffin polymer and then sectioned at 3 microns. Sections were stained with hematoxylin and eosin on automated equipment.

For diagnostic purposes, the degrees of hyperchromatination and cytoplasmic vacuolation were scored separately on each slide. The slides were randomized for scoring sequence, and one person scored all slides without the knowledge of treatments at the time of scoring. A set of standard slides of various scores was used a reference to ensure consistency. A score of 0 indicated no hyperchromatic cells or vacuolation under 400X magnification, and the score increased with the presence of hyperchromatic cells or vacuolation. A score of 1 indicated minimal hyperchromatination or vacuolation (few tiny vacuoles).   A score of 2 indicated mild hyperchromatination or vacuolation (frequent tiny vacuoles). A score of 3 indicated mild hyperchromatination or vacuolation (few large vacuoles also present). A score of 4 indicated

diffused hyperchromatination or vacuolation (frequent large vacuoles). A score of 5 indicated profound hyperchromatination or vacuolations observed thourghout the whole epithelium.

The other halves of the bladders, designated for ultrastructural studies under TEM and SEM, were fixed in general fixative for at least 24 hours, washed in 0.1 M sodium cacodylate, post-fixed in 0.1M sodium cacodylate containing 1% osmium tetroxide (OsO_4), and washed in 0.1M sodium cacodylate. Dehydration was performed by placing samples in increasing concentrations of graded ethanol (15%, 30%, 50%, 70%, 95%, and 100%) for 10 minutes each, followed by submersion in propylene oxide for 15 minutes.

For TEM, samples were embedded in a 50:50 solution of propylene oxide:Poly/Bed 812. To determine the areas for thin sectioning, thick sections (1.0 μm) were cut and tri-stained (Methylene blue stain in sodium borate, Azure II stain in water, and basic fuschin in sodium borate) (Humphrey and Pittman 1974). The thick sections were observed under a light microscope to identify areas with transitional epithelium.   Selected areas were cut into thin sections (60 nm thick), and stained with uranyl acetate and lead citrate. The thin sections were examined with a Zeiss 10CA transmission electron microscope (Zeiss, West Germany).

For SEM, samples were dried using a Ladd Critical Point Dryer model 28000 (Ladd Research Industries, Burlington, Vermont), mounted onto an SEM specimen stub, and sputter-coated with gold in an SPI-Module™ Sputter Coater (SPI Supplies, West Chester, Pennsylvania). The specimens were examined in a Philips Scanning Electron Microscope 505 (Philips, Eindhoven, Netherlands).

Trizol® stripping and mRNA extraction of urinary bladder transitional cells. Selective collection of urinary bladder transitional cells for RNA extraction has been described (Sen *et al.*

2005). The bladder was ligated, rinsed, and then filled with cold Trizol® solution for 10 min.

The cell lysate was aspirated, flash frozen and stored at -70°C. The stripped bladder was fixed in

formalin for light microscopic confirmation that only the urothelium was removed. Total RNA

was extracted from Trizol/cell lysate according to Trizol® manufacturer's protocols. Fresh

Trizol® solution and chloroform were added to thawed samples sequentially, followed by

centrifugation. The clear supernatant (the RNA-containing aqueous phase) was transferred into a

clean microcentrifuge tube and cold isopropanol was added. Samples were incubated at -70°C

overnight. After thawing, samples were centrifuged to precipitate the RNA. The RNA pellet

was washed with 70% diethylpyrocarbonate ethanol and air dried. The RNA was resuspended in

RNase-free water and stored at -70°C. Total RNA yield from each bladder ranged from 21.7 μg

to 90.8 μg, based on absorbance at the 260 nm wavelength on a spectrophotometer.

Real time RT PCR for gene expression. Real time RT PCR was performed in 384-well

plates using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster

City, CA) according to the manufacturer's instructions. In a one-step RT PCR reaction, 200 ng

of total RNA were subjected to cDNA synthesis and subsequently amplified during 40 PCR

cycles (48°C for 30 min, 95°C for 10 min , followed by 40 cycles of 95°C for 15 sec, and 60°C

for 60 sec). Amplification reactions were carried out in triplicate using TaqMan® One-Step RT-

PCR Master Mix. Primer and probe sequences are listed in **Table 1**. TATA-box binding protein

(TBP) was chosen as a reference gene because it demonstrated consistent expression across all

treatment groups. The probe for TBP was labeled with the VIC reporter dye, and probes for the

four genes of interest (ATM, ERCC3/XPB, Polβ, and XRCC1) were labeled with FAM (6-

carboxyfluorescein). The primers and probes for TBP were designed using Primer Express v2.0

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software (Applied Biosystems).   The primers and probes for XRCC1 and Polβ were from TaqMan® Pre-Developed Assays for Gene Expression – Rat (ABI), and all other primers and probes were ABI Assays-on-Demand™ Gene Expression products.

Standard curves for each gene were constructed using three serial dilutions of starting samples. From the standard curves, the relative input for each gene was determined (i.e. normalized to the reference gene, which was used to be called "housekeeping gene"). The average relative input from triplicates was designated as the expression level of each gene.

Statistical analysis. For water consumption, food consumption and body weight gain, the numbers were adjusted by body weight. The effects of arsenic concentration, time, and interaction of arsenic concentration and time on group means of body weight, body weight gain, food consumption and water consumption were evaluated by repeated measure analysis and Wilk's Lambda approach. The histological changes (vacuolation and hyperchromatin) of urinary bladder were analyzed by Cochran-Mantel-Haenszel method in which both lesion scores and DMA(V) treatments were ranked.

The effect of DMA(V) on gene expression was tested by multiple linear regression analysis with a maximal random likelihood model. To verify the association between accumulated arsenic exposure (expressed as DMA(V) intake per kg BW) and gene expression levels, the expression of reference gene (TBP) and starting RNA amount for RT PCR were controlled in the model. The assumptions of multiple linear regression analysis were verified.

A *p* value less than 0.05 was considered significant. All statistical analyses were performed using a SAS computer program version 8.02 or 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

General conditions

All animals survived the treatment and there were no clinical signs of intoxication. Up to 100 ppm DMA(V) did not affect body weight, body weight gain, or food consumption (data not shown). F344 rats exposed to 40 and 100 ppm DMA(V) increased their water consumption (**Fig. 1**). As a result, rats in the 100 ppm group had 4.6 times greater total arsenic intake as compared to those of the 40 ppm group, and 169 times those of the 1 ppm group (**Table 2**).

Histological changes of urinary bladder

Dimethylarsinic acid exposure increased cytoplasmic vacuolation and nuclear hyperchromatin in the rat transitional epithelium. Each bladder was given two scores, one each for vacuolation or hyperchomatin, and a higher score indicated a higher quantity of the vacuolation or hyperchromatin. Although vacuolation and hyperchromatin were present in all DMA(V) treatment groups, the scores of vacuolation and hyperchrmatin were highest in rats exposed to 100 ppm DMA(V) (**Fig. 2**). Furthermore, the scores were increased with DMA(V) concentration in a dose-dependent manner, as tested by Cochran-Mantel-Haenszel method.

Ultrastructural changes of transitional epithelium

SEM. Normal transitional epithelium with large, flat, polygonal superficial cells covered in microridges were present in control rats and rats treated with up to 40 ppm DMA(V) [**Figs 3 (a) and (c)**]. In rats exposed to 100 ppm DMA(V), transitional epithelium had widely distributed sites of necrotic and exfoliated cells [**Fig. 3 (b)**], consistent with morphologic evidence of cytotoxicity. Superficial transitional cells from rats treated with 100 ppm DMA(V) were round, with variable size and covered in polymorphic microvilli [**Fig. 3 (d)**]. Although the bladders

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were fixed at various stages of expansion, which prevented the use of cobblestone-like appearance and elevation of cells as indicators of hyperplasia, the surface structures (microridges and microvilli) were not affected. Based on the polymorpholic microvilli observed, rats exposed to 100 ppm DMA(V) had signs of regeneration and hyperplasia.

TEM. The transitional epithelium is typically composed of one layer of superficial cells facing the bladder lumen, one to three layers of intermediate cells (which appeared six to eight cells thick in a fully contracted bladder), and one layer of basal cells situated on top of the basement membrane.

Transitional epithelium from rats exposed to 1 or 4 ppm DMA(V) were not different from control (data not shown), but rats exposed to 40 or 100 ppm DMA(V) had increased vacuolation and decreased numbers of mitochondria in the transitional epithelium (**Fig. 4**). Vacuolation was more prominent in the superficial cells than in the intermediate or basal cells.

Morphological changes to the mitochondria were variable and appeared to be the main source of the vacuoles identified by light microscopy. Mitochondria in transitional cells from rats treated with 40 ppm DMA(V) were swollen with loss of cristae (**Fig. 4 (c, d)**). Transitional cells from rats treated with 100 ppm had almost no recognizable mitochondria in the superficial cells (**Fig. 4 (e, f)**).

Gene expression

The expressions of the four tested target genes (ATM, ERCC3, Polβ, and XRCC1) were calculated by relative quantification using standard curves of the same primer and probe set. The relative expression of each gene to TBP, the reference gene, was plotted against DMA(V) intake, and the average of each DMA(V) treatment group was also graphed (**Fig. 5**).

After controlling for TBP expression and total RNA, there was no association between gene expression and DMA(V) intake adjusted by body weight, as tested by multiple linear regression analysis. This indicated that the expressions of ERCC3, ATM, Polβ, and XRCC1 in the bladder transitional cells were not altered by DMA(V).

DISCUSSION

Dimethylarsinic acid at concentrations that produced no apparent clinical toxicity induced morphological changes in urinary bladder transitional epithelium, a target of arsenic carcinogenesis, but no alterations in the expression of DNA repair genes. While there have been previous reports of vacuolation, hyperchromatin, and hyperplasia in the transitional epithelium (Cohen *et al.* 2001; Sen *et al.* 2005; Shen *et al.* 2006), the present study first showed organelle damage, particularly mitochondrial damage, after DMA(V) exposure in a dose-response manner. The mRNA levels of DNA repair genes involved in excision repair or double strand break repair (ATM, ERCC3/XPB, Polβ, and XRCC1) were not altered by DMA(V) in transitional cells.

Rats exposed to up to 100 ppm DMA(V) showed no clinical signs of intoxication; however, water consumption was increased in rats exposed to 40 or more ppm of DMA(V) in the water. This is consistent with previous studies using F344 rats and DMA(V) (Nishikawa *et al.* 2002; Wei *et al.* 2002; Wei *et al.* 1999). In addition to increases in water consumption and urine volume, increases in calcium excretion in the urine and calcification in the kidney (but not in the bladder) were reported in F344 rats given DMA(V) in Purina diet (Arnold *et al.* 1999). In the present study, similar to rats given Altromin diet (Arnold *et al.* 1999), rats given Teklad 2018 SC diet showed increased water consumption and no calcification in the kidney after DMA(V) exposure (unpublished data). In contrast to F344 rats, Sprague-Dawley rats did not increase their water consumption when given DMA(V) in drinking water (unpublished data). F344 rats also

did not increase water consumption when given MMA(V) or trimethylarsine oxide in drinking water (Nishikawa *et al.* 2002). The cause of DMA(V) specific increases in water consumption in F344 rats remains unclear.

DMA(V) increased vacuolation, hyperchromatin, and mitochondrial swelling in the rat urothelium. Superficial cells were more severely damaged than the intermediate or basal cells, suggesting that urine in the bladder lumen, rather than blood from the submucosa or muscle, may be the main source of DMA(V) exposure. Alternatively, superficial cells may be more sensitive to DMA(V) effect than other cell layers. The observation of mitochondrial swelling in the bladder by TEM supports the hypothesis that arsenic specifically targets mitochondria, as seen in other cell types and purified mitochondria (Bustamante *et al.* 2005; Liu *et al.* 2005; Miller *et al.* 2007; Santra *et al.* 2007). Low concentrations of As(III) stimulated cytochrome c release from mitochondria and led to apoptosis, whereas high concentrations of As(III) directly targeted the mitochondrial respiratory chain and led to necrosis (Bustamante *et al.* 2005). In the present study, 100 ppm DMA(V) induced severe damage to mitochondria, likely rendering these organelles unable to support energy-dependent apoptosis. This could lead to necrosis, rather than apoptosis, consistent with previous reports of urothelial toxicity from high concentrations of DMA(V) (Cohen *et al.* 2001; Shen *et al.* 2006). Rats exposed to low concentrations of DMA(V) have increased apoptosis indexes in the urothelium (Kinoshita *et al.* 2007). These data are consistent with previous descriptions of gene expression from rats similarly exposed (Sen et al., 2005) in that the animals treated with 100 ppm did not have prominent differential gene expression compared to lower doses due to greater toxicity and cell death.

Vacuoles formed from various membranous components, i.e. mitochondria, endoplasmic reticula, and Golgi apparatus, can be indistinguishable. It is possible that other membranous

components, in addition to mitochondria, also contributed to DMA(V)-induced vacuoles. For example, dilated rough endoplasmic reticulum tends to de-granulate and appear similar to dilated smooth endoplasmic reticulum, a vacuole with no specific characteristics. Because no intermediate degrees of swelling were observed in the endoplasmic reticula or the Golgi apparatus, it is unlikely that they were major sources of vacuoles in the present study.

Increased hyperchromatin was present in the transitional epithelium of F344 rats exposed to DMA(V) in a dose-response manner. Because hyperchromatin indicates active production of DNA, it can be a sign of proliferation, which is consistent with our SEM observation. In mice, DMA(V)-induced hyperchromatin has been observed in the lung, but not in the liver (Nakano *et al.* 1992). The observed hyperchromatin in the lung was suspected to be caused by DMA(V)-induced DNA-protein crosslinks (Nakano *et al.* 1992), which has been reported in DMA(V)-treated cultured cells (Kato *et al.* 1994; Yamanaka *et al.* 1995; Yamanaka *et al.* 1993). Regarding arsenic-induced DNA-protein crosslinks in the urinary bladder, these have been reported in As(III)-exposed mice (Tice *et al.* 1997), but were not observed by us in rats sacrificed 1 day after 1 week exposure to 100 ppm DMA(V) in the drinking water (unpublished data).

Arsenic has been shown to inhibit DNA damage repair *in vivo* (Tran *et al.* 2002) and *in vitro*, including cultured lung and skin cells, targets of arsenic carcinogenesis (Schwerdtle *et al.* 2003; Wu *et al.* 2005). The decreased DNA repair may be due to decreased DNA repair gene expression. Concurrent observation of decreases in DNA repair gene expression and increases in expression of oxidative stress response genes in the cultured skin keratinocytes (Hamadeh *et al.* 2002) suggested decreased DNA repair in the presence of oxidative stress and possible oxidative DNA damage. Recently, decreased DNA repair and decreased expression of nucleotide excision repair genes were observed in the lymphocytes of humans drinking arsenic-contaminated water

in the USA (Andrew *et al.* 2006; Andrew *et al.* 2003). The mRNA concentrations of ERCC1, XPF, and XPB, as well as protein concentration of ERCC1, were lowered by arsenic exposure (Andrew *et al.* 2006; Andrew *et al.* 2003). In studies using microarray, decreased expression of DNA repair genes was seen in As(III)-treated human keratinocytes (Bae *et al.* 2002; Hamadeh *et al.* 2002).

To investigate whether DMA(V) targets DNA repair genes in the urinary bladder, we used real time RT PCR to measured mRNA of ATM, ERCC3/XPB, XRCC1, and Polβ in transitional cells collected from rats exposed to DMA(V). These tested genes were chosen because either their expressions or their functions have been affected by arsenic in other cell types. ATM (involved in DNA double strand break repair) was critical in cellular response to As(III) in primary porcine aortic endothelial cells (Tsou *et al.* 2006). The mRNA levels of ERCC3/XPB (in nucleotide excision repair) and XRCC1 (in base excision repair) were reported to be affected by inorganic arsenic exposure (Andrew *et al.* 2006; Andrew *et al.* 2003; Bae *et al.* 2003; Bae *et al.* 2002). The expression of Polβ was suspected to be affected by arsenic because the ligation step of base excision repair was inhibited by As(III) exposure, while purified Polβ protein was insensitive to inorganic arsenic treatments (Hu *et al.* 1998; Lynn *et al.* 1997). Our results showed that DMA(V) did not affect the mRNA level of ATM, ERCC3/XPB, XRCC1, or Polβ in the transitional cells. The differences in arsenic effects on the expression of DNA repair genes between the present study and previous studies may be due to the forms of arsenic and cell-type specific response to arsenic.

Based on these gene expression observations, it is possible that DMA(V) does not affect excision repair in the rat urinary bladder. Cell type specific responses to arsenic effects on DNA repair have been reported in As(III)-exposed mice. When mice were given As(III) in the

drinking water and benzo[a]pyrene topically on the skin, benzo[a]pyrene-induced DNA adducts were increased by As(III) exposure in the lung, but not in the skin (Evans *et al.* 2004). Moreover, when FVB/N mice carrying the G11 PLAP transgene were used to detect frameshift mutations, increased mutation was only detected in the skin, but not in lung or urinary bladder, of mice receiving both As(III) in water and benzo[a]pyrene on skin (Fischer *et al.* 2005).

Alternatively, DMA(V) may affect DNA repair in the urinary bladder without affecting baseline mRNA expression of repair genes. For example, DMA(V) may only lower damage-induced increases in DNA repair gene expression in the bladder, similar to As(III) in keratinocytes (Bae *et al.* 2002). Furthermore, arsenic may affect DNA repair without altering gene expression at mRNA level. Possible non-translational influence of arsenic on DNA repair includes (1) indirect inhibition of repair enzymes through oxidative stress, (2) direct inhibition of enzymes by binding with enzymes, releasing zinc from enzymes, or crosslinking enzymes, and (3) post-translational modification of enzymes.

Indirect inhibition of repair enzymes through oxidative stress is possible in the bladder, because DMA(V)-induced oxidative stress is associated with bladder cytotoxicity (Kinoshita *et al.* 2007; Wei *et al.* 2005), and arsenic-induced reactive oxygen/nitrogen species could damage DNA repair enzymes (Chien *et al.* 2004). Regarding direct inhibition of enzymes by arsenic, recent studies showed that certain arsenicals inhibit purified DNA repair enzymes at very low concentrations, supporting the hypothesis that arsenic inhibits DNA repair by inactivating repair enzymes. For example, purified XPA was inactivated by MMA(III) and DMA(III) in low µM range by releasing zinc in the zinc finger domain (Schwerdtle *et al.* 2003), and poly(ADP-ribose) polymerase was also inhibited by As(III) in low µM range (Yager and Wiencke 1997). Furthermore, DMA(V) is known to induce DNA-protein crosslinks, including DNA-H1 histone

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crosslinks (Yamanaka *et al.* 1993). Because H1 histone is needed for poly(ADP-ribosyl)ation, DNA-H1 histone crosslinks could interfere the ligation step of DNA repair. Additionally, DMA(V) may cause crosslinks between DNA and repair enzymes and consequently inactivate repair enzymes (Yamanaka *et al.* 1993). Post-translational modification of enzymes has been reported in inorganic arsenic-treated bladder cells. In human bladder cell lines, As(III) affected protein phosphorylation (Yuan *et al.* 2002) and ubiquination (Bredfeldt *et al.* 2004), which could affect the activity and half-life of proteins, respectively. These modifications of proteins involved in DNA repair, such as Mre 11, would affect DNA repair.

DMA(V) in drinking water did not alter baseline mRNA levels of DNA repair genes in the transitional cells of F344 rats. While these results did not provide supporting evidence for the hypothesis that DNA repair inhibition as a mode of action for DMA(V) carcinogenesis in the urinary bladder, the present data cannot exclude the possibility that DMA(V) affects DNA repair in the bladder through other mechanisms, such as alterations in damage-induced DNA repair gene expression and modifications on repair enzymes. However, DMA(V) in the drinking water induced vacuolation, hyperchromatin, and mitochondrial damage in rat urinary bladder transitional epithelium in a dose-response manner, suggesting that mitochondria are a target of DMA(V) toxicity.

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Table 1. Primer and probe sequences.

Reference Gene		Primer and Probe Sequence
TBP	TATA-Box Binding Protein	F: GCTGGGCTTCCCAGCTAAGT
		R: CACAGCTCCCCACCATGTTC
		P: VICCTTAGACTTCAAGATCCMGBNFQ
Target Genes		**Primer and Probe Sequence**
ATM	Ataxia Telangectasia Mutant	F: CAGTGAAGGTTGCTGGCAGTTAT
		R: CGTATCAGAGAACCGCGCTAA
		P: 6FAMATGGACAGATGAAGGCMGBNFQ
ERCC3/ XPB	Excision Repair Cross-Complementing group 3/ xeroderma pigmentosum B	F: CCCACTTTTCAACGCTTCAG
		R: CCAGGCAGTCAATCTTCAACTG
		P: 6FAMAAGTGACATCCACCCTGTAMGBNFQ
Polβ	Polymerase β	*CCTGGGGGTCACTGGGGTTGCTGGG
XRCC1	X-ray Repair Cross-Complementing group 1	*GCCCTCCCGGAGGTACCTCATGGCA

F: Forward primer, R: Reverse primer, P: Probe. * The sequences of primers and probe are proprietary information owned by ABI. The ABI-reported sequence that lies within the amplicon is listed.

Table 2. Average daily and accumulated DMA(V) intake of F344 rats exposed to DMA(V) in drinking water are expressed as group means ± standard deviations. The body weight of each week is the average of body weights at the beginning and the end of that week.

DMA(V) in water	Week 1		Week 2		Week 3		Week 4	
	Daily (µg/kg BW/day)	Accumulated (µg/kg BW)	Daily (µg/kg BW/day)	Accumulated (µg/kg BW)	Daily (µg/kg BW/day)	Accumulated (µg/kg BW)	Daily (µg/kg BW/day)	Accumulated (µg/kg BW)
0 ppm	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1 ppm	1.1 ± 0.1	7.8 ± 0.9	1.1 ± 0.1	15.3 ± 1.5	1.0 ± 0.1	22.5 ± 2.4	1.0 ± 0.1	29.2 ± 3.1
4 ppm	4.3 ± 0.4	29.8 ± 3.0	4.1 ± 0.7	58.7 ± 7.3	3.8 ± 0.5	85.6 ± 10.5	3.6 ± 0.5	110.7 ± 13.9
40 ppm	42.2 ± 4.6	295.6 ± 32.0	41.8 ± 5.3	588.1 ± 64.4	47.5 ± 7.0	920.3 ± 101.2	55.5 ± 5.0	1308.5 ± 91.5
100 ppm	107.1 ± 13.2	749.5± 92.6	105.8 ± 15.3	1489.8 ± 177.3	135.2 ± 25.6	2435.93 ± 26.4	172.0 ± 39.8	3640.1 ± 493.1
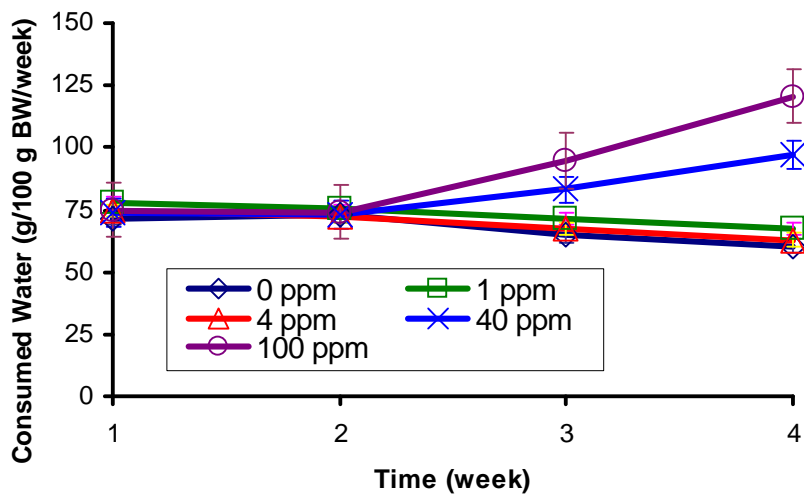
FIG. 1. Water consumption of F344 rats was significantly greater in rats treated with 40 and 100 ppm DMA(V), compared to control. The weekly water consumption was calculated by dividing total water consumption in one week by the average body weight at the beginning and the end of that week. Y error bars = standard deviations.

(a)
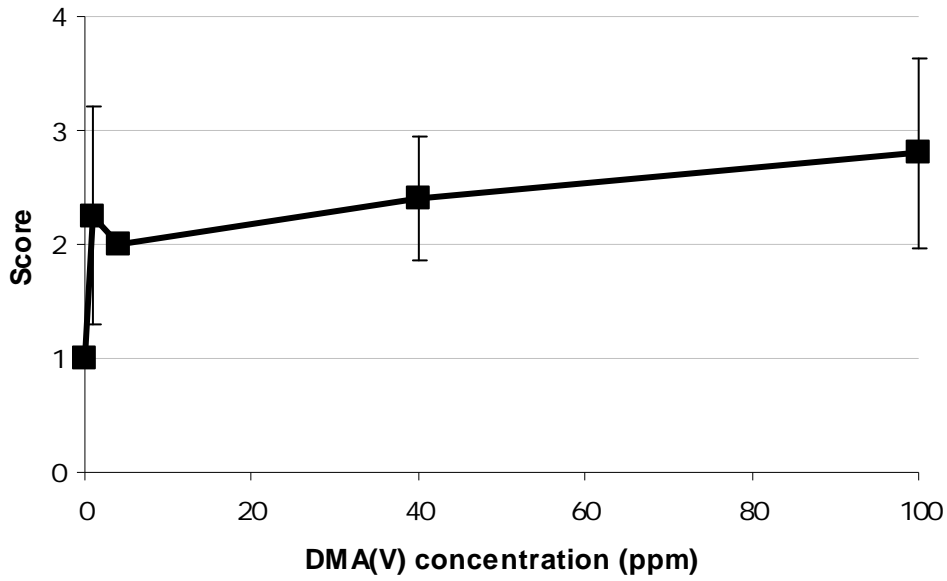
(b)
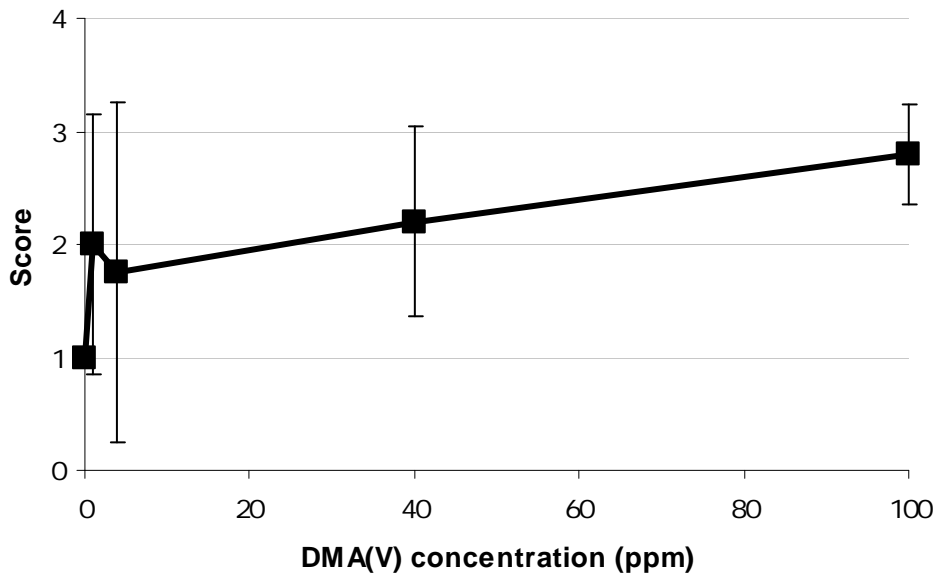
FIG. 2. Urinary bladder transitional epithelium had significantly increased vacuolation **(a)** and hyperchromatin **(b)** after DMA(V) treatment in a dose-dependent manner. Y error bars = standard deviations.
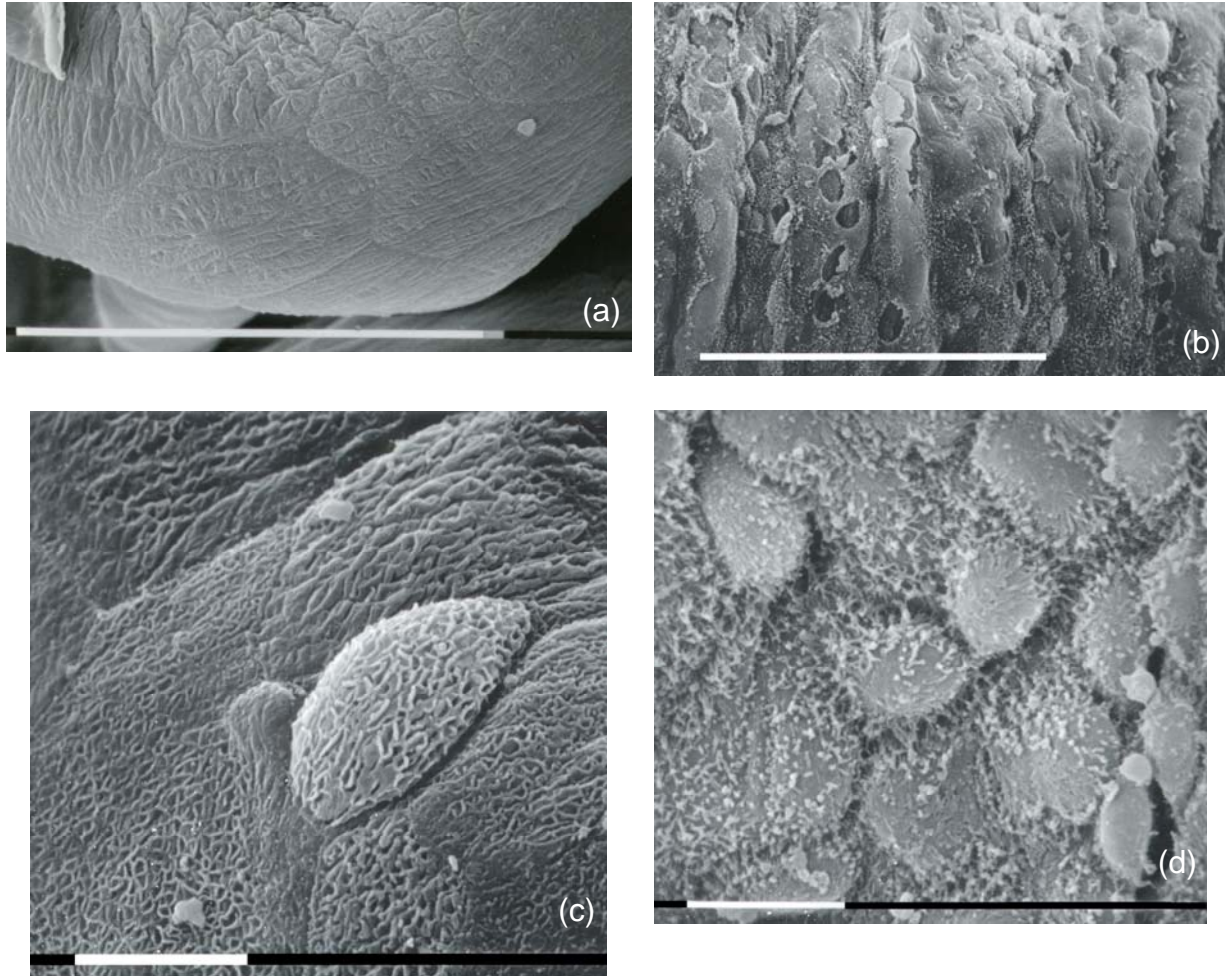
FIG. 3. (**a**) While control rats had normal transitional epithelium, including flat, polygonal cells, (**b**) rats exposed to 100 ppm DMA(V) had sites of necrotic and exfoliated cells (arrows) in the transitional epithelium. (**c**) The surface of transitional epithelium from rats exposed to 40 ppm DMA(V) had normal micro-ridges. (**d**) Transitional epithelium from rats exposed to 100 ppm had round cells covered in polymorphic microvilli. White bar = 100 μm in (a) and (b); 10 μm in (c) and (d). Original magnification: (a) 680x, (b) 625x, (c) 2620x, (d) 2500x.

(a)
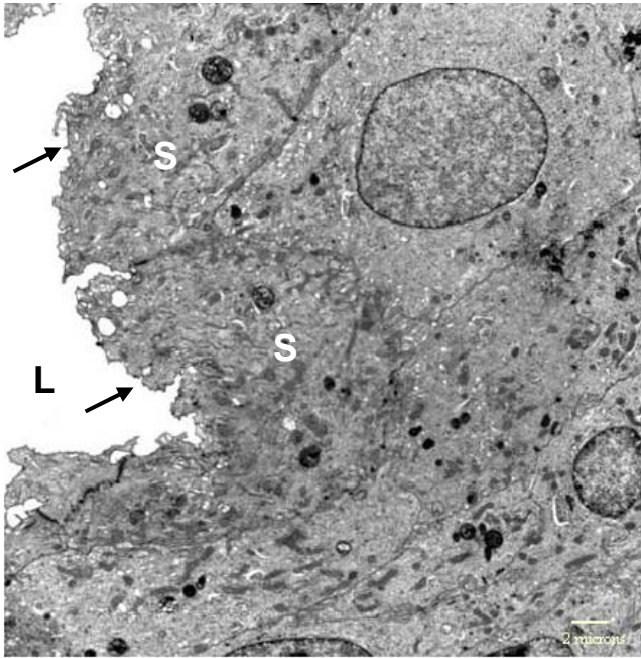
(b)
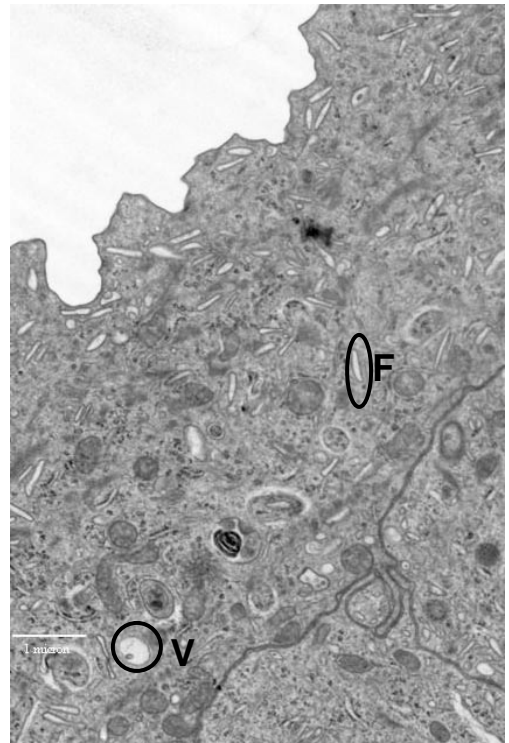
(c)
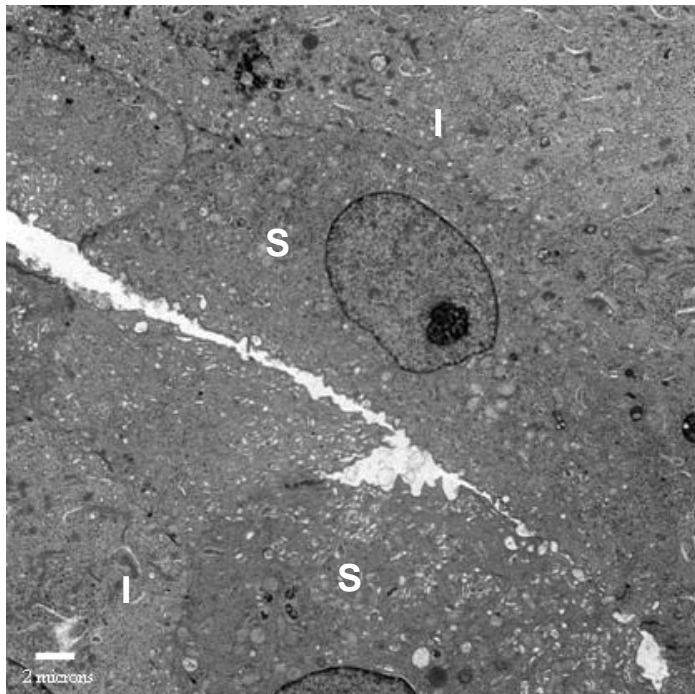
(d)
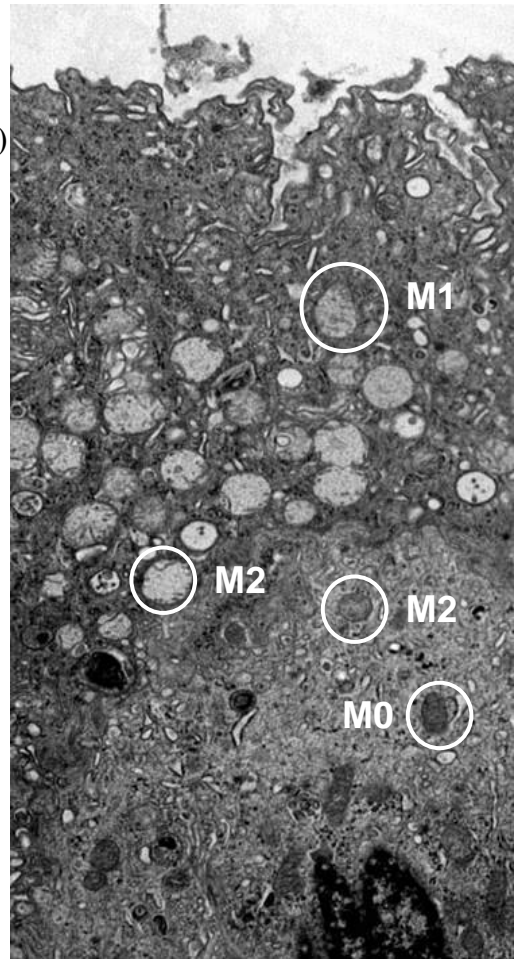
(e)
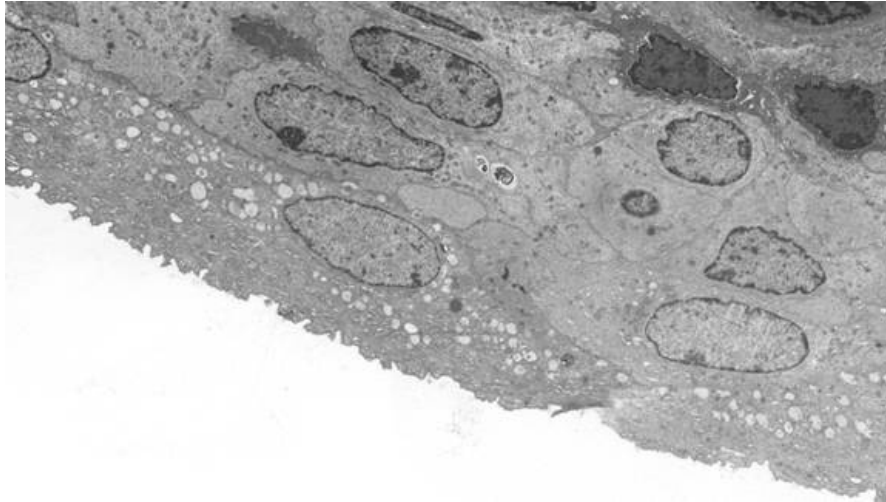
(f)
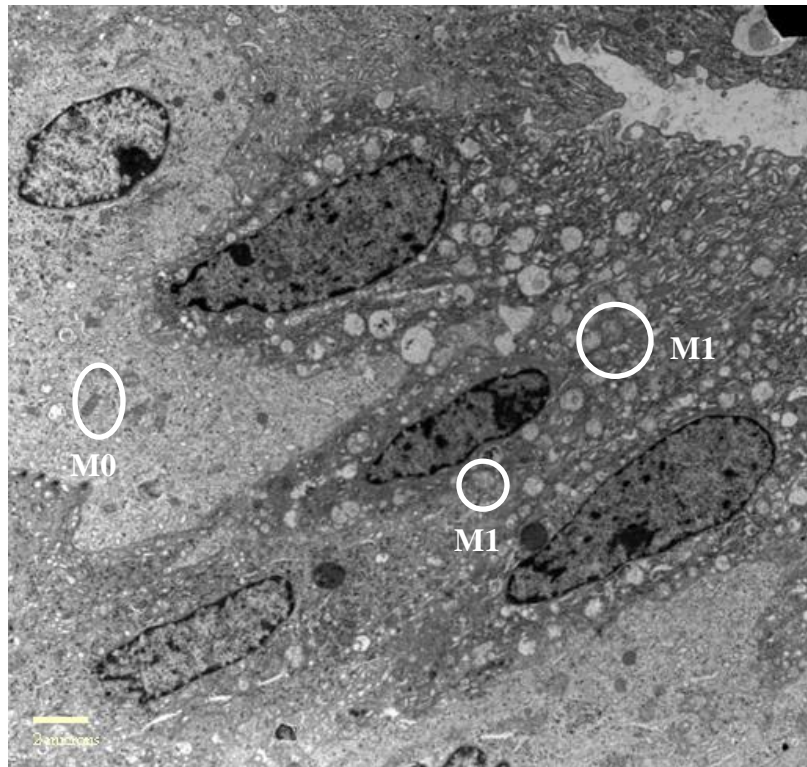
FIG. 4. Bladder transitional epithelium from control rats (0 ppm) (**a-b**) or DMA(V)-exposed

rats (**c-f**). In transitional epithelium of the control rats, the superficial cells (S) have a scalloped

luminal surface (arrows) characteristic of transitional cells (**a**). L = lumen. Almond-shaped thick

walled fusiform vesicles (F) are formed by asymmetric membrane plaques in a contracted

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bladder **(b)**. Transitional epithelium from rats treated with 40 ppm DMA(V) had swollen

mitochondria and increased numbers and areas of vacuoles were present **(c-d).** The vacuoles

appeared more abundant in superficial cells (S) than intermediate cells (I) **(c)**. The severity of

mitochondrial changes ranged from mild swollen with nearly normal-looking cristae (M0), to

severely swollen with disturbed cristae (M1) or almost no cristae (M2) **(d)**. Transitional

epithelium from rats treated with 100 ppm DMA(V) had profound vacuolation of the superficial

cells **(e)**. Although mitochondria in the superficial cells were swollen (M1), intact mitochondria

(M0) were present in the intermediate cells **(f)**.
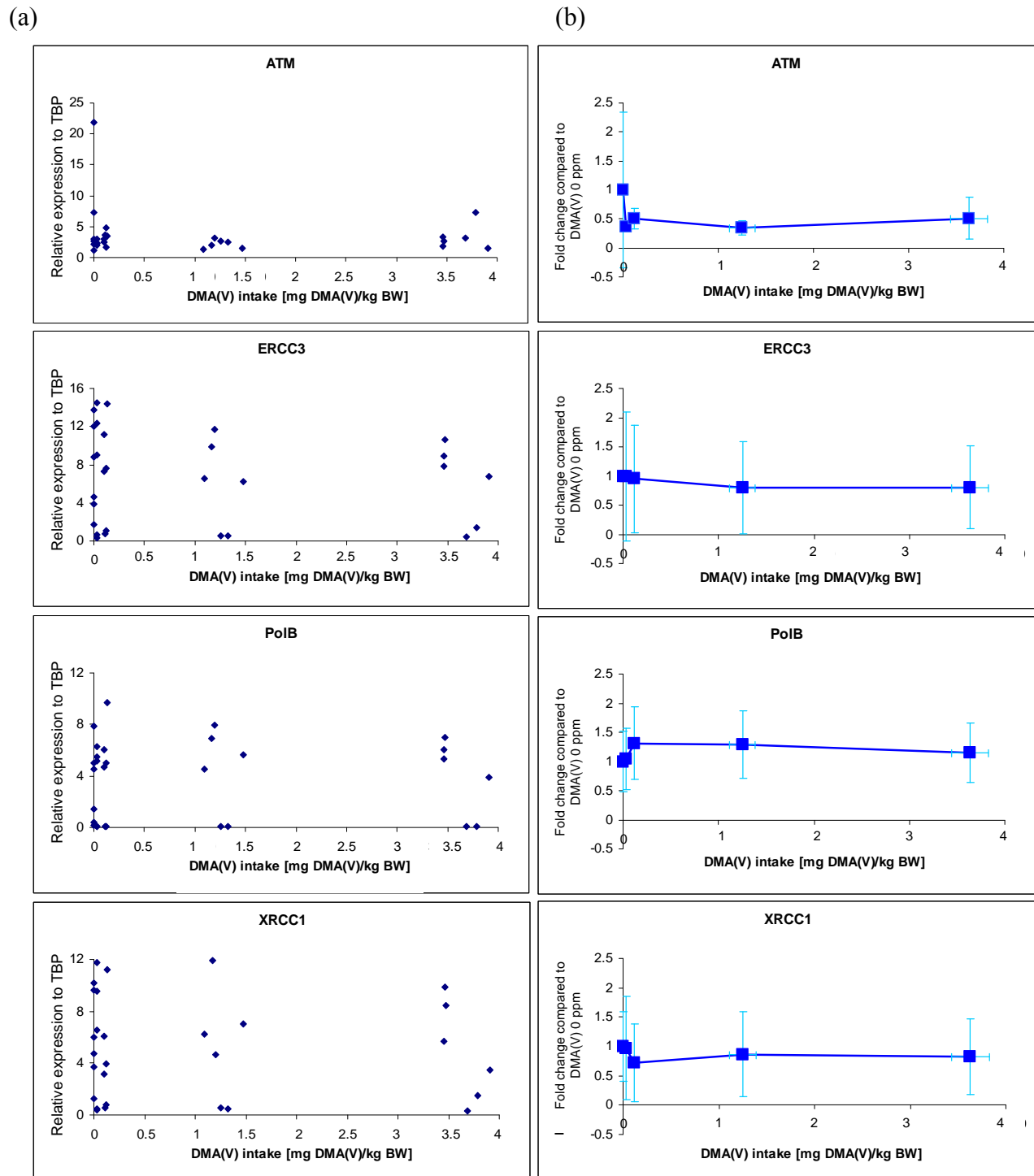
FIG. 5. The expression of DNA repair genes in transitional cells were not altered by up to 100 ppm DMA(V) in drinking water. (a) Expression of each rat normalized to the expression of the

reference gene, TBP. (b) Expression of each DMA(V) concentration group expressed as n-fold change to the average of 0 ppm group. Y error bars are standard deviations of gene expression, and X error bars are standard deviations of DMA(V) intake.

Chapter 6   Arsenate and dimethylarsinic acid in drinking water did not affect DNA damage repair in urinary bladder transitional cells or micronuclei in bone marrow

(preparing for submission; presently being reviewed by co-authors)

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ABSTRACT

Arsenic is a recognized human skin, lung, and urinary bladder carcinogen, and may act as a cocarcinogen in the urinary bladder (with cigarette smoking) and skin (with UV light exposure). Possible modes of action of arsenic carcinogenesis/cocarcinogenesis include induction of DNA damage, inhibition of DNA damage repair, interference with cell cycle control, decreases in apoptosis, alteration in DNA methylation, and changes in bio-metabolism of other chemicals. Arsenic-inhibited DNA damage repair has been reported in cultured keratinocytes and pulmonary epithelial cells, mouse skin, and lymphocytes from arsenic-exposed people. We investigated the effects of arsenic in drinking water on DNA damage repair in urinary bladder transitional cells and on micronucleus formation in bone marrow. F344 rats were given 100 ppm arsenate [As(V)] or dimethylarsinic acid [DMA(V)] in drinking water for 1 week. The *in vivo* repair of a single oral gavage of cyclophosphamide-induced DNA damage, and the *in vitro* repair of *in vitro* hydrogen peroxide- or formaldehyde-induced DNA damage were measured by the Comet assay. DMA(V) did not affect cyclophosphamide-induced DNA damage. Neither DMA(V) nor As(V) inhibited the *in vitro* repair of hydrogen peroxide- or formaldehyde-induced DNA damage, and neither increased the micronucleus frequency or elevated the cyclophosphamide-increased micronucleus frequency. These results suggest arsenic carcinogenesis/cocarcinogenesis in the urinary bladder may not be via DNA damage repair inhibition. This is the first report of arsenic effects on DNA damage repair in the urinary bladder.

Key words: methylated arsenic, DNA damage repair, micronucleus, urinary bladder. inorganic arsenic

INTRODUCTION

Arsenic, a human carcinogen, is a widely distributed natural metalloid, and is used in man-made products such as herbicides. The primary arsenic exposure to the general population is through drinking water. Arsenic, namely arsenite [As(III)] and arsenate [As(V)], in drinking water increases incidences of cancers in lung (squamous and small cell carcinoma) (Guo et al., 2004), skin (Bowen's disease, basal and squamous cell carcinoma) (Guo et al., 2001; Centeno et al., 2002), and urinary bladder (transitional cell carcinoma) (Guo et al., 1997; Chiou et al., 2001).

The study of arsenic carcinogenesis is complicated. The metabolism of inorganic arsenic into various arsenicals, tissue-specific distribution of the arsenicals, and arsenical-dependent toxicities and mechanisms by which adverse effects are mediated are just a few factors that need to be considered (Mass et al., 2001; Schwerdtle et al., 2003b; Kligerman and Tennant, 2006; Wang et al., 2007b). For example, humans and rodents metabolize inorganic arsenic into monomethylarsonic acid [MMA(V)], monomethylarsonous acid [MMA(III)], dimethylarsinic acid [DMA(V)], and dimethylarsinous acid [DMA(III)]. In mice gavaged with As(V), DMA(V) concentrations were highest in the urinary bladder and lung while inorganic arsenic concentrations were highest in the kidney (Hughes et al., 2003). Trivalent arsenicals are more toxic than their pentavalent counterparts, with MMA(III) and DMA(III) being the most toxic. While humans exposed to inorganic arsenic have increased incidents of urinary bladder cancer, in laboratory animals, significantly increased urinary bladder cancer has only been observed in F344 rats exposed to DMA(V) (Wang et al., 2002).

Several modes of action of arsenic carcinogenesis have been proposed. They include (1) DNA damage repair inhibition, (2) oxidative stress induction, (3) signal transduction pathway modulation, (4) cell proliferation increases (altered growth factors and regenerative

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proliferation), (5) chromosomal abnormality induction, and (6) DNA methylation alteration (Kitchin, 2001; Yang and Frenkel, 2002; Schoen et al., 2004). These possible modes of actions are not mutually exclusive. For example, arsenic-induced oxidative stress can lead to chromosomal abnormalities (Yih and Lee, 1999; Ho et al., 2000). Furthermore, the mode of action of arsenic carcinogenesis is believed to be tissue-specific. For instance, the urinary bladder may have a different mode of action than other organs because of the mixture of arsenic metabolites in the urine (Rossman, 2003).

Because arsenic is a strong comutagen and cocarcinogen, it has long been hypothesized that arsenic interferes with DNA damage repair, and therefore enhances the mutagenicity and carcinogenicity of DNA-damaging agents. Arsenic-inhibited DNA damage repair has been reported in cultured skin and lung cells (Yamanaka et al., 1997; Wu et al., 2005), but arsenic effects on DNA repair have not been studied in urinary bladder cells. Decreases in DNA repair gene expression and DNA repair enzyme activities were observed in cultured cells exposed to noncytotoxic concentrations of arsenic (Andrew et al., 2003b; Hartwig et al., 2003; Clewell et al., 2006). Decreased DNA repair gene expression occurred concurrently with increased expression of oxidative stress response genes (Hamadeh et al., 2002), suggesting decreased DNA damage repair at the presence of oxidative stress and possibly oxidative DNA damage. Recently, decreases in DNA damage repair and DNA repair gene expression were observed in lymphocytes collected from arsenic-exposed humans (Andrew et al., 2003a; Andrew et al., 2006).

The view that decreased DNA damage repair may contribute to urinary bladder cancer risk is supported by the association between DNA repair gene polymorphism and bladder cancer risk (Chen et al., 2004; Sanyal et al., 2004; Garcia-Closas et al., 2006) and by decreases in DNA

repair in carcinogen-treated bladder cells compared to normal cells (Yoshimi et al., 1989). Also, decreased expression of DNA repair genes was associated with bladder cancer progression (Kawakami et al., 2004; Korabiowska et al., 2004). Arsenic-decreased DNA damage repair in urinary bladder cells could explain, at least partially, the higher bladder cancer risk in smokers than that in non-smokers in the arsenic-exposed population (Steinmaus et al., 2003; Karagas et al., 2004; Chen et al., 2005) because cigarettes contain mutagenic components, and the urine of smokers is more mutagenic than the urine of nonsmokers (Bowman et al., 2002).

Studies of F344 rats exposed to DMA(V) in diet or water (Arnold et al., 1999; Cohen et al., 2001; Cohen et al., 2002; Wei et al., 2002; Cohen et al., 2006) provide convincing evidence for the following mode of action of DMA(V)-induced bladder cancer (Sams II et al., 2007). First, DMA(V) is reduced to DMA(III), and DMA(III) causes cytotoxicity in the urothelium. Consequently, regenerative proliferation occurs and leads to hyperplasia and eventually bladder tumors. These events are plausible in humans, but do not exclude other modes of action, such as oxidative stress (Wei et al., 2005), chromosomal abnormalities (Moore et al., 2002), or DNA damage repair inhibition.

One of the purposes of this study was to investigate if one-week oral exposure to DMA(V) or As(V) inhibits DNA damage repair in urinary bladder transitional cells, a target cell of arsenic carcinogenesis. Three types of repair were studied: (1) *in vivo* repair of cyclophosphamide (CP)-induced DNA damage, which includes DNA strand breaks and DNA-DNA crosslinks, (2) *in vitro* repair of hydrogen peroxide (H_2O_2)-induced DNA damage, which is mainly oxidative DNA damage, and (3) *in vitro* repair of formaldehyde-induced DNA-protein crosslinks.

We also investigated whether *in vivo* exposure of DMA(V) or As(V) increases micronucleus (MN) frequencies in the bone marrow. A MN is the result of either chromosomal breakage or

chromosome malsegregation. Humans exposed to arsenic through drinking water showed increased MN frequencies in various tissues/cells, and MN have been suggested as a biomarker of arsenic exposure (Moore et al., 1997; Tian et al., 2001; Basu et al., 2004).

MATERIALS AND METHODS

Chemicals

Arsenic (IARC, 1987a, 2004), CP (IARC, 1987b), and formaldehyde (IARC, 2006) are human carcinogens. Beuthanasia and sodium hydroxide (NaOH) are also hazardous to human health and should be handled with care.

For animal treatment, DMA(V) [$(CH_3)_2As(O)OH$] (CAS no. 75-60-5O), purity > 99 %, was purchased from Sigma, St. Louis, Missouri. As(V) in the form of sodium hydrogen arsenate heptahydrate [$Na_2HAsO_4 \cdot 7H_2O$] (CAS no. 10048-95-0), purity > 98.5 %, was also from Sigma. CP (CAS no. 6055-19-2), purity > 98%, was purchased from MP Biomedicals, Aurora, Ohio. Isoflurane was from Abbott Animal Health, North Chicago, Illinois, and Beuthanasia was obtained from Schering-Plough Animal Health Corporation, Union, New Jersey.

For the Comet assay, William E medium, trypsin, ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), dimethyl sulfoxide (DMSO), NaOH, and Trizma base were purchased from Sigma, as was formaldehyde, 36.5-38%. Fetal bovine serum (FBS) was from Cambrex Bio Science Walkersville Inc., Walkersville, Maryland and Atlanta Biologicals, Lawrenceville, Georgia. H_2O_2 was from Fisher Scientific, Suwanee, Georgia. Proteinase K (Catalog no. 25530-049), L-glutamine (Gibco), and SYBR gold (Molecular Probe) were obtained from Invitrogen, Grand Island, New York. Comet LMAgarose (low melting temperature agarose) and Comet Lysis Solution were purchased from Trevigen, Gaithersburg, Maryland. Ethanol (200 proof ethyl alcohol) was from AAPER, Shelbyville, Kentucky.

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For the MN assay, FBS (Catalog no. BW14-501C) and methanol were from Fisher Scientific, and Na_2EDTA was from Sigma. Acridine orange (Molecular Probe) was obtained from Invitrogen.

Animals and animal care

Animal use and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee for Animals Used in Research and Testing, prior to the initiation of the study.

Female F344 rats, age 5-9 weeks old, were purchased from Harlan Teklad (Madison, Wisconsin). F344 rats are the only identified laboratory animals that develop urinary bladder transitional cell carcinoma from arsenic in drinking water (Wei et al., 2002) or diet (Arnold et al., 2006). Females were chosen because their urinary bladders are more susceptible to arsenic toxicity than males (van Gemert and Eldan, 1998; Arnold et al., 1999; Shen et al., 2006). All rats were quarantined and acclimated for at least 2 weeks. Polycarbonate shoebox-style cages with Alpha Dry bedding (Shepherd Specialty Papers, Watertown, Tennessee) were used to minimize dust, because both DMA(V) and CP metabolites were excreted in the urine (de Jonge et al., 2005; Shen et al., 2006). The rats were housed in a temperature controlled room with a 12-hr light/dark cycle, and single-housed at least one week before the beginning of *in vivo* dosing. Teklad 2018 SC diet (Harlan Teklad, Madison, Wisconsin) and tap water in plastic water bottles with stainless steel sipper tubes with stoppers were available *ad libitum*.

Animal treatments and experiment design

The experiment consisted of eight animal treatment groups, established to provide data to test three types of DNA damage repair, with six rats in each group, with the exception of the negative control groups, G with four rats and H with three rats (**Table 1**). The number of animals per

group was determined based on preliminary studies. Rats in Groups G and H (tap water and then 1 or 5 days of recovery), negative controls, were expected to have the same levels of DNA damage and were designed to be pooled for the *in vitro* repair study to minimize the number of animals. Animals were treated with a 7-day exposure via drinking water [tap water, 100 ppm DMA(V), or 100 ppm As(V)], a single oral gavage of CP (11.75 mg CP/kg BW, equivalent to 1/8 LD_{50}) at the end of 7-day exposure, and recovery (1 or 5 days of tap water). Previous studies showed that 1 week exposure to 100 ppm DMA(V) did not affect food consumption, water consumption, or body weight in F344 rats (Cohen et al., 2002; Wang et al., 2004). The dose of CP (1/8 LD_{50}) was determined by preliminary experiments to be non-cytotoxic to urinary bladder transitional cells; furthermore, CP-induced DNA damage in transitional cells was detected 24 h after the gavage of CP, and recovered within 5 days (unpublished data). Solutions of DMA(V) (100 ppm) and As(V) (100 ppm) were made fresh weekly with tap water to mimic human water sources (Huff et al., 1998), and stored at room temperature. The CP solution (23.5 mg/10 ml deionized water) was made fresh weekly and stored at 4 ºC in the dark to minimize degradation (Beijnen et al., 1992).

The experiment was performed in 6 blocks, based on our capacity for surgery and Comet assays. Each block included one rat from groups A to F and I, and one rat from either Group G (blocks 2, 4, 6) or H (blocks 1, 2, 3, 5), with the exception that block 2 included rats from groups G and H. Following quarantine and acclimation, rats were assigned to one of the six blocks by age, so all rats in a block were the same age. For blocks 2 and 3, as well as for blocks 4 and 5, rats were first assigned into blocks by weight stratification. Within each block, rats were randomly assigned to animal treatment groups.

To study DMA(V) effects on *in vivo* repair of CP-induced DNA damage, urinary bladder transitional cells were collected after animal treatments in all Groups and subjected to the alkaline Comet assay based on previously developed methods (Wang et al., 2007a). Groups G and H (tap water, 1 or 5 day recovery) were for background DNA damage, and Groups E and F (tap water + CP, 1 or 5 day recovery) provide DNA repair control data. Groups A and B [DMA(V) + CP, 1 or 5 day recovery] were our test groups. Groups C and D [DMA(V), 1 or 5 day recovery] and Group I [As(V), 1 day recovery] provided information on the genotoxicity of selected arsenic treatments.

To study the effects of DMA(V) and As(V) on *in vitro* repair of DNA damage, transitional cells were collected from rats exposed to DMA(V) or As(V) with 1 day recovery (Groups C and I, test groups) and rats exposed to tap water (Groups G and H, negative control). Collected transitional cells were treated with H_2O_2 or formaldehyde (*in vitro* dosing) to induce DNA damage, and DNA damage levels were measured immediately after *in vitro* dosing, or after 4 h of *in vitro* recovery. Hydrogen peroxide-induced DNA damage was measured by the standard alkaline Comet assay, and formaldehyde-induced DNA damage was measured by the alkaline Comet assay with and without Proteinase K for detecting DNA-protein crosslinks (Wang et al., 2007a).

To study DMA(V) and As(V) effects on MN frequencies, bone marrow was collected from rats exposed to DMA(V), As(V), or DMA(V)+CP after a 1-day recovery period (Groups C, I and A), to tap water (Groups G and H, negative control), and to tap water + CP with a 1-day recovery period (Group E, positive control). Comparing transitional cells from rats exposed to DMA(V) or As(V) (Groups C and I) to cells from rats exposed to tap water (Groups G and H) provided data on whether DMA(V) or As(V) in drinking water induced MN, while comparing transitional

cells from rats exposed to DMA(V) and DMA(V)+CP (Groups C and A) provided data on whether DMA(V) increased CP-induced MN.

Comet assay on transitional cells to detect DNA damage and repair

Methods for harvest of suitably viable urinary bladder transitional cells and detailed protocols for the Comet assay in these cells were previously developed (Wang et al., 2007a). Briefly, the bladder was rinsed, inflated with trypsin and Na_2EDTA, and incubated for 30 minutes prior to being gently scraped to remove transitional epithelium. The transitional cells were resuspended in William E medium supplemented with 10% FBS and 2 mM L-glutamine (W+S), centrifuged, and tested for cell viability by trypan blue exclusion assay.

Transitional cells collected from rats in Groups C, G, H, and I were divided into three aliquots. These were treated with either PBS (negative control), 100 mM H_2O_2, or 5 mM formaldehyde for 10 min on ice (*in vitro* dosing). Half the cells in each *in vitro* dosing were resuspended in W+S and incubated at 37 ºC for 4 hr to recover prior to being subjected to the Comet assay. Formaldehyde-treated and untreated cells were subjected to the alkaline Comet assays with and without Proteinase K. H_2O_2-treated and untreated cells were subjected to standard alkaline Comet assay. Transitional cells collected from rats in groups A, B, D, E, and F were treated with PBS for 10 min on ice and then subjected to the standard alkaline Comet assay. All samples were tested for cell viability before proceeding to Comet assay.

Cells were resuspended in LMAgarose at 37 ºC, and were loaded onto FLARE slides. For the standard alkaline Comet assay, the slides were lyzed for 1 hr, and unwound in alkaline solution (pH >13) for 30 min. Electrophoresis with alkaline solution was performed at 40 Volt (1 V/cm) and 300 mA for 30 min. For alkaline Comet assays with and without Proteinase K, the lysis was overnight, and slides were incubated with Proteinase K (1 mg/ml in TE buffer, pH 8.0)

or TE buffer for 2 hr at 37 ºC. The unwinding step was 20 minutes, and electrophoresis was at 32 Volt (0.8 V/cm) and 300 mA for 30 min.

After electrophoresis, slides were neutralized and dried. Slides were randomized, stained with SYBR gold, and scored by a person without the knowledge of treatments using image-analysis software, Komet 5.5 (Kinetic Imaging Ltd, Liverpool, UK). For each sample, 100 Comets were scored for each Comet assay condition. The percentage of DNA in the tail was chosen as the indicator of DNA migration. DNA-protein crosslinks were calculated by subtracting the percentage of DNA in the tail without Proteinase K from the percentage of DNA in the tail with Proteinase K.

Micronucleus assay on bone marrow

MN frequencies were measured in the bone marrow of rats exposed to water (Groups G and H) and all rats that had one day recovery time after chemical exposure (groups A, C, E, and I). The left femur was removed from the euthanized rat, and both ends (epiphyses) of the femur were removed. The bone marrow was flushed with FBS with 25 mM EDTA, which was added to prevent cell clumping. Cells were pelletized by centrifugation at 220 G for 5 min at room temperature and resuspended with approximately 1 ml FBS with 25 mM EDTA. On a glass slide, a bone marrow smear was made by spreading a 5 µl cell suspension behind a second slide. Three smears were made from each rat. The smears were air dried, fixed with 100% methanol for 10 min, and air dried again. Slides were coded, stained by acridine orange, and scored under a fluorescent microscope. Two slides from the same rat were examined by two people independently without the knowledge of treatments. Each person scored 1000 polychromatic erythrocytes (reticulocytes; immature erythrocytes) per slide for MN frequencies, and 2000

erythrocytes per slide for the percentage of polychromatic erythrocyte (polychromatic erythrocytes among the total erythrocyte population).

Statistical analysis

Results are expressed as the mean ± standard deviations, and $p < 0.05$ was considered significant. All data were tested using SAS 9.1.3 from SAS Institute Inc., Cary, NC, except the percentages of polychromatic erythrocytes, which were tested using Statgraphics[®] Plus 5.1 statistical package from Manugistics, Rockville, MD.

For testing DNA damage repair, DNA damage was expressed as percentages of DNA in the tail in Comet assays. For testing *in vivo* repair of CP-induced DNA damage, analysis of variance (ANOVA) and the Tukey post-hoc test were used on arcsine transformed percentages of DNA in the tail. For testing *in vitro* repair of H_2O_2-induced DNA damage ANOVA was used on untransformed percentages of DNA in the tail. For testing *in vitro* repair of formaldehyde-induced DNA damage, the arcsine transformed percentages of DNA in the tail were tested by ANOVA and contrast test.

For MN frequencies, ANOVA and the Tukey post-hoc test were used on arcsine transformed data. Percentages of polychromatic erythrocytes in bone marrow were subjected to ANOVA and Duncan's multiple comparison post test.

RESULTS

Cell viability of urinary bladder transitional cells

After the designated animal treatments (**Table 1**), transitional cells collected from all rats had higher than 60% viabilities as measured by the trypan blue exclusion assay, with the exception of cells from one rat each in Groups A (26% viability) and I (55% viability). The extreme low viability (26%) may have been due to insufficient trypsin and EDTA inflation during the cell

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collection process, and samples with less than 60% viability were excluded. Overall, the viability results suggested that the animal treatments were noncytotoxic to the bladder.

While transitional cells collected from rats in Groups A, B, D, E, and F were not further treated *in vitro*, cells collected from rats in Groups C, G, H, and I were treated with H_2O_2 or formaldehyde and then underwent 0 or 4 hours of 37 ºC incubation for recovery. Cells collected from Groups C, G, H or I showed higher than 70% viability after the H_2O_2 or formaldehyde treatments, indicating that these *in vitro* dosings were noncytotoxic. Furthermore, most cells remained higher than 70% viability after 4 hours of recovery, with only a few samples with 53% to 67% viability.

DNA damage repair in urinary bladder transitional cells

(1) *In vivo* CP-induced damage

Cells collected from all rats were tested by the standard alkaline Comet assay. DNA damage levels were estimated by DNA migration, expressed as percentages of DNA in the tail (**Fig 1**). None of the i*n vivo* dosing regimens significantly increased DNA migration after a 1-day *in vivo* recovery (Groups A, C, E, G, and I), indicating either that none of the *in vivo* exposures to DMA(V), DMA(V)+CP, H_2O+CP, or As(V) increased DNA damage or that the damage was repaired with 24 h. There was also no difference in the levels of DNA damage between 1-day and 5-day recoveries within each *in vivo* dosing, indicating either no delayed presence of DNA damage or a complete repair of DNA damage within 1 day.

(2) *In vitro* H_2O_2-induced damage

To test whether DMA(V) or As(V) affects oxidative DNA damage repair, cells collected from rats in Groups C, G, H, and I (*in vivo* dosing of DMA(V), As(V), or tap water) were tested for the repair of *in vitro* H_2O_2-induced DNA damage. Cells were treated with or without H_2O_2,

after which half the cells were allowed to recover in H_2O_2-free medium for 4 h before being subjected to the standard alkaline Comet assay (**Fig 2**).

To test whether H_2O_2 increased DNA damage and whether any *in vivo* dosing increased cellular sensitivity to H_2O_2 genotoxicity, DNA migrations from all samples with 0 h *in vitro* recovery (with or without H_2O_2 treatment) were tested by ANOVA. As expected, H_2O_2 significantly increased DNA damage. None of the 7-day *in vivo* exposures to DMA(V) or As(V) significantly affected the cellular sensitivity to H_2O_2 genotoxicity.

To test whether repair of H_2O_2-induced DNA damage was affected, DNA migrations from samples treated with H_2O_2 after 4 h *in vitro* recovery were subjected to ANOVA. There were no significant *in vivo* dosing effects, indicating DNA damage levels after 4 h of recovery were the same among H_2O_2–treated cells collected from rats exposed *in vivo* to DMA(V), As(V), or tap water. These data showed that repair of *in vitro* H_2O_2-induced DNA damage was not affected by 7-day *in vivo* exposures to DMA(V) or As(V).

(3) *In vitro* formaldehyde-induced damage

Cells collected from rats in Groups C, G, H, and I were also tested for repair of *in vitro* formaldehyde-induced DNA-protein crosslinks. Cells were treated with or without formaldehyde, after which half the cells were allowed to recover in formaldehyde-free medium for 4 h. Comet assays with and without Proteinase K were performed, and results are shown in **Fig 3**. As expected, the effects of formaldehyde treatment (*in vitro* dosing), *in vitro* recovery, and Proteinase K treatment were significant, indicating that these treatments affected the DNA migration. However, the effect of *in vivo* dosing was not statistically significant, indicating that neither As(V) nor DMA(V) affected the DNA migration.

To measure DNA-protein crosslinks, DNA migrations in Comet assays with and without Proteinase K were compared. In cells collected from rats exposed to tap water (Groups G and H), formaldehyde significantly decreased DNA migration when no Proteinase K was used, but not when Proteinase K was used at 0 h. This indicated that DNA-protein crosslinks were induced by formaldehyde. After 4 h of recovery, formaldehyde-treated cells from rats exposed to tap water did not have significant differences in the DNA migrations in Comet assays with and without Proteinase K, indicating the lack of DNA-protein crosslinks. Because DNA migration was also significantly decreased within 4 h when no Proteinase K was used, it can be inferred that the observed decrease in DNA-protein crosslinks was due at least partially to the repair of strand breaks, alkaline labile sites, or incomplete excision repair sites.

In the cells collected from rats exposed to As(V) or DMA(V), formaldehyde-induced decreases in DNA migration were not significant. Similarly, there were no significant differences between samples treated with and without Proteinase K at any time point, except in formaldehyde-treated cells from rats exposed to DMA(V) at 4 h recovery.

MN and polychromatic erythrocytes in bone marrow

The MN frequencies were increased in bone marrow collected from rats exposed to CP (Group E; positive control) or DMA(V)+CP (Group A), compared to these from rats exposed to tap water (Groups G and H; negative control) (**Fig 4**). The MN frequencies were not significantly different between samples from rats exposed to DMA(V)+ CP and CP (Groups A and E), indicating an absence of additive or synergetic effects between DMA(V) and CP on MN formation. MN frequencies in the bone marrow from rats exposed to DMA(V) or As(V) (Groups C and I) were not significantly different from those of rats exposed to tap water (Groups G and H). Both the exposures to CP (positive control) and DMA(V)+CP decreased erythrocyte

production, based on the significantly lower percentages of polychromatic erythrocytes in bone marrow collected from rats exposed to CP or DMA(V)+CP (Groups E and A), compared to the negative control (Groups G and H) (**Fig 5**). Neither DMA(V) nor As(V) affected erythrocyte production in rats.

DISCUSSION

Arsenic inhibition of DNA damage repair has been reported in various cell types, but it has not been previously studied in urinary bladder transitional cells, a major target of arsenic carcinogenesis/cocarcinogenesis. In this study, we measured arsenic effects on *in vivo* and *in vitro* exposures to DNA damaging agents in transitional cells freshly collected from rats. The use of newly harvested transitional cells provided several benefits in accurately assessing arsenic effects compared to studies using whole bladders or bladder cell lines. First, arsenic carcinogenesis is cell-type specific, so the use of only transitional cells avoids potentially misleading information from other types of cells in the bladder. Secondly, urothelial cell lines, such as HUC-1 and UROtsa cell lines, may have inactivated p53 protein due to transformation with SV40 large T antigen (Rossi et al., 2001; Su et al., 2006). Studies showed that cells without functional p53 proteins were more sensitive to arsenic and have different cellular responses than cells with functional p53 (Liu et al., 2003; Kircelli et al., 2007). The present study showed that urinary bladder transitional cells from rats exposed to DMA(V) through drinking water did not show altered cellular sensitivity to *in vivo* CP- or *in vitro* formaldehyde-induced DNA damage, or their repair of *in vitro* H_2O_2-induced DNA damage. Similarly, transitional cells from rats exposed to As(V) through drinking water did not show altered sensitivity to *in vitro* formaldehyde-induced DNA damage or the repair of *in vitro* H_2O_2-induced DNA damage.

Furthermore, neither DMA(V) nor As(V) exposure induced MN in the bone marrow, and DMA(V) did not increase CP-induced MN.

Various DNA damage repair pathways are responsible for the mending of specific types of DNA damage (Hartwig et al., 2002). Previously reported arsenic-inhibited DNA damage repair pathways include (1) base excision repair pathway (Le and Weinfeld, 2004), (2) nucleotide excision repair pathway (Tran et al., 2002; Schwerdtle et al., 2003a; Danaee et al., 2004; Wu et al., 2005; Andrew et al., 2006), and (3) non-homologous end joining or homologous recombination pathway. The mismatch repair pathway was probably not affected by arsenic because micro-satellite instability was not altered in DMA(V)-induced rat bladder transitional cell carcinoma (Wei et al., 2003). Our observation, in which neither DMA(V) nor As(V) affected the repair of H_2O_2-induced DNA damage, suggested that base excision repair was not affected by arsenic in the urinary bladder transitional cells.

Formaldehyde-induced DNA-protein crosslinks are believed to be removed partially by spontaneous hydrolysis (Quievryn and Zhitkovich, 2000) and by nucleotide excision repair, proteasome-assisted proteolysis followed by nucleotide excision repair, or homologue recombination repair with nucleotide excision repair components (Barker et al., 2005). The enzymes involved in removing formaldehyde-induced DNA-protein crosslinks include excision repair cross complementing group 1 (ERCC1), xeroderma pigmentosum group A (XPA), XPF, XPG (all in the nucleotide excision repair pathway), and proteasome (Barker et al., 2005). Although both DMA(V) and its metabolite, DMA(III), decreased nucleotide excision repair in cultured human lung cells (Schwerdtle et al., 2003a), DMA(V) exposure through drinking water did not affect the repair or formation of formaldehyde-induced DNA-protein crosslinks in our study using urinary bladder transitional cells.

The general mechanisms of arsenic-induced DNA damage repair inhibition have been investigated by several groups. Possible mechanisms included direct inhibition on repair enzymes (through binding to the enzymes or releasing zinc from the zinc finger structures of the enzymes), decreases in DNA repair enzyme and cell cycle control gene expression, alterations in signal pathways of DNA repair, and post-translational modification of repair enzymes (Li and Rossman, 1989; Yager and Wiencke, 1997; Wang et al., 2001; Huang et al., 2004; Kitchin and Wallace, 2005; Witkiewicz-Kucharczyk and Bal, 2006; Klein et al., 2007; Pu et al., 2007). The fidelity of DNA damage repair, measured by the accuracy of new DNA synthesis, was not affected by arsenic (Tkeshelashvili et al., 1980). Arsenic-generated reactive oxygen and nitrogen species may also lead to DNA damage repair inhibition (Mei et al., 2002; Gurr et al., 2003; Chien et al., 2004).

Arsenic, including DMA(V), has been reported to induce DNA damage. While MMA(III) and DMA(III) are highly genotoxic and can directly and indirectly (through oxidative stress) damage DNA, other forms of arsenic are not very genotoxic and may only indirectly damage DNA through arsenic-induced oxidative stress (Mass et al., 2001; Kligerman et al., 2003; Soto-Reyes et al., 2005). In rats and mice, DNA single strand breaks were increased in the lung after an oral gavage of DMA(V) at a higher than LD_{50} dose, and the DNA damage was repaired within 24 hours after the gavage (Yamanaka et al., 1989). In cultured bladder cells, As(V) and DMA(V) were the least potent in inducing DNA damage among 6 trivalent and pentavalent arsenicals (Wang et al., 2007b). In the present study, urinary bladder transitional cells collected from rats 1 day after 1-week exposure to DMA(V) or As(V) did not show increases in DNA damage. In studies by Cohen et al. (2001, 2002), increased necrosis and exfoliation in the urothelium were observed under scanning electron microscope after 1-week exposure to 100

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ppm DMA(V), and necrotic and exfoliated (severely damaged or dead) cells are expected to have more DNA damage than healthy cells. It is plausible that our data reflected either a recovery from DMA(V)-induced DNA damage within 24 hours or the result of excluding exfoliated cells in the urine. Because the goal of this study was to investigate whether arsenic inhibits DNA damage repair as a mode of action of arsenic carcinogenesis/cocarcinogenesis, we focused on the cells that have potential to develop into cancer, and therefore excluded the exfoliated cells in the urine.

The effects of arsenic on the MN frequency depend on the arsenic species. Inorganic arsenic in drinking water [a mixture of As(III) and As(V)] increased MN frequencies in various tissues/cells of humans (Tian et al., 2001; Basu et al., 2004). As(III) increased bone marrow MN frequencies in mice and rats (Patlolla and Tchounwou, 2005; Lewinska et al., 2007), but As(V) did not so do in mice (Palus et al., 2006). The effects of DMA(V) on MN were only previously studied *in vitro*, and DMA(V) did not increase MN frequency until highly cytotoxic concentrations were reached (Noda et al., 2002; Seoane et al., 2002; Dopp et al., 2004; Colognato et al., 2007). Our finding, in which neither As(V) nor DMA(V) exposure through drinking water increased MN frequency in rat bone marrow, is consistent with previous reports. The arsenic species that directly induce MN are not clear. Dopp et al (2004) showed that MN were induced *in vitro* by DMA(III) and MMA(III), but not by inorganic arsenic, MMA(V), or DMA(V). However, injections of dimethylarsinous iodide, which is presumed to form DMA(III) by hydrolysis in aqueous solution, did not increase MN in mouse peripheral blood (Kato et al., 2003). Additionally, while neither DMA(V) nor dimethylarsinous iodide injections increased MN frequencies, MN frequencies were increased by co-injections of either (1) DMA(V) and reduced glutathione (GSH) or (2) dimethylarsinous iodide and GSH (Kato et al., 2003). Because

dimethylarsine was observed when DMA(V) and GSH were present, the authors suggested free radicals generated from the reaction of dimethylarsine with molecular oxygen, and not DMA(III) itself, may be the direct cause of MN.

As(III) was reported to further increase MN induced by benzo(a)pyrene (Lewinska et al., 2007) in mice. The possible mechanism of this increase may be the generation of oxidative stress and inhibition of DNA repair (McDorman et al., 2002; Lewinska et al., 2007). Arsenic may also increase or decrease the metabolism of benzo(a)pyrene (Kann et al., 2005; Noreault et al., 2005) and consequently the levels of benzo(a)pyrene active metabolites and induced DNA damage (Perez et al., 2003). Although DMA(V) could increase cytochrome P450 (Brown et al., 1997), which could in turn increase metabolism of CP to more toxic metabolites (Hengstler et al., 1997), DMA(V) exposure through drinking water did not increase CP-induced bone marrow MN in our study.

In summary, F344 rats exposed to 100 pm DMA(V) or As(V) in the drinking water for 1 week did not show alterations in the sensitivity to CP- or formaldehyde-induced DNA damage, in the repair of H_2O_2-induced DNA damage in the urinary bladder transitional cells. Neither DMA(V) nor As(V) exposure increased MN frequencies in the bone marrow, and DMA(V) did not increase CP-induced MN. Other mechanisms, such as oxidative stress, increases in cell proliferation, and alteration in metabolism of other chemicals, may contribute to arsenic carcinogenesis/cocarcinogenesis in the urinary bladder.

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Table 1. Study design. All rats were given tap water, 100 ppm DMA(V), or 100 ppm As(V) in drinking water for 7 days. At the end of 7-day treatment, certain rats received a single oral gavage of CP. Rats were given tap water during the recovery period. Cells collected from rats in Groups A, B, D, E, and F received no additional treatment. Portions of cells collected from rats in Groups C, G, H and I were treated with H_2O_2 or formaldehyde for 10 minutes on ice, and then some cells were allowed to recover in medium without H_2O_2 or formaldehyde at 37 ºC for 4 h.

	For *in vivo* repair		For *in vitro* repair		For MN
Group	*In Vivo* Dosing	*In Vivo* Recovery	*In Vitro* Dosing	*In Vitro* Recovery	Bone Marrow
A	DMA(V) + CP	1 day	--	--	MN
B	DMA(V) + CP	5 days	--	--	--
C	DMA(V)	1 day	H_2O_2, Formaldehyde	0 h, 4 h	MN
D	DMA(V)	5 days	--	--	--
E	Tap water + CP	1 day	--	--	MN
F	Tap water + CP	5 days	--	--	--
G	Tap water	1 day	H_2O_2, Formaldehyde	0 h, 4 h	MN
H	Tap water	5 days	H_2O_2, Formaldehyde	0 h, 4 h	MN
I	As(V)	1 day	H_2O_2, Formaldehyde	0 h, 4 h	MN
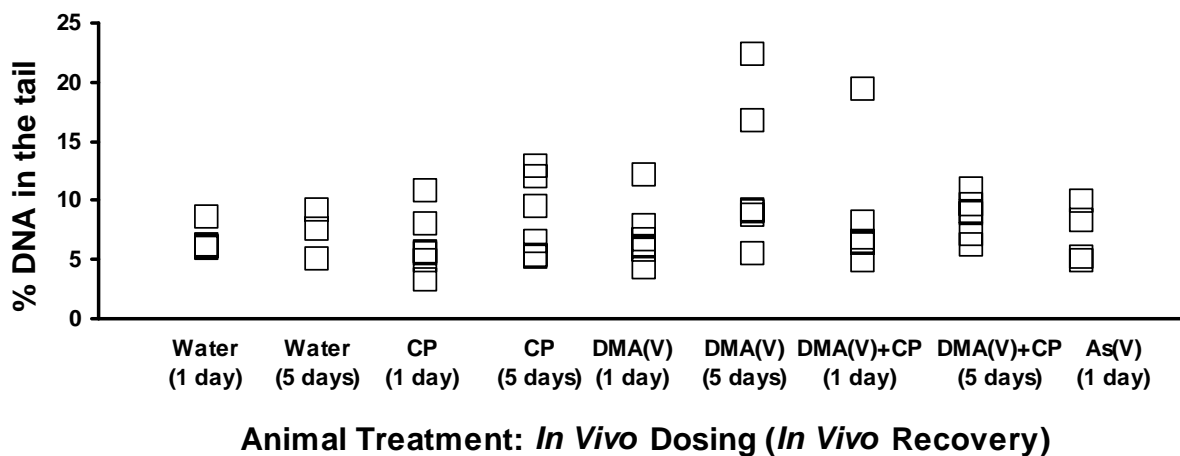
Fig 1. DNA damage levels in the urinary bladder transitional cells were not increased by animal treatments, and DNA damage levels were the same after 1 day and 5 day recovery. Each square indicates the mean percentage of DNA in the tail from 100 comets of one rat.
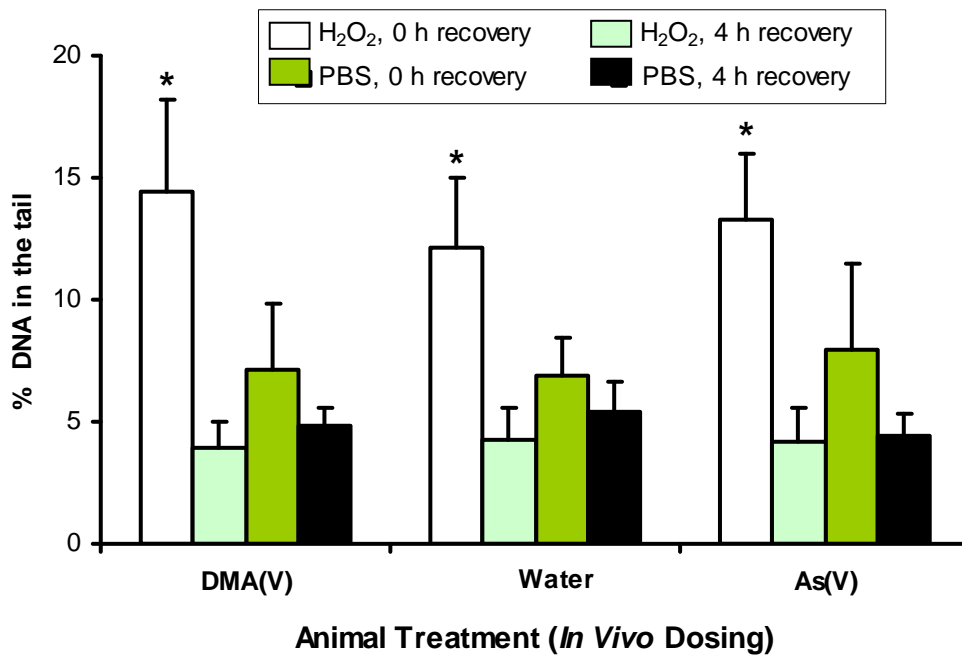
Fig 2. The repair of *in vitro* H_2O_2-induced DNA damage was not affected by *in vivo* DMA(V) or As(V) exposure. Urinary bladder transitional cells from rats exposed to 100 ppm DMA(V) (Group C), 100 ppm As(V) (Group I) in drinking water for 7 days had the same levels of DNA damage as cells from rats exposed to tap water (Groups G and H), as measured both before and after *in vitro* H_2O_2 treatment. * indicates significant difference from PBS-treated cells at the same recovery time.

(a)

0 h Recvoery

(b)
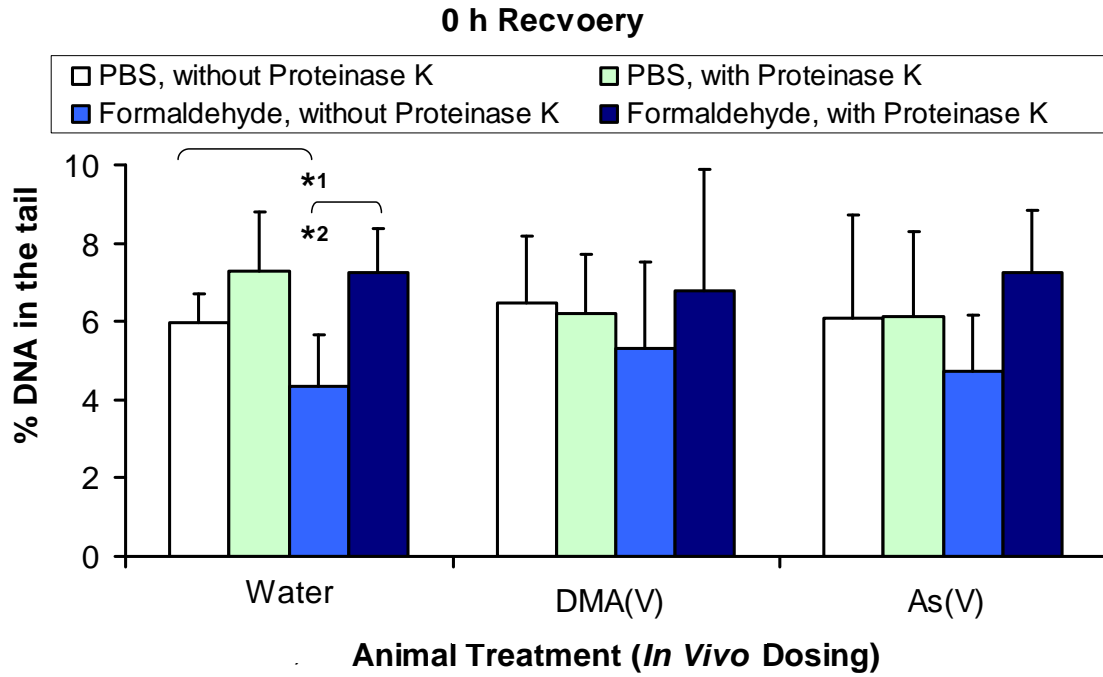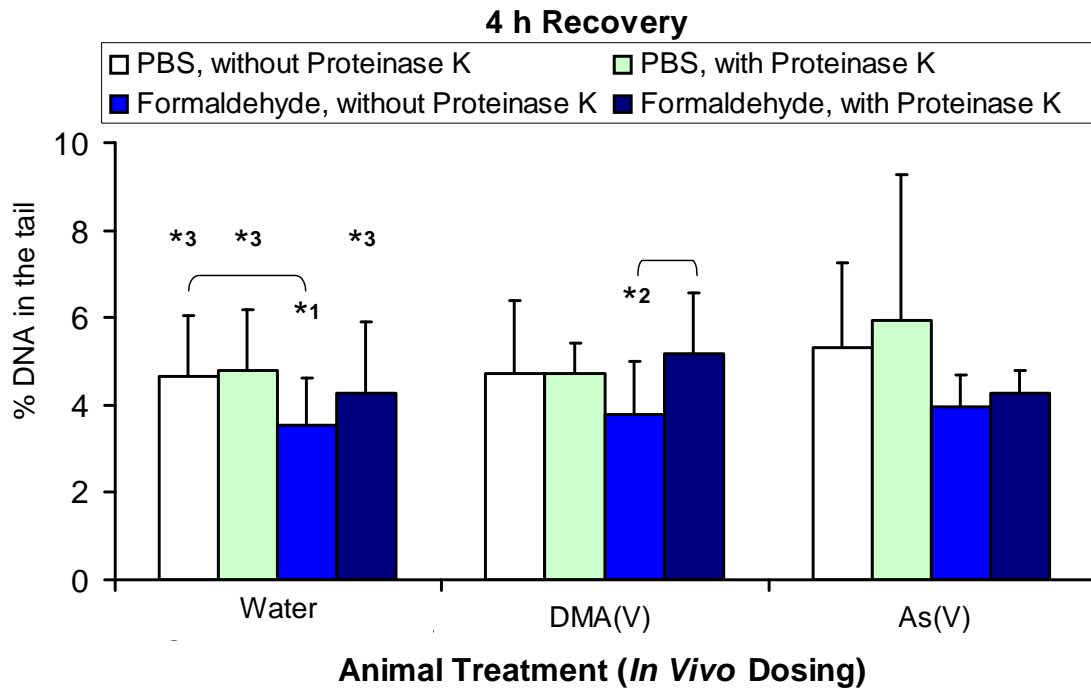
4 h Recovery

Fig 3. DNA migration of formaldehyde-treated and control cells after 0 hour (**a**) or 4 hours (**b**) of recovery. The animal treatments were the same as in Fig 2. *1 indicates significant difference from PBS-treated cells (same Comet assay, at the same recovery time). *2 indicates significant difference from Comet assays with Proteinase K (same sample, at the same recovery time). *3 indicates significant difference from the same sample at the same Comet assay test at 0 h recovery.
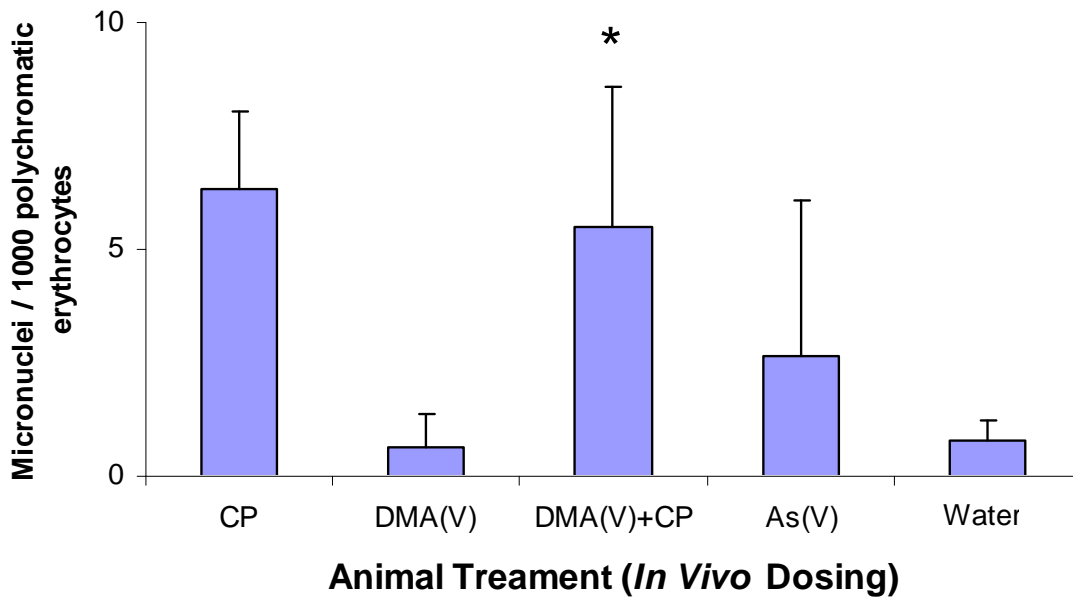
Fig 4. The frequencies of MN were not affected by DMA(V) or As(V). CP (positive control) and DMA(V)+CP exposure (Groups E and A) significantly increased MN frequencies compared to tap water exposure (Groups G and H). The MN frequencies were not significantly different among rats exposed to CP and DMA(V)+CP, or among rats exposed to DMA(V), As(V) and tap water (Groups C, I, and G and H). * indicates significant difference from rats exposed to tap water.
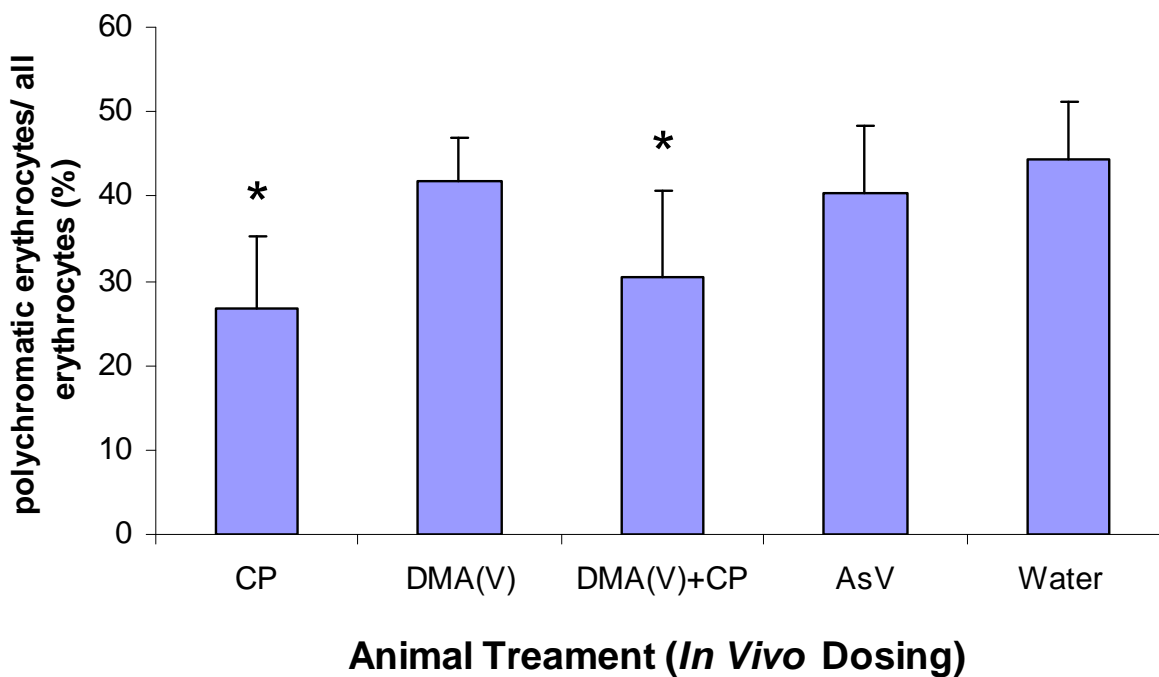
Fig 5. The percentages of polychromatic erythrocytes were not affected by DMA(V) or As(V). CP (positive control) and DMA(V)+CP exposure (Groups E and A) significantly decreased polychromatic erythrocyte percentages compared to tap water exposure (Groups G and H), and the polychromatic erythrocyte percentages were not significantly different between CP and DMA(V)+CP exposures. The polychromatic erythrocyte percentages were not significantly different among rats exposed to DMA(V), As(V) and tap water (Groups C, I, and G and H). * indicates significant difference from rats exposed to tap water

Chapter 7   Summary

Arsenic in drinking water increases the risk of skin cancers, lung cancers, and urinary bladder transitional cell carcinoma, particularly in smokers. One of the possible modes of action of arsenic carcinogenesis and cocarcinogenesis (with UV light and cigarette smoking) is inhibition of DNA damage repair, which has been extensively studied in inorganic arsenic-exposed human keratinocyte cultures and reported in lymphocytes of arsenic-exposed humans. Dimethylarsinic acid (DMA(V)) is the main detected arsenic metabolite in the urine of humans exposed to arsenic in the drinking water, and the only form of arsenic that induces bladder cancer in laboratory animals, namely F344 rats.   Using F344 rats exposed to DMA(V) we conducted studies to investigate arsenic effects on DNA repair in urinary bladder transitional cells.

The development of methods to specifically study transitional cells from rats is described in Chapters 3 and 4 of this dissertation. It is important to study transitional cells *in vivo* or in freshly collected cells, because arsenic carcinogenetic effects are cell type specific, and bladder cell lines may have inactivated p53 gene and therefore respond to arsenic differently as compared to cells with functional p53 gene. A modified Trizol method was developed to ensure only transitional cells were lysed for RNA extraction, and resultant real time RT PCR and microarray results reflect transitional cell gene expression. An enzymatic stripping method was developed to selectively harvest transitional cells suitable for Comet assay. Transitional cells harvested by the presented method retained their DNA repair capacity. Furthermore, cells harvested from each rat bladder were sufficient for at least six Comet assays by protocols developed in our laboratory, and routinely used for 12 Comet assays or more (as described in Chapter 6).

Chapter 5 describes the test of the hypothesis that arsenic decreases the expression of DNA repair genes in the bladder. The mRNA levels of DNA repair genes were measured in the transitional cells of F344 rats exposed to up to 100 pm dimethylarsinic acid (DMA(V)) in drinking water for 4 weeks. Real time RT PCR results showed that mRNA levels of DNA repair genes involved in excision repair or double strand break repair [Ataxia Telangectasia mutant (ATM), X-ray repair cross-complementing group 1 (XRCC1), excision repair cross-complementing group 3/xeroderma pigmentosum B (ERCC3/XPB), and DNA polymerase β (Polβ)] were not changed by the DMA(V) exposures. These results suggested that either DMA(V) does not affect DNA repair in the bladder or DMA(V) affects DNA repair without affecting baseline mRNA levels of repair genes in the bladder. For example, arsenic may lower damage-induced increases in repair gene expression or affect repair enzymes.

The DNA damage repair capacity in transitional cells of rats exposed to DMA(V) or arsenate (As(V)) is reported in Chapter 6. Exposures to DMA(V) did not alter transitional cells sensitivity to *in vivo* cyclophosphamide- or *in vitro* formaldehyde-induced DNA damage, or the repair of *in vitro* H_2O_2-induced DNA damage. Exposures to As(V) also did not affect the cellular sensitivity to *in vitro* formaldehyde-induced DNA damage or the repair of *in vitro* H_2O_2-induced DNA damage. Additionally, neither DMA(V) nor As(V) exposure induced micronucleus frequency in the bone marrow, and DMA(V) did not increase cyclophosphamide-induced micronucleus frequency. These data suggested that DNA damage repair in the urinary bladder was not affected by exposures to DMA(V) or arsenate.

While our experimental data did not provide evidence that supports the hypothesis of DMA(V) or arsenate affecting DNA damage repair in urinary bladder transitional cells, the results should not be interpreted as disproving that arsenic affects DNA repair in transitional

cells. In the experiments of arsenic effects on *in vitro* repair of DNA damage, cells were collected from rats that had one day recovery time. Although DMA(III) bound to hemoglobin would not be cleared from the body within 24 hours, the excretion of arsenic from urine may be minimal at 24 hours after the cessation of arsenic exposure. In other words, transitional cells collected from rats 1 day after the cessation of arsenic exposure may have recovered from arsenic effects to some degree.

In further studies of arsenic effects on DNA damage repair in transitional cells, the following experiments/considerations would provide valuable information. (1) The metabolism of the DNA damaging agent may be affected by arsenic. Consequently, the calculation of DNA repair capacity would be more accurately based on both the levels of active DNA damaging agent(s) and DNA damage in the target cells than that based on DNA damage levels alone. (2) Long-term exposure to arsenic may have a different response from short-term exposure. It would be informative to study cells/animals exposed to both low carcinogenic concentrations and non-carcinogenic concentration for months. (3) Cigarette smoking and arsenic exposure have more than addictive effects on increasing urinary bladder cancer. Using an inhalation study (which is not available at our facility) with rats exposed to arsenic in drinking water may shed light on the mode of action of arsenic-associated bladder cancer.

In summary, this dissertation reported the first studies of arsenic effects on the DNA damage repair in the urinary bladder, a target of arsenic carcinogenesis and cocarcinogenesis. Present studies showed that transitional cell mRNA levels of DNA repair genes involved in excision repair or double strand break repair (ATM, ERCC3/XPB, Polβ, and XRCC1) were not altered by exposures to DMA(V) in drinking water. Exposures to DMA(V) or As(V) in drinking water also did not affect the sensitivity or the repair of *in vivo* cyclophosphamide- or *in vitro*

formaldehyde- or H_2O_2-induced DNA damage in transitional cells. Current evidence does not support arsenic inhibition on DNA repair as a mode of action of arsenic carcinogensis or cocarcinogensis in the urinary bladder. Other mechanisms, such as oxidative stress, increases in cell proliferation, and alterations in the metabolism of other chemicals, may contribute to arsenic carcinogenesis and cocarcinogenesis in the urinary bladder.

Appendix 1   Gene expression profiling of responses to dimethylarsinic acid in female F344 rat urothelium.

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The present study is one of the collaborative projects of Amy Wang's dissertation experiment II with F344 rats exposed to dimethylarsinic acid in drinking water for 4 weeks. Amy designed the animal study, treated rats, collected exposure data and tissues, and extracted RNA from most bladder and some kidney samples. The bladder RNA was first used for real time RT PCR experiment (reported in Chapter 5) and the rest was for microarray test. Dr. Sen performed the microarray part and analyzed the data. Amy performed a morphology study (light microscopy, SEM and TEM) on separate bladders (reported here and in Chapter 5).

ABSTRACT

Gene expression profiling has been shown to be useful for identifying underlying mechanisms of toxicity, determining patterns of biological response, and elucidating candidate markers of exposure and response. Inorganic arsenic (iAs) is a human carcinogen and epidemiologic evidence implicates it in the development of urinary bladder cancer. Dimethylarsinic acid (DMA), the major excreted metabolite of iAs in humans, is a known rat bladder carcinogen. To examine the changes associated with DMA exposure, microarray analysis of the urothelium was performed in female F344 rats exposed to non-toxic and toxic doses of DMA in their drinking water for 28 days. A novel method for isolating predominantly urothelial cells was developed. Gene expression profiling of the urothelium using a custom 2-dye spotted array revealed that DMA treatment modulated the expression of transcripts of genes that regulate apoptosis, cell cycle regulation and the oxidative stress response. Expression of genes mapping to pathways involved in cancer control processes were also altered after DMA exposure. Morphological data suggested a dose dependent increase in cellular toxicity. Significant changes in differential gene expression were present after all treatments event at doses where standard toxicological responses were not detectable. The greatest perturbation in gene expression was present in rats after treatment with 40 ppm DMA. Doses which produced no histologic or ultrastructural evidence of toxicity (non-toxic) could be differentiated from toxic doses based on the expression of a subset of genes, which control cell signaling and the stress response. These reported changes in gene expression show similarities between the mechanisms of action of DMA in vivo and those previously described for iAs in vitro. These data illustrate the utility of transcriptional profiling and its potential in predicting key mechanistic pathways

involved in toxicity and as a time efficient tool to inform the mode of action analysis in risk assessment.

1. INTRODUCTION

Epidemiologic studies have shown an association between consumption of inorganic arsenic (iAs) throughdrinking water and an increased risk of bladder cancer (NRC, 1999). The complete mechanism by which iAs induces cancer remains unknown, partly because the animal models for iAs carcinogenesis have been limited. However, the major metabolite of iAs in humans and rodents, dimethylarsinic acid (DMA) has been demonstrated to be a bladder carcinogen in rats. There have been many more studies investigating the toxicity and metabolism of iAs, than the organic forms of arsenic. Once viewed simply as a detoxification product of iAs, evidence now indicates that DMA can produce toxicity in multiple organs (Wanibuchi et al., 1996; Wei et al., 1999, 2002). Chronic exposure to DMA in drinking water (50 or 200 ppm) or diet (100 ppm) causes a dose dependent increase in urinary bladder tumors of rats (Wei et al., 1999; Cohen et al., 2002). Mechanistic studies in vivo suggest that DMA(III) a metabolite of DMA, rather than DMA itself, plays a significant role in urothelial cytotoxicity and regeneration (Cohen et al., 2002). In vitro studies investigating the mode of action of DMA suggest that its metabolites, DMA(III) and possibly dimethylarsine, are the active compounds and that these metabolites can produce genotoxic effects (Andrewes et al., 2003; Kligerman et al., 2003; Kenyon and Hughes, 2001; Nesnow et al., 2002). Multiple studies have provided evidence that DMA itself does not react with DNA, rather its toxicity and eventual carcinogenic potential are manifested in association with an increased proliferative effect due to regenerative hyperplasia following toxicity and necrosis (Arnold et al., 1999).

The urinary bladder is one of the major human target organs for iAs induced cancer. The metabolites of ingested iAs (e.g. MMA(V), DMA and other organic arsenicals) are present in

high concentration in urine and persistently exposed to the urothelium. The normal bladder urothelium is a stratified transitional epithelium composed of the basal, intermediate and superficial cell layers. The urothelial cells exhibit a tendency for neoplastic transformation and are the cells of origin for transitional cell carcinoma, the most common form of bladder cancer (Southgate et al., 1999). DMA induced urothelial toxicity is marked by vacuolization of the epithelium, which can progress to necrosis and ulceration (Cohen, 1989).

Gene expression analysis using total RNA of bulk tissue cannot assign specific messages to particular cell types. Differentiating the target cell population from a background heterogeneous cell population is critical in understanding the specific molecular events that take place during a xenobiotic response in a particular target cell. In the present study, a novel approach to examine gene expression profiling of the target cell population is used to discern the molecular events that take place in the rat bladder following sub-chronic exposure to DMA in the drinking water. Gene expression profiling has been used previously to reveal indicators of subtle injury induced by a low dose of a substance that does not cause overt toxicity as defined by conventional criteria of toxicology (e.g., abnormal clinical chemistry and histopathology) (Heinloth et al., 2004). With this in mind, we chose exposure doses of 1 and 4 ppm of DMA, which have previously not been tested in vivo. Short-term exposure of DMA in the rat results in toxicity of the bladder, with eventual formation of bladder tumors in a 2-year bioassay. The higher doses of 40 and 100 ppm of DMA have been shown to cause toxicity of the bladder in vivo in the male rat (exposure through drinking water) and in the female rat (exposure through diet at comparable daily doses) (Wei et al., 1999; Cohen et al., 2002). We discuss our results in the context of a toxic response as elicited by DMA in the urothelium following a 28-day exposure.

2. MATERIALS AND METHODS

2.1. Chemicals

Dimethylarsinic acid was purchased from Electron Microscopy Science, Fort Washington, Pennsylvania. The chemical form was sodium cacodylate-trihydrate [$(CH_3)2AsO_2Na·3H_2O$], and had a purity of 99.52%, with inorganic As at 0.0030%. (Note: the use of the sodium salt is different from previously reported studies, which used DMA.)

2.2. Animal treatment and sample collection

Female 9-week-old F344 rats were purchased from Harlan, Madison, WI, USA. Rats were housed singly in polycarbonate shoebox-style cages with paper bedding. The temperature and humidity were monitored continuously and a 12 h light–dark cyclewas maintained. Teklad 2018 SC diet and water in plastic water bottles with stainless steel sipper tubes and rubber stoppers were available ad libitum. Following a 1-week acclimation period, during which time the rats were not administered DMA, the rats received DMA at concentrations of 0, 1, 4, 40 and 100 ppm (0, 0.1, 0.4, 4.7, 13 mg/kg/day, calculated from the nominal concentrations) in the drinking water for 28 days. Rats were randomly assigned in to the five groups by body weight, each group consisting of six rats. The drinking water solutions were changed three times per week and the water consumption was measured at each water change. All rats survived the 4-week treatment without signs of over toxicity. Rats were euthanized by CO_2 asphyxiation. The bladders were removed immediately after death and processed for mRNA extraction or morphology studies.

2.3. RNA preparation, labeling and hybridization

After 28 days of treatment, the rats were euthanized by CO_2 asphyxiation and the abdominal wall was incised to expose the urinary bladder. The bladders were removed within 2 min after the

animal died. Following the removal of the bladder, all the subsequent steps were carried out

rapidly without any interruptions to minimize autolysis. The procedure was adapted from Hester

et al. (2002). The bladder was tied off adjacent to the trigone and removed. Urine, if present, was

withdrawn from the bladder and the bladders were flushed with 1ml of ice cold RNAse free PBS

to remove exfoliated cells in the urine and bladder surface. Following the removal of the rinse

solution, 1ml of ice-cold Trizol (Invitrogen, Carlsbad, CA, USA) solution was injected into the

urinary bladder and the bladder was allowed to incubate for 10 min. The Trizol/cell lysate

solution was aspirated from the bladder and placed into a microfuge tube for flash freezing in

liquid nitrogen and storage at −70 ◦C. The urothelial layer was effectively removed to the

basement membrane using this protocol (Fig. 1). Total RNA was isolated from the thawed

Trizol/cell lysate solution according to maufacturer's instructions. The purity and concentration

of the RNA was measured on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA,

USA). The RNA isolated from the control animals was not sufficient to generate enough labeled

transcript to perform the competitive hybridization using the two dye labeling protocol for four

animals per dose group (i.e. 16 animals). Therefore the RNA from six control animals was

pooled, amplified and labeled with Cy3 dUTP. To maintain uniformity, the RNA from all

bladders of individual F344 treated rats (four animals per dose group) were individually

amplified and labeled with Cy5 dUTP. The protocol included synthesis of first and second strand

cDNA starting with 10 μg total RNA using Ambion's MessageAmpTM aRNAkit (Austin, TX,

USA). The cDNA was next subjected to in vitro transcription (IVT) with aminoallyl labeled

dUTP (Amersham Bioscience, Palo Alto, CA, USA). The modified aminoallyl labeled aRNA

was coupled to modified Cy3 and Cy5 dyes. The samples were fragmented and applied to the

glass microarray slide overnight at 42 ◦C. The custom printed microarrays containing 4395

70mer rat oligo probes (Qiagen, Valencia, CA, USA) were printed on contract by the Duke

Center for Genome Technology, DNA Microarray Center. Following hybridization, the slides

werewashed, dried by centrifugation, and scanned soon after on an Axon® scanner (Axon

Instruments, Union City, CA, USA).

2.4. Analysis of fluorescence and data analysis

Each hybridization was scanned using the GenePix 4000B microarray scanner (Axon

instruments, Union City, CA, USA). Array features were identified and assigned background

corrected red and green fluorescence intensity values using GenePixPro® v4. image analysis

software using the default settings. Normalization to correct for experimental variation was

performed using the GeneSpring software v5.2 (Silicon Genetics, Redwood City, CA, USA).

Only genes that were present in all samples were further analyzed. Genes that showed significant

differential expression across all dose groups upon treatment with DMA were filtered by analysis

of variance (p-value <0.01). To reduce the number of false positives, multiple testing correction

using the Benjamini Hochberg test was applied. Clustering of data was used to create heat map,

similarity was measured by standard correlation. The biologically relevant networks perturbed

following DMA exposure were investigated using the Ingenuity® software (Ingenuity, Mountain

View, CA, USA).

2.5. Morphology

A separate set of animals exposed to the same conditions were used for morphologic analysis of

the urinary bladder. For morphological analysis, each bladder was cut into two halves

longitudinally. One half was fixed in 10% neutral buffered formalin and processed by routine

methods for examination by light and the other half fixed in glutaraldehyde and processed by routine methods for transmission electron microscopy.

3. RESULTS

The choice of female F344 rats was based on their greater sensitivity to DMA-induced toxicity of the urothelium compared to males and their lack of a seminal coagulum, which is a frequent occurrence in male rat urinary bladder (Cohen et al., 2001, 2002; Arnold et al., 1999). Previous studies testing DMA toxicity in drinking water were performed on male rats (Wei et al., 1999). The calculated mean daily consumption of DMA was 0, 0.1, 0.4, 4.7, 13 mg/kg/day, or 0, 1, 4, 40 and 100 ppm, respectively, were based on the nominal concentration and measured daily water consumption (data not shown) in the present study and was comparable to previous studies (Wei et al., 1999). Doses of 40 and 100 ppm were chosen to represent cytotoxic doses of DMA to the urothelium. The doses of 1 and 4 ppm were chosen to represent non-toxic exposure in rats and are known not to cause tumors or preneoplastic lesions in bioassays. Since transcriptional changes can be sensitive to xenobiotic exposure even at low dose, we anticipated that we would identify changes more suitable for extrapolation to relevant human doses. In order to better describe the transcriptional changes in the target cells a novel method was developed to isolate the urothelial cell layer from the urinary bladder (adapted from Hester et al., 2002). The presence of the intact basement membrane was evidence that the Trizol treatment was successful in removal of the majority of the urothelium (Fig. 1). Total RNA isolated from the bladders using this protocol ranged between 20 and 70μg per bladder.

3.1. Morphology

A dose dependent increase in cytoplasmic vacuolation and hyperchromasia of the urotheliumwas observed (Fig. 2). No necrosis was visible under light microscopy. Most changes were seen in the superficial cells with intermediate cells being mildly affected and no evident morphologic changes to the basal cells. By light and transmission electron microscopy, no alterations were detected after treatment with 1 or 4 ppm for 28 days. After 40 and 100 ppm treatment, urothelial cells showed signs of cell swelling, appearance of cytoplasmic vacuoles and a decreased number of mitochondria. These changes were more pronounced after 100 than 40 ppm DMA. In summary, doses of 1 and 4 ppm of DMA produced no morphologic evidence of toxicity, while 40 and 100 ppm did.

3.2. Gene expression changes of DMA treated urothelium

Two-color microarray gene expression profiling was used to examine the transcriptional effects of DMA on the urothelium. Following normalization of the gene expression data, statistically significant genes that changed expression in each treatment group were identified. Five hundred and ten genes were significantly (ANOVA, $p < 0.01$) altered upon exposure to DMA. Of these, 38% (196 transcripts) were up regulated and 9% (47 transcripts) were down regulated by three-fold or greater. Clustering of this subset of genes showed a distinct treatment related response to DMA, which were present for all treatments including 1 ppm group (Fig. 3). The majority of the gene alterations were associated with the functional categories of apoptosis, cell cycle regulation, adhesion, growth factor and hormone receptors, signal transduction, and stress response (Table 1). Transcripts coding for proteins such as thioredoxin (Txn), thioredoxin peroxidase (Prdx1), glutathione *S*transferase (Gst), glutaredoxin (Glrx1) and glutathione peroxidase (Gpx1), which are modulated in response to increased levels of reactive oxygen species, had increased

expression in DMA treated rats. An increase was observed in the expression of metallothionein 1 (mt1), a metal binding protein that is induced by several heavy metals and stress as a response to toxicity. Increased expression of two ferritin related genes (Fth1 and Ftl1) was also observed. Ferritin is a protein that stores iron and releases it in a controlled fashion. Transcripts for other stress related proteins such as heat shock proteins (Hsp70, Hsp10, Hsp60), hemeoxygenase 2 (HO-2) and stress inducible molecular chaperone (GrpE#1) were all increased in response to DMA. These changes are consistent with the oxidative stress response that has been reported following DMA exposure both in vitro and in vivo (Cohen et al., 2001, 2002). Gene expression associated with proliferation control was altered in response to DMA treatment (Table 1).

These expression changes include several growth factors, epidermal growth factor receptor (Egfr), transforming growth factor α (Tgfα), insulin like growth factor 2 (Igf2), c-fos induced growth factor (Figf) and vascular endothelial growth factor (Vegf). These changes are consistent with the increased cell proliferations that have been reported in the bladder epithelium following administration of DMA to female F344 rats (Arnold et al., 1999). Additionally, modulation in the cell cycle regulation pathways were bserved by altered expression of genes including Cdc25a, a key initiator of mitosis, Cdc37, a cell cycle regulator and Cdkn2a which is involved in negative regulation of cell cycle. Gadd45α, which is important for DNA repair and cell cycle arrest at the G2-M checkpoint, was increased four-fold upon treatment. Caspase3 (casp3), which is involved in apoptosis showed a two-fold induction upon exposure to DMA. Bcl-2 associated death agonist (Bad) and Bcl-2 associated X protein (Bax) were up regulated two-fold upon exposure to DMA in the bladder. The other major functional changes in gene expression observed in this study included those involved in signal transduction modulators (Ywhaq, Gprk2, Rab10, Arf6), growth

factors and hormone receptors (Grn, Erbb3, Inhbe, Tshr), transcription modulators (Irf1, Mbtps1, Hoxa1, Atf4) and cellular adhesion and structural modification (Itgb1, Emb, Celsr3, Cdh8) (Table 1).

Biological networks that might be perturbed following treatment with DMA were further investigated using the Ingenuity pathway analysis tool. An interrogation by the software revealed that of the 510 significantly altered genes, a number of genes mapped to pathways involved in ERK/MAPK signaling (important in growth control and development), cell cycle regulation (G1/S checkpoint regulation andG2/Mdamage checkpoint regulation), PI3K/Akt signaling (important in cell survival), Wnt/β-catenin signaling (involved in surface receptor signaling), and apoptosis (Table 2). These pathways are also known to be involved in the carcinogenic process.

3.3. Dose response

A traditional dose response across all treatment groups as observed in traditional toxicology studies was not observed in the global expression profiles of the genes, however a dose pattern emerges hen combined gene expression data from non-toxic doses (1 and 4 ppm) are compared to the combined gene expression data from toxic doses (40 and 100 ppm). Several genes (Txn, Cdh1, cap1, Hspa5, Ramp1, Rcn and Hf10) involved in diverse functions such as oxidative stress response, cell signaling, cell adhesion and DNA binding were up regulated by greater than two-fold in the toxic doses as compared to the non-toxic doses (Table 3) although a treatment effect was observed even at the lowest dose of 1 ppm. Comparison of the expression profile of the control animals with that of the animals exposed to 1 ppm DMA in their drinking water showed that 11% (503 transcripts) of all genes were significantly affected by exposure to DMA. Of these 41% showed an increase in expression and 6% a decrease in expression by at least three-

fold. Functional analysis of these genes resulted in verification of changes in cell cycle regulation and proliferation, apoptosis and oxidative stress response, all molecular functions that have been previously associated with toxicity of arsenicals. Similar results were seen when the urothelium of animals exposed to 4 and 40 ppm DMA were analyzed. However, after 100 ppm treatment with DMA, many fewer genes involved in apoptosis, cell cycle regulation, proliferation, and the oxidative stress response had altered expression (Table 4). The present study indicates that after 28 days of treatment, exposure to 40 ppm DMA caused the most perturbation in genes involved in processes that can be linked to a toxic response (Table 4).

4. DISCUSSION

Toxicity is often preceded by and results in alterations in gene expression and these changes are a far more sensitive, characteristic and measurable endpoint than frank cytotoxicity itself. Measurement of genome wide gene expression patterns of an organism after toxicant exposure is informative and complementary to traditional methods (Nuwaysir et al., 1999). This study reports on the genome wide effects of DMA in the target urothelium of the rat after in vivo exposure and identifies genes and molecular networks previously not described for DMA induced toxicity. An attempt has been made to link the molecular changes with the phenotypic changes that are associated with DMA induced urothelial toxicity. The non-toxic doses of 1 and 4 ppm do not elicit a detectable toxicological response in the in vivo system. However, with the use of the sensitive microarray technology, we were able to detect gene level changes at these lower non-toxic doses. Gene expression profiling at the lower doses revealed indicators of potential adverse effects to the bladder, which were not detectable by traditional morphological methods. The changes that occur at the lower doses may identify precursor effects in the toxic

and carcinogenic process that proceeds following short and long term DMA exposure, respectively in the rat bladder. These precursor effects, when combined with the cellular toxicity observed at higher doses may contribute to the formation of bladder tumors upon long-term exposure to DMA.

The general profile of gene expression that emerges from this study provides insights into the mechanisms by which DMA might exert its toxic effects. Gene expression changes after DMA exposure resulted in substantial perturbation of normal regulatory processes in the urothelium of the female F344 rats. The microarray data revealed 510 genes that were significantly altered in the urothelium upon treatment with DMA. Based on the number of genes altered and modulation in biological networks at the individual doses, it appears that the greatest level of molecular changes occurred after 28 day treatment with 40 ppm DMA. However, the morphological changes representative of cytotoxicity as observed in this study and in previously reported studies are greater at 100 ppm than at 40 ppm. This difference in dose response appears to be dependent on the endpoint measured. The differences in dose response as observed between the morphological and molecular endpoints can be explained by the fact that enhanced toxicity present following treatment with 100 ppm DMA likely results in the degradation of cellular components including RNA which could result in a decrease in the altered transcript numbers at the high dose.

DMA perturbs cells by a complex alteration of expression of stress response genes, signaling molecules, transcription factors, adhesion molecules, cell cycle regulators, proliferation and apoptosis inducers and repressors. Many of the genes and functional pathways identified in this

study have been shown previously to play a role in iAs mediated toxicity. In addition to several

previously reported genes modulated by iAs, such as Gadd45α, Casp3, Hsp70, Sod1, we also

identified genes that had not previously been linked to iAs or DMA-induced toxicity and/or

carcinogenicity (Cdh8, Pdcd8, Cdk2na, Fth1, Arf6). These changes are consistent with the kind

of cellular insult a toxicant and/or a carcinogen might induce. The cellular response to DMA

treatment appears to be manifold and the resultant aberrant gene expression indicates the

complex nature of its effect. The salient feature of the present study was that we were able to

detect these changes simultaneously in the target cell for bladder cancer following in vivo

exposure to DMA and at low doses in a way previously not reported after in vivo exposure.

A subset of genes were identified that could potentially be used to differentiate toxic from non-

toxic doses of DMA. These include genes that are involved in diverse functions such as stress

response (Txn, Hsp70), cell adhesion (Cdh1), cell signaling (Cap1) and DNA binding (H1-0).

Thioredoxin is a protein-disulfide reductase enzyme that is important for redox signaling during

apoptosis and cell growth. Other stress response genes that are upregulated upon exposure to

DMA in the bladder epithelium included Gstp1, Sod1, Gpx1, HO-2, Prx1, Cat, Mt1 and Hsp70.

Existing data supports the role of oxidative damage and cell proliferation in DMA induced

cytotoxicity in the urothelium of rats (Wei et al., 2005, 1999; Cohen et al., 2002). DMA

administration has also been reported to cause an elevation in the levels of 8-OHdG, a biomarker

of DNA oxidation in the bladder in rats and mice (Wei et al., 2002; Yamanaka et al., 2001). In

cultured human epithelial cells (HeLaS3) oxidative DNA damage was observed following both

short term and long term exposure of DMA, although the effect was much more severe with the

trivalent metabolites (Schwerdtle et al., 2003). The increased formation of 8-OHdG in the serum

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and urine of metallothionein null mice as compared to the wild type mice suggests that metallothionein may play some protective role during DMA damages by acting as an antioxidant (Jia et al., 2004). Increased levels of enzymatic reactive oxygen scavengers, such as superoxide dismutase, catalase, and glutathione peroxidase, have been shown to modulate the genotoxicity of iAs in keratinocytes (Schuliga et al., 2002). In vitro studies have demonstrated the generation of free radicals as a source of oxidative stress may play a role in iAs-induced carcinogenesis (Kitchin, 2001; Kitchin and Ahmad, 2003; Nesnow et al., 2002; Schoen et al., 2004). Increased expression of Fth1 and Ftl1 suggest higher cellular amounts of free iron, which is typically associated with increased ferritin synthesis (Ahmad et al., 2000). This is consistent with previous studies which have shown that dimethylated arsenic species like DMA(III) and DMA release redox active iron from ferritin which is capable of causing DNA damage (Ahmad et al., 2000; Kitchin and Ahmad, 2003).

The perturbation of several genes involved in oxidative stress response in the bladder epithelium upon exposure to DMA is in agreement with the existing literature. The formation of reactive oxygen species following exposure to DMA as reported previously and the associated increased expression of stress related genes as observed in this study, suggest that oxidative stress could play a role in the DMA induced rat bladder toxicity. However, since trivalent arsenic species are known to bind with sulfhydryl groups in proteins (Lu et al., 2004), the possibility of a direct interaction between arsenic and several of the antioxidant genes such as metallothionein, thioredoxin and the related peroxidases cannot be ruled out.

In response to the injury associated with cellular stress, a variety of cellular pathways are activated to maintain cellular integrity and to protect the cell against deleterious effects. Arsenic accumulation in the urinary bladder epithelium causing activation of specific signaling pathways that lead to chronic increased cell proliferation is known (Luster and Simeonova, 2004). The effect of iAs on cellular signal transduction, especially MAPK pathways have been extensively studied (Yang and Frenkel, 2002; Guyton et al., 1996; Lau et al., 2004; Qian et al., 2003). Oxidative stress may initiate the activation of the MAPK pathways (Liu et al., 1996). Activation of Pkc and Egfr are thought to be required in iAs induced MAPK signaling. The Pkc isoenzymes are involved in a variety of cellular responses including apoptosis, cell growth, differentiation and transformation. Several of these Pkc isoforms showed differential expression in response to DMA treatment in the bladder. Exposure of mice to iAs in drinking water induces EGF receptor and ERK activation in bladder cells in vitro (Simeonova et al., 2000; Ludwig et al., 1998). The present study identified several pathways that were involved in the intracellular perturbations of the urothelium following exposure to DMA. An increase in Egfr signaling and activation of the ERK/MAPK pathway is observed upon DMA exposure in the rat bladder. NF-κB is a transcription factor that mediates a variety of cellular processes, including cell-cell interaction, intracellular communications and initiation and activation of carcinogenesis. A number of stimuli are known to activate NF-κB including environmental stressors, toxic metals, intracellular stresses and UV light. NF-B inhibition potentiates arsenite induced G2/M cell cycle arrest in human bronchial epithelial cells (Chen et al., 2001). In the present study, Tgf-α, Egfr, Pkcδ Hdac2, all members of the NF-κB signaling pathway, had increased expression by 1.5-, 2.8-, 3.5- and 4.5-fold, respectively, after DMA treatment.

Loss of cell cycle control is a key phenomenon in the process of carcinogenesis. iAs has been observed to damage DNA and induce cell cycle arrest at G1 or at the G2/M phase (Huang et al., 2004). iAs induced G2/M phase arrest is reported to occur via the induction of GADD45α, a checkpoint protein (Tran et al., 2002). Our study shows that DMA is involved in the modulation of various cell cycle regulation genes. Cdk2na, which is a negative regulator of cell proliferation and is involved in cell cycle arrest, has decreased expression. In addition, the DNA damage response gene, growth arrest and DNA damage inducible transcript (Gadd45α), had increased expression by four-fold. The induction of cell cycle regulating genes such as Cdc37, Egfr and Tgf-α observed in the present study are consistent with the role of DMA in promoting cell proliferation. They are also in agreement with the previously reported modes of action for iAs induced toxicity and carcinogenicity. It is known that arsenic influences cellular signal transduction such as the MAPK signaling pathway, which in turn leads to the activation of transcription factors AP-1 and NF-κB which in turn altered gene expression (Qian et al., 2003; Yang and Frenkel, 2002). DMA exposure in vivo also alters the regulation of ERK/MAPK signaling cascade along with other closely related pathways such as NF-κ B, Wnt/β-catenin and PI3K/Akt.

Apoptosis is another critical function of the cell, alteration of which has been associated with cancer development. iAs induced apoptosis in liver epithelial cells appears to be associated with cell cycle arrest at the G2/M phase and dependent upon cellular glutathione levels (Sakurai et al., 2002, 2004). Dimerization of Bad with either Bcl-2 or Bcl-xL influences the susceptibility of the cell to a death signal and Bax activation is associated with oxidative stress induced apoptosis (Cheng et al., 1997). The addition of inhibitors of caspase 3, caspase 8 and caspase 9 has been

shown to significantly reduce DMA induced apoptosis in liver epithelial cells (Sakurai et al., 2004). Cellular glutathione is required for DMA induced apoptosis to occur, and activation of cellular caspases after conjugation of DMA with cellular glutathione appears to be of mechanistic significance (Sakurai et al., 2004). Bad, Bax, Casp3, Casp7 and Cytc, all involved in the apoptotic pathway, were modulated uponDMAexposure in the bladder. The mitogenic ERK cascade is activated by arsenite via a Ras dependent pathway (Chen et al., 2001). At high dose and short exposure times, arsenite stimulates ERK via the Ras, Raf and MEKsignaling cascades (Ludwig et al., 1998). DMA has also been shown to cause apoptosis in the urinary bladder epithelium of metallothionein null mice (Jia et al., 2004). In the present study, Pyk2, 14-3-3, Bad, 4EBp1 and eIF4E, all members of the ERK/MAPK signaling cascade, had increased expression in response to treatment with DMA while the kinases, Pkc and p90RSK had decreased expression. These observations may suggest a role for apoptosis in DMA-induced toxicity in vivo in the rat as well.

Our study is the first report that catalogs the genes and pathways perturbed following DMA exposure in vivo. In addition, gene expression profiling of the target cell for DMA-induced toxicity after in vivo exposure enabled us to identify potential adverse affects at doses lower than those at which traditional toxicological end points can be measured. A previous study reported that DMA did not have a consistent modifying effect on gene expression in the rat bladder epithelium (Wei et al., 2005). This discrepancy could be due to the difference in the treatment regimen. Their study reported on rats dosed at 100 ppm at which there is enhanced cytotoxicity and we observed a decline in the number of genes differentially expressed at this high dose.

We have attempted to link our gene expression data to what is already known or proposed regarding the biology of DMA and related arsenical toxicity from past studies. To better understand, interpret and generate new knowledge about the toxicity of a chemical compound, it is necessary to compare microarray data to phenotypic changes identified from traditional toxicology studies. The results of this study provide evidence for a role for proliferation, oxidative stress response and apoptosis as part of the process of DMA induced urothelial toxicity in vivo and this is in agreement with existing evidence. Also, since arsenicals are known to have multiple affects on the cell including binding to sulfhydryl groups on proteins, multiple effects on the cell leading to cytotoxicity are possible. These data are limited in their scope to determine if the gene changes observed are the cause of toxicity or a result of toxicity. However, these findings could provide useful information in future functional studies with DMA. Also identification of genes whose expression is specifically modified by DMA exposure may provide a better understanding of the mechanisms of action and allow for development of biomarkers in mechanistic and risk assessment based studies. These early changes provide insights into mechanisms underlying the development of DMA induced toxicity. The data from this study suggests that transcriptional profiling is useful in predicting key mechanistic pathways and a cost and time efficient tool to inform the mode of action analysis in risk assessment.

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Table 1. Genes that were significantly different between control and DMA treated samples, grouped based on their functions.

Genbank ID	Common Name	Symbol	Fold change
Adhesion			
NM_012811	milk fat globule-EGF factor 8 protein	Mfge8	1.6
NM_012834	Cartilage oligomeric matrix protein	Comp	2.6
U44129	Lectin-mannose binding protein	Lman1	2.9
NM_012830	Cd2 antigen	Cd2	4.1
M88469	F-spondin	Sponf	-1.9
AB049056	Brain link protein 1	Bral1	-2.6
AJ009698	embigin protein	Emb	2.7
U72660	Ninjurin	Ninj1	2.2
NM_017318	Cell adhesion kinase beta	Ptkb2	2.5
NM_031832	IgE binding protein	Lgals3	4.2
NM_017022	Integrin, beta 1	Itgb1	2.4
L07127	Pancreatitis associated protein	Pap	-3.4
U57362	Procollagen type XII alpha 1	Coll2a1	-5.0
AB011528	Cadherin Receptor	Celsr3	-6.9
AF177679	Cadherin 8	Cdh8	-9.4
Apoptosis			
NM_022698	Bcl-2 associated death agonist	Bad	2.0
NM_017059	Bcl2-associated X protein	Bax	2.3
NM_030826	Glutathione peroxidase 1	Gpx1	3.4
M80601	Programmed cell death 2	Pdcd2	3.2
NM_012647	Sodium channel, voltage-gated, type II	Scn2a1	-5.0
NM_017312	Bcl-2-related ovarian killer protein	Bok	2.6
AB041723	Programmed cell death 8 (apoptosis-inducing factor)	Pdcd8	1.9
NM_019232	Serum/glucocorticoid regulated kinase	Sgk	2.1
NM_012922	Caspase 3	Casp3	2.0
Cell cycle regulation			
X83579	Cyclin dependent kinase 7	Cdk7	1.5
NM_012671	Transforming growth factor, alpha	Tgfa	1.5
NM_031511	Insulin-like growth factor II	Igf2	-1.6
D16236	Cell division cycle 25A	Cdc25A	-1.3
D26564	Cyclin dependent kinase 37	Cdk37	2.4
NM_031550	Cyclin dependent kinase inhibitor 2A	Cdk2na	-2.3
NM_031761	C-fos induced growth factor	Figf	-3.7
NM_031609	Neuroblastoma, suppression of tumorigenicity 1	Nbl1	2.2
AF187818	Epidermal growth factor receptor	Egfr	2.8
NM_031030	Cyclin G-associated kinase	Gak	3.5
U34843	Cell cycle progression related D123	D123	3.8
NM_017326	Calmodulin 2	Calm2	2.9
NM_024127	Growth arrest and DNA damage inducible transcript 45 alpha	Gadd45a	4.0
AF000578	Cell division cycle 5-like	Cdc5l	2.6
AF306457	RAN, member RAS oncogene family	Ran	3.6
Stress			
J03752	Rat glutathione S-transferase mRNA, complete cds	Mgst1	3.1
NM_030826	Glutathione peroxidase 1	Gpx1	3.4
L29427	Glutathione-S-transferase	Gstp1	3.6
X02904	Glutathione S-transferase	Gstp2	3.5
NM_012966	Heat shock 10 kD protein 1	Hspe1	3.6
M14050	Heat shock 70kD protein 5	Hspa5	2.8
NM_022229	Heat shock protein 60 (liver)	Hsp60	2.6
NM_012520	Catalase	Cat	2.3
NM_017050	Superoxide dimutase 1, soluble	Sod1	4.1

U73525	Thioredoxin 2	Txn2	2.7
NM_017169	Peroxiredoxin 2	Prdx2	3.4
AF311055	Thioredoxin	Txn2	4.6
NM_024487	Stress-inducible chaperone mt-GrpE#1	Grpel1	2.8
NM_012848	Ferritin subunit H	Fth1	4.0
NM_022500	Ferritin light chain 1	Ftl1	4.5
J00750	Metallothionein	Mtla	2.5

Growth factor and hormone receptor

AF187065	Nerve growth factor receptor assoc.protein	Ngfrap1	2.0
NM_017113	Granulin	Grn	3.7
NM_017218	Avian erythroblastosis oncogene B 3	Erbb3	1.4
NM_031815	Activin beta E	Inhbe	-2.2
NM_022847	Progesterone receptor	Pgr	1.9
NM_017058	Vitamin D receptor	Vdr	-2.8
M34842	Thyroid stimulating hormone receptor	Tshr	-4.0

Signal transduction

NM_013053	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	Ywhaq	3.0
M83681	RAB3D, member of RAS oncogene family	Rab3D	1.4
NM_023950	RAB7, member RAS oncogene family	Rab7	3.0
M93271	Branched chain keto acid dehydrogenase kinase	Bckdk	-2.2
NM_017276	Guanosine diphosphate (GDP) dissociation inhibitor 3	Gdi3	2.6
AF034577	Pyruvate dehydrogenase kinase	Pdk4	-2.2
NM_022928	G protein-coupled receptor kinase 2	Gprk2	-2.2
NM_019250	Ral guanine nucleotide dissociation stimulator	Ralgds	2.6
NM_022507	Protein kinase C, zeta	Prkcz	3.5
M83680	GTPase Rab14	Rab14	2.1
AF306457	RAN, member RAS oncogene family	Ran	3.6
AF139492	Syndapin 2	SdpII	2.2
NM_013022	RhoA - binding serine/threosine kinase alpha	Rock2	2.6
NM_017359	Ras-related protein rab10	Rab10	3.9
NM_022396	Guanine nucleotide binding protein	Gng11	1.8
NM_031090	Ras-related protein	Rab1	2.9
NM_017229	Phosphodiesterase 3B, cGMP-inhibited	Pde3b	-2.2
NM_024152	ADP-ribosylation factor 6	Arf6	3.8

Transcription factors

AF094821	Membrane-bound transcription factor protease, site 1	Mbtps1	2.0
NM_012866	Nuclear transcription factor Y gamma	Nfyc	1.6
NM_012591	Interferon regulatory factor 1	Irf1	2.3
AB012233	Nuclear factor I/C	Nfic	2.4
NM_022858	HNF-3/forkhead homolog-1	Hfh1	2.5
NM_013075	Homeo box A1	Hoxa1	-2.1
U91679	ETS domain transcription factor Pet-1	Pet1	-3.3
NM_022588	Metastasis associated 1	Mta1	2.5
NM_031139	Transcription factor USF2	Usf2	3.8
NM_024403	Activating transcription factor ATF-4	Atf4	3.6
AF061817	Prolactin regulatory element binding	Preb	2.9
NM_031042	General transcription factor IIF	Gtf2f2	2.0
NM_012581	Homeobox gene A2	Hoxa2	-4.8

Table 2. Pathway profiling of genes that were significantly different between control and DMA treated samples.

Pathway	Genbank ID	Gene name (fold change)	Function
Apoptosis	NM_031107	p90RSK (−1.2)	Regulation of size, development process
	NM_022698	Bad (2.0)	Apoptosis, cell death
	NM_017059	Bax (2.3)	Apoptosis, cell death, proliferation, tumorigenesis
	NM_012839	Cytc (3.7)	Electron transport, induction of apoptosis
	NM_019152	Calpain (3.0)	Positive regulation of proliferation
	NM_022260	Caspase 7 (3.0)	Apoptosis, cell death, morphology
	NM_012922	Caspase 3 (2.0)	Apoptosis, cell death, proliferation
	NM_021755	Lamin A (2.0)	Cell morphology
Cell Cycle (G1/S)	NM_031550	Cdkn2a (−2.3)	Cell cycle arrest, oncogenesis, negative regulation of cell proliferation
	D16236	Cdc25A (−1.2)	Cell division
	AF321130	Hdac (4.5)	Development, apoptosis
(G2/M)	NM_031550	Cdkn2a (−2.3)	Proliferation, cell death, apoptosis
	NM_031107	p90RSK (−1.2)	Regulation of cell size, development process
	NM_024127	Gadd45α (4.0)	Survival, apoptosis, cell cycle arrest
	NM_013054	14-3-3(β, ε, ζ) (3.3)	Protein binding
	X83579	Cdk7 (1.5)	DNA repair, cytokinesis
ERK/MAPK	NM_017318	Pyk2 (2.5)	Positive regulation of cell proliferation
	NM_013053	14-3-3(η, θ, ζ) (3.0)	

Pathway	Genbank ID	Gene name (fold change)	Function
	AB020615	Pkc(αβγδε) (−1.8)	
	NM_031107	p90RSK (−1.2)	
	NM_022698	Bad (2.0)	
	U05014	4Ebp1 (2.8)	Apoptosis, development
	X83399	eIF4E (2.1)	Development, cell death, size, morphology
PI3K/Akt	NM_053826	Pdk1 (1.6)	
	D26564	Cdc37 (2.4)	Cell proliferation, tumorigenesis, development
	NM_022698	Bad (2.0)	Apoptosis, cell death
	U05014	4Ebp1 (2.8)	Apoptosis, development
	X83399	eIF4E (2.1)	Development, cell death, size, morphology
	NM_017039	Pp2a (2.8)	
Wnt/Beta Catenin	NM_031334	Cadherin (3.1)	Cell adhesion, tumor suppressor
	NM_017039	Pp2a (2.8)	
	U77583	Ck1 (3.3)	
	NM_031550	Cdkn2a (−2.3)	Proliferation, cell death, apoptosis
NF-κB	NM_012671	Tgf-α(1.5)	Apoptosis, proliferation, cell migration
	NM_022507	Pkcζ(3.5)	Apoptosis, cell cycle progression, tumorigenesis
	AF187818	Egfr (2.8)	Proliferation, migration, invasion, transformation
	AF321130	Hdac (4.5)	Histone deacetylation

Table 3. Genes that differentiate the toxic doses (40 and 100 ppm) from the non-toxic doses (1 and 4 ppm) of DMA.

Genbank ID	Common name	1 and 4 ppm (fold change)	40 and 100 ppm (fold change)	Function
AF311055	Thioredoxin	3.1	6.8	Stress response
NM_031334	E-cadherin	2.1	4.5	Cell Adhesion
NM_022383	Cyclase-associated protein homologue	2.0	3.9	Cell signaling
M14050	Heat shock 70 kDa	2.0	4.0	Stress response
NM_031645	Receptor (calcitonin) activity modifying protein 1	4.3	8.5	Cell signaling
AJ001929	Reticulocalbin	1.9	3.7	Stress response
NM_012578	Histone H1-0	1.8	3.3	DNA binding

Table 4. Dose response as observed based on the number of genes altered in different functional categories after treatment with four different DMA concentrations.

Gene function	1 (ppm)	4 (ppm)	40 (ppm)	100 (ppm)
Apoptosis	9	12	19	6
Cell cycle regulation	7	8	24	5
Oxidative Stress	3	3	2	1
Cell proliferation	5	6	7	1
Oncogene	7	2	10	0
Growth factors	8	14	12	1
Total	39	45	74	14
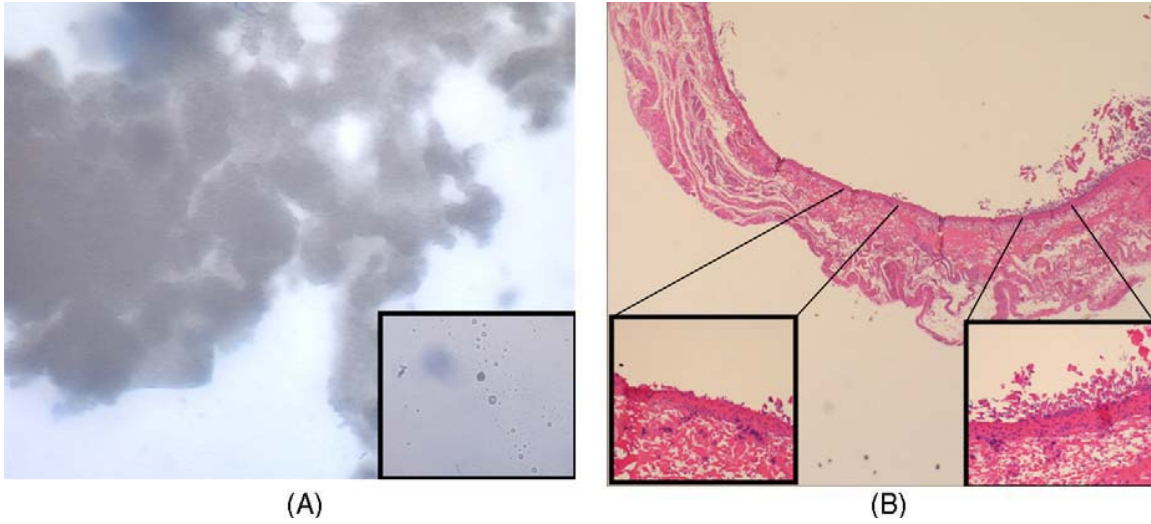
Fig. 1. Isolation of the urothelium. (A) Material removed from the bladder following trizol infusion showing large clumps of urothelial cells, insert depicts individual cells. (B) H and E stain of bladder following treatment with trizol showing an intact basement membrane with most of the urothelial cells removed, insets depict areas at higher magnification.
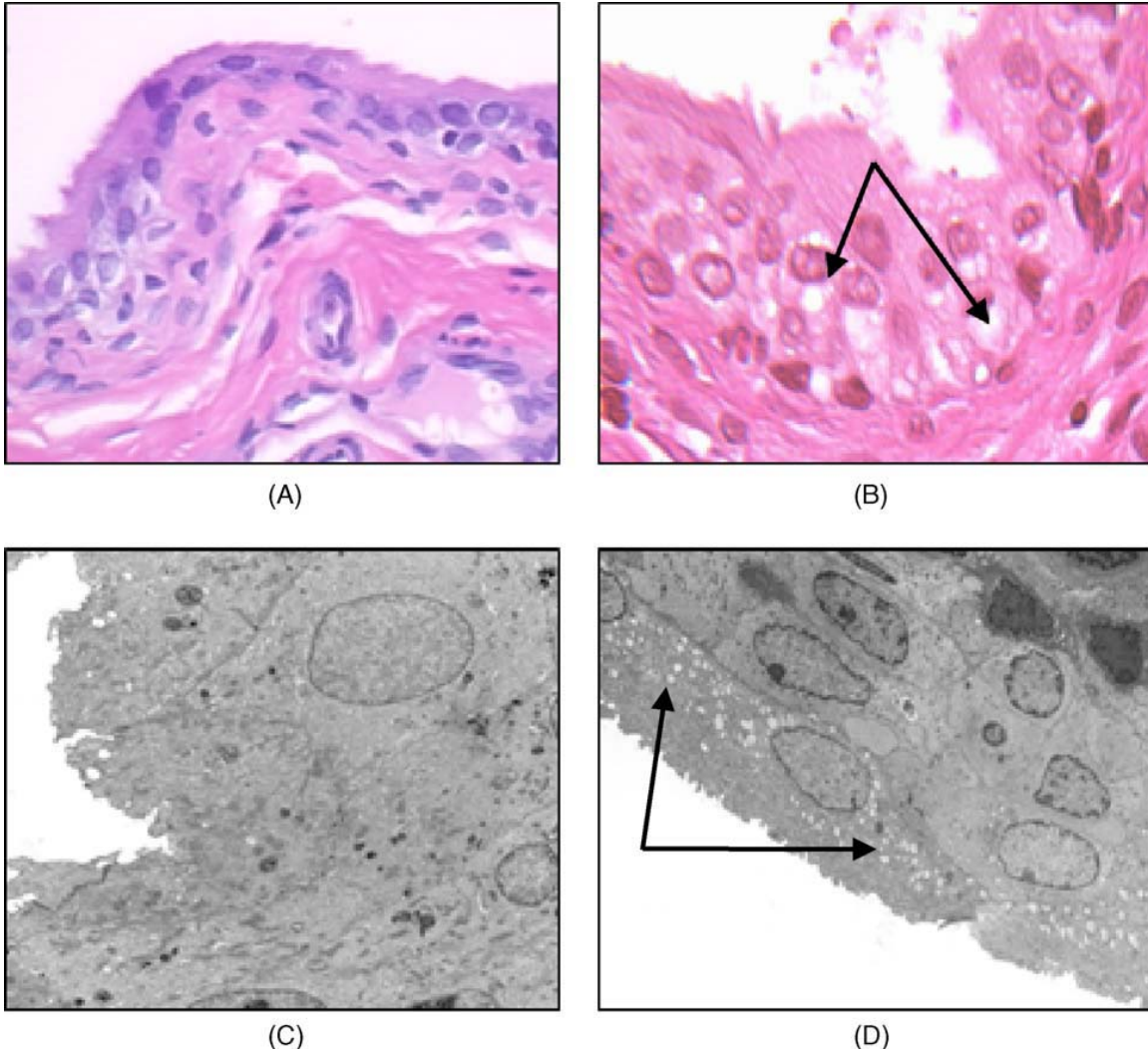
Fig. 2. Light (A, B) and transmission (C–E) electron microscopic images of the bladder epithelium following exposure to DMA. (A, C) control, (B, D) 100 ppm. Arrows point to areas of increased vacuolization, an indicator of urothelial cytoxicity.
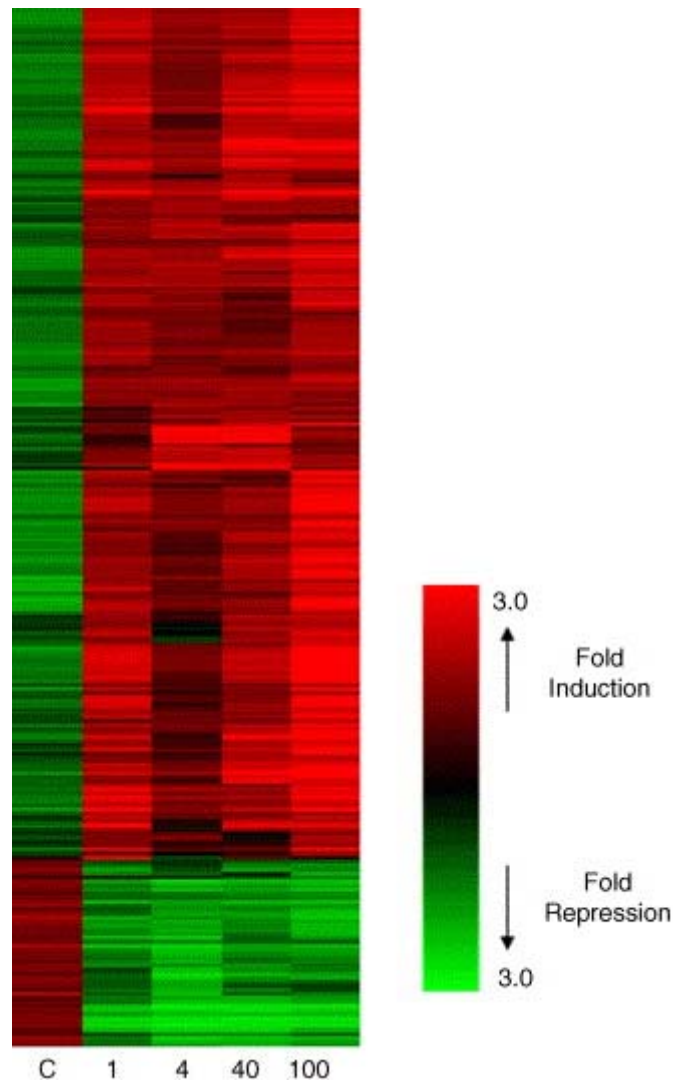
Fig. 3. Transcriptional response of the urothelium to 1, 4, 40 and 100 ppm DMA as compared to control to illustrate a treatment response at toxic and non-toxic doses. Gene tree generated by hierarchical clustering of the 510 DMA responsive genes. The color scale indicates the mean fold change of DMA induced gene expression (based on four biological replicates).

Appendix 2   Glutathione levels were not affected by up to 100 ppm dimethylarsinic acid in water for four weeks in F344 rats

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The present study is one of the collaborative projects of Amy Wang's dissertation experiment II with F344 rats exposed to dimethylarsinic acid in drinking water for 4 weeks. Amy designed the animal study, treated rats, collected exposure data, set up the collection stations, and lead students to collect lung, liver, kidney and bladder for glutathione study following a carefully designed protocol for minimal oxidation. Dr. Kitchin measured the glutathione concentrations.

INTRODUCTION

Arsenic-induced oxidative stress has been observed in various *in vitro* and *in vivo* studies, and has been suggested as a mode of action of arsenic carcinogenesis (For a review of arsenic and oxidative stress, see Shi *et al.* 2004; for reviews of mechanisms of arsenic carcinogenesis, see Kitchin 2001 and Rossman 2003). Reactive oxygen species were induced by inorganic arsenic and methylated arsenic exposure in various cellular systems, and superoxide anion (O_2-) was likely the primary species induced by arsenic. The involvement of nitric oxide from arsenate exposure, on the other hand, appeared to be cell type and arsenic concentration dependent (Shi *et al.* 2004).

While the evidence of increased oxidative stress after arsenic exposure is unambiguous, the sources of arsenic-induced oxidative stress remain unclear. At least five contributing mechanism have been proposed: (1) Based on the arsenic metabolism pathway, arsenic may cycle between oxidative states and produce radicals (Lantz 2001; Shi *et al.* 2004). For example, the oxidation of arsenite to arsenate can produce hydrogen peroxide (H_2O_2) in physiological conditions. (2) Arsenic may interact with and reduce intracellular levels of antioxidants (Lantz 2001). (3) Mitochondria may be the main source of arsenite-induced oxidative stress. Arsenite may activate NADP/NADPH oxidase, which catalyzes the production of superoxide (Rossman 2003; Shi *et al.* 2004). (4) Methylated arsenicals release free iron from ferritin, and free iron can promote production of hydrogen radicals (·OH) (Ahmad *et al.* 2000; Ahmad *et al.* 2002). (5) Dimethylated arsenic peroxide radicals and trivalent dimethylated arsenic, rather than active oxygen species, may be responsible for the induction of oxidative damage (Yamanaka and Okada 1994; Yamanaka *et al.* 2003).

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Dimethylarsinic acid [DMA(V)] is a major metabolite detected in the urine of humans exposed to inorganic arsenic, and induces urinary bladder cancer in F344 rats after a 2 year exposure via diet (at 100 ppm) or drinking water (at 50 and 200 ppm) (van Gemert and Eldan 1998; Wei *et al.* 2002). Oxidative stress induced by DMA(V) was observed in the kidney and liver, in addition to the urinary bladder of rats. For example, DMA(V)-induced increases of 8-hydroxy-2'- deoxyguanosine (8-OHdG) were reported in the kidney of NCI-Black-Reiter rats given DMA(V) through oral gavage, once a day, 5 days a week, for a period of 4 weeks. The highest 8-OHdG level was at the dose of 10 mg/kg body weight, not 20 mg/kg (Vijayaraghavan *et al.* 2001). In a study using an Ito model, F344 rats were given a single i.p. injection of diethylnitrosamine at a dose of 200 mg/kg body weight as an initiator. Starting 2 weeks thereafter they received arsenicals in the drinking water, or no supplement as a control, for 6 weeks. Increased 8-OHdG was observed in the livers of three arsenic-treated groups (100 ppm of monomethylarsonic acid, DMA(V) and trimethylarsine oxide), compared to the controls (Nishikawa *et al.* 2002). In F344 rats exposed to DMA(V) in drinking water for 104 weeks, increased cyclooxygenase-2 expression, a molecular marker of oxidative stress, was seen in 17 of 18 (94%) transitional cell carcinomas, 4 of 4 (100%) bladder papillomas, and 39 of 47 (83%) hyperplasias (Wei *et al.* 2002). Increased 8-OHdG formation in F344 rat urinary bladder was seen at as early as 2 weeks after treatment with 200 ppm DMA in the drinking water (Wei *et al.* 2002). Recently, Wei *et al.* reported that sodium salt of vitamin C, an antioxidant, decreased dietary DMA(V)-induced urinary bladder cytotoxicty and proliferation, indicating oxidative stress is involved in DMA(V) toxicity in bladder. They suggested that protein and/or lipid may be the targets of DMA(V) induced oxidative stress, due to the lack of consistent changes at mRNA level, as detected by microarray and real time RT PCR (Wei *et al.* 2005).

Similarly, oxidative stress was detected in various tissues of mice after oral DMA(V) exposure (van Gemert and Eldan 1998; Yamanaka *et al.* 2001b; Jia *et al.* 2004). Oxidative stress was detected usually after a few weeks of exposure or shortly after a single exposure to DMA(V). HRS mice exposed to 400 ppm DMA(V) in drinking water for 4 weeks showed increased amounts of 8-oxo-2'-deoxyguanosine (8-oxodG) in the lung and liver, and a non-significant increase in the bladder (van Gemert and Eldan 1998). Six and 9 hours after an acute oral exposure of 720 mg/kg DMA(V) (oral LD_{50} for mice is 1.2 g/kg), ddY mice showed increased 8-oxodG levels in liver, lung, and bladder (Yamanaka *et al.* 2001b). Similarly, increased formation of 8-OHdG in murine serum and urine was seen 24 hours after a single oral dose of DMA(V) (188, 375 or 750 mg/kg body weight) (Jia *et al.* 2004).

While drinking water is the main exposure route for humans to arsenic, very few animal studies using drinking water are available. Since exposure routes may affect kinetics and toxicity of arsenic, the present experiments were designed to evaluate whether DMA(V) in drinking water induces oxidative stress after a subchronic exposure. The levels of reduced form of glutathione (GSH) were measured in the liver, lung, kidney, and urinary bladder of F344 rats exposed to up to 100 ppm DMA(V) for 4 weeks. GSH was chosen because a decrease in GSH is an indicator of oxidative stress, and has been reported in rats exposed to DMA(V) through oral gavage (Brown *et al.* 1997), in rats i.p. injected with arsenite (Maiti and Chatterjee 2001), as well as in humans exposed to high environmental levels of arsenic (Shi *et al.* 2004).

MATERIALS AND METHODS

Chemicals, animals and treatments, and terminal necropsy

See Chapter 5, Materials and Methods.

Sample collection

Lung, liver, kidney and bladder were collected for GSH measurement. All solutions were ice-cold, and all the procedures, except weighing and homogenization, were done on ice. Briefly, tissue was weighed, minced, and washed with B-7 buffer (5.4 mM KCl, 136 mM NaCl, 20 mM HEPES, 5 mM DTE, 4 mM EDTA, and 0.8 mM B-6, at pH 7.5) to remove blood. Tissue was then transferred into a polystyrene tube containing 4-times tissue weight of B-7 buffer and homogenized. Eight ml of GSH freezing solution (20 mM EDTA, 50 mM HEPES) were deoxygenated by bubbling nitrogen gas for at least 5 minutes, until the time to add the homogenate. One ml of homogenate was transferred into a new popypropylene tube containing deoxygenated GSH freezing solution and 1 ml 50% TCA. The tube was flash frozen, kept on dry ice, and stored at -80°C.

Glutathione measurement

Glutathione level was measured by condensation with ophthalaldehyde exactly as published earlier (Brown *et al.* 1997).

Statistical analysis

Student's *t*-test was used to evaluate raw data and $p<0.05$ was considered significant.

RESULTS

No differences in GSH levels were observed in lung, liver, or kidney from rats exposed to DMA(V) in drinking water for 4 weeks at concentrations of 0, 40 or 100 ppm (**Table 1**), whereas the values in 0 ppm group were similar to historical controls (Brown *et al.* 1997). Urinary bladders showed a wide variance of GSH levels within each treatment group (data not shown), and GSH changes in the bladder were inconclusive.

Glutathione was not measured in tissues in the 1 or 4 ppm DMA(V) groups, since low concentrations of DMA(V) were less likely to affect GSH as compared to high concentrations of DMA(V).

DISCUSSION

There was no difference in the GSH levels, an indicator of oxidative stress, in the lung, liver or kidney of female F344 rats exposed to DMA(V) in drinking water for 4 weeks at concentrations up to 100 ppm. These observations were likely due to the subchronic dosing regimen in the present study. The accumulated total doses of DMA(V) over 4 weeks in the present study were compartible to the single doses of DMA(V) that induced GSH level changes (Brown *et al.* 1997), and animals may have increased GSH production as an adaptation to our 4-week-long DMA(V) treatments (Sakurai *et al.* 2004b).

In Brown *et al.* 1997, female SD rats were given DMA(V) by oral gavage at 21 and 4 hours prior to sacrifice. At 129 and 387 mg/kg of DMA(V), GSH concentrations were decreased in the lung, but not in the liver. In the present study, the total DMA(V) intakes (0, 137.3, and 376.4 mg/kg in 0 , 40, and 100 ppm groups, respectively) were similar to the Brown's study (1997) gavage doses. However, the present doses were accumulated over 4 weeks. Consequently, no GSH change was observed in female F344 rat lung, liver or kidney. The possibility remains that the present treatments were non-cytotoxic to lung, liver and kidney, which is in agreement with the absence of morphological changes in the kidney in classical histological examination (data not shown).

Additionally, rats exposed to up to 100 ppm DMA(V) may have adapted to the treatment within the 4 week period. Long term exposure to arsenic may modulate the GSH system differently from acute exposure. At acute exposure of arsenic, a rapid, but transient, increase of

oxidative stress may decrease GSH, as free radicals can oxidize GSH into GSSH, an oxidized form of glutathione. In the mean time, arsenic-induced free radicals can increase production of GSH via affecting gene expression and activities of GSH-related enzymes (Schuliga *et al.* 2002). Cytotoxic concentrations of DMA(V) have been reported to decrease cellular GSH levels temporarily, and to increase GSH levels with continuous exposure (Sakurai *et al.* 2004b). The effects of arsenic on GSH level are dependent on arsenic concentration and the cellular response to arsenic attacks (Shi *et al.* 2004).

While increased GSH levels with the continuous exposure to DMA(V) have been reported as an adaptive response (Sakurai *et al.* 2004b), increased GSH levels may not be the primary basis for arsenic tolerance acquired after long term exposure to low concentrations of arsenite. For example, a chronic arsenite-exposed rat liver epithelial cell line (CAsE-TRL 1215) showed 4.4 fold higher DMA(V) LD_{50} as compared to control cells without changes in cellular GSH or metallothionein levels (Romach *et al.* 2000). Reduced cellular deposition of arsenic was the primary cause of CAsE-TRL 1215's arsenic tolerance. On the other hand, a chronic arsenite-exposed human prostate epithelial cell line (CAsE-PE) showed elevated basal GSH levels and glutathione S-transferase activity (Brambila *et al.* 2002), and arsenic tolerance was abated by both GSH depletion and inhibition of GSH activity (Brambila *et al.* 2002). Similar to CAsE-TRL 1215, CAsE-PE accumulated less intracellular arsenic than its parental cell line. The decreased arsenic deposition in CAsE-PE resulted from higher arsenic efflux as compared to the control cells. Inhibition of multi-drug resistance gene (Mdr1) and multi-drug resistance protein (Mrp1) transporters abolish the arsenic tolerance, but the expression of mdr1 and mdr2 genes was not altered in CAsE-PE. In the case of the human prostate epithelial cell line, arsenic

tolerance involved increases in GSH levels and glutathione S-transferase activity that allow for more efficient arsenic efflux by MRT1 and MDR1 genes.

Glutathione provides protection against cytotoxicity (Ochi *et al.* 1994; Sakurai *et al.* 2002) and clastogenicity (Oya-Ohta *et al.* 1996) of arsenite, arsenate, monomethylarsonic acid and trimethylarsine oxide. However, GSH may contribute to DMA(V)-induced cytotocity and clastogenicity, in contrast to the protection against DMA(V)-induced oxidative stress. *In vitro* studies showed that GSH depletion reduced DMA(V) cytotoxicy (Ochi *et al.* 1994; Hirano *et al.* 2004), DMA(V) clastogenicity (Oya-Ohta *et al.* 1996) and DMA-induced apoptosis (Sakurai *et al.* 2002). Furthermore, *in vivo* studies showed that co-injection of GSH and DMA(V) in mice induced micronuclei in reticulocytes, while injection of DMA(V) or GSH alone did not (Kato *et al.* 2003). Results from i*n vitro* studies using various inhibitors of GSH and GSH-related enzymes suggested that DMA(V) may require intracellular GSH to induce apoptosis (Sakurai *et al.* 2002).

The classical biotransformation pathway of arsenic (see Chapter 1, Figure 3) requires GSH as an electron donor in reducing pentavalent arsenicals into trivalent arsenicals as well as in the methylation step. In addition, GSH is thought to form an arsenic-glutathione complex (Kato *et al.* 2003; Hayakawa *et al.* 2004). In one of the possible metabolic pathways of DMA(V), GSH interacts with DMA(V) and forms dimethylarsinate-glutathione complex [DMA(III)-SG] and dimethylarsinous acid [DMA(III)], which in turn form dimethylarsine and radicals (Kato *et al.* 2003; Hayakawa *et al.* 2004). Although it is agreed that DMA(III) and dimethylarsine are more toxic than DMA(V), the relative toxicity of other forms of arsenicals in this pathway [DMA(III)-SG, dimethylarsine radicals, and dimethylarsenical peroxy radicals] may be dependent on the testing system. *In vitro* DMA(III)-SG had much stronger cytotoxicity than DMA(V) (Sakurai *et*

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al. 2006), and may be responsible for directly inducing apoptosis in DMA(V)-exposed rat liver epithelial cells (TRL 1215 cell line) (Sakurai *et al.* 2002). On the other hand, mice i.p. or i.v. injected with DMA(V) or dimethylarsinous iodine, which is presumed to from DMA(III) in aqueous solution, did not show increased reticulocyte micronuclei, while co-administration of GSH increased micronuclei. These *in vivo* data suggested that the radicals (dimethylarsine radical and dimethylarsenical peroxy radical) generated by the reaction of dimethylarsine with oxygen, rather than DMA(III) itself or DMA(III)-SG, may cause the formation of reticulocyte micronuclei (Kato *et al.* 2003).

In summary, the present study showed that DMA(V) up to 100 ppm in drinking water for 4 weeks did not alter GSH levels in liver, lung, or kidney of F344 rat. This absence of change may have resulted from low toxicity (no morphological changes in the kidney) and/or from an adaptive response after weeks of exposure. These results, however, do not exclude the possibility of oxidative stress involvement in DMA(V) toxicity, because arsenic-induced GSH changes may be cell type specific as well as arsenic concentration and exposure time dependent.

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Table 1. Glutathione concentrations were not altered by DMA(V) treatment in F344 rat liver, lung, or kidney. Values are mean ± standard deviation. The numbers of rats were 7, 9, and 10 in 0, 40, and 100 ppm DMA(V) groups, respectively.

Treatment	Total DMA(V) intake (mg/kg)	GSH (µM/g)		
		Liver	Lung	Kidney
0 ppm DMA(V)	0	7.95±1.30	4.97±1.08	5.08±0.93
40 ppm DMA(V)	137.3±14.9	7.85±1.41	5.53±1.48	5.52±1.01
100 ppm DMA(V)	376.4±45.1	7.85±2.68	4.73±1.46	5.20±1.36

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EDUCATION

Doctorate of Philosophy, Toxicology, Aug. 2007
Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM)
Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia
Dissertation: Arsenic in drinking water caused ultra-structural damage in urinary bladder but
    did not affect expression of DNA damage repair genes or repair of DNA damage in
    transitional cells
Advisor: Steven D. Holladay, Ph.D.

Master of Science, Veterinary Medical Sciences, Aug. 2000
VMRCVM, Virginia Tech, Blacksburg, Virginia
Thesis: The Effects of Mercuric Chloride on Cultured Atlantic Spotted Dolphin (Stenella
    plagiodon) Renal Cells and the Role of Selenium in Protection
Advisor: Carl J. Pfeiffer, Ph.D.

Bachelor of Veterinary Medicine, June 1996
National Taiwan University, Taipei, Taiwan
Class president, 1994 and 1996
Case studies: (1) Canine type II diabetes, and (2) Squamous carcinoma in a rabbit

RESEARCH SKILLS

- Real time RT PCR for gene expression
- Comet assay (single cell gel electrophoresis) for DNA damage
- Flow cytometry and fluorescent dyes for apoptosis and cell cycle status
- Light microscopy, and transmission and scanning electron microscopy for morphology
- Cell culture
- In vivo rat models

LICENSURE AND CERTIFICATION

- License to practice veterinary medicine in Taiwan
- Certified for handling radioactive material by Virginia Tech
- Certified for Good Laboratory Practice (GLP)
- Permanent resident of the United States of America (U.S.A.)

PUBLICATIONS

A. Wang, J.L. Robertson, S.D. Holladay, A.H. Tennant , A.J. Lengi, S.A. Ahmed, W.R. Huckle,
    and A.D. Kligerman (2007) Measurement of DNA damage in rat urinary bladder
    transitional cells by the Comet assay: improved procedures for selective harvest of
    transitional cells and detailed Comet protocols. **Mutat. Res.** (in press)

A. Wang, S. Holladay, D.C. Wolf, S.A. Ahmed and J.L. Robertson (2006) Reproductive and developmental toxicity of arsenic in rodents: a review. **Int. J. Toxicol.** 25(5), 319-331

B. Sen, **A. Wang**, S.D. Hester, J.L. Robertson, D.C. Wolf (2005) Gene expression profiling of responses to dimethylarsinic acid in female F344 rat urothelium. **Toxicol.** 215 (3), 214-26

A. Wang, D. Barber, and C.J. Pfeiffer (2001) Protective effects of selenium against mercury toxicity in cultured Atlantic spotted dolphin (Stenella plagiodon) renal cells. **Arch. Environ. Contam. Toxicol.** 41 (4), 403-409

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ABSTRACTS

A. Wang, S.D. Holladay, A.D. Kligerman, J.L. Robertson. Dimethylarsinic acid, a major metabolite of inorganic arsenic, in drinking water inhibited DNA-protein crosslink repair in urinary bladder transitional cells. VMRCVM 19th Annual Research Symposium, Blacksburg, Virginia. May 15-16, 2007

K. Bailey, S.D. Hester, **A. Wang**, J.L. Robertson, D.C. Wolf, B. Sen. Comparison of gene expression in kidney and urinary bladder from rats treated with dimethylarsinic acid. Society of Toxicology 44th Annual Meeting, New Orland, Louisiana. Mar. 6 - 10, 2005

A. Wang, D.C. Wolf, S. Holladay, T. Caceci, J. Robertson. Short term exposure of dimethylarsinic acid (DMA(V)) via drinking water altered cellular and subcellular morphology of urinary bladder of female F344 rats. VMRCVM 16th Annual Research Symposium, Blacksburg, Virginia. June 17 - 18, 2004

B. Sen, **A. Wang**, S.D. Hester, J.L. Robertson, D.C. Wolf. Gene expression can differentiate carcinogenic from non-carcinogenic doses of dimethylarsinic acid (DMA[V]) in the transitional epithelium of the urinary bladder from female F344 Rats. The 23rd Annual Meeting of Toxicologic Pathology, Salt Lake City, Utah. June 13 - 17, 2004.

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J. Robertson, W. Huckle, **A. Wang**, A. Safaai-Jazi, W. Peng, Y. Zhang, W. Spillman, and K. Meissner. Utilization of unique Raman laser spectroscopic signature for detection of neoplastic disease in animals and humans. VMRCVM 15th Annual Research Symposium, Blacksburg, Virginia. June 5 - 6, 2003

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A. Wang, C.J. Pfeiffer, and D. Barber. Selenium protection against mercury toxicity in dolphin cells *in vitro*. 17th Annual Research Symposium of Virginia Tech, Blacksburg, Virginia. Mar. 26, 2001

D.C. Pfeiffer, **A. Wang**, and C.J. Pfeiffer. Lingual ultrastructure of the long-finned pilot whale (*Globicephala melas*). The 8th annual Atlantic Coastal Dolphin Conference, Wilmington, North Carolina. Mar. 24 - 26, 2000

A. Wang, L. Sharova, K. Carlson, and C.J. Pfeiffer. Effects of mercuric chloride on Atlantic spotted dolphin (*Stenella frontalis*) renal cells. The 13th Biennial Conference on the Biology of Marine Mammals, Maui, Hawaii. Nov. 28 - Dec. 3, 1999

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A. Wang, C.J. Pfeiffer, and L. Sharova. Effects of mercury on the cell proliferation and morphology of spotted dolphin (*Stenella frontalis*) cell line. Atlantic Coastal Dolphin Conference, Virginia Beach, Virginia. Mar. 19 - 21, 1999

OTHER PUBLICATIONS AND PUBLICATION-RELATED EXPERIENCE
Ad hoc reviewer, Toxicological Sciences, Oxford University Press, San Diego, California, 2003-2004.

Contributing author, Benesse Challenge magazine, Fukutake Publication Co., LTD. Taipei, Taiwan. 1997
- The monthly magazine and audio cassette are targeted to 1st to 3rd grade children for providing an interesting method of learning about nature

<u>Editor and Consultant</u>, Eastern Publication Company, Ltd., Taipei, Taiwan. 1993 - 1994
- Editor and consultant for *The Life of Birds*, a book of Sciences Cartoon Encyclopedia, a series of children's books, and winner of *Golden Tripod Award* for outstanding publications
- Provided information on bird biology, behavior, and bird-watching

GRANTS
- $250 from Graduate Research Development Project, Spring 2003. Awarded by Virginia Tech Graduate Student Assembly, Virginia Tech
- $2175 for Master's Thesis study, Fall 1999. Awarded by the Research Focus Unit for Environmental Medicine and Toxicology, VMRCVM
- $2380 for Master's Thesis study, Spring 1999. Awarded by the Department of Biomedical Sciences and Pathobiology, VMRCVM

SCHOLARSHIPS AND AWARDS
- Third Place Presentation Award (oral presentation competition) at the VMRCVM 19th Annual Research Symposium, 2007. Awarded by VMRCVM.
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- Bern Schwetz Student Travel Award (first place), 2005. Awarded by National Capital Area Chapter of Society of Toxicology
- Young Investigator Award (poster competition), 2004. Awarded by Society of Toxicologic Pathology
- Bern Schwetz Student Travel Award, 2004. Awarded by National Capital Area Chapter of Society of Toxicology
- Travel Fund Program Award, 2004. Awarded by Graduate Student Assembly of Virginia Tech
- Student Travel Award, 2003. Awarded by Society of Toxicological Pathology
- Student Travel Award, 1999. Awarded by Society for Marine Mammalogy
- Commencement Speaker, 1996. Selected by National Taiwan University
- President's Award, 1993. Awarded by National Taiwan University to top 5% students in each academic class
- Liu's Scholarship, 1993. Awarded by Liu's Memorial Foundation for distinct academic progress
- Ministry of Education Scholarship, 1992. Awarded by Taiwan government

RESEARCH EXPERIENCE
<u>Predoctoral Fellow/Ph.D Research,</u> Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, Virginia. Aug. 2000 – present
- Initiated and coordinated multi-laboratory research projects between U.S. Environmental Protection Agency (EPA) at Research Triangle Park, North Carolina (Molecular Toxicology Branch, Cancer Biology Branch, and Cellular Toxicology Branch) and Virginia Tech (Center of Comparative Oncology, Clinical Research Laboratory, Biochemistry/Pharmacology Laboratory, and Toxicology Laboratory)
- Developed an enzymatic method to selectively harvest urinary bladder transitional cells

- Designed and conducted research to measure arsenic effects on cellular and sub-cellular structures, DNA damage, DNA damage repair, and repair gene expression
- Collaborated with researchers at U.S. EPA to study arsenic effects on global gene expression, comparative gene expression between organs, oxidative stress, oxidative DNA damage, and chromosomal damage
- Utilized image analysis software
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Student Volunteer, Environmental Carcinogenesis Division, U.S. EPA, Research Triangle Park, North Carolina. May - July 2001; Aug. - Sept. 2003
- Ran real time RT PCR after extracting RNA
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Graduate Research Assistant, DNA Sequencing Facility, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, Virginia. Aug. - Dec. 2000
- Independently sequenced DNA samples submitted from various laboratories

Graduate Research Assistant, Electron Microscope Laboratory, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, Virginia. Aug. 1999 - May 2000
- Developed computerized inventory system specifically for electron microscope laboratory
- Developed and printed photographs taken under transmission electron microscope
- Assisted in other electron microscope related work

CLINICAL EXPERIENCE

Volunteer Veterinarian, Taiwan Cetacean Stranding Network, Taipei, Taiwan. Oct. 1996 - Dec. 1999
- Performed necropsies on stranded and by-caught cetaceans
- Provided medical advice
- Translated parts of *Marine Mammals Ashore* and other books from English to Chinese
- Assisted in organizing conferences and overseas workshops

Part-time Veterinary Clinician, National Taiwan University Veterinary Hospital, Taipei, Taiwan. Nov.1996 - June 1998
- Treated cats and dogs, including emergency and rehabilitation service

Veterinary Assistant, Pao-An Animal Hospital, Taipei, Taiwan. 1994 -1995
- Assisted in surgery, blood and fecal testing, and animal nursing
- Performed orchidectomy (neuter) and ovariohysterectomy (spay) of stray dogs at animal shelters

TEACHING EXPERIENCE

Instructor, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg, Virginia. May - Aug. 2003
- Instructed veterinary students in laboratory animal care, good laboratory practice, and experimental design as part of summer oncology training program

Graduate Assistant, Department of Biomedical Sciences and Pathobiology, VMRCVM,
    Blacksburg, Virginia. Jan. 2001 - Dec. 2004
- Assisted in teaching material preparation and laboratory lectures; gave review sections for
  Veterinary Histology, a core curriculum class for first year veterinary students
- Assisted in laboratory lectures for Veterinary Pathology, a core curriculum class for first
  year veterinary students
- Graded quizzes and assignments in Veterinary Neurobiology course

Speaker, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg,
    Virginia. Feb. 2000
- Lectured "Introduction to Manatee Biology and Medicine" in Marine Mammal
  Biomedicine course

Instructor, Department of Biomedical Sciences and Pathobiology, VMRCVM, Blacksburg,
    Virginia. June 1999 - Aug. 2000
- Instructed a veterinary student in cell culture technique, experimental design, and data
  analysis as part of a summer fellowship program
- Instructed a technician in cell culture technique

Graduate Teaching Assistant, Department of Biomedical Sciences and Pathobiology,
    VMRCVM, Blacksburg, Virginia. Jan. - June 1998
- Graded quizzes and assignments in Veterinary Neurobiology course

PROFESSIONAL MEMBERSHIPS
Society of Toxicology
- Chairperson for Student Symposium on Effective Presentation in Society of Toxicology
  43rd Annual Meeting, July 2003 - Mar. 2004
- Student Liaison to Continuing Education Committee, Mar. 2002 - Sept. 2003
- Chairperson for Lunch with an Expert in Society of Toxicology 42nd Annual Meeting,
  Mar. 2002 - Apr. 2003
- Chairperson for student and postdoctoral fellow mixer in Society of Toxicology 42nd
  Annual Meeting, Mar. 2002 - Apr. 2003
- Member, Feb. 2002 - present

Society of Toxicology, National Capital Area Chapel
- Student representative, Nov. 2001 - Apr. 2003
- Organized a one-day workshop for students, which became an annual event
- Student vice-representative, Apr. 2000 - Nov. 2001
- Member, Apr. 2000 - present

Society of Toxicologic Pathology, 2000 - present
Society for Marine Mammalogy, 1999 - 2003
International Association for Aquatic Animal Medicine (IAAAM), 1999 – 2003
Taipei Veterinary Medical Association, Taipei, Taiwan, 1996 - 1998

AFFILIATIONS
Veterinary Medicine Science Graduate Student Association, Virginia Tech
- Co-chairperson of Education Committee, Aug. 2000 - Aug. 2002
- Organized average 3 seminars per semester
- Member, Jan. 1999 - present

International Club, Virginia Tech
- Publicity Director, Aug. 2000 - Jan. 2001
- Member, Aug. 2000 - 2003

Chinese Student Association, Virginia Tech
- Acting President, Jan. - Aug. 1999
- Vice President, June - Dec. 1998
- Member, Jan. 1998 - present

China Youth Corps, Taiwan, Volunteer Medical Assistant, Feb. - Aug. 1996

Association for Public Studies, National Taiwan University
- Director of the Executive Board, Sept. 1994 - Sept. 1995
- Chairman of Orientation Committee, Feb. - Sept. 1994
- Chairman of Farewell Committee, Sept. 1993 - Sept. 1994
- Member, Feb. 1992 - June 1996

Taiwan Museum, Taiwan, Volunteer Guide, June - Sept. 1992
- Volunteer guide for the animal section

Parliamentary Procedure Study Camp, National Taiwan University, Counselor, Feb. 1992

CONTINUING EDUCATION
My Sister's Keeper Professional Development Retreat.
Virginia Tech, Blacksburg, Virginia. Mar. 26, 2007

Woman's Leadership Conference for Students
Virginia Tech, Blacksburg, Virginia. Mar. 18, 2006

Fundamentals of Nanotechnology: Chemistry, Exposure, Health/Environmental Assessments and
     Societal Impacts Continuing Education Course
Society of Toxicology, New Orleans, Louisiana. Mar. 6, 2005

Clinical Pathology – the Granddaddy of Biomarkers Continuing Education Course
Society of Toxicology, New Orleans, Louisiana. Mar. 6, 2005

Phototoxicity: Current Concepts, Experimental Designs, and Regulatory Expectations
     Continuing Education Course
Society of Toxicology, New Orleans, Louisiana. Mar. 6, 2005

Writing in the Sciences Workshop
National Capital Area Chapter, Society of Toxicology, Bethesda, Maryland. Nov. 3, 2004

Tools for Functional Genomics Continuing Education Course
Society of Toxicology, Baltimore, Maryland. Mar. 21, 2004

Herbals and Dietary Supplements in Athletic Performance Enhancement: Fact vs. Fiction
        Continuing Education Course
Society of Toxicology, Baltimore, Maryland. Mar. 21, 2004

Good Laboratory Practices Course
National Health and Environmental Effects Research Laboratory (NHEERL) Trainee
        Organization, U.S. EPA, Research Triangle Park, North Carolina. Sept. 10, 2003

Digital Microscopy and Analysis Continuing Education Course
Society of Toxicologic Pathology, Savannah, Georgia. June 15, 2003

Writing Successful Grants (While Avoiding Common Proposal Pitfalls) Workshop
Virginia Tech, Blacksburg, Virginia. May 23, 2003

Essential Informatics for Toxicologists Continuing Education Course
Society of Toxicology, Salt Lake City, Utah. Mar. 9, 2003

Epigenetics of Cancer Continuing Education Course
Society of Toxicology, Salt Lake City, Utah. Mar. 9, 2003

Internal Dosimetry: Measurement of DNA Damage as an Indicator of Internal Exposure to
        Genotoxicants Continuing Education Course
Society of Toxicology, Nashville, Tennessee. Mar. 17, 2002

How to Present Effectively Workshop
National Capital Area Chapter, Society of Toxicology, Washington, District of Columbia. Dec.
        12, 2002

Training the Future Professional Workshop
Research and Graduate Studies, Blacksburg, Virginia. Apr. 29, 2000

Presentation Skill Workshop
Center of Excellence Undergraduate Teaching, Blacksburg, Virginia. Mar. 11, 1998

Continuing Education in Veterinary Medicine
University of California at Davis, Davis, California. Aug. 8 - Sept. 2, 1994
  - 120 hours of lecture and laboratory on small animals

LANGUAGE SKILLS
  - Fluent in speaking and writing English and Mandarin/Chinese