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Research Article

Arsenate and Dimethylarsinic Acid in Drinking Water did not Affect DNA Damage Repair in Urinary Bladder Transitional Cells or Micronuclei in Bone Marrow

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Arsenic is a human skin, lung, and urinary bladder carcinogen, and may act as a cocarcinogen in the skin and urinary bladder. Possible modes of action of arsenic carcinogenesis/cocarcinogenesis include oxidative stress induction and inhibition of DNA damage repair. 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 cyclophosphamide (CP)-induced DNA damage resulting from a single oral gavage of CP, and the in vitro repair of hydrogen peroxide (H_2O_2)- or formaldehyde-induced DNA damage, resulting from adding H_2O_2 or formaldehyde into cell medium, were measured by the Comet assay. DMA(V) effects were not observed on either CP-induced DNA damage induction or on DNA repair. Neither DMA(V) nor As(V) increased the H_2O_2- or formaldehyde-induced DNA damage, and neither inhibited the repair of H_2O_2-induced DNA damage. Neither DMA(V) nor As(V) increased the micronucleus frequency, nor did they elevate micronucleus frequency resulting from CP treatment above the level observed by the treatment with CP alone. These results suggest that arsenic carcinogenesis/cocarcinogenesis in the urinary bladder may not be via DNA damage repair inhibition. To our knowledge this is the first report of arsenic effects on DNA damage repair in the urinary bladder. Environ. Mol. Mutagen. 50:760–770, 2009. Published 2009 by Wiley-Liss, Inc.*

Key words: arsenic; DNA repair; urothelium; micronucleus; comet assay

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 inorganic arsenic in the forms of arsenite [As(III)] and arsenate [As(V)], in drinking water increases the 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], and urinary bladder (transitional cell carcinoma) [Chiou et al., 2001]. In addition, arsenic may act as a cocarcinogen in the urinary bladder (with cigarette smoking) [Chiou et al., 2001] and skin (with UV light exposure) [Rossman et al., 2004].

The study of arsenic carcinogenesis is complicated. The metabolism of inorganic arsenic into various organic

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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 [Kligerman and Tennant, 2006; Wang et al., 2007b]. For example, humans and rodents metabolize As(V) into As(III), and 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, but 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. In humans, increased incidences of urinary bladder cancer were seen after exposure to inorganic arsenic, but in laboratory animals, significantly increased incidences of urinary bladder cancer have only been observed in F344 rats exposed to DMA(V) [Wang et al., 2002]. Rats can tolerate much higher doses of inorganic arsenic than humans, and this is at least partially because of the binding of DMA(III) to red blood cells in rats [Lu et al., 2004; Cohen et al., 2007].

Several modes of action of arsenic carcinogenesis have been proposed. They include (1) oxidative stress induction, (2) DNA damage repair inhibition, (3) signal transduction pathway modulation, (4) cell proliferation increases (altered growth factors and regenerative proliferation), (5) induction of chromosomal abnormalities, and (6) DNA methylation alteration [Kitchin, 2001; Schoen et al., 2004]. These possible modes of action are not mutually exclusive. For example, arsenic-induced oxidative stress can lead to chromosomal abnormalities [Ho et al., 2000]. Furthermore, the mode of action of arsenic carcinogenesis is believed to be tissue-specific. For instance, arsenic may have a different mode of action in the bladder than in other organs because of the mixture of arsenic metabolites in the urine [Rossman, 2003].

Because arsenic is reported to be 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 [Vogt and Rossman, 2001; Rossman et al., 2004]. Arsenic-inhibited DNA damage repair has been reported in cultured skin and lung cells [Schwerdtle et al., 2003; Wu et al., 2005], but arsenic effects on DNA repair have not been studied in urinary bladder cells. Decreases in expression of DNA repair genes were observed in cultured cells exposed to noncytotoxic concentrations of arsenic [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 repair of arsenic-induced oxidative damage. Recently, decreases in DNA damage repair and DNA repair gene expression were observed in lymphocytes collected from arsenic-exposed humans [Andrew et al., 2003; Andrew et al., 2006].

The view that decreased DNA damage repair may contribute to urinary bladder cancer risk is supported 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, but not necessarily activity, 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 as compared to nonsmokers in the arsenic-exposed population [Chiou et al., 2001] 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 [Cohen et al., 2001; Wei et al., 2002] provide evidence for the following mode of action of DMA(V)-induced bladder cancer [Sams et al., 2007]. First, DMA(V) is reduced to DMA(III), and DMA(III) causes cytotoxicity in the urothelium [Cohen et al., 2001, 2002; Sams et al., 2007]. 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 the present study was to investigate if 1-week oral exposure to DMA(V) or As(V) inhibits DNA damage repair in urinary bladder transitional cells, a target cell of arsenic carcinogenesis. DMA(V) was chosen because it can induce bladder cancer in rats, and As(V) was investigated as it is one of the common forms of arsenic that humans ingest in drinking water. Three types of DNA damage 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 cells of various tissues, and MN have been suggested as a biomarker of arsenic exposure [Moore et al., 1997; Feng et al., 2001; Tian et al., 2001; Basu et al., 2004; Chakraborty et al., 2006].

MATERIALS AND METHODS

Chemicals

Arsenic [IARC, 2004], CP [IARC, 1987], 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 treatments, DMA(V) [$(CH_3)_2As(O)OH$] (CAS no. 75-60-5O), purity > 99%, was purchased from Sigma, St. Louis, MO. 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, OH. Isoflurane was from Abbott Animal Health, North Chicago, IL, and Beuthanasia was obtained from Schering-Plough Animal Health Corporation, Union, NJ.

For terminal surgeries to harvest cells, Isoflurane (CAS no. 26675-46-7) was purchased from Abbott Animal Health, North Chicago, IL. Beuthanasia [390 mg pentobarbital sodium (barbituric acid derivative) and 50 mg phenytoin sodium per ml of Beuthanasia] (CAS no. 8024-20-2) was obtained from Schering-Plough Animal Health Corporation, Union, NJ.

For the Comet assay, Williams' medium E, 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, Walkersville, MD and Atlanta Biologicals, Lawrenceville, Ga. 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, NY. Comet LMAgarose (low melting temperature agarose), Comet Lysis Solution, and FLARE slides (each slide has three sample loading areas precoated with agarose) were purchased from Trevigen, Gaithersburg, MD. Ethanol (200 proof ethyl alcohol) was from AAPER, Shelbyville, KY.

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 purchased 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 before the initiation of the study.

Female F344 rats, age 5–9 weeks old, were purchased from Harlan Teklad (Madison, WI). 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 [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, TN) 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 1 week before the beginning of in vivo dosing. Teklad 2018 SC diet (Harlan Teklad, Madison, WI) and tap water in plastic water bottles with stainless steel sipper tubes with stoppers were available *ad libitum*.

Animal Treatments and Experimental Design (See Table I)

The experiment consisted of eight animal treatment groups, established to provide data to test the response to three types of DNA damage. With the exception of the negative control groups G, with four rats, and H, with three rats, all other groups consisted of six rats (Table I). 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) 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 via drinking water for 7 days with either 100 ppm DMA(V) [Groups A through D], or 100 ppm As(V) [Group I]. The concentration of DMA(V) was chosen because of the increase in bladder transitional cell carcinoma in rats exposed to 100 ppm DMA(V) in 2 year exposure studies [Wei et al., 1999; Arnold et al., 2006], and the As(V) concentration was chosen to provide comparison to DMA(V) exposure. A single oral gavage of CP (11.75 mg CP/kg BW, equivalent to 1/8 LD_{50}) was given at the end of 7-day exposure to DMA(V), and animals were allowed to recover for either 1 day [Group A] or 5 days [Group B] before the terminal surgery to collect urinary bladder cells and bone marrow. During the surgery, the rat was anesthetized by isoflurane inhalation, and the urinary bladder was treated and removed (see below Comet assay with transitional cells to detect DNA damage and repair and [Wang et al., 2007a]). The rat was then euthanized with an intracardiac injection of Beuthanasia, and the bone marrow was harvested. 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., 2001]. The dose of CP (1/8 LD_{50}) was determined by preliminary experiments to be noncytotoxic to urinary bladder transitional cells based on trypan blue exclusion assay;* furthermore, CP-induced DNA damage in transitional cells was detected 24 hr after the gavage of CP, and was not observed 5 days later as measured by alkaline Comet assay (unpublished data). Solutions of DMA(V) (100 ppm) and As(V) (100 ppm) were made weekly with tap water to

TABLE I. 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 the 7-day treatment, certain rats received a single oral gavage of CP (Groups A, B, E, and F). Rats were given tap water during the recovery period. Urinary bladder transitional cells collected from rats in Groups A, B, D, E, and F received no additional treatment. Portions of transitional 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°C for 4 h. For measurements of MN and polychromatic erythrocytes frequencies, bone marrow were collected from negative control rats (Groups G and H) and rats exposed to DMA(V) +CP (Group A), DMA(V) (Group C), CP (positive control, Group E), or As(V) (Group I) 1 day after the last in vivo dosing. *indicates the group from which bone marrow was collected after in vivo dosing and in vivo recovery (without in vitro dosing or recovery)

	For in vivo repair		For in vitro repair	
Group	In vivo dosing	In vivo Recovery (days)	In vitro dosing	In vitro Recovery (hrs)
A*	DMA(V) + CP	1	—	—
B	DMA(V) + CP	5	—	—
C*	DMA(V)	1	H_2O_2, Formaldehyde	0, 4
D	DMA(V)	5	—	—
E*	Tap water + CP	1	—	—
F	Tap water + CP	0	—	—
G*	Tap water	1	H_2O_2, Formaldehyde	0, 4
H*	Tap water	5	H_2O_2, Formaldehyde	0, 4
I*	As(V)	1	H_2O_2, Formaldehyde	0, 4

*While trypan blue exclusion assay is widely used and often recommended for viability checks before the Comet assay, it is a less rigorous estimation of cell survival than the clonal survival assay [Komissarova et al., 2005]. Therefore it is possible that the treatment [CP, H_2O_2, formaldehyde, or DMA(V)] could have caused a lower viability when measured in more sensitive assays, such as the clonal survival assay, than in the trypan blue exclusion assay.

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 weekly and stored at 4°C in the dark to minimize degradation [Beijnen et al., 1992].

The experiment was performed in six blocks, based on our capacity for surgery and the number of commensurate Comet assays that could be performed. 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. After quarantine and acclimation, rats were assigned to one of the six blocks by age, so all rats in a block were the same age. 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) were for background DNA damage at each time point, and Groups E and F (tap water + CP, 1 or 5 day recovery) provided 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 hr of in vitro recovery. Both the H_2O_2 and formaldehyde in vitro dosings were in non-cytotoxic, but genotoxic ranges. H_2O_2-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 bone marrow from rats exposed to DMA(V) or As(V) (Groups C and I) to bone marrow 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 bone marrow from rats exposed to DMA(V) or DMA(V)+CP (Groups C and A) provided data on whether DMA(V) increased CP-induced MN.

Comet Assay With Transitional Cells to Detect DNA Damage and Repair

Methods for selective harvest of viable urinary bladder transitional cells and detailed protocols for the Comet assay in these cells were previously developed [Wang et al., 2007a]. To prevent UV light-induced DNA damage, all procedures (except after dehydration by ethanol and before staining by SYBR gold) were done under UV-free light sources. Briefly, the bladder was rinsed, inflated with a solution containing trypsin and Na_2EDTA while the rat was under anesthesia. The inflated bladder was then separated from the rat and incubated for 30 min before being gently scraped to remove transitional epithelium. The transitional cells were resuspended in Williams' E medium supplemented with 10% FBS and 2 mM L-glutamine (W+S), centrifuged, and tested by trypan blue exclusion assay for an indicator of cell viability.

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 from each in vitro dosing were resuspended in W+S and incubated at 37°C for 4 hr to recover from the H_2O_2, formaldehyde, or PBS (negative control) treatments before 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 the 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.

Cells were resuspended in LMAgarose at 37°C and were loaded onto FLARE slides. For the standard alkaline Comet assay, the cells were lysed for 1 hr, and the DNA was unwound in alkaline solution (pH >13) for 30 min. Electrophoresis with alkaline solution was performed at 40 V (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°C. The unwinding step was 20 min, and electrophoresis was at 32 V (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 knowledge of treatments using image-analysis software Komet 5.5 (Kinetic Imaging, 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 in Comet assays. 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.

Bone Marrow MN

MN frequencies were measured in the bone marrow of rats exposed to water (Groups G and H), and all rats that had 1 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 containing 25 mM EDTA, which was added to prevent cell clumping. Cells were pelleted by centrifugation at 220 *g* for 5 min at room temperature and resuspended with ~1 ml FBS containing 25 mM EDTA. A bone marrow smear was prepared on a glass slide 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 with acridine orange, and scored using a fluorescent microscope. Two slides from the same rat were examined by two people independently without knowledge of treatments. Each person scored 1,000 polychromatic erythrocytes (reticulocytes; immature erythrocytes) per slide for MN frequencies, and 2,000 erythrocytes per slide for the percentage of polychromatic erythrocytes (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, Cary, NC, except the percentages of polychromatic erythrocytes, which were tested using Statgraphics® Plus 5.1 statistical package from Manugistics, Rockville, MD.

For testing in vivo repair of CP-induced DNA damage, analysis of variance (ANOVA) and the Tukey posthoc test were used on arcsine transformed percentages of DNA in the tail in Comet assays. For testing in vitro repair of H_2O_2-induced DNA damage ANOVA was used on untransformed percentages of DNA in the tail in Comet assays. For testing in vitro repair of formaldehyde-induced DNA damage, the arcsine transformed percentages of DNA in the tail in Comet assays were tested by ANOVA and contrast test.

For MN frequencies, ANOVA and the Tukey posthoc test were used on arcsine transformed data. Percentages of polychromatic erythrocytes

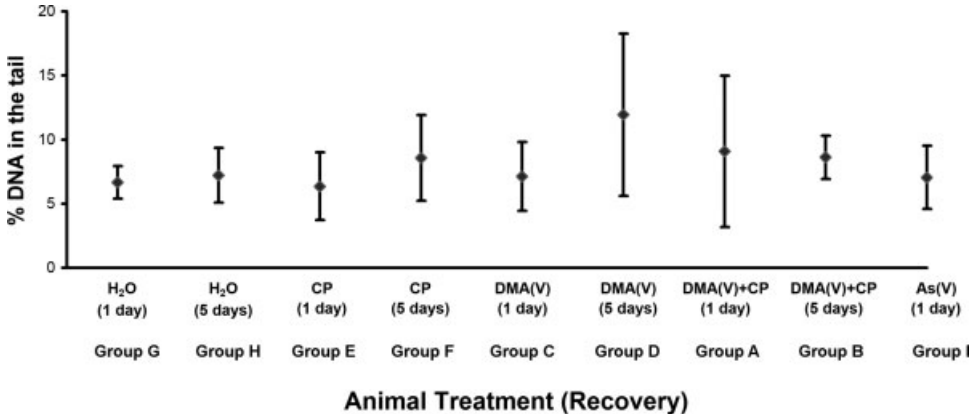

Fig. 1. DNA migration in the alkaline Comet assay without Proteinase K in the urinary bladder transitional cells was the same among rats exposed to in vivo dosings of water (negative control), CP, DMA(V), DMA(V)+CP, or As(V). DNA migration level after 5-day recovery following the in vivo dosing was not different from the DNA migration level after 1-day recovery of the same in vivo dosing. The results showed that neither DMA(V) nor As(V) increased DNA damage in transitional cells, or that the damage was repaired within 1 day. Each diamond indicates the average percentage of DNA in the ''Comet tail'' for each group, and the error bar indicates standard deviation.

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 I), 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 low viability (26%) may have been because of insufficient trypsin and EDTA treatment during the cell collection process, and samples with less than 60% viability were excluded. Overall, the viability results suggested that the animal treatments were at worst only mildly cytotoxic 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 hr of 37°C 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 essentially noncytotoxic. Furthermore, most cells had greater than 70% viability after a 4-hr recovery, with only a few samples with 53 to 67% viability.

DNA Damage Repair in Urinary Bladder Transitional Cells

In Vivo CP-Induced Damage

None of the in vivo dosing regimens significantly increased DNA migration in the alkaline Comet assay after a 1-day in vivo recovery (Groups A, C, E, G, and I) (Fig. 1), 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 within 24 hr. There was also no difference in DNA migration in the Comet assay between 1 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 24 hr.

In Vitro H_2O_2-Induced Damage

In the test of 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 hr before being subjected to the standard alkaline Comet assay (Fig. 2).

In the test of whether H_2O_2 increased DNA damage and whether any in vivo dosing increased cellular sensitivity to H_2O_2 genotoxicity, DNA migrations in the Comet assay from all samples with 0 hr in vitro recovery (with or without H_2O_2 treatment) were tested by an 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.

In the test of whether repair of H_2O_2-induced DNA damage was affected by in vivo exposure to As(V) or DMA(V), DNA migrations in the Comet assay from samples treated with H_2O_2 after 4 hr in vitro recovery were subjected to an ANOVA. There were no significant in vivo dosing effects, indicating DNA damage levels after 4 hr of recovery were the same among H_2O_2-treated cells

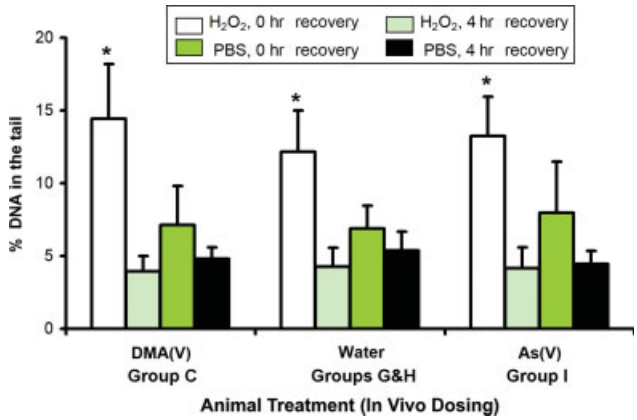

Fig. 2. The repair of in vitro H_2O_2-induced DNA damage in urinary bladder transitional cells was not affected by DMA(V) or As(V) exposures through drinking water. Transitional cells from rats exposed to 100 ppm DMA(V) (Group C), 100 ppm As(V) (Group I) in drinking water for 7 days had the same levels of DNA damage as cells from rats exposed to tap water (Groups G and H). After in vitro exposure to H_2O_2, DNA damage levels in all four groups were the same. * indicates significant difference from PBS-treated cells at the same recovery time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

collected from rats exposed in vivo to DMA(V), As(V), or tap water. These data showed that no effects from 7-day in vivo exposures to DMA(V) or As(V) on the repair of in vitro H_2O_2-induced DNA damage were observed.

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 hr. Comet assays with and without Proteinase K were performed, and the results are shown in Figure 3. The bulky protein in DNA-protein crosslinks decreases DNA migration rate in Comet assay, so cells with DNA-protein crosslinks show decreased DNA migration in Comet assay. Proteinase K removes protein and frees the previously protein-crosslinked DNA to migrate at a regular rate, but Proteinase K does not affect DNA or DNA-DNA crosslinks. Therefore, the differences in DNA migration in Comet assays with and without Proteinase K provide an indication of DNA-protein crosslink levels. In our study, the effects of formaldehyde treatment (in vitro dosing), in vitro recovery, and Proteinase K treatment were significant, indicating that these treatments affected DNA migration in Comet assays through the induction of DNA-protein crosslinks, and their subsequent removal by Proteinase K. However, the effects of in vivo dosing with the arsenicals were not statistically significant, indicating that neither As(V) nor DMA(V) affected the DNA migration in Comet assays.

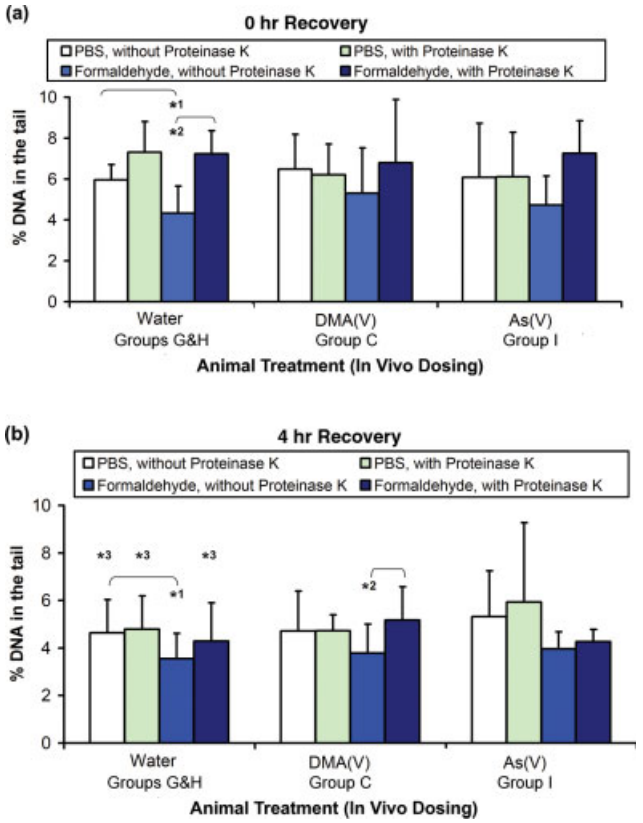

Fig. 3. DNA migration in formaldehyde-treated and control urinary bladder transitional cells after 0 hr (**a**) or 4 hr (**b**) of recovery. The animal treatments were the same as in Figure 2. In negative controls (Groups G and H), formaldehyde decreased DNA migration in the Comet assay without Proteinase K at both 0- and 4-hr recoveries. When Proteinase K was used there were significant increases in DNA migration in the Comet assay at 0-hr recovery. This suggested the presence of DNA-protein crosslinks after formaldehyde treatment. In DMA(V)- and As(V)-treated rats (Groups C and I), in vitro formaldehyde caused decreases in DNA migration in the Comet assay without Proteinase K, but these did not reach statistical significance. *1 indicates a significant difference from PBS-treated cells (same Comet assay, at the same recovery time), *2 indicates a significant difference from Comet assays with Proteinase K (same sample, at the same recovery time), *3 indicates a significant difference from the same sample in the same Comet assay test at 0-hr recovery. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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 hr. This indicated that DNA-protein crosslinks were induced by formaldehyde. After 4 hr of recovery, formaldehyde-treated cells from rats exposed to tap water did not exhibit significant differences in DNA migrations in Comet assays with and without Proteinase K, indicating the lack of DNA-protein crosslinks. Because DNA migration in the Comet assays was also significantly decreased within 4 hr 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 in Comet assays without Proteinase K, and increases after Proteinase K treatment did not reach the 0.05 level of significance. However, the same trends (slight decreases in DNA migration in formaldehyde-treated cells as compared to PBS-treated cells, and slight increases in DNA migration by Proteinase K treatment in formaldehyde-treated cells as compared to no Proteinase K treatment) were seen in those animals drinking tap water. The exact reasons are unknown but may be because of the slightly higher variances seen in the animals on DMA(V) and As(V).

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) as compared to the MN frequencies in those 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 vs. CP alone (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 or A, respectively) as 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 the present 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, may have inactivated p53 protein because of 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 [Kircelli et al., 2007]. Rats used in our study have normal expression of wild-type p53, and the status of p53 was not tested in the primary cell cultures of urinary bladder transitional cells. 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 reduced 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 reduced 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 repair of specific types of DNA damage. Previously reported arsenic-inhibited DNA damage repair pathways include (1) base excision repair (BER) [Le and Weinfeld, 2004], (2) nucleotide excision repair (NER) [Wu et al., 2005; Andrew et al., 2006], and (3) nonhomologous end joining or homologous recombination. The mismatch repair pathway was probably not affected by arsenic because microsatellite instability was not altered in DMA(V)-induced rat bladder transitional cell carcinoma [Wei et al., 2002]. Our observation that neither DMA(V) nor As(V) affected the repair of H_2O_2-induced DNA damage suggests that BER in the urinary bladder transitional cells was not significantly affected by 100 ppm of these arsenicals. Alternatively, effects on the BER pathway could be transitory and thus not seen in cells removed and treated in vitro with DNA damaging agents in our study. It is also possible that DNA repair in urinary bladder transitional cells is only affected by a longer-term exposure to arsenic than the 1-week exposure in this study. Similarly, formaldehyde-induced DNA-protein crosslinks are believed to be removed partially by spontaneous hydrolysis [Quievryn and Zhitkovich, 2000] and by NER, proteasome-assisted proteolysis followed by NER, or homologous recombination repair with NER components [Barker et al., 2005]. Although both DMA(V) and its metabolite, DMA(III), decreased NER in a cultured human lung carcinoma cell line, A549 cells [Schwerdtle et al., 2003], in our study DMA(V) exposure through drinking water did not affect the in vitro repair or formation of formaldehyde-induced DNA-protein crosslinks in urinary bladder transitional cells.

In Schwerdtle's study [Schwerdtle et al., 2003], A549 cells were exposed to DMA(V) for 16 hr, and then to

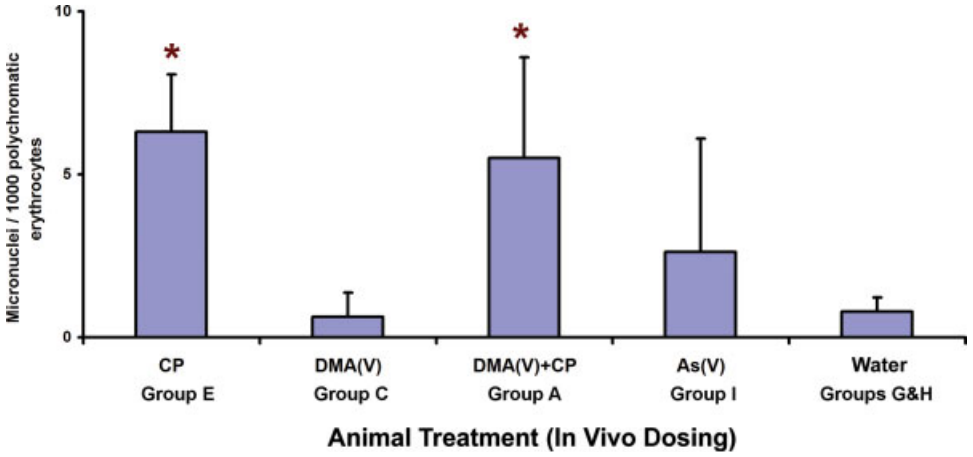

Fig. 4. The frequencies of MN in bone marrow were not affected by 7-day exposures to DMA(V) or As(V) through drinking water. CP (positive control, Group E) and DMA(V)+CP exposures (Group A) significantly increased MN frequencies as compared to tap water exposure (negative control, Groups G and H). The MN frequencies were not significantly different among rats exposed to CP and DMA(V)+CP, indicating that DMA(V) did not enhance CP effects in inducing MN. The MN frequencies were also not significantly different among rats exposed to DMA(V) (Group C), As(V) (Group I) and tap water, demonstrating that neither DMA(V) nor As(V) increased MN. * indicates a significant difference from negative controls [rats exposed to tap water (Groups G and H)]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

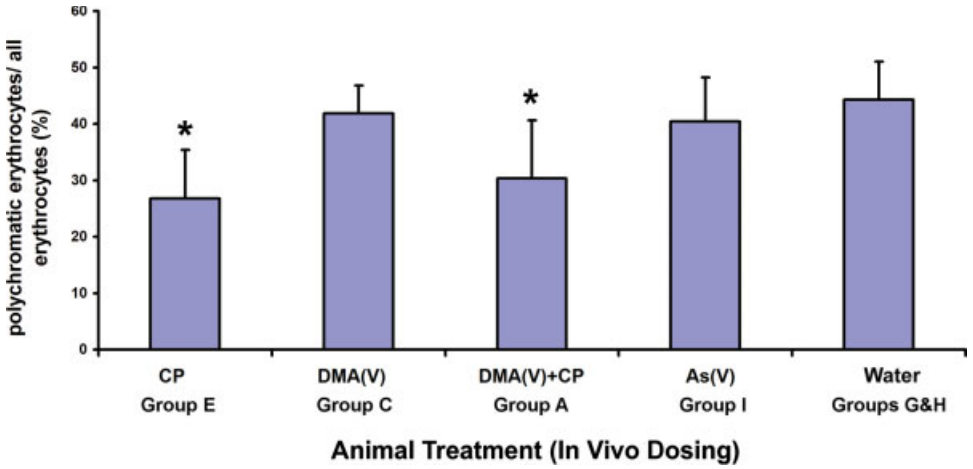

Fig. 5. Erythrocyte production was not affected by 7-day exposure to DMA(V) or As(V) in drinking water. Exposures to CP (positive control, Group E) and DMA(V)+CP (Group A) significantly decreased polychromatic erythrocyte percentages as compared to tap water exposure (negative control, Groups G and H), and the polychromatic erythrocyte percentages were not significantly different between CP and DMA(V)+CP exposures, suggesting that DMA(V) did not augment CP-induced decreases in erythrocyte production. The polychromatic erythrocyte percentages were not significantly different among rats exposed to DMA(V) (group C), As(V) (Group I) and tap water(Groups G and H), showing that neither DMA(V) nor As(V) decreased erythrocyte production. * indicates a significant difference from negative controls [rats exposed to tap water (Groups G and H)]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(+)-antibenzo[a]pyrene-7,8-diol 9,10-epoxide (BPDE) for 2 hr, followed by 8 hr of recovery in the presence of DMA(V). The repair of BPDE-DNA adducts was decreased at 250 μM DMA(V). Meanwhile, 30 min exposure to 10 mM DMA(V) caused only a slight increase in zinc release from synthesized XPA peptide (XPAzf) representing the zinc finger domain from xeroderma pigmentosum group A protein (XPA, a human zinc finger protein, involved in the recognition/incision step of NER), and no effect on the repair activity of isolated Fpg protein (an *Escherichia coli* zinc finger protein, involved in initiating BER). It cannot be ruled out that NER component(s) responsible for observed decreased NER in A459 cells were not involved in the removal of DNA-protein crosslinks, which could contribute to the lack of inhibition of DNA-protein crosslinks seen in our study. Compared to our study [100 ppm (0.72 μM) DMA(V) in water for 1 week and recovery for 24 hr in the absence of DMA(V)],

Schwerdtle's study used a much shorter exposure of a higher concentration of DMA(V) [16 hr of exposure to 250 μM DMA(V), and then 8 hr recovery in the presence of DMA(V)]. When female F344 rats were given 100 ppm DMA(V) in water for 14 days, dimethylated arsenicals were ~10 ppm in urine and less than 1 ppm in urinary bladder, while trimethylated arsenicals were higher than 90 ppm in urine and approximate 3 ppm in urinary bladder [Adair et al., 2007]. This suggested that urinary bladder cells were exposed to significantly less than 100 ppm DMA(V) in our study. While out study did not show inhibition of DNA repair, it does not necessarily contradict Schwerdtle's study (2003).

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 hr 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 six 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], increased necrosis and exfoliation in the urothelium were observed under scanning electron microscope after 1-week exposure to 100 ppm DMA(V), and necrotic and exfoliated (severely damaged or dead) cells may 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 hr through DNA repair or loss of heavily damaged cells via exfoliation.

The effects of arsenic on the MN frequency depend on the arsenic species and exposure doses. Inorganic arsenic in drinking water [a mixture of As(III) and As(V)] increased MN frequencies in various tissues/cells of humans (exfoliated bladder cells in China [Tian et al., 2001], Chile [Moore et al., 1997], India [Basu et al., 2004], and USA [Warner et al., 1994]; buccal mucosal cells in China [Feng et al., 2001; Tian et al., 2001] and India [Chakraborty et al., 2006]; airway epithelial cells in sputum in China [Tian et al., 2001]). In these studies, the concentrations of arsenic in water in exposed or high exposure groups ranged from 66.75 μg/l [Chakraborty et al., 2006], 215 μg/l [Basu et al., 2004] to 500–1000 μg/l [Feng et al., 2001; Moore et al., 1997; Tian et al., 2001] or even above 1.3 mg/l [Warner et al., 1994], and the arsenic concentrations in low exposure or control ranged from 15 μg/l to no exposure. As(III) increased bone marrow MN frequencies in mice that received As(III) at 50 mg/l in drinking water for 7 days [Lewinska et al., 2007] and rats that were intraperitoneally injected with As(III) at 5–20 mg /kg body weight /day for 5 days [Patlolla and Tchounwou, 2005; Lewinska et al., 2007], but the frequency of MN was not increased by As(V) in mice exposed to 50, 200 or 500 μg As(V)/l in water for 12 months [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 after exposure at 5 mM for 1 hr, 10 times the cytotoxic concentration (500 μM for 1 hr), in Chinese hamster ovary (CHO-9) cells [Dopp et al., 2004]. Although rats show a higher tolerance to arsenic toxicity and higher arsenic (namely DMA(III)) binding to red blood cells than humans, rats are still considered a good animal model for arsenic toxicity/carcinogenicity studies [Sams et al., 2007]. Furthermore, F344 rats are the only laboratory animals which have shown arsenic-induced urinary bladder cancer. 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 Chinese hamster ovary (CHO) cells 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 coinjections 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.

In summary, F344 rats exposed to 100 ppm 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, or in the repair of H_2O_2-induced DNA damage in 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. It also cannot be ruled out that longer exposures to lower concentrations of arsenic, closer to human exposure conditions than our 1 week exposure study, may cause DNA repair inhibition in urinary bladder transitional cells.

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