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Running title: Arsenic decreases DNA repair

Key words: arsenic, arsenite, DNA repair, ERCC1, molecular epidemiology, nucleotide excision repair

Abbreviations:
2-AAAF - 2-acetoxyacetylaminofluorene
BAP - benzo(a)pyrene
ERCC1 - excision repair cross complement 1
h – hours
MCL - maximum contaminant level
μg/L - micrograms per liter
μM – micromolar
PAH – polycyclic aromatic hydrocarbon
UVR - ultraviolet radiation

Article descriptor: DNA repair

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Abstract
The mechanism(s) by which arsenic exposure contributes to human cancer risk is unknown. However, several indirect co-carcinogenesis mechanisms have been proposed. Many studies support the role of arsenic in altering one or more DNA repair processes. The current study utilizes individual level exposure data and biologic samples to investigate the effects of arsenic exposure on nucleotide excision repair in two study populations, focusing on the excision repair cross complement 1 (ERCC1) component. We measured drinking water, urinary or toenail arsenic levels, and obtained cryo-preserved lymphocytes on a subset of individuals enrolled in epidemiologic studies in New Hampshire, U.S. and Sonora, Mexico. Additionally, in corroborative laboratory studies we examined the effects of arsenic on DNA repair in a cultured human cell models. Arsenic exposure was associated with decreased expression of ERCC1 in isolated lymphocytes at the mRNA and protein levels. In addition, lymphocytes from arsenic-exposed individuals showed higher levels of DNA damage, as measured by a Comet assay, both at baseline and following a 2-acetoxyacetylaminofluorene (2-AAAF) challenge. In support of the *in vivo* data, arsenic exposure decreased ERCC1 mRNA expression and enhanced levels of DNA damage following a 2-AAAF challenge in cell culture. These data provide further evidence to support the ability of arsenic to inhibit the DNA repair machinery, which is likely to enhance the genotoxicity and mutagenicity of other directly genotoxic compounds, as part of a co-carcinogenic mechanism of action.

Background

Arsenic is an established lung, skin and bladder carcinogen (IARC 2004), however the carcinogenic mechanisms are currently under investigation. Based primarily on studies of highly exposed populations in Taiwan and elsewhere, the U.S. EPA recently reduced the maximum contaminant level (MCL) standard for arsenic in drinking water from 50 μg/L to10 μg/L. At the lower end of the dose-response curve, the biologic effects and magnitude of disease risk in the human population remain unknown (Abernathy et al. 1999). However, a growing number of laboratory studies, both in cell culture and in experimental animals, have demonstrated biological effects of arsenic at very low levels equivalent to those below the new 10 μg/L standard. These effects include endocrine disruption, altered cell signaling, altered cell cycle kinetics, alterations in proliferative response, and other effects that may be associated with carcinogenesis and other disease processes (reviewed in (Rossman 2003)). Thus, it is important to understand the potential adverse effects of such exposure in the human population.

An estimated 2% of the drinking water serving US households contains 2 μg/L arsenic or more (NRC 2001). Approximately 40% of households in the state of New Hampshire are served by unregulated private wells, with homeowner financed, optional contaminant testing. Moreover, until recent studies revealed the extent of geologic arsenic contamination of drinking water in the state, arsenic was not part of the standard laboratory water testing panel. Case-control studies of bladder and skin cancer conducted in the New Hampshire population have detected evidence of elevated cancer risks. For bladder cancer, an excess risk was observed primarily among smokers exposed to arsenic in the drinking water, supporting hypotheses that these levels of arsenic are co-carcinogenic (Karagas et al. 2001, 2004). Likewise, drinking water in the Southwestern US and Northern Mexico contains arsenic, at concentrations above the new MCL of 10 μg/L (Meza et al. 2004).

The precise mechanism of arsenic co-carcinogenesis is unknown. It has been difficult to detect genotoxic effects of arsenic *per se* at environmental levels (ATSDR 1999; IARC 2004; Rossman 2003). However, many studies support the role of arsenic in altering one or more DNA repair processes (Gomez-Caminero et al. 2001). Arsenic has been shown to potentiate the genotoxicity of other organic mutagen-carcinogens, particularly polycyclic aromatic hydrocarbons (PAHs) including benzo(a)pyrene (BAP) and ultraviolet radiation (UVR) (ATSDR 1999; Rossman 2003). Rats treated with arsenic and BAP sustained adduct burdens longer than rats treated with BAP alone, suggesting impairment of DNA repair by arsenic as a possible mechanism (Tran et al. 2002). A study using human fibroblasts found that low (2.5 μM, approximately 180 μg/L) concentrations of arsenite reduced nucleotide excision repair efficiency, and incision frequency in particular, following UVR exposure (Hartwig et al. 1997). Results of another study in cultured human fibroblasts indicated that arsenic exposure reduced DNA repair capacity as measured by the Comet assay (Curnow et al. 2001). The effects of arsenic are strongly dose-, time-, and species-dependent (Barchowsky et al. 1999; Gomez-Caminero et al. 2001). In particular, several arsenic-induced effects exhibit a biphasic characteristic. For example, low (at or below 1-2 μM) doses of arsenic in cell culture increase cell proliferation and enhance endocrine signaling, whereas higher but still non-cytotoxic doses (2-5 μM) suppress these same pathways (Barchowsky et al. 1999). Likewise, patterns of altered gene expression as detected by DNA microarray studies demonstrated very different patterns at low versus higher doses (Andrew et al. 2003b). Thus, while animal and cell culture studies provide controlled model systems for mechanistic studies of arsenic, it is critical to understand which of these findings translate into cellular, molecular and clinical effects in actual human exposure situations, and the role of these in pathophysiological processes such as carcinogenesis. In our preliminary study of human lymphocytes from individuals exposed to drinking water arsenic, arsenic exposure was correlated in a strongly dose-dependent manner to

decreased expression of three nucleotide excision repair genes: *ERCC1*, *XPB*, *XPF* (Andrew et al. 2003a).

Objectives

The objective of this investigation was to evaluate our preliminary observation of an association between arsenic exposure, focusing on *ERCC1* gene expression levels in a larger number of individuals with exposure data and biologic samples. In addition to gene expression, the current study investigates the effects of arsenic exposure at both the protein and DNA repair functional levels. We then extended our investigation into another population exposed to similar levels of arsenic in Mexico and also performed i*n vitro* arsenic experiments to validate our results in a controlled system.

Materials and Methods

Human Populations:

New Hampshire - We selected subjects from an ongoing epidemiologic study of bladder cancer in New Hampshire (Karagas et al. 1998, 2004). Selection was based on high or low arsenic exposure from individuals on whom we had collected cryopreserved lymphocytes. Within this subset (*n*=37), the drinking water arsenic levels of the low exposure group averaged 0.7 μg/L, range (0.007 –5.3 μg/L), while the high exposure group averaged 32 μg/L, range (10.4 – 74.7 μg/L). Data on subject's exposure history were available through a personal interview covering demographic information, history of tobacco use and other lifestyle factors. Informed consent was obtained from each participant and all procedures and study materials were approved by the Committee for the Protection of Human Subjects at Dartmouth College.

Subjects agreed to provide a venous blood sample that is drawn into cell prep tubes (CPT) containing citrate and a lymphocyte isolation gradient. Blood tubes are maintained at 4°C and sent to the study laboratory for processing and analysis. No later than 24 hours following the blood draw, the lymphocytes collected in CPT tubes containing sodium citrate are isolated according to the manufacturers instructions using standard buoyant density centrifugation methods. Following centrifugation, first plasma is removed, aliquoted and frozen at -80°C, then the mononuclear cells are removed by pipette and cryopreserved (-120˚C) using freezing media at a controlled rate of 1˚C per minute. This procedure has previously been demonstrated to ensure approximately 90% viability after thawing (Wei et al. 1994).

Additionally, toenail clipping samples collected at the time of interview were analyzed for arsenic and other trace elements by Instrumental Neutron Activation Analysis (INAA) at the University of Missouri Research Reactor, using a standard comparison approach as described previously (Cheng et al. 1995). The detection limit for arsenic measured by INAA is approximately 0.001 μg/g. A water sample from the current household drawn into commercially washed (mineral free) high density polyethylene bottles that meet EPA standards for water collection (I-Chem) were analyzed for arsenic concentration using an Agilent 7500c Octopole ICPMS in the Dartmouth Trace Element Analysis Core Facility (REFS).

Sonora, Mexico - Subjects were recruited in 2004 from several towns in the Yaqui Valley of Sonora, Mexico by contact through local healthcare officials, after attending an informational meeting in their hometowns, as described previously (Meza et al. 2004). The current study involved a subset of subjects who provided biologic samples (n=16). They ranged in age from 23 to 63 years of age and were in good health (self reported and by physical examination). All subjects gave their informed consent, as approved by the Human Subjects Committee of the University of

Arizona and the Ministry of Public Health of Sonora State. Physical data and data on the health status, cigarette smoking, dietary habits and other variables were collected by questionnaire and physical examination. Individuals from the town of Esperanza were exposed to drinking water arsenic levels from two wells measured multiple times, with a combined mean of 43.3 ± 8.4 μg As/L. A comparable group of individuals from another town, Col. Allende, were exposed to lower levels of arsenic from one well of, 5.5 ± 0.20 μg As/L. This well water was the sole source of drinking water for these subjects. Blood collection and processing was performed using similar methods to those described above for New Hampshire.

First morning void urine samples were obtained in 100 mL polypropylene bottles and kept on ice. Within six hours, cooled samples were taken to Institute Technologic of Sonora and kept frozen at –40°C. The accumulated samples were then shipped on dry ice to the University of Arizona (Tucson, AZ, USA) where the samples were stored at – 80°C until the analysis of total arsenic and arsenic species was performed as described previously (Meza et al. 2005). The detection limits were 0.42-1.08 μg/L for arsenic compounds.

Cell Line:

Jurkat lymphoblast cells were used as a controlled *in vitro* system to evaluate the effects of arsenic on DNA damage and repair. Cells were grown in suspension in RPMI medium containing L-glutamine with 10% fetal bovine serum (Atlanta Biologicals; Norcross, GA) and 1% penicillin-streptomycin (Cellgro). Cells were exposed to sodium arsenite (Sigma; St. Louis, MO) (0.01 – 10 μM, which is equivalent to 0.75 – 750 μg/L) for a period of 24 h before harvesting and RNA isolation for gene expression analysis. Cells were exposed in culture to 0 or 1 μM (equivalent to 75 μg/L) arsenic as sodium arsenite for 24 hours prior to performing the Comet assay described below.

Laboratory Analyses:

Gene expression analysis - RNA was harvested using Trizol reagent (Gibco/BRL, Life Technologies; Gaithersburg, MD) followed by DNase digestion using DNAfree (Ambion Inc.; Austin, TX) according to the manufacturer's instructions and quantitated by spectrophotometric absorbance at 260 nm. Real-time Reverse Transcription - Polymerase Chain Reaction (RT-PCR) was performed using gene specific primers and reagents (ABI; Foster City, CA) using the ABI PRISM sequence detection system and software. Briefly, total RNA (0.5 μg) was reverse transcribed using 100 U M-MLV reverse transcriptase in a mixture with oligo-dT and dNTPs according to the instructions provided with the Qiagen Omniscript kit (Qiagen; Valencia, CA). Samples were reverse transcribed in a MJ Research PTC-100 thermocycler (MJ Research Inc., Watertown, MA) for 60 min at 44^{o}C and the reaction terminated by heating to 95^{o}C for 10 min. Expression of *ERCC1* (excision repair cross-complementing rodent repair deficiency, complementation group 1; GenBank GeneID 2067) was assessed by real-time PCR using 10 ng total RNA, 400 nM primers, 200 nM probe and TaqMan Universal PCR Master Mix (ABI). The sequence for the ERCC1 primer probe set is: Forward- CAGGACTTCGTCTCCCGGT, Probe- TCTGGAACAGCTCATCGCCGCA, Reverse- GCATAAGGCCAGATCTTCTCTTG. Relative quantitation was performed using a standard curve consisting of serial dilutions of pooled sample cDNA from the same source as the test RNA with each plate. Relative expression levels of each gene were normalized to *18s* rRNA or *GAPDH* (ABI; Foster City, CA).

Protein levels - The level of ERCC1 protein was assessed by immunoblotting using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to resolve proteins from whole cell lysates. Lymphocytes were rinsed with ice cold stop buffer (10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 5 mmol/L EGTA, 100 mmol/L NaF, 200 mmol/L sucrose, 100 μmol/L Na-orthovanadate, 5 pyrophosphate, 4 μg/ml leupeptin, 4 μg/ml soybean trypsin inhibitor, 1 mmol/L

benzamidine, 20 μmol/L calpain inhibitor 1, 100 mU/ml aprotinin, and 100 μmol/L phenylmethylsulphonylflouride). The stop buffer was then replaced with a minimal volume of 2x SDS-PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% (w/v) bromophenol blue). The lysates were boiled for 5 min and clarified by centrifugation at 13,000 rpm for 10 min. Equal amounts of cell lysate are resolved by electrophoresis on 8 -12% SDS-polyacrylamide gels. Electrophoresis was performed at constant voltage (200 V), then the resolved proteins were transferred from the polyacrylamide gel to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore; Bedford, MA) by semi-dry transfer (Hoeffer Semiphor; San Fransisco, CA) for 1 h at constant current (32 mA/minigel) using transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.01% sodium dodecyl sulfate). To eliminate nonspecific interactions of antibodies with the membrane, the PVDF membrane was blocked with TTBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% milk (w/v) for 1 h at room temperature or overnight at 4^{o}C. The membrane was incubated with the primary ERCC1 antibody (Neomarkers, Lab Vision Co., Fremont, CA) diluted 1:200 in TTBS overnight at 4^{o}C. The membrane was washed 3X with TTBS and incubated with horse radish peroxidase (HRP)-linked sheep anti-mouse IgG (1:2000 in TTBS) (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min - 1 h at room temperature. After 3 washes with TTBS, protein bands were visualized by enhanced chemiluminescence using the Renaissance system (NEN Life Sciences, Boston, MA) and film (Lumi-Film, Roche Molecular Biochemicals, Indianapolis, IN).

DNA damage and repair assessment - The single cell gel electrophoresis or comet assay, is widely used to measure DNA damage and repair in primary human lymphocytes by measuring strand breaks and apurinic sites (Baltaci et al. 2002; De Silva et al. 2000; Rajaee-Behbahani et al. 2001; Schmezer et al. 2001). We divided the lymphocyte sample from each individual into parts to assess damage at several time-points. We assessed baseline DNA damage levels as well as the capacity of

the lymphocytes to repair damage induced by an *in vitro* challenge with 2-acetoxy-acetyl-aminoflourine (2-AAAF) (Midwest Research Inst.; Kansas City, MO), the reactive and genotoxic metabolite of 2-acetylaminofluorene (2-AAF). Alkaline labile 2-AAAF adducts are primarily repaired through the nucleotide excision repair pathway (van Steeg 2001). Aliquots of lymphocytes were challenged for 2 hours *in vitro* with 4 μM 2-AAAF. A subset of 2-AAAF challenged lymphocytes were incubated for an additional 4 hours to allow for DNA repair of the lesions. Comet analysis was performed using Trevigen materials and protocols. Briefly, cells were mixed with agarose and spread over a warmed, pre-coated microscope slide. The agarose was allowed to solidify for 30 minutes at 4ºC, followed by immersion in pre-chilled Lysis Solution for 45 minutes or overnight. Next the slides were placed in freshly prepared Alkaline Solution, pH>13 for 30 minutes at room temperature. The slides were then washed twice by immersion in 1X TBE buffer for 5 min. Electrophoresis was carried out in alkaline buffer for 20 minutes at 1 volt per cm (measured electrode to electrode) in the dark. Lastly, the slides were dipped into 70% ethanol for 5 minutes, allowed to dry completely, and stained with SYBR green (Trevigen Inc.; Gaithersburg, MD). Image analysis of each cell was performed to quantify the length of the comet and the intensity of staining. All cells were analyzed using a fluorescence microscope coupled to the MD Biotech comet assay image analysis system (Morgantown, WV). The Olive tail moment is a unitless measure of DNA damage that was calculated as described previously using the quantity of migrated DNA multiplied by the distance between the comet head and the center of gravity of the DNA in the comet tail (Olive et al. 1990). The quantity of migrated DNA is the fraction of the DNA that has migrated from the head. The quantity of DNA is assessed as the DNA staining intensity subtracted from the background intensity. We scored the tail moment of all cells in a given well. Each point represents an average of 50 lymphocytes per individual (for human studies) or culture (for *in vitro* studies) from at least 3 - 9 individuals or 6 cultures.

Statistical analysis - Statistical analysis for gene expression and immunoblotting was performed using the ANOVA procedure with Newman-Keuls post-test, unpaired t-test, or linear regression procedures in GraphPad PRISM software. We consider *p* values less than 0.05 to be statistically significant. Statistical computations and graphics for Comet analysis were done using the statistical package S-Plus 6.2. We plotted the mean Olive tail moment with 95% confidence interval (CI) for each treatment group as a function of time. We performed an unpaired two-sided t-test to compare the low and high arsenic groups at each time point. Linear regression was used to assess the slopes of the lines. Corresponding *p* values are indicated.

Results

Human Studies:

Demographic characteristics of the study populations are provided in Table 1. A larger percentage of the subjects in Mexico were female. In addition, the Mexican population tended to be younger, with a mean age of 37, as compared to a mean age of 64 in New Hampshire subjects. Approximately one third of each population consisted of smokers.

As shown in Figure 1, analysis of both arsenic-exposed populations combined indicated that individuals exposed to drinking water arsenic concentrations >6 μg/L (n=11) had lower *ERCC1* gene expression levels than those with ≤5 μg/L arsenic (n=42) (p<0.05). The Mexican population alone had a lower *ERCC1* level among individuals exposed to arsenic >6 μg/L, although this was not statistically significant. Likewise a lower *ERCC1* level was observed in the NH individuals exposed to >6 μg/L arsenic that reached statistical significance (p<0.05).

We further assessed arsenic exposure using available internal biomarkers of arsenic level. However, different biomarkers were used in the two populations, which prevented pooling. Measurements included toenail arsenic for the New Hampshire population, and urinary arsenic for the Mexican population. These markers correlate with drinking water arsenic concentration (Karagas et al. 2000). Linear regression analysis of the New Hampshire population indicated an inverse association between toenail arsenic levels and *ERCC1* expression (r^2 =0.4, p<0.05). *ERCC1* expression decreased with increasing inorganic urinary arsenic level (As III + As V), but not total urinary arsenic (which may include organic arsenic), although this was not statistically significant (r^2=0.08, p=0.3). In the NH population, we found no difference in *ERCC1* expression level according to cancer status (p=0.8). For both populations, *ERCC1* expression did not significantly differ by smoking status, age, or gender (data not shown).

We went on to investigate whether the decreased gene expression levels that we observed in human lymphocytes exposed to arsenic in New Hampshire translated to changes in protein levels. Immunoblots using an ERCC1 antibody indicated lower levels of ERCC1 protein among individuals exposed to drinking water arsenic levels above 5 μg/L ($p<0.05$), although there was some inter-individual variation in expression (representative blot shown in Figure 2A, quantification shown in Figure 2B).

Additionally, we hypothesized that arsenic exposure would be associated with correspondingly higher DNA damage levels and reduced DNA repair function. We analyzed human lymphocytes from a subset of New Hampshire residents exposed to low (<0.7 μg/L) or high (>=13 - 93 μg/L) levels of drinking water arsenic using the comet assay (Figure 3). We detected higher levels of DNA damage, as indicated by larger Olive tail moments, for lymphocytes analyzed at baseline from individuals exposed to high levels of drinking water arsenic *in vivo* compared with lower level exposures (time 0 h, $p<0.05$). Analysis of these cells at 2 hours following 2-AAAF challenge demonstrated a dramatic increase in DNA damage but did not reveal any statistically significant differences in the amount of damage at time 2 h by arsenic exposure status ($p=0.25$). However, at the 6 hour time-point, after the 4 hour repair period, significantly higher levels of DNA damage remained in lymphocytes from individuals exposed to high compared with low levels of arsenic *in vivo* ($p<0.05$) (Figure 4). Control lymphocytes that did not receive *in vitro* challenge showed similar levels of DNA damage at the 6 hour time-point (Figure 4). The difference in slopes of the low and high arsenic lines was not statistically significant.

To further confirm the hypothesis that arsenic exposure inhibits *ERCC1* expression, we repeated these experimental treatments using a human lymphoblast cell line. As shown in Figure 5, arsenic suppressed *ERCC1* expression in the treated cells in a dose-responsive manner, beginning at

the 0.1 μM (approximately 7 μg/L) dose, with statistically significant decreases at or above 1 μM (p<0.05) compared with unexposed controls.

We further investigated the hypothesis that arsenic exposure *in vitro* would decrease DNA repair function using the lymphoblast cell line. Arsenic exposed and unexposed cells had similar levels of baseline DNA damage at time = 0 h (Figure 6). Arsenic-exposed cells challenged with 2-AAAF for 2 hours showed significantly higher levels of DNA damage than 2-AAAF-challenged cells that had not been exposed to arsenic (time = 2 h) (p<0.05, Figure 6). DNA damage for both arsenic-exposed and –unexposed, 2-AAAF-challenged cells decreased during the 4 hour repair period (Figure 6). Nevertheless, DNA damage for arsenic-exposed cells remained significantly higher than the non- exposed cells at the 6 hour time-point (p<0.05, Figure 6).

Discussion

Elucidating the mechanism of arsenic carcinogenicity has been challenging, in part due to the dose-, time- and species-specificity of its biologic effects (Barchowsky et al. 1999; Gomez-Caminero et al. 2001). Our early study supported previous *in vitro* work showing disruption DNA repair expression by arsenic. In the present study, we extended our findings to two different human populations at the gene expression, protein and DNA repair functional levels. Thus, our data provide both human *in vivo* and *in vitro* data to support the hypothesis that arsenic inhibits DNA repair processes (ATSDR 1999; Hartwig 1998), and that this has the potential to affect subsequent exposure to other genotoxic and mutagenic agents.

The effects of arsenic on DNA damage and repair have been evaluated almost exclusively in experimental systems. Previous *in vitro* studies demonstrated that arsenic specifically interferes with the repair of DNA photolesions (Yang et al. 1992) and cross-linking agents (Yager and Wiencke 1993). Another study using human fibroblasts found that low (2.5 μM) concentrations of arsenite reduced nucleotide excision repair efficiency, and incision frequency in particular, following UVR exposure (Hartwig et al. 1997). Results of additional studies in cultured human fibroblasts indicated that arsenic exposure reduced DNA repair capacity and specifically inhibited the repair of UV-induced pyrimidine dimer-related DNA damage in lymphoblastoid cells as measured by the Comet assay (Curnow et al. 2001; Danaee et al. 2004; Yager and Wiencke 1993).

Differences in gene expression results between these *in vitro* studies may be explained by differences in arsenic dose since the effects of arsenic are highly dose-dependent. In our study, treatment of lymphocytes with more than 1 μM sodium arsenite *in vitro* decreased *ERCC1* gene expression. The circulating levels of arsenic achieved in mice after administering 0.625 nM arsenic / kg ip are approximately equivalent to the 5 μM *in vitro* dose and do not cause overt signs of toxicity (Soucy et al. 2003). In contrast, acutely toxic doses of arsenic induced stress response

pathway genes as well as *ERCC1* gene expression in the livers of mice injected with 100 – 300 μM As/kg (Liu et al. 2001). Low concentrations of arsenic induce cell proliferation, angiogenesis, hormone signaling, and NF-kB dependent transcription, and do not appear to activate MAP kinase signaling or other stress response pathways. In contrast, high doses of arsenic induce apoptosis and activate MAP kinases, ERK and p-38, as well as stress- and heat shock-mimetic responses, inhibition of proliferation and apoptosis (Barchowsky et al. 1999). While decreased expression of ERCC1 may be partly responsible for the decreased DNA repair function associated with arsenic exposure, we recognize that other pathway members may be involved and future investigation will be needed to elucidate all factors involved. Since there are other environmental and genetic factors that can influence DNA repair, we would not expect complete concordance between arsenic exposure and expression or function on an individual level. Nevertheless, our *in vitro* studies demonstrate the effects of arsenic within a controlled experimental system. Further work to identify genotypes that modify the influence of arsenic exposure on DNA repair is needed.

In a human population, we previously found that drinking water arsenic exposure at levels between 5 and 75 μg/L was associated with decreased mRNA expression of nucleotide excision repair pathway genes in lymphocytes from exposed individuals (Andrew et al. 2003a). Based on those preliminary results we then followed up with the current study which utilizes a larger human population in New Hampshire. In addition to enlarging the sample size, examination of a second population in Mexico, exposed to moderate levels of arsenic, supported our New Hampshire population results. While we observed decreased *ERCC1* expression in both populations, the difference did not reach statistical significance in the Mexican population. The New Hampshire study had more extreme levels of exposure (Mexico: 5.5 – 43 μg/L; New Hampshire 0.007 – 75 μg/L), but more likely the Mexico study had a smaller sample size and lacked statistical power. To our knowledge, there are no other studies that have evaluated the association between arsenic

exposure and DNA repair gene expression or protein levels in human populations, particularly at arsenic levels that are in the range that is routinely found in the U.S.

In addition, our Comet analysis provides functional DNA repair data in an arsenic exposed human population. These data support previous observations of decreased DNA repair capacity following arsenic exposure *in vitro*. In prior work, arsenite was shown to inhibit DNA repair and act as a co-genotoxin for the direct-acting alkylating agent, methyl methanesulphonate (MMS), the indirect-acting PAH, benzo(a)pyrene (BaP), and UV-induced pyrimidine dimers in white blood cells and fibroblasts (Curnow et al. 2001; Danaee et al. 2004; Hartmann and Speit 1996; Okui and Fujiwara 1986). As observed previously by others, our *in vitro* arsenic exposure appeared to inhibit the fast component of DNA repair since the difference is observable after challenge (Figure 6, time = 2 h) (Danaee et al. 2004). This difference in DNA migration remained significantly higher in the arsenic exposed group after the repair period (Figure 6, time 6 h). Thus, our study and others consistently report that arsenic exacerbates DNA damage induced by other mutagens.

Whether inorganic arsenic can directly induce DNA damage by itself is more controversial and previous studies of DNA damage and mutagenesis by physiological levels of inorganic arsenic have been inconsistent (Mass et al. 2001; Schwerdtle et al. 2003; Sordo et al. 2001; Yih and Lee 2000). Our *in vitro* comet data did not show an increase in DNA migration following 24 h treatment with 1 μM arsenic alone (Figure 6, time = 0 h), but we did observe higher DNA damage levels at baseline in cells harvested from individuals exposed to drinking water arsenic at levels ≥ 13 μg/L compared to those with low levels of arsenic (<1 μg/L). The basis for this increase remains to be determined; however, higher levels of DNA damage in these lymphocytes following 2-AAAF challenge, and the slower repair kinetics of this damage, suggest that the higher baseline levels may be a result of arsenic-inhibited repair and exposure to other DNA damaging agents.

In summary, our studies of arsenic exposure *in vitro* and our novel work using *in vivo* arsenic exposure in two human populations support the hypothesis that arsenic exposure decreases DNA repair capacity. Further, our data demonstrate decreased expression of the nucleotide excision repair pathway member, ERCC1, and decreased repair following 2-AAAF challenge. These results support the theory that arsenic can act through a co-carcinogenic mechanism of action, exacerbating the genotoxicity and mutagenicity of other compounds.

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Tables

Table 1. Selected Characteristics of the New Hampshire and Mexican populations.

	New Hampshire *n*=37	Mexico *n*=16	Pooled population *n*=53
Gender			
Male	73%	44%	64%
Female	27%	56%	36%
Age			
≤50	8%	81%	30%
>50	92%	19%	70%
Current Smoking			
Yes	32%	38%	34%
No	68%	62%	66%
Water Arsenic			
≤5 μg/L	89%	56%	79%
>6 μg/L	11%	44%	21%

Figure Legends

Figure 1. *ERCC1* gene expression by drinking water arsenic level. *ERCC1* expression levels are shown pooled and separately for individuals from the NH and Mexican populations by drinking water arsenic concentration. *ERCC1* levels for 53 individuals were assessed by RT-PCR and normalized to 18s or GAPDH as described in Methods. Values are means ± SEM. An asterisk (*) denotes statistical significance compared to the ≤5 μg/L group at $p<0.05$.

Figure 2. Drinking water arsenic exposure > 5 μg/L is associated with decreased ERCC1 protein levels in human lymphocytes from the New Hampshire population. **A**) Immunoblot of protein extracts from human lymphocytes obtained from a subset of 8 individuals was assessed using antibody to ERCC1 or β-actin. **B**) The ratio of ERCC1 to β-actin band densities from the immunoblot shown in A. are graphed by drinking water arsenic concentration. Values are means ± SD. An asterisk (*) denotes statistical significance compared to the control group at $p<0.05$.

Figure 3. Comet assay on human lymphocytes obtained from New Hampshire subjects exposed *in vivo* to low (top row) or high (bottom row) drinking water arsenic levels. Cells were analyzed at baseline (column 1), after in vitro challenge with 2-AAAF (column 2), and after a 4 hour repair period (column 3).

Figure 4. Comet assay results quantified using image analysis were associated with *in vivo* arsenic exposure in human lymphocytes from the New Hampshire population. Cells from 12 individual subjects were each divided into 3 subsets and analyzed immediately after harvest (time = 0 h), after a 2 hour *in vitro* challenge with 4 uM 2-AAAF (time = 2 h), and following a 4 hour repair period

(time = 6 h). The level of DNA damage is expressed as the Olive tail moment, as described in Methods.

Figure 5. *In vitro* arsenic exposure decreases *ERCC1* expression in a cultured Jurkat lymphoblast cell line. Cells were harvested after exposure to arsenic (0.01 – 10 μM) for 24 h. *ERCC1* mRNA expression level was assessed by RT-PCR, quantitated using a standard curve using known amounts of cDNA and normalized to 18s rRNA, as described in Methods. Data are graphed on a log 10 x-axis scale. Values are means ± SD. An asterisk (*) denotes statistical significance compared to the control group at $p<0.05$.

Figure 6. *In vitro* arsenic exposure modifies DNA damage and repair function in a cultured Jurkat lymphoblast cell line. Cultured cells were exposed *in vitro* to 0 or 1 μM arsenic for 24 hours (n=6 independent cultures). Cells from each culture were analyzed by single cell gel electrophoresis immediately after harvest (time = 0 h), after a 2 hour *in vitro* challenge with 4 μM 2-AAAF (time=2 h), and following a 4 hour repair period (time= 6 h). The level of DNA damage is expressed as the tail moment, as described in Methods. Values represent mean ± 95% confidence interval. An asterisk (*) denotes statistical significance compared to the control group at $p<0.05$.

Figures

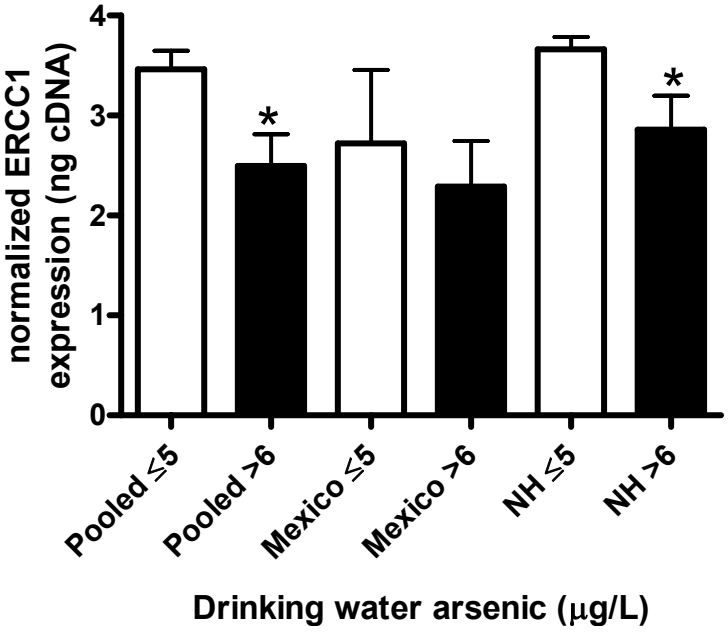

Figure 1.

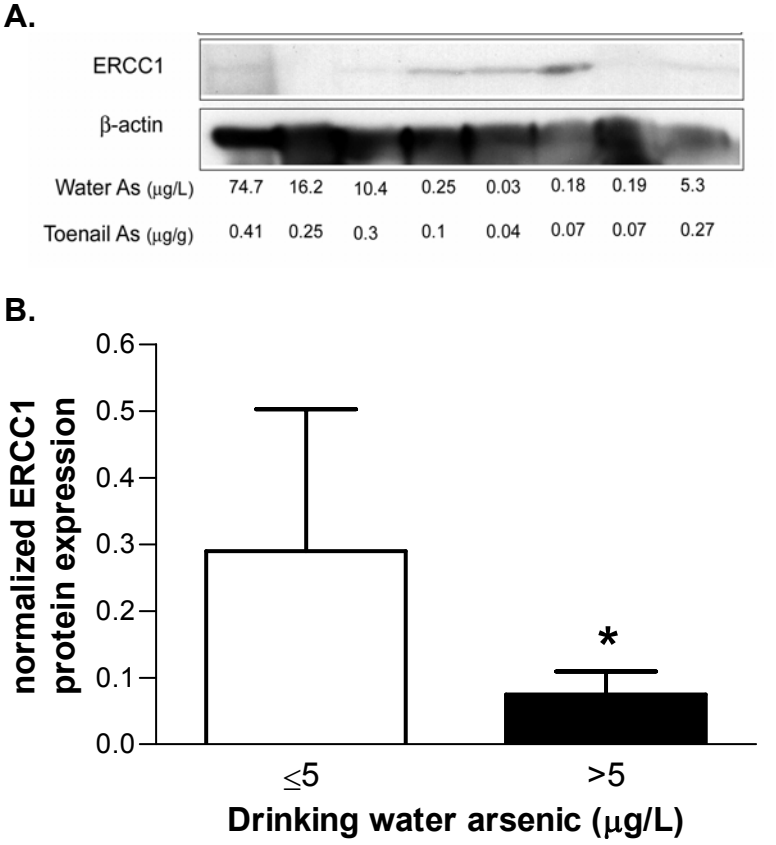

Figure 2.

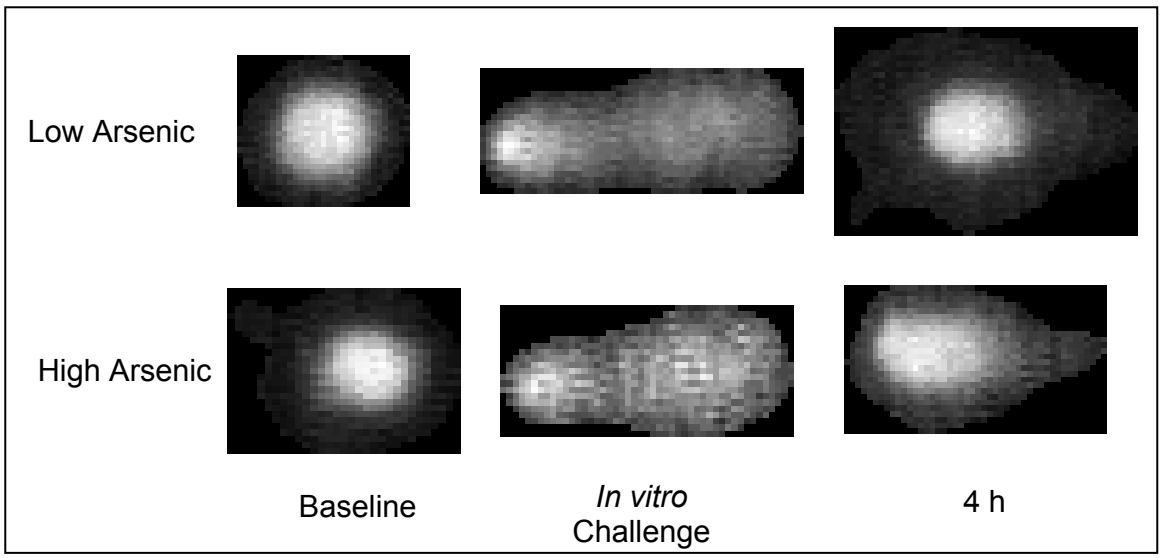

Figure 3.

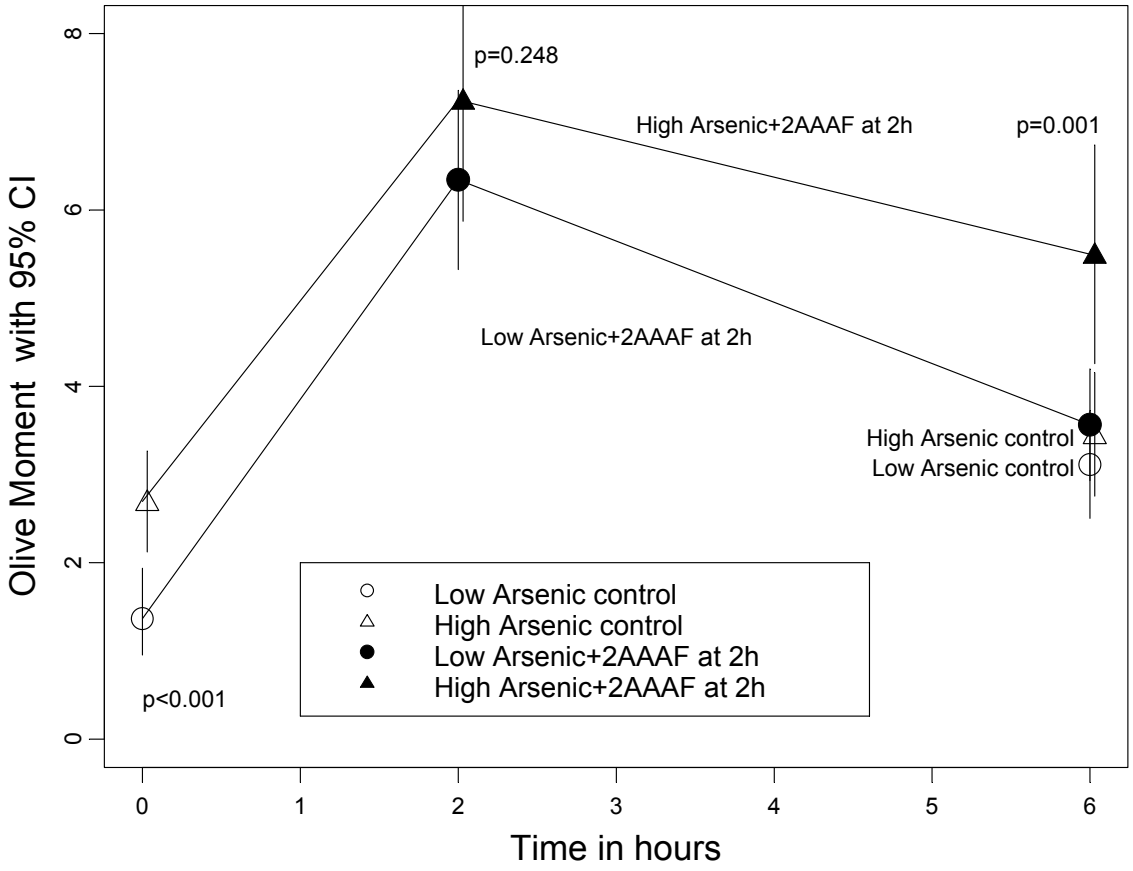
Olive Moment with 95% CI
Time in hours
p=0.248
p=0.001
p<0.001
High Arsenic+2AAAF at 2h
Low Arsenic+2AAAF at 2h
High Arsenic control
Low Arsenic control
Low Arsenic control
High Arsenic control
Low Arsenic+2AAAF at 2h
High Arsenic+2AAAF at 2h

Figure 4.

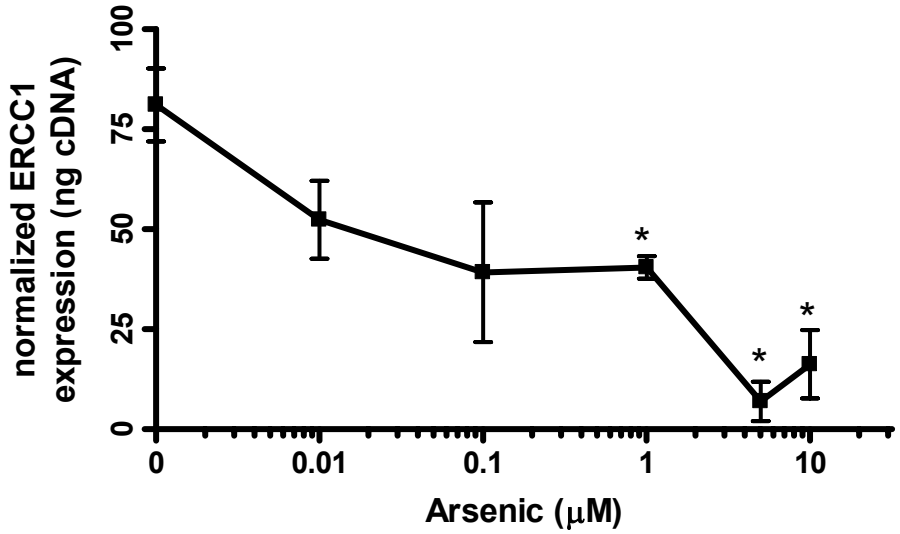

Figure 5.

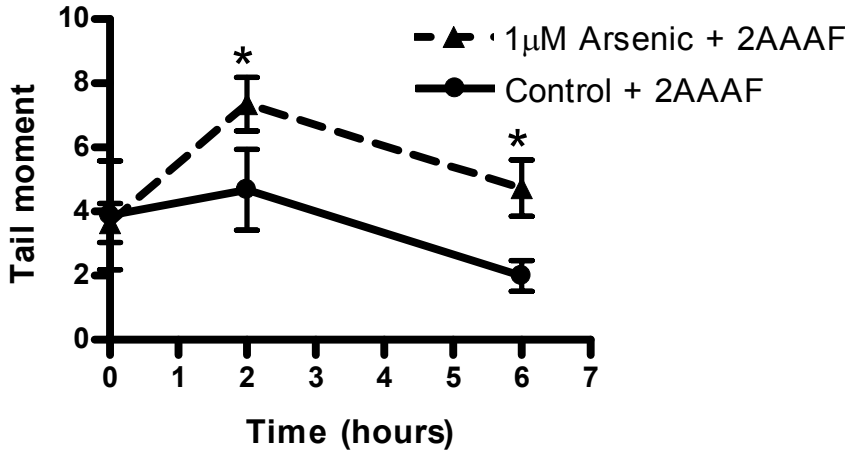

Figure 6.