Abstract

Arsenic is a naturally occurring metal to which humans are exposed through air, food, soil, and water. Chronic exposures to arsenic have been reported to be associated with a variety of adverse health effects including cardiovascular and pulmonary disease, neurological and developmental effects, and cancers of the skin, bladder, kidney, lung, and liver. The groundwater in Ba Men, located in Central West Inner Mongolia, China is naturally contaminated with arsenic at concentrations ranging from 0.3 µg/l to 1446 µg/l. Various adverse health effects in this region, including skin hyperkeratosis and other chronic diseases, have been linked to high-arsenic exposure from the drinking water. A pilot study was designed to detect internal arsenic levels using biomarkers of exposure, urine, nails, and hair samples, and to evaluate DNA and chromosomal damage in buccal cells. Arsenic was detected in the drinking water samples of the 19 exposed study subjects with a mean level of 527.5 ± 23.6 µg/l, while the 13 control subjects had a mean level of 4.4 µg/l ± 1.0. Arsenic detected in the internal exposure biomarkers for the exposed group compared to the control group was statistically significant for urine samples (632.7 µg/l and 28.5 µg/l, respectively, p < 0.0001), nail samples (32.02 µg/g and 3.36 µg/g, respectively, p < 0.0001), and hair samples (12.42 µg/g and 0.798 µg/g, respectively, p < 0.0001). Biomarkers of effects were examined using the micronucleus assay to detect chromosomal abnormalities and the DNA laddering assay to detect DNA fragments. MN induction in the high-arsenic exposed group was 3.4 fold higher (2.21MN/1000 buccal cells) compared with the control group (0.65MN/1000 buccal cells) (statistically significance at p < 0.01). DNA fragments containing <100bp in length were seen in 89% of the individuals in the exposed group compared to 15% in the control
group (p<0.0001). Correlations were determined to establish relationships between the arsenic in the drinking water, internal exposure markers, and biomarkers of effects. The biological exposure markers (urine, nail, and hair samples) all statistically correlated with the water arsenic levels. The strongest correlation was between the urine samples and water arsenic levels (Spearman r = 0.8397, p < 0.0001). The strongest correlation among the internal exposure markers was between urine and nail samples (Spearman r = 0.7776, p < 0.0001). The biomarkers of effects were statistically correlated with the arsenic water levels and all three internal exposure markers. The strongest correlation among the internal exposure makers and the biomarkers of effects was between the hair samples and DNA fragments (Spearman r = 0.7318, p < 0.0001). The two biomarkers of effects were statistically correlated with one another (Spearman r = 0.5238, p < 0.01). These results indicate that the individuals living in Ba Men are chronically exposed to arsenic via drinking water and reveal evidence of the genotoxic effects of arsenic in human cells. Arsenic levels measured in urine, nail, and hair samples can be useful as biomarkers of exposure. Micronuclei and DNA fragmentation patterns can be potentially useful as biomarkers of effects for assessing chromosome damage and DNA damage in human populations chronically exposed to arsenic via drinking water sources.
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I. Introduction

Arsenic is an element that has been in folklore for thousands of years. Dating back to 387 B.C., it was cited by Theophrastus of Erebos (Goyer, 1991). Arsenic was a widely used poison during the middle ages and was frequently called the “inheritance powder” because of its widespread use in homicides. Arsenic is considered a toxic metal and is ubiquitous in our environment. It has been measured in air, water, and soil samples. Arsenic compounds are naturally occurring in the earth’s crust and can contaminate water sources through natural leaching processes into ground water or through mining processes where the by-products are not contained properly (e.g. ore mining and smelter operations) (Goyer, 1991 and U.S. EPA, 1998). Elevated arsenic concentrations in water and soils can also be increased by the use of arsenic in certain industries such as wood preservation and agriculture (U.S. EPA, 1998).

Because of the wide spread use of arsenic as a criminal weapon (poison), it was one of the first poisons to be isolated and detected in human body tissues and fluids around the 1700’s (Goyer, 1991). The primary route of arsenic exposure in humans is ingestion of contaminated water and foods. Chronic human exposure to arsenic has been linked to skin cancers through ingestion and to lung cancers through inhalation (Tseng et al, 1968, Enterline et al, 1995). Based on extensive epidemiological evidence, the International Agency for Research on Cancer (IARC) has classified arsenic as a known human carcinogen (IARC, 1987). The United States Environmental Protection Agency has also identified arsenic as a group A “known human carcinogen” (U.S. EPA 1998 and
U.S. EPA, 1993). More recently, attention has turned to the possibility that chronic exposure to arsenic through drinking water sources may be associated with internal cancers, such as lung, bladder, liver, and kidney (Chiou et al, 2001, Smith et al, 1998, Ferricchio et al, 2000, Steinmaus et al, 2000, Morales et al, 2000 and Hopenhayn-Rich et al, 1996). Along with the association of both internal and skin cancers from chronic exposure with arsenic, other non-cancer endpoints such as cardiovascular and neurological effects have been reported (Hertz-Picciotto et al, 2000 and Yue-zhen et al, 1992). In West Bengal, India, the concentration of arsenic in drinking water sources is extremely high and the population here has been shown to exhibit signs of chronic arsenic toxicity including non-cancer manifestations such as abnormal electromyographys, gastrointestinal symptoms, enlarged livers and other liver problems (elevated liver enzymes and NCP-fibrosis), thickening of the palms and soles, and hyperpigmentation of the skin (Guha Mazumder et al, 1998).

**Arsenic Metabolism and Toxicity**

Arsenic is a very reactive and complex element that forms many inorganic and organic compounds and either of these forms (species) can exist in the trivalent or pentavalent valence state. Human exposure is mainly through water and food sources. Typically, arsenic-contaminated water contains inorganic arsenic compounds while food usually contains the organic forms of arsenic. Historically, the most toxic form was considered to be the trivalent, inorganic form, arsenite. The inorganic arsenicals, both
trivalent and pentavalent, are the predominant forms found in water (U.S. EPA, 1998). Inorganic salts and acids of arsenic ingested in water undergo a metabolic pathway in the human body known as methylation. This pathway (see Figure 1) begins with the inorganic arsenate (As$^V$O$_4^{3-}$) (usually in the pentavalent state because of stability) reduced to inorganic arsenite (As$^{III}$O$_3^{3-}$). Step 2 involves the enzymatic oxidative methylation of the arsenic compound to form methylarsonic acid (CH$_3$As$^V$O$_3^{2-}$), which is a pentavalent methylated species. Step 3 involves the reduction of this species to a trivalent monomethylated species, methylarsonous acid (CH$_3$As$^{III}$O$_2^{2-}$) prior to the enzymatic oxidative methylation for a second time, which yields dimethylarsinic acid ((CH$_3$)$_2$As$^V$O$_2^{+}$) (Styblo et al, 1999).

This metabolism of arsenic requires reduction of As$^V$ to As$^{III}$ prior to the enzymatic addition of methyl groups to the arsenicals. The reduction of arsenic to a trivalent state is preformed by endogenous thiols such as glutathione (GSH) or by As$^V$ reductases (Lin et al, 2002). In 1995, Zakharyan et al reported two different enzyme activities purified from rabbit liver cytosol that along with S-adenosyl-L-methionine (SAM) and a thiol were able to methylate both arsenite and monomethylarsonate (MMA) (1995). These enzyme activities were designated arsenite methyltransferase and methylarsonite methyltransferase, respectively, but were not sequenced (Zakharyan et al, 1995). However, a sequence was recently reported from a protein isolated from rat liver cytosol that catalyzes the transfer of a methyl group from SAM to trivalent arsenicals producing methylated and dimethylated arsenicals (Lin et al, 2002). This novel protein has been designated S-adenosyl-L-methionine (AdoMet): arsenic(III) methyltransferase
Figure 1: The metabolic pathway of inorganic arsenic. The proposed enzyme responsible for catalyzing the reaction is a methyltransferase. Glutathione (GSH) is the reducing agent and S-Adenosylmethionine (SAM) is the methyl donor. This figure shows inorganic arsenic (As\(^{\text{V}}\)) in the pentavalent state being metabolized into the organic species dimethylarsinic acid ((CH\(_3\))\(_2\)As\(^{\text{III}}\)).
(Lin et al, 2002). The enzyme responsible for this metabolic pathway in human cells has not yet been identified or sequenced but research is ongoing in this field.

The biomethylation of arsenic has long been considered a detoxification pathway, however, another toxic form has been identified in human urine, monomethylarsonous acid (CH$_3$As(OH)$_2$), which contains the trivalent arsenic species (Aposhian et al, 2000). This suggests that methylation may not be considered a detoxification pathway of arsenic in humans. The biomethylation of arsenic compounds is thought to occur mainly in the liver (Styblo et al, 1999). A report published in 1999 refers to the inability of investigators to detect arsenic methyltransferase activity in extracts of surgically removed human livers and there are no data available from other human tissues describing the methylation of arsenic (Zakharyan et al, 1999). However, the capability to methylate inorganic arsenic in vitro has been shown using primary human cell cultures and rat hepatocytes (Styblo et al, 1999).

Acute, high-concentration arsenic poisoning in humans can cause death. However, there are still uncertainties associated with chronic exposures to arsenic at lower concentrations (compared to poisoning events) and the observed health effects, such as cancer and non-cancer effects seen throughout the world. The possible mode of action for arsenic carcinogenicity has been widely studied over the past decade and includes several genotoxic effects such as comutagenicity with such mutagens as alkylating agents and UV radiation by interfering with DNA repair processes (enzymes), alterations in DNA methylation status, oxidative stress, and increases in cellular proliferation. Another possible mode of action addressed in this report is the possibility that arsenic carcinogenicity can be expressed through genetic alterations, such as
chromosomal alterations and clastogenetic effects. The true nature of arsenic carcinogenicity is still not clear and may involve any or all of the hypothesized modes to date (National Academy of Sciences, NRC report, 1999).

World and U.S. Arsenic Exposures

Several parts of the world utilize drinking water sources that are contaminated with high levels of arsenic. A very early publication by Tseng et al showed that villages in a particular region of Taiwan had community well water that was contaminated with arsenic ranging from 0.01 to 1.82 mg/l. There are areas in India where tens of thousands of people get their drinking water from deep wells that have arsenic levels between 0.05 mg/l and 3.2 mg/l of arsenic (Guha Mazumder et al, 1998). This problem in India has garnered much attention by both the scientific community, as well as, the popular media at large. In South America, arsenic contaminated drinking water has been studied in a small northern region in Chile, where the arsenic levels range from 0.040-0.870 mg/l over the past fifty years (Smith et al, 1998). In a central Argentina province, the arsenic concentrations in some towns' drinking water have been reported between 0.040 and 0.533 mg/l (Hopenhayn-Rich et al, 1996). People living in the Zimapan valley, located in a small central Mexican town, have been exposed to arsenic concentrations ranging from trace (less than 0.014mg/l) to 1.09 mg/l (Armienta et al, 1997).
According to the World Health Organization (WHO), the preferred level of arsenic in water is ≤ 0.01 mg/l and the maximum permissible limit acceptable by WHO is 0.05 mg/l (Basu et al., 2001). In the United States, the drinking water standard, or Maximum Contaminant Level (MCL), for arsenic has historically been 0.050 mg/L. This standard was determined without the use of scientific risk assessment methodologies; rather it was adopted from the old U.S. Public Health Service recommendation of 1942 (U.S. EPA, 1998). Regulation of arsenic under the Safe Drinking Water Act (SDWA) has been mandated to the U.S. Environmental Protection Agency (U.S. EPA). This agency promulgated a new arsenic rule under the Clinton Administration on January 22, 2001, which established an enforceable MCL for arsenic of 0.01 mg/L in “non-transient non-community water systems, and to community water systems” (Federal Register, January 22, 2001). However, when the new Bush Administration gained control of the White House, this final rule was rescinded pending further review of the science and further public input of the proposed rule. (Federal Register, May 22, 2001). After months of further scientific review and a call for more public comment in the Federal Register (October 5, 2000), the U.S. Environmental Protection Agency announced on October 31, 2001 that the new MCL for arsenic in drinking water will be set at 0.01 mg/L and the agency will maintain the original compliance date of 2006. (U.S. EPA Press Release, October 31, 2001). Despite the announcement of the new MCL, there is still a great deal of uncertainties related to the human health effects of arsenic at low levels. Under the SDWA, the agency is also required to review and revise, as appropriate, the national primary drinking water standard for arsenic every 6 years.
The focus of this human exposure and health effect research is on geological arsenic as opposed to industrial or agricultural sources. Agricultural and industrial sources may potentially contribute to the contamination of drinking water through point sources, however our research does not address these sources. Arsenic deposits in the United States occur mainly in the Southwest (i.e. Nevada, New Mexico and Utah), where there are unique geological strata in the Earth's crust which contain arsenic-rich minerals. Other geographically significant pockets of arsenic are scattered throughout the U.S., i.e. Wisconsin, New Hampshire, Michigan, and Alaska.

These arsenic-enriched mineral deposits are found in higher concentrations in soils than in rocks and are largely associated with soils and groundwater affected by geothermal activity (Thornton and Farago, 1997). Groundwater is used by many Americans in the Southwest as a water source through private wells or community wells by many smaller municipalities in these same regions. In the U.S., most studies involving arsenic exposure have come from occupational settings or environmental exposures from industrial or agricultural point sources (Enterline et al, 1995, Tollestrup et al, 1995, Wong et al, 1992 and Hwang et al, 1997). Few U.S. studies have been conducted to ascertain health effects associated with geological arsenic exposures through drinking water sources (Warner et al, 1994, Karagas et al, 1998 and Lewis et al, 1999). Some major impediments with studying U.S. populations with geological arsenic contaminated drinking water supplies are related to low levels of arsenic concentrations, few health effects reported, small number of individual exposed, transient populations and confounders such as diets that contain arsenic compounds (e.g. seafood).
Arsenic Exposure in Inner Mongolia, China

It is because of these inherent difficulties within the U.S. based population that researchers have sought out international populations to study health effects related to arsenic contaminated drinking water. We have chosen to study a population in China because of its' unique characteristics (i.e., drinking water is the primary source of arsenic exposure, wide range of arsenic exposures with individuals showing health effects related to arsenic exposures, non-transient population with individual rather than community wells). The autonomous region in China known as Inner Mongolia is located in the northern part of the country, see Figure 2. Inner Mongolia has four cities and eight regions with Bayingnormen (Ba Men) as one of the eight regions (Ma et al, 1999). Ba Men is our study site, geographically located between the Lang Shan Mountains and the Huang He River (Yellow River) in western Inner Mongolia. These mountains contain arsenic-rich minerals and the runoff from them creates the groundwater supply for Ba Men. Also, the area in Ba Men where the ground water collects was at one time a sunken lake, which allows the arsenic-rich ground water to stagnate. These geological features contribute to the Ba Men region’s ground water contamination. The ground water in this area has arsenic levels ranging from 0.05mg/L to 1.8mg/L (Ma et al, 1999).

The people living in Ba Men use well water as their primary water source. In 1978, the residents of Ba Men switched from shallow (3-5m) shared wells to deeper, hand pumped, private wells which are more likely to contain arsenic (Ma et al, 1999). In the Ba Men arsenic endemic area, wells at a depth of 15-30m have been shown to contain the highest arsenic levels (Ma et al, 1999). In this region, a study conducted in 1995
Figure 2 is a map showing the arsenicism endemic region in Inner Mongolia, China. The insert shows the location of Inner Mongolia (blue) and the arsenicism endemic region (red) in relation to the entire country of China.
showed that drinking water is the main source of arsenic intake for the population, with an average daily intake of approximately 2 liters per person (Yu et al, 1995). Ba Men is an agricultural region where wheat, corn, potatoes, sunflowers and, fruit are the primary crops grown (Ma et al, 1999). However, no pesticides containing arsenic are used in this region and no industries emit or discharge any arsenic by-products because most of the industries are agriculturally based (Ma et al, 1999). In this 1995 study, other possible arsenic sources were tested and the analysis of soils, crops (wheat, corn, vegetables), and surface water from the Huang He River (used for irrigation purposes) all showed arsenic levels below the Chinese standards (Chinese standards: soil – 13mg/kg; grain – 0.6mg/kg; vegetables – 0.5mg/kg; surface water – 0.05mg/L) (Sun et al, 1995).

The residents in Ba Men have used deep, private wells for over 20 years, which has chronically exposed them to arsenic. In Inner Mongolia, it is estimated that some 300,000 residents distributed in 543 villages live in the arsenic endemic region in Ba Men where the arsenic levels in groundwater exceeds 0.05mg/L (Ma et al, 1999). The first case of arsenicism, which includes skin hyperkeratosis, hyperpigmentation, depigmentation, and other arsenic-related skin lesions, in Inner Mongolia was reported to health officials in 1990 and there has been an increasing trend each year since. By 1994, through clinical investigations, arsenicism had been confirmed in 1,774 patients in this endemic area of Inner Mongolia.

The population in the arsenic endemic region of Ba Men was selected to investigate health effects related to arsenic exposure via drinking water based on factors that would assist in a more accurate risk assessment. These unique factors are: (1) drinking water is the primary source of arsenic contamination; (2) the arsenic
concentrations in the wells in this region show a wide range and the exposed residents show a high prevalence of arsenicism; (3) individual exposure can be assessed because most families have their own private, hand pumped, wells; (4) a stable population with low migration patterns; (5) unlike the Taiwanese population (Tseng et al, 1968) or other studied populations in developing regions with arsenicism, this study site has a population with a good nutritional status, so poor nutrition is probably not a confounder in this study; and (6) Chinese investigators have already accumulated exposure and health effects data from this population (Yu et al, 1995, Sun et al, 1995 and Ma et al, 1995). It is because of the vast amounts of data already obtained by the Chinese scientists and the ongoing research in this region that the U.S. EPA has entered into a five-year cooperative agreement with the Chinese researchers at the Institute of Endemic Diseases for Prevention and Treatment in Inner Mongolia. The collaboration between both of these research institutes will focus on the health effects of chronic arsenic exposure from drinking water using a multidisciplinary approach encompassing biomarkers of exposure and effects, as well as more traditionally applied epidemiological methods.

Biomarkers

Since there are uncertainties in the dose-response relationship associated with arsenic exposure at low doses, research is needed to fill these voids. Biomarkers may be a valuable tool to assess human arsenic exposure and health effects at low doses. As a pilot study, a small set of environmental and human samples were collected in Ba Men. The
biomarkers measured in our study included internal markers of exposure, which measured the arsenic levels in the urine, nails, and hair and biomarkers of effects, which assess DNA fragmentation patterns and elevated micronuclei frequencies in buccal cells. In addition, the concentrations of arsenic in the drinking water were assessed as the total arsenic levels. Characterizing exposure and demonstrating the relationship with biomarkers of effects was an important feature of this study.

The biomarkers of effects (DNA fragmentation and micronuclei) were examined in buccal cells, which are human epithelial cells in the oral mucosa. Buccal cells were chosen as surrogate cells because they are obtained non-invasively, and are easy and inexpensive to collect (Marchand et al, 2001). Buccal cells may not be the target cells for arsenic toxicity because oral cancers have not been observed in any arsenic exposed human populations. However, hyperpigmentation in buccal mucosa was observed among the Ba Men residents exposed to high arsenic concentrations in the drinking water (unpublished data). Buccal cells provide a good surrogate cell type to examine possible genotoxic effects and/or possible exposure from arsenic because these cells are directly exposed upon ingestion of arsenic contaminated drinking water. Exfoliated buccal cells can be used as an ex vivo measurement of genotoxic damage through staining of cells for the presence of micronuclei or as a source of DNA to examine DNA fragmentation patterns, using the DNA laddering assay. Most studies reported in the literature examined micronuclei formation in urothelial cells with some studies reporting on the extent of micronuclei induction in buccal cells associated with chronic arsenic exposure in humans (Smith et al, 1993, Warner et al, 1994, Biggs et al, 1997, Moore et al, 1997, Gonsebatt et al, 1997, and Tian et al, 2001). It has been reported that substantial amounts
of DNA can be isolated from buccal cells ranging from 2-10μg of DNA (Garcia-Closas et al, 2001). DNA isolated from these cells can be assessed for damage using the DNA laddering assay. One study to date has assessed DNA fragmentation patterns in human exfoliated buccal cells as a biomarker of DNA damage using the DNA laddering assay, as well as, the TUNEL assay in a chronically exposed arsenic population (Feng et al, 2001).

The DNA laddering assay assesses DNA damage by examining a feature that is considered the hallmark biochemical indicator of apoptosis, DNA fragmentation. This assay however, only identifies the end point, DNA fragmentation (degradation), and does not examine, in full, the entire pattern of DNA fragmentation resulting from apoptosis (Walker et al, 1999). Apoptosis, or programmed cell death, is essential in the normal development of cells, which includes genetic processes needed for the death and disposal of old or damaged cells. There are studies that have linked the apoptosis pathway with arsenic exposure in vitro using human protomyelic leukemia cells (HL-60)(Ochi et al, 1996) and in animal studies (Constan et al, 1996).

Studies suggest that arsenic can be used as a therapeutic treatment of some cancers, especially acute promyelocytic leukemia (APL) (Lu et al, 1999, Zheng et al, 1999, and Huang et al, 2000). These studies use the DNA fragmentation assay to provide evidence of the induction of apoptosis within human cells. The exact nature and primary targets of arsenic-induced cytotoxicity, however, are still unknown. One study published in 2001 suggests that some arsenic species may be direct-acting genotoxicants based on analysis of DNA damage by the DNA nicking assay and the Single Cell Gel Assay (Mass et al, 2001). To date, most of the DNA fragmentation studies on arsenic-induced
apoptosis have been conducted in vitro or in vivo in animal models. Our lab was first to publish on DNA fragmentation in human epithelial cells (Feng et al, 2001).

The micronucleus assay developed in the early 1970's by Schmid and co-workers was primarily used for the in vivo screening of chemicals by analyzing chromosome-breaking effects (Schmid, 1975). Micronuclei are actually lagging fragments that were included in the daughter cells and transformed into one or more secondary nuclei, which are smaller than the original nucleus. This happens when in anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles (Schmid, 1975). Micronuclei were first detected in myeloblasts, myelocytes, erythroblasts, and young erythrocytes in bone marrow smears from mammals treated with chromosome-breaking agents.

In the early 1980's Stich et al applied this technique to exfoliated human cells (1983). Stich argued that buccal and urinary bladder mucosa cells could reflect cytogenetic anomalies that occur in the dividing cells of the basal epithelial layers (1983). Micronuclei survive as the cells move from the basal layer to the surface of the mucosa of the oral cavity and urinary bladder and the frequency of these micronucleated exfoliated cells provide a measure of the genotoxic damage in the human mucosa (Stich, 1983). Stich published a paper in the early 1980's looking at the frequency of micronuclei in betel quid chewers and tobacco chewers (Stich, 1983). While mouth cancers are not primarily the cancers of interest with arsenic exposure, we expect that buccal cells should be good surrogates because most of the cancers of interest arise in the epithelial cell layers. Consistent with this expectation, Smith et al suggest that buccal cells may be a good biomarker for arsenic exposure as early as 1993 based on arsenic's clastogenic
properties. However, most published reports on micronuclei studies associated with arsenic exposure are mainly studies conducted on urothelial cells (Biggs et al, 1997, Moore et al, 1997 and Moore et al, 1997) with relatively few studies conducted using buccal cells. Gonsebatt et al reported on exfoliated cells obtained from the oral mucosa and from the urine in a Mexican-based population exposed to arsenic via drinking water showing elevated micronuclei frequencies in the high arsenic exposed population for both buccal and urothelial cells (1997). In 1994, a published paper by Warner et al looked at both buccal cells and urothelial cell micronuclei formation in a population that chronically ingests arsenic contaminated water in Nevada showing elevated micronuclei frequencies in the urothelial cells but not in the buccal cells (1994). And Aposhin et al published a paper in 1997 where buccal cells showed elevated micronuclei frequencies in a small sample (n=9) of subjects in a Chilean population exposed to high levels of arsenic in their drinking water.
Objectives of this Study

In collaboration with Chinese investigators, we have conducted a pilot study to assess the feasibility of collecting environmental and human samples for assessing arsenic exposure and health effects in Ba Men, Inner Mongolia. The objectives of this study are the following: (1) to determine whether non-invasively obtained samples (i.e. urine, nails, and hair) can be used to assess internal dose measurements of arsenic, (2) to determine if arsenic as detected in urine, nails, and hair can be used to assess human exposure to arsenic by determining the relationships between the arsenic levels in the water and each of these internal dose markers, (3) determine if internally measured (from urine, nail, and hair samples) arsenic levels are associated with DNA fragmentation and/or elevated micronuclei frequencies in human epithelial cells (buccal cells), and (4) establish correlations between biomarkers of exposure and biomarkers of effects in this population.
II. Materials and Methods

Study Subjects

Thirty-two study subjects from villages located in Ba Men, Inner Mongolia participated in this study. The high-arsenic exposure group subjects were from the Fenzidi Village in Lin He and the Shibaqitu Village in Wu Yuan County. The control group subjects were from the Long Sheng Village in Lin He. The control group subjects were exposed to low levels of arsenic, which were representative of the background arsenic levels measured in this region. Water was collected from individual wells to determine the arsenic levels of exposure in this population. Urine, nail, and hair samples were collected from all study subjects to assess internal arsenic levels. Buccal cells were collected to assess chromosome damage using the micronucleus assay and the DNA laddering assay, to assess DNA fragmentation.

Questionnaires were administered to all subjects to obtain demographic information, water consumption, history of well use, diet, smoking, alcohol consumption, occupation, pesticide-use, and medical information. The study subjects (see Table 1) are all farmers and include 19 individuals (5 females and 14 males) in the high-arsenic exposure group, and 13 individuals (5 females and 8 males) in the control group. In the high-arsenic exposed group there are 10 nonsmokers and 9 smokers and in the control group there are 9 nonsmokers and 4 smokers. The study subjects in this region all have very similar socioeconomic status, diet, education, and lifestyle, and all have lived in this region for the last 10 years or longer. The high-arsenic exposure group that was selected
all showed signs of arsenicism, characteristic of skin lesions such as skin hyperkeratosis, hyperpigmentation and/or depigmentation, and included 16 subjects with hyperkeratosis, hyperpigmentation, and depigmentation, 2 subjects with hyperpigmentation and depigmentation, and 1 subject with only depigmentation. For human subject protection, this study was conducted according to the recommendations of the World Medical Association Declaration of Helsinki (World Medical Association Declaration of Helsinki, 1989). All study subjects gave informed consent to participate in this study. The research protocol was approved by the U.S. Environmental Protection Agency for International research projects.

Water Sample Collection and Analysis

Drinking water samples were collected in acid-washed containers from the water tanks of the study subjects’ homes for 5 consecutive days in separate containers. Individuals living in this region use water tanks in their homes as storage facilities for the water after it is pumped from the wells. Water samples were transported on ice packs via air to our lab in Chapel Hill, N.C. The samples were stored at -80°C until aliquots were shipped on dry ice via air to the University of Alberta in Edmonton, Canada for analysis. Total arsenic levels were determined using the hydride generation/atomic fluorescence spectrometry detection method (Le, 1998).
Urine Sample Collection and Analysis

Urine samples were collected from the first and second voids of the day for 5 consecutive days. These samples were centrifuged at 500x g for 10 minutes to remove exfoliated urinary bladder cells (which were discarded). The samples were frozen and shipped on ice packs to the U.S. via air for storage and analysis. Storage in the U.S. was at -80°C until samples were shipped via air on dry ice to the University of Alberta in Edmonton, Canada for analysis. The determination of the total arsenic levels was done by HPLC separation and hydride generation/atomic fluorescence spectrometry detection described by Le and Ma (1998).

Hair Sample Collection, Preparation and Analysis

Hair samples were collected by trained technicians. A 0.2- 0.3-gram hair sample was collected by taking end clippings from the front, back, and each side of the individual’s head. These samples were placed in a labeled ziplock bag and shipped to the U.S. EPA via air. Hair samples were sent to the University of Alberta for analysis. The hair was prepared by freezing approximately 20-30mg in liquid nitrogen then grinding it into a fine powder. Typically, a 20mg ground sample was weighed into a beaker containing 10ml of a nitric, sulfuric acid mixture (3:1 mixture). The sample was allowed to digest with gentle heating until the solid material was completely dissolved and the solution appeared clear. This solution was diluted with deionized water for subsequent
analysis. To detect low levels of arsenic in the samples, the acid was evaporated and the sample solution reconstituted in 5ml of deionized water for subsequent analysis.

The analysis of arsenic concentration in each sample was carried out with this digested sample solution using hydride generation/atomic fluorescence spectrometry detection (HGAFS). Studies of recovery, standard reference materials (oyster tissue and water), calibration, interference, reproducibility, and detection limit were carried out to confirm the suitability of the method. This procedure allows for the determination of total arsenic in the hair sample (Le, 1998).

Nail Sample Collection, Preparation and Analysis

Nail samples were collected by trained technicians. A 0.3-0.5-gram nail sample was collected by taking clippings from all fingers and toes of each subject. The finger and toenails were mixed together and placed in a ziplock bag. The bag was labeled as nails and shipped to the U.S. EPA via air. Nail samples were cleaned with distilled, deionized water then sent to the University of Alberta for analysis. The nails were prepared by freezing approximately 20-30mg in liquid nitrogen then grinding into a fine powder. Typically, a 20mg ground sample was weighed into a beaker containing 10ml of a nitric, sulfuric acid mixture (3:1 mixture). The sample was allowed to digest with gentle heating until the solid material was completely dissolved and the solution appeared clear. This solution was diluted with deionized water for subsequent analysis. To detect
low levels of arsenic in the samples, the acid was evaporated and the sample solution reconstituted in 5ml of deionized water for subsequent analysis.

The analysis for the arsenic concentration in each sample was carried out with this digested sample solution using hydride generation/atomic fluorescence spectrometry detection (HGAFS). Studies of recovery, standard reference materials (oyster tissue and water), calibration, interference, reproducibility, and detection limit were carried out to confirm the suitability of the method. This procedure allows for the determination of total arsenic in the nail sample. (Le, 1998).

**Buccal Cell Collection**

Buccal cells were collected from each subject for 5 consecutive days, matching the collection times for both water and urine. All subjects were instructed to clean their mouth prior to sample collection to remove food debris. A trained technician used a soft toothbrush to gently scrape the inner cheeks (buccal mucosa) of each subject. Then the toothbrush was swirled rapidly in a centrifuge tube containing Saccomanno’s cytology fluid (a fixative containing 39% ethanol, 3.0% polyoxyethylene, and 2.0% isopropanol). The buccal cells were centrifuged to obtain cell pellets, which were washed once with fresh Saccomanno’s fluid. The samples were transported on “blue ice packs” to the laboratory at the U.S. Environmental Protection Agency in North Carolina via air for analysis. The buccal cells were stored at -20°C until assayed.
DNA Laddering Assay

DNA Isolation:

DNA was isolated from buccal cells of the study subjects. During the following DNA isolation steps all tubes, tips, and supplies were autoclaved if possible or solutions purchased and prepared RNAase/DNAase free. The buccal cells were diluted in Saccommano fluid (Shandon, Pittsburgh PA) to a concentration of approximately 1 x 10^6 cells/ml. From the original storage tubes of scraped buccal cells, 2 ml of volume was removed and placed in a 15ml centrifuge tube. These cells were spun at 600x g for 10 minutes, supernatant was discarded, and the cells washed with 1ml of RNAase/DNAase free 1x PBS. The cells were again collected by centrifugation at 600x g for 10 minutes and again the supernatant discarded. The cell pellet was reconstituted in 400µl of 1x PBS and transferred to a 1.5ml Eppendorf tube. From this point on in the isolation process we used a modified version of the protocol in the Qiagen QiAmp DNA blood mini kit. To the cells in the 1.5ml tube, 400µl of buffer AL (lyseing buffer provided in kit) and 25µl of 40mg/ml Proteinase K solution were added and then the tubes were carefully mixed by inversion for approximately 3-4 minutes. The cells were incubated in a water bath at 37°C for 60 minutes. Then, 20 µl of RNase A (25mg/ml) was added to each tube and the tubes were incubated a second time at 37°C for an additional 60 minutes. Finally, the tubes were placed in a 56°C water bath for an additional 10 minutes. Following the incubations, 210µl of ice cold 100% ethanol was added to each tube and the tubes were gently inverted to mix the samples. From the Qiagen kit, a spin column was placed into a collection tube then 600µl of each sample was placed into a spin column and centrifuged
at 8700x g for 2.5 minutes. The collection column was changed and the remaining amount of sample (~600μl) was placed in the same spin column and centrifuged again at the identical speed. After the DNA had been captured on the column it was washed using the reagents (wash buffers #1 and #2) provided in the kit. 500μl of the wash buffer #1 was added to each spin column, a new collection tube was placed under the column and centrifuged at 8700x g for 2.5 minutes. A second wash was done in the same manner using the wash buffer #2. After the washing steps, to ensure all excess liquid was removed from the columns, a new collection tube was placed under the spin column and it was centrifuged at 12,100x g for 3 minutes. The DNA was eluted from the column using an elution buffer provided by the kit (AE elution buffer). The elution buffer labeled AE was heated to 56°C in a water bath and the columns were placed into new collection tubes labeled with the corresponding sample number. 150μl of heated AE buffer was placed into the column and the columns were allowed to stand at room temperature for 1 minute, then the columns were placed into the 56°C water bath for 10 minutes. Following the incubation, the columns were centrifuged at 8700x g for 2 minutes. The collection tubes with the DNA in the AE buffer were set aside and new, labeled 1.5ml Eppendorf tubes were placed under the columns and an additional 150μl of heated AE buffer was added to the column. The columns were allowed to stand at room temperature for 1 minute then centrifuged at 8700x g for 2 minutes. The 2 tubes were combined for a total volume of 300μl of AE buffer containing DNA. The DNA was precipitated using 5M NaCl at a ratio of 1:10, so 30μl of 5M NaCl was added to each sample tube, and absolute ethanol at a ratio of 1:1, so 300μl of 100% ethanol was added
to each sample tube. The tubes were capped and inverted for several minutes then placed into a -20°C freezer overnight.

**DNA Quantitation:**

The DNA was recovered by centrifugation at 13000x g, at 4°C, for 30 minutes. The supernatant was removed and the DNA was washed with 70% ethanol and centrifuged again at the above specifications. The supernatant was again removed and the inside of the Eppendorf tubes were dried carefully with cotton tipped applicators. The tubes were further allowed to dry at room temperature to get rid of excess alcohol. According to the pellet size between 12 and 22μl of AE buffer was added to reconstitute the DNA pellet for storage. For larger pellets associated with the cell line used as controls, 40-100μl of AE buffer was added to the DNA pellet. A 2μl aliquot was taken from each sample and diluted in 998μl distilled, de-ionized water for quantitation in the UV/VIS Spectrophotometer. The remaining sample volumes were stored in 10μl aliquots at -80°C until gel electrophoresis. The 1ml (1:500) DNA dilution was read in a spectrophotometer at 230nm, 260nm, 280nm, and 320nm. The following calculations were made from these reading:
**DNA quantity:** 260nm reading \( \times \) Factor of 25

The Factor of 25 was derived from the equation: \(\text{Abs} = abc\), assuming a value of 1 for the absorbance.

\[
\text{Abs} = \text{Absorbance at 260nm} \\
a = \text{extinction coefficient at 260nm (20 \mu l/\mu g/cm for double-stranded DNA)} \\
b = \text{wavelength path length (1cm path length for cuvette)} \\
c = \text{concentration (DNA diluted 1:500 of stock solution)}
\]

**DNA Purity:** Absorbance ratio between 230/260 should be between 0.3 and 0.5 to rule out protein contamination. Absorbance ratio between 260/280 should be between 1.7 and 2.0 to rule out RNA contamination.

These calculations were made and recorded. The total DNA recovery calculation was made as well. This is important in determining the proper volume of DNA to add in each well during electrophoresis. We want approximately 2\(\mu g\) of DNA placed in each well for the DNA laddering assay to ensure enough DNA to detect if laddering occurs.

**Gel Electrophoresis:**

A 1\% agarose gel was prepared using 0.3g of Agarose-1000 (Metaphor brand from FMC Bioproducts, Rockland, ME) and 30ml of 1xTBE buffer (Tris-borate, EDTA buffer solution). The 1\% agarose gel solution was heated for 60 seconds in a microwave oven to dissolve the agarose and it was allowed to cool at room temperature several minutes until the temperature reaches approximately 60\(^{\circ}\)C, then 3\(\mu l\) of a 1x nucleic acid
stain (Gel Star brand from BioWhittaker Molecular Applications, Rockland, ME) was added to the liquid and mixed gently as not to create bubbles. The liquid agarose was poured into a gel mold of approximately 7x10cm dimensions and an 8-tooth comb was used to create the wells. The gel was allowed to harden for 45 minutes at room temperature before loading samples. The DNA samples were loaded at a concentration of approximately 2μg of DNA per well and a final volume of only 10μl per well. This was accomplished by determining what volume of sample equates to 2μg of DNA, adding AE buffer to a final volume of 9μl before adding the required 1μl of loading dye. The freshly cast agarose gel was placed into a gel electrophoresis box (Kodak Biomax QS710, Eastman Kodak, Rochester, NY) and enough 1xTBE was added to cover the entire gel by 1cm of buffer. The combs were removed and the 10μl sample was added to each well. Each gel was run with a DNA molecular weight standard marker containing 100-1000 base pairs (New England Biolabs, Beverly, MA) in position number 8 for reference. The gels were electrophoresed for 50-60 minutes at 72 volts or until the loading dye had migrated approximately 5-6 cm.

The gels were trans-illuminated using a UV light box. The samples were scored as positive or negative according to the presence or absence of DNA fragments <100bp in length. The intensity of the <100bp fragments were scored, as well, using a subjective scoring system of high (+++), medium (++), and low (+) according to the band intensity as viewed under the UV light. The gels were photographed using a Kodak camera.
Micronucleus Assay

Slides were prepared using buccal cells collected from study subjects. A 200μl aliquot of exfoliated buccal cells at 1 x 10^6 per milliliter was spun in a cytopsin (Cytospin 3, Shandon, Pittsburgh, PA) at 700rpm for 7 minutes to get a monolayer of cells attached to the glass microscope slides. A Feulgen fast-green staining procedure was used to visualize the nuclear regions and the cytoplasm as described by Belien et al (1995). After cells were affixed to the glass slides, they were fixed in methanol for 10 minutes, then hydrolyzed in 5N HCl at 27°C for 40 minutes. Cells were then stained with fresh, room temperature, Schiff’s reagent (Sigma, St. Louis, MO) for 50 minutes and washed in tap water for 15 minutes. The slides were counterstained with 0.1% fast-green (Sigma, St. Louis, MO) for 20 seconds, washed, dehydrated with ethanol, and cleared with xylene, then a cover slip was added.

Micronuclei were scored in accordance with criteria reported by Tolbert et al (1992). Cells that were smeared, clumped, overlapped, without intact nuclei or with necrotic nuclei were not scored. Micronuclei in cells of acceptable quality were defined as (1) having a rounded or oval shape and showing a smooth perimeter of the membrane, (2) being less than one-third the diameter of the main nucleus but large enough to discern shape and color, (3) showing positive Feulgen staining with intensity, texture, color, and refractation similar to that of the main nucleus, (4) being in the same focal plane as the nucleus, and (5) not overlapping with the nucleus. At least 3000 buccal cells per sample were scored on coded slides using a Zeis Axioskop microscope (Zeis, Germany). Scoring of micronuclei was performed by a trained pathologist (Defa Tian) and
crosschecked by a second trained analyst (Barbara Collins). The analysts who scored micronuclei did not have any prior knowledge on subjects’ identification or arsenic exposure. Concurrence between the two analysts was expected for a positive micronuclei result.
Statistical Analysis

Statistical analysis was done using the Prism software from GraphPad Software, Inc (San Diego CA). The external exposure to arsenic was measured in the well water samples. Internal exposure markers analyzed consisted of urine, nails, and hair. The mean and standard error of the mean were calculated and reported here. Statistical significance for the internal exposure markers was determined using the nonparametric Mann-Whitney rank-sum test with alpha set at 0.05. The effect of arsenic exposure on buccal cells was measured using the micronuclei frequencies and the DNA fragmentation results. Because neither of the effect markers measured were normally distributed, the nonparametric Mann Whitney rank sum test was used for both the micronuclei frequency data and the DNA fragmentation intensity data to evaluate the statistical significance between the exposed and control groups. A second measure of DNA fragmentation was also evaluated as the percent of subjects that showed positive results for DNA fragments smaller than 100bp. Fisher's exact (two-tailed) test was used to evaluate the statistical significance of the difference between the arsenic exposed group and the controls for this assay. Since smoking may be a potential confounder in the induction of micronuclei (Stich et al, 1984, Sarto et al, 1997) and a possible DNA damaging agent, the data were analyzed for all subjects and segregated by smoking status. No other breakouts were attempted because of the small sample size in this study. Associations between the water data and each of the internal exposure markers and effect markers, as well as relationships between the biomarkers of effects were assessed. The two-tailed Spearman
rank correlation coefficient was used to measure these associations with the statistical significance set at alpha = 0.05.
III. Results

Information and characteristics about the study subjects are shown in Table 1. The characteristics of the study subjects are similar between the high-arsenic exposed and controls when comparing the mean age, tobacco and alcohol use, and vegetable consumption. Study subjects in both the high-arsenic exposed group and the control group lived in this region for a similar duration. The high-arsenic group all \( n=19 \) showed health effects (skin lesions) compared to none in the control group. The high-arsenic exposed subjects were exposed to a mean arsenic concentration of \( 527.5 \mu g/l \) in the drinking water in contrast to the control group’s mean exposure of \( 4.4 \mu g/l \). Figure 3 shows the arsenic concentration in the drinking water with the high-arsenic exposed group significantly higher than the control group \( p<0.0001 \) for all study subjects.

Arsenic was also detected at high levels in the urine, nail, and hair samples of exposed subjects with mean values of \( 632.7 \mu g/l, 32.02 \mu g/g, \) and \( 12.42 \mu g/g, \) respectively, while the controls were \( 28.5 \mu g/l, 3.36 \mu g/g, \) and \( 0.798 \mu g/g, \) respectively, see figures 4-6. For these internal dose markers, the high-arsenic exposed group is significantly higher than the controls for all study subjects \( p<0.0001, p<0.0001, p<0.0001 \) for urine, nail and hair, respectively). The arsenic levels measured in the water samples were similar to that measured in the urine samples, \( 527.5 \mu g/l \) and \( 632.7 \mu g/l, \) respectively. For subjects segregated by smoking status, the only internal exposure marker that showed a statistical difference between smokers and nonsmokers for arsenic concentration was the urine samples \( p<0.05 \). The arsenic content for the high-arsenic exposed group in nail
samples shows over a 2.5-fold increase in arsenic concentration per gram of tissue compared with the hair samples.

Along with the exposure biomarkers, we measured two effect biomarkers, micronuclei (MN) and DNA fragmentation in oral epithelial cells (buccal cells). Figures 7a and 7b show buccal cells stained for the presence of typical micronuclei, regions with nuclear material stain a pinkish-red while the cytoplasm stains a light blue-green. Figure 8 shows a DNA laddering assay gel representing high arsenic-exposed subjects and controls and is typical of the results seen between the exposed and control study subjects. Figures 9 and 10 show the results of the micronuclei frequency and DNA fragmentation, respectively. The MN frequencies showed a statistically significant difference between the high-arsenic exposed subjects and the controls (p<0.01) for all subjects and for nonsmokers (p<0.001) using the Mann Whitney rank test. Micronuclei induction was highest in the arsenic high-exposed group of nonsmokers, 2.48 ± 0.53 (mean ± SEM) when compared with all subjects, (2.21 ± 0.36) and smokers, (1.91 ± 0.50). The difference for the smokers between the arsenic high-exposed and controls was not statistically significant (p=0.414).

The DNA fragmentation results show statistical difference for the mean intensity of the bands less than 100 base pairs in length between the high-arsenic exposed subjects and the controls in all subjects (p<0.0001), nonsmokers (p<0.01), and smokers (p<0.01), see figure 10. The mean intensity is more than two-fold higher in the high-arsenic exposed group when compared to the control group for nonsmokers, all subjects, and smokers, with the smokers in the high-arsenic exposed group showing the highest intensity values. DNA fragmentation results are also shown in figure 11 and reflect the
percentage of subjects with positive results for the presence of DNA fragments smaller than 100bp in size. For all subjects, 17 out of 19 subjects (89%) in the high-arsenic exposed group have DNA fragments that are less than 100 base pairs in length, while only 2 out of 13 (15%) showed fragments less than 100 base pairs in length for the control group (p<0.0001, Fisher’s exact test). Among the smokers, 100% (9/9) of the subjects in the high-arsenic exposed group had DNA fragments less than 100 base pairs in length. This may suggest smoking is a confounding factor but our small sample size makes it difficult to confirm.

Internal exposure biomarkers of arsenic in the urine, nails, and hair correlate well with the arsenic levels in the well water samples as reported in table 2. The Spearman r-values for urine, nails, and hair, irrespective of smoking status, are 0.8397 (p<0.0001), 0.6729 (p<0.0001), and 0.7698 (p<0.0001), respectively. Among the internal exposure biomarkers of arsenic levels, urine, nails, and hair were all statistically correlated. However, urine and nails showed the strongest correlation among the internal exposure biomarkers (Spearman r = 0.7776, p<0.0001).

For the biomarkers of effect, MN frequencies statistically correlated with arsenic exposure (water, Spearman r = 0.4271, p = 0.0148) and with all of the exposure biomarkers (urine, nails, and hair). The strongest statistical correlation for the MN frequencies for all subjects was with the internal exposure biomarker, hair (Spearman r = 0.7057, p<0.0001). In smokers, the MN frequencies were not statistically correlated with the arsenic exposure or any internal exposure biomarker (urine, nails, hair). However, MN frequencies for nonsmokers show correlations with statistical significance for arsenic exposure (Spearman r = 0.6142, p=0.0051) and with all of the internal exposure biomarkers.
biomarkers (urine, nails, hair). The strongest statistical correlation for the MN frequencies in nonsmokers was with the internal exposure biomarker, hair (Spearman r = 0.8051, p<0.0001).

The DNA fragmentation results are statistically correlated with arsenic exposure (water, Spearman r = 0.6683, p<0.0001) and for all of the internal exposure biomarkers (urine, nails, and hair). The strongest correlation for the DNA fragmentation results for all subjects was with the internal exposure biomarker, nails (Spearman r = 0.7318, p<0.0001). For smokers, the DNA fragmentations results were not statistically related to the nail arsenic levels (Spearman r = 0.4705, p = 0.1047), however, in nonsmokers they were statistically correlated (Spearman r = 0.8148, p<0.0001). It is also interesting that the DNA fragmentation results for nonsmokers were not statistically correlated with the water arsenic levels (Spearman r = 0.4219, p = 0.0720), but there were statistically significant relationships between the DNA fragmentation results and all of the internal exposure markers (urine, nails, hair).

Table 2 also shows correlations between the two effect biomarkers, MN frequencies and DNA fragmentation. DNA fragmentation significantly correlates with MN frequencies (Spearman r = 0.5238, p=0.001) in all subjects, but when the data is segregated according to smoking status, only nonsmokers show a good association between DNA fragmentation and frequency of micronuclei induction (Spearman r = 0.6435, p=0.003).
### Table 1. Study Subject Information

<table>
<thead>
<tr>
<th>Parameters</th>
<th>High-ArsenicExposed</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic in drinking water (µg/L)</td>
<td>527.5 ± 23.6</td>
<td>4.4 ± 1.0</td>
</tr>
<tr>
<td>Total subjects (n)</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Sex (n)</td>
<td>14 males, 5 females</td>
<td>8 males, 5 females</td>
</tr>
<tr>
<td>Mean age ± SD (yr)</td>
<td>38 ± 15</td>
<td>38 ± 13</td>
</tr>
<tr>
<td>Smoking status (n)</td>
<td>10 nonsmokers&lt;sup&gt;a&lt;/sup&gt;, 9 smokers&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 nonsmokers&lt;sup&gt;a&lt;/sup&gt;, 4 smokers&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average cigarettes/day smokers</td>
<td>23 ± 7</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Smoking years</td>
<td>25 ± 13</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>Green vegetables (servings/week)</td>
<td>3</td>
<td>2.8</td>
</tr>
<tr>
<td>Alcohol Consumption (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Some</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>None</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Duration of arsenic exposure (yr)</td>
<td>17 ± 4</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Skin lesions (n)</td>
<td>19</td>
<td>none</td>
</tr>
<tr>
<td>(Hyperkeratosis and/or pigmentation abnormalities)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Nonsmokers have never smoked.

<sup>b</sup>Smokers are current smokers.
Figure 3 shows total-arsenic levels measured in water samples over a 5 day period for all 32 study subjects and segregation by smoking status. Each water sample was analyzed separately for a daily total-arsenic value over the 5 day collection period and the 5 measurements were averaged to obtain an observed value for each study subject.

Data shown are mean±SEM, Mann Whitney test, 2-tailed
Statistical significance (alpha set at 0.05) between exposed and control groups are also reported.
Figure 4 shows total-arsenic levels measured in urine samples over a 5 day period for the first and second morning voids including all 32 study subjects and segregation by smoking status. Each urine sample (1st and 2nd void separately) was analyzed separately for a total-arsenic concentration over a 5 day collection period and the 10 measurements were averaged to obtain an observed value for each study subject.

Data shown are mean±SEM, Mann Whitney test, 2-tailed
Statistical significance (alpha set at 0.05) between exposed and control groups are also reported.
Figure 5 shows total-arsenic levels measured in nail samples for all 32 study subjects and segregation by smoking status.

Data shown are mean±SEM, Mann Whitney test, 2-tailed
Figure 6 shows total-arsenic levels measured in hair samples for all 32 study subjects and segregation by smoking status.

Data shown are mean±SEM, Mann Whitney test, 2-tailed
Figures 7a (top) and 7b (bottom) are pictures of buccal cells from arsenic exposed individuals. The buccal cells are Feulgen stained and show typical micronuclei. Both photomicrographs are 400x magnification showing nuclear regions stained a pinkish-red color while the cytoplasm is counterstained with fast green and appears light blue-green.
Figure 8 shows the DNA laddering assay gel with DNA form individuals with arsenic in the drinking water verses the controls. Lane “M” contains the 1000 base pair DNA marker.
Figure 9 shows micronuclei per 1000 buccal cells for all 32 study subjects and segregation for smoking status.

Data shown are mean±SEM, Mann Whitney test, 2-tailed
Figure 10 shows band intensities associated with DNA fragments < 100bp in length for all 32 study subjects and segregation for smoking status.

Data shown are mean ± SEM, Mann Whitney test, 2-tailed
Figure 11 shows percent of study subjects with DNA fragments <100bp in length. Included are all 32 study subjects and segregation by smoking status.

Fisher's Exact test, 2-tailed
Table 2. Correlations\(^a\) between Arsenic Exposure, Exposure Biomarkers (Urine, Nails and Hair), Effect Biomarkers (Micronuclei and DNA Fragments)

<table>
<thead>
<tr>
<th></th>
<th>Water</th>
<th>Urine</th>
<th>Nails</th>
<th>Hair</th>
<th>MN smk</th>
<th>MN non</th>
<th>Frag smk</th>
<th>Frag non</th>
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<tbody>
<tr>
<td>Water (^b)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Urine (^b)</td>
<td>0.8397</td>
<td>1.00</td>
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<td></td>
<td>&lt;0.0001</td>
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<td>Nails (^b)</td>
<td>0.6729</td>
<td>0.7776</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>Hair (^b)</td>
<td>0.7698</td>
<td>0.7612</td>
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<td>MN (^c)</td>
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<td></td>
<td>0.0148</td>
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<td>Frag (^d)</td>
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<tr>
<td></td>
<td>&lt;0.0001</td>
<td>0.0002</td>
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<tr>
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<td>------</td>
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<td></td>
<td>0.6659</td>
<td>0.2872</td>
<td>0.2742</td>
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<tr>
<td>MN non (^f)</td>
<td>0.6142</td>
<td>0.5479</td>
<td>0.6939</td>
<td>0.8051</td>
<td>------</td>
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<td></td>
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<tr>
<td>Frag smk</td>
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<td>0.4705</td>
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<td>------</td>
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<td>0.0018</td>
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<td>Frag non</td>
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<td>0.0478</td>
<td>&lt;0.0001</td>
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<td>0.9731</td>
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\(^a\) Spearman \(r\), two tailed with \(\alpha = 0.05\)
\(^b\) Water, Urine, Nails, and Hair are reported in all subjects
\(^c\) "MN" stands for Micronuclei Frequency in all subjects
\(^d\) "Frag" stands for DNA Fragment <100bp intensity in all subjects
\(^e\) "smk" stands for Smoker defined as a current smoker
\(^f\) "non" stands for Nonsmokers defined as having never smoked
Bold values emphasize statistically significance (\(p<0.05\))
Figure 12 shows the correlation between urine and nail samples for all 32 study subjects.
Figure 13 shows the correlation between urine and hair samples for all 32 study subjects.
Correlation between Arsenic Levels in Urine and MN Frequencies

Spearman \( r = 0.41 \)
\[ p < 0.05 \]

Figure 14 shows the correlation between urine and MN for all 32 study subjects.
IV. Discussion

The focus of this technical report is aimed at assessing arsenic exposure via drinking water and possible relationships between drinking water, internal dose as measured by biomarkers of exposure, and biomarkers of effects in the individuals living in the village of Ba Men in Inner Mongolia, China. We measured total arsenic levels ranging from 409.4 to 640 µg/l with a mean of 527.5 µg/l for those wells classified in the high-arsenic group compared with the control group exposed to low levels of arsenic ranging from 0.4 to 4.9 µg/l (mean of 4.4 µg/l). The arsenic drinking water levels observed here are consistent with some of the high arsenic levels that exist in various other sites in the world. In India and Bangladesh, a recent report of arsenic concentrations in tube wells in Bangladesh showed that 5% of the 34,000 water samples analyzed had levels of 500 µg/l or greater and in the same report the wells in West Bengal, India showed that 1.1% of 101,000 water samples were equal to or greater than 500 µg/l (Rahman et al, 2001). In Chile, the concentrations have been measured as high as 860 µg/l, and in Mexico measurements of 408 µg/l have been reported (Ferreccio et al, 2000, Gonsebatt et al, 1997). A few published reports in the United States have concentrated on areas with high arsenic exposures particularly in Utah and Nevada. These reports have shown arsenic levels as high as 620 µg/l and with a mean level of 1312 µg/l, respectively (Calderon et al, 1999 and Warner et al, 1994). Previous exposure assessments in Inner Mongolia, China, Ma et al have reported arsenic levels as high as 1800µg/l (1999).
Assessing individual exposures to arsenic is important in relating the possible health effects associated with ingestion of arsenic along a dose continuum (i.e. low to high). Development and utilization of internal biological markers of exposure will play an important role in determining health effect outcomes associated with arsenic exposures. The relationship between arsenic levels, intake, and three biomarkers of exposure and two biomarkers of effect were assessed.

For the three internal biomarkers of exposure examined in this study, we found the highest concentration of arsenic in urine followed by the nail samples and hair. Urine is the most widely used internal exposure biomarker for arsenic (Biggs et al., 1997, Calderon et al., 1999, and Karagas et al., 2001). A disadvantage of using urine is that it only provides a measurement of recent arsenic exposure as it is cleared from the body by the kidneys in a matter of days (usually 3-4 days) (Karagas et al, 2001). In our study, we measured a mean total-arsenic concentration in well water samples over a 5-day period of 527.5 μg/l with a mean total-arsenic concentration in urine samples of 632.7 μg/l. The high levels of total-arsenic observed in the drinking water and the increased urinary output of total-arsenic in our population agree with other studies showing similar arsenic levels in the drinking water compared with the measured arsenic in urine samples. Studies in Chile and Mexico show arsenic levels in the drinking water with mean values of 632μg/l and 410μg/l in the exposed groups and measured urinary arsenic levels with mean values of 582.4μg/l, 300μg/l in the same group, respectively (Biggs et al., 1997 and Olguin et al., 1983). The elevated concentration of arsenic found in the urine in our study may be explained two ways. First, the intake of arsenic may be concentrated prior to excretion, which would cause an increase in urinary output of arsenic. Second, in a
chronically exposed population like the one in Ba Men, it can be assumed that individuals were constantly exposed to arsenic via drinking water and they may reach an internal steady state (Biggs et al, 1997). If in fact the study participants reach a steady state, then we may expect to detect excretion patterns at a fairly constant rate, i.e. the measured total-arsenic levels in the water will be similar in concentration as that measured in the urine samples. Along with high arsenic exposure levels, detectable levels of urinary arsenic are found in populations exposed at low levels of arsenic via drinking water sources. Karagas et al reported on a population exposed at low levels with the mean arsenic concentration in water of 2.72 µg/l and the measured urinary arsenic concentrations of 9.89 µg/l.

Our urine results suggest that smoking may be a confounder when using urine as an internal exposure marker. In graph 4, the urine arsenic levels in the smokers of the high-arsenic exposed group are two-fold higher than the levels measured for the nonsmokers in that same group. This possible synergistic effect observed in our study between smoking and arsenic exposure shows statistical significance between the high-arsenic exposed group in smokers versus nonsmokers in that same group (p<0.05). These results are consistent considering one of the known pollutants measured in tobacco smoke is arsenic. However, a more reasonable explanation in this pilot study is a smaller sample (n=9) size, when the subjects were stratified by smoking status, and an observed high variability of arsenic levels within the urine samples.

Because some of the ingested arsenic is metabolized and excreted in the urine in a matter of days, other internal exposure biomarkers are needed to evaluate chronic exposure to arsenic. Deposition of arsenic in the germinal nail and hair matrix occurs a
short time after ingestion (Henke et al, 1982). This deposition in both the germinal nail matrix and the hair bulb occurs within minutes after the absorption of arsenic into the bloodstream because of the rich blood supply to both of these areas (Shapiro, 1967). The element arsenic has an affinity for the sulphhydryl groups of keratin, which allows for the accumulation of arsenic in both the nails and hair (Karagas et al, 2000).

Collecting nails is a convenient, noninvasive way to measure chronic arsenic exposure. Fingernail growth has been reported to be 4-5mm per month while toenail growth is slower at about 1.1mm per month (Henke et al, 1982). Because of this slow growth rate for nails, especially the extremely slow growth rate of the toenails, and the deposition of arsenic shortly after ingestion, it seems reasonable to use nails to assess chronic exposures to arsenic. (Karagas et al, 2000). Along with the accumulation, Garland et al have shown that toenail measurements of arsenic levels using instrumental neutron activation analysis can be reproduced over a 6-year period (Garland et al, 1993).

Henke et al have reported on normal arsenic levels detected in various tissues and organs and state that measurements of arsenic levels in nail samples are 0.2-0.3 μg/g (1982). This “normal” value is what might be detected in individuals who are not highly exposed to arsenic from any source and is a value reported in the forensic literature. In our study, we have measured arsenic concentrations well above this norm with arsenic concentrations elevated to a mean of 32.02 μg/g in nails, a 100-fold increase. Most studies measuring arsenic concentrations in nail samples have focused on individuals exposed to much lower levels of arsenic in drinking water. Karagas et al have reported water arsenic concentrations of 3.51 and 2.72 μg/l with corresponding nail arsenic concentrations of 0.13 μg/g and 0.11 μg/g, respectively (2001). A higher nail arsenic
level was reported in a Mexican study showing a nail arsenic level of 4.55μg/g ± 2.25
(mean ± SD). This value is still considerably lower than in our population but the
Mexican population was only exposed to three-quarters the amount of arsenic via
drinking water compared to our study (Olguin et al, 1983). Another study examining
arsenic exposure and fingernail levels reports on high arsenic levels in the fingernails, but
the route of exposure was inhalation. In this study, the arsenic exposure levels in the air
are reported as high as 35μg/m³ corresponding to a measured arsenic level in the
fingernail sample of 16.0μg/g (Agahian et al, 1990). It is apparent that the arsenic levels
observed in the nail samples for our population are some of the highest (2-6 fold higher)
reported to date.

Hair samples have also been used to evaluate body burden of arsenic. Armienta
et al in a study conducted in Mexico, where the water arsenic levels ranged from 14 μg/l-
1090 μg/l, showed significant differences in arsenic levels in hair samples from
individuals that consumed water with 500 μg/l or greater compared with the individuals
consuming lower levels of arsenic in the water (14 μg/l) (1997). Henke et al have
reported on what they called normal levels of arsenic detected in human head hair to be
0.13 –3.71 μg/g. The growth rate of human hair is about a half an inch (13mm) per
month or about 0.4-0.5mm per day (Shapiro, 1967). In our study, we measured high
levels of arsenic in the hair samples, a mean concentration of 12.4 μg/g, that correspond
with high levels of arsenic concentrations in the water samples compared with control
levels detected in hair samples of 0.798 μg/g. A study conduct in Mexico on a
population exposed to water containing 410 μg/l only showed an arsenic concentration
for the hair of 1.24 μg/g, which is almost 12-fold lower than our reported arsenic level in
hair (Olguin et al, 1983). Also, Olguin et al referenced two other reports on arsenic levels in hair samples showing levels of 1.16 µg/g and 4.2 µg/g, both of which are almost 12-fold and 3-fold less than our observed values, respectively. Again, our population in Ba Men has been exposed to a higher level (almost 25% more arsenic compared with the Olguin study) of arsenic in the drinking water and for numerous years (17 years), while the Olguin et al study doesn’t report on the overall length of exposure in there population from drinking arsenic contaminated water. The Olguin et al study doesn’t attempt to discuss any variation in water arsenic levels in their report. Their results may be influenced by daily or monthly variation in water arsenic content. For our population, the variation in arsenic water content is not extreme (data not reported in this report).

Hair may be a good surrogate to assess chronic exposures to arsenic, however it is thought that hair is more likely to encounter other external arsenic contaminants, such as airborne arsenic particles (Armienta et al, 1997 and Garland et al, 1993). The method by which we analyzed hair wasn’t able to differentiate between internal and external arsenic contamination. Hair analysis for arsenic contamination requires a more complicated process, such as sectional analysis, which allows for the determination between internal versus external arsenic contamination (Henke et al, 1982). Our results of the nail and hair arsenic levels detected in this study indicate that nail arsenic levels are more than twice that measured in the hair samples. This result is interesting and may be due to the larger surface area corresponding to a possible higher amount of keratin in the nail samples as compare to the hair samples. The growth rate of nails (finger) has been reported as about half that of hair, which may also account for the higher concentration seen in the nail samples. The sample of nails taken may have been more concentrated
with arsenic because of this slower (half as much) growth rate. A third explanation may be attributable to the chemical composition of each relatively structure-less site, whereby the nails may have a higher content of keratin containing more sulphhydryl groups (No reference in support of this claim).

The internal biomarkers of exposure (urine, nails, and hair) correlated well with the water concentrations in our pilot investigation. The nail, hair and urine samples were all statistically significant in the high-arsenic group as compared to the control group (p<0.001, p<0.0001, p<0.0001) and all three biomarkers correlated well with water analysis, \( r = 0.6729 \), p<0.0001 for nail v/s water, \( r = 0.7698 \), p < 0.0001 for hair v/s water, and \( r = 0.8397 \), p < 0.0001 for urine v/s water. Figures 12 and 13 are examples of two correlations performed on these data using the urine, nail, and hair sample arsenic levels.

Other studies have looked at these biomarkers of exposure at lower levels of exposure. Karagas et al showed a good correlation between toenail arsenic concentrations and water arsenic concentrations at low levels (\( r = 0.46 \), p<0.001) (2000). Karagas et al reported that the highest correlation was among those with water concentrations at or above 1 \( \mu \)g/l (\( r = 0.65 \), p<0.001) (2000). The authors followed up this study with a second study again correlating low arsenic water concentrations with both toenails and urine samples over a 3-5 year period, showing water arsenic concentrations >1 \( \mu \)g/l correlates with both urinary arsenic (0.46, p=0.029) and toenail arsenic (0.64, p=0.006) (Karagas et al, 2001). Calderon et al measured drinking water arsenic levels between 8-620 \( \mu \)g/l in a Utah-based population, showed that urinary arsenic
concentrations may be an adequate way of estimating an individuals' internal arsenic exposure (1999).

The biomarkers of effects reported in this technical report are micronuclei (MN) for assessing chromosome damage and DNA fragmentation for assessing DNA damage in buccal cells. The micronuclei frequencies per 1000 buccal cells were 3.4 fold higher in exposed individuals (2.21 MN/1000 buccal cells) than in the controls (0.65 MN/1000 buccal cells) and the relationship between the exposed and controls was statistical significant (p< 0.01). Our reported values are consistent with other published reports looking at micronucleated buccal cells associated with ingestion of arsenic via drinking water (Gonsebatt et al, 1997 and Aposhian et al, 1997). Gonsebatt et al reported a 4-fold increase in micronucleated exfoliated oral mucosa cells showing a significant increase in MN cells in the exposed individuals (2.21 MN/1000 buccal cells) who ingested water containing arsenic levels of 408 µg/l compared with the controls (0.58 MN/1000 buccal cells) (water arsenic level = 29.9 µg/l) (1997). In this report, the authors noted that this was true only for the males in this Mexican population and explained their results with the work/water consumption habits of the males (i.e. males drank more water) (Gosenbatt et al, 1997). Aposhian et al reported a 5-fold increase in micronucleated buccal cells (1.72 MN/1000) for those individuals consuming drinking water with higher arsenic concentrations (593 µg/l) than among the controls (0.30 MN/1000 buccal cells) (water arsenic levels = 21 µg/l) (1997). Although these results reported by Aposhian et al need to be confirmed in a larger study as this published study is based on the very small sample size used to evaluate the prevalence of micronucleated buccal cells in this
population. Another study reported on MN frequencies per 1000 cells in both children and women in an arsenic exposed population drinking water with about 200 μg/l of arsenic. This study showed a significant difference in the exposed individuals (both children and women) as compared with the control population, however, the reported MN were in peripheral blood lymphocytes and the numbers of MN scored were about 15-fold higher in the arsenic exposed group compared to our results in the high-arsenic exposed group reported here. This may be due to the use of a more sensitive surrogate marker, the peripheral blood lymphocytes, because the reported values in the control population in their study were also 15-fold higher compared with our control values.

Effects attributed to arsenic exposure can be evaluated using the micronucleus assay because arsenic acts as a clastogen and is genotoxic (Basu et al, 2001). The exact nature by which micronuclei develop in these exfoliated cells is not clear. Exfoliated buccal cells arise from the epithelial basal cell layer through cellular division and chromosomal damage that occurs during cellular division in vivo can be detected. Micronuclei may arise from acentric chromatid or chromosome fragments and arsenic has been associated with spindle disruption (Basu et al, 2001) and/or may produce stressors that interact with the DNA repair processes to damage the chromosome structures resulting in fragments (Tian et al, 2001).

We also stratified our data for analysis according to smoking status for the MN results. It was interesting to see the overall mean frequency of MN formation was higher in nonsmokers exposed to high levels of arsenic (2.48 MN/1000 cells) compared to smokers (1.91 MN/1000 cells). This result seems counter to my a priori hypothesis that higher levels of arsenic in drinking water may have an additive or synergistic effect with
smoking in the formation of MN. However, this hypothesis may still be valid because
the synergistic effect of both smoking and arsenic exposure through drinking water may
force these cells into apoptosis or necrosis resulting in cell death. So, scoring for the
formation of MN in the buccal cells of smokers may be underrepresented here due to this
synergistic effect of both exposures. Also in smokers, there wasn’t a significant
difference between high-arsenic exposed individuals and controls. MN formation in the
control group was 2-fold higher compared to the MN scored in the control, nonsmoking
group. So, smoking in the control group may be a confounder in these results for the
formation of micronuclei. However, these MN results between smokers and nonsmokers
in this population need to be confirmed because of the limited number of smoking
subjects in this study.

In our study, the high arsenic exposed individuals showed DNA fragments <100
base pairs in length in 89% of subjects (17/19) whereas the DNA extracted from the
controls had only 15% (2/13) (see Figure 11). Because exfoliated oral mucosa cells may
be terminally differentiated cells, one would expect to see an increase in apoptosis
(necrosis) in these exfoliated buccal cells. However, the controls don’t show a similar
amount of DNA fragments (laddering patterns) as would be expected if this were the
case. Also, in our sampling technique, we tried to rinse off most of the exfoliated buccal
cells and collect more cells from deeper layers in the oral mucosa. This is the reason why
we had a trained technician scrape the inner cheek for buccal cells. It is also interesting
to note that we saw DNA fragments which were <100bp in length, where typical
fragments associated with apoptosis are usually 180 base pairs in length, which are
associated with a histone because of the enzymatic cleavage of the DNA during this
process (Wyllie, 1980, White, 1996, and Walker et al, 1999). Of course, this pilot study only examined a limited number of samples and these results need to be confirmed in a much larger sample size. Feng et al, from our lab, published the only results in the literature to date showing DNA fragments in buccal cells were related to high arsenic levels in drinking water (2001). Mass et al examined the potential for arsenic (especially the methylated trivalent form) to be a direct-acting DNA damaging agent (2001). In two separate assays, DNA was damaged without exogenously adding enzymes or chemical activation systems. The authors point out that arsenicals may be carcinogenic and/or genotoxic by multiple modes of action, several published papers show that inorganic arsenic in the trivalent form inhibits tubulin polymerization and inhibits DNA ligase II or other enzymes involved in repair (Mass 2001, Basu 2001). DNA damage may be due to direct or indirect interactions with the DNA and/or because of inhibition of DNA repair processes.
V. Summary

The water used for drinking in this region (Ba Men) of Inner Mongolia, China is contaminated with high levels of arsenic, ranging from 203 µg/l to 1446 µg/l in the exposed group in this study, while the control group had a water arsenic concentration ranging from 0.3 µg/l to 9.8 µg/l. Urine, nail, and hair samples were collected and all three were statistically associated with the high levels of arsenic in the drinking water. The highest levels of arsenic were detected in the urine followed by the nails and the hair samples (arsenic in urine>arsenic in nails>arsenic in hair) in this population. Based on the results of these three internal exposure markers in this pilot study, I would choose to collect and measure chronic arsenic levels in nail samples as a measure of internal exposure, specifically the toenail samples. Toenails are easily obtained and analyzed for arsenic levels and give a reliable measure of chronic arsenic exposure through ingestion. They have a very slow growth rate and are less likely to be contaminated with arsenic by other exposure routes, such as airborne.

Health effect biomarkers used in this pilot study were the induction of MN and the presence of DNA fragments. Both of these markers of effect were statistically related with the high arsenic levels detected in the drinking water. MN formation was 3.4 fold higher in the high-arsenic exposed group compared to the controls and the presence of DNA fragments was 89% in the high-arsenic exposed group compared to 15% in the controls. As well, both of these biomarkers of effect were statically associated with all three of the internal exposure markers reported in this population.
VI. Conclusions

The results of this pilot study showed promise for using urine, nails, and hair as internal exposure biomarkers to assess arsenic exposure and for the use of the MN assay and DNA laddering assay as biomarkers of effects in human cells. A major purpose of this pilot study was to examine the feasibility of conducting a larger scale biomarker study in this region of China and incorporating these three internal exposure biomarkers and the two human health biomarkers reported on here. It is important when assessing arsenic exposure via drinking water to measure the concentration of arsenic in the water samples, as well as get an idea of the internal exposure with the use of internal exposure biomarkers. All three of the internal exposure biomarkers proved to be potentially useful markers of internal exposure in this population, which was exposed chronically to high levels of arsenic via drinking water supplies. As well, the biomarkers of effects used in this study showed that the use of noninvasive buccal cells was a practical and potentially useful surrogate cell.

Even though we can’t determine the mechanism of DNA fragmentation and induction of micronuclei associated with arsenic exposure in this pilot investigation, we can state that they were associated with high arsenic levels in the drinking water supplies. Care should be used when drawing major generalities about this pilot study due to the limited number of samples. Because of these results, a larger study was carried out to assess the dose-response relationship of arsenic exposure in this population over a high, medium, and low range of exposures. This larger study will attempt to answer questions of dose-response relationships associated with chronic arsenic exposure via drinking
water in humans, as well as allow us to better evaluate the interaction of smoking and arsenic ingestion via drinking water as it relates to this population.
References


Federal Register: May 22, 2001 (Volume 66, Number 99) 28341-28350. 40CFR parts 9, 141, 142. National Primary Drinking Water Regulations; Arsenic and Clarifications to Compliance and New Source Contaminants Monitoring; Delay of Effective Date; Final Rule.


