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Arsenic, Organic Foods, and Brown Rice Syrup

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BACKGROUND: Rice can be a major source of inorganic arsenic ([As.sub.i]) for many subpopulations. Rice products are also used as ingredients in prepared foods, some of which may not be obviously rice based. Organic brown rice syrup (OBRS) is used as a sweetener in organic food products as an alternative to high-fructose corn syrup. We hypothesized that OBRS introduces As into these products.

OBJECTIVE: We determined the concentration and speciation of As in commercially available brown rice syrups and in products containing OBRS, including toddler formula, cereal/energy bars, and high-energy foods used by endurance athletes.

METHODS: We used inductively coupled plasma mass spectrometry (ICP-MS) and ion chromatography coupled to ICP-MS to determine total As ([As.sub.total]) concentrations and As speciation in products purchased via the Internet or in stores in the Hanover, New Hampshire, area.

DISCUSSION: We found that OBRS can contain high concentrations of As; and dimethylarsenate (DMA). An "organic" toddler milk formula containing OBRS as the primary ingredient had [As.sub.total] concentrations up to six times the U.S. Environmental Protection Agency safe drinking water limit. Cereal bars and high-energy foods containing OBRS also had higher As concentrations than equivalent products that did not contain OBRS. [As.sub.i] was the main As species in most food products tested in this study.

CONCLUSIONS: There are currently no U.S. regulations applicable to As in food, but our findings suggest that the OBRS products we evaluated may introduce significant concentrations of [As.sub.i] into an individual's diet. Thus, we conclude that there is an urgent need for regulatory limits on As in food.

KEY WORDS: arsenic, baby formula, brown rice syrup, cereal bars, energy bars, organic foods, speciation. Environ Health Perspect 120:623-626 (2012). http://dx.doi.org/10.1289/ehp.1104619 [Online 16 February 2012]

Arsenic (As) is an established carcinogen based on studies of populations consuming contaminated drinking water (Smith et al. 2002). Recently, attention has focused on As exposure from food, in particular fruit juices (Rock 2012) and rice (Stone 2008). Rice may contain As in total concentrations up to 100-400 ng/g, including both inorganic As ([As.sub.i]) and the organic species dimethylarsenate (DMA) (Williams et al. 2005), with much lower concentrations (relative to DMA) of monomethylarsenate (MMA) also occasionally detected. Total As ([As.sub.total]) in rice and relative proportions of DMA and As; differ both geographically (Meharg et al. 2009) and as a function of genetic and environmental controls (Norton et al. 2009).

[As.sub.i] is more toxic than DMA or MMA (Le et al. 2000), and food regulatory limits, where they exist, are based on [As.sub.i]. Infants fed rice cereal at least once daily may exceed the daily As exposure limit of 0.17 [micro]g/kg body weight per day based on drinking water standards (Meharg et al. 2008b). Rice products such as cereals and crackers (Sun et al. 2009) and rice drinks (Meharg et al. 2008a) are potentially significant dietary sources of As. Infants and young children are especially vulnerable because their dietary As exposure per kilogram of body weight is 2-3 times higher than that of adults [European Food Safety Authority (EFSA) 2009].

DMA is a metabolite of [As.sub.i]. Although considered less toxic than [As.sub.i], its toxicological potential has not been studied extensively. The presence of DMA in rice is likely due to natural soil microbial processes; however, DMA was used as a pesticide before being banned by the U.S. Environmental Protection Agency (EPA) in 2009 (U.S. EPA 2009). Organic food consumers may therefore object to its presence in organic foods even in the absence of direct evidence of human health effects of DMA.

In the United States, organic brown rice syrup (OBRS) is used as a sweetener as a healthier alternative to high-fructose corn syrup in products aimed at the "organic foods" market. Added sugar is often the main ingredient in infant and toddler formula, and the addition of sucrose to a main-brand organic formula was the feature of a popular press article in relation to possible childhood obesity (Moskin 2008). Many products--including some baby milk formulas, cereal bars, and high-energy performance products for athletes--list OBRS as the major ingredient. Brown rice is usually higher in both [As.sub.total] and [As.sub.i] than white rice because [As.sub.i] is localized to the aleurone layer, which is removed when rice is polished, whereas DMA passes into the grain (Carey et al. 2011; Sun et al. 2008). Ranges of As concentration in rice products, including OBRS, are similar to As concentrations in brown rice (Signes-Pastor et al. 2009).

We posit that consumers of organic food products are generally attempting to make educated eating choices and that this consumer group would be particularly interested to know if, and to what extent, OBRS introduces [As.sub.i], DMA, and MMA into these products. We therefore measured [As.sub.total] and As speciation in three commercially available brown rice syrups, 15 infant formulas without OBRS, 2 toddler formulas with OBRS, 29 cereal bars (13 with OBRS), and three flavors of a high-energy performance product.

Materials and Methods

We purchased three commercial OBRSs from local or online stores. For one syrup, two bottles of the same product (from different lots) were tested. Fifteen infant formulas and two toddler formulas (initially purchased as part of a parallel study on As content of formulas and infant foods), as well as 29 cereal bars and three energy shot blocks were all purchased from local stores in the Hanover, New Hampshire, area.

Sample preparation. All samples were analyzed for [As.sub.total]) and selected samples were extracted for As species. For formulas, [As.sub.total] was determined after closed vessel microwave digestion (MARSXpress; CEM Corp., Matthews, NC) with Optima HN[O.sub.3]. Approximately 0.25 g formula was digested in 2 mL 50% HN[O.sub.3] (nitric acid). The samples were heated at 180 [degrees]C for 10 min, allowed to cool, and then diluted to 10-25 mL with deionized water. Cereal bars and energy blocks were homogenized using a ceramic knife and were not dried before digestion. A subsample was digested in 2-3 mt. Optima HN[O.sub.3] and heated at 95 [degrees]C for 30 min. The digested sample was diluted with deionized water to 25-50 mL. This digested sample was diluted a further 10 x before analysis to reduce the acid concentration in the sample to < 5%. All digestions and dilutions were recorded gravimetrically. Samples were extracted for As speciation using 1% HN[O.sub.3] and open-vessel hearing in a microwave digestion unit following a heating profile of 55 [degrees]C for 5 min, 75 [degrees]C for 5 min, and 95 [degrees]C for 20 min (Foster et al. 2007; Huang et al. 2010). An aliquot of the extracted sample was then centrifuged at 13,300 rpm for 30 min; an aliquot of that supernatant was further spin filtered at 10 kDa.

[As.sub.total] and As speciation. [As.sub.total] was determined by inductively coupled plasma mass spectrometry (ICP-

MS; model 7700x; Agilent, Santa Clara, CA) using helium as a collision gas at a flow rate of 4.5 mL/min. Samples were analyzed by either external calibration or the method of standard additions. As speciation of the 1% HN[O.sub.3] extracts was determined by ion chromatography coupled to ICP-MS using a Hamilton PRP X100 anion exchange column (Hamilton Company, Reno, NV) and a 20 mM ammonium phosphate eluant at pH 8. Formulas were evaluated in triplicate, and 5% duplicate and duplicate spikes were performed for the cereal bars and energy blocks.

We used NIST Standard Reference Material (SRM) 1568a rice flour (National Institute of Standards and Technology, Gaithersburg, MD) as a quality control material for both [As.sub.total] measurements and As speciation. Although As species are not certified for SRM 1568a, reproducible consensus values have been demonstrated in many studies (Meharg and Raab 2009; Raab et al. 2009; Williams et al. 2005). We determined Astoial in SRM 1568a to be 279 [+ or -] 31 ng/g (mean [+ or -] 1 SD; n = 6); the certified value is 290 [+ or -] 30 ng/g. For As speciation (n = 5), we determined DMA to be 186 [+ or -] 21 ng/g, MMA to be 9.4 [+ or -] 3.7 ng/g, and [As.sub.i] to be 101 [+ or -] 15 ng/g, which are in the range reported by other studies.

Data analyses. Given our calculated values for As speciation in the formulas, we estimated As concentrations (micrograms per liter) of reconstituted formulas assuming that one scoop of powdered formula weighs 8.75 g and that one scoop of formula is added to 60 mL As-free water to make 2 fluid ounces of formula. We then estimated daily intake of As species for a baby weighing 6 kg and 9 kg, assuming consumption of six 4-ounce bottles of milk formula each day, and compared this with "safe" levels estimated for consumption of drinking water containing [As.sub.i] at the U.S. EPA and World Health Organization (WHO) maximum contaminant limit of 10 [micro]g/L (Meharg et al. 2008b).

Table 1. As concentration and As speciation for three OBRSs.

	[As.sub.total] [ng/g		Speciation analysis			
Sample	(mean [+ or -] 1 SD)]	[As.sub.i] (%)	DMA(%)	MMA(%)	Sum of As species (ng/g)	
A,lot 1	78 [+ or -] 6	89	7	4	81	
A, lot 2	94 [+ or -] 8	84	12	4	94	
B	136 [+ or -] 3	91	6	3	118	
C	406 [+ or -] 6	51	46	3	294	

Analyses were performed in triplicate.

Results and Discussion

Rice syrups. [As.sub.total] concentrations in three rice syrups (and from two lots of one of the syrups) ranged from 80 to 400 ng/g (Table 1). As; was 80-90% of [As.sub.total] for two of the three syrups; for the third syrup, only 50% of [As.sub.total] was [As.sub.i]. However, because this syrup was much higher in [As.sub.total], it also had the highest [As.sub.i concentration of the syrups. All syrups had detectable MMA, ranging from 3 to 4% of [As.sub.total], but the major organic As species for each syrup was DMA. Our results are similar to those of Signes-Pastor et al. (2009) who reported dry weight [As.sub.total] concentrations of 80, 100, 120, and 330 ng/g in four rice syrups, with 71% [As.sub.i] and 85% extraction efficiency in the highest As syrup. Moreover, given these authors' estimate of 15% moisture content for the syrups, we estimate that the actual contribution to As concentration in food products that include OBRS as the dried product--such as toddler formulas--would be approximately 1.15 times the concentration listed in Table 1.

Baby formulas. We analyzed 17 different formulas. Average [As.sub.total] concentrations in the 15 infant formulas that did not contain OBRS were relatively low, in the range of 2-12 ng/g (Jackson et al. 2012). 'Those results were consistent with two other studies of As in infant formula (Ljung et al. 2011; Vela and Heitkemper 2004). However, the As concentrations in the two toddler formulas that listed OBRS as the primary ingredient (one dairy-based and one soy-based) were > 20 times the As concentrations in infant formulas that did not contain OBRS (Figure 1A). The proportion

of [As.sub.i] varied among products and among lots of the soy-based formula, but the concentration of As; in the reconstituted formulas with OBRS was either just below (dairy, 8-9 [micro]g/L) or 1.5-2.5 times above (soy) the current U.S. drinking water standard (10 [micro]g/L). In addition, the OBRS formulas contained 19-40 [micro]g/L DMA and trace levels of MMA. Expressed as daily As intake per kilogram of body weight, the exposure of infants and toddlers drinking OBRS-containing milk products is even more apparent (Figure 1B). Using web-based search engines, we found only these two toddler formulas that used OBRS, so the number of infants using this formula is presumably a very low percentage of U.S. formula-fed infants.

Infants, in a phase of rapid development, are especially vulnerable to contaminants, and emerging data suggest that As exposure early in life may pose risks not only during childhood but also in adult life (Vahter 2009). This suggests that we need to pay particular attention to the potential for As exposure during infancy. The standards and guidelines for daily intake of As are currently a matter of debate (Meharg and Raab 2009; Meharg et al. 2008b). The WHO established a provisional maximum tolerable daily intake (PMTDI) guideline of 2.1 [micro]g/kg/day in 1983 (Food and Agriculture Organization of the United Nations/WHO 1983). For an infant weighing either 6 or 9 kg, both of the OBRS formulas would be above this value based on [As.sub.total]; for a 6-kg infant, the soy formulas would be above the guideline based only on [As.sub.i]. It should be noted that the WHO 1983 PMTDI is based on a safe drinking water limit of 50 [micro]g/L rather than the current limit of 10 [micro]g/L [European Food Safety Authority (EFSA) 2009; Meharg and Raab 2009]. Currently, only China has a limit for As in food; an [As.sub.i] limit of 150 ng/g for rice (Zhu et al. 2008). Although the OBRS toddler formulas would not exceed this limit on average, [As.sub.total] and [As.sub.i] concentrations of these OBRS formulas are cause for concern.

Cereal and energy bars. OBRS is also a popular sweetener for many cereal/energy bars and high-energy athletic performance products. Our web-and store-based market survey of 100 bars indicated that about 50% contain either OBRS (31%), other rice products (5%), or both (14%). We tested 29 bars and three types (flavors) of an energy product obtained from a local supermarket. The results for the cereal/energy bars are shown in Table 2. All of the bars had detectable [As.sub.total] with a range of 8-128 ng/g. The 7 bars that did not list any rice product among the top five ingredients were among the 8 lowest As-containing bars we tested. The remaining bars listed at least one of four rice products (OBRS, rice flour, rice grain, and rice flakes) in the first five ingredients and had Aston' concentrations ranging from 23 to 128 ng/g.

We analyzed As speciation in 12 of the rice-containing bars. Of the 12 bars, 11 contained [As.sub.i] concentrations > 50%, with an average of 70% [As.sub.i]. All organic As was DMA. The percent recovery (sum of As species as a percentage of [As.sub.total]) ranged from 67% to 124%; however, some of this variability is because the bars were not dried before analysis and were analyzed "as is," with limited homogenization using a ceramic-bladed knife. The amount of As; ingested when eating one of these bars is a function of the As concentration of the bar and the size (weight) of the bar. The bars we analyzed ranged in weight from 28 to 68 g; at the upper limit of bar weight and [As.sub.i] content, an individual bar contained up to 4 [micro]g [As.sub.i]. For example, bar 27 weighed 45 g and contained 101 ng/g [As.sub.total] and 79% [As.sub.i], equating to an [As.sub.i] content of 3.6 [micro]g.

Energy shot blocks. We also analyzed As concentration and speciation in three high-energy products for endurance athletes known as "energy shot blocks," each of which contained OBRS. Although an educated consumer might be aware of the potential for rice to contain As (and therefore know that products containing rice ingredients might also contain As), the energy shot blocks are gel-like blocks, so it would not be immediately apparent to the consumer that these too are rice-based products.

The As concentration in one of the energy shot blocks containing OBRS was 84 [+ or -] 3 ng/g [As.sub.total] (n = 3), which was 100% [As.sub.i]. The other two energy shot blocks were very similar to one another in [As.sub.total] concentrations (171 [+ or -] 3.6 ng/g, mean [+ or -] SD; n = 6) and speciation (53% [As.sub.i]). No MMA was detected in the energy shot blocks. All three flavors contained 2.5-2.7 [micro]g [As.sub.i] per 30-g serving. The manufacturer recommends consuming up to two servings (60 g) per hour during exercise, so an endurance athlete consuming four servings during a 2-hr workout would consume approximately 10 [micro]g [As.sub.i] per day, equal to the [As.sub.i] intake resulting from consumption of 1 L of water at the current U.S. EPA and WHO limit of 10 [micro]g/L. Athletes consuming the two flavors containing 171 ng/g [As.sub.total] would also consume 2.5 [micro]g DMA per 30-g serving.

Conclusions

Food is a major pathway of exposure to As for most individuals (EFSA 2009). Rice and rice products can contribute to an

individual's [As.sub.i] exposure (Meharg et al. 2008a, 2008b; Williams et al. 2005). There is a growing body of information about As concentration and speciation in rice in the peer-reviewed literature and thus in the public domain, but much less information is available on rice-based food products. Rice products are used in a variety of foods, including gluten-free products and, as we show here, in products where OBRS is used as an alternative to high-fructose corn syrup. The formulas containing OBRS--which could be the sole sustenance for an individual over a critical period of development--can result in consumption of milk with As concentrations much higher than the drinking water standard, yet there are no U.S. regulations to deal with this particular scenario. Similarly, endurance athletes who consume 4 servings of OBRS-containing energy shot blocks (manufacturer-recommended maximum for 2 hr of physical activity) may be exposed to as much as 10 [micro]g [As.sub.i] and 20 [micro]g [As.sub.total] in a single day. Moreover, the major As species in the overwhelming majority of food products we have tested is the more toxic [As.sub.i], a finding that, although noted in other studies (Sun et al. 2009), is particularly troubling given the nonthreshold relationships between cancer risk and exposure to [As.sub.i] (National Research Council 2001).

ACADEMIC JOURNAL ARTICLE *Environmental Health Perspectives*

Unventilated Indoor Coal-Fired Stoves in Guizhou Province, China: Cellular and Genetic Damage in Villagers Exposed to Arsenic in Food and Air

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Unventilated Indoor Coal-Fired Stoves in Guizhou Province, China: Cellular and Genetic Damage in Villagers Exposed to Arsenic in Food and Air

Zhang, Aihua, Feng, Hong, Yang, Guanghong, Pan, Xueli, Jiang, Xianyao, Huang, Xiaoxin, Dong, Xuexin, Yang, Daping, Xie, Yaxiong, Peng, Luo, Li, Jun, Hu, Changjun, Li, Jian, Wang, Xilan, Environmental Health Perspectives

BACKGROUND: Inorganic arsenic (iAs) is a well-known human carcinogen recognized by the World Health Organization and the International Agency for Research on Cancer. Currently, most iAs studies in populations are concerned with drinking water and occupational arsenicosis. In Guizhou province, arsenicosis caused by the burning of coal in unventilated indoor stoves is an unusual type of exposure. Because the poisoning mechanism involved in arsenicosis is as yet unknown and no effective therapy exists, progress has been slow on the prevention and therapy of arsenicosis.

OBJECTIVES: We examined the relationship between arsenic (As) exposure from the burning of coal in unventilated indoor stoves and genetic damage in humans, using cellular and molecular indices. We selected villagers from Jiaole township, Guizhou province, China, who had been exposed to milligram levels of As daily via food and air contaminated by the burning of As-containing coal in unventilated indoor stoves.

RESULTS: The As-exposed subjects from Jiaole were divided into four groups according to skin lesion symptoms: nonpatients, mild, intermediate, and severe arsenicosis. Another 53 villagers from a town 12 km from Jiaole were recruited as the external control group. In the four groups of exposed subjects, As concentrations in urine and hair were 76-145 [micro]g/L and 5.4-7.9 [micro]g/g, respectively. These values were higher than those in the external control group, which had As concentrations of 46 [micro]g/L for urine and 1.6 [micro]g/g for hair. We measured sister chromatid exchange and chromosomal aberrations to determine human chromosome damage, and for DNA damage, we measured DNA single-strand breaks and DNA-protein cross-links. All measurements were higher in the four exposed groups compared with the external control group. DNA repair was impaired by As exposure, as indicated by the mRNA of

O-6-methylguanine-DNA methyltransferase (MGMT), X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1), and, to a lesser extent, by the mis-match repair gene hMSH2 mRNA. The expression of mutant-type p53 increased with aggravation of arsenicosis symptoms, whereas the expression of p16-INK4(p16) decreased. p53 mutated at a frequency of 30-17% in the carcinoma (n = 10) and precarcinoma (n = 12) groups. No mutation was found in p16, although deletion was evident. Deletion rates were 8.7% (n = 23) and 38.9% (n = 18) in noncarcinoma and carcinoma groups, respectively.

CONCLUSIONS: The results showed that long-term As exposure may be associated with damage of chromosomes and DNA, gene mutations, gene deletions, and alterations of DNA synthesis and repair ability.

KEY WORDS: arsenic, coal, genetic damage, toxicity. Environ Health Perspect 115:653-658 (2007). doi:10.1289/ehp.9272 available via http://dx.doi.org/ [Online 9 January 2007]

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Although inorganic arsenic (iAs) is a well-known human carcinogen recognized by the World Health Organization (2001) and the International Agency for Research on Cancer (1987), the mechanism of carcinogenicity are not clear. Because of significant differences in arsenic (As) metabolism between experimental animals and humans, the use of animal models to evaluate the carcinogenic effects iAs has not been established successfully (Goering et al. 1999). For decades, As has been considered a nongenotoxic carcinogen because it is only weakly active or, more often, completely inactive in bacterial and mammalian cell mutation assays (Hei and Filipic 2004). Recently, increasing evidence has shown that As is a strong, dose-dependent gene and chromosomal mutagen that is capable of inducing mostly multilocus deletions (Hei et al. 1998). Experiments in mammalian cells have also shown that this induction was significantly reduced in the presence of antioxidant enzymes (Liu et al. 2002).

As early as 1976, villagers from Guizhou province in southwestern China were reported to be suffering severe symptoms of arsenicosis (Zhou et al. 1993), which was attributed to exposure to high levels of As in food, especially in corn and chili peppers, and to a lesser extent by breathing As-laden air (Finkelman et al. 2003). Villagers mined local low-grade As-containing coal from abundant, small local coal pits, with As-coal concentrations mostly in the range of hundreds of milligrams per kilogram (Finkelman et al. 1999; Zheng et al. 1999). Corn and chili peppers were dried over unventilated indoor stoves used for every day cooking and for heating during the winter months. Repeated surveys of As-coal and medical examinations have identified nine towns in four counties in southwest Guizhou province as having high levels of As in food and air (Zhang et al. 2000a). Approximately 200,000 people within the four counties were at risk of exposure to high levels of As; 3,000 cases of arsenicosis were diagnosed in the late 1990s, with approximately 2,000 of these cases in Xinren county alone (Liu et al. 2002). Over the years, various measures adopted by local governments, such as shutting down the coal pits containing high levels of As and installation of ventilated stoves, have been only minimally effective until a health education campaign was implemented in 2005 (An et al. 2005).

New cases of arsenicosis have been identified each year since 1990, although there was evidence of reduction in exposure as early as 1998. Between 1998 and 2004, collected coal samples had As concentrations of 92-816 mg/kg (Huang et al. 2002). The average concentration in indoor air during that time was 0.087 [+ or -] 0.045 mg/[m.sup.3] (n = 22), which is lower than that in indoor air in 1991 [0.46 [+ or -] 0.30 mg/[m.sup.3] (n = 18); Zhang et al. 2000a; Zhou et al.1993]. Urinary As concentrations declined from 130.6 [+ or -] 121.2 [micro]g/L (n = 167) in 1998 to 97.0 [+ or -] 76.1 [micro]g/L (n = 43) in 2004. This finding coincided with the decrease of As concentrations in indoor air. In the exposed population, skin lesions were common. Other damage included lung dysfunction, neuropathy, and nephrotoxicity. The prevalence of hepatomegaly was 20%. Approximately 200 people have died from the effects of the most severe As poisoning, which included liver cirrhosis, ascites, and liver and skin cancers. Treatment of individuals who have arsenicosis was difficult because of a long exposure time of more than 30 years (Zhou et al.1993) and the high level of As exposure in the population.

In this article, we report the results of a series of investigations between 1998 and 2004, which included a large number of individuals with arsenicosis. To look for cellular and molecular biomarkers of exposure, we collected blood and skin samples from villagers who had been exposed to As. We analyzed the effects of As exposure on chromosome and DNA damage, DNA synthesis and repair, and tumor suppressor gene mutations. One of our goals was to identify molecular biomarkers for early diagnosis that may be applicable to populations exposed to much lower levels of As, usually found in drinking water.

Materials and Methods

Subjects. We consulted a database maintained by the Guizhou Provincial Office of Endemic Disease to identify populations exposed to As (Yang et al. 1998). All subjects in our study resided in Jiaole township in Xinren county and gave informed consent. Our team examined the target population in 1998, with a total of 184 villagers who had been exposed to As agreeing to participate in our study. Arsenicosis symptoms were categorized based on the degree of symptoms: nonpatient (n = 19), mild (n = 49), intermediate (n = 54), and severe (n = 62). Symptoms were classified according to the Chinese National Arsenicosis Diagnosis Standard protocol (Yu et al. 2007). Two control groups were included in this study. First, non-patients (n = 19), defined as individuals showing no symptoms of arsenicosis, from Jiaole, were designated the internal control group. The second, designated the external control group, comprised 53 villagers from Ma Jiatun township (approximately 12 km from Jiaole) who did not use coal containing high levels of As (Table 1).

Biological samples. Blood and skin samples were collected each year from 1998 to 2001 from villagers burning As-containing coal in unventilated indoor stoves. Biosamples were collected from the same villagers at each collection time; however, we could not always collect samples from all subjects at each collection time, which resulted in missing data.

In 1998 we obtained 193 blood samples (external, n = 25; internal, n = 13; mild, n = 44; intermediate, n = 49; severe, n = 62) in which to study cellular chromosome damage (Table 1). In the same year, skin samples were collected from 70 subjects who were treated surgically to alleviate pain related to arsenicosis. These samples were used to measure of p53 and p16-INK4 (p16) gene expression (Table 2). In 1999 we obtained a second batch of blood samples from 156 patients, 19 internal controls, and 41 external controls in which to measure DNA damage (Table 1). In 2001 a third batch of blood samples was collected from 70 subjects for analysis of p53 and p16 mutations (Table 2). Also, in 2001, a second batch of skin samples from 61 subjects who had undergone surgery was obtained for DNA gene repair study (Table 3). On the basis of dermal pathology, the subjects from 2001 study were categorized into four groups: group A, general pathological changes (hyperplasia) with 22 subjects belonging to mild (n = 8), moderate (n = 7), severe (n = 7) arsenicosis groups; group B, hyperkeratosis with 22 subject belonging to mild (n = 3), moderate (n = 7), and severe (n = 12) arsenicosis group; group C, precancerous lesion with 12 subjects drawn from moderate (n = 5) and severe (n = 7) arsenicosis groups; and finally, group D, cancerous lesion with 18 subjects including squamous carcinoma, basal cell carcinoma, and Bowen disease from mild (n = 1), moderate (n = 3), and severe (n = 14) arsenicosis groups.

Urine samples were collected in acid-washed plastic containers. Concentrated hydrochloric acid (1 mL HCl to 100 mL urine) was added to prevent bacterial growth (Concha et al. 1998). The samples were then frozen and stored at -80[degrees]C (Crecelius et al. 1986). Collected skin tissue was flash frozen in liquid nitrogen and stored at -80[degrees]C (He et al. 2004). Blood samples were kept on dry ice before being transported to our laboratory and kept frozen at -80[degrees]C.

Biomarkers of biological response. We chose biomarkers of mutation and abnormal gene expression because they can indicate molecular changes before the occurrence of cancer and because of their potential usefulness in early diagnosis (Klaassen 2001). We used a standard protocol, and the parameters and analytical procedures are described in detail in the citations above.

Chromosome and DNA damage. Sister chromatid exchanges (SCEs), chromosomal aberrations (CAs), and micronuclei (MN) (Table 1) in peripheral blood were blind counted (Xie et al. 1999). Spontaneous DNA synthesis (DS) and unscheduled DNA synthesis (UDS) were assayed with a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA; Zhang et al. 2000c). DNA-protein cross-links (DPCs) were measured by an [.sup.25.I]-post labeling assay (Zhang et al. 2000c). DNA single-strand breaks (SSBs) were determined by single-cell gel electrophoresis (SCGE; Zhang et al. 2000b) and the yield of these breaks was measured using the DNA COMET assay (Figure 1).

Expression of P53 and P16 proteins. It is well known that wild-type P53 protein is a nuclear phosphoprotein coded by the p53 tumor suppressor gene. If the DNA is damaged, wild-type p53 shuts off cell duplication and initiates a "suicide system" to kill those cells with damaged DNA. In contrast, if wild-type p53 mutates into mutant-type p53, cells with damaged DNA may enter the S phase prematurely and produce CAs (Vogelstein 1990). P16 protein is a known inhibitor of cyclin-dependent kinase 4 (CDK4) that binds to CDK4 in the [G.sub.1] phase of the cell cycle to down-regulate its activity. It inhibits the progression of cells, especially those with DNA damage from [G.sub.1] to S phase. As a result, cell division and growth are reduced (Serrano 1993; Sherr 1993).

The immunohistochemical technique is well established for use in clinical histopathological diagnosis (Chen et al. 2005; Editorial Board of the Chinese Journal of Pathology 1996). The expression of P53 and P16 (Table 2) was measured by immunohistochemical methods in skin tissue samples, most of which (n = 70) were collected in 1998 (Hong et al. 2000; Hu et al. 2003). The mouse monoclonal P53 and P16 antibodies and EnVision+ System kit were purchased from Dako (Dako North America Inc., Carpinteria, CA, USA). In our study the quantitative data for these percentages were transformed into two qualitative types: positive or negative. For P53 and P16 protein, a subject with more than 1% positive cells was regarded as positive.

Mutation of p53 and p16 genes. Polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP), as well as PCR cloning and sequencing, were used to detect the mutation of p53 exons 5-8 and p16 exon 2 in 60 peripheral blood samples of subjects (Table 2; Figure 2) collected in 2001 (Pan et al. 2004;). Point mutation of p53 occurs mainly in exons 5-8 (Hasegawa et al. 1995; Vet et al. 1996; Xu et al. 2001); The PCR primers for p53 were as follows: first pair of primers--forward, 5'-GTGAGGGGGCTCTACACAAG-3'; reverse, 5'-ACCAGCGTGTCCAGGAAG-3'; second pair of primers--forward, 5'-CTTCCTGGACACGCTGGT-3'; reverse, 5'-GTCCTCACCTGAGGGACCTT-3'.

Point mutation of p16 gene occurs mainly in exon 2 (Kamb et al. 1994; Nobori et al. 1994). To obtain a suitable length of segment for nondenatured polyacrylamide gel, p16 was amplified by two pair primers, the primer sequences are are as follows: first pair of primers--forward, 5'-GTGAGGGGGCTCTACACAAG-3'; reverse, 5'-ACCAGCGTGTCCAGGAAG-3'; second pair of primers--forward, 5'-CTTCCTGGACACGCTGGT-3'; reverse, 5'-GTCCTCACCTGAGGGACCTT-3'. The PCR mixture contained 1 x PCR buffer; dATP,dCTP, dGTP, dTTP (2 [micro]M each); primers (100 nM); Mg[Cl.sub.2] (1.5 mM); Taq DNA polymer (2 U); and template DNA (200 ng) in a final volume of 50 [micro]L. The PCR program was set for an initial denaturation at 95[degrees]C for 5 min, 35 cycles of denaturation at 94[degrees]C for 50 sec, annealing at 56-65[degrees]C (depending on the type of primer pair used) for 40 sec, extension at 72[degrees]C for 50 sec, and final extension at 72[degrees]C for 5 min. PCR products were electrophoresed on 1.5% agarose gels and observed under ultraviolet illumination. Five microliters of PCR product was mixed with 5 [micro]L of denatured solution, which was denatured at 95[degrees]C for 5 min and kept on ice for 5 min. Similarly, a PCR product was added to 8% nondenatured polyacrylamide gel and electrophoresed with 150 V of constant voltage for 3 hr. After electrophoresis the gel was removed and stained with silver.

PCR products were inserted into vector pMD 18-T, then transformed to the competent DH5[alpha] cell, prepared in LB medium containing AMP100. Plasmid DNA was extracted using E.Z.N.A Plasmid Miniprep Kit I (Omega Bio-Tek, Inc., Doraville, GA, USA); recombinant DNA was screened with EcoRI, HindIII, and sequenced in Takara Biotechnology Co. (Dalian, China).

Expression of DNA repair gene. Expressions of O-6-methylguanine DNA methyltransferase (MGMT), X-ray repair complementing defective repair in Chinese hamster cells 1(XRCC1), and mis-match repair gene (hMSH2) mRNA (Table 3) were analyzed by in situ hybridization in 61 skin tissue samples. The samples were from arsenicosis patients who had undergone surgery in 2001 (Chen et al. 1999; Zhang et al. 2005). Tissue samples were analyzed as follows: The sections were treated with 3% hydrogen peroxide, digested in proteinase, and prehybridized at 37[degrees]C. Sections were then hybridized overnight and blocked in paraffin or mineral oil. Mouse biotin-antidigoxin antibody was applied for 60 min at 37[degrees]C, followed by treatment with streptavidin-biotin-peroxidase complex (Wuhan Boster Bioengineering Ltd., Wuhan, Hubei, China) for 20 min at 37[degrees]C, with biotin-peroxidase for 20 min at 37[degrees]C, and stained with DAB (Wuhan Boster Bioengineering Ltd., Wuhan, Hubei, China). The sections were then refiltered, dehydrated, cleared and enveloped and visualized using a microscope. The in situ hybridization kit, DAB stain kit, and in situ hybridization special cover-slices were purchased from Wuhan Boster Biological Engineering Limited Corp. (Wuhan, China). After in situ hybridization, positive samples exhibited brownish yellow inclusions in the cytoplasm. The percentage of samples was calculated as follow: positive cases / detected cases x 100%. Skin samples from control groups were taken from nonarsenicosis patients receiving undergoing other medical procedures at that hospital at that time.

Statistical analyses. SPSS (version 11.0, http://www.spss.com) was used for analysis of variance (ANOVA), linear correlation and regression, and the chi-square test. Results were reported as statistically significant when $p < 0.05$.

Results

Arsenic exposure and chromosome and DNA damage. For the external control group, the average concentrations of urinary As and hair As were 46 [micro]g/L and 1.6 [micro]g/g, respectively (Table 1). Urinary As concentrations in the four exposed groups were 2-3 times that in the external control group, with $p < 0.01$. Compared with the external control

group, hair As concentrations were 3-5 times that in the exposed groups, with $p < 0.01$. It is worth noting that concentrations of urinary As (~ 76 [micro]g/L) and hair As (~ 5.4 [micro]g/g) in the internal control group, that is, subjects without diagnosable arsenicosis symptoms, were statistically significantly ($p < 0.05$) lower than those in the three other groups with mild, moderate, or severe arsenicosis (Table 1). In addition, both urinary and hair As concentrations increased as the degree of arsenicosis increased.

Consistent with the statistically significant difference of As exposure, SCE ratio, which is an indicator of chromosome damage, was found to be significantly different in all four exposed groups compared with the external control group ($p < 0.05$ or $p < 0.01$; Table 1). In addition, the SCE ratio of the severe arsenicosis group was higher than that of the internal control group ($p < 0.05$; Table 1). CAs were found to be significantly different in the three patients groups compared with the external control ($p < 0.01$; Table 1). For MN, only the severe arsenicosis group was significantly different from the external control group, internal control group, and the other two exposed groups (Table 1).

Unscheduled DNA synthesis increased gradually with increasing As concentrations in urine and hair (Table 1), whereas spontaneous DNA synthesis showed a dramatic decrease from approximately 1,150 cpm for the external control group to approximately 200 and 300 for all four exposed groups. DNA-protein crosslinks increased with increasing exposure (Table 1). DPCs in the internal control group was much higher than that in the external control group ($p < 0.01$; Table 1). It increased remarkably in the moderate and severe groups, compared with the two control groups and the mild arsenicosis group ($p < 0.05$ or $p < 0.01$; Table 1). DNA COMET tail length (Figure 1) increased with increasing As exposure when external control, nonpatient, mild, moderate, and severe arsenicosis groups were compared.

Arsenic exposure and abnormal expressions of P53 and P16 proteins. In patients with arsenicosis, skin pathology was classified according to four groups. The positive rate in P53 protein expression was 89% (16/18), 73% (8/11), 33% (7/21) and 17% (3/18) for the carcinoma, precarcinoma, hyperkeratosis, and common pathological changes groups, respectively (Table 2). The high positive rates in the carcinoma and precarcinoma groups were statistically different from those in the common pathological changes and hyperkeratosis groups ($p < 0.05$ or $p < 0.01$; Table 2). The positive rates of P16 protein expression were 33% (6/18), 36% (4/11), 68% (15/22), and 79% (15/19) for the carcinoma, precarcinoma, hyperkeratosis, and common pathological changes groups, respectively. The low positive rate in the carcinoma group was statistically different from those in all other groups; there is also a significant difference between the precarcinoma group and the common pathological changes group (Table 2).

Arsenic exposure and p53 gene mutation. A striking difference in the p53 mutation frequency was found between the carcinoma (30%) and precarcinoma groups (17%) compared with the hyperkeratosis (0%) and common pathological changes (0%) groups ($p < 0.05$; Table 2). Sequencing p53 identified the following mutation types and sites: at codon 143 (GTG[right arrow]GTA) in two cases, which were silent mutations; at codon 146 (TGG[right arrow]CGG) in one case, resulting in a change from tryptophan to arginine in the protein; at codon 151 (CCC[right arrow]TCC) in two cases, resulting in a change from proline to serine in the protein (Table 4; Figure 2). No mutation was found on p16 exon 2.

Arsenic exposure and abnormal expression of DNA repair genes. The positive rates of all DNA repair genes, that is, MGMT, XRCC1, and hMSH2 mRNA, increased gradually as the arsenicosis symptoms became milder (Table 3). The positive rates for MGMT mRNA were 18, 42, 69, and 73% for the carcinoma, precarcinoma, hyperkeratosis, and common pathological changes groups, respectively. The carcinoma group displayed statistically lower positive rates than those of the common pathological changes group ($p < 0.01$) and the hyperkeratosis group ($p < 0.05$) but not lower than those of the precarcinoma group. Similar trends were observed for two other DNA repair genes with respect to As exposure and symptoms. In this case the low positive rate of XRCC1 mRNA in the carcinoma group (27%) was significantly lower than that for the common pathological changes group (77%; $p < 0.01$). The low positive rate of hMSH2 mRNA (36%) for the carcinoma group was not statistically significant from the other groups.

Discussion

Our results clearly demonstrate that in a population exposed to As primarily through consumption of food cooked on coal-burning unventilated indoor stoves and, to a much lesser extent, inhalation of the contaminated air from these stoves, cellular and genetic material exhibited changes in most cases consistent with the As exposure level and symptoms. In individuals with arsenicosis, DNA was damaged, DNA repair was impaired, and mutations or deletions of p53 and p16 were evident. Our findings suggest: a) SCE and CAs are sensitive biomarkers for human chromosome damage induced by As exposure; b) DNA single-strand breaks and DPCs can be used to monitor DNA damage in populations exposed to As; c) p53 mutations and p16 deletions were associated with carcinogenicity due to As exposure;

and d) the decrease in DNA repair may be another important mechanism in arsenicosis carcinogenicity.

Biomarkers of As-induced human chromosome damage. As-induced chromosome damage appeared to occur earlier than clinical symptoms, as shown by the higher SCE, CA, and MN values in the exposed groups compared with the external control group. However, among the three indicators of chromosome damage, the MN seems to be the least sensitive because the difference is not statistically significant between the internal control, mild, and intermediate arsenicosis groups compared with the external control (Table 1). SCE was found to be the most sensitive indicator of arsenic exposure because it was the only biomarker that displayed a statistically significant difference between the internal and the external control groups (Table 1). The CAs was less sensitive than SCE, but more sensitive than MN (Table 1). Both mammals and humans exposed to As had increased frequencies of MN and CAs, which led to SCE abnormalities (Wilson et al. 2002). Gurr et al. (1993) found that when sodium arsenite was used to treat [G.sub.2]-phase Chinese hamster ovary cells, chromosome condensation and breakage were induced, indicating MN development during interphase.

Biomarkers of DNA damage. The present study also showed that SCGE, a sensitive and common assay, could be used to monitor DNA damage of populations exposed to As. Arsenic caused DNA single-strand breaks in As-exposed populations, with a wide range of symptoms. The breaks occurred notably earlier than clinical symptoms (Figure 1). This is consistent with DNA damage determined by SCGE in human lymphocytes cultured in vitro with a high concentration of arsenate (0.2-1.5 mmole/L) (Hartmann and Speit 1994). Furthermore, our results and those from cell culture studies (Li et al. 2001) showed that As induced DNA strand breaks and exhibited a dose-response relationship.

DNA-protein cross-links are thought to be important genotoxic lesions induced by environmental agents and carcinogens, such as ultraviolet light (Smith 1962), formaldehyde (Cosma et al. 1988), and cis- or trans-platinum (II) diammine dichlorides (Banjar et al. 1984). Unlike strand breaks and other DNA lesions that are readily repaired, DPCs are relatively persistent in the cells (Lei et al.1995; Oleinick et al.1987). Because of a poor repair capacity, DNA-protein complexes still exist during DNA replication, which may cause loss of important genetic material and even inactivate tumor suppressor genes (Costa 1991; Lei et al.1995). In our study, the extent of DNA-protein cross-linking in the internal control groups and the three exposed groups was signifcantly higher than that in the external control group. There exists a positive relative relationship between DPC and As poisoning (Table 1). This suggests that DNA-protein cross-linking can be used to monitor genetic damage from As exposure. The formation of DPCs may be one of the mechanisms that induces mutation and perhaps cancers in arsenicosis patients.

Gene mutations, deletions, and As exposure. p53 mutations were found by PCR-SSCP in the peripheral blood samples of arsenicosis patients with precarcinomas and carcinomas. The mutation frequencies, 17 and 30% for the precarcinoma and carcinoma groups, respectively, were lower than those reported previously in tumor tissues of arsenicosis cancer patients from Taiwan (Hsu et al. 1998; Shibata et al. 1994). The mutation frequency of p53 exons 5-8 was determined by PCR-SSCP to be 62%, or in 8 of 13 bladder cancer patients exposed to As in drinking water from the Black Foot disease area in Taiwan (Shibata et al. 1994). The mutation frequencies of p53 exons 2-11 were 28.6%-55.6% in skin cancer patients with Bowen's disease, basal cell carcinoma, or squamous cell carcinoma, again from the Black Foot disease area in Taiwan (Hsu et al. 1998). These differences in DNA mutation frequencies may be attributed to sample type: peripheral blood versus tumor tissue. Several considerations prompted us to investigate DNA samples from peripheral blood: a) Arsenic is harmful to many organs and can induce to tumorigenesis. b) Tumorigenesis has multiple stages and often occurs over a long period. Monitoring mutations in blood samples taken over time provides a better opportunity than tissue samples for investigating gene mutations and tumorigenesis, as tissue samples are usually more difficult to obtain.

The results of cloning and sequencing showed that the mutation sites were found at codon 143 (GTG[right arrow]GTA) in two cases, which was a silent mutation; at codon 146 (TGG[right arrow]CGG) in one case, which resulted in a change from tryptophan to arginine in the protein; and at codon 151 (CCC[right arrow]TCC) in two cases, which resulted in a change from proline to serine in the protein (Figure 2). Hsu et al. (1998) reported similar results in 16 skin cancer patients in Taiwan with p53 mutations; 38% of the mutations were G:C[right arrow]A:T transitions and 25% silent. The sites of p53 mutations were mainly exons 5 and 8.

In contrast to p53 mutation, no mutation was observed for p16 in the peripheral blood samples of 60 patients, including 5 patients with p53 mutations, even in the mutation hotspot exon 2 (Kamb et al. 1994; Nobori et al. 1994). We recognize that mutations of p16 may have been observed if we had analyzed more samples. Previously, expression of P16 protein was observed in tumor tissues, but in our study we used peripheral blood samples. Furthermore, other exon mutations or inactivation mechanisms such as deletion or methylation could have inactivated p16 (Lu et al. 2004; Zhao et al. 2003). We have recently observed p16 exons 1 and 2 deletions using multi-PCR in peripheral blood of arsenicosis patients (n =

41) from blood samples obtained in 2004 (Bin et al. 2006). In noncarcinoma patients (n = 23), 8.7% exhibited p16 deletions. In comparison, 38.9% of carcinoma patients (n = 18) showed p16 deletions. This difference was statistically significant ($p < 0.05$).

DNA synthesis, repair, and As carcinogenicity. Arsenic exposure has a profound impact on DNA synthesis. This is best illustrated by a 4-fold decrease in the DNA synthesis rate when the external and internal control groups were compared (Table 1). The unscheduled DNA synthesis showed a rather gradual increase as As exposure increased and symptoms worsened. It is plausible that As combines with the sulfhydryl group of DNA polymerase and repairase, which inhibits the activity of DNA polymerase and repairase (Meng 1998).

Our study also found that As exposure has a profound negative impact on DNA repair, decreasing positive expression for XRCC1, MGMT, and hMSH2 mRNA with the increasing severity of skin lesion in arsenicosis patients (Table 3). The expression product of XRCC1 is essential in repairing DNA defects caused by ionizing radiation and alkylating agents. XRCC1 is involved in base excision and single-strand break repair (Whitehouse et al. 2001). Cells with XRCC1 deletion were found to be sensitive to ionization radiation and had a high SCE ratio (Zheng 1999). MGMT is a highly specific repair enzyme involved in repairing the base at the site of guanine O6 by transferring the alkylated base to the cysteine residue (Estellen et al. 2000). The natural expression of MGMT is a key in maintaining DNA stability of the DNA structure and repairing DNA alkylation damage. hMSH2 mRNA can repair a variety of DNA damage, including alkyl-base mismatches, insertions or deletions, and structural abnormalities, which lowers the mutation frequency and maintains stability of DNA structure.

Our study showed that long-term As exposure may be associated with chromosome and DNA damage, gene mutations, deletions, and alterations in DNA synthesis and repair. However, the mechanim(s) by which cellular and genetic damage leads to cancer remains unclear and further research is needed.