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Protective Activity of Hesperidin and Lipoic Acid Against Sodium Arsenite Acute Toxicity in Mice

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ABSTRACT

The objective of the present work was to evaluate the toxic effects of sodium arsenite, As(III), in mice and the protective effect of 2 antioxidants, hesperidin and lipoic acid, against the observed As(III)-induced toxicity. In each study, mice were assigned to 1 of 4 groups: control, antioxidant, antioxidant + arsenite, and arsenite. Animals were first injected with the vehicle or 25 mg antioxidant/kg BW. After 30 minutes they received an injection of 10 mg arsenite/kg BW or 0.9% NaCl. Two hours after the first injection, the liver, kidney, and testis were collected for histological evaluation. Liver samples were also taken for quantification of arsenic. In mice exposed only to As(III), various histopathological effects were observed in the liver, kidneys, and testes. In mice pretreated with either hesperidin or lipoic acid, a reduction of histopathologic effects on the liver and kidneys was observed. No protective effects were observed in the testes for either of the 2 studied antioxidants. In conclusion, hesperidin and lipoic acid provided protective effects against As(III)-induced acute toxicity in the liver and kidneys of mice. These compounds may potentially play an important role in the protection of populations chronically exposed to arsenic.

Keywords. Hesperidin; lipoic acid; sodium arsenite; histopathology; mice; liver; kidney; testis.

INTRODUCTION

Inorganic arsenic compounds are well known for their high toxicity. Today, chronic low-dose arsenic exposure via drinking water presents a major concern, because it affects human populations and is associated with various forms of cancer (Goering et al., 1999; Huff, Chan, and Nyska, 2000). Then, the sporadic ingestion of high levels of inorganic arsenic still occurs very often in highly contaminated areas (Smith et al., 1998; Tseng, 2002) or as a result of suicidal and homicidal attempts (Bartolome et al., 1999; Fanton et al., 1999). Sodium arsenite is one of the most harmful inorganic arsenic compounds because of its high reactivity against endogenous thiol groups, especially dithiols (Knowles and Benson, 1983). Once absorbed, however, arsenite is biotransformed into various methylated metabolites. This has long been regarded as a detoxification process because the pentavalent methylated arsenic metabolites, monomethylarsonic acid (MMAsV) and dimethylarsinic acid (DMAsV), are much less toxic and are excreted more readily than inorganic arsenic (Buchet, Lauwerys, and Roels, 1981; Yamauchi and Yamamura, 1984; Marafante et al., 1987; Yamauchi, Yamato, and Yamamura, 1988).

The metabolic detoxification concept has been questioned by the recent finding that a toxic trivalent methylated arsenical, monomethylarsonous acid (MMAs(III)), which is a prominent arsenic metabolite in the bile of rats (Gregus, Gyurasics, and Csanaky, 2000), mice, hamsters, and rabbits (Csanaky and Gregus, 2002), is often detectable in low amounts in the urine of humans consuming arsenic-contaminated water (Aposhian et al., 2000; Mandal, Ogra, and Suzuki, 2001). Comparative toxicity assays between arsenite, arsenate, and synthetic MMAs(III) revealed that the latter compound is more toxic to microorganisms (Cullen, 1989), rats (Styblo et al., 2000), hamsters (Petrick, 2001), as well as to human hepatocytes (Petrick et al., 2000; Styblo et al., 2000). Thus, while formation of the pentavalent methylated arsenic metabolites can indeed be regarded as a detoxification process, the production of MMAs(III) is rightly considered to be toxificant, and thus the production of this metabolite, if significantly high, may contribute to the toxicity of arsenic (Vahter and Concha, 2001).

The four main areas of research on the cellular mechanisms of arsenic toxicity are: mutation inductions and chromosomal aberrations; altered signal transduction, cell-cycle control, cellular differentiation, and apoptosis; alterations in gene expression; and direct damage through oxidative stress (Agency for Toxic Substances and Disease Registry, 2000). None of these mechanisms are exclusive, and oxidative stress has been shown to influence all of them, directly or indirectly (Ercal, Gurer-Orhan, and Aykin-Burns, 2001). Indeed, several recent studies relate arsenic toxicity with the fact that metabolism is associated with an increase in the formation of reactive species and oxidative stress (Barchowsky, 1999; Yamanaka et al., 2001; Chen et al., 2002). Arsenite has been shown to enhance metallothionein-II and ferritin H mRNA levels in HeLa cells (Guzzo et al., 1994) and to stimulate production of heme oxygenase in cultured mammalian cells (Applegate, Luscher, and Tyrrell, 1991), which may result from an adaptation to an ongoing oxidative stress process. An increase in the fluorescence intensity of dichlorofluorescein

diacetate has also been observed in human fibroblasts and Chinese hamster ovary cells exposed to arsenite (Lee and Ho, 1995; Wang et al., 1996), indicating the production of reactive oxygen species.

In human populations chronically exposed to arsenic (e.g., Taiwan) pathological effects have also been related to a low antioxidant capacity in the plasma of individuals (Wu et al., 2001). Although the mechanism of chronic arsenic toxicity is not fully known, studies of populations in Taiwan (Tseng, 2002), Chile (Smith et al., 2000), and Mexico (Cebrian et al., 1983) living in areas of endemically high arsenic (0.3 to 0.8 mg/liter drinking water) have shown at least 1 of the following toxic signs: hyperpigmentation, keratosis, skin cancer, or blackfoot disease, a vascular disease that leads to gangrene in the extremities. A review by Peraza and coworkers (1998) has pointed out the fact that people from other parts of the world exposed to similar arsenic levels (e.g., United States) did not exhibit these toxic effects. This has led to the assumption that other factors (not only exposure level) may play important roles in the expression of arsenic toxicity, including differences in dietary habits and nutritional status. In fact, undernourished populations, especially those suffering from vitamin deficiencies, have greater susceptibility to arsenic (Valentine et al., 1994; Peraza et al., 1998). Moreover, if we consider that most cases of human exposure to arsenic compounds result from natural environmental sources, like drinking water, in which it is difficult to remove the source of contamination, then inclusion of protective factors in the diet may be useful to prevent arsenic toxicity. In vitro, it has already been observed that antioxidants can reduce toxic arsenite-induced features such as sister chromatid exchange in human lymphocytes (Nordenson and Beckman, 1991), the induction of micronuclei in XRS-5 cells (Wang and Huang, 1994), apoptosis in Chinese hamster ovary cells (Wang et al., 1996), and cytotoxicity in human fibroblasts (Lee and Ho, 1995) and MGC-803 cells (Chen et al., 2002).

Recently, Ramanathan and coworkers observed that α-tocopherol and ascorbic acid prevent lipid peroxidation and protect the antioxidant system in arsenite-intoxicated rats (Ramanathan et al., 2003). Thus, the evaluation in mice of potential protection against the toxicity of inorganic arsenite may constitute an important contribution to the knowledge of the interaction between diet and arsenic toxicity. Lipoic acid is a well known dithiolic antioxidant, present in several vegetables, especially spinach, tomato, and broccoli, but also in animal tissues (Lodge et al., 1997), and has been proposed to be a potential therapeutic agent in the treatment or prevention of different pathologies, including diabetes, polyneuropathy, cataracts, and neurodegeneration, in which oxidative stress is involved (Packer, Witt, and Tritschler, 1995). Hesperidin is a flavanone abundant in citrus fruits, such as grapefruit and oranges, showing effective antioxidant and anti-inflammatory activities, as well as vascular protection properties (Garg et al., 2001). Therefore, these 2 compounds, in theory, are good candidates for modulation of arsenite toxicity. In the present study we identify the main histopathological features of arsenite toxicity in mice and the protection capacity of lipoic acid and hesperidin against arsenite toxicity in the liver, kidney, and testis, which constitute target organs with metabolic, excretion, and reproductive functions.

MATERIALS AND METHODS

Chemicals: Sodium arsenite (As(III); NaAsO$_2$), lipoic acid (C$_8$H$_{14}$O$_2$S$_2$), and hesperidin (C$_{18}$H$_{34}$O$_{15}$) were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Animals and Treatments: Male ICR(CDI) mice (7 weeks old) were obtained from Harlan Interfauna Iberica (Barcelona) and kept for acclimatization (1 week) at $22 \pm 1°C$ with a 12-hour light/dark cycle. Housing and experimental treatment of the animals were in accordance with National Institute of Health guidelines (Institute of Laboratory Animal Resources, 1996). Animals were fed with standard rodent chow and allowed free access to tap water except on the injection day, when animals were deprived of food. Mice ($n = 6$ per group) received 2 intraperitoneal (ip note) injections according to the experimental design described in Table 1.

First, animals were injected with the vehicle or 25 mg antioxidant/kg body weight. Thirty minutes later, these animals were injected with 10 mg arsenite/kg body weight or 0.9% NaCl. A 7.5% NaHCO$_3$ solution was used as vehicle for lipoic acid and saline (0.9% NaCl) for hesperidin and As(III). The dose selection for As(III) (10 mg/kg body weight) was based on a recent study where an LD$_{50}$ of 17 mg/kg body weight was found for wild-type mice (Liu et al., 2002). The intraperitoneal route was chosen in an attempt to avoid the potential confounding factors on As absorption through the gastrointestinal tract and to compare parenteral arsenic effects with previous reports in the literature, although performed on different animal species (Flora et al., 1997; Sarkar et al., 2003). The dose selection for hesperidin was based on a study indicating an effective anti-inflammatory effect after a single 25 mg/kg dose given intragastrically to mice (Garg et al., 2001). The dose selection for lipoic acid was also based on references to similar administration levels of lipoic acid (25–100 mg/kg, single ip dose) to study the effects on antioxidant enzymes and to suppress elevated lipid peroxidation elicited by cisplatin (Moini, Packer, and Saris, 2002). Moreover, a metabolic study of labelled lipoic acid showed that mice excreted only 50% of the labelled dose within the first 24 hours after a single oral dose of 30 mg/kg body weight. Therefore, by administering arsenite 30 minutes

TABLE 1.—Experimental design for antioxidant and arsenite administration to mice.

Group (n = 6)	NaHCO$_3$ 7.5%	LA 25 mg/kg	H 25 mg/kg	NaCl 0.9%	As(III) 10 mg/kg
I—(control mice)	†			‡	
II—(control mice)				†‡	
III—(LA)		†		‡	
IV—(H)			†	‡	
V—(LA + As(III))		†			‡
VI—(H + AsIII)			†		‡
VII—(As(III))	†				‡
VIII—(As(III))				†	‡

I (control mice): mice injected with NaHCO$_3$ 7.5% and NaCl 0.9%; II (control mice): mice injected with NaCl 0.9%; III (LA): mice injected with lipoic acid 25 mg/kg and NaCl 0.9%; IV (H): mice injected with hesperidin 25 mg/kg and NaCl 0.9%; V (LA + As(III)): mice injected with lipoic acid 25 mg/kg and arsenite 10 mg/kg; VI (H + As(III)): mice injected with hesperidin 25 mg/kg and arsenite 10 mg/kg; VII (As(III)): mice injected with NaHCO$_3$ 7.5% and arsenite 10 mg/kg; VIII (As(III)): mice injected with NaCl 0.9% and arsenite 10 mg/kg. (†) first injection; (‡) second injection (30 minutes after).

after the antioxidant treatment, we assure high plasma levels of antioxidant. By adopting the same protocol of administration for both antioxidants (ip route, single dose) we are able to compare their protective potency. Two hours after the first injection, animals were sacrificed by cervical dislocation under CO_2 anesthesia and the liver, kidney, and testis were collected.

Total Arsenic in the Liver: Arsenic was quantified in the liver samples of mice from all groups. The method used was atomic absorption spectrometry with electrochemical atomization, as implemented previously for animal kidneys (Soares, Bastos, and Ferreira, 1995) and adapted in the present study for liver samples. A representative sample of liver (0.2 g) was minced with polypropylene (PP) material previously rinsed with 15% HNO_3 and ultrapure water, transferred to PP-decontaminated tubes, and dried in an oven at $\pm 60°C$ for several days. The dried samples were reduced to powder in a Teflon container, and about 500 μg transferred to another Teflon container, which, after addition of 2 ml of HNO_3 and 250 μl of H_2O_2, was closed for digestion overnight in an oven at 85–90°C. The digested solution was transferred to a decontaminated tube and diluted to a convenient volume. Arsenic quantification was carried out in a Perkin-Elmer HGA-850 furnace installed in a Model AAnalyst 300 spectrometer with deuterium arc background correction, equipped with an AS-800 autosampler. The analyses were performed using Perkin-Elmer HGA Tubes with Integrated Platform. The adopted ashing and atomization temperatures were 1,200°C and 2,200°C, respectively.

Metal Standards were prepared daily from 1,000 mgl^{-1} solutions (Tritisol, Merck) in HNO_3 (0.2% v/v). All the acids used were of Suprapure grade (Merck). The chemical modifier used in As measurements was a mixture of $Mg(NO_3)_2$ and $Pd(NO_3)_2$.

Histological Analysis: Freshly removed fragments were fixed by immersion in Bouin's solution, dehydrated in an ethanol series, and embedded in paraffin wax for histological procedures. Organs were cut in order to obtain representative sections of both left and right kidney and testis as well as of all liver lobules. Paraffin sections (6 μm thick) were stained with hematoxylin and eosin. Pathological examinations were conducted in a "blind" procedure way. Each slide was assessed for particular histological alterations. Each liver slide was screened for the presence of inflammatory cells infiltration, dilatation of the interhepatocyte space, and hemorrhagic clots. Disorganization of the hepatic parenchyma and centrilobular hepatocyte swelling were assessed on all centrilobular regions of each slide. Incidence results were allocated to 1 of 4 semiquantitative categories: − absent in all slides, + present in less than 25% of the slides, ++ present in 25 to 75% of the slides, and +++ present in more than 75% of the slides. The same procedure was adopted for evaluation of tubular epithelium vacuolation and presence of interstitial blood in the kidney. Seminiferous tubules vacuolation and tubular atrophy were analyzed in the testis. Hepatocyte vacuolation was assessed in each slide by counting the number of vacuolated cells among 100 cells analyzed (transect of a representative area), and the results were allocated to similar categories as described previously (% of vacuolated cells).

RESULTS

Necropsy observation of all tested animals showed no macroscopic lesions, and no differences were registered in the relative weights of the liver, kidney, and testis of the animals from the different group treatments. All organs in the control animals (groups I and II) displayed normal architecture, where typical aspects were clearly evident (Figures 1A and 2A). Animals from groups III (lipoic acid) and IV (hesperidin) showed no histological differences when compared with groups I and II, except in group III, where medium-low levels of vacuolation and disorganization were observed in the liver (Table 2). All animals exposed to As(III), including those pretreated with antioxidants (groups V and VI), showed histological alterations in the liver, kidney, and testis (Figures 1B–1D, 2B–2D, and 3A–3C). Animals from groups VII (NaHCO$_3$ + arsenite) and VIII (NaCl + arsenite) showed no histological differences when compared.

Histological alterations in the liver include the presence of inflammatory cells infiltration (Figures 1B–D), centrilobular
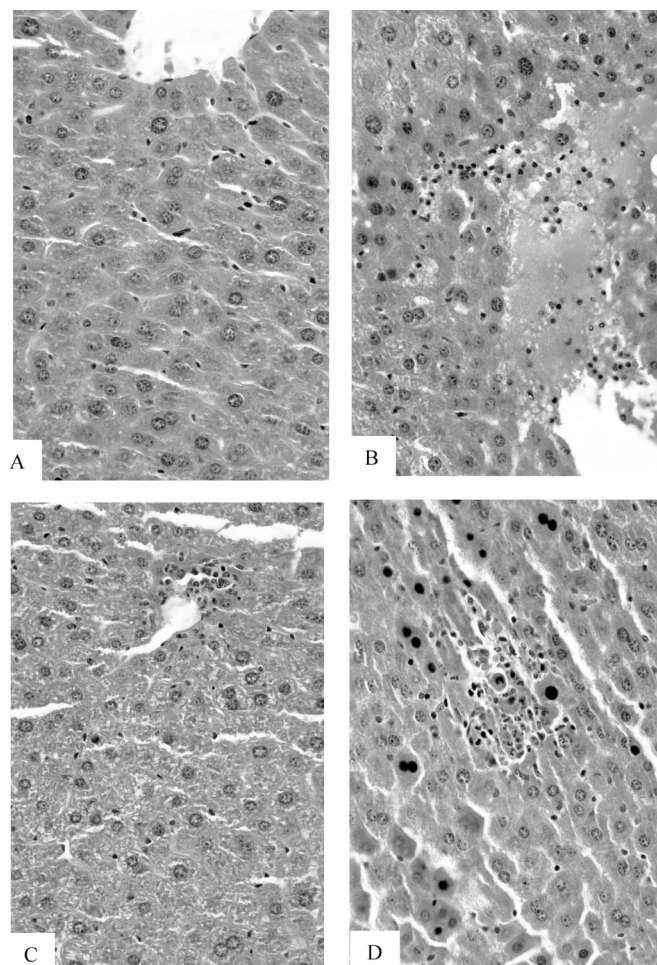

FIGURE 1.—Liver histopathology of (A) control group, (B) 10 mg/kg As(III)-treated mice, (C) LA + As(III)-treated mice, (D) H + As(III)-treated mice. Hepatic cells of control group show normal morphology. Liver from As(III)-treated mice reveals inflammatory cells infiltrates, vacuolation of the hepatocytes, fibrin deposition, and loss of typical hepatic cords organization. Hematoxylin-eosin stain.
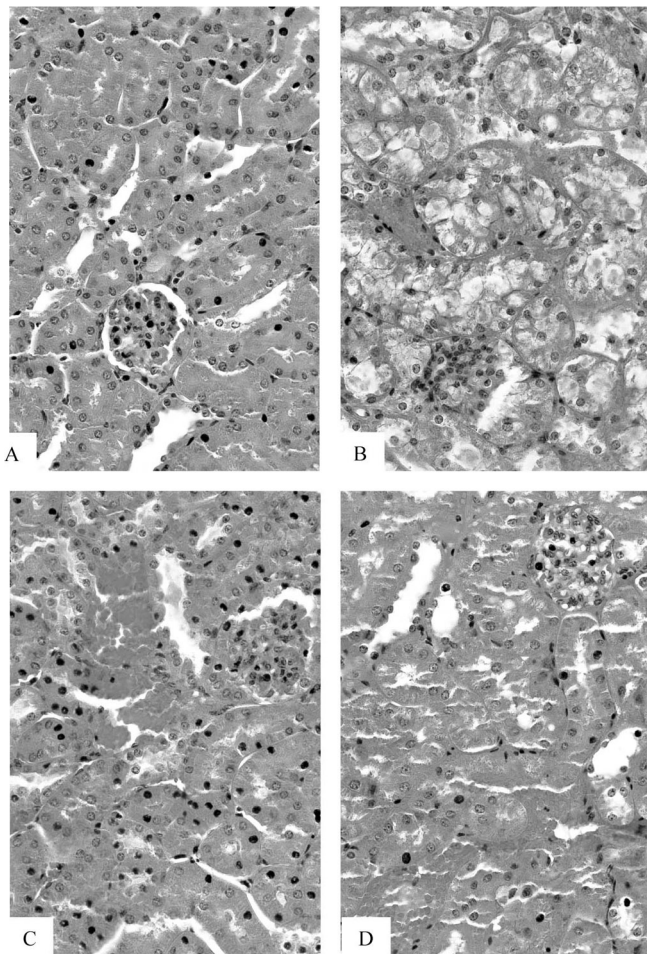
FIGURE 2.—Kidney histological aspects of (A) control group, (B) 10 mg/kg As(III)-treated mice, (C) LA + As(III)-treated mice, (D) H + As(III)-treated mice. Kidney of control, group shows normal morphology. Kidney from As(III)-treated mice reveals vacuolation of the cortical tubule cells and intravascular fibrin deposition. C and D—Antioxidants show protective effects against As(III)-induced toxicity. Hematoxylin-eosin stain.

TABLE 3.—Total arsenic quantification in the liver.

Group	As (μg/g dry liver)
V—(LA + As(III))	71.36 ± 2.279
VI—(H + As(III))	64.29 ± 2.867
VII—(As(III))	66.34 ± 2.312
VIII—(As(III))	68.41 ± 1.119

In groups I, II, III, and IV (not exposed to arsenite) the arsenic levels were below the detection limit (0.5 μ g/L).

swelling (Figure 1B), hepatocyte vacuolation (Figure 1B), parenchyma disorganization, dilatation of the interhepatocyte space, and hemorrhagic clots (Table 2). Comparison of the hepatic histological aspects observed in animals from groups VII and VIII (arsenite only) and those in group V (lipoic acid + arsenite) showed that lipoic acid pretreatment diminishes the incidence of inflammatory cells infiltration, centrilobular hepatocyte swelling, and the presence of hemorrhagic clots (Table 2; Figures 1B–C). Comparison of groups VII and VIII with group VI (hesperidin + arsenite) showed that hesperidin pretreatment was able to diminish the incidence of inflammatory cells infiltration (more efficiently than lipoic acid), centrilobular hepatocyte swelling, and hepatocyte vacuolation, and completely hindered hemorrhagic clots (Table 2; Figures 1B and D). Both lipoic acid and hesperidin, in the assayed doses, were unable to alter histological aspects of the disorganization of the hepatic parenchyma and dilatation of the interhepatocyte space induced by arsenite (Table 2).

Total arsenic quantification in the liver showed no significant differences (Student's t-test) between groups exposed to arsenite only (VII, VIII) and groups pretreated with antioxidants and then exposed to arsenite (V, VI) (Table 3). In animals from groups not exposed to arsenite the arsenic levels were below the detection limit of the method (0.5 μg/L) (Soares, Bastos, and Ferreira, 1995).

Arsenite exposure (groups VII and VIII) produced pronounced renal histopathology, evidenced by tubular cell vacuolation and the presence of interstitial blood

TABLE 2.—Major histological alterations observed in the liver, kidney, and testis of mice exposed to different treatment conditions.

Histological alterations	Group					
	I/II (Control)	VII/VIII (As(III))	III (LA)	V (LA + As(III))	IV (H)	VI (H + As(III))
Liver						
Inflammatory cells infiltration	− (0/12)	+ + + (11/12)	− (0/6)	++ (5/6)	− (0/6)	+ (2/6)
Centrilobular hepatocyte swelling	− (0/12)	++ (9/12)	− (0/6)	+ (2/6)	− (0/6)	++ (2/6)
Hepatocyte vacuolation	− (0/12)	++ (8/12)	++ (2/6)	++ (3/6)	− (0/6)	+ (1/6)
Parenchyma disorganization	− (0/12)	++ (9/12)	++ (2/6)	+ + + (4/6)	− (0/6)	+ + + (4/6)
Dilatation of the interhepatocyte space	− (0/12)	+ (5/12)	− (0/6)	+ (2/6)	− (0/6)	+ (2/6)
Hemorrhagic clots	− (0/12)	++ (6/12)	− (0/6)	+ (2/6)	− (0/6)	− (0/6)
Kidney						
Tubular epithelium vacuolation	− (0/12)	+ + + (10/12)	− (0/6)	+ (2/6)	− (0/6)	+ (2/6)
Interstitial blood	− (0/12)	++ (6/12)	− (0/6)	++ (2/6)	− (0/6)	+ (1/6)
Testis						
Seminiferous tubules vacuolation	− (0/12)	++ (8/12)	− (0/6)	++ (4/6)	− (0/6)	++ (4/6)
Tubular atrophy	− (0/12)	+ (3/12)	− (0/6)	+ (1/6)	− (0/6)	+ (2/6)

Incidence of lesions among animals from different experimental groups ($n/6$ or $n/12$; n = number of animals displaying lesions) and severity of lesions in the liver, kidney, and testis: – absent in all slides; + present in less than 25% of the slides; ++ present in 25 to 75% of the slides; + + + present in more than 75% of the slides.
I (control mice): mice injected with NaHCO$_3$ 7.5% and NaCl 0.9%; II (control mice): mice injected with NaCl 0.9%; III (LA): mice injected with lipoic acid 25 mg/kg and NaCl 0.9%; IV (H): mice injected with hesperidin 25 mg/kg and NaCl 0.9%; V (LA + As(III)): mice injected with lipoic acid 25 mg/kg and arsenite 10 mg/kg; VI (H + As(III)): mice injected with hesperidin 25 mg/kg and arsenite 10 mg/kg; VII (As(III)): mice injected with NaHCO$_3$ 7.5% and arsenite 10 mg/kg; VIII (As(III)): mice injected with NaCl 0.9% and arsenite 10 mg/kg.
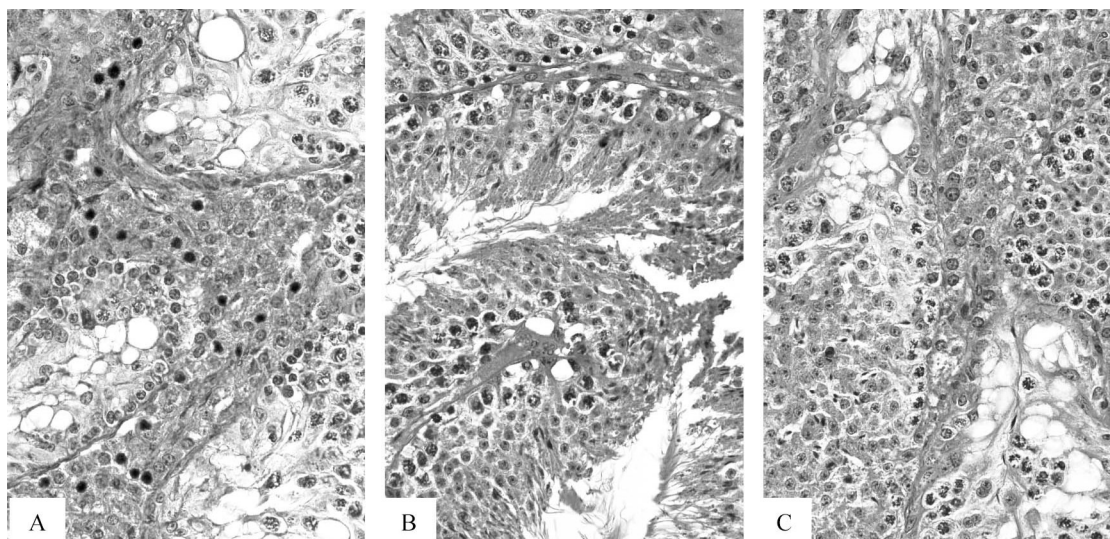
FIGURE 3.—Testis histological aspects of (A) 10 mg/kg As(III)-treated mice, (B) LA + As(III)-treated mice, (C) H + As(III)-treated mice. Testis from As(III)-treated mice reveals vacuolation of the seminiferous tubule cells. B and C—Antioxidants were unable to protect this organ against As(III)-induced toxicity. Hematoxylin-eosin stain.

(Figures 2B–D). Animals from groups V and VI showed minor aspects of tubular cell vacuolation when compared to animals from groups VII and VIII, which means that lipoic acid and hesperidin pretreatment produced protective effects in this organ against arsenite toxicity (Table 2; Figures 2B–D). Hesperidin also showed vascular protection capacity by diminishing the presence of interstitial blood in the kidney (Table 2).

Testes of all arsenite-exposed animals (groups V, VI, VII, and VIII) showed vacuolation and atrophy of seminiferous tubules at similar levels (Table 2). Neither lipoic acid nor hesperidin treatment was able to protect the testis from arsenite aggression under the adopted protocol (Figures 3A–C).

DISCUSSION

Arsenic is a major environmental toxicant and a human carcinogen, which is recognized to induce cardiovascular disease, diabetes, hypertension, anemia, peripheral neuropathy, and skin, liver, and kidney damage in chronically exposed individuals (ATSDR, 2000). Acute arsenic poisoning is usually responsible for gastrointestinal, hepatic, hematological, renal, central neurological, and cardiovascular lesions. The severity of poisoning depends on the arsenic species and the absorbed dose (Ellenhorn, 1997). On the other hand, nutritional status may play an important role in the expression of arsenite toxicity (Peraza et al., 1998). Therefore, it is essential to understand which dietetic factors contribute to the exacerbation or attenuation of arsenite toxicity. The present work, consisting of a single administration of antioxidants prior to the single administration of arsenite, constitutes a rapid and clear way to infer about the possibility of lipoic acid and hesperidin as candidates for modulation of arsenite toxicity. The adopted protocol in the present work eliminates confusing factors that can happen during subchronic administration (e.g., alteration in water and food ingestion), which could complicate the understanding of the results.

With the liver, we observed a pattern of histological alterations in arsenite-administered animals that has also been described by Liu and coworkers (2000) after chronic exposure of metallothionein-I/II in knockout mice and of arsenite or arsenate, either through drinking water for 48 weeks or through repeated sc injections (5 days/week) for 15 weeks, in the corresponding wild-type mice. These authors described fatty vacuolation, liver degeneration, inflammatory cell infiltration, and a few areas of focal necrosis in livers of mice receiving arsenite. Santra and coworkers (2000) conducted a 3- to 15-month assay in which they exposed male BALB/c mice to drinking water collected from an arsenic-contaminated well (3.2 mg As/ L) with a familiar human poisoning history, and found several degrees of lesions, from hepatocellular degeneration and mononuclear cell collection in the initial stage, to fatty degeneration (macrovesicular steatosis), hepatocellular necrosis, Kupffer cells proliferation, and hepatic fibrosis at 15 months of exposure.

Another study performed by Flora and coworkers (1997), in which rats were coexposed to arsenic trioxide (100 ppm ip, daily) and ethanol (10% in drinking water) for 6 weeks, demonstrated decreased eosinophilea in midzonal and centrilobular hepatocytes, compared to peripheral hepatocytes, and necrosis in more than 50% of the liver lobules, with the presence of inflammatory cells, mainly macrophages in the necrotic areas. These studies indicate that chronic exposure to different inorganic arsenic compounds (arsenite, arsenic trioxide, or arsenate) produces characteristic pathology in the liver, including fatty infiltration, liver degeneration, inflammatory cell infiltration, and focal necrosis. Our results also showed that acute exposure to arsenite in mice produces almost immediate histological alterations identical to those produced after chronic exposure, as described before. Fatty infiltration is a frequent cellular response to toxicity, and its prevalence in the liver is particularly common because this organ has an important role in lipid metabolism. The mechanism for this pathological change after exposure to arsenite

is unclear, but may involve the inhibition of lipid excretion out of the cell. S-adenosyl-methionine is a major intracellular methyl group donor and is critical in the conversion of phosphotidylethanolamine to phosphotidylcholine, an essential component of specific lipoproteins that transport fat out of the liver (Hirata et al., 1980). Arsenic may exacerbate fatty accumulation by reducing or diverting methyl-donor pools and altering intracellular methylation reactions. This mechanism can be a possible explanation for the induction of fatty vacuolation in the liver by arsenic.

We have also observed dilatation of the space between hepatic cords (Table 2) that is often related to observations of hepatocyte swelling, dilatation of sinusoidal capillaries, and bile canaliculi, which contribute to the alteration of the normal structure of the hepatic lobule and parenchyma disorganization (Albores et al., 1996; Flora et al., 2002).

Some of the histological alterations observed in the present work may result from inflammation. In fact, the presence of inflammatory cells in the liver of mice and rats treated with arsenite has been associated with aspects of focal hepatocellular degeneration or necrosis (Flora et al., 1997; Liu et al., 2000; Santra et al., 2000). Also, Liu and coworkers (2000) observed that the presence of inflammatory cells was accompanied by enhanced levels of inflammatory cytokines (interleukin-1β, interleukin-6, TNF-α) in the serum of mice exposed to arsenite and postulated that these cytokines could be excreted by those inflammatory cells located in the liver and could play a role in arsenic-induced hepatotoxicity.

Hesperidin exhibits antioxidant and anti-inflammatory properties (Galati et al., 1994; Garg et al., 2001). It is noteworthy, considering the proinflammatory activity of arsenical compounds, reported in the literature and displayed in our study, that hesperidin turned out to be a good agent for the desired protective activity. In the present study, hesperidin also evidenced prominent vascular protection capacity in the liver of arsenic-treated mice. This hesperidin feature has been described in clinical studies in which this compound is used as a supplement in patients suffering from blood vessel disorders, including fragility and permeability complaints (Allegra et al., 1995; Amiel and Barbe, 1998).

In this study, lipoic acid was less efficient than hesperidin in protecting liver from arsenite action, particularly on inflammation and hemorrhagic lesions. This can be explained by their different mechanisms of action in the organism. In fact, whereas lipoic acid acts mainly by its antioxidant properties as a radical scavenger and by interacting with other antioxidants (Packer, Witt, and Tritschler, 1995; Moini, Packer, and Saris, 2002a), hesperidin has shown multiprotective effects: on the vascular system, anti-inflammatory action, enzyme modulation, platelet and cell aggregation inhibition, antioxidant effect, and immunomodulatory activity (Garg et al., 2001). These wider protective effects of hesperidin can explain its higher protection capacity against lesions evoked by arsenite when compared with lipoic acid, especially at inflammatory cells infiltration and hemorrhagic clot levels.

In addition, the hypothetical interference of lipoic acid in the methylation of arsenite should be considered in the discussion of its less protective capacity when compared to hesperidin. In fact, the detoxifying role of arsenic methylation is very controversial. During this process, which occurs mainly in the liver, glutathione or reduced lipoic acid are necessary

to produce mono-(MMA) and dimethylated (DMA) arsenic metabolites.

This is considered by some researchers to be a detoxification mechanism because methylation reduces the amount of arsenic retained in tissues by increasing the water solubility of arsenite (Goering et al., 1999). Other researchers disagree because MMA may be the most toxic intracellular form of arsenic due to its ability to induce enzyme inhibition, oxidative stress, and DNA damage (National Academy of Sciences, 2001). If this is true, lipoic acid properties could contribute to the exacerbation of the toxicity of arsenite. On the other hand, lipoic acid is capable of regenerating high levels of glutathione and other cellular reductants (Packer et al., 1995), so it should be a good candidate for protection against arsenite. In our experimental conditions toxicity exacerbation was not observed, but protective effects also were not verified, at least at the hepatocyte vacuolation and parenchyma disorganization levels. In addition, by itself, lipoic acid produced some alterations that have never been described before. Indeed, some degree of disorganization of the hepatic parenchyma and vacuolation of the hepatocytes was observed, which may be related to changes in glycogen metabolism.

It is noteworthy that it was recently shown that lipoic acid increases glycogen levels in perfused rat liver (Anderwald et al., 2002) and stimulates glucose uptake into 3T3-L1 adipocytes by increasing intracellular oxidant levels and/or facilitating insulin receptor autophosphorylation, presumably by oxidation of critical thiol groups present in the insulin receptor beta-subunit (Moini et al., 2002b). Whether lipoic acid induces the uptake of glucose by mouse hepatocytes and interferes with glycogen metabolism warrants further investigation. In a recent review Moini and coworkers (2002) focused on the antioxidant and pro-oxidant activities of the pair lipoic acid (LA)/dihydrolipoic acid (DHLA). These investigators have pointed out the fact that LA and/or DHLA may directly or indirectly cause oxidation of cellular proteins and thereby modulate biological processes with beneficial or harmful effects depending on the biological system. Also, in an in vivo study performed in mice to evaluate the effects of lipoic acid administered intraperitoneally on the toxicity of cadmium (Bludovska et al., 1999), it was found that the administration of lipoic acid alone enhanced lipid peroxidation. This observation corroborates the hypothesis of some pro-oxidant effects of lipoic acid. Nevertheless, its ability to diminish the incidence of inflammatory cells infiltration, centrilobular hepatocyte swelling, and the presence of hemorrhagic clots in the liver shows that this compound has some protective capacity against arsenite toxicity, which can be attributed to its antioxidant activity (Packer, Witt, and Tritschler, 1995; Biewenga, Haenen, and Bast, 1997).

Total arsenic quantification in the liver revealed that all animals exposed to arsenite acquired hepatic contents similar to those of of arsenic, which indicates that both antioxidants were unable to alter arsenic distribution in the organism. Besides that, with this quantification we assure that the pathologic alterations observed do not result from differences or errors in experimental procedures and that arsenic was actually present in the organism, exerting its toxicity.

The kidney is the main organ responsible for arsenic elimination, and it is considered a target organ for this element

and its metabolites (Goering et al., 1999; ATSDR, 2000). Nevertheless, clinical reports of arsenic-induced kidney cell toxicity are considerably fewer than those of other metals, such as lead and cadmium; nevertheless, there are some reports of increased rates of nephritis and nephrosis among persons drinking arsenic-contaminated well water, and elevated rates of renal cancer have been reported in the United States (Lewis et al., 1999) and Chile (Smith et al., 1998) among persons exposed to arsenic-contaminated well water. With respect to the kidney, our results are once again in accordance with the studies of Liu and coworkers (2000) in which arsenite was shown to induce tubular cell vacuolation and tubular atrophy in metallothionein-I/II knockout mice and the corresponding wild-type mice. In a different approach, Hirata and coworkers (1990) pretreated hamsters with buthionine sulfoximine (BSO), a glutathione synthesis inhibitor, resulting in acute renal failure with oliguria and prominent tubular necrosis 1 hour after ingestion of sodium arsenite (5 mg As/kg). In another study by Liu and coworkers (2002), the mdr1a/1b mice (knockout mice that lack the mdr1-type drug-transporting P-glycoproteins in the liver, kidney, small intestine, and brain (Schinkel et al., 1997)) and wild-type FVB mice were given sodium arsenite (12–19 mg/kg, sc), and 24 hours later, the kidneys showed degeneration, including vacuolation, interstitial congestion, and focal necrosis of the proximal tubules.

Some investigators have observed that the antioxidants ascorbic acid and α-tocopherol are able to modulate arsenic toxicity in the kidney (Ramanathan et al., 2003). Our study follows the same purpose of screening different compounds with antioxidant capacity in order to identify which of them displays higher protection against arsenic toxicity. Hesperidin and lipoic acid had not yet been assessed for modulation of arsenite toxicity in mice. We have observed that hesperidin and lipoic acid were able to reduce the tubular cell vacuolation induced by arsenite, but only hesperidin showed the capacity to diminish vascular lesions, which can be related to its antioxidant and anti-inflammatory properties. Referring to lipoic acid, other authors have described protective effects in rats against ischaemic acute renal failure (Takaoka et al., 2002), angiotensin II–induced renal injury (Mervaala et al., 2003), and adriamycin-induced lipid peroxidation (Malarkodi, Balachandar, and Varalakshmi, 2003). Maritim, Sanders, and Watkins (2003) observed that an appropriate pharmacological dose of lipoic acid (10 mg/kg ip once daily for 14 days) reduced oxidative stress in STZ-induced diabetic Sprague–Dawley rats (Maritim, Sanders, and Watkins, 2003). In the present study, we demonstrated that in mice the previous administration of lipoic acid prevents the toxicity induced by single arsenite exposure at the kidney level. The evidence of protective effects of hesperidin and lipoic acid against the injury produced after acute exposure to arsenite, at the given liver and kidney levels, points to the need to proceed with experiments on longer treatment protocols (several weeks).

With respect to the testis, the main lesions observed in the present study consisted of vacuolation and atrophy of seminiferous tubules. In in vitro studies, incubation of mice organs with As(III) and S-adenosyl-methionine, indicate that the testis had the highest As(III)-methylation activity, followed by the kidney, liver, and lung, which seems to be related to a major arsenite methyltransferase-specific activity in mouse testis (Aposhian, 1997; Healy et al., 1998). Our results have shown that arsenite induces seminiferous tubule vacuolation and atrophy, indicating degeneration of germ cells. Accordingly, recent studies performed by Sarkar and coworkers (2003) in rats have demonstrated major damage on spermatogenesis induced by repeated ip injections of arsenite, including degeneration of germ cells with consequent diminished numbers of sperm cells in the epididymis. Pant and coworkers (2001) have given As(III) to mice (53.39–533.90 micromol per liter of drinking water for 35 days) and noticed abnormalities in sperm count, motility, and morphology at the higher dose levels. In our experimental conditions, lipoic acid and hesperidin were unable to alter the observed histological features elicited on the testis by arsenite.

In conclusion, hesperidin and lipoic acid exhibit protective effects against sodium arsenite-induced acute toxicity in liver and kidney of mice. These compounds may potentially play an important role in the protection of populations chronically exposed to arsenic.

REFERENCES

Albores, A., Cebrian, M. E., Garcia-Vargas, G. G., Connelly, J. C., Price, S. C., Hinton, R. H., Bach, P. H., and Bridges, J. W. (1996). Enhanced arsenite-induced hepatic morphological and biochemical changes in phenobarbital-pretreated rats. *Toxicol Pathol* **24**, 172–80.

Allegra, C., Bartolo, M. Jr., Carioti, B., and Cassiani, D. (1995). An original microhaemorheological approach to the pharmacological effects of Daflon 500 mg in severe chronic venous insufficiency. *Int J Microcirc Clin Exp* **15**(Suppl 1), 50–4.

Amiel, M., and Barbe, R. (1998). Study of the pharmacodynamic activity of Daflon 500 mg. *Ann Cardiol Angeiol* (Paris) **47**, 185–8.

Anderwald, C., Koca, G., Furnsinn, C., Waldausl, W., and Roden, M. (2002). Inhibition of glucose production and stimulation of bile flow by R(+)-alpha-lipoic acid enantiomer in rat liver. *Liver* **22**(4), 355–62.

Aposhian, H. V. (1997). Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Annu Rev Pharmacol Toxicol* **37**, 397–419.

Aposhian, H. V., Gurzau, E. S., Le, X. C., Gurzau, A., Healy, S. M., Lu, X., Ma, M., Yip, L., Zakharyan, R. A., Maiorino, R. M., Dart, R. C., Tircus, M. G., Gonzalez-Ramirez, D., Morgan, D. L., Avram, D., and Aposhian, M. M. (2000). Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chem Res Toxicol* **13**(8), 693–7.

Applegate, L. A., Luscher, P., and Tyrrell, R. M. (1991). Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res* **51**(3), 974–8.

Agency for Toxic Substances and Disease Registry (ATSDR) (2000). *Toxicologic Profile for Arsenic* (update). U.S. Department of Health & Human Services, Washington, D.C.

Barchowsky, A., Klei, L. R., Dudek, E. J., Swartz, H. M., and James, P. E. (1999). Stimulation of reactive oxygen, but not reactive nitrogen species, in vascular endothelial cells exposed to low levels of arsenite. *Free Radic Biol Med* **27**, 1405–12.

Bartolome, B., Cordoba, S., Nieto, S., Fernandez-Herrera, J., and Garcia-Diez, A. (1999). Acute arsenic poisoning: clinical and histopathological features. *Br J Dermatol* **141**(6), 1106–9.

Biewenga, G. P., Haenen, G. R., and Bast, A. (1997). The pharmacology of the antioxidant lipoic acid. *Gen Pharmacol* **29**(3), 315–31.

Bludovska, M., Kotyzova, D., Koutensky, J., and Eybl, V. (1999). The influence of alpha-lipoic acid on the toxicity of cadmium. *Gen Physiol Biophys* **18**, 28–32.

Buchet, J. P., Lauwerys, R., and Roels, H. (1981). Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethylarsonate, or dimethylarsonate in man. *Int Arch Occup Environ Health* **48**, 71–9.

Cebrian, M. E., Albores, A., Aguilar, M., and Blakely, E. (1983). Chronic arsenic poisoning in the north of Mexico. *Hum Toxicol* **2**(1), 121–33.

Chen, A., Cao, E. H., Zhang, T. C., and Qin, J. F. (2002). Arsenite-induced reactive oxygen species and the repression of α-tocoferol in the MGC-803 cells. *Eur J Pharmacol* **448**, 11–8.

Csanaky, I., and Gregus, Z. (2002). Species variations in the biliary and urinary excretion of arsenate, arsenite and their metabolites. *Comp Biochem Physiol* **131**, 355–65.

Cullen, W. R. (1989). The metabolism of methylarsine oxide and sulfide. *Appl Organometal Chem* **3**, 71–8.

Ellenhorn, M. J. (1997). Arsenic. In *Ellenhorn's Medical Toxicology: Diagnosis and Treatment of Human Poisoning* (M. J. Ellenhorn, ed.), pp. 1538–43. Williams & Wilkins, Baltimore.

Ercal, N., Gurer-Orhan, H., and Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. *Curr Top Med Chem* **1**, 529–39.

Fanton, L., Duperret, S., Guillaumee, F., Miras, A., Vallon, J. J., and Malicier, D. (1999). Fatal rhabdomyolysis in arsenic trioxide poisoning. *Hum Exp Toxicol* **18**, 640–1.

Flora, S. J., Dubey, R., Kannan, G. M., Chauhan, R. S., Pant, B. P., and Jaiswal, D. K. (2002). Meso 2,3-dimercaptosuccinic acid (DMSA) and monoisoamyl DMSA effect on gallium arsenide induced pathological liver injury in rats. *Toxicol Lett* **132**, 9–17.

Flora, S. J., Pant, S. C., Malhotra, P. R., and Kannan, G. M. (1997). Biochemical and histopathological changes in arsenic-intoxicated rats coexposed to ethanol. *Alcohol* **14**, 563–8.

Galati, E. M., Monforte, M. T., Kirjavainen, S., Forestieri, A. M., Trovato, A., and Tripodo, M. M. (1994). Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. *Farmaco* **40**(11), 709–12.

Garg, A., Garg, S., Zaneveld, L. J., and Singla, A. K. (2001). Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytother Res* **15**(8), 655–69.

Goering, P. L., Aposhian, H. V., Mass, M. J., Cebrian, M., Beck, B. D., and Waalkes, M. P. (1999). The enigma of arsenic carcinogenesis: role of metabolism. *Toxicol Sci* **49**, 5–14.

Gregus, Z., Gyurasics, A., and Csanaky, I. (2000). Biliary and urinary excretion of inorganic arsenic: monomethylarsonous acid as a major biliary metabolite in rats. *Toxicol Sci* **56**, 18–25.

Guzzo, A., Karatzios, C., Diorio, C., and DuBow, M. S. (1994). Metallothionein-II and ferritin H mRNA levels are increased in arsenite-exposed HeLa cells. *Biochem Biophys Res Commun* **205**(1), 590–5.

Healy, S. M., Casarez, E. A., Ayala-Fierro, F., and Aposhian, H. (1998). Enzymatic methylation of arsenic compounds. V. Arsenite methyltransferase activity in tissues of mice. *Toxicol Appl Pharmacol* **148**, 65–70.

Hirata, M., Tanaka, A., Hisanaga, A., and Ishinishi, N. (1990). Effects of glutathione depletion on the acute nephrotoxic potential of arsenite and on the arsenic metabolism in the hamster. *Toxicol Appl Pharmacol* **106**, 469–81.

Hirata, F., Toyoshima, S., Axelrod, J., and Waxdal, M. J. (1980). Phospholipid methylation: a biochemical signal modulating lymphocyte mitogenesis. *Proc Natl Acad Sci USA* **77**(2), 862–5.

Huff, J., Chan, P., and Nyska, A. (2000). Is the human carcinogen arsenic carcinogenic to laboratory animals? *Toxicol Sci* **55**, 17–23.

Institute of Laboratory Animal Resources (ILAR) (1996). *Guide for the Care and Use of Laboratory Animals.* National Academy Press, Washington, DC.

Knowles, F. C., and Benson, A. A. (1983). The biochemistry of arsenic. *Trends Biochem Sci* **8**, 178–80.

Lee, T. C., and Ho, I. C. (1995). Modulation of cellular antioxidant defence activities by sodium arsenite in human fibroblasts. *Arch Toxicol* **69**, 498–504.

Lewis, D. R., Southwick, J. W., Ouellet-Hellstrom, R., Rench, J., and Calderon, R. L. (1999). Drinking water arsenic in Utah: a cohort mortality study. *Environ Health Perspect* **107**(5), 359–65.

Liu, J., Liu, Y., Goyer, R. A., Achanzar, W., and Waalkes, M. P. (2000). Metallothionein-I/II null mice are more sensitive than wild-type mice to the hepatotoxic and nephrotoxic effects of chronic oral or injected inorganic arsenicals. *Toxicol Sci* **55**, 460–7.

Liu, J., Liu, Y., Powell, D. A., Waalkes, M. P., and Klaassen, C. D. (2002). Multidrug-resistance mdr1a/1b double knockout mice are more sensitive than wild type mice to acute arsenic toxicity, with higher arsenic accumulation in tissues. *Toxicology* **170**(1–2), 55–62.

Lodge, L., Handelman, G. J., Konishi, T., Matsugo, S., Mathur, V. V., and Packer, L. (1997). Natural sources of lipoic acid: determination of lipoyllysine released from protease-digested tissues by high performance liquid chromatography incorporating electrochemical detection. *J Appl Nutr* **49**, 3–11.

Malarkodi, K. P., Balachandar, A. V., and Varalakshmi, P. (2003). Protective effect of lipoic acid on adriamycin induced lipid peroxidation in rat kidney. *Mol Cell Biochem* **247**(1–2), 9–13.

Mandal, B. K., Ogra, Y., and Suzuki, K. T. (2001). Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic-affected areas in West Bengal, India. *Chem Res Toxicol* **14**(4), 371–8.

Marafante, E., Vahter, M., Norin, H., Envall, J., Sandström, M., Christakopoulos, A., and Ryhage, R. (1987). Biotransformation of dimethylarsinic acid in mouse, hamster and man. *J Appl Toxicol* **7**, 111–7.

Maritim, A. C., Sanders, R. A., and Watkins, J. B. 3rd. (2003). Effects of alpha-lipoic acid on biomarkers of oxidative stress in streptozotocin-induced diabetic rats. *J Nutr Biochem* **14**(5), 288–94.

Mervaala, E., Finckenberg, P., Lapatto, R., Muller, D. N., Park, J. K., Dechend, R., Ganten, D., Vapaatalo, H., and Luft, F. C. (2003). Lipoic acid supplementation prevents angiotensin II-induced renal injury. *Kidney Int* **64**(2), 501–8.

Moini, H., Packer, L., and Saris, N. E. (2002a). Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. *Toxicol Appl Pharmacol* **182**(1), 84–90.

Moini, H., Tirosh, O., Park, Y. C., Cho, K. J., and Packer, L. (2002b). R-alpha-lipoic acid action on cell redox status, the insulin receptor, and glucose uptake in 3T3-L1 adipocytes. *Arch Biochem Biophys* **397**(2), 384–91.

National Academy of Sciences (2001). *Arsenic in Drinking Water.* National Academy Press Washington, DC.

Nordenson, I., and Beckman, L. (1991). Is the genotoxic effect of arsenic mediated by oxygen free radicals? *Hum Hered* **41**, 71–3.

Packer, L., Witt, E. H., and Tritschler, H. J. (1995). alpha-Lipoic acid as a biological antioxidant. *Free Radic Biol Med* **19**(2), 227–50.

Pant, N., Kumar, R., Murthy, R. C., and Srivastava, S. P. (2001). Male reproductive effect of arsenic in mice. *Biometals* **14**(2), 113–7.

Peraza, M. A., Ayala-Fierro, F., Barber, D. S., Casarez, E., and Rael, L. T. (1998). Effects of micronutrients on metal toxicity. *Environ Health Perspect* **106** (Suppl 1), 203–16.

Petrick, J. S., Ayala-Fierro, F., Cullen, W. R., Carter, D. E., and Aposhian, H. V. (2000). Monomethylarsonous acid (MMAIII) is more toxic than arsenite in Chang human hepatocytes. *Toxicol Appl Pharmacol* **163**, 203–7.

Petrick, J. S., Jagadish, B., Mash, E. A., and Aposhian, H. V. (2001). Monomethylarsonous acid (MMA III) and arsenite: LD50 in hamsters and in vitro inhibition of pyruvate dehydrogenase. *Chem Res Toxicol* **14**, 651–6.

Ramanathan, K., Shila, S., Kumaran, S., and Panneerselvam, C. (2003). Ascorbic acid and alpha-tocopherol as potent modulators on arsenic induced toxicity in mitochondria. *J Nutr Biochem* **14**(7), 416–20.

Santra, A., Maiti, A., Das, S., Lahiri, S., Charkaborty, S. K., and Mazumder, D. N. (2000). Hepatic damage caused by chronic arsenic toxicity in experimental animals. *Clinical Toxicol* **38**, 395–405.

Sarkar, M., Chaudhuri, G. R., Chattopadhyay, A., and Biswas, N. M. (2003). Effect of sodium arsenite on spermatogenesis, plasma gonadotrophins and testosterone in rats. *Asian J Androl* **5**, 27–31.

Schinkel, A. H., Mayer, U., Wagenaar, E., Mol, C. A., van Deemter, L., Smit, J. J., van der Valk, M. A., Voordouw, A. C., Spits, H., van Tellingen, O., Zijlmans, J. M., Fibbe, W. E., and Borst, P. (1997). Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci USA* **94**(8), 4028–33.

Smith, A. H., Arroyo, A. P., Mazumder, D. N., Kosnett, M. J., Hernandez, A. L., Beeris, M., Smith, M. M., and Moore, L. E. (2000). Arsenic-induced skin lesions among Atacameno people in Northern Chile despite good nutrition and centuries of exposure. *Environ Health Perspect* **108**(7), 617–20.

Smith, A. H., Goycolea, M., Haque, R., and Biggs, M. L. (1998). Marked increase in bladder and lung cancer mortality in a region of Northern Chile due to arsenic in drinking water. *Am J Epidemiol* **147**(7), 660–9.

Soares, M. E., Bastos, M. L., and Ferreira, M. (1995). Determination of arsenic, cadmium and lead in porcine and bovine kidneys by electrothermal atomic absorption spectrometry. *Analyst* **120**, 2367–70.

Styblo, M., Del Razo, L. M., Vega, L., Germolec, D. R., LeCluyse, E. L., Hamilton, G. A., Reed, W., Wang, C., Cullen, W. R., and Thomas, D. J. (2000). Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Arch Toxicol* **74**, 289–99.

Takaoka, M., Ohkita, M., Kobayashi, Y., Yuba, M., and Matsumura, Y. (2002). Protective effect of alpha-lipoic acid against ischaemic acute renal failure in rats. *Clin Exp Pharmacol Physiol* **29**(3), 189–94.

Tseng, C. H. (2002). An overview on peripheral vascular disease in blackfoot disease-hyperendemic villages in Taiwan. *Angiology* **53**(5), 529–37.

Vahter, M., and Concha, G. (2001). Role of metabolism in arsenic toxicity. *Pharmacol Toxicol* **89**, 1–5.

Valentine, J. L., Cebrian, M. E., Garcia-Vargas, G. G., Faraji, B., Kuo, J., Gibb, H. J., and Lachenbruch, P. A. (1994). Daily selenium intake estimates for residents of arsenic-endemic areas. *Environ Res* **64**(1), 1–9.

Wang, T. S., and Huang, H. (1994). Active oxygen species are involved in the induction of micronuclei by arsenite in XRS-5 cells. *Mutagenesis* **9**, 253–7.

Wang, T. S., Kuo, C. F., Jan, K. Y., and Huang, H. (1996). Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. *J Cell Physiol* **169**, 256–68.

Wu, M. M., Chiou, H. Y., Wang, T. W., Hsueh, Y. M., Wang, I. H., and Lee, T. C. (2001). Association of blood arsenic levels with increased reactive oxidants and decreased antioxidant capacity in a human population of northeastern taiwan. *Environ Health Perspect* **109**, 1011–7.

Yamanaka, K., Takabayashi, F., Mizoi, M., An, Y., Hasegawa, A., and Okada, S. (2001). Oral exposure of dimethylarsinic acid, a main metabolite of inorganic arsenics, in mice leads to an increase in 8-Oxo-2′-deoxyguanosine level, specifically in the target organs for arsenic carcinogenesis. *Biochem Biophys Res Commun* **287**, 66–70.

Yamauchi, H., and Yamamura, Y. (1984). Metabolism and excretion of orally administered dimethylarsinic acid in the hamster. *Toxicol Appl Pharmacol* **74**, 134–40.

Yamauchi, H., Yamato, N., and Yamamura, Y. (1988). Metabolism and excretion of orally and intraperitoneally administered methylarsonic acid in the hamster. *Bull Environ Contam Toxicol* **40**, 280–6.