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Effect of arsenic in endochondral ossification of experimental animals

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Abstract

Arsenic (As) toxicity is a global health problem affecting millions of people, the most toxic forms being Arsenites [As(III)] and Arsenates [As(V)]. Arsenic intoxication can occur through different exposure routes. The aim of the present work was to determine the effect of As on endochondral ossification and bone remodeling in experimental animals, by means of biochemical, histologic, and histomorphometric determinations.

Sixteen male Wistar rats, 100 g body weight (b.w.), were divided into two groups: experimental group ($n = 8$), treated with 10 mg/l of $NaAsO_2$ in their drinking water, receiving 0.21 mg/kg b.w./day during 45 days; and control group ($n = 8$) remained untreated. On day 45, blood samples were obtained by cardiac puncture to perform hematologic blood counts and biochemical determination. The animals were killed, the tibiae, femurs, kidneys and livers were resected, fixed in formalin and processed histologically. Tibia and femur sections were obtained and stained with H&E. The following histomorphometric parameters were determined on tibia and femur sections: bone volume (BV/TV), thickness of growth plate cartilage (GPC.Th) and thickness of hypertrophic zone (HpZ.Th).

Biochemical determinations showed that experimental animals exhibited neutrophilia and a decrease in lymphocytes and monocytes. As levels were below 1 μg/dl in both groups. The femur sections of the experimental group showed (1) a statistically significant increase in total growth cartilage plate thickness ($p < 0.05$) at the expense of the hypertrophic zone ($p < 0.05$); (2) subchondral trabecular bone sealed to the growth plate with a non-significant increase in primary spongiosa bone volume. These results suggest that As alters endochondral ossification.
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Keywords: Arsenic; Endochondral ossification; Bone histomorphometry

Introduction

Arsenic toxicity is a global health problem affecting millions of people (Ratnaike, 2003). Among the different forms of As found in the environment, inorganic As is the most dangerous. Arsenite [As(III)] is usually more toxic than arsenate [As(V)]. In the

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environment, arsenic combines with oxygen, chloride and sulfur, and other metals forming inorganic compounds of As. Once it enters an animal or a plant, it combines with carbon and hydrogen forming organic compounds. The main source of arsenic in drinking water is arsenic-rich rocks through which arsenic is filtered, and certain industrial processes. The As cycle has been altered as a consequence of human interference, and due to this the concentrations of As have increased, thus contaminating the environment and living organisms. As-based compounds are employed as fertilizers and pesticides in agriculture, for wood preservation, in the glass and ceramics industry, in mining and manufacture of copper and lead alloys, as feed supplements to cattle, and they are also present in certain foods, tobaccos, wines and medicinal drugs. Seafood is rich in As, and is an important component of many people's diet. Carbon combustion and metal smelting are the main sources of As in the air (Van Deuren et al., 1996).

Arsenic intoxication can occur through different exposure routes, including ingestion of contaminated drinking water, inhalation and accidental skin contact. As has a toxic effect on around 200 enzymes, especially those related with cell metabolism and DNA synthesis and repair (Ratnaike, 2003). Hydroarsenicism is caused by the intake of small amounts of As in drinking water over long periods of time, usually more than 10 years, and features alterations of the skin and visceral organs. Arsenic-related melanosis of the trunk, resembling leukomelanodermia, can also present on the neck and back. Leukoplakia badges are also frequent. One of the distinctive features of Arsenicism is hyperkeratosis of the palms and soles, and the presence of squamous and basal cell carcinomas. In addition, finger and toe nails exhibit longitudinal striae where the As accumulates (Tello, 1951).

India, China, South America and certain regions of the US are among the most affected sites (Vogt and Rossman, 2001). Argentina is one of the most affected countries due to contamination of underground water from soils and rocks that are rich in arsenic. The maximum contaminant level for As established by the World Health Organization (WHO) is 0.01 mg/l, and concentrations ranging between 0.10 and 0.18 mg/l are considered high. Different regions studied in our country were found to have As levels ranging between 0.10 and 0.8 mg/l (Besuschio, 1999).

There are no experimental studies in the literature on the effect of As on bone tissue and endochondral ossification *in vivo*. Regarding As-related bone diseases, an epidemiologic study performed in Lancashire, UK, showed association between hydroarsenicism and cases of Paget's disease of bone in a population of cotton factory workers (Lever, 2002).

In vitro studies on cell cultures of mouse macrophages showed that low doses of arsenite induce an increase in hydrogen peroxide and differentiation of pre-osteoclastic cells, whereas high concentrations increase levels of hydrogen peroxide, trigger caspase-dependent apoptosis of pre-osteoclasts, through the release of oxygen reactive species (Szymczyk et al., 2006).

There is no conclusive evidence in the literature supporting the theory that arsenite has a deleterious effect on the skeleton. Arsenate and phosphate have analogous behavior; they compete depositing in the hydroxyapatite crystals of bone and converting to arsenite, the most toxic form of inorganic As, inside the cell (Szymczyk et al., 2006).

The increase in As in the environment and the lack of access to drinking water in rural areas with As-contaminated water, as well as the increase in As in urban areas demonstrate the need to conduct further studies on the effects of As, not only on the skin and viscera, which have been extensively studied, but also on bone remodeling.

The aim of the present study was to evaluate the effect of arsenic on bone biology in experimental animals, by means of biochemical, histologic and histomorphometric parameters.

Materials and methods

Animals

Sixteen male Wistar rats weighing 100 g were assigned to one of two groups. Animals in the experimental group, As group ($n = 8$), were given 10 mg/l of $NaAsO_2$ (Carlo Ebba, Milan) in their drinking water (distilled water), so that each animal received 0.21 mg/kg day, for 45 days. Control animals ($n = 8$) were given regular distilled drinking water. The animals were fed a normal protein diet and water ad libitum. Housing conditions included eight animals per cage, 21–24 °C temperature, 52–56% humidity, and 12-h light–dark cycles. The National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23 Rev 1985) were observed. The animals were examined and weighed weekly.

Biochemical and histomorphometric evaluation

At the end of the experiment (day 45), a blood sample was obtained from each animal by cardiac puncture in order to perform blood counts and biochemical determination of plasma calcium, phosphorus, alkaline phosphatase, urea, creatinine, glucemia and As. As determinations were performed using high-performance liquid chromatography (HPLC) AA-G Hidruros.

The animals were killed, and the tibiae, femurs, livers and kidneys were resected. Tibiae and femurs were

measured using a Vernier caliper. The samples were then fixed in buffered 10% formaldehyde solution for 48 h, decalcified in ethylendiaminotetracetic acid (EDTA, Sigma) pH 7.4 for 30 days and processed histologically for embedding in paraffin. Longitudinal sections of the tibiae and femurs were obtained and stained with H&E. The histomorphometric determinations were performed on digital microphotographs of the sections, using Image Pro Plus 4.5 software. The following histomorphometric parameter was measured on the longitudinal sections of the tibiae:

Bone volume (*BV/TV*) (%): The bone volume of a specific area of subchondral trabecular bone tissue was measured. This area was then divided into two sectors, A and B, each measuring 800 μm^2, sector A consisting of primary spongiosa and sector B of secondary spongiosa (Fig. 1). Total bone volume (A + B) and primary spongiosa bone volume (A) were evaluated.

The following parameters were measured on the femur sections:

Thickness of growth plate cartilage (GPC.Th) and thickness of hypertrophic zone (HpZ.Th) (Fig. 2).

The thickness of the growth plate and hypertrophic zone was calculated as the mean of seven different measurements performed at seven locations randomly chosen on each section.

The results were statistically analyzed using ANOVA.

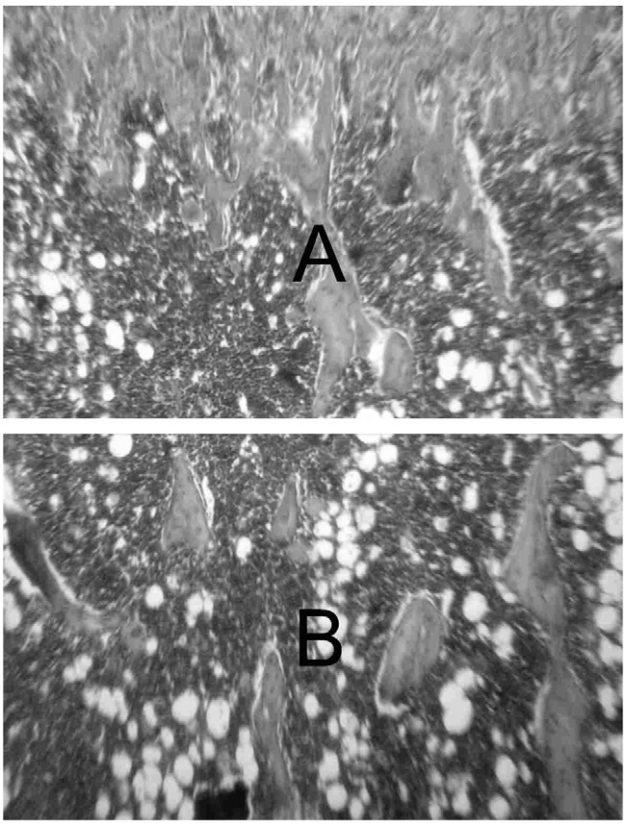

Fig. 1. Area divided into two sectors, A and B. Sector A consisting of primary spongiosa and sector B of secondary spongiosa. Total bone volume (A + B) and primary spongiosa bone volume (A) were evaluated.

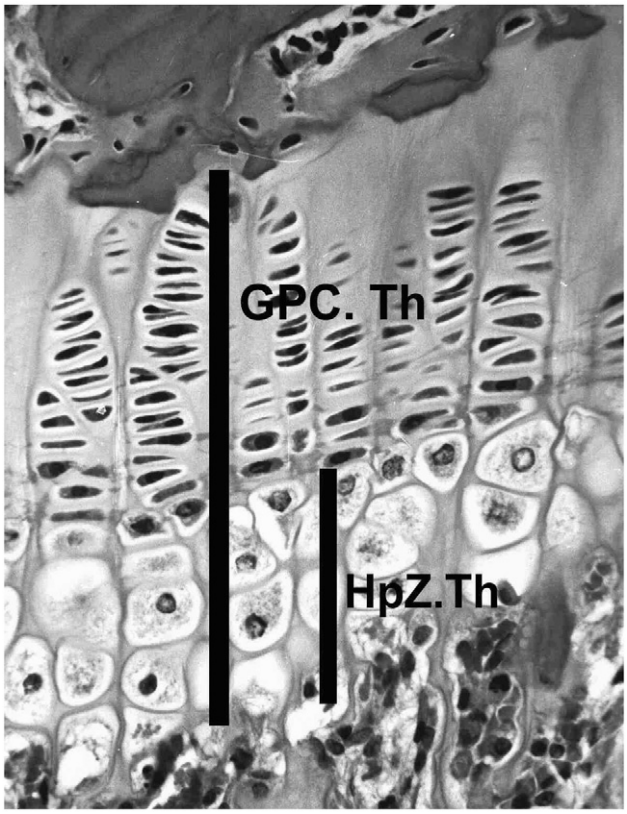

Fig. 2. Thickness of growth plate cartilage (GPC.Th) and thickness of hypertrophic zone (HpZ.Th).

Results

Animal body weight

No significant differences were observed between groups when comparing final body weight (C: 320 ± 20 g; As: 307 ± 35 g) or tibia (C: 37.2 ± 0.93 mm; As 37.5 ± 1.44 mm) length.

Biochemical and hematologic data

Biochemical and hematologic data are shown in Table 1. A significant increase in neutrophils and a significant decrease in lymphocytes and monocytes were observed. Arsenic levels were below 1 μg/dl in both the experimental and control groups.

Liver and kidney histology

Kidney sections of As-treated animals exhibited venous congestion in the cortical area. Likewise,

Table 1. Biochemical and hematologic data.

	Control	Experimental	*p*
Alkaline phosphatase (IU/l)	360±101	347±73	NS
Phosphate (mg/dl)	9.95±3.38	9.95±1.07	NS
Calcium (mg/dl)	10.7±0.60	10.1±0.46	NS
Urea (g/l)	0.54±0.04	0.54±0.06	NS
Creatinine (mg/l)	5.65±0.63	5.10±0.55	NS
Glucose (g/l)	1.79±0.46	1.77±0.44	NS
GB (μl)	5075±1089	4687±1033	NS
Neutrophils (%)	10.5±5.65	22.6±4.10	$p<0.05$
Lymphocytes (%)	84.4±8.31	73.9±4.9	$p<0.05$
Eosinophils (%)	0.87±0.99	2.00±2.13	NS
Monocytes (%)	4.62±2.06	1.37±0.52	$p<0.05$
Basophils (%)	0	0	

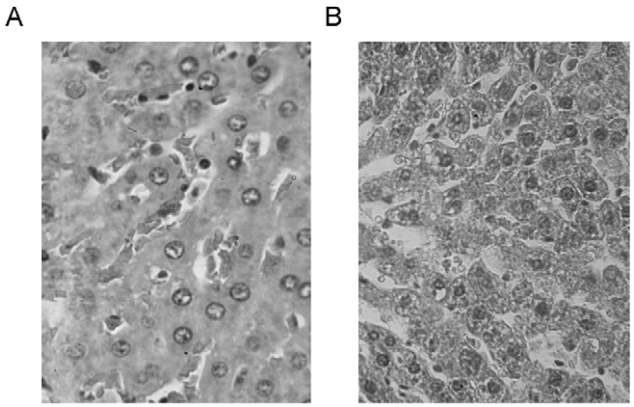

Fig. 3. Control (A) and experimental (B) liver. Experimental sections show passive chronic congestion, increase in the thickness of the Disse space and hepatocyte atrophy.

experimental liver sections showed passive chronic congestion, increase in the thickness of the Disse space and hepatocyte atrophy (Fig. 3A and B).

Tibia histology and histomorphometry

Histologic sections of the tibiae corresponding to the As group evidenced growth cartilage plate sealed with trabeculae immediately below the metaphyseal cartilage (Fig. 4A and B) with no statistically significant increase in primary spongiosa bone volume as compared with controls.

A statistically significant increase in the thickness of the growth plate cartilage (GPC.Th) and of the hypertrophic zone (HpZ.Th) was observed in As-treated animals. The histomorphometric results are shown in Table 2.

Discussion

The results of the present experimental study show that intoxication with undetectable amounts of arsenic causes an increase in the thickness of the growth plate cartilage, which is sealed with mixed trabeculae, indicating inhibition of endochondral ossification.

Heavy metals are stable elements that cannot be metabolized, and therefore pass through the food chain and are finally ingested by humans (bio-accumulation). The most common harmful metals are lead, copper, aluminum, arsenic, cadmium, mercury, uranium and nickel. There are heavy metals in the air, drinking water, and foods, as well as in chemicals used in the industry. The main routes of entry to the body are inhalation, ingestion and percutaneous absorption. A large number

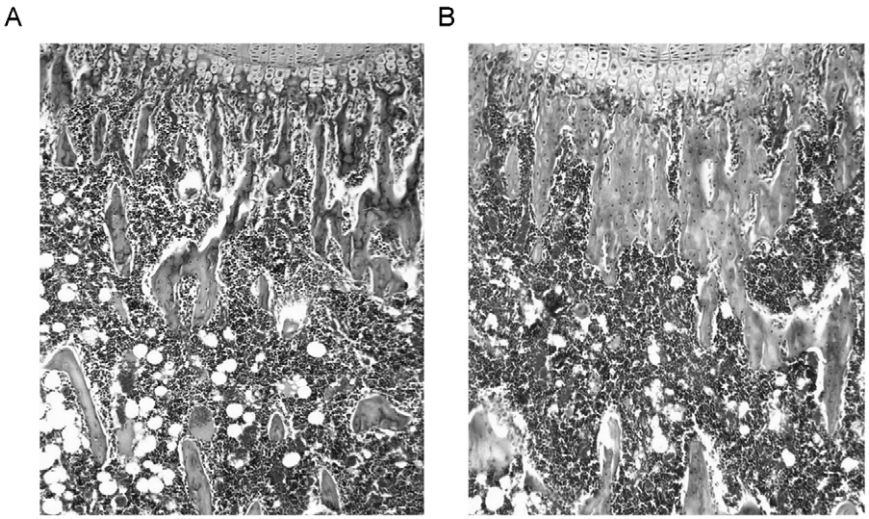

Fig. 4. Control (A) and As (B) histologic section of the tibiae corresponding to the As group shows growth cartilage plate sealed with trabeculae immediately below the metaphyseal cartilage (B).

Table 2. Histomorphometric evaluation.

	Control	Experimental	*p*
Bone volume (BV/TV) (%)	20.0±3.7	21.0±6.15	NS
Bone volume (primary spongiosa) (BV/TV) (%)	27.9±4.8	35.3±14.9	NS
Thickness of growth plate cartilage. GPC.Th (μm)	196.9±17.8	222.7±24.9	$p<0.05$
Thickness of hypertrophic zone (HpZ.Th) (μm)	64.6±8.3	79.9±12.9	$p<0.05$

of these metals interact with bone cells, causing damage to the skeletal system (Holz et al., 2007). The skeleton is the largest target organ of divalent cations, including heavy metals.

The effect of a number of metals on bone has been reported in the literature. Lead intoxication affects osteoblasts, osteoclasts and chondrocytes, is associated with osteoporosis and inhibition of endochondral ossification (Vahter et al., 2007; Carmouche et al., 2005) and has been found inside hydroxyapatite crystals (Hamilton and O'Flaherty, 1995) in areas of mineralization and bone growth, where it remains until the bone is resorbed (Hicks et al., 1996). Cadmium is found in foods, and in high doses causes osteomalacia and osteoporosis (Noda et al., 1991). Toxicity of aluminum is also well documented, and has been shown to cause osteomalacia in renal patients (Sebes et al., 1984; Daimon, 2003). Other metals such as lithium and uranium cause osteopenia and inhibit bone formation, respectively (Lewicki et al., 2006; Ubios et al., 1991, 1995).

It is well documented that long-term exposure to arsenic through drinking water naturally containing high levels of arsenic causes endemic regional chronic hydroarsenicism, which is associated with risk of diabetes, vascular disease and cancer. The present study is the first to describe alterations in endochondral ossification in experimental animals intoxicated with arsenic.

In the present study, the As group presented an increase in the thickness of the growth cartilage and the hypertrophic zone, and trabeculae sealed to the cartilage. These findings are in agreement with previous experimental studies on renal failure and iron overload performed at our laboratory, which showed that the presence of growth cartilage plate sealed to trabeculae is associated with an inhibition of endochondral ossification (Mandalunis and Ubios, 2005).

The present work also shows alterations in histologic features of the liver and kidneys. Several studies have reported that As causes damage to the liver, which ranges from structural alterations to hepatocyte apoptosis and necrosis (Boscolo et al., 1982; Arteel et al., 2008; Bashir et al., 2006). Regarding kidney damage, Sinha et al. (2008) reported alterations in kidney tissue and changes in serum levels of urea and creatinine.

Hematologic studies have shown an increase in the number of neutrophils and a decrease in the number of lymphocytes and monocytes. Both experimental (Sikorski et al., 1991) and clinical studies (Meng and Meng, 2000; Gonsebatt et al., 1992; Soto Peña et al., 2006) have reported the immunotoxic effects of As, showing inhibition of T lymphocyte proliferation and suggesting that As may cause damage to immune cells impairing their capacity to respond to pathogens and transformed cells.

Sakurai et al. (2005, 2006) found that arsenite affects monocyte differentiation to macrophages *in vitro*, since it inhibits the colony-forming stimulator (CFS), forming in smaller, non-adhesive macrophages. These findings are consistent with reports by Lemaire et al. (2006), showing that As induces a rapid loss of adhesion "*in vitro*".

In a study using cultures of macrophages that differentiate to osteoclasts in the presence of RANKL Szymczyk et al. (2006) found that AsO_2 produces H_2O_2 and that the quantity produced depends on the concentration: in low doses it stimulates H_2O_2 production inducing osteoclast differentiation, whereas in high concentrations the quantity of H_2O_2 increases leading to apoptosis of osteoclasts.

In vivo studies have demonstrated that arsenite alters macrophagic functions, such as adhesion or phagocytosis (Sengupta and Bishayi, 2002), and decreases immunologic response (Bishayi and Sengupta, 2003).

Remodeling of bone trabeculae is performed by the coordinated action of osteoblasts and osteoclasts. Osteoclasts are multinucleated cells of monocytic origin that resorb bone tissue.

Taking into account the fact that osteoclasts belong to the mononuclear phagocytic system and therefore differentiate from monocytes, it could be posited that the inhibition of bone remodeling may be related to osteoclast deficiency or dysfunction. Our results showed that the number of circulating monocytes decreased significantly in the experimental group. However, no decrease in the number of osteoclasts was observed. It is possible that the existing osteoclasts were not able to adhere properly to the bone matrix, thus inhibiting resorption and remodeling of subchondral bone, causing subchondral trabecular bone to seal to the cartilage.

Further studies should be conducted to investigate whether the observed inhibition on endochondral ossification is associated with a direct effect of As on osteoclast function or an indirect effect, which is mediated by metabolic alterations of the liver, kidneys and bone marrow.

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