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The protective effect of boric acid on aluminum-induced hepatotoxicity and genotoxicity in rats

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Abstract: The efficacy of boric acid (BA) was examined on liver marker enzymes in aluminum (Al)-treated rats. Also, a liver micronucleus assay was performed to evaluate the genotoxicity in hepatocytes. With these aims, Sprague-Dawley rats were randomly separated into 10 groups of 5 animals. Aluminum chloride (5 mg/kg body weight (b.w.) AlCl₃) and BA (3.25, 13, 36 and 58.5 mg/kg b.w.) alone were administered with injections to the experimental animals. Furthermore, the animals were also treated with Al for 4 consecutive days followed by BA exposure for 10 days. The rats were anesthetized after Al and BA injections and the levels of serum enzymes were determined. Hepatocytes were isolated for counting the number of micronucleated hepatocytes (MNHEPs). After exposure to Al, the enzymatic activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) significantly increased. Furthermore, this metal caused a significant increase in MNHEPs’ incidence. In contrast, the applications of BA doses did not cause any adverse effect on the above parameters. Moreover, pretreatments with BA significantly modulated the toxic effects of Al.

Key words: Aluminum, boric acid, liver, micronucleus assay, rat, serum enzymes

Sıçanda alüminyum ile uyarılmış hepatotoksisite ve genotoksisite üzerine birik asidin koruyucu etkisi

Özet: Bu çalışmada, alüminyum ile muamele edilmiş sıçanlarda birik asitin (BA) karaciğer markör enzimleri üzerine etkisi incelendi. Ayrıca hepatositlerde genotoksisite değerlendirmesi için karaciğer mikronukleus testi uygulandı. Bu amaçlarla, Sprague-Dawley sıçanları rastgele on gruba ayrıldı ve her bir grupta beş hayvan yeraldı. Alüminyum klorid (5 mg/kg vücut ağırlığı (v.a.) AlCl₃) ve birik asit (3,25, 13, 36 ve 58,5 mg/kg v.a.) tek başlarına injeksionlar ile deneysel hayvanlara verildi. Bunun yanı sıra, ön günün BA maruz kalmayı takiben dört gün boyunca da hayvanlar alüminyum ile muamele edildi. Alüminyum ve BA enjeksionundan sonra bayıltılmış sıçanlardan alınan kanlardak serum enzim düzeyleri tespit edildi. Mikronukleusu hepatositlerin (MNHEPs) sayısını hesaplamak için de hepatositler izole edildi. Alüminyumla maruz kalmayı takiben, alkin fosfataz (ALP), aspartat aminotransferaz (AST), alanin aminotransferaz (ALT) ve laktat dehidrojenaz (LDH) enzim aktiviteleri belirgin olarak yükseldi. Ayrıca, bu metal MNHEPs oranında belirgin bir yükselmeye neden oldu. Aksine, BA dozlarnın uygulanması yukarıdaki parametreler üzerinde herhangi bir etkisiz etkiye sebep oldu. Üstelik BA ile ön muameleler alüminyumun toksik etkilerini düşürdü.

Anahtar sözcükler: Alüminyum, birik asit, karaciğer, mikronukleus testi, sıçan, serum enzimleri
Introduction

Aluminum (Al) is the third most common element on the Earth's crust. The almost ubiquitous presence of Al has so heavily contaminated our environment that exposure is virtually inescapable (1). The extensive use of this metal (food and occupational exposure) was questioned due to its toxic effects (2). Moreover, Al toxicity occurred as a result of aluminum containing pharmaceutical products such as aluminum based phosphate binders or antacid intake (3). Al has pro-oxidative effects in vitro and in vivo (4). Thus, Al provoked cardiotoxicity, nephrotoxicity, and neurotoxicity (3,5). Furthermore, Al induced hepatic dysfunctions, DNA cross-linking in rat ascites hepatoma cells (6), MN and sister chromatid exchange (SCE) formations in human peripheral blood lymphocytes (7-10).

Boron is a trace mineral for plants, animals, and humans (11). It probably strengthens the antioxidant defense mechanism by a yet unknown mechanism (12). Research findings suggest that physiological amounts of supplemental dietary boron affect a wide range of metabolic parameters in animals (13). It is released in the form of BA that is water soluble and biologically available. BA is found in an array of consumer goods including fireproofing for fabrics and wood, insecticides, and in many cosmetics and personal care products as well (14). It is absorbed from the gastrointestinal tract into the blood stream (15) and plays an important role in improving arthritis, plasma lipid profiles, and brain function (11,16). Boronated agents with hypolipidemic, anti-inflammatory, or anticancer properties are also developed (16). Short-term mutagenicity studies clearly revealed that boron compounds are not genotoxic. BA was found negative for mutagenicity in the Escherichia coli Sd-4 and Salmonella typhimurium assays (17,18). Moreover, results in mammalian genotoxicity test systems were all negative. BA did not induce chromosome aberrations (CAs) or increase the frequency of SCEs in Chinese hamster ovary (CHO) cells (18). BA did not induce chromosomal or mitotic spindle abnormalities in bone marrow erythrocytes in the MN assay in Swiss-Webster mice (22). Besides these findings, BA did not alter the incidence of SCEs over control cells (23,24) but induced structural and total CAs (24). It was also determined that boron mines did not have genotoxic and carcinogenic effects on boron miners and did not increase chromosomal anomalies (25). Therefore, BA remains a very interesting research topic due to its equivocal and relatively unknown useful action, role in the treatment of various diseases, and interactions with other elements.

The liver is a critical organ that contains most of the accumulated metals and where toxic effects can be expected (26,27). Al accumulated higher in the liver than in the brain, muscle, heart, or lung (28). Hence, in recent years, it has become especially important to examine useful antidotes against the toxic effects of Al. The supplementation with free radical scavengers (e.g. BA) may protect animals from the harmful effect of Al. The role of BA in liver against aluminum-induced changes has not so far been studied. Usually, the extent of hepatic damage is assessed by the increased level of cytosolic enzymes such as ALT, AST, and ALP (29,30). Liver MN assays are particularly useful for the detection of the in vivo genotoxic effects of many chemicals by providing the necessary metabolic activation pathways (31). In this study, our major goal was to elucidate the in vivo potential protective role of BA against Al-induced hepatotoxicity and genotoxicity in rats. Thus, here we focused on changes in serum enzymes as a marker associated with hepatic dysfunction and alterations in MN formations in hepatocytes as genotoxic endpoint. With these aims, the present study was designed to determine levels of liver enzymes and rate of MNHEPs formations in rats after exposure to 3.25, 13, 36, and 58.5 mg/kg b.w. concentrations of BA and 5 mg/kg b.w. AlCl3.

Materials and methods

Animals

The experiment was carried out on male Sprague-Dawley rats, 8 weeks old, weighing 150-200 g. The
animals were kept on a 12-h light-dark cycle and allowed free access to food and water. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (32).

**Experimental design**

The animals were randomly divided into 10 groups: (1) controls, which were intact with no previous experimental history; (2) rats that received 5 mg/kg b.w. AlCl$_3$ (CAS No. 7784-13-6, Merck*) injection intraperitoneally (ip) for 4 days; (3, 4, 5, and 6) rats that received 3.25, 13, 36, and 58.5 mg/kg b.w. BA (CAS No. 10043-35-3, Sigma*) ip for 10 days; (7, 8, 9, and 10) rats were treated with AlCl$_3$ for 4 consecutive days followed by different doses of BA exposure for 10 days. The doses were selected according to the literature data (33-35). There were 5 rats in each group. After the following injections of all chemicals, the animals were anesthetized with ether.

**Biochemical studies**

Blood samples were collected into serum separator tubes (Microtainer; Becton Dickinson, Franklin Lakes, NJ, USA), allowed to stand (75-90 min), centrifuged (3000 rpm, 5 min), serum harvested, and stored at –20 °C. The following parameters were measured by an automated biochemical analyzer (Olympus AU 2700) with Bayer testing kits (Bioclinica): ALP, AST, ALT, and LDH.

**Liver MN assay**

The liver MN assay was done by using the method of Suzuki et al. (31). Isolated hepatocytes from rats were prepared by the collagenase perfusion technique (36). The liver was perfused through the hepatic portal vein with calcium-free Hanks balanced salt solution to remove blood for about 10 min at a flow rate of 2.5 mL/min. As soon as the liver became grayish brown in color, a second buffer solution containing collagenase (Hank's balanced salt supplemented with 4 mM calcium chloride and 0.5 mg collagenase/mL) was perfused at the same rate until the liver appeared to have broken up. Then the liver minced into 3- to 4-mm pieces with a sterile scalpel. Following mechanical dissociation, the cells were filtered through gauze and centrifuged at 1350 rpm for 5 min. The hepatocyte pellets were suspended in 10% neutral buffered formalin and stored under refrigeration. Immediately prior to evaluation, 10-20 μL of hepatocyte suspension was mixed with an equal volume of acridine orange (AO)–DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride) stain solution (AO, 0.5 mg/mL; DAPI, 10 μg/mL) for fluorescent staining. Approximately 10-20 μL of the mixture was dropped onto a glass slide and covered with a cover glass. Samples of well-isolated hepatocytes were evaluated with the aid of a fluorescence microscope counting the number of MNHEPs in 2000 hepatocytes for each animal. MNHEPs were defined as hepatocytes with round or distinct MNs that stained like the nucleus, with a diameter 1/4 or less than that of the nucleus, and confirmed by focusing up and down, taking into account hepatocyte thickness.

**Statistical analysis**

Biochemical and cytogenetic data were analyzed using one-way analysis of variance (ANOVA) and Fischer’s least significant difference (LSD) and Duncan’s tests to determine whether any treatment significantly differed from the controls or each other. On the other hand, the 4 data elements are required for the application of the Kastenbaum and Bowman method (37). These are experimental error, the maximum difference between treatments that is desired to detect, a significance level, and power. With this information, the tables provided by Bowman and Kastenbaum were used to determine the differences in the incidence of MNHEPs. Results presented as mean ±SD values and the level of 0.05 was regarded as statistically significant.

**Results and discussion**

Activity levels of serum marker enzymes were found elevated markedly in rats treated with AlCl$_3$ (Tables 1 and 2). No such changes were observed in control rat samples. As is evident from the tables, BA doses alone (3.25, 13, 36, and 58.5 mg/kg b.w.) did not change the activity levels of ALP, AST, ALT, and LDH. The pre-treatment with BA could bring a significant decrease in the activity levels of these enzymes when compared to AlCl$_3$ group. Moreover, the levels of all ALT and AST enzyme samples showed similarity with control values after treatments together with BA and AlCl$_3$. The ALP (3.25 and 13 mg/kg b.w. BA) and
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LDH (3.25 mg/kg b.w. BA) enzyme activities were normalized by low doses of BA exposure. Our results clearly revealed that BA presented useful effects on the activities of enzymes as directly related to the dose for LDH and ALP and without depending on dose for ALT and AST against AlCl₃-induced liver damages.

The conclusions of the present study reveal that BA inhibits the leakage of liver marker enzymes into circulation and, therefore, limits the damages caused by AlCl₃ toxicity. In the past, no proof was available to support these data. It is known that the swelling and necrosis of hepatocytes results in the release of liver enzymes into the circulating blood (38). This was associated with massive centrilobular necrosis, ballooning degeneration, and cellular infiltration of the liver (39). The chemicals as Al induce these findings (40,41) and overall perturbations observed in the metabolic profile of serum demonstrate the impairment in the liver metabolism (42). Rahman
et al. (43) reported that the increase in the activities of different enzymes in blood might be due to the necrosis of liver, this showing the stress condition of the treated animals. At this point, our study clearly supports that liver damage is induced by Al administration. As a matter of fact, the elevation in transaminase is encountered in conditions causing hepatocellular damage, loss of functional integrity of the cell membrane, and necrosis such as in chemically induced liver injury and elevation in enzymes (44,45). Thus, it will be relevant to briefly mention the importance of enzymic changes as manifestations of tissue toxicity. Elevations are seen in the liver and myocardial injury, and a rise in serum AST and ALT is more specific and predominant in the liver and myocardial injury, respectively. The modulations in transaminase are also influenced by the degree of hepatic decompensation of cell necrosis (46). A significant increase in ALP could occur in parenchymal liver disorders such as hepatitis and cirrhosis, and striking elevation is encountered with extrahepatic biliary tract (mechanical) obstruction or with intrahepatic (functional cholestasis) (47). Again, the LDH in serum as a biological marker for liver damage increases (48). Cell necrosis leads to a rise in the concentration of the LDH enzyme in serum and tissue. The LDH released into the medium provides an index of cell death and membrane permeability to LDH, and an increase in LDH activity in the medium occurs as a result of cell membrane disintegration and enzyme leakage (49-51). The present study also demonstrates that AlCl$_3$ significantly affects LDH level in serum. The evidence reveals that the activities of LDH are used as a marker of Al toxicity (52). Thus, it is obvious that the increase in degenerative effects of AlCl$_3$ becomes more prominent with the increase in the LDH level. Results from previous investigations indicate that BA does not affect liver weight and histology (53). Our study also reveals that hepatocytes retain their capacity to normal function after BA alone exposures. Moreover, BA against Al presents useful effects on the activities of enzymes as directly related to the dose (LDH and ALP) or without depending on dose (ALT and AST).

No single mechanism emerges to explain all the systemic effects of Al. One of the mechanisms involves free radical-induced oxidative cell injury in Al toxicity (52). As a matter of fact, interactions between oxidative stress and hepatic damage may accelerate the progression of chronic hepatodegenerative disorders, including enzymes increase induced by Al (10,42). In contrast, increasing antioxidant capacity plays an important role as a hepatoprotective (54). Therefore, there is great interest in the clinical roles of BA. It is noteworthy that even in small amounts BA helps protect nucleic acids from damage by free radicals and reactive oxygen species that can be generated during normal metabolism as well as through exposure to toxins and pollutants (23,55). However, it should be noted that boron itself is not an antioxidant, but is reported to strengthen the antioxidant defense system of the tissues (56,57). Al has high affinity for proteins, carbohydrates, and polynucleotides and may exist as a reversible macromolecular complex of cation/polyanion. If the cation/polyanion ratio exceeds a threshold value, an irreversible complex can be formed, giving rise to inhibitory effect of Al. Once in the liver, Al increases cells’ iron uptake and oxidative status (58). The liver is the chief site of iron storage, containing 98% of total iron and a large abundance of transferrin receptors (TfRs). Thus, it produces toxic effects by modifying iron homeostasis and interfering with iron regulatory proteins (59). BA does not affect the parameters of carbohydrate and protein metabolism (60). Besides, BA can protect biomembranes (56). On the other hand, Al-induced polynuclear ions can also produce membrane changes and aggregate biomolecules (61). Again, the chemicals such as Al induce the process of lipid peroxidation in the cell membrane (62), a principal cause of hepatotoxicity (63). Lipid peroxidation is linked with the excess generation of reactive oxygen species, which may be contributed to by exogenous or the endogenous sources. Boron (as borax) can help to maintain the oxidant/antioxidant balance of the liver as a result of the decreased generation of lipid peroxidation (12). Additionally, Al intake inflicts membrane associated proteins (Na-K ATPase, and PKC) (64). In contrast, it has been proposed that boron contributes to living systems by acting indirectly as a proton donor and that it exerts an influence on cell membrane structure and function (65). Although the absolute essentiality of boron for plants is well documented, studies to date have not shown it to be unequivocally essential for either animals or humans.
Table 3 shows the results of the liver MN assay in Sprague-Dawley rats. AlCl₃ induced a statistically significant increase in formations of MNHEPs although BA did not cause any alterations dependent upon the number of doses administered. Moreover, BA (3.25 and 13 mg/kg b.w.) modulated the increased MNHEPs rates by AlCl₃ (Figure 1A, B, and C).

The bone marrow and liver MN assays were recommended for evaluating systemic genetic toxicity of chemicals (66). Using the liver instead of the bone marrow is important for the following reasons: (I) many chemicals are metabolized in the liver but not in the bone marrow, (II) short-lived metabolites generated in the liver (or other tissue) may not allow efficient exposure in bone marrow, (III) bone marrow is not a target organ for some classes of carcinogens (21). Thus, in the present study, we assessed the liver MN assay. The results of in vitro studies in mammalian and bacterial systems have not revealed mutagenic effects of aluminum compounds (67,68) although some genotoxicity studies have shown significant increases in MN formations and structural chromosome aberrations in human lymphocytes by Al (7,9,69). Manna and Das (70) reported the genotoxic effects of intraperitoneally injected aluminum chloride in mice. Agency for Toxic Substances and Disease Registry (71) also suggested that further genotoxicity studies, particularly in vivo exposures of Al, would be useful for verifying the results of the Manna and Das (70)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of animals</th>
<th>MNHEP (%)/2000 HEP, mean ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>5</td>
<td>0.38 ± 0.08a</td>
</tr>
<tr>
<td>AlCl₃ (5 mg/kg b.w.)</td>
<td>5</td>
<td>0.94 ± 0.51c</td>
</tr>
<tr>
<td>BA (3.25 mg/kg b.w.)</td>
<td>5</td>
<td>0.30 ± 0.17a</td>
</tr>
<tr>
<td>BA (13 mg/kg b.w.)</td>
<td>5</td>
<td>0.35 ± 0.20a</td>
</tr>
<tr>
<td>BA (36 mg/kg b.w.)</td>
<td>5</td>
<td>0.53 ± 0.28ab</td>
</tr>
<tr>
<td>BA (58.5 mg/kg b.w.)</td>
<td>5</td>
<td>0.49 ± 0.22ab</td>
</tr>
<tr>
<td>AlCl₃ + BA (3.25 mg/kg b.w.)</td>
<td>5</td>
<td>0.43 ± 0.19a</td>
</tr>
<tr>
<td>AlCl₃ + BA (13 mg/kg b.w.)</td>
<td>5</td>
<td>0.51 ± 0.24ab</td>
</tr>
<tr>
<td>AlCl₃ + BA (36 mg/kg b.w.)</td>
<td>5</td>
<td>0.82 ± 0.37bc</td>
</tr>
<tr>
<td>AlCl₃ + BA (58.5 mg/kg b.w.)</td>
<td>5</td>
<td>1.04 ± 0.58c</td>
</tr>
</tbody>
</table>

Figure 1. Sample photomicrographs of hepatocytes treated with (A) BA (13 mg/kg b.w.), (B) AlCl₃ (5 mg/kg b.w.) and (C) AlCl₃ + BA (58.5 mg/kg b.w.). Arrows show micronuclei. The cells were stained with AO-DAPI and viewed with epiluminescent fluorescent lighting; ×1000.
study and for evaluating other potential end points of genotoxicity. At this point, the findings of our study support the limited in vivo reports. As a matter of fact, it is established that a significant increase in the rates of MNHEPs occurs after in vivo Al exposure. It is also determined that that BA exhibits antigenotoxic properties against AlCl₃. Existing data suggest that genotoxicity is not an area of concern following exposure to boron compounds in humans and animals (72). Likewise, negative results in a large number of in vitro and in vivo mutagenicity assays indicate that boron compounds, especially boric acid and borax, are not genotoxic (23,24,25,55). Furthermore, recent findings show that these compounds exhibit anti-oxidant and anti-genotoxic properties against toxic chemicals including titanium and thioacetamide (12,55). Very little is known about the biological effects of boron exposure in animal cells (73). Although the physiological function of the boron in hepatocytes is unknown, a hypothesis is that Al-induced genetic damage can be prevented by inductive effects of boron compounds on antioxidant capacity, because the toxic effects of Al appear to be mediated, at least in part, by free radicals (74). As known, genetic damage mainly develops related to oxidative stress.

It is noteworthy that the toxic effect of Al on human health cannot be ruled out either, and thus exposure to Al should be monitored and limited. Foremost, the detrimental effects of Al as a risk factor for developing tissue damages should be decreased as far as possible. In the present study, the boron-related remedy was especially important against aluminum-induced hepatic dysfunction and genetic damage. Thus, BA can be firstly proposed to prevent Al toxicity as a nutritional supplement or a functional food component.

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