*Int. J. Morphol.,*
*30(3)*:1106-1114, 2012.

# Boron as Testicular Toxicant in Mice (*Mus domesticus*)

## El Boro como Tóxico Testicular en Ratón (*Mus domesticus*)

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**BUSTOS-OBREGÓN, E. & OLIVARES, C.** Boron as testicular toxicant in mice (*Mus domesticus*). ***Int. J. Morphol., 30(3)***:1106-1114, 2012.

**SUMMARY:** It has been reported that boron causes changes in various systems, including the male reproductive system. Residents in some towns in northern Chile were consuming a few years ago in the drinking water 20 times more than the amount established as permissible limit by WHO. This study evaluates the effects in an animal model of high intake of boron on the testis. Boron was administered in the drinking water. Twenty male mice (*Mus domesticus*), sexually mature, were used, divided into 2 groups: the experimental group was given Boron at a dose of 12 mg/L, and the control group 0.6 mg/L, for 42 days. Sections of testis were obtained for: HE staining (Morphometry and Histopathology), Immunohistochemistry (Cox-2), Mallory and Picrosirious stain (evaluation of tunica albuginea). The results indicate that ingestion of a dose of 12 mg Boron/L produces vacuolization, tubular epithelial desquamation and tamponade. Morphometry revealed decreased tubular diameter and epithelial height and lumen diameter and increased interstitial area in the exposed group. Immunodetection of COX-2 was positive in high percentage of tubules in the intoxicated group. The tunica albuginea was thinner, with decreased percentage of type I collagen fibers and an increase in the percentage of type III collagen fibers in animals exposed to boron in contrast to the control group. Exposure to critical levels of boron produces severe histopathological changes in the testis, altering morphometric parameters and causing overexpression of Cox-2. Finally, evaluation of collagen fibers suggests that Boron produced a degradation of the collagen of the tunica albuginea, causing a decrease in the thickness of it and altering the percentage ratio collagen I/collagen III, a process called collagenolysis.

**KEY WORDS: Boron; Mouse; Testicular structure; Collagen I/III; Infertility.**

## INTRODUCTION

In recent years there has been an increased concern about the harmful effects that many chemicals have on male reproduction, so that chemical contamination and testicular function have been studied. These toxic substances can act as testicular toxicants, usually associated with social habits or living conditions. This is why there is concern regarding potential adverse effects of various environmental pollutants, due to observations of impaired development and reproduction in wildlife populations and in humans exposed to chemicals.

An example of this was experienced some years ago in northern Chile, where the population was consuming high levels of boron in drinking water (Vitali, 2008).

**Boron.** Boron is a chemical element and a trace element, which is present in the environment in solid or liquid form, such as borates, boric acid, boric oxide and salts (Crespo, 2001). Trace elements are those which, although present in very small amounts in body tissues, are essential nutrients, and perform certain functions necessary to sustain life (Alarcon-Corredon, 2009).

Boron is also employed in the manufacture of glass and cosmetics (Martin, 2004). In Chile boron resources are mainly located in the north of the country, specifically in evaporitic basins known as salt flats (Chong *et al*., 2000).

Boron enters the environment mainly through the weathering of rocks, volatilization of boric acid found in seawater, and by volcanic activity. Furthermore, to a much lesser extent, boron is released into the environment through anthropogenic activities such as agriculture, waste management, sewage and fossil fuel use (Butterwick *et al*., 1989). Recent studies show that boron accumulates in plants, insects and fish, but nevertheless does not biomagnify in

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aquatic food chains (Saiki *et al*., 1993). Boric acid has been used in medicine as an antiseptic eye; borated components have been used to extend and preserve food palatability. Recently, these uses have been discontinued in some countries due to the recognition of the toxic effects of borated agents (Conor, 2004).

Studies indicate that ingestion of boron in humans is 0.2 to 0.6 mg/day in water, and 1.2 mg in food, where the highest concentration of boron in drinking water agreed by the World Health Organization is 0.6 to 1 mg/L. Maximal boron ingest per day is 1.5 to 2.0 mg, amounts over these limits are considered critical or toxic levels that can damage health. Amounts of boron in the water for human use in the city of Arica in recent years have ranged from 2.0 mg / L in the north to 12.0 mg/l in the southern sector of the city (Espinoza-Navarro *et al.*, 2007)

The overall clinical effects of administration of a single high oral dose (250-350 mg/kg live weight) of boric acid (LD50, approximately 400-700 mg boron/kg of body weight in rats and mice), include depression, ataxia, occasional convulsions, hypothermia, and red-purple skin and mucous membranes (Pfeiffer *et al.*, 1945; Weir & Fisher, 1972).

Inorganic borates and boric acid are absorbed from the gastrointestinal tract, mucous membranes and damaged skin. This diffuses rapidly into tissues and concentrates in bone, teeth and nails. The boric acid is not metabolized, and is eliminated unchanged in urine (Martin). Chronic exposure to the element has been shown by studies in laboratory animals that significantly affect reproduction (Creasy & Foster, 2010).

It has been demonstrated by exposure to chronic and subchronic oral dose of boric acid or borax in laboratory animals, that male reproductive tract is a constant target of the effects of toxicity. Basically, testicular lesions were observed in rats, mice and dogs under the administration of boric acid or borax in food or drinking water (Dieter, 1994; Ku *et al*., 1993; National Toxicology Program, 1987; Weir & Fisher).

The first clinical signs of toxicity in dogs is a decrease in the size of the scrotum, as well as decreased absolute and relative testicular weight. After subchronic exposure, the histopathological effects range from inhibition of spermiation (Linder *et al*., 1990; Treinen & Chapin, 1991), to the degeneration of the seminiferous tubules, with a variable loss of germ cells, even reaching the complete absence of them. Atrophy and reversible or irreversible loss of fertility may result. Despite the loss of fertility, libido or sexual behavior of the animal are not affected (Linder *et al*.).

Studies in rats show that exposure to boron produces histopathological changes in the tubular tissue, showing tubular atrophy, tubular epithelial vacuolization and tamponade (Bustos-Obregón *et al*., 2007).

The testicle is surrounded by a thick connective tissue capsule, the tunica albuginea (Geneser, 2002). The connective tissue of the tunica albuginea, as in many organs and tissues, contains in its structure collagen fibers synthesized by fibroblasts, responsible for the architecture of the testicular parenchyma.

The connective tissue of the tunica albuginea, is described as a compact fibrous tissue with intersecting septa (connective tissue modeling), with a predominance of fibrillar collagen fraction, which meets primarily a mechanical function, designed to withstand stresses in two prevalent directions in the parenchyma or wrap it with protective purposes. It presents very thick collagen bundles arranged tightly. The cells are mainly represented by fibroblasts, which are situated between the different fibrillar bundles (Mery, 1979).

Collagen fibers are the most characteristic structures and abundant connective tissue prototype. Formations are of indeterminate length, usually cylindrical but sometimes are ribbonlike, with no tendency to form networks. Their diameter ranges from 1 to 12 microns (μm) in loose connective tissues, but can reach more than 100 μm in tighter tissues. These fibers have longitudinal striations because they are constituted by thin collagen fibrils, which have a diameter of 0.2 to 0.5 microns. These fibers are held together by an amorphous substance, a glycoprotein. Electron microscopy shows that the fibrils are composed of thinner formations; the microfibril, from 200 to 2000 Angstroms (Å) in diameter, which are responsible for the collagen fiber birefringence when examined in the polarizing microscope. From a functional standpoint, the tunica albuginea plays an important role in maintaining anatomical testicular parenchyma, where spermatogenesis takes place.

**Oxidative Stress**. These mechanisms can be caused by various stimuli such as chemicals, oxygen deprivation, infectious agents, among others, which can generate the same or similar substances to those generated in inflammatory processes, as is the isoform COX-2. It is primarily responsible for the production of prostaglandins produced during inflammation. Previous studies have indicated that COX-2 is involved in the reduction of steroid hormones (Zirkin, 2005). It has also been suggested that cyclooxygenase-2 may play an important role in the testis when spermatogenesis is impaired (Frungieri *et al.*, 2002; Wang *et al.*, 2003).

Ecotoxicology studies show that different elements found naturally in our environment are toxic to our body, especially to the reproductive system. In the case of organophosphorus insecticides and some heavy metals like lead, which causes decreased sperm counts and disequilibrium between pro-and antioxidant factors, eliciting oxidative damage to cells (Bustos-Obregon & Hartley, 2008).

The effect of trace elements in the environment is an important public health problem. There is considerable literature concerning the general toxicology of these elements. However little attention has been given to the adverse effect of these elements in male reproduction, although the information exists, stressing the continued decline in semen quality worldwide (Dindyal, 2004). This may be due to urbanization and industrialization. In the male infertility clinic, growing evidence suggests that some environmental trace elements may be associated with a subtle and chronic exposure to environmental chemicals in the air, water and food.

The purpose of this study is to evaluate the testis following administration of high doses of boron, in an animal model similar to humans inhabiting the north of Chile, that for many years were affected by a high intake of boron in drinking water.

## MATERIAL AND METHOD

**Experimental design**. Twenty sexually mature male CF-1 mice *Mus domesticus* (30-40 g) were used. One group (ten mice) drank Santiago's tap water (0.6 mg Boron/L), and the other ten mice were exposed to drinking water containing 12 mg Boron/L for 42 days (one complete spermatogenic cycle of 33.2 days, plus one cycle of 8.5 days), from 85 days of age until 127 days old, simulating Arica's tap water human intake. Animals were kept under standard animal room conditions, and sacrifice was done according to the bioethical norms of the Medical School.

**Histological Technique**. The testes were removed and fixed in alcoholic Bouin's fluid, and then processed for paraffine embedding and staining with HE, Mallory, immunohistochemistry for COX-2, or Picrosirious Red (polarized light).

**Histopathology.** Testicular sections HE stained were examined at 1000x, recording plugging tubular epithelial vacuolization, epithelial desquamation and percentage ratio of interstitium/tubules.

**Morphometry**. Digitalized testicular sections, photographed at 400x, were used to measure epithelial height (μm) tubular diameter (μm), tubular lumen diameter (mm) and interstitial area (%), counting 100 tubules per mouse. Images were processed for analysis using Image Tool 3.0.

**Immunohistochemistry**. Testicular sections were processed according to Corominas (1997), and the technical recommendations of the primary antibody anti-COX-2 manufacturer (Santa Cruz Biotechnology), contrasted with light hematoxylin.

**Evaluation of Tunica Albuginea**. In Mallory stained testicular sections, thickness of the tunica albuginea was measured (using Image Tool 3.0). Testicular sections stained with Picrosirius Red were examined by polarized light microscopy (Zeiss Axiostar Plus Mod), according to Junqueira *et al.* (1979).

Collagen type I, form thick and tight fibers, with an intense yellow to red birefringence. Type III collagen fibers form thin fibers, composed of thin fibrils, freely arranged, with a weak birefringence of a greenish color (Junqueira *et al.*).

To quantify this different birefringence of collagen fibers, Image Pro 6.0 Software was used, obtaining a percentage of the different colors on a photograph captured in the polarized light microscope. A similar configuration of the software to Meruane *et al.* (2011) was used. It quantifies the yellow and red as collagen I and green as collagen III, to establish the percentage among them.

**Statistic**. The results were expressed in % or microns (μm) ± SD, using the t-Student statistical test to find significance ($p < 0.05$) in the results.

## RESULTS

**Effect of Boron treatment on testicular histopathology**. Histopathological analysis revealed a significant increase in abnormalities of the seminiferous tubules in the Boron group compared to the control group (%± SD, $p<0.05$) (Table I).

Table I. Effect of Boron treatment on testicular histopathology.

| Abnormality (%Tubules) | Control | Boron treated mice |
|---|---|---|
| Vacuolization | 1.8 ± 0.78 | 11.7 ± 4.60 * |
| Tubular cell desquamation | 2.8 ± 1.03 | 12.2 ± 4.40 * |
| Tamponade | 3.5 ± 0.84 | 23.7 ± 7.67 * |

(*: $p < 0.05$).

**Effect of Boron tratment on morphometric parameters**. Morphometric evaluation of tubular diameter, luminal diameter, epithelial height and interstitial area, reveal significant differences ($p<0.05$) between the control and Boron group (Table II).

**Inmunohistochemical COX-2 Expression.** Showed a significantly greater percentage of immunoreactive tubules ($p <0.05$) in the Boron exposed group compared to control group (Table III).

**Evaluation of the thickness of the tunica albuginea (Mallory staining)**. Tunica albuginea thickness showed a significant decrease ($p <0.05$) in Boron treated mice (Table IV; Fig. 1).

**Evaluation of the collagen fibers of the tunica albiginea by pricrosirius red staining.** The evaluation of the collagen fibers of the connective tissue of the tunica albuginea, by Picrosirius staining, observed in polarized light microscopy, revealed a significant decrease of collagen type I fibers ($p<0.05$), and a significant increase in collagen type III fibers ($p <0.05$), in the group exposed to boron (Table V; Fig. 2).

Color sample analysis, using Image Pro 6.0, of the Picrosirius stained sections, examined by polarized light microscopy, permitting collagen area measurement. It was found that Control animal had 13.35% of image area for collagen I, and 0.019% for collagen III, while Boron exposed animal had 11.57% of image area for collagen I, and 2.10% for collagen III.

Table II. Effect of Boron treatment on morphometric parameters.

| Morphometric parameter | Control | Boron treated mice |
|---|---|---|
| Tubular diameter (µm) | 189 ± 2.25 | 155 ± 3.47 * |
| Luminal diameter (µm) | 60.2 ± 1.16 | 78.1 ± 1.24 * |
| Epithelial height (µm) | 61.0 ± 1.12 | 50.7 ± 0.89 * |
| Interstitial area (% area) | 14.4 ± 1.00 | 29.2 ± 6.57* |

(*: $p < 0.05$)

Table III. Immunohistochemical COX-2 Expression.

| Immunohistochemical expression | Control | Boron treated mice |
|---|---|---|
| IHQ COX-2 (% tubules) | 1.3 ± 0.5 | 11.3 ± 3.2* |

(*: $p < 0.05$)

Table IV. Evaluation of the thickness of the tunica albuginea (Mallory staining).

| Morphometric parameter | Control | Boron treated mice |
|---|---|---|
| Tunica albuginea thickness (µm) | 107.6 ± 28.4 | 29.70 ± 9.1* |

(*= $p < 0.05$)

Table V. Evaluation of the collagen fibers of the tunica albuginea by picrosirius red staining.

| Collagen Type (% fibers) | Control | Boron treated mice |
|---|---|---|
| Collagen I | 11.25 ± 3.54 | 7.82 ± 3.02* |
| Collagen III | 0.23 ± 0.17 | 2.27 ± 0.85 * |

(*= $p < 0.05$).

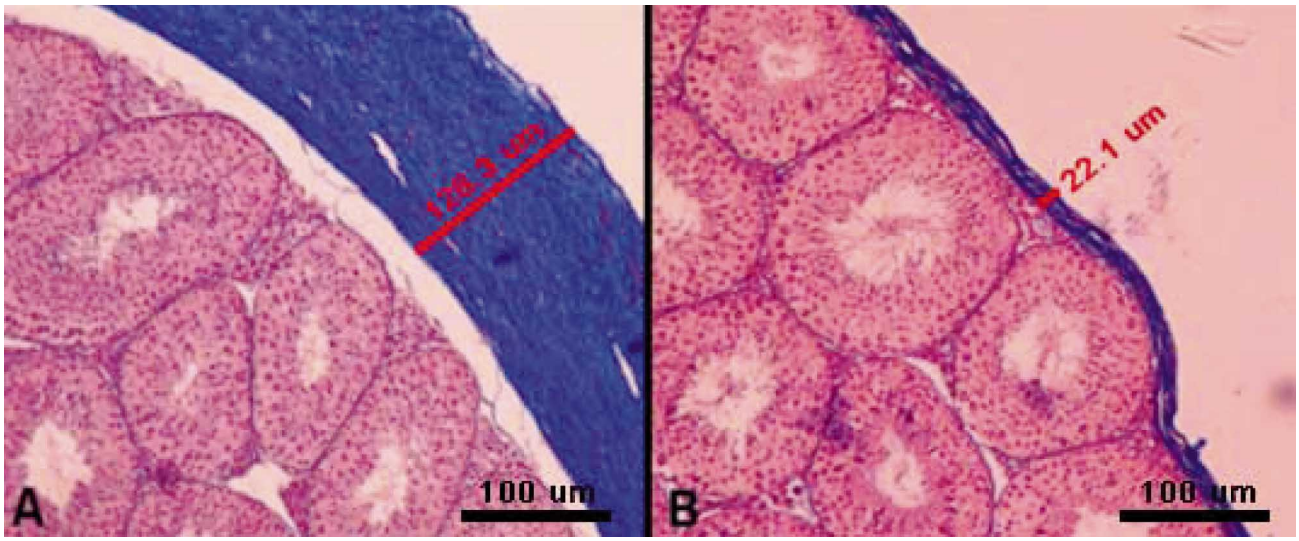


Fig. 1. Mallory stained micrographs, showing the different thickness of the tunica albuginea in the control group (A) compared to the Boron treated group (B). Bar = 100 µm.

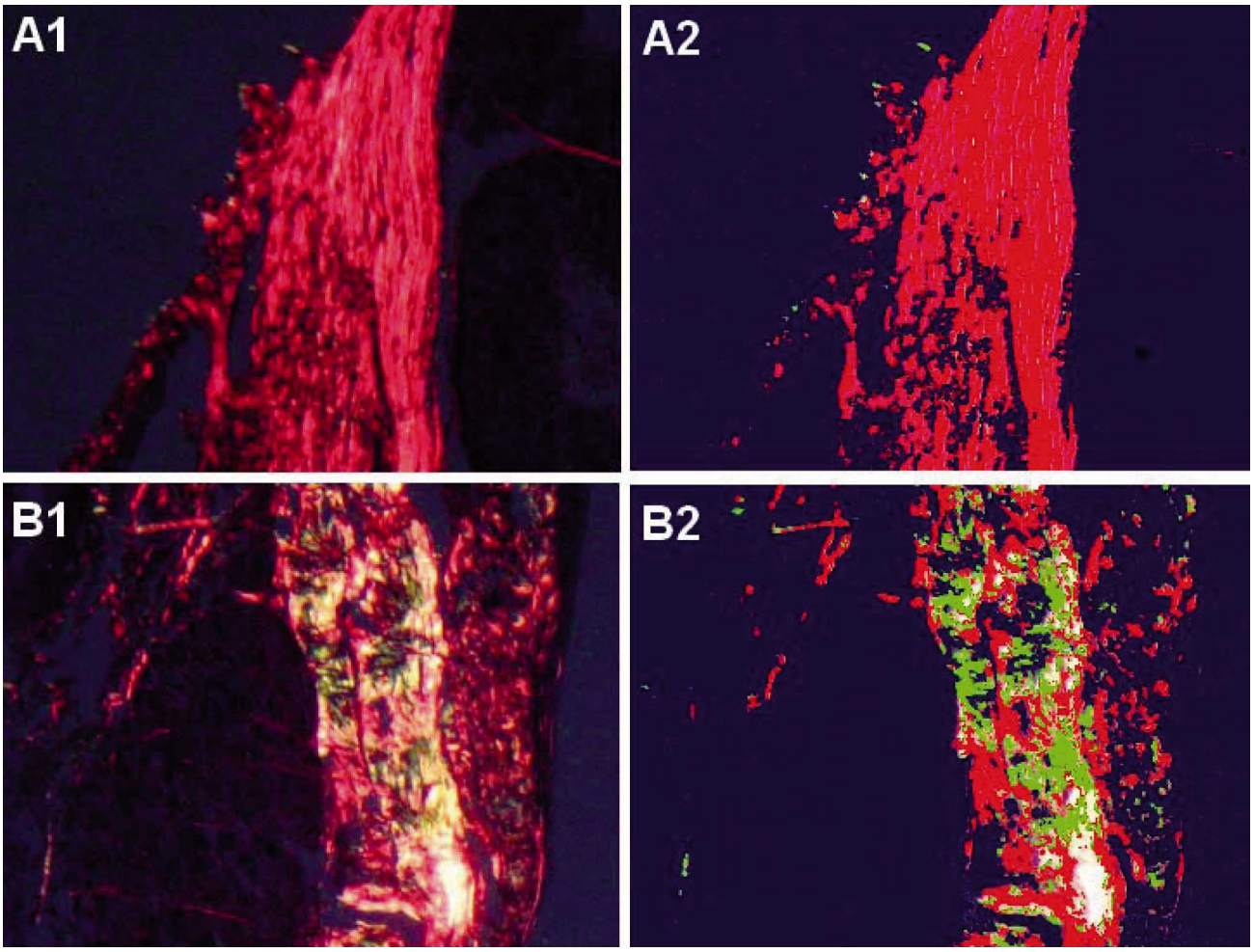


Fig. 2. Micrograph showing the birefringence of the collagen fibers of the tunica albuginea of control group in the polarized light microscope (A1), and then analyzed by Image Pro 6.0 (A2). Birefringence in polarized light microscopy of the collagen fibers of the tunica albuginea of the boron treated group (B1), and then processed by Image Pro 6.0 (B2).

## DISCUSSION

Analyzing the situation of people in northern Chile some years ago, morphometric histological findings were consistent with previous studies in rats by Dixon *et al*. (1976). In addition, due to a previous study showing histopathological effects of boron on mouse liver, at a dose of 12 mg/L in drinking water affects liver connective tissue, and the relationship collagen I/III fibers, Mallory and Picrosirius Red staining was used to evaluate tunica albuginea damage.

**Morphometric and Histopathological analysis**. A significant damage to the testes of animals exposed to 12 mg of boron/L, is evidenced by a decrease in epithelial height of the tubules. These changes can be explained by reduced proliferation of spermatogenic cells. In turn, it depends on Interstitial cells paracrine function through the action of testosterone on sustentacular cells and germ cells. All this alterations were seen as a disruption of androgen status (decrease in circulating testosterone levels), associated with a lower epithelial height, disorganization of the epithelium and germ cell detachment in rats exposed to boric acid (Chapin & Ku, 1994; Naghii *et al*., 2011). The decrease in epithelial height in the group exposed to boron may also be due to increased apoptotic index (Blanco-Rodriguez & Martinez-García, 1998), which can be related to the presence of vacuolization found, suggesting loss of specific junctions between sustentaculars cells and germ cells development. This cell loss may be directly related to the number of tubules showing desquamation and subsequent tamponade.

Atrophy was observed in more than half of the mice intoxicated with boric acid, thus explaining the relative increase of interstitial area in boron treated animals, which may be due to the activity of prostaglandins in a possible inflammatory process characterized by edema, which is consistent with studies done in rats exposed to Boron, and other metalloids such as arsenic.

Spermatogenesis in mammals requires the action of a number of peptides, steroids and hormones. These hormonal messengers are critical not only for the regulation of germ cell development but also for the proliferation and somatic cell function and thus for the proper development of the testis. Therefore, studies are needed to assess the androgenic status in animals treated with boron, to understand better the disruption of the blood-testis barrier, and its androgen dependency (Meng *et al.*, 2005). Since the testes is the nest for spermatogonial development, cells that maintain the epithelium in physiological condition (sustentacular cells and interstitial cells), when affected directly or indirectly, cause alteration in the spermatogenic process, with a decrease in quality of the ejaculate.

Further studies are needed to assess whether histopathological alterations and changes in morphometric parameters observed by chronic exposure to critical levels of boron, affect long-term fertility.

**Immunohistochemistry**. Boron elicited overexpression of COX-2, which is found in epithelial cells of the distal part of cauda epididymis and vas deferens. COX-2 is the isoform that is expressed in some tissues, only in pathological processes, such as inflammation and cellular stress, which can lead to cell death (Mandelker, 2011). Inflammation causes low sperm quality and could even lead to infertility in animals (Kanokwan, 2011). Overexpression of COX-2, together with increase of the intersticial area, could be taken as an inflammatory process, although in tissue sections of animals exposed to boron no inflammatory leukocyte infiltration was found.

May be chemical damage due to boron, through free radical production, as in the case of other metalloids such as arsenic, exerts oxidative stress in cells causing DNA damage through the production of superoxide and hydrogen peroxide (Patrick, 2003), and producing an overexpression of COX-2, which may be due to decreased intracellular levels of arachidonic acid caused by its destruction by the action of free radicals, and increases the production of PGs. In turn, it has been shown that testosterone synthesis is profoundly affected by the production of prostaglandin F2a (PGF2) whose synthesis is catalyzed by the enzyme cyclooxygenase-2 from arachidonic acid (Wang *et al*.), being both the enzyme cyclooxygenase-2 and PGF 2a-classified as inhibitors of steroidogenesis (Gunnarsson *et al.*, 2004; Frungieri *et al.*, 2006).

The COX-2 is expressed in the epithelium of the vas deferens and inhibits smooth muscle contraction (Ruan *et al*., 2008). Considering this, the overexpression of COX-2 could eventually generate a blockage of fluid through the vas deferens by inhibiting the contraction of its wall, affecting the seminiferous tubules, which may increase in diameter due to increased retrograde pressure. This may also be related to the presence of tubular blockage, since this could generate an accumulation of epithelial desquamation in the lumen.

It has been demonstrated that inhibitors of COX-2 have a positive effect on sperm quality, associated with an increase in the volume of the ejaculate in humans (Gambera *et al.*, 2007). This would be useful in further studies to evaluate the security of non-steroidal anti-inflammatory drugs in the testes of animals exposed to boron.

Critical levels of exposure to Boron for a long period of time could produce carcinogenesis, following the processes of cell damage by free radicals generated by cell death, due to membrane damage with subsequent overexpression of COX-2, and prostaglandins produced that could increase the number of abnormal cells, which normally undergo programmed cell death (apoptosis).

**Evaluation of tunica albuginea by Mallory and Picrosirius red stain**. It shows a decrease in its thickness in boron treated animals, denoting connective tissue degradation by boron. There is an increased amount of type III collagen fibers and a decreased amount of type I collagen fibers, compared to the control group. This indicates that Boron produces an alteration in the ratio of collagen fibers I/III (collagenolysis). Studies in mice indicate that this tissue damage is followed by fibrosis (Shahzeidi *et al*., 1993; Ala-Kokko *et al*., 1987).

Collagen plays a vital role in maintaining structural integrity and tissue function. Picrosirius Red staining can be of value for studying collagen degradation. This is consistent both with a study in mice, in that the evaluation of the effects of boron at a dose of 12 mg/L had an impact on the consistency of liver connective tissue, where deposition of collagen decreased after exposure to Boron, and also produces a change in collagen type I to collagen type III. Changes in other tissues have been reported which involves collagenolysis, with a consequent weakening of tissue architecture because of the role of collagen as support (Bustos-Obregon *et al.*, 2008).

It has been established that collagen microfibrils are composed of macromolecules of different thickness, called tropocollagen. Chemically the tropocollagen is composed of 30% glycine, 25% by proline or hydroxyproline and the rest by other aminoacids. The latter is unique to collagen (Müller-Esterl, 2008), and because the pathological collagen degradation in the body leads to an elimination of a large amount of hydroxyproline in the urine in some diseases (Hernandez & Morales, 1985), it would be important to have further studies to supplement the data provided by this study.

Any damage in testicular collagen network architecture may compromise testicular function and, therefore, normalcy of spermatogenesis.

**CONCLUSIONS**. This study demonstrates that chronic exposure to 12 mg boron/L in adult mice induces histological changes such as vacuolization, epithelial desquamation, tubular tamponade, and morphometric changes such as decreased tubular diameter, epithelial height and increased luminal diameter and interstitial area.

Chronic exposure to critical levels of boron in adult mice induces the expression of COX-2.

Boron has an impact on the consistency of the connective tissue of the tunica albuginea (collagenolysis), probably resulting in testicular architectural damage and disfunction.

**BUSTOS-OBREGÓN, E. & OLIVARES, C.** El boro como tóxico testicular en ratón (*Mus domesticus*). ***Int. J. Morphol., 30(3)***:1106-1114, 2012.

**RESUMEN:** Se ha reportado que el Boro provoca alteraciones en diversos sistemas, incluido el sistema reproductor masculino. Los habitantes de algunas localidades del norte de Chile estuvieron hasta hace algunos años consumiendo en el agua de bebida 20 veces más que la cantidad establecida como límite permisible por la OMS. El presente estudio evalúa en modelo animal los efectos del alto consumo de Boro sobre el testículo, administrado en el agua de bebida. Se utilizaron veinte ratones (*Mus musculus*) machos, sexualmente maduros, divididos en 2 grupos; al grupo experimental se administró Boro a una dosis de 12 mg/L, y al grupo control 0.6 mg/L, durante 42 días. Posteriormente se obtuvieron secciones de testículo para: Tinción H-E (Morfometría e Histopatología); Inmunohistoquímica (Cox-2); Tinción de Mallory y Picrosirious Red (evaluación de túnica albugínea). Los resultados indican que la ingestión de Boro a dosis de 12 mg/L produce vacuolización, descamación epitelial y taponamiento tubular. La morfometría revela disminución en el diámetro tubular y altura epitelial, así como aumento del diámetro luminal y del área intersticial en el grupo expuesto. La inmunodetección de COX-2 resultó positiva en gran porcentaje de túbulos en el grupo intoxicado. La túnica albugínea, demostró menor grosor, así como una disminución en el porcentaje de fibras colágenas tipo I y un aumento en el porcentaje de fibras colágenas tipo III en los animales expuestos a Boro en contraste con el grupo control. La exposición a niveles críticos de Boro genera alteraciones histopatológicas severas en el testículo, alterando parámetros morfométricos y provocando la sobreexpresión de COX-2. Finalmente, la evaluación de fibras colágenas sugiere que el Boro produjo una degradación del colágeno de la túnica albugínea, provocando una disminución en el espesor de ésta y alterando la relación porcentual Colágeno I/Colágeno III, proceso llamado colagenólisis.

**PALABRAS CLAVE: Boro; Ratón; Estructura testicular; Colágeno I/III; Infertilidad.**

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Received: 22-05-2012
Accepted: 06-07-2012