Mechanism of the Testicular Toxicity of Boric Acid in Rats: In Vivo and In Vitro Studies

Warren W. Ku and Robert E. Chapin

Developmental and Reproductive Toxicology Group, National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

High-dose boric acid (BA) exposure produces testicular lesions in adult rats characterized by inhibited spermatogenesis (IS) that may progress to atrophy. In vivo and in vitro studies addressed possible mechanisms. In vivo, boron tissue disposition was examined, since no detailed data existed, and relevant boron concentrations for in vitro studies needed to be set. Since BA induces riboflavinuria and also affects calcium/phosphorus homeostasis, and testis zinc appears essential for normal testis function, we examined BA effects on flavin status and testis levels of phosphorus (P), calcium (Ca) and zinc (Zn). Data showed that the testicular toxicity and central nervous system (CNS) hormonal effect were not due to selective boron accumulation in testis or brain/hypothalamus, with testis boron concentrations at approximately 1 to 2 mM; that riboflavin deficiency is not involved, due to both the absence of overt signs of deficiency and effects on tissue flavin content during BA exposure; and that changes in testis P, Ca and Zn levels did not precede atrophy, and are therefore unlikely to be mechanistically relevant. In vitro studies addressed the hallmarks of the BA testicular toxicity: the mild hormone effect, the initial IS, and atrophy. No effect of BA on the steroidogenic function of isolated Leydig cells was observed, supporting the contention of a CNS-mediated rather than a direct hormone effect. Since increased testicular cyclic adenosine monophosphate (cAMP) produces IS, and a role for the serine proteases plasminogen activators (PAs) in spermatogenesis has been proposed, we examined in vitro BA effects on both Sertoli cell cAMP accumulation and PA activity, respectively. Results showed that the IS is not due to BA effects on either process. To address the atrophy, we evaluated BA effects in Sertoli-germ cell cocultures on: Sertoli cell energy metabolism, since lactate, secreted by Sertoli cells, is a preferred energy source for germ cells; and also on DNA/RNA synthesis, since germ cells synthesize DNA/RNA and BA impairs nucleic acid synthesis in liver, and may do so in testis. The most sensitive in vitro endpoint was DNA synthesis of mitotic/meiotic germ cells, with energy metabolism in Sertoli or germ cells affected to a lesser extent. The in vitro effect on DNA synthesis was manifest in vivo as a decrease in the early germ cell/Sertoli cell ratio prior to atrophy in testes from BA-exposed rats. Overall, these combined studies revealed some changes offering a plausible explanation for the atrophy aspect of the BA testicular lesion. However, the mechanism for the IS is still undefined, and should be the subject of future work.

— Environ Health Perspect 102(Suppl 7):99–105 (1994)

Key words: boric acid, toxicity, rats, testis, cultured cells

Introduction

High-dose boric acid exposure produces testicular lesions in adult rats characterized by inhibited spermatogenesis (IS) that may progress to nonrecoverable atrophy, depending on the dose (1–4). The mechanism for the apparent selective testicular toxicity of boric acid remains obscure, and has not been thoroughly studied. One mechanism could be decreased testosterone. Rats fed boric acid exhibited slightly reduced basal serum testosterone levels, and secretagogue challenge studies suggested that the decrease was CNS-mediated (1,5). These data are relevant because IS is thought to reflect a change in hormone status (6), although other processes likely are involved. However, a relationship between the mild boric acid-mediated decreases in testosterone and the progression to testicular atrophy is unlikely, since spermatogenesis can be maintained in the presence of significantly reduced intratesticular testosterone (7). Altogether, the inconsistencies for a hormonal mechanism imply that boric acid may exert some direct effects on the testis.

In an effort to address possible mechanisms, studies were carried out both in vivo and in vitro. The selection of the evaluation parameters was based on either the known effects of boric acid in other biological systems or processes suggested to be involved in normal spermatogenesis (to address boric acid-induced atrophy) and spermatogenesis that might be affected by boric acid.

In vivo studies, all in Fischer (F344) rats, focused on boron tissue disposition and the evaluation of some selected parameters in rats fed boric acid for various time periods. Boron tissue disposition would identify sites of boron accumulation as well as set relevant boron concentrations to be used in in vitro studies. In evaluating in vivo parameters, the objective was to identify events which occurred prior to the onset of testis lesion expression, and thus were of possible mechanistic relevance. The effects of in vitro boric acid exposure on some selected properties of various testicular cell culture systems addressed the hallmarks of the boric acid testicular toxicity: the mild hormone effect, the initial inhibited spermatogenesis (IS), and atrophy. In vitro studies allowed us to evaluate and identify potential target cells and/or target processes in the absence of complicating factors encountered in vivo. The objective of this approach was to identify effects in vitro which occur at the highest boric acid concentrations in in vivo studies.

For each parameter, the appropriate background and rationale are given, followed by the methods, results, and brief discussion. Finally, we provide a summary of the salient findings of the current
Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

In Vivo Studies

Boron Tissue Disposition

Detailed data on the tissue disposition of boron in the rat, including accessory sex organs and the brain, were lacking. Initial studies examined boron tissue disposition in reproductive, accessory sex organs, and other selected tissues in rats fed boron acid (8). The objective was to determine if selective accumulation of boron in reproductive tissues, accessory sex organs and/or the brain might correlate with the apparent selective testicular toxicity. Also, knowledge of testis boron concentrations would be necessary in setting relevant concentrations to use in in vitro studies. Rats were fed 9000 ppm boron acid (1575 ppm boron, ~68 mg boron/kg body weight/day) for up to 7 days. Tissues were excised at various times, and boron analyzed by inductively coupled argon plasma (ICAP; 9) emission spectrometry. Tissues appeared to reach steady-state boron levels by 3 to 4 days. The data also showed that neither the testicular toxicity nor the slight CNS hormonal effect associated with boron acid exposure can be explained on the basis of selective accumulation of boron in the testis or brain/hypothalamus: the tissue/plasma ratios in these tissues were approximately one (Figure 1). The range of testis boron concentrations was between approximately 1 and 2 mM.

Tissue Flavins

boric acid exposure induces riboflavinuria in rats (10), and thus could potentially result in riboflavin deficiency. Riboflavin (vitamin B2) is the precursor to flavin adenine dinucleotide (FAD) and mononucleotide (FMN), both essential coenzyme derivatives. To investigate whether riboflavin deficiency was associated with chronic boric acid exposure, the flavin content in testis, liver, and brain were evaluated during testis lesion expression. Rats were fed 9000 ppm boric acid for up to 28 days. The FAD/FMN content in liver, brain, and testes were determined fluorometrically at various times (11). No significant and consistent changes with time in testis flavin content were observed prior to and during testis lesion expression (Table 1). Furthermore, no changes in flavin content in liver or brain were observed (unpublished, data not shown). Testicular pathology has been reported only under conditions of severe riboflavin deficiency and consequent with severe body weight loss and significant systemic changes (12). These changes were not observed in boric acid-treated rats. The absence of both overt systemic changes and effects on tissue flavin levels suggest that riboflavin deficiency is not a significant etiologic factor in the boric acid testicular lesion.

Serum Clinical Chemistries and Testis Phosphorus, Calcium, and Zinc

There are indications that boron affected calcium and phosphorus homeostasis both in human (13) and in animal toxicity studies (14,15). In addition, zinc (Zn) is suggested to be an essential metal in sexual maturation and normal testis function (16), with depressions in testis Zn associated with chemically induced testicular toxicity for other toxicants (14,15). Selected serum clinical chemistries, including Ca and inorganic phosphorus, and testis levels of P, Ca, and Zn were measured during boric acid exposure (2). Rats were fed various boric acid concentrations (3000–9000 ppm) for up to 9 weeks. At weekly intervals, rats were bled, then euthanized. Serum clinical chemistries were performed, and testis phosphorus, Ca, and Zn content analyzed by ICAP. No consistent changes with dose and time were observed for serum sodium, potassium, calcium, or glucose, and there were only variable and minor increases in serum chloride (<5%) (data not shown). However, slight but significant decreases in serum inorganic phosphorus (7–14%) and creatinine (8–28%) were observed that worsened with dose and time (data not shown). The decreases in serum inorganic phosphorus were consistent with previous observations of decreased plasma Pi and increased urinary inorganic phosphorus excretion during boric acid exposure in dogs and cattle (15,19). The significance of the slight decreases in serum creatinine during exposure is unknown. There was no gross or histological evidence of kidney damage as a result of boric acid exposure. Although serum inorganic phosphorus was decreased, testis levels of phosphorus, as well as Ca and Zn, showed no significant and consistent changes with dose and time that preceded testis lesion development (data not shown). Changes in the levels of these elements were only associated with the loss of

Table 1. Testis flavin content during boric acid exposure.

| Day | Treatment group | Testis lesion | FAD (μg/g ± SE) | FMN (μg/g ± SE) |
|-----|----------------|--------------|----------------|----------------|
| 2   | Control        | –            | 1.9 ± 0.02     | 0.8 ± 0.02     |
| 4   | 9000 ppm       | –            | 1.9 ± 0.02     | 0.8 ± 0.02     |
| 7   | Control        | –            | 1.9 ± 0.03     | 0.6 ± 0.02     |
| 10  | 9000 ppm       | IS a         | 1.8 ± 0.10     | 0.5 ± 0.02     |
| 28  | Control        | –            | 1.9 ± 0.05     | 0.7 ± 0.02     |
| 28  | 9000 ppm       | IS           | 1.8 ± 0.03     | 0.4 ± 0.01 c   |

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

Mechanistic studies and postulate the direction future studies might take.
post-spermatogonial germ cells during atrophy and are therefore unlikely to be mechanistically important.

**In Vitro Studies**

The effects of *in vitro* boric acid exposure on some selected properties of various testicular cell culture systems (20) were evaluated to address the hallmarks of the boric acid testicular toxicity: the mild hormone effect, the initial IS, and atrophy. To address the hormone changes, boric acid effects on the steroidogenic capacity of purified adult Leydig cells were evaluated. Since intratesticular (i.t.) administration of dibutyryl cyclic AMP (dbcAMP) has been shown to produce IS (21), the effects of boric acid on Sertoli cell cAMP levels were also evaluated. In addition, boric acid effects on the stage-specific secretion of plasminogen activator (PA) activity—a serine protease suggested to be involved in the spermatogenesis process (19)—were evaluated in cultured seminiferous tubules. To address the atrophy, we evaluated boric acid effects on morphology/germ cell attachment, energy metabolism, and DNA/RNA synthesis in primary co-cultures of Sertoli and germ cells from 28- to 30-day-old rats (23). In all studies, culture systems were exposed to boric acid at 0.1 to 10 mM, which is one to two orders of magnitude above and below the concentrations found *in vivo*, for various times up to 72 hr.

**Leydig Cell Testosterone Production**

Rats fed boric acid exhibit slightly reduced basal serum testosterone levels (1), suggesting a mild hormone effect. Thus, we evaluated the direct effects of *in vitro* boric acid exposure on basal and human chorionic gonadotropin (hCG)-stimulated testosterone production in purified adult Leydig cells (20). Leydig cells were isolated from adult rats (24), exposed to boric acid, challenged with hCG, and medium testosterone was measured by radioimmunoassay (RIA). No changes in basal or hCG-stimulated testosterone production were observed following a 24-hr exposure to concentrations of boric acid 5- to 10-fold those used in *in vivo* studies (Figure 2). Thus, no effect of boric acid on the steroidogenic function of isolated Leydig cells was observed, supporting the contention of a CNS-mediated rather than a direct effect. The lack of an effect on steroidogenesis is consistent with the histologically normal appearance of Leydig cells during boric acid exposure (2), although other functional effects cannot be ruled out.

**Intracellular cAMP Accumulation in Co-Cultures**

Studies by Gravis (21) showed that i.t. injection of dbcAMP to hamsters interrupted tubulobular complex formation and inhibited spermatiation. IS is the primary lesion seen following boric acid exposure (1,2). Also, the cAMP second messenger system is impaired by known or putative testicular toxicants (25-28). Therefore, we examined the effect of boric acid on follicle stimulating hormone (FSH)-stimulated intracellular cAMP accumulation in co-cultures (20). Specifically, we addressed whether the IS was the result of boric acid-mediated increases in Sertoli cell cAMP levels. Co-cultures were pretreated with boric acid, incubated with FSH and boric acid, then intracellular cAMP determined by RIA. The results showed a significant decrease (52%) in FSH-induced intracellular cAMP accumulation only at 10 mM boric acid, which is 5- to 10-fold above the highest

---

**Figure 2.** Effect of boric acid on Leydig cell testosterone production. Adult Leydig cells were isolated, exposed to boric acid for 20 hr, then challenged with human chorionic gonadotropin (hCG) for 3 hr in the presence of control or boric acid-containing medium. Three hour basal and hCG-stimulated testosterone in Leydig cell medium were measured by RIA. Basal testosterone levels = 0.98 ± 0.15 (SE) ng testosterone/1.5 × 10^6 cells. Values are the mean ± SE from two separate experiments, n = 4 replicates/experiment.

**Figure 3.** Effect of boric acid on secreted tissue-type (t-PA) (A) and urokinase-type (u-PA) (B) plasminogen activator (PA) activity in cultures of stage-pooled rat seminiferous tubules. Cultures of tubule segments comprising stages II to VI, VII to VIII, IX to XII, and XIII to I of the seminiferous cycle were prepared, exposed to boric acid (1-10 mM), and secreted t-PA and u-PA activity assayed using specific chromogenic substrates. Values are the mean ± SE from three separate experiments, n = 3 replicates/experiment.

---

**Volume 102, Supplement 7, November 1994**
boron concentrations in in vivo studies (data not shown). This suggests that the initial IS during boric acid exposure is not the result of increased intracellular cAMP leading to impaired-tubulobulbar-complex formation. This is also consistent with the ultrastructurally observed presence of intact tubulobulbar complexes in boric acid-treated rats and the continued formation of residual bodies in boric acid-treated rats displaying IS (1). The observation that FSH-stimulated cAMP accumulation was decreased only above relevant toxicologic in vivo concentrations suggests that this change is not mechanistically critical.

Plasminogen Activator Activity in Cultured Seminiferous Tubules

Plasminogen activators (PAs) are serine proteases with broad trypsinlike activity. Sertoli cells synthesize and secrete both tissue-type (t-PA) and urokinase-type (u-PA) PAs in a stage-specific manner (29,30). Studies in cultured tubule segments at various stages of the seminiferous cycle showed that the highest levels of PA activity were at stages VII and VIII, with the lowest activity at all other stages. PAs are postulated to play a role in the complex cell migration/tissue reorganization that occurs during critical stages of spermatogenesis (22). In addition, and pertinent to boric acid toxicity, a role in the facilitation of the release of mature spermatids by Sertoli cells has been suggested (22). Interestingly, boric acid and its n-alkyl boronic acids inhibit serine protease activity in purified enzyme systems (31). This led us to speculate that the IS seen after boric acid exposure may be the result of inhibited or decreased PA activity. Cultures of tubule segments comprising stages II to VI, VII to VIII, IX to XII, and XIII to I of the seminiferous cycle were prepared from adult rats (32), exposed to boric acid in vitro as above, and the activity of secreted t-PA and u-PA assayed using specific chromogenic substrates (33). No boric acid-associated changes were observed in secreted t-PA or u-PA activity from any stage pool (Figure 3). There were also no changes in PA activity in untreated conditioned media when boric acid was directly added to the assay incubation to final concentrations up to 10 mM (data not shown). These data suggest that inhibition of PAs is probably not the mechanism for the IS seen during boric acid exposure.

Morphology of Sertoli-Germ Cell Co-Cultures

Co-cultures of the somatic Sertoli cells and early germ cells have been used to study various aspects of the seminiferous tubule compartment of the testis (23). The detection of early morphologic changes and/or germ cell detachment in Sertoli-germ cell co-cultures exposed to boric acid might serve to identify a target cell. Thus, the effects of boric acid on cell morphology and germ cell detachment in co-cultures were evaluated (20). Co-cultures prepared on chamber slides were exposed to boric acid for up to 72 hr, then fixed and stained with hematoxylin/ eosin. No overt morphologic changes or germ cell loss were observed in co-cultures exposed to up to 10 mM boric acid.

Energy Metabolism in Co-Cultures

Spermatogenesis is an energy-demanding process, with developing germ cells requiring metabolic support from the somatic Sertoli cells. Exogenous lactate is the preferred energy substrate for germ cells and is essential for maintaining the metabolic activities of developing spermatocytes and spermatids (34). Thus, we evaluated the effect of boric acid on the ability of Sertoli cells in co-culture to secrete lactate into the culture medium (20). Additional measures of intermediary metabolism in co-cultures (medium pyruvate/cellular adenosine triphosphate [ATP]) were also examined. Lactate and pyruvate from co-culture-conditioned media and cellular ATP levels were determined as described (35). Co-cultures continuously exposed to boric acid for up to 72 hr showed significant decreases (30–60%) in medium lactate and pyruvate (3 and 10 mM) (Figure 4), and slight but significant decreases (10–17%) in cellular ATP only at 10 mM (data not shown).

These data suggest that boric acid affects some aspect of energy metabolism. Glycolysis would be the logical metabolic site of action. Borate is known to competitively and reversibly inhibit the activity of purified glyceraldehyde-3-phosphate dehydrogenase through boration of the cofactor nicotinamide adenine dinucleotide (NAD) (36). Consequently, it is possible that this or other NAD-requiring enzymes may be affected. boric acid may thus affect germ cell function and/or maturation by decreasing the availability of utilizable energy substrates through an indirect effect on glucose metabolism in Sertoli cells. However, the decreases in lactate and pyruvate in co-cultures occurred at boric acid concentrations slightly greater than those found in in vivo studies (3- to 5-fold) and therefore may not be mechanistically relevant. Unfortunately, there are insurmountable difficulties in evaluating this mechanism in vivo, due to both contamination of Sertoli cells with other cell types and postmortem artifacts (37).

DNA/RNA Synthetic Activity in Co-Cultures

Spermatogenesis involves continued proliferation and differentiation of the seminiferous epithelium. In the testis, both mitotic (spermatogonial) and meiotic (post-sper-
matogonal) germ cells undergo DNA as well as RNA synthesis (38,39), with most of the DNA synthesis occurring in spermatogonia and preleptotene spermatocytes (38). Sertoli cells also possess a high rate of RNA synthesis in vivo (40). Boric acid can impair nucleic acid synthesis in rat liver (38,39), and might do so in testis. To examine this possibility, we evaluated the effects of boric acid on DNA and RNA synthetic activity in Sertoli–germ cell co-cultures (20). Co-cultures were pretreated with boric acid, then labeled for 24 hr with \(^{3}H\)-thymidine (\(^{3}H\)-TdR to label DNA) and \(^{3}H\)-uridine (\(^{3}H\)-UdR to label RNA) in the presence of boric acid. Adherent post-spermatogonial germ cells (GC) were removed from the Sertoli cell/spermatogonia (SC/SG) monolayer, and the two cell fractions processed separately for determination of acid-insoluble radioactivity. 

\[^{3}H\]-TdR/\(^{3}H\)-UdR radioactivity was measured in the same cell fraction and normalized to the amount of DNA. Nucleotide precursor incorporation studies showed significant boric acid-mediated decreases (24–70%) in DNA synthetic activity in co-cultures, with the effects more apparent in the SC/SG fraction (Figure 5). This was the most sensitive parameter evaluated, with significant decreases (24–44%) noted between 1 and 3 mM boric acid, which is at or near the highest boron concentrations found in in vivo studies. In addition, these effects occurred in the absence of any changes in RNA synthetic activity and DNA content in either the SC/SG or GC fraction (data not shown). The intracellular acid-soluble 

\[^{3}H\]-TdR radioactivity (representing free precursor) was determined in the SC/SG fraction, and was found to be decreased 25 to 35% as a result of boric acid exposure (data not shown). This suggests that the decrease in DNA synthetic activity may in part be the result of decreased TdR transport into the cells.

Immunostaining for the thymidine analog 5-bromo-2’-deoxyuridine (BrdU) was done to identify the cells in co-culture displaying DNA synthetic activity. BrdU labeling was mostly limited to some of the germ cells overlaying Sertoli cells in co-culture, with no labeling of Sertoli cells in the monolayer (Figure 6).

Thus, it is postulated that boric acid affects the DNA synthetic activity of both mitotic (spermatogonial) and meiotic (post-spermatogonial) germ cells. Tres and Kierszenbaum (43) identified DNA synthetic activity in spermatogonia, preleptotene, and leptotene spermatocytes in Sertoli–germ cell co-cultures. It is likely that the DNA synthetic activity of all of these germ cell types is affected by boric acid. It is interesting to note that the boric acid effects on DNA synthetic activity occurred in the absence of overt changes in morphology and DNA content. These observations, along with suggestions of decreased TdR transport, suggest that the DNA synthetic rate may be affected rather than an inhibition through the induction of cytotoxic DNA damage. This is consistent with previous in vitro studies which showed that boric acid is not mutagenic and oncogenic, and is cytotoxic only at high concentrations (44).

These changes in DNA synthesis may explain at least the atrophy aspect of the

![Figure 5. Effect of boric acid on thymidine (\(^{3}H\)-TdR) incorporation into the Sertoli cell/spermatogonial (SC/SG) (A) and germ cell (GC) (B) fractions of Sertoli germ cell co-cultures. Co-cultures were pretreated with control or boric acid-containing medium (0.1–10 mM) for 24 hr, then labeled for 24 hr with \(^{3}H\)-TdR/\(^{3}H\)-UdR in the presence of the same media (total boric acid exposure = 48 hr). Adherent post-spermatogonial germ cells (GC) were removed from the SC/SG monolayer, and the two cell fractions processed separately for determination of acid-insoluble radioactivity. Only \(^{3}H\)-TdR data are presented here. Data are from a representative experiment of 4. Values are the mean ± SE, n = 4 replicates. * Significant difference from control, p<0.05, Dunnett’s multiple comparison test.](image)

![Figure 6. 5-Bromo-2’-deoxyuridine (BrdU) labeling in Sertoli-germ cell co-cultures. Untreated co-cultures were exposed to 10 μM BrdU in culture medium for 24 hr. Immunohistochemical detection of BrdU incorporated into cellular DNA was carried out. Top: Close-up of overlying layer of germ cells in a co-culture labeled with BrdU. Some of the adherent germ cells showed BrdU labeling (arrowheads); Bottom: Same field as the top section with a focus on the Sertoli cell monolayer. No BrdU labeling of Sertoli cell nuclei was observed (small arrowheads). Bar = 50 μm.](image)

| Treatment group | Leptotene spermatocyte/Sertoli cell ratio, (n) \(^{a}\) | % Control |  
|----------------|---------------------------------|---------|
| Control        | 3.67 ± 0.14 (5)                 |         |
| 9000 ppm boric acid | 2.02 ± 0.28 (4) | 55      |

\(^{a}\) Week 3 of exposure. \(^{a}\) For each rat (n), the number of leptotene spermatocytes and Sertoli cells in stage IX tubules in a representative cross-section of testis were counted, and the ratio determined. Values are the mean ± SD. A minimum of 500 leptotene spermatocytes were counted /rat. * p<0.05, Student’s t-test.
boric acid lesion seen in vivo. To determine if there were any in vivo changes in germ cell kinetics consistent with these in vitro effects, the number of early germ cells was evaluated in testes from boric acid-exposed rats. Interestingly, the leptotene spermatocyte/Sertoli cell ratio in stage IX tubules was decreased by 45% in rats exposed to 9000 ppm boric acid for 3 weeks (Table 2). This suggests some perturbation in the production and/or maturation of early germ cells, consistent with the in vitro effect noted here. Furthermore, and more important, these changes in vivo preceded epithelial disorganization and the eventual germ cell sloughing leading to atrophy. This suggests that this mechanism offers a plausible explanation for the atrophy seen after boric acid exposure.

Summary and Future Directions

Studies in vivo and in vitro were carried out to address possible mechanisms for the testicular toxicity of boric acid in rats. In vivo, studies examining boron tissue disposition showed that neither the testicular toxicity nor the slight CNS hormonal effect associated with boric acid exposure can be explained on the basis of selective accumulation of boron in the testis or brain/hypothalamus, respectively. Secondly, examination of tissue flavin levels during boric acid exposure showed that riboflavin deficiency was not a significant etiologic factor in the boric acid testicular lesion. Lastly, slight but significant decreases in serum inorganic phosphorus and creatinine were observed that worsened with dose and time of boric acid exposure. Despite the decreases in serum Pi, changes in testis levels of phosphorus, Ca, and Zn were only associated with the loss of post-spermatogonial germ cells during atrophy. Since these element changes were concurrent with atrophy, they are unlikely to be important from a mechanistic viewpoint.

The effects of in vitro boric acid exposure on selected aspects of cultured testicular cells were also evaluated to address the mild hormone effect, initial IS, and the atrophy. No direct effect of boric acid on the steroidogenic function of purified Leydig cells was observed, supporting the contention of a CNS-mediated effect rather than a direct effect. In vitro data suggest that the initial IS seen after boric acid exposure was not the result of increased cAMP levels or inhibited or decreased PA activity. The data do suggest an effect of boric acid on the DNA synthetic activity of mitotic and meiotic germ cells and to a lesser extent on some aspect of energy metabolism in Sertoli and/or germ cells. The effect on DNA synthesis suggests that boric acid may interfere with the production and/or maturation of early germ cells, thus promoting the testicular atrophy seen in boric acid-exposed rats. This was supported by the observed decrease in the early germ cell/Sertoli cell ratio in testes from boric acid-exposed rats prior to atrophy. While these changes may explain the atrophy aspect of the boric acid lesion seen in vivo, they do not identify the mechanism of the IS. Despite the lack of boric acid effects on PA activity, the possibility that the IS seen after boric acid exposure results from decreased proteolytic activity or altered cellular distribution of proteases at critical stages of spermatogenesis is worth pursuing. It has recently been shown that cyclic protein-2 (CP-2), secreted in greatest amounts by rat Sertoli cells from stage VI to VII seminiferous tubules, is the proteolytic form of the cysteine protease cathepsin L (45). It has been proposed that CP-2/cathepsin L may facilitate spermatid movement into the tubule lumen by the degradation of adhesion molecules that bind spermatids to Sertoli cells (45). We are pursuing the possibility that the boric acid-induced IS results from either impaired activation of the proteolytic core enzyme, decreased or inhibited proteolytic activity, or altered cellular distribution of CP-2/cathepsin L. Preliminary evidence suggests that boric acid does not directly affect cathepsin activity or the activation of pro-cathepsin L in seminiferous tubule fluid (data not shown). Alternatively, decreased proteolytic activity could result from increased levels of expression of protease inhibitors. Interestingly, a major broad spectrum protease inhibitor is found in the seminiferous tubule and is secreted by Sertoli cells: α1-macroglobulin (46). The possibility that the boric acid-induced IS is the result of increased expression or altered cellular distribution of α1-macroglobulin will also be evaluated. Thus, there are still some approaches that may prove useful in explaining the boric acid-induced IS. In summary, the in vivo and in vitro effects of boric acid on some selected biochemical parameters in the testis have been characterized, with some of the observed changes offering a plausible mechanistic explanation for the atrophy aspect of the testicular toxicity. Nonetheless, we suspect that boric acid most likely affects a number of cellular sites/processes that, in concert, lead to a disruption in spermatogenesis.

REFERENCES

1. Treinen KA, Chapin RE. Development of testicular lesions in F344 rats after treatment with boric acid. Toxicol Appl Pharmacol 107:325–335 (1991).
2. Ku WW, Chapin RE, Wine RN, Gladen BC. Testicular toxicity of boric acid (BA): relationship of dose to lesion development and recovery in the F344 rat. Reproductive Toxicol 7:305–319 (1993).
3. Linder RE, Strader LT, Rehberg GL. Effect of acute exposure to boric acid on the male reproductive system of the rat. J Toxicol Environ Health 31:133–146 (1990).
4. Chapin RE, Ku WW. The reproductive toxicity of boric acid. Environ Health Perspect 102(Suppl 7):87–91 (1994).
5. Fail P. Testicular and pituitary endocrine response is altered by boric acid in male rodents. Abstract presented at the Symposium on the Health effects of Boron and its Compounds. 16–17 September 1992, Irvine, California.
6. Russell LD, Malone JP, Karpas SL. Morphological pattern elicited by agents affecting spermatogenesis by disruption of its hormonal stimulation. Tissue Cell 13:369–380 (1981).
7. Rommers FFG. How much androgen is required for maintenance of spermatogenesis? J Endocrinol 116:7–9 (1988).
8. Ku WW, Chapin RE, Moseman RF, Brink RE, Pierce KD, Adams KY. Tissue disposition of boron in male Fischer rats. Toxicol Appl Pharmacol 111:145–151 (1991).
9. Moseman RM. Chemical disposition of boron. Environ Health Perspect 102(Suppl 7):113–117 (1994).
10. Roe DA, McCormick DB, Lin RT. Effects of riboflavin on boric acid toxicity. J Pharm Sci 61:1081–1085 (1972).
11. Burch HB. Fluorometric assay of FAD, FMN, and riboflavin. Methods Enzymol 3:960–962 (1957).
12. Goldsmith GA. Riboflavin deficiency. In: Riboflavin (Rivlin RS, ed). New York:Plenum, 1975:221–244.
13. Nielsen FH, Hunt CD, Mullen LM, Hunt JR. Effect of dietary boron on mineral, estrogen, and testosterone metabolism in postmenopausal women. FASEB J 1:394–397 (1987).
14. Seal BS, Weeth HJ. Effect of boron in drinking water on the male laboratory rat. Bull Environ Contam Toxicol 25:782–789 (1980).
15. Weeth HJ, Steth CF, Hanks DR. Boron content of plasma and urine as indicators of boron intake in cattle. Am J Vet Res 42:474–477 (1981).
16. Reeves PG. Zinc deficiency and dipeptidyl carboxypeptidase activi-
Testicular Toxicity of Boric Acid

17. Oishi S, Hiraga K. Testicular atrophy induced by phthalic acid monoesters: effects of zinc and testosterone concentrations. Toxicology 15:197–202 (1980).

18. Maitani T, Suzuki KT. Effect of cadmium on essential metal concentrations in testis, liver and kidney of five inbred strains of mice. Toxicology 42:121–130 (1986).

19. Pfeiffer CC, Hallman LF, Gersh I. Boric acid ointment: a study of possible intoxication in the treatment of burns. JAMA 128:266–274 (1945).

20. Ku WW, Shih LM, Chapin RE. The effects of boric acid (BA) on testicular cells in primary culture. Reproductive Toxicology 7: 321–331 (1993).

21. Gravis CJ. Ultrastructural observations on spermatozoa retained within the seminiferous epithelium after treatment with dibutyl cyclic AMP. Tissue Cell 12:309–322 (1980).

22. Lacroix M, Smith FE, Fritz IB. Secretion of plasminogen activator by Sertoli cell enriched cultures. Mol Cell Endocrinol 9: 227–236 (1977).

23. Ku WW, Chapin RE. The preparation and use of Sertoli–germ cell co-cultures from 28-day old rats. In: In vitro Biological Systems: Methods in Toxicology, vol 1, part A (Tyson CA, Frazier JM, eds). New York: Academic Press, 1993; 451–454.

24. Klinefelter GR, Hall PF, Ewing LL. Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. Biol Reprod 36:769–783 (1987).

25. Wiebe JP, Salhanick AI, Myers KL. On the mechanism of action of lead in the testis: in vitro suppression of FSH receptors, cyclic AMP and steroidogenesis. Life Sci 52:1997–2005 (1993).

26. Lloyd SC, Foster PMD. Effect of mono-(2-ethylhexyl) phthalate on follicle-stimulating hormone responsiveness of cultured rat Sertoli cells. Toxicol Appl Pharmacol 95:484–489 (1988).

27. Heindel JJ, Chapin RE. Inhibition of FSH-stimulated CAMP accumulation by mono(2-ethylhexyl) phthalate in primary rat Sertoli cell cultures. Toxicol Appl Pharmacol 97:377–385 (1989).

28. Heindel JJ, Keith WB. Specific inhibition of FSH-stimulated CAMP accumulation by D₆-tetrahydrocannabinol in cultures of rat Sertoli cells. Toxicol Appl Pharmacol 101:124–134 (1989).

29. Lacroix M, Parvinen M, Fritz IB. Localization of testicular plasminogen activator in discrete portions (stages VII and VIII) of the seminiferous tubule. Biol Reprod 25:143–146 (1981).

30. Heitzle JA, Waller EK, Fritz IB. Hormonal stimulation alters the type of plasminogen activator produced by Sertoli cells. Biol Reprod 34:895–904 (1986).

31. Antonov VK, Ivanina TV. n-Alkyl boronic acids as bifunctional reversible inhibitors of α-chymotrypsin. FEBS Lett 7: 23–25 (1970).

32. Parvinen M, Ruokonen A. Endogenous steroids in rat seminiferous tubules. Comparison of different stages of the epithelial cycle isolated by transillumination-assisted microdissection. J Androl 3:211–220 (1982).

33. Karlan BY, Clark AS, Littlefield BA. A highly sensitive chromogenic microtiter plate assay for plasminogen activators which quantitatively discriminates between the urokinase and tissue-type activators. Biochem Biophys Res Commun 142:174–154 (1987).

34. Jutte NHPM, Grootoedeg J, Rommerts FG, van der Molen HJ. Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. J Reprod Fert 62:399–405 (1981).

35. Chapin RE, Gray TJB, Phelps JL, Dutton SL. The effects of mono-(2-ethylhexyl)-phthalate on rat Sertoli cell-enriched primary cultures. Toxicol Appl Pharmacol 92:467–479 (1988).

36. Wolny M. Effect of borate on the catalytic activities of muscle glyceraldehyde 3-phosphate dehydrogenase. Eur J Biochem 80:551–556 (1977).

37. Free MJ. Carbohydrate metabolism in the testis. In: The Testis, vol II (Johnson AD, Gomes WR, Van Demark D, eds). New York: Academic Press, 1970; 125–192.

38. Monezi V. Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using triitated thymidine. J Cell Biol 14:1–18 (1962).

39. Monezi V, Geremia R, D’Agostino A, Boitani C. Biochemistry of male germ cell differentiation in mammals: RNA synthesis in meiotic and postmeiotic cells. Curr Top Dev Biol 12:11–36 (1978).

40. Galdieri M, Monezi V. Ribosomal RNA synthesis in spermatogonia and Sertoli cells of the mouse testis. Exp Cell Res 80: 120–126 (1973).

41. Weser VU. Influence of borate and germanate on the biosynthesis of RNA. Hoppe-Seyler’s Z Physiol Chem 349:989–994 (1968).

42. Dani HM, Saini HS, Allag IS, Singh B, Sareen K. Effect of boron toxicity on protein and nucleic acid contents of rat tissues. Res Bull (N.S.) of the Punjab University 22:229–235 (1971).

43. Tres LL, Kierszenbaum AL. Viability of rat spermatogenic cells in vitro is facilitated by their co-culture with Sertoli cells in serum-free hormone supplemented medium. Proc Natl Acad Sci USA 80:3377–3381 (1983).

44. Landolph JR. Cytoxicity and negligible genotoxicity of borax and borax ores to cultured mammalian cells. Am J Ind Med 7:31–43 (1985).

45. Erickson-Lawrence M, Zabludoff SD, Wright WW. Cyclic protein-2, a secretory product of rat Sertoli cells, is the proenzyme form of cathespin L. Mol Endocrinol 5:1789–1798 (1991).

46. Cheng CY, Grima J, Stahler MS, Guglielmotti A, Silvestrini B, Bardin CW. Sertoli cell syntheses and secretes a protease inhibitor, α₂-macroglobulin. Biochemistry 29:1063–1068 (1990).