Investigation of protective effects of lithium borate on spermatogenesis and testes histopathology against cadmium-induced acute toxicity in rats

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Abstract: In this study, protective effects of lithium borate (LTB) on spermatogenesis as well as histopathological and immunohistochemical findings of testes in experimentally induced acute Cadmium (Cd) toxicity in rats were determined. Twenty-eight male Wistar albino rats, were used, weighing 200–220 g. Rats were randomly divided into 4 groups: Control, Cd, LTB, and LTB + Cd. Rats were anesthetized with ketamine at the end of the sixth day, blood was taken from their hearts, and the rats were decapitated. Typically, the control and LTB groups exhibited similar values. Compared with those observed for the control group, the sperm morphology (i.e. abnormal sperm count) increased for the Cd group, while the FSH, LH, total testosterone levels, sperm motility, and density decreased in a statistically significant manner. Clearly, no adverse effects of LTB on the sperm motility, density, and sperm morphology (i.e. abnormal sperm count) were observed, but LTB decreased the negative effects of Cd toxicity. Abnormal disturbances in the head and tail areas of the sperms increased; thus, the total abnormal sperm rate increases, leading to the decreased fertilization capacity. The histopathological examination of testicular tissues revealed severe haemorrhage and hyperaemia in intertubular intervals, tubule atrophy, severe degenerative and necrotic changes in spermatocytes, tubule wall thinning, and necrosis in basal germ cells. In immunohistochemically Caspase-3, 8-OHdG, and COX-2 staining, changes in the control and LTB groups were not detected, while the Cd toxicity group exhibited severe expression in the testis tissue. Histopathological and immunohistochemical changes were significantly decreased in the LTB + Cd group compared to the Cd group. In conclusion, in this study it was determined that LTB has protective effects on Cd-induced testicular toxicity in rats.

Key words: Acute cadmium toxicity, hormones, spermatogenesis, histopathological findings, lithium borate, rat

1. Introduction

Cd, a highly toxic heavy metal, and Cadmium (Cd) compounds are extremely common in nature. Although Cd does not exhibit a well-known physiological role in higher organisms, it exhibits carcinogenic and mutagenic effects. It is a first-class carcinogen, and all its doses are toxic (Kay et al., 1986; Méndez - Armenta and Rios, 2007; Modi et al., 2019; Thompson and Bannigan, 2008). Cd is widely used in several industrial areas (Cannino et al., 2009; Joe et al., 2011). The accumulation of Cd in the atmosphere increases due to environmental contamination (Cannino et al., 2009; Marcano et al., 2009). Cd enters the body through the respiratory, inguinal, and digestive systems (Cannino et al., 2009; Marcano et al., 2009). Deficiency of proteins, calcium, and iron in nutrition leads to the increased absorption of Cd from the intestines. Virtually any type of Cd vapor can be absorbed in the lungs. Acute Cd toxicity may occur in a short time period due to exposure to high-dose Cd toxicity and may lead to hepatic and testicular damage. Cd toxicity leads to the main lesions in the testes, including oedema, testicular haemorrhage, and necrosis with the destruction of seminiferous tubules (Gupta et al., 1967; Mason et al., 1964). A correlation between the fertility and Cd toxicity has been reported (Acharya et al., 2008). Molecular mechanisms of Cd toxicity have not been completely explained thus far. Chronic Cd toxicity can be caused by prolonged exposure to low amounts of Cd, leading to kidney damage and otoxicity (Yari et al., 2010). Generally, chronic Cd poisoning leads to the colouring of teeth and bones as well as diseases of
the respiratory system and kidneys (Gökalp et al., 2005). In Cd toxicity, death occurs as a result of coagulopathic bleeding, metabolic acidosis, and cardiovascular collapse within a few hours (Baldwin et al., 1999; Gökalp et al., 2005; Himeno and Aoshima, 2019; Modi et al., 2019).

Boron is commonly found in the earth's crust. Several nutrients and spring water are rich in boron (Becker et al., 1997; Moore, 1997; Naghii and Saman, 1996). A daily average boron uptake of 1.0–1.28 mg/day for adults has been reported (Celikezen et al., 2014; Türkez et al., 2012). Boron and boron compounds, such as lithium borate (LTB), are extremely effective for the prevention of oxidative stress from metal toxicity. These compounds exhibit antioxidant activity as described previously. Boron and boron compounds are preferred for the production of eye-watering, mouthwashes, and irrigant solutions and drugs due to their antiseptic and antiepileptic properties. In addition, boron compounds are predominantly used in cologne, perfume, shampoo, and baby powder production processes. In addition, boron neutron capture treatment is known for treating cancer. Boron is used for the treatment of brain cancer as it permits the selective clearance of cancer cells from the environment while simultaneously exerting a minimal effect on healthy cells. Furthermore, boron is also used for the treatment of osteoporosis, menopause, and allergic diseases, as well as in psychiatry and magnetic resonance devices (Oto et al., 2017). Accidental poisoning due to the oral ingestion of an excess amount of borates or boric acid has been reported (Dixon et al., 1976; Weir and Fisher, 1972). Lethal doses of boric acid in infants, children, and adults are 2–3 g, 5–6 g, and 15–20 g, respectively. In lethal cases, lesions are observed on the liver, kidney, central nervous and gastrointestinal systems, and skin. Death occurs as a result of respiratory failure. The inhalation of boron leads to respiratory irritation, cough, and difficulty in breathing. High doses of boron exert toxic effects, but exhibit therapeutic effects. Hence, therapeutic effects of boron on the health and fertility in humans and animals have been investigated in several studies (Dixon et al., 1976; Yildirim et al., 2018 a,b).

Factors such as race, age, muscle activity, region, season, environmental temperature, care, and nutrition affect fertility and spermatogenesis. Some studies have been conducted to determine the effects of boron and boron compounds in heavy metal toxicities (Celikezen et al., 2014; Dixon et al., 1976; Oto et al., 2017; Türkez et al., 2012; Yildirim et al., 2018 a,b). However, these studies are limited, insufficient, and irrelevant, and they do not provide sufficient and detailed information about the effects of LTB on the acute Cd toxicity in rats. Hence, in this study, protective effects of LTB on some reproductive hormones, spermatogenesis, testes histopathology and immunohistochemistry in rats exposed to acute Cd toxicity are investigated, thereby contributing to the literature.

2. Materials and methods

2.1. Animal and experimental design

The experimental protocol was approved by the Committee on the Ethics of Animal Experiments at Ataturk University (Permit Number: 12- 216/2018). During the study, the rats' comfort and good care were provided in accordance with ethical rules.

In this study, 28 male Wistar albino rats, about 200–220 g were used. Rats were randomly divided into 4 groups as control and 3 experimental groups. In this study, CdCl2.5H2 (Cadmium chloride pentahydrate, CAS No. 7790 – 78 - 5, Sigma) was used as the Cd source, and Lithium borate was used as the source of LTB. All the materials were prepared by dissolving in physiological serum.

Control group: The control group received a standard pellet feed, drinking water, and physiological serum by intraperitoneal (IP) injection for 5 days.

Cd group: The physiological serum was administered for 5 days. One hour after the administration of the physiological serum on the fifth day, this group received a single dose of 0.025 mmol/kg of Cd by IP injection.

LTB group: This group received 15 mg/kg/day of LTB orally for 5 days.

LTB + Cd Group: This group received 15 mg/kg/day of LTB by oral administration for 5 days. After 1 h of LTB administration on the fifth day, 0.025 mmol/kg of Cd was administered by IP injection.

2.2. Sperm examination, serum FSH and LH analysis

All rats in the study were anesthetized with ketamine at the end of the sixth day, blood was taken from their hearts, and the rats were decapitated. LH and FSH in serum samples were measured by the chemiluminescence microparticle immunological analysis using an Abbott ARCHITECT C 16200 modular routine device. The results obtained were expressed in IU/mL. Testosterone was measured by the same method on the Abbott ARCHITECT I 4000 SR routine analyser in accordance with the kit package insert. The results were expressed as mmol/L. Sperm density and motility were detected by a method reported by Sonmez et al. (2005) with some modifications. Morphologically abnormal sperm cells were evaluated by the method reported by Turk et al. (2008).

2.3. Histopathological and immunohistochemical examination

The animals were euthanized at the end of the 6 experimentation days by decapitation, and testes tissues were quickly removed for histopathology. The testes tissues were fixed in 10% neutral buffered formaldehyde for 48 h. These tissues were embedded in paraffin blocks after the routine tissue procedure. These tissues were sectioned at 5-μm thickness, stained with H&E, and examined under a
light microscope (Olympus BX51 optical microscope and Olympus DP25 digital camera, Japan). The tissues were scored as negative (-), slight (+), moderate (++), or severe (+++), according to the histopathologic findings.

Immunohistochemical studies were performed according to the kit procedure. 3 (Diaminobenzidine (DAB) was used as the chromogen. The primary antibodies were Caspase 3, 8 - hydroxydeoxyguanosine (8-OHdG), and cyclooxygenase 2 (COX - 2) (Catalogue no: sc - 271759, sc - 393871, sc - 514489, Santa Cruz, USA), respectively. The sections were evaluated as negative (-), mild (+), moderate (++), and severe (+++).

2.4. Statistical analysis

Statistical analysis of FSH, LH, total testosterone, and sperm motility and density were presented as mean ± standard derivation (X ± SD). ANOVA and Tukey tests were used for comparison between groups. Histopathological examination of semiquantitatively obtained data Kruskal–Wallis test was used for the analysis of the differences between the groups. In addition, for the comparison of 2 groups Mann–Whitney U test was used. SPSS version 20 was used for the statistical analysis (IBM Corp., Armonk, NY, USA). The data were expressed that was considered statistically significant as mean ± SD (P < 0.05).

3. Results

Tables 1–3 summarize the results obtained for the spermatological parameters and hormone levels of the groups, and showed macroscopic findings (Figure 1), and histopathological findings (Figure 2–5 and Table 4).

The values for the control and LTB groups were typically similar. The values for the Cd and LTB + Cd groups were outside the range of change for the values of healthy rats. The values for the LTB + Cd group were better than those obtained for the Cd group, and the values were similar to the mean values and physiological variation limits. Compared with those observed for the control group, the sperm morphology (abnormal sperm count) increased for the Cd group, while the FSH, LH, total testosterone levels, sperm motility, and density decreased in a statistically significant manner. Clearly, no adverse effects of LTB on the sperm motility, density, and sperm morphology (abnormal sperm count) were observed, but LTB decreased the negative effects of Cd (Tables 1–3).

3.1. Macroscopic findings

Control group: Testicular tissues exhibited normal anatomical appearance (Figure 1–A).

Cd group: Severe haemorrhage and oedema were detected in the testes (Figure 1–B).

Table 1. FSH, LH, and testosterone levels of all groups.

| Groups (n=10) | FSH (IU/mL)       | LH (IU/mL)       | Total Testosterone (mmol/mL) |
|---------------|-------------------|------------------|-----------------------------|
| Control       | 0.4750 ± 0.0071\(^a\) | 0.0033 ± 0.0005\(^a\) | 9.80 ± 0.86\(^a\) |
| Cd            | 0.3200 ± 0.0054\(^b\) | 0.0007 ± 0.0005\(^b\) | 4.54 ± 0.093\(^b\) |
| LTB           | 0.4980 ± 0.0082\(^a\) | 0.0034 ± 0.0005\(^a\) | 10.30 ± 0.88\(^a\) |
| LTB + Cd      | 0.4000 ± 0.0054\(^a\) | 0.0022 ± 0.0008\(^b\) | 6.54 ± 0.093\(^b\) |

The difference between the values of different letters in the same column is significant, \(^a,b,c,d\): P < 0.001

Table 2. Mean sperm motility and density values in all groups.

| Groups (n=10) | Sperm Motility (%) | Sperm Density \((\times 10^6)\) |
|---------------|--------------------|-------------------------------|
| Control       | 75.25 ± 5.45\(^a\) | 92.50 ± 8.23\(^a\) |
| Cd            | 32.75 ± 4.89\(^b\) | 47.60 ± 5.02\(^b\) |
| LTB           | 78.70 ± 4.66\(^a\) | 93.56 ± 9.22\(^a\) |
| LTB + Cd      | 49.50 ± 3.72\(^b\) | 59.00 ± 5.51\(^b\) |

The difference between the values of different letters in the same column is significant, \(^a,b,c,d\): P < 0.001, \(^a,c\): P < 0.0001

Table 3. Sperm morphology (abnormal sperm) values of all groups.

| Groups (n=10) | Anormal sperm ratio (%) |
|---------------|-------------------------|
|               | Head | Tail | Total |
| Control       | 9.0 ± 2.37\(^a\) | 12.25 ± 2.45\(^a\) | 21.25 ± 2.28\(^a\) |
| Cd            | 24.52 ± 4.48\(^c\) | 45.33 ± 6.68\(^b\) | 69.85 ± 6.49\(^b\) |
| LTB           | 9.33 ± 2.38\(^a\) | 11.25 ± 2.32\(^a\) | 20.58 ± 2.25\(^a\) |
| LTB + Cd      | 15.60 ± 3.89\(^a\) | 30.35 ± 5.50\(^a\) | 45.95 ± 5.50\(^a\) |

The difference between the values of different letters in the same column is significant, \(^a,b,c\): P < 0.001, \(^a,c\): P < 0.0001
No pathological findings were observed in testicular tissues (Figure 1–C).

LTB + Cd group: Mild haemorrhage was detected in testes (Figure 1–D). Table 4 summarizes the macroscopic findings.

3.2. Histopathological findings
Control group: The histological appearance of testis tissues was normal (Figure 2–A).
Cd group: Severe haemorrhage and hyperaemia in the intertubular intervals, tubule atrophy, severe degenerative, and necrotic changes in spermatocytes, tubule wall thinning, and basal germ cell necrosis were detected in testicular tissues (Figure 2–B).
LTB group: Tubular and interstitial tissues in testes exhibited a normal histological appearance (Figure 2–C).
LTB + Cd group: Mild oedema in interstitial intervals, while the degeneration of spermatocytes in some tubules was detected, and necrotic cells were not observed (Figure 2–D). Table 4 summarizes histopathological findings.

3.3. Immunohistochemical findings
Control group: When the testis tissues were immunohistochemically examined, expressions of Caspase-3, 8-OHdG, and COX-2 were negative (Figure 3–A, 4–A, 5–A).
Cd group: Severe hyperaemia in the intertubular intervals, tubule atrophy, severe degenerative, and necrotic changes in spermatocytes were observed (Figure 3–B, 4–B, 5–B).
LTB group: When the testis tissues were immunohistochemically examined, expressions of Caspase-3, 8-OHdG, and COX-2 were not observed (Figure 3–C, 4–C, 5–C).
LTB + Cd group: Mild cytoplasmic Caspase-3 and 8-OHdG expressions at spermatocytes were observed, and mild degree COX-2 expression in intertubular intervals was detected (Figure 3–D, 4–D, 5–D). Table 4 summarizes the immunohistochemical findings.

4. Discussion
Cd is an industrial and environmental pollutant that is ubiquitous in the environment. Cd accumulates in animal and human tissues, leading to significant health problems. The metal industry, battery production, contaminated food and water, polluted air and tobacco inhalation lead to Cd exposure to humans. The International Agency for Research on Cancer has defined Cd as a first-class carcinogen (Himeno and Aoshima, 2019). Cd causes oxidative stress via the formation of several free radicals such as superoxide and nitric oxide in the organs. Cd binds to sulphhydryl groups of proteins and causes tissue damage, membrane structure deterioration, and structural

Figure 1. Testicular tissue, normal anatomical appearance (A), oedema, hyperaemia, congestion and haemorrhage (B), normal anatomical appearance (C), moderate oedematous and mild hyperaemic (D).
disorder. The typical effects of Cd toxicity in cells include a) epigenetic changes in the expression of DNA, b) inhibition of cell metabolism, and c) deterioration in the renal proximal tubule structure (Bernhoft, 2013). Experimental and environmental exposure to Cd revealed some effects of severe atrophy in the testes, loss of renal function, hepatic damage, respiratory and digestive system disorders, and anaemia. In addition, toxic effects on various cells include damage to the nucleus membrane and mitochondria crystals, chromatin condensation, and eventually cell death (Meeker et al., 2008; Rahim et al., 2013; Schwartz and Reis, 2000; Sarkar et al., 2013).

Boron is an essential micro-element for plants, humans, and animals. Boron enters the human and animal body by mouth, by inhalation, and through the skin. Boron is rapidly and completely absorbed by the gastrointestinal tract and passes into the bloodstream (Usuda et al., 1998). However, the bioaccumulation of boron by consumption via the food chain does not occur, and it plays an important role in the regulation of body minerals such as calcium and vitamin D, protects the bone structure via the prevention of the decrease of calcium and magnesium, improves arthritis and the plasma lipid profile, and contributes to increased learning skills in children (Devirian and Volpe, 2003). In addition, disinfectant, anti-inflammatory, or anticancer properties were determined. Boron is also used to treat cancer (Barranco et al., 2009).

Factors such as race, age, sex, pregnancy, lactation, muscle activity, region, season, environment temperature, care, and nutrition effect on spermatogenesis. Although studies investigating effects of Cd on the reproductive performance have been reported, protective effects of
LTB on acute Cd toxicity have not been investigated. In this study, Tables 1–3 summarize the hormone levels that are effective in spermatogenesis, sperm motility, density, and morphology, and Table 4 and Figure 2–5 show the histopathological structure of the testes and immunohistochemical findings.

Studies have reported that Cd decreases the level of hormones related to the reproductive performance in rats. In addition, Cd toxicity disrupts the testis function, decreases sperm motility and amount, and causes infertility (Yang et al., 2006). These results are similar to those obtained for the Cd group herein. In this study, no change in the FSH, LH, and testosterone levels for the control and LTB group values was observed. However, values for the LTB + Cd group exhibited a slight decrease; this decrease was less than that of observed for the Cd group, related to the protective effect of LTB. Cd toxicity adversely affected the sperm motility, density, and morphology in rats. The motility rate was significantly reduced, and the abnormal sperm rate was significantly increased. The decrease in the testosterone level, sperm motility, and sperm density, as well as the increase in the abnormal sperm rate, was possibly related to the damage to tubules and Leydig cells. In the histopathological findings, there are severe destructive tests in Figure 1–5 and Table 4.

It was reported that empty in the lumens of tubules in terms of active sperm, observed severe degeneration of germ cells and spermatocytes in tubules wall, found congestion and oedema in intertubular intervals, stained darkly the nuclei of spermatids and found many apoptotic cells (Akinloye et al., 2006; De Souza Predes et al., 2016). In this study, the histopathologic examination of testis tissues for the Cd group revealed severe haemorrhage and hyperaemia in intertubular intervals, basal germ cell necrosis, and tubule wall thinning, corresponding to severe degenerative and necrotic changes in spermatocytes.

Figure 3. Negative Caspase-3 expressions in testes tissues of control and LTB groups (A and C), severe Caspase-3 expression in spermatocytes of Cd group (arrowheads) (B), mild Caspase-3 expression in spermatocytes (arrowheads) of LTB + Cd group (D), IHC - P, Bar: 20 µm.
Extremely mild degeneration at spermatocytes and absence of necrotic cells were determined for the LTB + Cd group.

Caspases are critical mediators of programmed cell death (apoptosis). Among the types of caspases, Caspase-3 is a death protease that catalyses the specific cleavage of several important cellular proteins, and it is frequently activated. This caspase, also known as the slaughter caspase, is the final stage that leads to apoptosis and does not undergo transformation. In this study, when Caspase-3 in the testis tissue was stained immunohistochemically, severe cytoplasmic localization in the spermatocytes of the Cd group was observed, and in the LTB + Cd group, this expression level was observed to be mild. Our findings supported those reported previously (Barranco et al., 2009; Kim et al., 2006).

Oxidative stress is imbalanced between free radicals and antioxidants in the body. Free radicals can cause large-chain chemical reactions in the body because they can react extremely easily with other molecules. These reactions are referred to as oxidation, which can be harmful to the body. 8-OHdG concentrations of a cell reveal a significant measure of oxidative stress. The 8-OHdG expressions have been reported to be severe in testis tissue in oxidative damage (Belhan et al., 2017; Saripinar et al., 2017). In this study, 8-OHdG expressions were immunohistochemically observed to determine the oxidative damage in testes. 8-OHdG expressions in the Cd group were severely observed. The expression of 8-OHdG in this toxicity is consistent with the literature (Belhan et al., 2017; Belhan et al., 2019).

COX-2 is present in inflammatory areas. COX-1 and COX-2 produce prostaglandins that contribute to pain,
Figure 5. Testes tissue, control and LTB groups, negative COX-2 expression (AC), Cd group, severe COX-2 expressions in damaged tubules and intertubular intervals (B), LTB + Cd group, mild COX-2 expression in interstitial tissue (arrowheads) (D), IHC - P, Bar: 20µm.

Table 4. Scores of macroscopic, histopathological and immunohistochemical findings in testes.

|                                | Control | Cd  | LTB  | LTB + Cd |
|--------------------------------|---------|-----|------|----------|
| Oedema in tests                | -       | +++ | -    | ++       |
| Haemorrhage in testes          | -       | +++ | -    | +        |
| Intertubular haemorrhage       | -       | +++ | -    | +        |
| Degeneration at spermatocytes  | -       | +++ | -    | ++       |
| Necrosis at Spermatocytes       | -       | ++  | -    | -        |
| Caspase-3                      | -       | +++ | -    | +        |
| COX-2                          | -       | +++ | -    | +        |
| 8-OHdG                         | -       | +++ | -    | +        |
fever, and inflammation. COX-2 enzymes are responsible for the release of prostaglandins after infection or injury. Prostaglandins exhibit several effects, one of which is the inflammation regulation of living organisms. Liu et al. (2018) have reported that the perivascular COX-2 expression increases in intertubular intervals of testis toxicity depending on the dose. In the study, the COX-2 expression was severely observed in intertubular intervals of the testis tissues in the Cd group. It was determined to be compatible with the literature reviews (Bustos - Obregón et al., 2013).

As a result, according to hormone analysis, sperm examination, histopathological and immunohistochemical findings, Cd toxicity causes infertility in rats via the damage to testes and suppression of FSH, LH, and testosterone levels. In addition, notably, LTB reduces the side effects of the acute Cd toxicity and exhibits a protective effect on the testis tissue.

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