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EFFECTS OF IN OVO ADMINISTRATION BORIC ACID ON TESTES IN CHICKEN: A HISTOLOGICAL AND STEREOLOGICAL STUDY

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ABSTRACT

Boron is reported to cause fertility disorders in birds. We investigated the potential adverse effects of boric acid administered during embryogenesis on testicular development and spermatogenesis after hatching and puberty.
The fertilized eggs were divided into four groups. Three groups were injected with different concentrations of boric acid (1000, 1500, or 2000 ppm) dissolved in 9% NaCl into the egg yolk, and the control group received only a 9% NaCl injection. The evaluations were conducted at two time points: post-hatching day 1 and 26 weeks of age. Testes of one-day-old- and 26-week-old birds were subjected to morphological and stereological evaluations to assess the potential effects of boric acid on testicular development. Effects of boric acid on spermatogenesis and semen quality parameters were also investigated in 26-week-old birds. Finally, plasma FSH, LH, and testosterone levels were analyzed in all birds.No statistically significant differences were noted between one-day-old chicks and 26-week-old birds regarding stereological parameters. Likewise, no significant differences were detected among groups concerning Sertoli, germ, and Leydig cells. Semen quality parameters did not reveal statistically significant differences, and similarly, plasma LH and testosterone levels did not statistically differ between the study and the control groups. Comb height and plasma FSH levels were increased in experimental groups, compared to the control in 26-week-old birds. A single administration of different boric acid doses did not impact the testicular morphological features after hatching and puberty. In-ovo boric acid exposure quantitatively altered the numbers of the Sertoli, germ, and Leydig cells, respectively, but revealed no adverse effect on spermatogenesis. This study might contribute to further research targeting boric acid's multifaceted effects on the dynamics of spermatogenesis.

Keywords: Boric acid, in-ovo, poultry, stereology, spermatogenesis

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INTRODUCTION

The boron (B) compounds are substantially dispersed into the environment due to the widespread use of borax and boric acid salts (H_3BO_3) in different industrial fields (Aktas*et al.,* 2017; Moss and Nagpal, 2003).Boric acid was previously reported to have impaired sperm production for the first time in the early 1970s (Cox, 2004; Lee *et al.,* 1978).Concordantly, boric acid was classified under Category 1B according to the European Union Hazard Statements, R60–61 (may damage fertility) established by the Globally Harmonized System (GHS) of classification and labeling of chemicals (Bolt *et al*., 2020).However, on the other hand, research focusing on the effects of boric acid on reproductive health reveal conflicting data. Boron at high concentrations had a substantial adverse impact on fertility in males in rodents, and induced 19th-step spermatid deposition within stage IX-XII seminiferous tubules, a decline in sperm count, and functionally abnormal spermatid production (Chapin and Ku, 1994; Ku *et al.,* 1993; Linder *et al.,* 1990). Testicular atrophy was also noted due to its chronic exposure, and atrophy proceeded even after the cessation of boric acid administration (Robbins *et al.,* 2010). Boron was also reported to have accumulated in the testes after subacute exposure in rats (Kocaturk *et al.,* 2005).On the other hand, contradictory data is available, indicating, for instance, that sufficient doses of boron exhibit an active cryoprotective effect on spermatozoa (Bucak *et al.,*

2021). Likewise, dietary boron supplementation improved semen quality parameters, immunity, and antioxidant defense mechanisms in male goats (Krishnan *et al.,* 2019). Similarly, no tissue injury was noted on the testicular tissue in rats, particularly on the seminiferous epithelium, regardless of borax dose and exposure period, and spermatogenesis was routinely maintained (Dixon *et al.,* 1976). Such controversial results have raised a debate on whether inorganic boron compounds might be justified to be categorized as fertility toxins (Bolt *et al.,* 2020).

Birds are oversensitive animal species, susceptible to environmental pollutants (Mekonen, 2017). Boron adversely affected blood, brain, and liver biochemistry, increasing the likelihood of behavioral disorders in ducklings (Hoffman *et al.,* 1990) and decreasing the hatching rate (Landauer, 1952). Studies claim that boron leads to malformations and fertility disorders in birds (Fail *et al.,* 1998; Hoffman *et al.,* 1990; Smith and Anders, 1989). On the other hand, a single dose of in ovo boron or sodium aluminosilicate application substantially stimulated embryogenesis and mineralization in local Turkiye breeds (King *et al.,* 1993).

The potential effects of boron on testes and spermatological parameters have yet to be documented in avian species. Based on the studies mentioned above, the effect of boric acid on fertility and testes is somewhat contradictory. Therefore, considering the reported negative impact of boric acid on various organs and tissues and the avian species' susceptibility to environmental pollutants, we designed the present study to investigate the potential effects of in-ovo-applied boric acid on testes and spermatogenesis after hatching and during puberty.

MATERIALS AND METHODS

Animals and Experimental Design: The Local Ethics Committee for Animal Research of the Istanbul University Faculty of Veterinary Medicine approved the study (2011/133). The fertilized eggs were incubated in the hatchery at 37.5ºC with a relative humidity of 60% and turned every three hours.

Four hundred Super Nick chickens fertilized eggs were provided from a local manufacturer (Hastavuk, Bursa, Turkiye) and divided into four groups under Completely Randomized Design (three experimental groups and one control; n=100). Fifteen eggs perished during manipulations, and the administrations were conducted with 385 fertilized eggs. The parameters were evaluated at two time-points: post-hatching day 1 and 26 weeks of age. Different doses of boric acid (1000, 1500, and 2000 ppm) dissolved in 9% NaCl were injected into the egg yolk of the experimental groups at both time points, and the controls received only 9% NaCl. Boric acid was injected into the egg yolk as a single application (100µl/egg) through a blind spot of the egg drilled with disposable insulin syringes, and then the tiny holes were sealed with wax (Aktas *et al.,* 2008) on the fourth day of incubation since gonad differentiation occurs approximately at the 6.5-7th days of incubation (Esener and Bozkurt, 2018). 263 of 385 fertilized eggs hatched. After hatching, males (n=120) were determined based on the length and texture of the wing feathers (American Poultry Association). The hatching rates are indicated in Table 1.The body weights of all male chicks were recorded initially at hatching. The protocol was continued with 120 male chicks, of which 10from each group were sacrificed at the first time-point (post-hatching day 1). Therefore another 40 birds in total, including the control and experimental groups, were sacrificed at week 26.

The hatching rate was assessed as percentage values on day 21, which is percentage hatchability = (number of chicks/total number of eggs) *100.

After hatching, the room temperature was adjusted to 33°C and decreased by two °C every week, and the birds were kept under a 14L:10D lighting regime during the experiment.

Forty birds (each group contained ten individuals) out of 123 were sacrificed on post-hatching day 1 to assess the acute effects of boric acid on testicular development. The remaining animals were raised free-range, with ad libitum access to feed and water until 26 weeks of age, to investigate the effects of boric acid on spermatogenesis.

All birds were subjected to xylazine (1 mg/kg) + ketamine HCl (20 mg/kg) anesthesia before blood sampling and testicular resection. Blood samples were intracardially harvested for hormone analyses, and blood plasma was stored at -20^{0}C until the analyses. Then the animals were intracardially perfused via mDF (modified Davidson's fluid) (Howroyd *et al.,* 2005), and the testicular tissues were removed. The testicular tissue samples were further fixed in mDF for 24 h. All birds were administered heparin (15 IU/g live weight) subcutaneously 30 min before anesthesia under the administration principles of intracardiac perfusion.

Morphological Evaluation of testes

Gonadosomatic indices: Gonadosomatic indices (weight of testis/body weight) and testis weight asymmetry (right testis weight/left testis weight) were calculated for each bird.

Comb size: Comb sizes were measured using a digital caliper (accuracy=0.01 mm). The height of the comb was manually measured from the cranium to the peak comb.

Tissue preparation and sectioning

One-day-old chicks: The right and left testes of one-day-old chicks were rapidly harvested right after sacrification and released from the epididymis, and the weights of the

testes were separately recorded. Left testes were fixed in 2.5% glutaraldehyde solution (in 0.15 M cacodylate buffer pH 7.4), routinely processed, and embedded in Epon 812. Semi-thin (1 µm) sections were obtained from the resin blocks by an ultramicrotome and stained with toluidine blue. Four sections were cut from each resin block of approximately 2.5 mm thickness every 500 µm interval for sampling (Esener and Bozkurt, 2018).

26-week-old birds: The body weights were recorded before sacrification. Right and left testes were instantly harvested after sacrification, the epididymis was removed, and the testes' weights were recorded. The tissues were fixed in mDF's solution for 24 h. Then, the left testis was dissected into 2-mm-thick samples perpendicular to its long axis (8-10 disks for each testis). Two separate disks selected for each animal were embedded into 2- hydroxy-methacrylate (Technovit 7100). Eight to ten serial sections of 25 µm thickness were obtained from each methacrylate block in every 200-µm section interval using tungsten steel knives, then stained with periodic acid-Schiff (PAS).

Stereological Analyses: Stereological measurements were performed using the Stereo Investigator Version 9 (MBF Bioscience USA) Image Analysis Program. A stereological workstation, including a digital camera, an automatically controlled specimen stage, a light microscope (Leica, DM400B), and a software program, were used to count cells.

Estimating absolute nuclear volume of Sertoli, germ, and Leydig cells in one-day-old chicks: The volume fraction of Sertoli, germ, and Leydig cells within testis volume on 1-µm-thick sections was determined by the standard point-counting method. Ten microscopic fields (100X, Apoplan) on four separate sections were evaluated for each animal. The counting was performed using a graticule of 396-point grids. The points falling onto Sertoli, germ, and Leydig cells were counted in each microscopic field, and the total number of the points was expressed as percentage values (=3960). The weight of the testis measured was converted to testis volume (cm^3) by the equation testis volume = testis mass (g) / specific weight. Each animal's absolute volume per testis was calculated by multiplying the percentage volume (volume density) by testis volume, assuring that shrinkage was minimal and the testis weight before and after fixation was comparable (Bozkurt *et al.,* 2009).

Determination of Sertoli cell numbers in one-day-old chicks: As previously described, the Sertoli cell number for each chick was determined by the nucleator method (Wreford, 1995). According to the method, nucleolus-nuclear border radii in four separate directions were measured for each nucleus of randomly selected 50-70 Sertoli cells, and then a mean value was calculated using the standard equation. The Sertoli cell number per testis per chick was obtained by dividing the absolute Sertoli cell nuclear volume by the mean Sertoli cell nuclear volume.

Estimating Sertoli, germ, and Leydig cell numbers in 26-week-old birds: The Sertoli, germ, and Leydig cell numbers for each testis were determined by the optic dissector method (Wreford, 1995). The germ cells were distinguished as spermatogonia, primary, round, and elongated spermatids according to their morphological features and localization in the tubule, assuming the number of nuclei equals the cell number. The tissue sections embedded in 25-µm-thick methacrylate were optically sectioned by a NA 1.40 100X objective. The counting was standardized by a pilot study. The numeric values of the grid sampling area (X-Y- axis; 1210000 μm^2), counting frame area (X-Y axis; 400 μm^2), upper guard zone of the counting frame (µm), and the dissector height (Z-axis) was uploaded to the software for each animal. The same parameters were utilized automatically for all sectioned samples. An unbiased counting frame (the area of a 400 μm^2-counting frame) was inserted onto the surfaces of the testicular tissue monitored on the screen. Establishing a frame avoids the "edge effect" and biased counting, enabling all nuclei profiles to be counted by the frame regardless of their size and shape, assuring an equal sampling for all particles. According to the principles of an unbiased counting frame, cells within the counting frame, or those intersecting only with the free but not restricted lines or their extensions, were marked with the cursor and counted. The upper surface of the section was adjusted to the focal point, and the upper guard zone was set to 3 µm to avoid potential surface imperfections. A sequential 15 µm of the section was moved downward in the Z-axis and scanned step-wise by the computerized and motorized scale (ensuring that the distance between adjacent optic areas was 1 µm). The nuclei were focused within the counting frame and counted when focused, and total Sertoli, germ, and Leydig cell numbers were estimated for each animal.

Semen analysis

Semen collection: Before sperm collection, the birds were familiarized with abdominal massage (initiated at 17 weeks) (Talebi *et al.,* 2018). The semen samples were assessed for volume, motility, concentration, viability, and morphological features according to the previously proposed method (Tabatabaei *et al.,* 2009). After collection, semen samples were incubated in a warm water bath at 37 °C. The criteria for high-quality sperm are as follows: semen volume = 0.2-0.6 ml, sperm concentration = $\geq 3 \times 10^9$ spermatozoa/ml, total motility = ≥80%, and morphology = ≤10% abnormal sperm.

Semen Volume: The volume (ml) of the semen collected via abdominal massage was measured using an automatic pipette.

Sperm Motility: The collected semen samples were instantly diluted (1: 200) in a modified Ringer solution (NaCl: 68 g, KCl: 17.33 g, CaCl: 6.42 g, MgSO4: 2.50 g, NaHCO3: 24.50 g and 10000 mL of distilled water), and pipetted onto a glass slide covered with glass cover. Sperm motility was estimated with at least 200 spermatozoa on ten microscopic fields at 400x by a phase-contrast microscope with a heating stage. The motility was defined as the percentage value of the spermatozoa with moderate to the rapid progressive movement. The motility was subjectively evaluated by an expert.

Sperm Concentration: The semen samples diluted in Hancock solution were placed onto a Thoma glass slide. The number of spermatozoa on the five large squares of a Thoma counting chamber was assessed at 400x, and the sum was multiplied by 10.000 to calculate the sperm concentration per 1 ml.

Sperm Morphology: A morphological evaluation provides a preliminary opinion concerning the potential fertility of spermatozoa. The morphological abnormalities are categorized in terms of the anatomic organelle of a spermatozoan, including the acrosome, head, midpiece, and tail. For this purpose, three drops of the specimens were added into the Eppendorf tubes containing 1 ml of Hancock solution (62.5 mL formalin (% 37) + 150 mL sodium saline + 150 mL buffer + 500 mL double distilled water). One drop of the mixture was placed onto a glass slide and covered with cover glass. A total of 200 spermatozoa were counted on each slide. The percentage values of acrosomal and total structural anomalies were evaluated by phase-contrast microscopy (100x HPF, with oil immersion).

Hormone Analyses: Plasma testosterone, LH, and FSH levels were analyzed by the ELISA method using commercial kits according to the manufacturer's instructions (Cusabio, China).

Statistical Analyses: Data were analyzed by the one-way analysis of variance (ANOVA) followed by a Duncan test using the SPSS 11.0 program. The level of significance was established at $P<0.05$.The hatching rate was compared by the chi-square test.

RESULTS

Hatching rate: No statistically significant difference (P=0.527) was noted (based on the chi-square test) between the groups regarding the hatching rate and male chicks (P=0.474) (Table 1).

Morphometric evaluations: Considering one-day-old chicks, no statistically significant difference was determined in body weight (P=0.082), weights of the left (P=0.644) and the right (P=0.248) testes, gonadosomatic index (P=0.863), and testis weight asymmetry (P=0.242) (Table 2).

Considering 26-week-old birds, no statistically significant difference was determined in body weight (P=0.136), weights of the left (P=0.516) and the right (P=0.349) testes, gonadosomatic index (P=0.802), and testis weight asymmetry (P=0.943) (Table 2). A statistically significant difference was noted regarding the comb height ($P\leq$ 0.001). The post-hoc Duncan test revealed differences between the control and experimental groups (Figure 1).

Stereological Analysis

One-day-old chicks: No statistically significant difference was noted between the experimental and the control groups regarding the absolute volume (mm^3) of Sertoli (P=0.363), germ (P=0.173), Leydig cells (P=0.098), and Sertoli cell number (P=0.179) (Table 3).

26-week-old birds: No statistically significant difference was noted between the experimental and the control groups regarding the number of Sertoli cells (P=0.393), spermatogonia (P=0.581), (primary (P=0.686), round (P=0.557), and elongated spermatids (P=0.666), and Leydig cells (P=0.521) (Table 4).

Sperm analysis: No statistically significant differences regarding semen quality parameters were detected between the experimental and control groups in 26-week-old birds ($P>0.05$) (Table 5).The semen samples were evaluated for volume (P=0.176), motility (P=0.559), concentration (P=0.204), viability (P=0.212), and morphological features (acrosome (P=0.750), head (P=0.625), middle (P=0.403), tail (P=0.651) (Table 5).

Hormone analysis: Regarding the LH (P=0.544) and testosterone (P=0.464) levels of one-day-old chicks and those of 26-week-old birds (P=0.203 and P=0.806, respectively), no statistically significant difference was noted between the experimental and control groups.

The plasma FSH (P=0.038) levels of 26-week-old birds were higher in all boric acid groups than in the controls (Table 6).

Table 1. Effect of in ovo-exposure to boric acid on indices of general toxicity in the domestic hatching fowl

Groups	Number of eggs	Number of Hatched chicks	Hatching rate %	Number of Hatched male chicks	Hatching rates (%) of male chicks
Control	88	62	70.45	27	43.55
1000 ppm	93	63	67.74	34	53.97
1500 ppm	103	65	63.11	29	44.62
2000 ppm	101	73	72.28	30	41.10
P values			*P*=0.527		*P*=0.474

Table 2. Effect of in-ovo-exposure to boric acid on testis development in one-day- and 26-week-old birds.

Groups	n	Body Weight (g)		Left testis Weight (g)		Right testis Weight (g)		Gonadosomatic Index (Left Testis Weight/Body Weight)		Testis Weight Asymmetry (Left Testis weight /Right Testis Weight)	
		Day one Mean±SE	26 weeks Mean±SE	Day one Mean±SE	26 weeks Mean±SE	Day one Mean±SE	26 weeks Mean±SE	Day one Mean±SE	26 weeks Mean±SE	Day one Mean±SE	26 weeks Mean±SE
Control	10	42.1±0.89	2025.0±23.81	0.0040±0.00046	14.68±1.086	0.0035±0.00025	14.40 ±0.90	0.000084±0.000006	0.0072±0.00054	0.947±0.0674	0.989±0.0237
1000 ppm	10	42.0±1.02	1980.±31.52	0.0036±0.00043	13.86±0.77	0.0039±0.00052	13.24±0.64	0.000092±0.000013	0.0070±0.00038	1.052±0.0691	0.967±0.0374
1500 ppm	10	39.3±0.54	1949.4±40.32	0.0033±0.00017	13.64±0.87	0.0030±0.00022	13.35±0.90	0.000077±0.000005	0.0069±0.00039	0.921±0.0739	0.983±0.0372
2000 ppm	10	41.9±0.93	1930.0±15.65	0.0035±0.00085	12.90±0.54	0.0030±0.00024	12.43±0.55	0.000085±0.000006	0.0066±0.00026	0.859±0.0564	0.966±0.0254
P values		*P*=0.082	*P*=0.136	*P*=0.644	*P*=0.516	*P*=0.248	*P*=0.349	*P*=0.863	*P*=0.802	*P*=0.242	*P*=0.943

Mean±SE, SE: standard error

Table 3. Nuclear volume density of Sertoli, germ, and Leydig cells and estimation of the number of Sertoli cells in the left testis of one-day-old chicks

Groups	n	Sertoli cell absolute nuclear volume (mm^3)	Germ cell absolute nuclear volume (mm^3)	Leydig cell absolute nuclear volume (mm^3)	Sertoli cell number
		Mean±SE	Mean±SE	Mean±SE	Mean±SE
Control	10	20846117 ±1624902.9	20148499 ±2139080.5	23654777 ±2731011.4	313806 ±25718
1000 ppm	10	18965699 ±965170.8	16707565 ±1483508.0	26007050 ±1471122.3	256336 ±12105
1500 ppm	10	17115801 ±1200096.8	16082973 ±1308894.4	20438672 ±2320632.7	244731 ±15096
2000 ppm	10	17987614 ±2145464.1	14991810 ±1531685.7	18479678 ±2010766.3	288316 ±36331
P values		*P*=0.363	*P*=0.173	*P*=0.098	*P*=0.179

Mean±SE, SE: standard error

Table 4. The numbers of Sertoli cells, Spermatogonia, Primary, Round, and Elongated Spermatids, and Leydig cells in 26-week-old birds

Groups	n	Sertoli cell	Spermatogonia	Primary Spermatid	Round Spermatid	Elongated Spermatid	Leydig cell
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Control	8	46206 ±23975	183534 ±109429	260171 ±159523	820386 ±436100	363379 ±147253	87414 ±47106
1000 ppm	8	28574 ±10011	90481 ±28545	87592 ±46595	389331 ±147797	173353 ±100514	42576 ±21853
1500 ppm	8	19302 ±5542	75509 ±18562	160371 ±49169	348837 ±84847	288000 ±86588	42023 ±10128
2000 ppm	8	10498 ±4592	74780 ±28909	133806 ±45726	361365 ±142432	217797 ±80043	28376 ±9388
P values		*P*=0.393	*P*=0.581	*P*=0.686	*P*=0.557	*P*=0.666	*P*=0.521

Mean±SE, SE: standard error

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Table 5. Sperm analysis in 26-week-old birds

Groups	n	Semen Volume (mm^3)	Motility (%)	Sperm Concentration (1/mL)	Sperm Viability (%)	Acrosome (%)	Head (%)	Midpiece (%)	Tail (%)
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Control	10	325±48.5	0.82±0.04	2300000000 ±352451730	0.767±0.040	4.40±0.64	4.5±0.62	3.8±0.74	3.9±0.70
1000 ppm	10	405±58.4	0.88±0.026	1580000000 ±184872328	0.863±0.024	3.30±0.80	4.8±0.61	4.2±0.59	4.7±0.67
1500 ppm	10	250±44.7	0.83±0.034	1830000000 ±287537437	0.821±0.031	3.60±0.82	4.2±0.65	5.4±0.83	4.2±0.65
2000 ppm	10	375±52.8	0.82±0.025	1600000000 ±192642444	0.808±0.028	3.50±0.78	5.4±0.78	4.5±0.50	3.5±0.70
P values		P=0.176	P=0.559	P=0.204	P=0.212	P=0.750	P=0.625	P=0.403	P=0.651

Mean±SE, SE: standard error

Table 6.Hormone analysis: Average concentrations of FSH, LH, and testosterone levels in one-day- and 26-week-old birds.

Groups	n	LH (mIU/ml)		Testosterone (ng/ml)		FSH (mIU/ml)
		Day one Mean±SE	26 weeks Mean±SE	Day one Mean±SE	26 weeks Mean±SE	26 weeks Mean±SE
Control	9	3.18 ±1.126	0.94 ±0.158	0.53 ±0.039	0.57 ±0.080	157,70±11,62[b]
1000 ppm	8	1.51 ±0,324	0.80 ±0.191	0.49 ±0.046	0.69±0.081	190,90±2,82[a]
1500 ppm	11	1.70 ±0.363	0.83 ±0.193	0.65 ±0.101	0.66±0.057	183,08±12,99[a]
2000 ppm	9	2.32 ±0.814	0.60 ±0.066	0.54 ±0.057	0.77 ±0.087	188,04±4,65[a]
P values		*P*=0.544	*P*=0.203	*P*=0.464	*P*=0.806	*P*=0.038

Mean±SE, SE: standard error

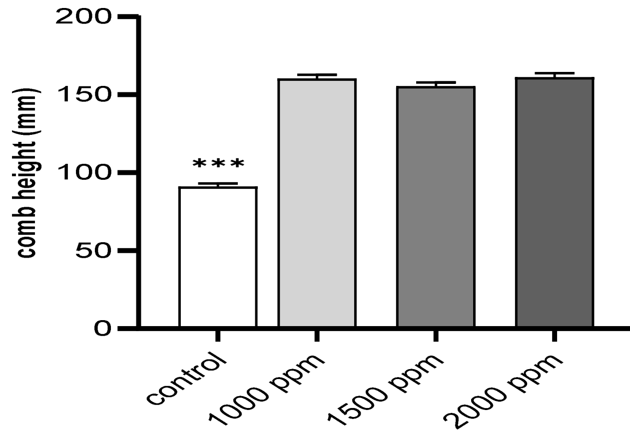

Fig 1. Comb height (mm) of 26-week-old birds. Data are presented as mean±SE.; ***: P ≤ 0.001 indicates the effects of boric acid treatment. Control group animals' comb height was lower than those of boric acid-treated groups.

DISCUSSION

Wildlife birds are primarily exposed to environmental pollutants; therefore, a bird embryo serves as a very efficient study model for investigating environmental pollutants. Layer birds secrete persistent lipophilic compounds into the egg through the egg yolk. Reproductive toxicity, including embryonal mortality and malformations, is a well-documented indicator of such pollutants in birds (Berg *et al.,* 1999).

The egg model of the present study was designed to investigate the potential effects of boron compounds on reproductive toxicity. In our preliminary study, three doses (500, 750, and 1000 ppm) of boric acid were injected into the egg, and none of the doses impacted the hatching rate. Therefore, in our main study, the dose of boric acid was established at a minimum level of 1000 ppm and continued with 1500 and 2000 ppm (=1 mg/ml, 1.5 mg/ml, 2 mg/ml in 0.9% NaCl respectively). However, these doses used in our study did not significantly affect the hatchability rate. The teratogenic effect of in-ovo injection of 2.5 mg of boric acid on the 13.5th day of embryogenesis was previously reported in different avian species (White Leghorns, White Minorcas, Black Minorcas, and Silver Grey Dorkings) (Landauer, 1952). On the other hand, the in-ovo injection of 0.15 mg of boric acid did not exhibit any teratogenic effect; on the contrary, it positively affected bone mineralization and growth in Mallard ducks (King *et al.,* 1993). Low doses (30 ppm) of dietary boric acid did not affect Mallard ducks. In contrast, intake of 300 and 1000 ppm of boric acid reduced the hatchability of fertile eggs and body weights (Smith and Anders, 1989). Therefore, boric acid reveals a dose-dependent effect on the hatching rate, which is compatible with our findings.

In our study, the doses used revealed no effect on body weights or the weights of testes, neither after hatching nor at puberty. To our knowledge, this is the first study investigating the effects of in-ovo injected boric acid on relevant parameters in birds. In a previous study conducted on rats, a diet supplemented with 3000, 4500, 6000, and 9000 ppm of boric acid for nine weeks exhibited a reduction in the testicular weights, compatible with the atrophic morphological changes (Ku *et al.,* 1993). Similarly, when the mice were exposed to 1.2 mg/L (0.035 mg B/kg/day) of boric acid through drinking water for five days, body weights were reduced by 28% (Aysan *et al.,* 2013). In another study, rats received 300 and 500 mg/kg (53 and 88 mg B/kg) of boric acid via oral gavage for 2-4 weeks. Both doses reduced the weights of the testes and epididymis and caused histopathological changes. The lowest observed adverse effect level (LOAEL) was determined as 53 mg B/kg/day (Fukuda *et al.,* 2000). Furthermore, rats were orally administered 5, 10, and 20 mg/kg/day of boric acid between the 6th and 21st days of gestation. Then the offspring were treated with the same doses of boric acid between the first and 28th days, and body weight was decreased with the highest boric acid concentration (Watson *et al.,* 2020). These studies mentioned above investigated the effects of the dietary intake of boric acid; however, the present study is an experimental design with in-ovo administration of boric acid, which revealed no impact either on body or testicular weights; therefore, despite the conflicting results in the literature, the in-ovo effect of boric acid are novel findings to be further investigated. Nevertheless, we consider that the effect of boric acid on the body and testes weights changes depending on the application method, dose, and species.

In our study, we detected a numerical decrease in the number and absolute volume of Sertoli cells and, thus, in the absolute volume of germ cells in one-day-old chicks with increasing doses of boric acid. Similarly, there is a numerical decrease in the number of Sertoli and Leydig cells, spermatogonia, and round spermatids in 26-week-old birds. However, these differences were not statistically significant due to high individual variation. In a previous study, moderate and high levels of boron were reported to have reduced the primary spermatocytes in ostrich (Wei *et al.,* 2021). Furthermore, Lee *et al.* (1978) previously documented the adverse effect of boric acid on these parameters in rodents. Significant germinal epithelium loss, reduction in the diameter of seminiferous tubules, and boron deposits were monitored in rats that received dietary 1000 and 2000 ppm of boron during a chronic exposure period of 30-60 days, and 60-day exposure to 2000 ppm boron resulted in depleted germ cells (On the other hand, no adverse effects with 500 mg/kg boric acid (88 mg B/kg/day) recognized as NOAEL (no-observed-adverse-effect level) were demonstrated on spermatogenesis and sperm quality in

male rats (Linder *et al.,* 1990). In another study, the lesions of the testes were evaluated in 32-week-old male rats after dietary exposure of 3000, 4500, 6000, and 9000 ppm of boric acid for nine weeks. 3000/4500 ppm inhibited spermiation, while 6000/9000 ppm caused testicular atrophy (Ku *et al.,* 1993). Although these studies focused mainly on chronic exposure to high doses of boric acid in rodents, the results are in accordance with the numerical decreases we detected in our study.

In the present study, boric acid quantitatively reduced semen concentration despite the insignificance in statistical evaluations due to high individual variations; however, sperm motility remained unchanged, and no morphological abnormalities were detected, which revealed that boric acid did not adversely affect semen quality parameters. There are a few reported studies concerning the subject, and to our knowledge, no studies were conducted on birds. Moreover, the results obtained from different species are controversial, as can be assumed based on the following studies. For instance, dietary intake of 1000, 4500, and 9000 ppm of boric acid reduced sperm motility in mice, and testicular atrophy was demonstrated with its median and high doses of boric acid (Chapin and Ku, 1994), which could be attributed to the acute toxicity of high doses of boric acid on gonads. Similarly, sperm count, motility, and morphology were adversely affected by the median and highest doses of boric acid in rats that were orally administered boric acid in three different doses (50, 150, and 500 mg/kg/day) for three weeks (Yoshizaki *et al.,* 1999). Likewise, the number of sperms and sperm motility were reduced after a four-week exposure with the two highest concentrations (250 and 500 mg/kg) via oral gavage and retention of step 19 spermatid within the stages IX-XI of spermatogenesis was monitored in rats (Kudo *et al.,* 2000). On the contrary, dietary boron administration exhibited favorable outcomes concerning spermatological parameters in rabbits (Elkomy *et al.,* 2015) and goats (Krishman *et al.,* 2019). On the other hand, no adverse effects of boric acid were detected regarding the relevant parameters in humans, and the spermatological parameters remained unchanged in the boron workers chronically exposed to boron (Robbins *et al.,* 2010), which was compatible with our findings. Similarly, in a study conducted on 1000 men working in the boron mines in Northeast China, boron intake by drinking water was assessed as 125 mg/kg, and no statistical significance was detected regarding semen properties except for a reduction in X/Y chromosome ratio (Sciallie*t al.,* 2010). Likewise, a study investigating the potential effects of boron on reproductivity was conducted on a population of workers recruited in the boron mines in Turkiye. No adverse effects were monitored concerning reproductive toxicity parameters, such as morphological abnormalities in spermatozoa, sperm motility and concentration, and blood hormone (FSH, LH, and testosterone) levels (Duydu*et al.,* 2011). We consider that despite the similarities in the findings of these studies with our results, it is improbable to propose consistency since the present study is experimental research investigating the single application of boric acid in the embryonic period, and the controversial findings are associated mainly with the utterly different exposure route and species-wise variations.

FSH binding activates at least five signalization cascades: cAMP-PKA, MAP kinase, phosphatidylinositol 3-kinase (pI3-K), calcium, and phospholipase A2 (PLA2), in Sertoli cells. Although it has been known that FSH affects Sertoli cells, the mechanism and direction of this effect on birds are not precise yet (Vizcarra *et al.,* 2022). In our study, FSH levels were increased in experimental groups, and the difference between the experimental and control group was statistically significant. Chronic exposure to high doses of boron added to drinking water increased FSH levels in ostrich (Wei *et al.,* 2021), which is also compatible with our findings, pointing out the dose-dependent increase in FSH expression. We found a statistical increase in FSH and a numerical increase in testosterone levels, while Sertoli cell numbers decreased. Changes in the number of FSH receptors on cells in the hypothalamic-hypophysial-testicular axis and on the Sertoli cells may explain this phenomenon. Further studies should be conducted to investigate the FSH receptor status to explain such a change in FSH expression. Elevations in serum FSH levels were previously associated with testicular atrophy in rats (Ku *et al.,* 1993). The decrease in Sertoli cell numbers likely causes a decrease in inhibin levels secreted from Sertoli cells, which induces the negative feedback mechanism between inhibin and FSH; therefore, a possible decrease in inhibin levels might have caused the relevant increase in plasma FSH levels. Increased FSH levels in our study may be associated with quantitatively decreased Sertoli cells.

The testosterone levels increased quantitatively in the experimental groups of 26-week-old birds, whereas LH levels of one-day- and 26-week-old birds were decreased with the increasing doses of boric acid. The absolute volume of Leydig cells in one-day-old chicks and the number of Leydig cells in 26-week-old birds were reduced in line with the plasma LH level, which may be associated with the feedback phenomenon between the number of Leydig cells and the plasma LH level, playing a significant role in the hormonal mechanisms of testes. However, in contrast to our data, the authors found increased LH levels in low boron groups. In another study, boric acid decreased plasma testosterone while increasing the FSH level in rodents (Fail *et al.,* 1998). The differences may be due to the application method, dose of boric acid, and species-specific differences.

The comb height and color are associated with sperm quality parameters, including sperm concentration, motility, and vitality. The comb color is positively correlated with sperm vitality. In contrast, the comb height is negatively correlated, and thus, birds with the tiniest red-colored combs exhibited the highest percentage value for live sperms (Navara *et al.,* 2012). In our study, even though the difference in comb height revealed a statistical significance ($P \leq 0.001$), no difference was noted regarding sperm vitality which might be associated with the non-hazardous effect of boric acid on fertility. Therefore, we may conclude that no effect of boric acid was monitored on sperm quality parameters even though plasma testosterone levels might have raised at certain intervals, manifested as an increase in the comb height.

Conclusion: The potential effects of boron and boron compounds on reproductivity and reproductive toxicity are still polemical. Whether the boron compounds widely found in the environment and nature might be justified to be included in the category of reproductive toxins is still in debate. Thus, the ongoing research concerning boron compounds is focused on developing in-vitro testing methods regarding reproductive toxicity.

The potential effects of boron directly on the testes of birds have yet to be investigated. In the present study, three separate non-teratogenic doses of boric acid administered before gonad differentiation revealed no adverse effects on testis morphological and stereological parameters after hatching and at puberty; yet, increased comb height, plasma FSH levels and quantitatively affected the number of Sertoli, germ, and Leydig cells even though a statistical significance could not be defined. This study might contribute to further research targeting boric acid's multifaceted effects on the dynamics of spermatogenesis.

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