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Dietary boron supplementation enhances sperm quality and immunity through influencing the associated biochemical parameters and modulating the genes expression at testicular tissue

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ABSTRACT

Introduction: Dietary boron improves immune and antioxidant status and calcium metabolism in mammals. However, till date the effects of dietary boron supplementation on male reproduction, especially on sperm production and sperm quality in farm animals are not documented.

Objective: The present study was aimed to investigate the influence of dietary boron on semen production, semen quality, immunity and molecular changes in the testis, blood and seminal plasma and to assess the inter-relationship with other minerals in male goats.

Methodology: The study was conducted in 21 adult male goats divided into 3 groups (control, boron and selenium supplemented groups, n = 7 each). In boron group, boron was supplemented at 40 ppm and in selenium group, selenium was supplemented at 1 ppm over and above the basal level. In control group, only the basal diet was fed without supplementary boron or selenium. The feeding trial was carried out for 60 days. Selenium was taken as a positive control for the dietary boron supplementation experiment. Following feeding trials, the sperm concentration, kinematics and functional attributes, immunity and molecular level changes in the testis, biomolecular changes in the blood and seminal plasma and also interrelationship with other minerals were studied.

Results: The average sperm concentration (million/ml) and the total sperm production (million/ejaculate) were significantly ($p < 0.05$) increased in boron supplemented group when compared to selenium and control groups. The boron levels in blood plasma ($r = 0.65$) and seminal plasma ($r = 0.54$) showed a positive correlation with sperm progressive motility. Blood and seminal plasma metabolic biomarker namely, aspartate aminotransferase (AST) ($p < 0.01$) was significantly lower in the boron and selenium supplemented group than control, while alanine aminotransferase (ALT) ($p < 0.05$) was significantly lower in the boron supplemented group than selenium and control group. There was a significant increase in the mRNA expression of serine proteinase inhibitor (*SERPIN*) and interferon γ (*IFNγ*) in the testis of boron supplemented than the control group. Boron supplementation up-regulated the immune-regulatory gene, interleukin 2 (*IL2*) and antioxidant gene, catalase (*CAT*) in the peripheral blood mononuclear cells (PBMC). On contrary, toll-like receptor 2 (*TLR2)* mRNA expression was significantly ($p < 0.05$) down-regulated in boron and selenium supplemented groups.

Conclusion: The study revealed that dietary boron supplementation increased the sperm output, sperm motility and enhanced the immune and antioxidant defense capacity in male goats. The improved semen quality can be attributed to enhanced expression of testicular *SERPIN*, a crucial protein for the regulation of spermatogenesis process.

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1. Introduction

Boron is essentially required for physiological functions of plant and animals. The functional significance of various macro and micro minerals was studied in animal production [1,2] but the beneficial role of boron is least understood [3,4]. The research on the beneficial role of boron in higher animals started in 1990s when the boron was found to be associated with bone disorders [5]. Though the definitive role of dietary boron on physiological functions has not been established in mammals, boron supplementation influences bone growth and maintenance, central nervous system, endocrine and reproductive functions [6]. Boron enhanced Interleukin 6 (IL-6) production in mammalian cultured cells [7]. It regulated the enzyme activity of serine protease [8]. Boron supplementation also improved immune and antioxidant status and calcium metabolism [3,9,10].

Boron has regulatory role in utilization of different macro and micro nutrients [11]. In calcium deficient conditions boron ameliorated oxidative stress and enhanced cellular and humoral immunity [3]. In rabbits, boron feeding improved total antioxidant capacity, sperm quality [12] and testosterone concentration [13]. Boron deficiency affects embryo development in *Xenopus laevis* [14] and Zebra fish [15]. However, at very high doses, boron might affect various systems including reproduction [16]. Perusal of literature revealed paucity of information of dietary boron on male reproduction, sperm production and sperm quality in farm animals. Till date, the effect of boron on male reproduction and mechanisms in regulating sperm production and function are not documented in ruminants. The present study was aimed to 1) investigate the influence of dietary boron on semen production, semen quality, immunity and molecular changes in the testis, blood and seminal plasma and 2) assess the interrelationship with other minerals in male goats.

2. Materials and methods

2.1. Experimental design and feeding trial

The study was conducted in 21 adult male goats divided into 3 groups of seven in each group (control, boron and selenium supplemented groups, n = 7). The goats were maintained in the Experimental Livestock Unit of ICAR-NIANP, Bangalore. The average weight (kg) of these goats were 27.82 ± 1.11, 27.72 ± 0.70 and 27.75 ± 0.79 in control, boron and selenium supplemented groups, respectively before the starting of the experiment. In boron group, boron (sodium tetra borate, SRL, Mumbai, LR grade) was supplemented at 40 ppm and in selenium group, selenium (Selplex, Altech, Bangalore) was supplemented at 1 ppm over and above the basal level in the diet. Boron and selenium were administered orally once daily before morning feeding. Care was taken to ensure complete ingestion of the minerals. In control group, only the basal diet was fed without supplementary boron or selenium. The feeding trial was carried out for 60 days. The basal diet fed commonly to all the groups consisted of 55% roughage and 45% concentrate. The dry matter as maize stover and concentrate mixture was offered to each animal at the rate of 3% of body weight. The concentrate mixture (CP- 20% and TDN- 70%) consisted of crushed maize grain 40%, soybean meal 30%, wheat bran 27.5%, mineral mixture (without added boron and selenium) 2% and common salt 0.5%. Maize stover was chaffed and fed as a source of roughage. The animals were provided with fresh and clean drinking water *ad libitum*.

2.2. Blood and semen sample collection

Semen samples were collected before and after the feeding minerals (boron and selenium) supplementation. Following feeding trials macro minerals such as, calcium (Ca), phosphorus (P), magnesium (Mg) and micro minerals such as, cobalt (Co), zinc (Zn), manganese (Mn), copper (Cu), boron (B) and selenium (Se) levels in the blood and seminal plasma were estimated. Semen collection was carried out using electro ejaculation method and collected semen was maintained at 37 °C and semen analyses were carried out within 15–20 min of collection.

2.3. Assessment of seminal attributes

2.3.1. Sperm kinematics

The motility and velocity parameters of neat semen were assessed using computer-assisted semen analyzer (CASA, Sperm Class Analyser, Microptic, Barcelona, Spain). Five consecutive digitalized images of 25 frames per second were captured using 10X negative phase-contrast objective (50i Nikon microscope, Nikon, Japan) and analyzed by classifying the spermatozoa based on average path velocity (VAP). The kinematic parameters were analysed by setting the following parameters: speed type-VCL, cell size-5–70 μm^2, spermatozoa speed with < 10, 10–35, 35–60 and > 60 μm/s were classified as immotile, slow, medium and fast motile, respectively. The various parameters obtained from CASA analysis were progressive forward motility, rapid progressive forward motility, total progressive motility, total motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), percentage of linearity (LIN), straightness (STR) and percentage of type A spermatozoa (amplitude of lateral head displacement = > 2.5 μm, STR = > 80%) as described earlier [17].

2.3.2. Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) in goat spermatozoa was assessed by JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′- tetraethyl benzimidazolyl carbocyanine iodide) staining [18]. One microlitre (1.53 mM in dimethyl sulfoxide) of JC-1 stain was added to 50 μl PBS (pH = 7.4) containing one million spermatozoa and incubated for 30 min at 37 °C in a dark room. Then, the samples were mixed gently and smeared on to glass slides and examined under epifluorescence microscope (Nikon Eclipse 50i; Nikon Corporation, Tokyo, Japan) with excitation filter of 510–560 nm and barrier filter of 505 nm in dark room. A minimum of 200 spermatozoa was observed at 100X magnification in all samples. The spermatozoa exhibiting more than 80% yellowish to orange fluorescence in the mid-piece due to formation of J-aggregates were considered as positive for MMP.

2.3.3. Sperm acrosome integrity

Acrosome integrity in the semen samples was assessed as per procedure described earlier [19] with minor modification. Ten million spermatozoa in 50 μl PBS were incubated at 37 °C for 20 min in dark by adding 0.8 μl carboxyfluorescein diacetate (CFDA) (4 mg/ml DMSO). Following this, 5 μl propidium iodide (PI) (0.27 mg/ml in PBS) was added to the sample and incubated for another 10 min at 37 °C. After incubation, the spermatozoa were washed twice by adding 2 ml of PBS (pH = 7.4) to the sample centrifuged at 1500 rpm for 5 min each. The supernatant was removed by leaving 100 μl of the sample containing spermatozoa in the tube. The cells were mixed well and the smear was made on to the glass slide and observed under the epifluorescence microscope (510–560 excitation, 505 emission). The spermatozoa showing complete green fluorescence were considered as acrosome intact and those showing partial or complete red nuclei were classified as acrosome integrity lost.

2.4. Macro and micro mineral estimation

The minerals such as B, Se, Ca, Mg, Zn, Co, Mn and Cu were estimated in seminal plasma, blood plasma, sperm and testis by mild acid digestion protocol. In brief, the plasma samples (1 ml) and 2% nitric acid (1 ml) were mixed well and final volume was made up to 10 ml with deionized water. The concentrations of all the elements of the samples were analysed using ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry, Optima 8000, Perkin-Elmer, USA) [20]. Plasma P was estimated calorimetrically [21].

2.4.1. Preparation of mineral extract from testes for ICP-OES

Mineral estimation in the testes was done as per the procedure described earlier [22]. A weighed quantity (2 g) of completely dried testes was taken in pre-weighed silica crucible and subjected to decarbonisation followed by ashing at 550–600 °C in muffle furnace for 5 h. The residue left in the crucible was considered as total ash, which was digested with 5 N HCl on a hot plate. After digestion, the samples were filtered through Whatman filter paper No. 41 in a volumetric flask. The samples were repeated washed with ultrapure water (Type1, 18.2 MΩ cm) till it was free from the acid and finally the volume was made up to 50 ml.

2.4.2. Preparation of mineral extract from sperm pellet for ICP-OES

Mineral estimation in the spermatozoa was done as per the procedure described earlier with slight modification [20]. Spermatozoa pellet obtained after centrifugation (2000 rpm for 5 min) was added with 1 ml of ultrapure water (Type 1) and 3 ml of concentrated nitric acid. The samples were boiled on a hot water bath till the appearance of clear solution. Final volume was made up to 10 ml with ultrapure water (Type 1).

2.5. Blood metabolites and biochemical estimation

2.5.1. Plasma triglycerides

The triglycerides in the blood plasma samples were estimated by adopting enzymatic method (GPO-PAP endpoint) [23] using kit (Autospan liquid gold triglycerides, GPO-PAP, End point assay, 72LS100). Triglycerides were hydrolyzed by lipoprotein lipase (LPL) to produce glycerol and free fatty acid. In presence of glycerol kinase, ATP phosphorylates glycerol to produce glycerol 3-phosphate and ADP. Glycerol 3-phosphate was further oxidized by glycerol 3-phosphate oxidase (GPO) to produce dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide coupled with 4-aminoantipyrine and 4-chlorophenol to produce red coloured quinoneimine dye. The absorbance of coloured dye was measured at 505 nm and is proportional to triglycerides concentration in the sample. Triglycerides concentration in plasma was calculated using the following formula: Triglycerides (mg/dl) = (Absorbance of test/Absorbance of standard) * 200

2.5.2. Alanine aminotransferase

ALT activity (IU/L) was determined in blood and seminal plasma [24] using kit (Autospan liquid gold, ALT (alanine transferase), Modified UV(IFCC), kinetic assay, 76LS200). Pyruvate so formed from L-Alanine and α-ketoglutarate is coupled with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) to form corresponding hydrozone, a brown coloured complex in alkaline medium, which was measured at 505 nm with UV–vis spectrophotometer (Cintra 10e, GBC, Australia). Plasma ALT activity (IU/L) was calculated using the following formula: ALT (IU/L) = {(Absorbance of test – Absorbance of control)/(Absorbance of standard – Absorbance of blank)} * Concentration of standard

2.5.3. Aspartate aminotransferase

AST activity (IU/L) was determined in blood and seminal plasma [24] using kit (Autospan Liquid gold, AST (Aspartate transaminase), Modified UV(IFCC), kinetic assay 77LS200). AST catalyzes the transamination of L-aspartate and α-ketoglutarate to form oxaloacetate and L-glutamate. Oxaloacetate so formed is coupled with 2, 4- dinitrophenyl hydrazine (2, 4-DNPH) to form corresponding hydrozone, a brown coloured complex in alkaline medium, which was measured at 505 nm with UV–vis Spectrophotometer. Plasma AST activity (IU/L) was calculated using the following formula: AST (IU/L) = {(Absorbance of test – Absorbance of control)/(Absorbance of standard – Absorbance of blank)} * Concentration of standard

2.6. Antioxidant enzyme activity

2.6.1. Estimation of catalase activity

The activity of catalase in seminal plasma of the goat was measured using the protocol of [25] with minor modifications. In brief, the reaction mixture was prepared in a test tube by adding 880 µl of potassium phosphate buffer (50 mM, pH7.0) and 100 µl of hydrogen peroxide (100 µM). To this reaction mixture, 20 µl of seminal plasma was added and the change in absorbance was measured for 3 min with 20 s interval at 240 nm. The non-enzymatic reaction rate was correspondingly assessed by replacing the sample with potassium phosphate buffer. The rate of change of absorbance was recorded for enzymatic and non-enzymatic samples. The enzyme activity was calculated and expressed as units/ml/minute.

2.6.2. Estimation of superoxide dismutase (SOD) activity

The SOD activities in the seminal plasma were estimated according to the method described earlier [26] with minor modification. In brief, the reaction mixture was prepared by adding 980 µl of 50 mM triscocodylic acid buffer with 1 mM EDTA and 10 µl of 20 mM pyrogallol. The seminal plasma (10 µl) was added to the above reaction mixture. The rate of change in the absorbance at 420 nm was recorded at every 20 s interval for 3 min for both enzymatic and non-enzymatic (blank) reactions.One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation. The enzyme activity was expressed as units/ml/minute.

2.6.3. Estimation of activity of glutathione reductase (GR)

The GR activities in the seminal plasma were measured [27] with slight modifications. In brief, the reaction mixture was prepared by adding 880 µl of potassium phosphate buffer (0.2 M, pH 7) along with 20 mM glutathione oxidised solution (50 µl) and 50 µl of NADPH (2 mM prepared with 10 mM HCl). The seminal plasma (20 µl) was added to the above reaction mixture. The rate of change in the absorbance at 340 nm was recorded for both enzymatic and non-enzymatic (blank) reactions for 5 min in 30 s interval. The enzyme units were calculated and expressed as units/ml/minute.

2.7. Relative expression of transcripts in the testis and PBMC

The RNA was isolated from the goat testis and PBMC using TRIzol method [28] and the quality of the isolated RNA was estimated using spectrophotometer (Nanodrop 1000, Thermo Scientific, USA). The primers were designed using Primer-blast software (https://www.ncbi.nlm.nih.gov/tool/primer-blast) (Suppplementary Table 1). The realtime PCR (Stepone Plus, Applied Biosystems, CA 92008 USA) was carried out in 20 µl reaction using SYBR master mix (K0221, Thermo Scientific, Maxima SYBR Green/Rox PCR Master Mix, USA). The relative quantification of the genes in the goat testis samples (*SPINK2, SERPIN, G6PD, HSP90, IFN*γ and *GH*) and in the PBMCs (*CAT, IL2* and *TLR2*) were estimated using *GAPDH* as the reference gene. The relative expression of these transcripts was calculated using $2^{-\Delta\Delta CT}$ [29].

2.8. Statistical analysis

The percent mean values for various sperm attributes were arcsine transformed and subjected to statistical analysis. One-way ANOVA and LSD Post-hoc test were used to analyze the statistical difference in various sperm functional attributes, biochemical parameters and body and testicular weights estimated among the supplemented and control groups. Pearson's correlation coefficient was used to assess the correlation between sperm motility and levels of boron in blood and seminal plasma. All the values were presented as mean ± SEM. The $p \leq 0.05$ was considered as statistically significant.

Table 1
Body weight of the goats before and after dietary mineral feeding and testicular weight.

Parameters	Control	Boron	Selenium
Body weight (kg; 0 day)	27.82 ± 1.11	27.72 ± 0.70	27.75 ± 0.79
Body weight (kg; 60 day)	28.26 ± 1.06	29.05 ± 0.72	29.45 ± 0.80
Testis weight (g)	169.00 ± 14.6	185.3 ± 11.80	174.5 ± 10.7

3. Results

Dietary supplementation of boron and selenium did not influence the overall body weight change or the testicular weight (Table 1). The boron-supplemented group had significantly ($p < 0.05$) higher total sperm motility (%) and total progressive motility (%) as compared to control and selenium supplemented groups (Fig. 1). The average sperm concentration (million/ml) in the ejaculate and per ml of the semen was also significantly ($p < 0.05$) increased in boron supplemented group when compared to selenium and control groups (Fig. 2). Spermatozoa viability, acrosome integrity and MMP did not vary between control and treatment groups.

Before the start of the supplement, there was no significant difference in the levels of macro and micro minerals between the control and treatment groups either in blood or seminal plasma. The boron supplemented group showed significantly ($p < 0.05$) higher levels of blood boron, zinc and phosphorus when compared to other groups. Similarly, in the seminal plasma the calcium and boron levels were also significantly ($p < 0.05$) higher in the boron supplemented group than the control and selenium supplemented groups (Table 2a & b). As compared to control, boron supplemented animals had 2.9 fold higher boron level in the seminal plasma. However, no significant difference in the testicular and spermatozoa boron level following boron supplementation was observed (Table 2c & d). Similarly, dietary boron did not alter the level of other macro and micro minerals in the testicular tissue or spermatozoa. The boron levels in blood plasma (r = 0.65) and seminal plasma (r = 0.54) showed a positive correlation with sperm progressive motility (Fig 3). Blood plasma and seminal plasma metabolic biomarker namely, AST ($p < 0.01$) was significantly lower in the boron and selenium supplemented group than control, while ALT ($p < 0.05$) was significantly lower in the boron supplemented group than selenium and control group. Dietary selenium supplemented group also had significantly low level of blood plasma AST than the control. The blood plasma triglycerides did not show any significant difference among the groups studied (Table 3). Among the antioxidant enzymes studied, glutathione reductase (mU/ml) level was significantly ($p < 0.05$) higher in seminal plasma of the boron-supplemented group

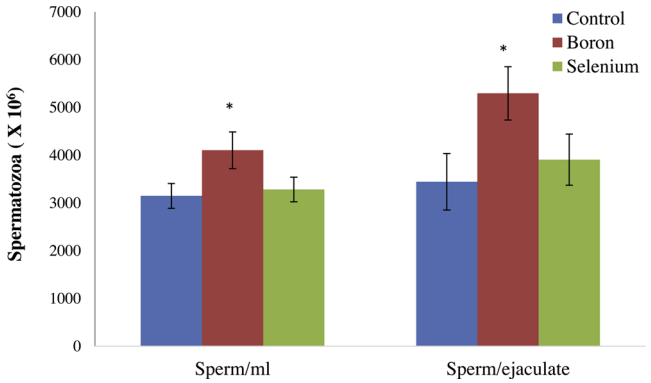

Fig. 2. Sperm concentration (millions) in semen of boron fed group when compared to selenium and control groups of goats. The dietary boron supplementation improved total spermatozoa output in the form of sperm/ml as well as sperm/ejaculate in goats.*$p < 0.05$.

(102.3 ± 14) than selenium (64.3 ± 16) and control (31.8 ± 08) groups (Fig. 4a). However, no significant differences in the seminal plasma SOD (Fig. 4b) and CAT (Fig. 4c) levels were observed among the groups.

The present study documented the presence of transcripts associated with serine protease inhibitors such as *SERPIN* and *SPINK2* in the goat testis. There was a significant ($p < 0.05$) increase in the expression of *SERPIN* (2.02 fold) and *IFN*γ (2 fold) in the testis of boron supplemented group (Fig. 5a). In the PBMC, the immune-regulatory gene, *IL2* (8.63 fold) and antioxidant gene, *CAT* (4.37 fold) expressions were significantly ($p < 0.05$) up regulated in boron supplemented group, whereas, *TLR2* (0.38 fold) expression was significantly ($p < 0.05$) down-regulated in boron and selenium supplemented groups. The expression of *CAT* and *IL2* were not affected by the selenium supplementation (Fig. 5b).

4. Discussion and conclusion

Supplementation of macro and microelements have beneficial effects on male reproduction in terms of increased sperm production, sperm quality and fertility [30,31]. However, the role of the novel trace mineral, like boron in farm animal reproductive physiology has not been not clearly documented. In present study, we have analyzed the dietary influence of boron on sperm production potential, sperm quality, molecular and biochemical effects in the testicular compartment, circulation and seminal plasma. Also, the association of boron on the levels of other macro and micro minerals as well as on sperm

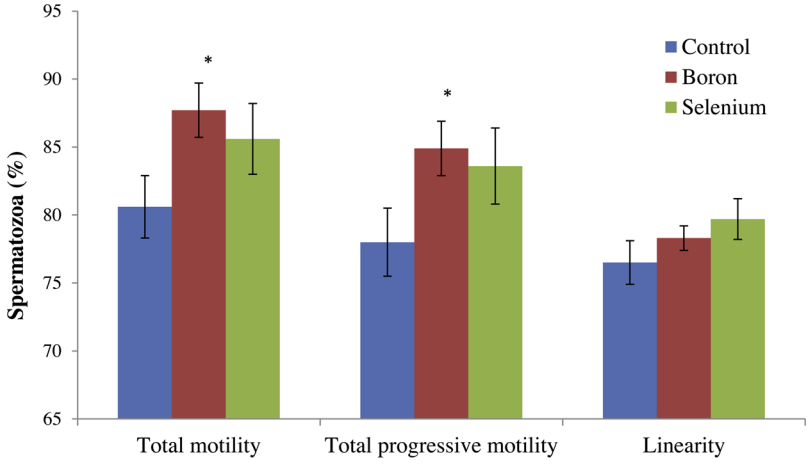

Fig. 1. The effect of dietary boron and selenium on sperm kinematics such as total motility, total progressive motility and linearity in goats. The semen samples were collected after 60 days of feeding the minerals and sperm kinematics was studied using computer assisted semen analyzer. The dietary boron supplementation improved total motile and progressive forward motile spermatozoa in goats. *$p < 0.05$.

Table 2
Macro and micro mineral profiles in the (a) blood plasma (b) seminal plasma (c) sperm and (d) testis of the goats supplemented with Boron and Selenium. These minerals were estimated in blood plasma, seminal plasma, spermatozoa and testis samples collected after 60 days of feeding.

Sl.no.	Sample	Groups	Ca (mg/dl)	Mg (mg/dl)	P (mg/dl)	Co (mg/l)	Zn (mg/l)	Mn (mg/l)	Cu (mg/l)	B (mg/l)	Se (mg/l)
a	Blood plasma	Control	8.0 ± 0.5	2.1 ± 0.2	4.1 ± 0.5^{b}	0.1 ± 0.01	0.9 ± 0.04^{b}	0.1 ± 0.0	0.8 ± 0.1	0.4 ± 0.03^{b}	0.2 ± 0.01
		Boron	8.9 ± 0.5	2.4 ± 0.2	5.1 ± 0.3^{a}	0.1 ± 0.02	1.2 ± 0.10^{a}	0.1 ± 0.0	0.8 ± 0.1	0.8 ± 0.05^{a}	0.2 ± 0.01
		Selenium	8.3 ± 0.3	2.1 ± 0.2	3.8 ± 0.3^{b}	0.1 ± 0.01	1.1 ± 0.07^{b}	0.1 ± 0.0	0.8 ± 0.1	0.3 ± 0.08^{b}	0.2 ± 0.02
b	Seminal plasma	Control	7.4 ± 0.9^{b}	5.7 ± 1.0	5.4 ± 0.6	0.7 ± 0.00	5.1 ± 1.5	0.07 ± 0.01	1.7 ± 0.4	1.5 ± 0.2^{b}	0.3 ± 0.1^{b}
		Boron	11.0 ± 0.9^{a}	6.4 ± 0.9	6.0 ± 0.4	0.7 ± 0.00	5.0 ± 1.5	0.05 ± 0.01	1.0 ± 0.4	4.4 ± 1.2^{a}	0.3 ± 0.2^{b}
		Selenium	8.1 ± 0.8^{b}	5.0 ± 0.8	6.3 ± 0.8	0.7 ± 0.00	4.8 ± 0.7	0.08 ± 0.01	1.6 ± 0.2	2.6 ± 0.3	0.8 ± 0.5^{a}
c	Sperm	Control	30.7 ± 10.7	24.6 ± 9.5	59.5 ± 16.0	4.0 ± 1.6	4.0 ± 0.4	0.29 ± 0.05	2.9 ± 1.0	2.0 ± 0.5^{a}	0.4 ± 0.1
		Boron	24.9 ± 10.4	23.3 ± 5.4	64.3 ± 14.1	3.2 ± 0.7	4.8 ± 0.6	0.30 ± 0.05	1.6 ± 0.3	1.4 ± 0.3^{a}	0.4 ± 0.1
		Selenium	23.1 ± 5.0	15.0 ± 3.0	42.8 ± 9.50	2.0 ± 0.6	4.6 ± 0.8	0.24 ± 0.03	1.4 ± 0.5	0.4 ± 0.1^{b}	0.4 ± 0.0
			Ca(%)	Mg (%)	P(%)	Co (ppm)	Zn (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)	Se (ppm)
d	Testis	Control	0.05 ± 0.02	0.08 ± 0.00	2.90 ± 0.17	0.36 ± 0.05	107.9 ± 4.87	3.13 ± 0.22	18.41 ± 4.50	8.58 ± 1.47	0.39 ± 0.04
		Boron	0.09 ± 0.03	0.08 ± 0.00	2.75 ± 0.16	0.24 ± 0.05	98.47 ± 3.92	2.92 ± 0.18	8.31 ± 0.38	6.21 ± 0.47	0.27 ± 0.05
		Selenium	0.04 ± 0.00	0.08 ± 0.00	2.81 ± 0.13	1.48 ± 0.07	88.85 ± 1.44	3.57 ± 0.20	6.74 ± 0.62	7.55 ± 0.88	0.25 ± 0.03

Values in a column with different superscripts (a,b) differ significantly ($p < 0.05$).

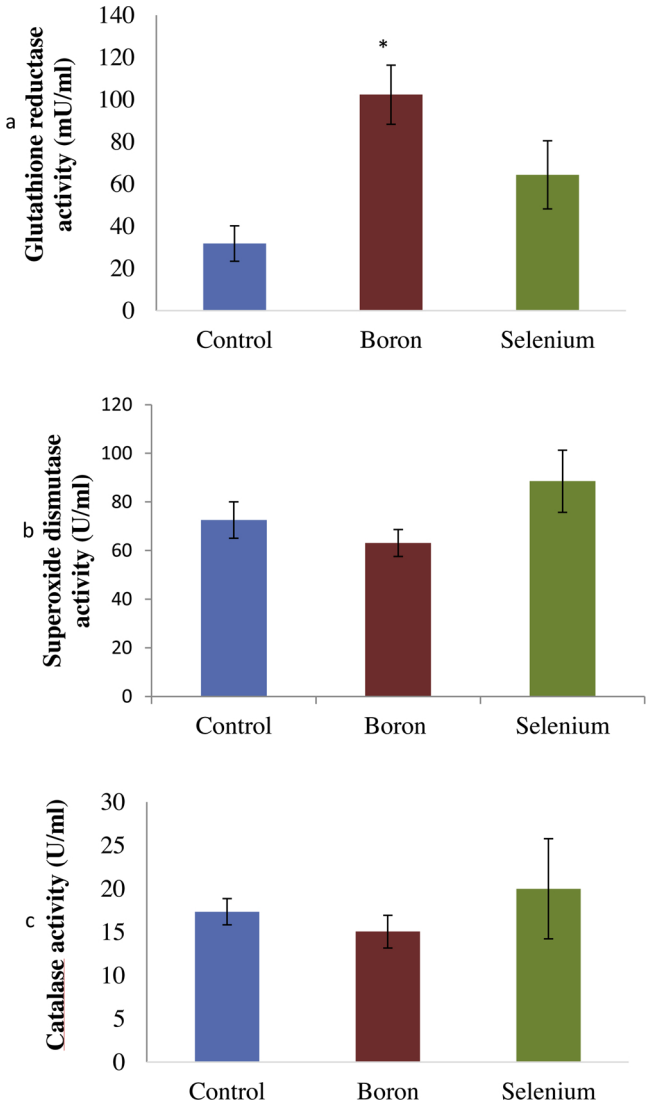

Fig. 3. Relationship between the sperm total progressive motility to the boron level in a) blood plasma and b) seminal plasma of goats. The blood and seminal plasma boron levels were significantly associated with progressive motile spermatozoa (%) in goats. $p < 0.05$.

attributes has been recorded in the present study. Selenium, an established mineral for improving the male reproductive performance was taken as a positive control for the dietary boron supplementation experiment.

Boron supplementation enhanced seminal attributes such as total sperm motility, total progressive sperm motility and total sperm output. Similar findings were observed in rabbit semen wherein boron feeding improved total sperm output and sperm concentration [12]. There was a significant positive correlation between seminal plasma and blood boron concentration with total progressive motility, indicating the beneficial effect of dietary boron on semen quality. So far, the influence of dietary boron on sperm motility was not documented in any ruminants in general and the goats in particular. The improved seminal attributes might be due to reduced oxidative stress in the testicular compartment [9] and beneficial effect of SERPIN, serine protease inhibitor proteins that favored spermatogenesis and steroidogenesis for improved quality sperm production. Serine protease inhibitor proteins protected semen against unwanted proteolysis in the reproductive tract apart from antimicrobial activity [32].

Boron supplementation augmented the immune status in goats as indicated by the increased level of antioxidant enzyme glutathione reductase in the seminal plasma and catalase in the peripheral blood. The higher total antioxidant capacity and lower MDA level have been documented upon boron feeding in rabbits [12]. Serine borate is a transition state inhibitor of gamma-glutamyl transpeptidase [33] and known to increase the intracellular glutathione level in cultured fibroblast cells [34]. The boron mediates antioxidant status through modulating SOD, GSH and catalase [7]. However, in the present study, we did not observe any significant changes in the seminal plasma SOD and CAT of boron supplemented groups. Dietary boron also increased the immunity status by increasing the testicular *IFN*γ when compared to control. Earlier study reported endogenous production of IFNα and γ by testicular cells and IFNγ protein was expressed constitutively in early spermatids [35] and IFN-α administration improved testicular spermatogenesis and increased the epididymal sperm concentration in the rat [36].

There was no difference in the body weight change in the boron supplemented group as indicated by growth. Though the dietary boron increased sperm output in the present study, there was no significant increase in testicular weight in the boron supplemented group suggesting that supplementation of boron promoted sperm output possibly by reducing sperm apoptosis. This mechanism is possible as the sperm protective *SERPIN* gene expression was higher in boron-supplemented group. Boron supplemented groups had higher blood levels of boron, zinc and phosphorus. The present study is in agreement with the earlier

Table 3
The levels of ALT (IU/L), AST (IU/L) and triglycerides (mg/dL) in blood and seminal plasma of goats supplemented with boron and selenium groups. These enzymes and triglyceride levels were estimated in blood and seminal plasma samples collected after 60 days of feeding.

Groups	ALT (IU/L)		AST (IU/L)		Triglycerides (mg/dL)
	Blood plasma	Seminal plasma	Blood plasma	Seminal plasma	Blood plasma
Control	30.34 ± 7.92[a]	41.67 ± 10.2[a]	27.78 ± 4.67[c]	32.83 ± 9.97[c]	25.17 ± 6.21
Boron	12.99 ± 2.56[b]	9.47 ± 2.3[b]	7.43 ± 4.12[d]	3.91 ± 1.29[d]	15.10 ± 2.79
Selenium	29.05 ± 7.64[a]	21.26 ± 6.7[a]	6.69 ± 2.78[d]	4.85 ± 2.59[d]	18.26 ± 3.46

Values in a columns with different superscripts differ significantly (a,b; $p < 0.05$) and (c,d; $p < 0.01$).

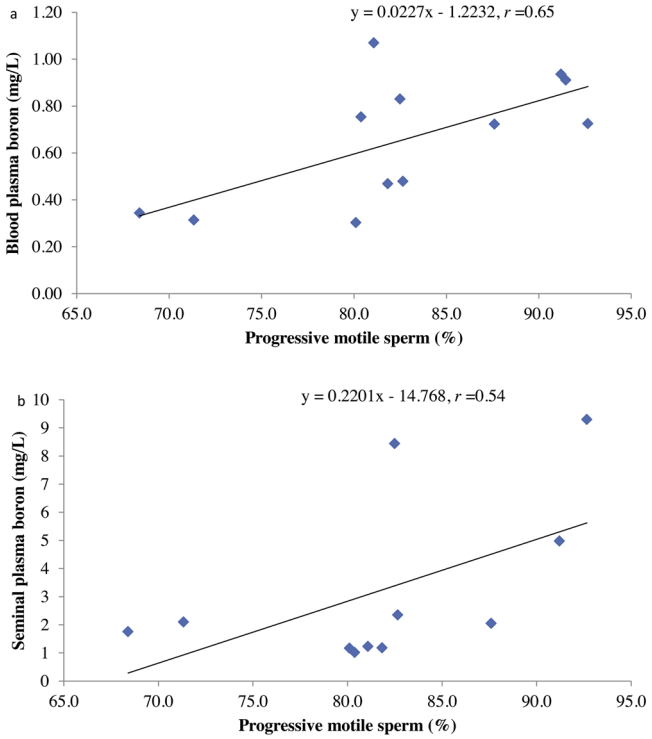

Fig. 4. Seminal plasma a) glutathione reductase activity, b) superoxide dismutase activity and c) catalase activity and in control, boron and selenium fed goats. Though the seminal plasma catalase and superoxide dismutase activities were not influenced, the glutathione reductase activity was significantly improved by boron supplementation in goats.*$p < 0.05$.

report wherein dietary boron increased the zinc concentration in the tibial bone and phosphorus level in the femoral bone of boron deficient rats [37]. The higher serum level of boron in the boron fed group in the present finding might be due to supplementation as reported in earlier study in rats [3]. In rats, boron feeding did not have any influence on the levels of serum minerals such as Zn and P, however, had antagonistic effect on Mn concentration [3].

Similarly, boron supplementation increased the concentration of seminal plasma minerals such as calcium and boron, however, did not change the concentration of other minerals namely Mg, P, Co, Zn, Mn, Cu and Se. Earlier study from this Institute and elsewhere also documented the influences of dietary boron on calcium metabolism in the bone of rat [3] and chicks [38]. In humans, dietary boron decreased the calcium excretion and increased testosterone concentration [39]. However, to the best of our knowledge, the mechanism influencing the increased concentration of calcium in seminal plasma upon boron supplementation has not been reported in any of the species.

In general, macro and micro mineral levels are higher in the seminal plasma as compared to the blood levels. The higher mineral levels in the seminal plasma have met the requirement of the sperm maturation and fertilization [40] as in the case of higher Zn level in seminal plasma was associated with good semen quality in humans [41]. Boron feeding in animals increased the levels of blood zinc and phosphorus. However, the mechanism by which the boron feeding improves blood zinc and phosphorus levels need to be studied.

We documented the expression of serine protease inhibitors such as *SERPIN* and *SPINK2* in the goat testis and the positive effect of dietary boron on the expression of *SERPIN* gene. The mechanism of action of the boron upregulating *SERPIN* in the testicular tissue needs to be elucidated. Organo compounds of boron (in the alkyl or arly forms) is proven to have serine protease inhibitor activity [42]. Serpin was reported earlier in the male reproductive tract and sperm of species such as rodents and crustaceans [43]. The Serpin acts as a sperm-decapacitating factor that protects sperm by inhibiting protease activity until sperm maturation and capacitation in the female reproductive tract [44,45]. The boron inhibits the serine proteases by forming a tetrahedral boron adduct that mimics those formed during normal substrate hydrolysis [46]. Dietary boron increased the serine protease inhibitor in the testis thus favoring the acrosome remodeling during spermatogenesis as acrosome is packed with serine protease, acrosin. Serpin functions as a scavenger of prematurely activated acrosin. The increased concentration of the spermatozoa in the boron supplemented group might be due to the protective role of Serpin favoring production of normal/viable spermatozoa or protecting spermatozoa from enzymatic digestion [47]. Whereas in human semen, other protease namely, ProMMP-9 was reported to have negative correlation with sperm concentration [48].

Dietary boron (40 ppm) did not accumulate both in the spermatozoa and the testicular tissue as the boron level did not differ in the control and treatment groups. However, following boron feeding, boron levels were significantly high in the blood circulation and seminal plasma. The probable source of boron in the seminal plasma could be from accessory sex glands or epididymal secretions. In humans, no adverse effects of boron on spermatozoa concentration, motility, morphology as well as blood hormones including follicle stimulating hormone, luteinizing hormone and testosterone have been reported [49]. Interestingly, the sperm DNA integrity was protected in the boric acid/borate exposed men indicating beneficial effect of boron [50]. Boron accumulation in semen over blood concentration is in accordance with a recent human study [51]. Such increase in the boron levels in biological fluids might have favored for the beneficial effect on semen quality and sperm production through molecular changes in the gene expression such as *SERPIN* and *IFN*γ. Earlier study is in accordance to our study wherein dietary boron in human at the rate of 8.41 mg/day did not cause accumulation in the body, but had anti-cancerous properties [52]. We also observed changes caused by dietary boron supplementation in the blood and seminal plasma levels of other macro and micro minerals, however, no alterations were observed in the sperm and testicular mineral levels.

Low levels of AST and ALT in the blood plasma may be attributed to the reduced stress in the dietary boron supplemented animals as these enzymes levels were high under stress conditions [53] and dietary boron has immune and antioxidant properties thereby reducing oxidative stress in animals [9]. Such action also might have increased the sperm production and function in the present study.

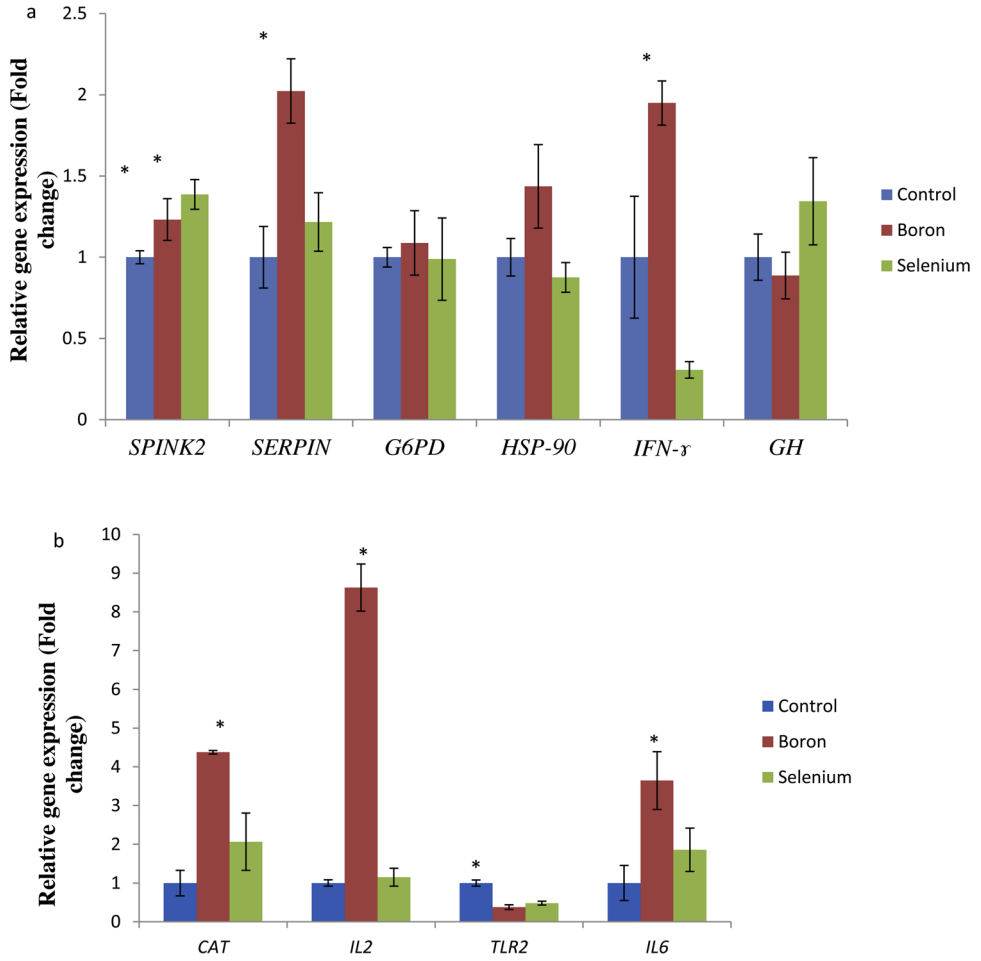

Fig. 5. Influence of dietary boron on the relative expressions of serine protease inhibitors (*SPINK2* and *SERPIN*), glucose metabolism (*G6PD*), stress response and immunity (*HSP90, IFNγ, IL2, IL6* and *TLR2*) and antioxidant enzyme (*CAT*) associated genes in testicular tissue (a) and peripheral blood mononuclear cells (b) of goats. *GAPDH* was used as a house keeping gene. The fold change in control group was considered as 1. In the testicular tissue, *SPINK2, SERPIN* and *IFNγ* and in PBMC, *CAT, IL2, TLR2* and *IL6* genes were upregulated in goats. $p < 0.05$.

At the PBMC level, dietary boron supplementation increased the *IL2* and catalase and decreased *TLR2* mRNA expression is suggestive of increased immune status and reduced oxidative stress. The significant increase in proinflammatory *IL2* gene expression in the present study indicates enhanced immune function in the boron fed animals [54]. Dietary zinc supplementation increased pro-inflammatory and antioxidant status by the elevation of cytokines such as *IL2* [55]. Similarly, upregulation of antioxidant enzyme catalase is suggestive of the reduced stress in dietary boron group and is in agreement with the decreased level of stress biomarker enzymes, AST and ALT. The low doses of boron (5–20 mg/l) increased catalase in the erythrocytes of the mice [56].

In the present study, decreased *TLR2* expression in the PBMC of the boron fed animals suggest the positive role of boron on semen production as the enhanced *TLR2* expression in the spermatozoa was associated with reduced sperm motility, sperm apoptosis, and impaired fertilization [57]. Overall, the present study suggest that dietary boron supplementation mediated the molecular level changes in male goat favored sperm output and quality sperm production.

Dietary boron supplementation increased the sperm output, sperm motility and enhanced the immune and antioxidant defense capacity in male goats. Further, it also increased the expression of positive regulators such as *SERPIN* and *IFNγ,* which are critical regulators of spermatogenesis process. Such changes at the testicular level coupled with antioxidant microenvironment improved reproductive function in male goats.

Conflict of interest

The authors declare no conflicts of interest

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.05.004.

References

[1] N.K.S. Gowda, R.U. Suganthi, V. Malathi, A. Raghavendra, Utilization of dietary minerals and blood biochemical values in lambs fed hydrated sodium calcium alumino silicate sorbent material at supplementary level, Small Rumin. Res. 69 (2007) 17–22.

[2] S. Selvaraju, K.S. Bhat, S.S. Archana, N.K.S. Gowda, B.B. Krishnan, I.J. Reddy, D.T. Pal, K.S. Roy, J.P. Ravindra, Profile of plasma biomolecules and minerals in various reproductive status of cattle and buffaloes, Indian J. Anim. Sci. 87 (2017)

1071–1076.
[3] T.V. Bhasker, N.K.S. Gowda, S. Mondal, P. Krishnamoorthy, D.T. Pal, A. Mor, S.K. Bhat, A.K. Pattanaik, Boron influences immune and antioxidant responses by modulating hepatic superoxide dismutase activity under calcium deficit abiotic stress in Wistar rats, J. Trace Elem. Med. Biol. 36 (2016) 73–79, https://doi.org/10.1016/j.jtemb.2016.04.007.
[4] M. Korkmaz, R. Turkmen, H.H. Demirel, Z.K. Saritas, Effect of boron on the repair of osteochondral defect and oxidative stress in rats: an experimental study, Biol. Trace Elem. Res. 187 (2019) 425–433, https://doi.org/10.1007/s12011-018-1381-3.
[5] F.H. Nielsen, Boron in human and animal nutrition, Plant Soil 193 (1997) 199–208, https://doi.org/10.1023/A:1004276311956.
[6] F.H. Nielsen, Historical and recent aspects of boron in human and animal health, Bor. Dergisi. 2 (2017) 153–160.
[7] C.D. Hunt, Dietary Boron: an overview of the evidence for its role in immune function, J. Trace Elem. Exp. Med. 16 (2003) 291–306, https://doi.org/10.1002/jtra.10041.
[8] C.D. Hunt, Regulation of enzymatic activity, Biol. Trace Elem. Res. 66 (1998) 205–225, https://doi.org/10.1007/BF02783139.
[9] T.V. Bhasker, N.K.S. Gowda, D.T. Pal, S.K. Bhat, P. Krishnamoorthy, S. Mondal, A.K. Pattanaik, A.K. Verma, Influence of boron supplementation on performance, immunity and antioxidant status of lambs fed diets with or without adequate level of calcium, PLoS One 12 (2017), https://doi.org/10.1371/journal.pone.0187203.
[10] I. Uluisik, H.C. Karakaya, A. Koc, The importance of boron in biological systems, J. Trace Elem. Med. Biol. 45 (2018) 156–162, https://doi.org/10.1016/j.jtemb.2017.10.008.
[11] T.A. Devirian, S.L. Volpe, The physiological effects of dietary boron, Crit. Rev. Food Sci. Nutr. 43 (2003) 219–231, https://doi.org/10.1080/10408690390826491.
[12] A.E. Elkomy, A.M.A. El-hady, O.A. Elghalid, Dietary boron supplementation and its impact on semen characteristics and physiological status of adult male rabbits, Asian J. Poult. Sci. 9 (2015) 85–96, https://doi.org/10.3923/ajpsaj.2015.85.96.
[13] M.R. Naghii, S. Samman, The effect of boron supplementation on the distribution of boron in selected tissues and on testosterone synthesis in rats, J. Nutr. Biochem. 7 (1996) 507–512, https://doi.org/10.1016/0955-2863(96)00102-7.
[14] D.J. Fort, R.L. Rogers, D.W. McLaughlin, C.M. Sellers, C.L. Schlekat, Impact of boron deficiency on Xenopus laevis, Biol. Trace Elem. Res. 90 (2002) 117–142, https://doi.org/10.1385/bter:90:1-3:117.
[15] R. Rowe, C. Eckhert, Boron is required for zebrafish embryogenesis, J. Exp. Biol. 202 (1999) 1649–1654.
[16] H.M. Bolt, Y. Duydu, N. Başaran, K. Golka, Boron and its compounds: current biological research activities, Arch. Toxicol. 91 (2017) 2719–2722, https://doi.org/10.1007/s00204-017-2010-1.
[17] S. Selvaraju, I.J. Reddy, S. Nandi, S.B.N. Rao, J.P. Ravindra, Influence of IGF-I on buffalo (*Bubalus bubalis*) spermatozoa motility, membrane integrity, lipid peroxidation and fructose uptake in vitro, Anim. Reprod. Sci. 113 (2009) 60–70, https://doi.org/10.1016/j.anireprosci.2008.08.011.
[18] S. Selvaraju, T. Sivasubramani, B.S. Raghavendra, P. Raju, S.B.N. Rao, D. Dineshkumar, J.P. Ravindra, Effect of dietary energy on seminal plasma insulin-like growth factor-I (IGF-I), serum IGF-I and testosterone levels, semen quality and fertility in adult rams, Theriogenology 78 (2012) 646–655, https://doi.org/10.1016/j.theriogenology.2012.03.010.
[19] S. Selvaraju, J. Ghosh, J.P. Ravindra, Prognostic value of various spermatological attributes as predictors of zona binding and zona penetration of buffalo (*Bubalus bubalis*) semen, Reprod. Domest. Anim. 44 (2009) 6–11, https://doi.org/10.1111/j.1439-0531.2007.00979.x.
[20] M. Katayama, S. Kaneko, K. Takamatsu, T. Tsukimura, T. Togawa, Determination of trace metals in human seminal plasma using inductively coupled plasma mass spectrometry and multivariate statistical analyses for sperm parameters, J. Mol. Biomark. Diagn. 04 (2013) 1–7, https://doi.org/10.4172/2155-9929.1000147.
[21] C.H. Fiske, Y. Subbarrow, The colormetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400, https://doi.org/10.1007/s13398-014-0173-7.2.
[22] T.V. Bhasker, N.K. Gowda, S. Mondal, D.T. Pal, H.P. Aithal, A.K. Pattanaik, S.V.R. Rao, S.K. Bhat, Boron supplementation influences bone mineralization by modulating expression of genes regulating calcium utilization, Anim. Nutr. Feed Technol. 17 (2017) 201–215, https://doi.org/10.5958/0974-181X.2017.00021.X.
[23] M.W. McGowan, J.D. Artiss, D.R. Strandbergh, B. Zak, A peroxidase-coupled method for the colorimetric determination of serum triglycerides, Clin. Chem. 29 (1983) 538–542.
[24] S. Reitman, S. Frankel, A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases, Am. J. Clin. Pathol. 28 (1957) 56–63.
[25] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126, https://doi.org/10.1016/S0076-6879(84)05016-3.
[26] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474, https://doi.org/10.1111/j.1432-1033.1974.tb03714.x.
[27] I. Carlberg, B. Mannervick, Purification and characterization of the flavoenzyme glutathione reductase from rat liver, J. Biol. Chem. 250 (1975) 5475–5480.
[28] S. Parthipan, S. Selvaraju, L. Somashekar, A.P. Kolte, A. Arangasamy, J.P. Ravindra, Spermatozoa input concentrations and RNA isolation methods on RNA yield and quality in bull (*Bos taurus*), Anal. Biochem. 482 (2015) 32–39, https://doi.org/10.1016/j.ab.2015.03.022.
[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[30] G.W. Preedy, S.L. Hill, J.S. Stevenson, R.L. Weaber, K.C. Olson, Injectable trace-mineral supplementation improves sperm motility and morphology of young beef bulls, Prof. Anim. Sci. 34 (2018) 1–9, https://doi.org/10.15232/PAS.2017-01667.
[31] K. Hemalatha, A. Arangasamy, S. Selvaraju, M.V. Krishnaiah, G.P. Rani, A. Mishra, N.M. Soren, I.J. Reddy, J.P. Ravindra, Effect of dietary supplementation of organic zinc and copper on in vitro semen fertility in goat, Small Rumin. Res. 161 (2018) 68–72, https://doi.org/10.1016/J.SMALLRUMRES.2018.02.003.
[32] M.A. Dietrich, M. Słowińska, H. Karol, M. Adamek, D. Steinhagen, A. Hejmej, B. Bilińska, A. Ciereszko, Serine protease inhibitor Kazal-type 2 is expressed in the male reproductive tract of carp with a possible role in antimicrobial protection, Fish Shellfish Immunol. 60 (2017) 150–163, https://doi.org/10.1016/j.fsi.2016.11.041.
[33] S.S. Tate, A. Meister, Serine-borate complex as a transition-state inhibitor of gamma-glutamyl transpeptidase, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 4806–4809, https://doi.org/10.1073/pnas.75.10.4806.
[34] S.P. Spielberg, J.D.B. Butler, K. MacDermot, J.D. Schulman, Treatment of glutathione synthetase deficient fibroblasts by inhibiting γ-glutamyl transpeptidase activity with serine and borate, Biochem. Biophys. Res. Commun. 89 (1979) 504–511, https://doi.org/10.1016/0006-291X(79)90658-2.
[35] N. Dejucq, I. Dugast, A. Ruffault, P.H. van der Meide, B. Jégou, Interferon-alpha and -gamma expression in the rat testis, Endocrinology 136 (1995) 4925–4931, https://doi.org/10.1210/endo.136.11.7588226.
[36] H. Hibi, K. Yokoi, M. Yamamoto, Effects of alpha-interferon on sperm production, concentration, and motility in the rat, Int. J. Urol. 4 (1997) 603–607.
[37] F.H. Nielsen, Dietary fat composition modifies the effect of boron on bone characteristics and plasma lipids in rats, Biofactors 20 (2004) 161–171.
[38] G. Yildiz, B. Hakan Koksal, Effects of dietary boric acid addition on growth performance, cholesterolemia, some carcass and tibia characteristics in different rearing periods in broiler chickens, Revue Med. Vet. 164 (2013) 219–224.
[39] F.H. Nielsen, C.D. Hunt, L.M. Mullen, J.R. Hunt, Effect of dietary boron on mineral, estrogen, and testosterone metabolism in postmenopausal women, FASEB J. 1 (1987) 394–397.
[40] J. Zhao, X. Dong, X. Hu, Z. Long, L. Wang, Q. Liu, B. Sun, Q. Wang, Q. Wu, L. Li, Zinc levels in seminal plasma and their correlation with male infertility: a systematic review and meta-analysis, Sci. Rep. 6 (2016) 22386, https://doi.org/10.1038/srep22386.
[41] D. Dissanayake, P. Wijesinghe, W. Ratnasooriya, S. Wimalasena, Relationship between seminal plasma zinc and semen quality in a subfertile population, J. Hum. Reprod. Sci. 3 (2010) 124–128, https://doi.org/10.4103/0974-1208.74153.
[42] C.D. Hunt, J.P. Idso, Dietary boron as a physiological regulator of the normal inflammatory response: a review and current research progress, J. Trace Elem. Exp. Med. 12 (1999) 221–233, https://doi.org/10.1002/(SICI)1520-670X(1999)12:3<221::AID-JTRA6>3.0.CO;2-X.
[43] Q. Li, L. Liu, Y. Wang, J. Xie, L. He, Q. Wang, Characterization and expression analysis of serpins in the Chinese mitten crab Eriocheir sinensis, Gene 575 (2016) 632–640, https://doi.org/10.1016/j.gene.2015.09.033.
[44] C.H. Lu, R.K.K. Lee, Y.M. Hwu, S.L. Chu, Y.J. Chen, W.C. Chang, S.P. Lin, S.H. Li, SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor, Biol. Reprod. 84 (2011) 514–525, https://doi.org/10.1095/biolreprod.110.085100.
[45] C. Chotwiwatthanakun, W. Santimanawong, P. Sobhon, S. Wongtripop, R. Vanichviriyakit, Inhibitory effect of a reproductive-related serpin on sperm trypsin-like activity implicates its role in sperm maturation of Penaeus monodon, Mol. Reprod. Dev. 85 (2018) 205–214, https://doi.org/10.1002/mrd.22954.
[46] S.C. Berry, A.L. Fink, A.B. Shenvi, C.A. Kettner, Interaction of peptide boronic acids with elastase: circular dichroism studies, Proteins Struct. Funct. Genet. 4 (1988) 205–210, https://doi.org/10.1002/prot.340040307.
[47] X. Zheng, M. Geiger, S. Ecke, I. Resch, U. Eberspächer, P. Donner, W.D. Schleuning, B.R. Binder, Serine protease inhibitors (Serpins) in human seminal plasma: concentrations and inhibition of acrosin, Elsevier, Fibrinolysis 8 (1994) 364–371.
[48] I. Tentes, B. Asimakopoulos, E. Mourvati, K. Diedrich, S. Al-Hasani, N. Nikolettos, Matrix metalloproteinase (MMP)-2 and MMP-9 in seminal plasma, J. Assist. Reprod. Genet. 24 (2007) 278–281, https://doi.org/10.1007/s10815-007-9129-6.
[49] Y. Duydu, N. Başaran, A. Üstündağ, S. Aydın, Ü. Ündeğer, O.Y. Ataman, K. Aydos, Y. Düker, K. Ickstadt, B.S. Waltrup, K. Golka, Assessment of DNA integrity (COMET assay) in sperm cells of boron-exposed workers, Arch. Toxicol. 86 (2012) 27–35.
[50] Y. Duydu, N. Başaran, A. Üstündağ, S. Aydın, Ü. Ündeğer, O.Y. Ataman, K. Aydos, Y. Düker, K. Ickstadt, B.S. Waltrup, K. Golka, Reproductive toxicity parameters and biological monitoring in occupationally and environmentally boron-exposed persons in Bandırma, Turkey, Arch. Toxicol. 85 (2011) 589–600.
[51] Y. Duydu, N. Başaran, S. Aydın, A. Üstündağ, C.Ö. Yalçın, H.G. Anlar, M. Bacanlı, K. Aydos, C.S. Atabekoğlu, K. Golka, K. Ickstadt, T. Schwerdtle, M. Werner, S. Meyer, H.M. Bolt, Evaluation of FSH, LH, testosterone levels and semen parameters in male boron workers under extreme exposure conditions, Arch. Toxicol. 92 (2018) 3051–3059, https://doi.org/10.1007/s00204-018-2296-7.
[52] M. Korkmaz, E. Uzgören, S. Bakirdere, F. Aydin, O.Y. Ataman, Effects of dietary boron on cervical cytopathology and on micronucleus frequency in exfoliated buccal cells, Environ. Toxicol. 22 (2007) 17–25, https://doi.org/10.1002/tox.20229.
[53] S. Nazifi, M. Saeb, E. Rowghani, K. Kaveh, The influences of thermal stress on serum biochemical parameters of Iranian fat-tailed sheep and their correlation with triiodothyronine (T 3), thyroxine (T 4) and cortisol concentrations, Comp. Clin. Pathol. 12 (2003) 135–139, https://doi.org/10.1007/s00580-003-0487-x.
[54] E. Jin, M. Ren, W. Liu, S. Liang, Q. Hu, Y. Gu, S. Li, Effect of boron on thymic cytokine expression, hormone secretion, antioxidant functions, cell proliferation, and apoptosis potential via the extracellular signal-regulated kinases 1 and 2 signaling pathway, J. Agric. Food Chem. 65 (2017) 11280–11291, https://doi.org/10.1021/acs.jafc.7b04069.

[55] A.S. Prasad, F.W. Beck, B. Bao, J.T. Fitzgerald, D.C. Snell, J.D. Steinberg, L.J. Cardozo, Zinc supplementation decreases incidence of infections in the elderly: effect of zinc on generation of cytokines and oxidative stress, Am. J. Clin. Nutr. 85 (2007) 837–844, https://doi.org/10.1093/ajcn/85.3.837.

[56] H. Türkez, F. Geyikoğlu, A. Tatar, S. Keleş, A. Özkan, Effects of some boron compounds on peripheral human blood, J. Biosci. 62 (2007) 889–896, https://doi.org/10.1515/znc-2007-11-1218.

[57] S. Hagan, N. Khurana, S. Chandra, A.B. Abdel-Mageed, D. Mondal, W.J.G. Hellstrom, S.C. Sikka, Differential expression of novel biomarkers (TLR-2, TLR-4, COX-2, and Nrf-2) of inflammation and oxidative stress in semen of leukocytospermia patients, Andrology 3 (2015) 848–855, https://doi.org/10.1111/andr.12074.