Reproduction in Domestic Animals

Reprod Dom Anim **49**, 1004–1007 (2014); doi: 10.1111/rda.12422
ISSN 0936–6768

Influence of Dietary Zinc on Semen Traits and Seminal Plasma Antioxidant Enzymes and Trace Minerals of Beetal Bucks

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Contents
Zinc (Zn) is a potent antioxidant and plays a key role in scavenging free radicals. We hypothesized that supplementation of Zn would reduce the oxidative damage, which is linked with poor sperm quality. Sixteen bucks of similar average age (2 years) and body weight (41 kg) were randomly divided into four groups viz., 1, 2, 3 and 4 supplemented with zinc sulphate into the diet at the rate of 0, 50, 100 and 200 mg/buck/day, respectively, for 3 months. At the end of the experiment, semen samples were collected and assessed. Seminal plasma was separated to find the concentration of superoxide dismutase (SOD), glutathione peroxidase (GPx), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and trace minerals (Zn, Cu, Mn and Fe). The results revealed that semen volume (1.85 ± 0.01 ml) and sperm motility (88.23 ± 5.77%) increased significantly ($p < 0.05$) in supplemented groups compared with the control specifically in group 3. SOD (10.66 ± 0.23 inhibition rate %) and GPx (23.55 ± 0.49 mU/ml) increased significantly ($p < 0.05$) in group 3 with no effect on AST and ALT. Among seminal plasma trace elements, no significant change ($p > 0.05$) was observed. From the present results, we concluded that zinc sulphate at the rate of 100 mg/buck/day improved semen traits and seminal plasma antioxidant capacity in Beetal bucks.

Introduction

There is a strong relationship between essential elements and spermatogenesis. Zinc (Zn) is found in high amount in male reproductive tract and semen (Chia et al. 2000). It plays an important role in the physiology of spermatozoa, and the deficiency of this element leads to several reproductive defects including testicular dysfunction, lower testicular weight and shrinkage of seminiferous tubules (Bedwal and Bahuguna 1994; Chia et al. 2000; Hadwan et al. 2012). Studies in animal models have shown that oral Zn supplementation improved physical characteristics of semen including sperm count and motility (Ghasemi et al. 2009; Rafique et al. 2010). Martin et al. (1994) concluded that a diet deficient in Zn may cause atrophy of the primary, secondary and accessory sex glands.

The study of free radicals and reactive oxygen species has become an important area of investigation in living cells (Khan 2011). Structurally, lipids are the major components of the spermatozoa, which are vulnerable to the attack of free radicals. Physiologically, the free radicals are required at the time of sperm capacitation and fusing of the sperm to ova (Agarwal et al. 2003). Generally, there is a balance between the creation and destruction of the free radicals; however, when their production exceeds the neutralizing ability of the body, oxidative stress occurs (Khan 2011). Fortunately, the seminal plasma has been endowed with antioxidant substances, which suppress the production of free radicals. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are important antioxidant enzymes of the seminal plasma, which scavenge the free radicals and convert them into stable products. Decreased concentration of SOD and GPx has been linked with poor sperm quality and reproductive performance (de Lamirande and Gagnon 1995; Yue et al. 2010).

Zn scavenges excessive production of superoxide radical, and thus, this element has antioxidant like activities (Gavella and Lipovac 1988). Chia et al. (2000) suggested that Zn may bind with the free radicals in the seminal plasma, produced by abnormal spermatozoa, and thus, the concentration of this element may be decreased. Zn is also part of copper–zinc SOD (Hadwan et al. 2012). Kumar et al. (2006) suggested that improved semen characteristics in cross-bred bulls may be due to the antioxidant potential of Zn.

Trace elements work as functional and structural cofactor in metal containing enzymes (Khan et al. 2012). Cu is a part of SOD and thus involved in scavenging free radicals (Chan et al. 1998; Khan 2011). It has been known that Mn deficiency may cause impaired or depressed reproductive efficiency. Mn is an essential part of SOD (Khan 2011) and its deficiency may adversely affect the seminal plasma SOD. Zn deficiency may cause gonadal dysfunction, low testicular weight and impaired growth of seminiferous tubules (Chia et al. 2000).

Till date, very little work has been done on supplementation of Zn on the reproductive efficiency of livestock species and the information is unavailable in Beetal bucks regarding the sperm quality and seminal plasma antioxidant capacity. Therefore, this study was designed to address the effect of different levels of dietary Zn on the semen quality and seminal plasma antioxidant enzymes and some of the trace minerals in Beetal bucks.

Materials and Methods

Animals feeding and management

The local committee of Use and Care of Animals at The University of Agriculture, Peshawar, Pakistan, approved this study. A total of 16 healthy male Beetal bucks at the age of 2 years having almost similar average body weight (41 kg) were selected at the Dairy Farm of The University of Agriculture, Peshawar, Pakistan. The diet offered to the bucks met or exceed the requirements of NRC (1985). All bucks were individually housed and provided the same quantity of

feed. Clean drinking water was provided *ad libitum*. Zn powder consisted of zinc sulphate was obtained from BA Traders (Lahore, Pakistan). After initial period of 1-week adaptation, bucks were randomly divided into four matching groups and supplemented with zinc sulphate at the level of 0, 50, 100 and 200 mg/buck/day. The treatments are referred as group 1, 2, 3 and 4, respectively. The experiment lasted for 3 months.

Semen evaluation

Semen from the experimental bucks was collected early in the morning using a teaser. One ejaculate from each buck was collected at the end of the experiment. Thus, a total of 16 semen ejaculates were evaluated in this experiment. Semen was collected with the help of artificial vagina by maintaining the temperature at 37°C. Immediately, after collection, semen was maintained in hot water bath at 37°C and subjected to evaluation. Volume of each ejaculate was recorded with the graduated collection tube. Sperm concentration was determined using Neubauer haemocytometer chamber. The percentages of motile sperm were estimated at low-power magnification (10×) using a compound microscope (Olympus CX 41; Olympus Corporation, Tokyo, Japan). The percentage of motile spermatozoa was assessed by diluting (1 : 1) a drop of semen with 0.1 M sodium citrate, pH 6.769. The mixture was transferred to a glass slide with a cover slip and observed under high magnification. The individual motility was recorded as the percentage of progressive motile sperm. Blom (1950) method was used to assess live and dead spermatozoa using an eosin–nigrosin blue staining mixture.

Separation of seminal plasma analyses

After initial evaluation, the rest of the semen was centrifuged at 700 × ***g*** for 15 min to separate seminal plasma. After separation, the seminal plasma was stored at −20°C for further analyses.

Determination of serum SOD and GPx

Seminal plasma SOD activity was assayed using an SOD Assay Kit (BioVision, Mountain View, CA, USA). This assay is based upon utilization of WST-1, a tetrazolium salt, which produces formazan dye upon reduction with a superoxide anion. The rate of the reduction with a superoxide anion is linearly related with the amount to the xanthine oxidase activity and is inhibited by SOD. Briefly, 20 µl sample was added into microplate designated as sample and blank 2. Then, 20 µl double-distilled water was put in blank 1 and 3. Two hundred microlitre WST working solution was pipetted in sample and each blank (1–3). Enzyme working solution was added at the rate of 20 µl in sample and blank 1. At the end, 20 µl dilution buffer was put in blank 2 and 3. The mixture was incubated at 37°C for 20 min. The SOD activity was measured after taking absorbance (A) using the following formula:

$$\mathrm{SOD}_{\mathrm{activity}} = \frac{(A_{\mathrm{blank1}} - A_{\mathrm{blank3}}) - (A_{\mathrm{sample}} - A_{\mathrm{blank2}})}{(A_{\mathrm{blank1}} - A_{\mathrm{blank3}})} \times 100.$$

The SOD activity was measured as the percentage of inhibition of the WST-1 reduction rate.

Seminal plasma glutathione peroxidase (GPx) activity was evaluated by spectrophotometry (IRMECO Model U2020, IRMECO QmbH, Geesthacht, Germany) at 340 nm with a commercial kit supplied by BioVision (Mountain View, CA, USA). GPx in the sample converts reduced glutathione to oxidized glutathione while reducing lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water. Briefly, reaction mixture was prepared by mixing 33 µl assay buffer, 3 µl 40 mM NADPH solution, 2 µl glutathione reductase and 2 µl reduced glutathione. The mixture was incubated for 15 min, and then, 10 µl cumene hydroperoxide was mixed. The absorbance was taken just after mixture and after 5 min. GPx activity was calculated using the following equation.

$$\text{GPx activity} : \mathrm{B}/(\mathrm{T2} - \mathrm{T1}) \times \text{sample dilution}.$$

The activity of GPx was expressed in mU/ml.

Determination of serum aspartate aminotransferase and alanine aminotransferase

Seminal plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with UV-Vis spectrophotometer using a commercial kit of Randox Laboratories Ltd. (BT29 4 QY; Crumlin, UK). For determination of AST, a mixture of 20 µl sample was mixed with 50 µl R1 (phosphate buffer, L-aspartate, α-oxoglutarate) and 100 µl distilled water. The mixture was incubated at 37°C for 30 min. To this mixture, 50 µl R2 (2, 4-dinitrophenylhydrazine) was added and incubated for 20 min at 20°C. After incubation, 1 ml R3 (sodium hydroxide) was added. Absorbance was taken after 5 min of incubation. Final concentration was determined from the standard curve. The same procedure of determination of ALT is same except R1 contains L-alanine instead of L-aspartate.

Mineral determination

The preparation of seminal samples for mineral analysis was carried out as described by Khan et al. (2012). Seminal plasma samples (100 µl) were taken separately in a 25-ml digestion flask, and 1 ml concentrated nitric acid was added into each flask. The contents of each flask were boiled for 5 min or till all the fumes were evaporated. After cooling, 0.5 ml perchloric acid was added in it. The content of flask was heated vigorously on hot plate till volume reduced to 1 ml. The contents were finally diluted up to 10 ml by adding redistilled water. These digested and diluted sample solutions were used for the estimation of trace elements.

Concentration of iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) was measured with the help of atomic absorption spectrophotometer.

Table 1. Mean ± SE semen characteristics of control and treated male Beetal bucks

Parameters	Group 1	Group 2	Group 3	Group 4
Semen volume (ml)	1.53 ± 0.08^{b}	1.13 ± 0.03^{c}	1.85 ± 0.01^{a}	1.90 ± 0.05^{a}
Sperm concentration (10^9/ml)	1.13 ± 0.16	1.50 ± 0.57	1.54 ± 0.11	1.23 ± 0.53
Sperm motility (%)	61.66 ± 3.10^{b}	78.33 ± 7.90^{a}	88.23 ± 5.77^{a}	87.33 ± 4.40^{a}
Dead sperm percentage (%)	4.36 ± 0.33	3.62 ± 0.12	3.33 ± 0.17	3.34 ± 0.22

Mean value having different superscript within the same row differ significantly ($p < 0.05$); Group 1–4: Dietary supplementation of zinc at the rate of 0, 50, 100 and 200 mg/buck/day, respectively.

Statistical analysis

Data were statistically analysed with the help of statistical software (version 8.1, STATISTIX, Tallahasee, FL, USA). One-way analysis of variance was applied to test the significance of four dietary treatments on the studied parameters (Steel et al. 1997). Means were separated by Duncan multiple-range test (Duncan 1955). p value <0.05 was considered to be statistically significant.

Results

The results indicated that semen volume and sperm motility increased significantly ($p < 0.05$) in treated groups compared with control (Table 1). No significant change ($p > 0.05$) was observed in sperm concentration and dead spermatozoa. The dead sperm concentration decreased in all treated groups. The highest sperm concentration was found in group 3 among the treated groups even not significantly ($p > 0.05$). In group 3, SOD and GPx concentration increased significantly ($p < 0.05$), without affecting AST and ALT concentration (Table 2). The concentration of SOD and GPx increased ($p < 0.05$) linearly in treated group 2 and 3 and then declined ($p < 0.05$) in group 4. No significant change ($p > 0.05$) was observed in Zn, Mn, Fe and Cu concentration (Table 3).

Table 2. Mean ± SE seminal plasma enzyme concentration of control and treated male Beetal bucks

Parameters	Group 1	Group 2	Group 3	Group 4
SOD (per cent inhibition)	6.40 ± 0.24^{c}	7.40 ± 0.25^{b}	10.22 ± 0.11^{a}	7.65 ± 0.13^{b}
GPx (mU/ml)	10.22 ± 0.16^{d}	13.39 ± 0.90^{c}	22.15 ± 0.11^{a}	19.96 ± 1.13^{b}
AST (U/l)	70.14 ± 0.11	68.13 ± 0.06	65.68 ± 0.28	68.12 ± 0.01
ALT (U/l)	62.68 ± 0.08	69.63 ± 0.08	69.44 ± 0.09	68.96 ± 0.03

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; GPx, Glutathione peroxidase; SOD, Superoxide dismutase.
Group 1–4: Dietary supplementation of zinc at the rate of 0, 50, 100 and 200 mg/buck/day, respectively.
Mean value having different superscript within the same row differ significantly ($p < 0.05$).

Table 3. Mean ± SE seminal plasma trace minerals (mg/kg) of control and treated male Beetal bucks

Parameters	Group 1	Group 2	Group 3	Group 4
Zn	5.53 ± 0.45	7.43 ± 0.12	8.22 ± 0.43	8.39 ± 0.13
Mn	13.35 ± 1.07	14.65 ± 0.01	15.75 ± 1.08	16.26 ± 1.26
Fe	3.44 ± 0.01	4.11 ± 0.12	4.26 ± 0.08	3.44 ± 0.31
Cu	0.22 ± 0.14	0.32 ± 0.05	0.26 ± 0.04	0.32 ± 0.07

Zn, Zinc; Mn, Manganese; Fe, Iron; Cu, Copper.
Group 1–4: Dietary supplementation of zinc at the rate of 0, 50, 100 and 200 mg/buck/day, respectively.
Mean value having different superscript within the same row differs significantly ($p < 0.05$).

Discussion

In this study, the effect of different levels of Zn supplementation on semen characteristics, seminal plasma antioxidant enzymes and trace minerals were evaluated. The result of the present study indicated that Zn at the level of 100 mg/buck/day produced optimum results regarding semen traits and seminal plasma characteristics. The present results are in agreement with previous researchers who observed almost similar findings in goat (Saleh et al. 1992), rabbits (Tharwat 1998) and bull (Kumar et al. 2006). In the present study, the semen volume and sperm motility increased significantly in the treated groups. Increased semen volume has been previously reported in goat and rabbit in response to supplementation of zinc sulphate (Saleh et al. 1992; Tharwat 1998). Semen volume is the combined secretion of testes, epididymis and accessory sex glands. Zn has been reported to stimulate the growth and secretion of accessory sex gland (Kumar et al. 2006). So, enhanced semen volume may be due to the dietary zinc sulphate supplementation. Kumar et al. (2006) reported improved sperm motility in the Zn-supplemented groups. Similar results were previously reported in men, sheep and rabbits (Tharwat 1998; Kendall et al. 2000; Wong et al. 2002). In the current study, the improved sperm motility may be due to increase in Zn containing enzymes (lactate dehydrogenase and sorbital dehydrogenase) and improved oxygen uptake by sperm or scavenging of free oxygen radicals (Kumar et al. 2006). In the present study, the dead sperm percentage did not change between the control and the treated groups. It has been postulated that Zn has no effect on the Sertoli cells which in turn control the normal and abnormal morphology of sperm during spermatogenesis (McDonald 2003; Kumar et al. 2006).

In the present study, the concentration of SOD and GPx increased significantly in group 3 supplemented with Zn at the rate of 100 mg/buck/day. Superoxide dismutase is an important antioxidant enzyme, which protects the body against the free radicals. Decreased SOD activity may be linked with increased production

of peroxide, which destroys nuclear materials and damages the protein and enzymes (Hong et al. 2010; Khan 2011). Zn has been known as antioxidant, which scavenges free radicals and prevents lipid peroxidation by inhibiting phospholipase (Eggert Kruss et al. 2002). It has been postulated that Zn increases metallothioneins enzymes and protects sulfhydryl group protein (Powell 2000). As Zn is also an integral structural part of the Cu-Zn-SOD (Khan 2011), it is inferred that higher Zn availability may increase the SOD activity (Goel et al. 2005). According to the opinion of Goel et al. (2005), no conclusive evidence is available for the protective effect of Zn on SOD and GPx, and the increase concentration of these enzymes may be either due to induced metallothione content or indirect reduction of oxygen-reactive species. Additionally, the higher dose (200 mg/buck/day) had deleterious impact on the seminal plasma antioxidant enzymes. The exact reason could not be ascertained from the available literature, we hypothesized that the higher level of Zn may have proxidant effect on SOD and GPx. In the present study, seminal plasma Zn and Mn increased linearly with the increasing level of zinc sulphate. The simple reason of the increased Zn concentration may be due to the supplementation of the zinc sulphate. Furthermore, with the supplementation of Zn, the seminal plasma Mn also increased non-significantly, in all the treated groups, suggesting that Zn may help in absorption of Mn. The increasing level of Zn supplementation had no concomitant increase in Cu and Fe concentration, which agrees with the previous findings (Yadrick et al. 1989).

Based on the present results, it is suggested that zinc sulphate at the rate of 100 mg/buck/day was an optimum dose to improve semen volume, motility and seminal plasma antioxidants (SOD, GPx) in Beetal bucks.

Conflict of interest

The authors have no conflict of interest.

Author contributions

Hafiz ur Rahman conducted this study. Muhammad Subhan Qureshi helped in the study and prepared the initial draft of the study Rifat Ullah Khan prepared the plane of work, edited and revised the paper.

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Submitted: 3 Jul 2014; Accepted: 11 Aug 2014

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