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Seminal Attributes, Antioxidant Defense System and Fertility Rate Reveal an Association in Beetal Goat Bucks

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Abstract

Semen attributes and their association with fertility in Beetal bucks was assessed. Three ejaculates per buck (n=14) were analyzed for sperm functional tests, malondialdehyde concentration (MDA), antioxidant enzymes. Seminal plasma was analyzed for testosterone and total proteins. A significant (p<0.05) difference in individual motility, viability, plasma membrane integrity (PMI), acrosome integrity (AI), MDA and antioxidant enzymes among the bucks was observed. There was also a significant (p<0.05) variation in total protein and testosterone levels in seminal plasma. A fertility rate of 100%, 83.3 %, 66.7 % and 50% was found in 3, 4, 5 and 2 bucks, respectively. Bucks were divided into high (G-I, 83.3-100% FR) and low (G-II, 50-66.7% FR) fertile groups. Average sperm concentration, individual motility and viability, were significantly (p<0.05) high in G-I compared to G-II. Average MDA concentration, super oxide dismutase, catalase and glutathione reductase were significantly (p<0.05) high in G-II compared to G-I. Average values of total protein and testosterone were significantly (p<0.05) higher in G-I and G-II, respectively. Study concluded that oxidative stress and testosterone level has an impact on semen quality and fertility of Beetal bucks.

Keywords: Sperm Attributes, Oxidative Stress, Fertility Rate, Beetal Bucks

Introduction

Caprines are considered to play an important role in uplifting the socio-economic standards of common and poor income communities. The Beetal breed reared in the Punjab region of India and Pakistan is used for milk and meat production. The success of a goat flock depends on the number of kids raised, weaned, and marketed each year. Although the genetic quality of a goat herd is important but reproductive traits in goat have low heritability (Gimenez, 2007). The fertility potential of a male is made on the basis of fertility rate in females mated, in spite of the fact that semen evaluation provides predictive information on expected performance of the male and insights into the fertilizing capacity of the preserved spermatozoa (Januskauskas and Zilinskas, 2002). There is a link between semen fertility and its measurable parameters (Januskauskas and Zilinskas, 2002). Evaluation of sperm viability, progressive motility, hypo-osmotic swelling test (HOST), acrosome integrity (AI) and morphological abnormalities reduce the economic and time constraints in field conditions. Value of any one of these parameters could provide a fairly good and adequate prediction of the other. Therefore, evaluation of semen parameters becomes highly imperative. Artificial insemination (AI) is an important procedure for wider dissemination of semen, limits the spread of sexually transmitted diseases and facilitates genetic improvement. Semen has to be extended and stored at 4°C or cryopreserved at -196ºC for AI. Therefore, it is of utmost importance to evaluate the semen before cryopreservation and even in the situation of natural mating.

One of the most important factors that constitute poor semen quality is oxidative stress, which results from lipid peroxidation (LPO). Concentration of polyunsaturated fatty acids (PUFA) in sperm membrane is generally high in small ruminant's sperm membrane than other species and in such situation, sperm becomes highly vulnerable to oxidative stress. Generation of reactive oxygen species (ROS) leads to loss in membrane integrity, impaired cell function along with motility and induce apoptosis (Gandini *et al*., 2000). An antioxidant system comprising of glutathione peroxidise (GPX), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), glutathione reductase (GRE) had been reported as a defence mechanism against LPO. Although a significant physiological role of ROS during normal sperm function has been reported (Kodama *et al*., 1996; DeLamirande *et al*., 1997), but when the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS create oxidative stress.Therefore, study was aimed to evaluate sperm attributes, LPO and antioxidant enzymes and to ascertain their relationship with fertility rate in Beetal goat bucks.

Materials and Methods

Experimental Animals, Housing and General Management

All the procedures were approved by Institutional ethical committee (GADVASU/2018/IAEC/46/06). All the experimental bucks were kept under loose housing system throughout the study period. Bucks were kept in a single pen with provision of about 60 ft^2 covered as well as open area per buck. Clean potable water was available to them round the clock. All animals were de-wormed and vaccinated as per schedule.

Experimental Design

Fourteen sexually mature Beetal bucks were randomly selected. Semen was collected twice a week using goat artificial vagina (IMV technologies). Experimental bucks in the age group of 1-2 years were kept under loose housing system. Bucks were fed green fodder twice and concentrate once daily. Before the start of the experiment, all animals were dewormed as per schedule in order to maintain uniform health. Six ejaculates per buck were analyzed for various sperm functional tests, LPO, antioxidant enzymes. Six goats were mated per buck for fertility rate (FR).

Sperm Attributes

Volume of semen was noted in a graduated tube and sperm concentration was calculated with the help of haemocytometer. Motility was noted by wet mount method. Briefly, a drop of semen on a slide, covered with a cover slip was observed microscopically using CCTV. A total of 200 motile and non-motile sperms were observed on the monitor and percent of motile spermatozoa was calculated. For viability, a drop of semen was mixed with a drop of 0.5 % aqueous eosin in normal saline, mixed for 60 sec followed by addition of a drop of 10 % nigrosin on a slide. Mixed for another 60 sec and a smear was prepared, dried and observed under a binocular microscope

(Olympus) at 1000x. About 200 sperms stained white (live) and pink (dead) were counted in different fields and percentage of live spermatozoa was calculated (Fig. 1a). HOST was performed to analyze the PMI in spermatozoa. Semen (10 µl) was mixed with 100 µl of 125 mOsmol (42.5 ml of 300 mOsmol and 57.5 ml DW) HOS solution and incubated at 37ºC for 30 min. One drop of incubated semen was placed on a slide, covered with cover slip and examined under bright field microscope at 400 x for coiled tailed spermatozoa. Similarly, 10µl semen was incubated in phosphate buffer saline (PBS) under similar conditions. A total of 200 spermatozoa with coiled and uncoiled tails in HOS and PBS were observed under different fields and percentage of coiled tailed spermatozoa was calculated. The number of coiled tailed spermatozoa in PBS was deducted from the number in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive spermatozoa (Fig. 1b). Acrosome integrity (AI) of spermatozoa was assessed using Giemsa stain (Watson, 1975). A smear of washed semen was prepared on a clean glass slide, air dried and fixed in methanol for 30 min. After drying, the smear was stained in Giemsa working solution (stock Giemsa stain 3 ml, 0.1 M phosphate buffer saline 2 ml, pH 7.4 and doubled distilled water (DDW, 35 ml) for 4 hrs. The slides were rinsed quickly in DDW, air dried and examined under oil immersion (1000x) of the bright field microscope. At least 200 spermatozoa with intact and damaged acrosome (partially or completely) were counted in different fields. Percentage of spermatozoa with intact acrosome was calculated (Fig. 1c).

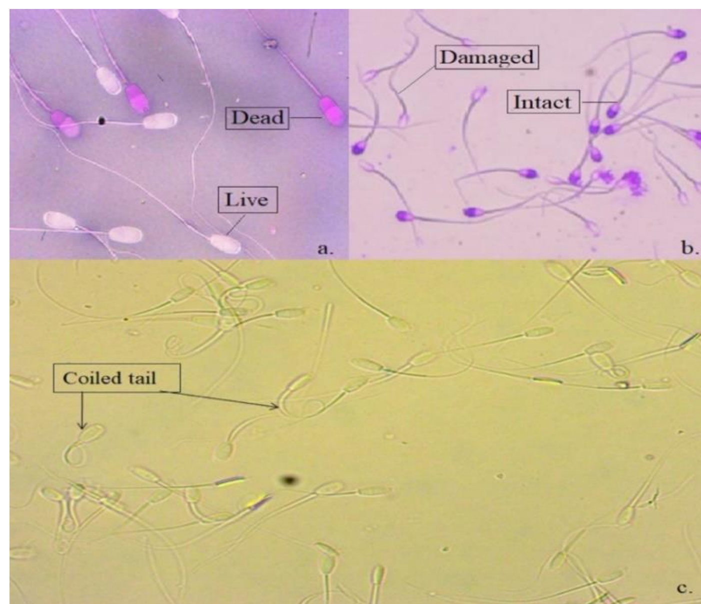

Figure 1: Showing viability (a), Plasma membrane integrity (b), acrosome integrity (c) in buck spermatozoa

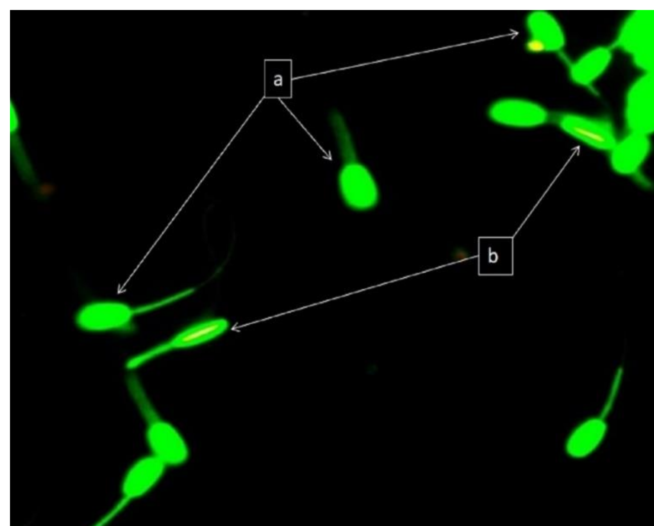

Figure 2: Showing DNA integrity in buck spermatozoa. Spermatozoa with double (a) and single (b) stranded DNA

DNA integrity was evaluated by the method of Lui & Baker (1992). About 200 µl of freshly washed sperm suspension was added to 400 µl of solution A (0.1% Triton-X-100 in 0.08 N HCl and 0.15 M NaCl) and mixed

gently for 30 sec. About 1.2 ml of ice-cold solution B (1 mM sodium EDTA, 0.15 M NaCl, 0.3 M $Na_2HPO_4.7H_2O$ and 0.1 M citric acid, pH 6.0) was added, mixed gently and equilibrated for 15 min. Finally, 10 µl of acridine orange (6.0 µg/ml of solution B) was gently mixed with semen placed on a glass slide and covered with coverslip. About 200 spermatozoa were evaluated under an epifluorescent microscope (400 x). The heads of the sperm cells with normal DNA integrity (double stranded) emitted green fluorescence, whereas those with denatured or single stranded DNA had orange or yellow fluorescence (Figure 2).

Antioxidant Defense System

Malondialdehyde (MDA) Concentration

MDA concentration was evaluated by the method of Buege and Steven, 1978. Washed sperm suspension (0.2 ml) was incubated with 0.1 ml of 150 mMTris HCl (pH 7.1) at 37°C for 20 min. After incubation 0.5 ml of 10 % Trichloroacetic acid and 1.0 ml of 0.375% Thiobarbituric acid were added and kept for 20 min in the boiling water bath. Thereafter, mixture was centrifuged for 15 min at 5000 rpm and absorbance of supernatant was taken at 532 nm. The molar extinction coefficient for (Malondialdehyde) MDA is 1.56 x 10^5 M-1.cm-1.

$$\text{MDA } (\mu M) = \frac{\text{OD x Volume of assay mixture} \times \text{Extinction coefficient}}{\text{Volume of sample}}$$

Enzyme Extraction

Semen was washed twice with PBS (pH, 7.4). Sperm pellet (1.0 x 10^9 sperm /2.0 ml) was suspended in Tris buffer (pH 7.5, 25 mM Tris, 2 mM Sodium EDTA, 2 mM DTT and 250 mM NaCl containing 1% Triton X-100). The sperm suspension was agitated at 25ºC for 30 min and then centrifuged at 9615 g at 4ºC for 20 min. Supernatant (enzyme extract, EE) was stored in aliquots at -20ºC till analysis of enzymes. Super oxide dismutase (SOD), Glutathione reductase (GRE) and catalase (CAT) were measured in EE by the method of Nishikimi *et al*. (1972), Krohne-Ehrich *et al*. (1977) and Goth (1991), respectively.

Superoxide Dismutase

The assay mixture consisting of 0.1 ml Nitro blue tetrazolium (312 µM) and 10 µl Phenazine methosulfate (120 µM) was incubated at 25°C for 10 min. Thereafter, 10 µl of EE was added and the reaction was initiated by addition of 0.1 ml of Nicotinamide adenine dinucleotide (936 µM). An increase in absorbance was recorded at 560 nm for 2 min at 1 min interval. A control was also run simultaneously. The SOD activity was calculated by the following formula:

$$\text{SOD } (\text{IU}/10^9 \text{ sperm/minute}) = \frac{\Delta T \times 2}{\Delta C}$$

Where, ΔT - Change in OD $_{\text{Test}}$ at 60 sec interval and ΔC - Change in OD $_{\text{Control}}$ at 60 sec intervals

Glutathione Reductase (GRE)

The assay mixture consisting of 100 µl potassium phosphate buffer (50 mM, pH 7.0), 25 µl potassium chloride (50 mM), 25 µl EDTA (1 mM), 25 µl glutathione disulfide (1 mM) and 12.5 µl BSA (0.05 %) were incubated at 25°C for 10 min. Thereafter, 25 µl of EE and 12.5 µl NADPH (1 mM) was added and increase or decrease in absorbance was recorded at 340 nm for 5 min at 1 min interval.

$$\text{GR } (\text{IU}/10^9 \text{spermatozoa/minute}) = \Delta T - \Delta C$$

Where, ΔT- Change in OD_{Test} at 60 sec interval and ΔC- Change in OD $_{\text{control}}$ at 60 sec intervals.

Catalase

The assay mixture consisting of 3 blanks: Blank 1) 100 µl substrate (65 µM H_2O_2) + 100 µl ammonium molybdate

(AM, 32.4 mM) + 20 µl EE; Blank 2) 100 µl substrate + 100 µl AM + 20 µl buffer (60 mM Sodium potassium phosphate buffer); Blank 3) 100 µl buffer + 100 µl AM + 20 µl buffer and EE) and sample; 20 µl EE + 10 µl H_2O_2 and reaction was stopped by the addition of 100 µl AM. The absorbance was recorded at 405 nm.

$$\text{KU}/10^9 \text{ spermatozoa} = \frac{\text{A (Sample)} - \text{A (Blank 1)} \times 27.1}{\text{A (Blank 2)} - \text{A (Blank 3)}}$$

Testosterone and Total Proteins in Seminal Plasma

Seminal plasma (SP) was procured by centrifugation of semen at 5000 rpm for 5 min. Testosterone was estimated in SP using an ELISA kit (Bioassay Technology Laboratories) as per manufacturer's instructions. Testosterone concentration was calculated from the standards run along with the samples and is expressed as ng/ml seminal plasma. Protein concentration was estimated in seminal plasma by the method of Lowry *et al*. (1951).

Fertility Rate Evaluation

Goats showing natural heat symptoms were mated in isolation with the prior selected buck according to mating history and pedigree record in order to avoid inbreeding depression. Proper record was maintained regarding breeding of each buck. Conception rate was confirmed ultra-sonographically after fifty days of mating. Relationship between sperm attributes and FR was observed.

Statistical Analysis

Significant differences among the bucks and groups based on fertility rate for semen attributes, LPO, antioxidant enzymes, testosterone, total proteins were tested by one-way ANOVA using SPSS 20 programme.

Results and Discussion

Analysis of Freshly Ejaculated Semen

Sperm Attributes

A significant ($p<0.05$) difference in mass activity, viability, PMI, AI and DNA-I was observed among the bucks (Table 1). However, differences in sperm concentration, ejaculate volume and initial motility were non-significantly ($p>0.05$) different among the bucks (Table 1). It revealed a significant buck effect on the semen quality. On the contrary, a few studies revealed insignificant buck effect on sperm concentration (Afroz, 2005 and Karim, 2008). Sultana *et al*. (2013) observed significant differences ($p<0.01$-0.05) in volume, sperm concentration, motility and viability among five bucks. Significant variation in initial motility among the bucks was also found (Islam *et al*., 2007; Das *et al*., 2006; Sultana *et al*., 2013). Variation in semen quality of Crossbred Buck (Saanen x Beetal) semen is also reported (Narwade *et al*., 2018). Average values of mass activity and ejaculate volume in14 bucks were 2.66±0.13 and 1.17±0.04 ml (Table 1).

Contrary to our observations, mass activity was reported as 3.66±0.2 (Mara *et al*., 2007) and 3.98±0.03 (Saraswat *et al*., 2012) in goats and Sirohi bucks respectively. Higher average sperm concentration (2.5 – 4.5 x 10^9/ml) was reported in buck semen (Nur *et al*., 2005) compared to our observations (1.49 – 2.9 x 10^9 /ml). Average motility was observed higher in Black Bengal goat semen (80.33 %, Dhar, 2007 and Siddiqua *et al*., 2016) than observed in Beetal goat buck semen (71.1±6.9%) during the present study, whereas, it was quite low in goat semen (62.5 %; Nur *et al*., 2005). Average viability was also reported higher (87.3±5.7 %, 87.58±0.96 % and 92.96 ± 0.99%) in fresh buck semen (Nur *et al*., 2005; Sultana *et al*., 2013 and Siddiqua *et al*., 2016) in comparison to present findings (73.4±1.7%). Plasma membrane plays an important role in maintaining the structural and functional integrity of spermatozoa. Proportion of HOS reacted spermatozoa in semen indicates the presence of spermatozoa with normal structural and functional integrity. Moreover, presence of acrosomal cap is of utmost importance in the fertilization process and has been highly related with frozen semen fertility (Saraswat *et al*., 2012). Values for PMI were comparatively low (23.41±1.03%) in Beetal buck semen than reported in fresh goat buck semen (53.7±11.3%; Nur *et al*., 2005).

Table 1: Sperm attributes (Mean ± SE) in freshly ejaculated semen of Beetal bucks throughout the year

Buck No.	Mass Activity	Volume (ml)	Motility (%)	Viability (%)	HOST (%)	Count (milion/ml)	Acrosome Integrity (%)	DNA Integrity (%)
1	2.3^{c} ±0.2	1.5±0.1	70±4.5	65.5^{c}±6.9	17.1^{bc}±3.2	1998.8±411.1	76.7^{bc}±2.9	90.8^{b}±1.2
2	3.3^{b} ±0.4	0.9± 0.1	79.2±6.9	82.9^{b}±2.9	23.6^{ab} ±3.7	2034.7±176.7	74.3^{c}±5.7	94.4^{ab}±1.5
3	3.3^{b} ±0.2	1.3±0.2	75.8±7.1	76.4^{bc}±6.1	18.6^{bc}±4.6	2539.2±439.5	79.6^{b}±4.0	92.7^{ab}±1.0
4	3.0^{b}±0.5	1±0.3	68.3±8.3	79.1^{bc}±5.8	15.4^{c}±5.4	1761±402.3	77.9^{b}±3.3	93.7^{ab}±0.6
5	3.7^{a}±0.3	1.4±0.2	81.7±6.9	82.6^{b}±1.9	26.7^{bc}±2.6	2921.7±453.7	85.1^{ab}±3.8	95.8^{a}±1.5
6	1.3^{c}±0.4	1.2±0.2	60±7.3	76.1^{bc}±3.3	21.4^{bc}±2.8	1990.4±197.1	84.6^{ab}±3.1	92.9^{ab}±2.4
7	2.7^{c}±0.5	0.9± 0.2	61.7±6.5	66.4^{c}±7.6	34.2^{a}±5.2	1490.4±353.3	89.9^{a}±3.9	92.8^{ab}±1.4
8	2.7^{c}±0.3	1.1±0.1	68.3±9.1	61.8^{c}±7.6	21±1.8	1880±667.9	75.4^{bc}±3.5	92.6^{ab}±0.2
9	2.2^{c}±0.7	1.2±0.2	73.3±4.9	66.7^{c}±6.5	27.4^{b}±3.8	2069.9±479.6	76.6^{bc}±3.3	89.8^{b}±0.8
10	1.8^{c}±0.2	1.2±0.1	61.7±4.1	73.5^{bc}±5.6	19.8±3.1	2039.2±598.4	77.6^{ab}±2.7	92.5^{ab}±1.1
11	2.5^{c}±0.6	1.2±0.1	66.7±9.2	69.3^{c}±5.8	23.9^{bc}±3.1	2222.1±166.7	79.5^{ab}±7.3	95.9^{b}±1.9
12	3.2^{b}±0.4	1.3±0.1	83.3±3.3	63.3^{c}±9.6	29.0^{b}±2.9	2627.5±543.5	70.6^{d}±6.4	93.6^{ab}±3.4
13	2.8^{c}±0.7	1.2±0.1	75.8±8.0	84.2^{a}±3.5	21.4^{bc}±2.0	2329.6±454.1	88.9^{ab}±1.6	94.6^{ab}±2.6
14	2.5^{c}±0.6	1.4±0.2	70±6.8	80.6^{ab}±3.3	28.6^{b}±3.6	2692.5±588.9	88.4^{ab}±1.4	90.1^{b}±1.4
Average	2.7± 0.1	1.2±0.1	71.1±6.9	73.5^{bc}±1.7	23.4^{bc} ±1.0	2185.5±118.1	80.4±1.2	93.1±0.4

Values are a mean of six replicates per buck; Superscripts (a,b,c,d) indicate significant differences among the bucks

Percentage of defected acrosome was 19.63 % in Beetal bucks, whereas, Nur *et al*. (2005) found only 7.2±2.9% spermatozoa with defected acrosomes in buck semen. Average DNA integrity was observed as 93.1±0.4% in Beetal buck semen, which was quite high and did not impair the semen quality. DNA fragmentation in spermatozoa is associated with poor semen quality, lowered fertilization rates, impaired pre-implantation, and poor pregnancy outcomes (Lin *et al*., 2008). Differences in values for sperm attributes among the present study and previous studies may be due to difference in breed and environment. Normal semen characteristics of an ejaculate of a mature buck should be 0.50 – 1.0 ml volume, 2 to 5 x 10^9 spermatozoa / ml, 70 to 90 % motile and 75 to 95 % viable spermatozoa. Sperm count/ml, individual motility and viability were in recommended range in 9, 8 and 7 bucks respectively.

Lipid Peroxidation (MDA Concentration) and Antioxidant Enzymes

The concentration of polyunsaturated fatty acids in sperm membrane is comparatively higher in small ruminants than other species, and hence renders the sperm more venerable to oxidative damage, generating from the ROS following LPO. Subsequently, it results in loss in membrane integrity, impaired sperm function and induction of apoptosis (Gandini *et al*., 2000). An antioxidant system glutathione (GSH), SOD, CAT, GRE and glutathione peroxidase (GPX) serves as an antioxidant defense mechanism against LPO of semen and has been considered to maintain sperm's functionality. MDA (µM/10^9 sperms) and SOD (IU/10^9 sperms), CAT (KU/10^9 sperms) and GRE (IU/10^9 sperms) revealed a significant ($P<0.05$) variation among the bucks (Table 2). Generation of ROS causes changes in sperm membrane fluidity, loss of membrane integrity and irreversible loss of sperm motility. However, protection to sperm functionality against the harmful effects of ROS is provided mainly by antioxidant enzymes (Koziorowska-Giluna *et al*., 2015). Contribution of each antioxidant system vary among species (Michael *et al*., 2008), however, there is a synergistic action between antioxidants. It can be predicted from the present observations that SOD, CAT and GRE synergistically acted to reduce the oxidation stress. GRE activity was low as compared to SOD and CAT in Beetal buck semen. It is well known that mammalian sperms tend to be low in GSH, GPX and GRE activity (Michael *et al*., 2008). Low oxidative stress may be responsible for within normal range of one or the other sperm attributes in 7-9 bucks (Table 1).

Table 2: Lipid peroxidation and antioxidant enzymes in spermatozoa of freshly ejaculated semen of Beetal bucks

Buck No.	MDA (µM/10^9sperms)	SOD (IU/10^9sperms)	CAT (KU/10^9sperms)	GRE (IU/10^9sperms)
1	29.8±9.3	447.3^{ab}±85.1	383.0^{ab}±92.9	26.0^{a}±12.6
2	45.2±20.1	369.0^{b} ±21.8	478.7^{ab}±235.5	11.3^{ab}±6.4
3	40.8±18.1	313.5^{b} ±58.3	329.3^{b}±115.4	8.2^{ab}±4.2
4	24.5±6.6	532.0^{ab} ±94.6	397.0^{ab}±88.1	13.8^{ab}±6.7
5	29.7±8.2	317.0^{b} ±102.4	181.2^{b} ±52.2	4.2^{b}±1.3
6	14.9±6.0	367.0^{b} ±36.1	142.0^{b}±58.4	2.7^{ab}±0.3
7	45.9±16.1	583.3^{ab} ±117.7	284.0^{b}±69.1	12.3^{ab}±4.7
8	36.3±8.4	509.5^{ab} ±184.2	714.8^{a}±149.7	14.7^{ab}±10.3
9	19.3±6.3	396.0^{ab} ±80.4	200.2^{b} ±45.4	16.6^{ab}±11.1
10	27.4±8.2	741.0^{a}±275.8	437.0^{ab}±87.7	5.5^{ab}±1.5
11	26.3±11.7	388.5^{ab} ±63.4	235.2^{b}±51.1	16.5^{ab}±6.2
12	17.8±3.8	390.0^{b} ±24.2	261.5^{b}±83.7	7.1^{ab}±3.5
13	55.8±34.1	332.7^{ab} ±72.6	275.8^{b}±98.5	15.3^{ab}±4.4
14	32.2±10.6	375.5^{ab} ±22.7	382.0^{ab}±104.4	5.5^{ab}±1.5
Average	31.9±3.7	433.1±30.1	335.8±30.4	11.4±1.7

Values are a mean of six replicates per buck; Superscripts (a, b) indicate significant differences among the bucks

Total Protein and Testosterone in Seminal Plasma

There was also a significant (p<0.05) variation in total protein (28.4±2.2 to 52.1±2.3 mg/ml) and testosterone levels (2.8±0.3 to 5.5±0.9 ng/ml) in SP of bucks. However, average values of total protein and testosterone were 38.2±1.2 mg/ml and 4.2±0.2 ng/ml, respectively. Testosterone level in SP of Damini bucks (Malik *et al* 2018) corresponds to our observation in the Beetal bucks (Table 3). Total protein in SP of Damini bucks (Malik *et al.,* 2018) was also close to the present observations. It is also revealed that breed, season and photoperiod had significant effect on testosterone level (Arrebola and Abeci, 2017).

Table 3: Total protein and testosterone in seminal plasma of Beetal bucks

Buck No.	Total protein (mg/ml)	Testosterone (µg/ml)
1	38.2^{ab} ±1.3	3.1^{c}±0.4
2	28.8^{c}±1.1	5.5^{a}±0.9
3	35.1^{ab} ±1.4	4.6^{ab}±0.7
4	40.1^{b}±1.7	4.1^{ab}±0.5
5	28.4^{c}±2.2	4.6^{ab}±0.5
6	43.0^{ab}±3.0	2.8^{d}±0.3
7	33.5^{ab}±2.1	5.0^{b}±0.6
8	40.2^{b} ±3.2	4.5^{ab}±0.4
9	34.6±1.9	4.9^{b}±0.9
10	42.7^{ab}±1.5	3.7^{c}±0.9
11	42.1^{b} ±0.7	2.9^{d}±0.1
12	38.2^{ab} ±2.4	4.2^{ab}±0.9
13	36.9^{ab} ±2.2	5.4^{a}±0.6
14	52.6^{a}±2.3	3.2^{c}±0.4
Average	38.2±1.1	4.2±0.2

Values are a mean of six replicates per buck; Superscripts (a, b) indicate significant differences among the bucks

Relationship of Fertility Rate with Sperm Attributes and Antioxidant Defense System

Fertility Rate of Bucks

Ultrasonography of 84 goats after 50 days of mating indicated pregnancy in 64 goats. A fertility rate of 100%, 83.3 %, 66.7 % and 50% was found for 3, 4, 5 and 2 bucks, respectively (Table 4). Sultana *et al* (2013) also observed significantly (P<0.05) highest non- return rates in one buck (87.31±7.99%) and lowest in one buck (63.41±6.72%). Non return rates of 87% and 81% were also observed in Norwegion dairy goats (Paulenz *et al*., 2003) and Jamnapari

bucks (Chauhan & Anand, 1990).

Table 4: Grouping of bucks based on fertility rate

Group-I		Group-II	
Buck No.	**Fertility rate (%)**	**Buck No.**	**Fertility rate (%)**
3	100	1	66.7
4	100	2	66.7
5	100	7	66.7
6	83.3	8	66.7
9	83.3	10	66.7
11	83.3	12	50
14	83.3	13	50
Average	90.4	Average	61.8

Relationship of Fertility Rate with Semen Attributes

Bucks were divided into two groups, G-I and G-II exhibiting 83.3-100% and 50-66.7% FR, respectively to find out the relationship of fertility rate with sperm attributes and antioxidant defense system. Average values of sperm concentration (10^9 sperms/ml), individual motility (%) and viability (%) were significantly ($p<0.05$) high in G-I (2.3±0.3, 75.8±7.2 and 75.8±6.2) as compared to G-II (2.06±0.46, 71.4±6.0 and 71.1±6.7) (Tables 5 & 6).

Table 5: Mean values for mass activity, volume and sperm count in fresh semen based on fertility rate

Buck No.	MA		Volume (ml)		Sperm count($X10^9$/ml)	
	G-I	G-II	G-I	G-II	G-I	G-II
3 (1)	3.3^{a}±0.2	2.3^{b}±0.2	1.3^{a}±0.2	1.5^{a}±0.1	2.5^{a}±0.4	1.9^{a}±0.4
4 (2)	3.0^{a}±0.5	3.3^{a}±0.4	1.0^{a}±0.3	0.9^{a}±0.1	1.7^{b}±0.4	2.0^{a}±0.1
5 (7)	3.7^{a}±0.3	2.7^{b}±0.5	1.4^{a}±0.2	0.9^{a}±0.2	2.9^{a}±0.4	1.5^{a}±0.3
6 (8)	1.3^{b}±0.4	2.7^{a}±0.3	1.2^{a}±0.2	1.1^{a}±0.1	1.9^{a}±0.1	1.8^{a}±0.6
9 (10)	2.2^{a}±0.7	1.8^{b}±0.2	1.2^{a}±0.2	1.2^{a}±0.1	2.1^{a}±0.4	2.0^{a}±0.5
11 (12)	2.5^{b}±0.6	3.2^{a}±0.4	1.2^{a}±0.1	1.3^{a}±0.1	2.2^{a}±0.1	2.6^{a}±0.5
14 (13)	2.5^{b}±0.6	2.8^{b}±0.7	1.4^{a}±0.2	1.2^{a}±0.1	2.7^{a}±0.5	2.3^{a}±0.4
Average	2.7^{a}±0.4	2.6^{a}±0.3	1.3^{a}±0.2	1.1^{a}±01	2.3^{a}±0.3	2.1^{b}±0.4

Values in superscript indicate significance ($p<0.05$) difference among two groups (a, b); Figures in parentheses are buck numbers of G-II; G-I: >83.33 – 100 % fertility rate; G-II: 50.0-66.66 % fertility rate

Table 6: Mean values for motility, viability, plasma membrane, acrosome and DNA integrity in fresh semen based on fertility rate

Buck No.	Motility (%)		Viability (%)		PMI (%)		AI		DNA-I	
	G-I	G-II	G-I	G-II	G-I	G-II	G-I	G-II	G-I	G-II
3 (1)	75.8^{a}±7.1	70.0^{b}±4.5	76.4^{a}±6.1	65.5^{b}±6.9	18.6^{a}±4.6	17.1^{a}±3.2	79.6^{a}±4.0	76.7^{a}±2.9	92.7±1.0	90.8^{a}±1.2
4 (2)	68.3^{b}±8.3	79.2^{a}±6.9	79.1^{b}±5.8	82.9^{a}±2.9	15.4^{b}±5.4	23.6^{a}±3.7	77.9^{a}±3.3	74.3^{a}±5.7	93.7^{a}±0.6	94.4^{a}±1.5
5 (7)	81.7^{a}±6.9	61.7^{b}±6.5	82.6^{a}±1.9	66.4^{a}±7.6	26.7^{b}±2.6	34.2^{a}±5.2	85.1^{b}±3.8	89.9^{a}±3.9	95.8^{a}±1.5	92.8^{a}±1.4
6 (8)	65.0^{a}±7.3	68.3^{a}±9.1	76.1^{a}±3.3	61.8^{b}±7.6	21.4±2.8	21.0^{a}±1.8	84.6^{a}±3.1	75.4^{b}±3.5	92.9^{a}±2.4	92.6^{a}±0.2
9 (10)	73.3^{a}±4.9	61.7^{b}±4.1	66.7^{b}±6.5	73.5^{a}±5.6	27.4^{a}±3.8	17.8^{a}±3.1	76.6^{a}±3.3	77.6^{a}±2.7	89.8^{a}±0.8	92.5^{a}±1.1
11 (12)	66.7^{b}±9.2	83.3^{a}±3.3	69.3^{a}±5.8	63.3^{b}±9.6	23.9^{b}±3.1	29.0^{a}±2.9	79.5^{a}±7.3	70.6^{b}±6.4	95.9^{a}±1.9	93.6^{a}±3.4
14 (13)	70.0^{b}±6.8	75.8^{a}±8.0	80.6^{a}±3.3	84.2^{a}±3.5	28.6^{a}±3.6	21.4^{b}±2.0	88.4^{a}±1.4	88.9^{a}±1.6	90.1^{a}±1.4	94.6^{a}±2.6
Average	75.8^{a}±7.2	71.4^{b}±6.0	75.8^{a}±4.6	71.1^{b}±6.2	23.1^{a}±3.7	21.7^{a}±3.1	81.7^{a}±3.7	79.1^{a}±3.8	94.0^{a}±1.4	93.1^{a}±1.6

Values in superscript indicate significance ($p<0.05$) difference among two groups (a, b); Figures in parentheses are buck numbers of G-II; G-I: >83.33 – 100 % fertility rate; G-II: 50.0-66.66 % fertility rate

There was no significant difference in mass activity, ejaculate volume, PMI, AI and DNA integrity among the groups (Table 5 and 6). It indicated that sperm attributes like sperm concentration, individual motility, viability, PMI and AI were insufficient to predict the sub-fertility of bucks. Gadea *et al*. (2004) was also of the same opinion that standard parameters like motility, morphology and sperm concentration are insufficient not only to predict fertility, but even to identify sub-fertile individuals. It is considered that selection of breeding bucks for either natural mating or artificial insemination relies on semen quality evaluation (Kumar *et al*., 2009). Therefore, semen evaluation may help in early detection of impaired fertility.

Relationship of Fertility with Antioxidant Defense System of Spermatozoa

Average MDA production (µM/10^9 spermatozoa), SOD (IU/10^9 spermatozoa), CAT (KU/10^9 spermatozoa) and GRE (IU/10^9 spermatozoa) were significantly ($p<0.05$) low in G-I (26.8±9.6, 336.2±65.4, 266.7±73.6 and 9.6±4.5) compared to G-II (36.9±14.3, 481±111.6, 404.9±116.7 and 13.2±6.2, Table 7).

Table 7: Mean values for Lipid per-oxidation and antioxidant enzymes in fresh semen based on fertility rate

Buck No.	MDA (µM/10^9 spermatozoa)		SOD (IU/109 spermatozoa)		Catalase (KU/109 spermatozoa		GRE (IU/109 spermatozoa)	
	G-I	G-II	G-I	G-II	G-I	G-II	G-I	G-II
3 (1)	40.8^a±18.1	29.8^b±9.3	313.5±58.3	447.3^a±85.1	329.3^b±115.4	383.0^a±92.9	8.2^b±4.2	26.0^a±12.6
4 (2)	24.5^b±6.6	45.2^a±20.1	532.0^a±94.6	369.0^b±21.8	397.0^b±88.1	478.7^a±235.5	13.8^a±6.7	11.3^a±6.4
5 (7)	29.7^b±8.2	45.9^a±16.1	317.0^b±102.4	583.3^a±117.7	181.2^b±52.2	284.0^a±69.1	4.2^b±1.3	12.3^a±4.7
6 (8)	14.9^a±6.0	36.3^a±8.4	367.0^b±36.1	509.5^a±184.2	142.0^b±58.4	714.8^a±149.7	2.7^b±0.3	14.7^a±10.3
9 (10)	19.3^a±6.3	27.4^a±8.2	396.0^b±80.4	741.0^a±275.8	200.2^b±45.4	437.0^a±87.7	16.6^a±11.1	5.5^b±1.5
11 (12)	26.3^a±11.7	17.8^b±3.8	388.5^a±63.4	390.0^a±24.2	235.2^a±51.1	261.5^a±83.7	16.5^a±6.2	7.1^a±3.5
14 (13)	32.2^b±10.6	55.8^b±34.1	375.5^a±22.7	332.7^b±72.6	382.0^a±104.4	275.8^b±98.5	5.5^b±1.5	15.3^a±4.4
Average	26.8^b±9.6	36.9^a±14.3	336.2^b±65.4	481^a±111.6	266.7^b±73.6	404.9^a±116.7	9.6^b±4.5	13.2^a±6.2

Values in superscript indicate significance ($p<0.05$) difference among two groups (a, b); Figures in parentheses are buck numbers of G-II; G-I: >83.33 – 100 % fertility rate; G-II: 50.0-66.66 % fertility rate

Therefore, these enzymes were more utilized by the bucks of G-I to reduce the oxidative stress. Increased levels of ROS have been correlated with decreased sperm motility (Eskanazi *et al*., 2003), increased sperm DNA damage (Armstrong *et al*., 1999), sperm cellular membrane lipid peroxidation (Aitken, 1994) and decreased efficacy of oocyte-sperm fusion (Agarwal *et al*., 2005). Excessive ROS levels impaired the motility and fertilization capacity of ram semen (Bucak *et al*., 2007). An important correlation between lipid peroxidation in the plasma membrane, production of MDA and loss of motility was also reported in caprine (Bucak *et al*., 2007). Therefore, higher production of MDA was also cause for low value of sperm attribute and fertility rate in G-II bucks during the present study.

Relationship of Fertility Rate with Seminal Plasma Total Proteins and Testosterone Level

Average values of total protein and testosterone were significantly ($p<0.05$) high in G-I (4.5±0.7 vs. 3.9±0.5 ng/ml) and G-II (41.1±2.0 vs 35.1±1.8 ng/ml), respectively (Table 8). Quality of spermatozoa is influenced by testosterone levels. Low testosterone levels may lead to decline in the quality of spermatozoa as testosterone plays an important role in sperm maturation (Cornwall, 2009). A correlation between testosterone levels with libido and sperm quality is also proved (Rachmawati *et al*., 2011). Angel-Garcia *et al*. (2014) concluded that testosterone injection to sexually inactive bucks provoke an increase in serum testosterone, which in turn induce sexual behavior and improvement of semen quality. Although, testosterone was significantly ($p<0.05$) low in G-I compared to G-II, but sperm count, motility and viability were in normal range of 4-5 and 3-4 bucks of G-I and G-II, respectively. Moreover, average sperm count, motility and viability were within normal range of both groups. Fertility rate also ranged from 50-66.6 % in G-II. It indicated that testosterone level of both the groups was optimum to maintain the sperm attributes within normal range and fertility rate of ≥50%.

Table 8: Mean values for testosterone and total protein in seminal plasma based on fertility

Buck No.	Testosterone (ng/ml)		Total protein (mg/ml)	
	G-I	G-II	G-I	G-II
3 (1)	$4.6^a±0.7$	$3.1^b±0.4$	$35.05^a±1.4$	$38.2^a±1.4$
4 (2)	$4.1^b±0.5$	$5.5^a±0.9$	$40.05^a±1.7$	$28.8^b±1.1$
5 (7)	$4.6^a±0.5$	$5.0^a±0.6$	$43.0^a±3.0$	$28.4^b±2.2$
6 (8)	$2.8^b±0.3$	$4.5^a±0.4$	$40.2^a±3.2$	$33.5^b±2.1$
9 (10)	$4.9^a±0.9$	$3.7^b±0.9$	$34.6^b±1.9$	$42.1^a±1.5$
11 (12)	$2.9^b±0.1$	$4.2^a±0.9$	$42.1^a±0.7$	$36.9^b±2.2$
14 (13)	$3.2^b±0.4$	$5.4^a±0.6$	$52.6^a±2.3$	$38.2^a±2.4$
Average	$3.9^b±0.5$	$4.5^a±0.7$	$41.1^a±2.0$	$35.1^b±1.8$

Values in superscript indicate significance ($p<0.05$) difference among two groups (a, b); Figures in parentheses are buck numbers of G-II; G-I: >83.33 – 100 % fertility rate, G-II: 50-66.66 % fertility rate

Conclusion

Study concluded that sperm's oxidative stress and SP testosterone levels influence semen quality vis à vis fertility of Beetal bucks. This study also revealed an importance of semen quality assessment based on various sperm function tests that may provide the guidelines for reproductive performance in buck evaluation.

Conflict of Interests

There is no conflict of interest.

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