



Status of beta defensin-1 in Indian goat breeds

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ABSTRACT

The present study was carried out to know the status of Beta Defensin-1 in goat semen before and after cryopreservation with beta defensin-1 supplemented semen diluent and in blood of different breeds of goat (Barbari, Jamunapari and Jakhrana). Goat semen (N-10) from each breed was collected by artificial vagina method. Immediately after collection, the volume, colour, consistency, and mass motility of ejaculate were assessed and were extended with Tris-Egg yolk-Fructose diluent having 10% (v/v) egg yolk and glycerol 6% (v/v). Samples were divided for estimation of beta defensin-1 and rest parts were cryopreserved with semen diluent having beta defensin-1 @ 10 ng/mL. Blood samples (N-30) were also collected from the same animal after semen collection. The samples were stored at -20°C until assayed. Plasma membrane of sperm was broken by freeze thaw followed by ultracentrifugation (20,000 × g for 5 min) at room temperature before ELISA test. The samples were diluted with Phosphate buffer (1:2) before analysis. The samples were analyzed using goat specific beta defensin-1 commercial kit (EO6D0419) as per the manufacturer's instruction. The result showed that with supplementation of beta defensin-1 in goat semen, diluent maintains the concentration of beta defensin-1 even after cryopreservation. There was significant decrease (P<0.05) in beta defensin-1 concentration in sample which had no supplement in semen diluent after cryopreservation. The supplementation of beta defensin-1 in goat semen diluent improved the post-thaw immune modulatory properties of semen.

Key words: Artificial Insemination, Beta defensin, Goat sperm, Immunomodulator, Semen cryopreservation

Defensins are antimicrobial peptides (AMPs) of the innate immune system produced by epididymis. Beta defensins are triple-stranded β -sheet structure and have a molecular weight between 3–6 kDa. Two beta-defensins have been identified in goat, viz. GBD1 and GBD2. Glycosyl Phosphatidyl Inositol (GPI) anchored glycoproteins are inserted into the plasma membrane during sperm residence in the caput of the epididymis, whereas the surface-associated glycoproteins continue to be added until the time of ejaculation (Martins *et al.* 2003). Defensins were originally thought only to contribute to the defense of the reproductive system from pathogen invasion (Hall *et al.* 2002). However, these are shown to be associated with specific sperm functions, including initiation of motility and capacitation (Zhou *et al.* 2004; Tollner *et al.* 2004). The mechanism of sperm transport to oocyte and evasion of immune surveillance within the hostile environment of the female reproductive tract is not fully known (Cao *et al.* 2010). The male fertility problem (10%) in farm animals is

due to poor immunological competence of spermatozoa and pregnancy losses (30%) by idiopathic and immunological origin. Beta defensin protects the sperm from immunological aggression (Li *et al.* 2001) of female reproductive tract. We can enhance the fertilizing ability of spermatozoa by increasing the production of beta defensins *in vivo*.

Human population will reach to 1.7 billion and goat population will be 216 million by 2050 (ICAR-CIRG Vision, 2050). Therefore to meet requirement of milk, meat and associated by products of ever increasing human population, it is imperative to increase goat productivity. It could be possible by increasing high productive descript (33%) goat population and reducing non-descript (67%) and less productive goats by promoting scientific goat farming. Artificial Insemination (AI) is the only solution to improve the quality and productivity per goat. There are 71 million breedable does and 17 million breeding bucks available in India as per 12th Livestock Census Report. Artificial insemination (AI) with frozen semen could be successfully used for preservation, *ex situ* conservation and propagation of goat germplasm (Kharche *et al.* 2013). If by adding immuno-modulator in goat semen, dilutor may lead to enhance the fertility of sperm then low sperm concentration per semen straw will be sufficient for AI and

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successful conception. So, the production of semen straws will be more per goat and can cover entire population of goat in this country. Thus the aim of present study was to know the status of beta defensin in goat semen and blood, so that the inclusion level can be decided to get better post thaw quality suitable for AI programme.

MATERIALS AND METHODS

Animal and their management: The adult bucks (N-30) of 2–4 years old of Barbari, Jamunapari and Jakhrana breed of Indian goat were selected for the study. The bucks were kept under semi-intensive system of management at experimental shed of Animal Physiology and Reproduction Division of this Institute.

Semen collection and cryopreservation: Goat semen ejaculates (N-10) from each breed were collected using artificial vagina method twice a week. Immediately after collection macroscopic (volume, colour, consistency) and microscopic (mass motility) evaluation of ejaculates were done. Semen samples were extended with Tris–Egg yolk–Fructose diluent (Tris 3.604 g; Citric acid 1.902 g; Fructose 1 g; Streptomycin 100 mg; Penicillin 100000 IU; Triple distilled water 100 ml; pH 6.75–6.8), having 10% (v/v) egg yolk and glycerol 6% (v/v). Samples were divided for estimation of beta defensin-1 and rest parts were cryopreserved with semen diluent having beta defensin-1 (Sigma Aldrich, USA) @ 10 ng/mL. Samples having mass motility >4 and progressive motility >70% were taken for this study as this quality of semen sample qualify for freezing process. Sperm concentrations were adjusted to 1×10^8 /ml and diluted semen was equilibrated at 5°C for 4 h before being frozen (Ranjan *et al.* 2009a; 2009b; 2014; 2015; 2017).

Blood Collection and storage: Blood samples (N-30) were also collected from the same animal after semen collection from each breed. The samples were stored at –20°C until assayed.

Estimation of beta defensin-1: Plasma membrane of sperm was broken by freeze thaw followed by ultracentrifugation ($20,000 \times g$ for 5 minute) at room temperature before ELISA test. The samples were diluted with Phosphate buffer (1:2) before analysis. The samples were analyzed using goat specific beta defensin-1 commercial kit (EO6D0419, Genxbio Pvt. Ltd.) as per the manufacturer's instruction.

Artificial Insemination in estrous goat: Intra-cervical AI was mainly used to get maximum benefits. For Intra cervical AI, the oestrous goat was lifted from back for clear visualization of genitalia. A lubricated glass vaginal speculum was inserted through vagina for visualization of cervical opening under sunlight. Then frozen thawed semen straw was inserted through vaginal speculum and go through cervical opening and semen was deposited there. AI should be done twice at 12 h interval. AI should be performed after 10–12 h of oestrous exhibition. The total time taken is 2–3 min.

Statistical analysis: Data were analyzed by SPSS data

analysis software package (SPSS, Chicago, IL, USA). The factorial model included the effect of breeds (Barbari, Jamunapari, Jakhrana) as independent variables and percent of beta defensin-1 concentration as dependent variables.

RESULTS AND DISCUSSION

Beta defensin-1 concentration (pg/ml) in neat semen was significantly higher ($P < 0.05$) in Barbari followed by Jamunapari and Jakhrana. The same trends were observed in sperm pellet and seminal plasma of different Indian goat breed. In contract, we found very high concentration of beta defensin-1 (pg/ml) in blood of Jakhrana followed by Barbari and Jamunapari Indian goat breed. The concentration was two to three times higher in seminal plasma than sperm pellet. The post thaw quality was significantly ($P < 0.05$) higher in 10 ng/ml beta defensin concentration than other concentrations so, 10 ng/ml beta defensin concentration in goat semen dilutor should be used for routine semen freezing protocol showed in our earlier result (Ranjan *et al.* 2018 unpublished). Supplementation of beta defensin-1 in goat semen diluent maintains the initial concentration of beta defensin-1 after cryopreservation. There was significant decrease ($P < 0.05$) in beta defensin-1 concentration in sample had no beta defensin-1 supplement in semen diluent after cryopreservation. The concentration of beta defensin decreased significantly 5–6 time lower than control group. The results were presented in Table 1 and Table 2.

The best combinations of semen straw having beta defensin @ 10 ng/mL in semen diluent were cryopreserved

Table 1. Concentration of beta defensin-1 in blood and semen before cryopreservation

Goat breed	Blood serum	Semen neat	Sperm pellet	Seminal plasma
Jakhrana	3143.54±156.47 ^a	638.44±27.14 ^c	241.18±44.85 ^c	434.27±31.87 ^b
Barbari	2640.88±128.44 ^b	881.72±79.58 ^a	508.67±56.77 ^a	1437.14±156.47 ^a
Jamunapari	2385.31±267.67 ^c	772.18±66.68 ^b	367.85±41.35 ^b	1321.37±141.35 ^a

Different superscripts (a,b,c) differed significantly within column ($P < 0.05$).

Table 2. Concentration of beta defensin-1 in semen before and after cryopreservation having 10 ng/mL beta defensin-1 in semen diluent

Goat breed	Semen neat	Post thaw semen having BD @ 0 ng/mL	Post thaw semen having BD @ 10 ng/mL
Jakhrana	638.44±27.14 ^c	85.26±12.26 ^b	509.63±40.36 ^b
Barbari	881.72±79.58 ^a	112.58±21.28 ^a	694.54±55.78 ^a
Jamunapari	772.18±66.68 ^b	124.56±26.35 ^a	704.71±64.38 ^a

Different superscripts (a,b,c) differed significantly within column ($P < 0.05$).

and used for AI in 20 Barbari goats. The kidding per cent was 35 on actual kids produced in Indian Barbari goat.

In mammals, the main site of β -defensin expression is the epididymis and secretion from here is believed to result in their detection on the plasma membrane of sperm (Yudin *et al.* 2005; Fei *et al.* 2012). We also observed beta defensin concentration in semen pellet and seminal plasma in different Indian goat breed. There was significantly higher concentration of beta defensin in Barbari followed by Jamunapari and Jakhrana breed of goat. In contrast, we found opposite trend in blood plasma, which may be due to hardy and wild nature of Jakhrana breed of goat compared to other breeds of goat. Jakhrana have significantly ($P < 0.05$) high beta defensin-1 concentration in blood in comparison to semen than Barbari and Jamunapari.

Jakhrana breed are more disease resistant than other breeds studied in this experiment. But the semen quality is poor in Jakhrana than Barbari and Jamunapari breed of goat. The lack of ability of the defensin-deletion sperm to bind to the oocyte, even under IVF conditions, may have several potential explanations: (i) disruption of the membrane composition and loss of non-defensin molecules essential for oocyte binding, (ii) loss of the defensin(s) that would normally interact with the oocyte and/or (iii) lack of sperm motility meaning a reduction of propulsive force. The β -defensin, *DEFB126*, was linked to the ability of sperm to penetrate hyaluronic acid gel (a mimic of female cervical mucus) and men homozygous for a frameshift mutation were found not to be infertile but to have a reduced chance of successful conception (Tollner *et al.* 2011). More recently, these defensins have also been shown to be associated with specific sperm functions, including initiation of motility and capacitation (Zhou *et al.* 2004; Tollner *et al.* 2004). Beta-defensin, uniformly spans the entire sperm surface and is not exclusive to a specific domain (Yudin *et al.* 2003).

The differentiation between self and non-self antigens is a critical property of the immune system and is the cornerstone for defending the body against invasion by pathogens (Medzhitov and Janeway 2002; Ribeiro *et al.* 2012). On the other hand, the ability of the female to tolerate male gametes is essential for the continuation of a species. Because sperm antigens have been shown to elicit a potent immunological response in the female, it stands to reason that sperm must have a shield that conceals or masks unique testicular and epididymal antigens on the sperm surface. It strongly suggests that *DEFB126* acts as a shield (Zasloff 2002; Colledge 2013).

Due to the complex cervical anatomy in goats, it becomes very difficult to pass the AI gun throughout the cervix. Therefore, the conception rate is highly correlated with depth of penetration. This technique is more suited in Indian goats and easy to perform. Although the laparoscopic AI involving deposition of frozen-thawed semen directly into the uterus generally results in 60–70% fertility (Abdelhakeam *et al.* 1991; Salamon and Maxwell, 1995), the conception rate of cryo-preserved semen following

trans-cervical AI (TCAI) is still very low (16–40%; Cseh *et al.* 2012; Kharche *et al.* 2013; Kumar and Naqvi 2014). We found 35% AI success rate based on actual kidding basis by using trans-cervical AI Technique in Indian Barbari goat breed. The premature capacitation as a consequence of freezing and thawing curtails the lifespan of spermatozoa having very shorter time to achieve fertilization compared to the fresh sperm. Therefore, further research efforts are required to develop better freezing protocols and diluents that minimize the ultra-structural and biochemical alterations in spermatozoa resulting from the freezing/thawing process, particularly if intended for TCAI; because spermatozoa have to survive longer to traverse through cervical mucosa before reaching the site of fertilization.

It can be summarized that there was significant decrease ($P < 0.05$) in beta defensin-1 concentration in sample had no beta defensin-1 supplement in semen diluent after cryopreservation. Supplementation of beta defensin-1 in goat semen diluent maintains the initial concentration of beta defensin-1 after cryopreservation.

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REFERENCES

- Abdelhakeam A A, Graham E F, Vazquez I A and Chaloner K M. 1991. Development of an extender for freezing; effects of osmotic pressure, egg yolk levels, type of sugars and the method of dilution. *Cryobiology* **28**: 43–49.
- Cao D, Li Y, Yang R, Wang Y, Zhou Y, Diao H, Zhao Y, Zhang Y and Lu J. 2010. Lipopolysaccharide-induced epididymitis disrupts epididymal beta-defensin expression and inhibits sperm motility in rats. *Biology of Reproduction* **83**: 1064–70.
- Colledge W H. 2013. Defending Sperm Function. *PLoS Genetics* **9**: 10
- Cseh S, Faigl V and Amiridis G S. 2012. Semen processing and artificial insemination in health management of small ruminants. *Animal Reproduction Science* **130**: 187–92.
- Fei Z, Hu S, Xiao L, Zhou J, Diao H, Yu H, Fang S, Wang Y, Wan Y, Wang W, He Y, Wang C, Xu G, Wang Z, Zhang Y and Fei J. 2012. mBin1b transgenic mice show enhanced resistance to epididymal infection by bacteria challenge. *Genes and Immunity* **13**: 445–51.
- Hall S H, Hamil K G and French F S. 2002. Host defense proteins of the male reproductive tract. *Journal of Andrology* **23**: 585–97.
- Kharche S D, Jindal S K, Priyadhrashini R, Kumar S, Goel A K, Ramachandran N and Rout P K. 2013. Fertility following frozen semen artificial insemination in Jamunapari goats. *Indian Journal of Animal Sciences* **83**(10): 1071–73.
- Kumar D and Naqvi S M K. 2014. Effect of time and depth of insemination on fertility of Bharat Merino sheep inseminated trans-cervical with frozen-thawed semen. *Journal of Animal Science and Technology* **56**: 8.
- Li P, Chan H C, He B, So S C, Chung Y W, Shang Q, Zhang Y D and Zhang Y L. 2001. An antimicrobial peptide gene found in the male reproductive system of rats. *Science* **291**: 1783–85.
- Martins S G, Miranda P V and Brandelli A. 2003. Acrosome

- reaction inhibitor released during *in vitro* sperm capacitation. *International Journal of Andrology* **26**: 296–304.
- Medzhitov R and Janeway C A Jr. 2002. Decoding the patterns of self and non self by the innate immune system. *Science* **296**: 298–300.
- Ranjan R, Goel A K, Ramachandran N, Kharche S D and Jindal S K. 2015. Effect of egg yolk levels and equilibration periods on freezability of Jamunapari buck semen. *The Indian Journal of Small Ruminants* **21**(1): 32–36.
- Ranjan R, Goel A K, Ramachandran N, Kharche S D, Gangwar C and Jindal S K. 2014. Comparison between normal and dual staining technique for evaluating acrosome status and viability in frozen thawed buck spermatozoa. *The Indian Journal of Small Ruminants* **20**(2): 50–53.
- Ranjan R, Priyadharsini R, Goel A K, Brejendra S, Kumar S, Kharche S D and Jindal S K. 2017. Effect of membrane stabilizer on the freezability of Buck semen. *Indian Journal of Animal Sciences* **87**(4): 435–36.
- Ranjan R, Ramachandran N, Jindal S K and Sinha N K. 2009a. Hypo osmotic swelling test in frozen thawed goat spermatozoa. *Indian Journal of Animal Sciences* **79**: 1022–23.
- Ranjan R, Ramachandran N, Jindal S K, Sinha N K, Goel A K, Kharche S D and Sikarwar A K S. 2009b. Effect of egg yolk levels on keeping quality of Marwari buck semen at refrigeration temperature. *Indian Journal of Animal Sciences* **79**(7): 662–64.
- Salamon S and Maxwell W M C. 1995. Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Animal Reproduction Science* **38**: 1–36.
- Tollner T L, Venners S A, Hollox E J, Yudin A I, Liu X, Tang G, Xing H, Kays RJ, Lau T, Overstreet J W, Xu X, Bevins C L and Cherr G N. 2011. A common mutation in the defensin DEFB126 causes impaired sperm function and subfertility. *Science Translational Medicine* **3**: 92.
- Tollner T L, Yudin A I, Treece C A, Overstreet J W and Cherr G N. 2004. Macaque sperm release ESP13.2 and PSP94 during capacitation: The absence of ESP13.2 is linked to sperm–zona recognition and binding. *Molecular Reproduction and Development* **69**: 325–37.
- Yudin A I, Generao S E, Tollner T L, Treece C A and Overstreet J W. 2005. Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti–sperm antibodies. *Biology of Reproduction* **73**: 1243–52.
- Yudin A I, Tollner T L, Li M W, Treece C A, Overstreet J W and Cherr G N. 2003. ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. *Biology of Reproduction* **69**: 1118–28.
- Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415**: 389–95.
- Zhou C X, Zhang Y L, Xiao L, Zheng M, Leung K M, Chan M Y, Lo P S, Tsang L L, Wong H Y, Ho L S, Chung Y W and Chan H C. 2004. An epididymis specific beta-defensin is important for the initiation of sperm maturation. *Nature Cell Biology* **6**: 458–64.