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Effect of beta defensin-1 on post-thaw quality of cryopreserved Barbari buck semen

R RANJAN[1], S P SINGH[2], K GURURAJ[3], S K JINDAL[4] and M S CHAUHAN[5]

ICAR-Central Institute for Research on Goats, Farah, Mathura, Uttar Pradesh 281 122 India

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The mechanism by which sperms reach the oocyte and evade 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. The epididymis produced peptides classified as 'defensins' which were originally thought only to contribute to the defense of the reproductive system from pathogen invasion. Defensins are antimicrobial peptides (AMPs) and first described in 1980s as components of the innate immune system. They have been classified based on structure into three class- alpha, beta and theta. 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. It helps in initiation of motility and capacitation of sperm (Tollner *et al.* 2011, Zhou *et al.* 2011). Beta defensin uniformly spans the entire sperm surface and is not exclusive to a specific domain (Yudin *et al.* 2003). They form a coat on sperm surface that provides protection from recognition by immune competent cells in *in vivo* model system as well as when challenged with antisperm antibodies *in vitro*. The ability of the female to tolerate male gametes is essential for the continuation of a species. Sperm antigens elicit a potent immunological response in the female, so sperm must have a shield that conceals or masks unique testicular and epididymal antigens on the sperm surface (Colledge 2013). Beta defensin protects the sperm from immunological aggression of female reproductive tract (Li *et al.* 2001). By increasing the production of beta defensin *in vivo*, we can enhance the fertilizing ability of spermatozoa. Moreover, fortification of beta defensin in semen dilutor may lead to use less number of spermatozoa per dose for artificial insemination. So, more number of semen straws can be prepared from the same goat and it will be cost effective. Thus the aim of present study was to improve the post thaw quality by adding immunomodulator (beta defensin-1) in goat semen dilutor so that the frozen goat semen straw can be used for Artificial Insemination Programme.

Present address: [1]Senior Scientist (dr_raviranjan @yahoo.co.in), [2]Scientist (spsinghmail1@gmail.com), [4]Principal Scientist and Head (jindalmathura@gmail.com), Animal Physiology and Reproduction Division; [3]Scientist (guruvet @gmail.com), Animal Health Division; [5]Director (director.cirg @icar.gov.in).

The adult Barbari bucks of 2–4 years old (10) were selected for the study. The bucks were kept under semi-intensive system of management. All the animals were handled as per Ethical rule. Institute Animal Ethical Committee (IAEC) had approved the work and all procedure was followed as per IAEC rule.

Ejaculates (60) from Barbari bucks 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 having mass motility ≥+3 and volume ≥0.5 ml were used for freezing.

Semen samples were extended with Tris-Citrate-Fructose yolk diluents (3.604 g Tris, 1.902 g citric acid, 1 g fructose, 100 mg streptomycin, 100000 IU penicillin, 100 ml triple distilled water; pH 6.8–6.9) having 10% (v/v) egg yolk and 6% (v/v) glycerol (Ranjan *et al.* 2009, 2015). The samples were diluted to maintain the sperm concentration 100–120 million/dose.

Semen sample selected were diluted with the dilutor having 0 ng/ml (control), 10 ng/ml, 20 ng/ml and 30 ng/ml of beta defensin-1 (M/s Sigma-Aldrich Chemicals Pvt. Ltd.) concentration and diluted semen samples were equilibrated at 5°C for 4 h before being frozen.

Semen was filled in French mini straws at 5°C after 4 h equilibration period and straws were sealed with polyvinyl alcohol powder. Straws were frozen in vapours of liquid nitrogen by keeping them 4 cm above the liquid nitrogen for 10 min. Finally straws were plunged and stored into liquid nitrogen container.

Post thaw semen (10 µl) was placed on a clean grease free warm slide (38°C) with cover slip and observed under 200× magnification of phase contrast microscope for assessing the progressive motility. The average values of 2 independent experts were considered for calculating the progressive motility. For calculating the live and dead sperm count, a method of Hancock (1951) using Eosin-Nigrosine stain was followed. Abnormal sperms were counted with

the same staining technique. Giemsa stain was used to assess the acrosomal integrity of frozen thawed buck spermatozoa as per Watson (1975) and Ranjan *et al.* (2014). Hypo-osmotic swelling test was carried out by Ranjan *et al.* (2009).

Data were analyzed by two-way ANOVA by SPSS package 16 (IBM® SPSS Statistics Software). The factorial model included the effect of beta defensin-1 concentration as independent variable and per cent post thawed motility and live sperm count, acrosome intact sperm and hypo-osmotic swelled sperm as dependent variables.

The effects of beta defensin-1 in goat semen extender were evaluated and the per cent of motile spermatozoa, live spermatozoa, hypo-osmotic swelled spermatozoa and acrosome integrity for each beta defensin-1 concentration were averaged. The effect of the different concentration of beta defensin-1 in diluents on post-thaw sperm quality is summarized in Table 1.

The progressive motility, live sperm count, acrosome integrity and hypo-osmotic swelling positive spermatozoa (Mean±SE) in fresh semen were 84.12±0.57, 85.27±0.58, 82.11±0.61 and 80.54±0.51 respectively. The parameters calculated in the present study were within acceptable limit for freezing protocol.

The progressive motility, live sperm count, acrosome integrity and hypo-osmotic swelling positive spermatozoa (Mean±SE) were highest in 10 ng/ml followed by 20 ng/ml, 0 ng/ml and 30 ng/ml of beta defensin-1 concentration. The data also revealed that the post-thaw quality differed significantly ($P<0.05$) with different concentration of beta defensin-1 used in the present study. The acrosome intact spermatozoa was non-significantly different ($P<0.05$) among control, 10 ng/ml and 20 ng/ml beta defensin-1 in the present study.

The post-thaw survival is restricted to approximately 50% of the sperm population even with best cryopreservation protocol. Our objective was to improve the post-thaw quality by adding immune-modulator in the goat semen dilutor so that the frozen goat semen straw can be used more efficiently for Artificial Insemination programme. There are 71 million breedable does and 17 million breeding bucks available in India as per 12th Livestock Census report. One buck by natural mating can cover maximum 50 does in a year, but by stored frozen semen, 3,000 does can be covered in a year. Therefore, to cover 71 million breedable does, we need 1.5–2 million bucks as compared to only 50,000 buck needed for frozen semen AI Technology. The epididymis produce peptides classified as 'defensins', which were originally thought only to contribute to the defense of the reproductive system from pathogen invasion (Hall *et al.* 2002). More recently, these defensins have also been shown to be associated with specific sperm functions, including initiation of motility and capacitation (Tollner *et al.* 2004, Zhou *et al.* 2004). Beta-defensin, uniformly spans the entire sperm surface and is not exclusive to a specific domain (Yudin *et al.* 2003). Sperm capacitation is required for egg fertilization and involves several cellular changes including loss of cholesterol from the sperm head and increased membrane hyperpolarization. Beta defensin proteins may inhibit these events to help prevent premature sperm capacitation. In addition, beta defensin might also block Ca^{2+} entry, which is required for the acrosome reaction (Yudin *et al.* 2003). Our results showed that beta defensin protect premature capacitation and acrosomal reaction. Fortification of beta defensin-1 as immuno-modulator in semen dilutor enhanced the post thaw quality of cryopreserved sperm as shown in our result. This will lead to use of less number of spermatozoa per dose for Artificial Insemination. So, more number of semen straws can be prepared from the same goat and it will be cost effective also.

SUMMARY

Goat plays a vital role in the economy of poor and marginal farmers and very little efforts have been taken for their genetic improvement at farm level. The greatest problem still existing with the cryopreservation of goat spermatozoa is that even with the best preservation techniques available to-date, post-thaw survival is restricted to approximately 50% of the sperm population. Defensins are antimicrobial peptides and 2 beta-defensins have been identified in goat C GBD1 and GBD2). They form a coat on sperm that provides protection from recognition by immune competent cells in *in vivo* model system as well as when challenged with antisperm antibodies *in vitro*. The present study was carried out to know the effect of beta defensin-1 in goat semen dilutor on post-thaw quality of Barbari buck semen. Ejaculates from Barbari bucks aged between 2–4 years were collected using artificial vagina method. Immediately after collection, the volume, colour, consistency, and mass motility of ejaculates were assessed and were extended with Tris-Egg yolk-Fructose diluent having egg yolk (10%) and glycerol (6%). Beta defensin was added in four concentrations (0 ng/ml-control, 10 ng/ml, 20 ng/ml and 30 ng/ml) in goat semen dilutor. Sperm concentrations were adjusted to 1×10^8 sperm/semen straw and diluted semen was equilibrated at 5°C for 4 h before being frozen. The post thaw motility (%) was 48.18±0.61, 52.15±0.57, 49.12±0.72 and 46.28±0.83 in different concentration of beta defensin (0 ng/ml-control, 10 ng/ml,

Table 1. Effect of different concentrations of beta defensin-1 in goat semen dilutor on post-thaw quality of Barbari buck semen

Beta defensin-1 (ng/ml)	Progressive motility (%)	Live sperm (%)	Acrosome intact (%)	HOS (%)
0 (control)	48.18±0.61^{c}	61.21±1.25^{c}	68.45±1.21^{a}	59.48±1.79^{b}
10	52.15±0.57^{a}	66.53±1.55^{a}	69.85±1.19^{a}	67.74±1.59^{a}
20	49.12±0.72^{b}	59.67±1.92^{c}	68.38±1.26^{a}	61.26±1.45^{b}
30	46.28±0.83^{c}	64.12±1.58^{b}	63.21±1.53^{b}	48.18±1.38^{c}

Different superscripts within a column differ significantly at $P<0.05$

20 ng/ml and 30 ng/ml), respectively. The corresponding values of live per cent sperm were 61.21±1.25, 66.53±1.55, 59.67±1.92 and 64.12±1.58, respectively. The corresponding values of acrosome integrity per cent were 68.45±1.21, 69.85±1.19, 68.38±1.26 and 63.21±1.53, respectively. The corresponding values of hypo-osmotic swelled sperm per cent were 59.48±1.79, 67.74±1.59, 61.26±1.45 and 48.18±1.38, respectively. The post thaw quality was significantly higher in 10 ng/ml beta defensin concentration than other concentrations. It can be concluded that 10 ng/ml beta defensin in goat semen dilutor can be used for routine semen freezing protocol for better post-thaw quality.

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