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Goat specific synthesized antimicrobial peptide *β-defensin* improves post-thaw sperm fertility

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β-defensin antimicrobial peptide (*βdef-AMP*) are 3-6 kDa three dimensional antiparallel β-sheet structure showing antimicrobial properties against different types bacteria like *Escherichia coli, Salmonella typhimurium and Candida albicans* (Ranjan *et al.* 2019). Defensin was formerly believed to protect the reproductive tract from pathogen invasion but also have motility-enhancing properties (Ranjan *et al.* 2018). Two goat specific β-defensin *GBD1* and *GBD2* were discovered which produce shields over sperm surface and therefore reduce the impact of immune attacks within the female reproductive system (Ranjan *et al.* 2018, Li *et al.* 2001). Antimicrobial peptides (AMPs) have bacterial selectivity, sensitivity, thermodynamic stability and proteolytic stability, when they were first utilised in boar semen extender (Schulze *et al.* 2016). Cryopreserving semen is highly effective approach for expediting the conservation and proliferation of superior germplasm. This method significantly accelerates efforts in enhancing and safeguarding breeds within the goat species and other animals (Medeiros *et al.* 2002). *β-defensin 1* enhanced sperm motility and capacitation after cryopreservation (Ranjan *et al.* 2021). The European Medicines Agency formulated a strategy to decrease the spread of antimicrobial resistance and to foster the creation of substitutes for antibiotics (Lee *et al*. 2013). The limitation on the utilization of antibiotics is linked to the prevalent occurrence of antibiotic-resistant strains in bacteria obtained from animal specimens, including instances such as seminal doses (Bussalleu and Torner 2013). Hence, the pursuit of substitutes for conventional antibiotics becomes imperative and AMPs emerge as a hopeful avenue. Although the extensive development of bacterial resistances stands as a primary constraint in antibiotic application, AMPs may be best replacement for antibiotics for semen cryopreservation (Speck *et al.* 2014). In our previous study, we have seen the significant effect of recombinant beta defensin 1 (hBD-1) on post thaw quality (Ranjan *et al.* 2018). The objective of this study was to examine the effects of goat specific synthesised β-defensin supplementation on antimicrobial efficacy, post-thaw sperm fertility and antioxidant capacity of buck semen.

Goat specific *βdef-AMP* was designed and synthesized from outsource (GenScript, New Delhi). All chemicals were purchased from Sigma-Aldrich else otherwise mentioned. Tris (3.6 g), citric acid (1.9 g), fructose (1.0 g) and triple distilled water (100 mL), hen fresh egg yolk (10% v/v), glycerol (6 % v/v), penicillin 100000 IU/mL and streptomycin 1 mg/mL were added in semen diluent for goat semen cryopreservation (Ranjan *et al.* 2022a). Total 60 ejaculates (six ejaculates from each buck) from 10 Barbari bucks were collected through the artificial vagina method twice a week in year 2023. Ejaculates from the same buck that had a mass motility on international standard of 4 or more were pooled and extended to 400 million/mL sperm concentration and divided into 4 equal groups. Diluted semen was supplemented with βdef-AMP with different concentrations: control, 10 µM, 15µM and 20 µM based on available literature in different species (Ranjan *et al*. 2018, Timonet *et al*. 2018, Khayamabed *et al*. 2020). The sperm progressive motility, live sperm count, hypo osmotic swelled sperm (HOS), acrosome integrity and Malondialdehyde (MDA) production was calculated. Equilibrated diluted semen samples were kept at 5ºC for 4 hours in the cold handling cabinet (IMV technologies, France) and filled in 0.25 mL French mini semen straws and sealed with polyvinyl alcohol powder. The filled and sealed semen straws were vapor frozen at 2cm above the liquid nitrogen (LN_2) level for 10 min. Subsequently, the semen straws were frozen using our established laboratory protocol into LN_2 container (Ranjan *et al.* 2022b). The cryopreserved sample was thawed at 37ºC for 1 min and clean dried with tissue paper. A total of 60 replicates were experimentally designed and data were calculated for analysis. The assessment of bacterial growth in cryopreserved sperm samples was carried out through Miles and Misra method on nutrient agar plates (Slack and Wheldon 1978). The microbial load was determinate by colony counts in the drop areas (Slack and Wheldon 1978, Datta 2021) (Fig. 1 A). Progressive sperm

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motility, Sperm viability (Fig. 1 B), membrane integrity (Fig. 1 C) was calculated (Ranjan *et al.* 2022b) and acrosomal integrity evaluated using FITC-PSA stain (Fig. 1 D). Lipid peroxidation assessment was carried out as per Zanganeh *et al*. 2013. Statistical analysis was done by General Linear Model of SPSS Package-16 (IBM® SPSS Statistics Software) by using One-way ANOVA and data were normalised before analysis. The percentage of post-thawed motility, live sperm count, acrosome intactness, HOS, MDA and bacterial load were all dependent variables in the factorial model, which also included the influence of *βdef-AMP* concentration as an independent variable. Statistics were considered to be statistically significant when differences had values (p<0.05) and the mean±SE was used to present the results.

The percentage of progressively motile sperm, live sperm, HOS and acrosome integrity were 83.64 ± 0.97, 90.80 ± 0.70, 86.63 ± 1.03 and 85.62 ± 1.01 respectively in fresh semen. The percent of malondialdehyde production was 10.54 ± 0.43 µmol/mL in neat semen.

The bacterial load in the control group exhibited a significantly higher level (p<0.05) compared to the other treatments. Conversely, the bacterial load at the concentration of 20 µM of *βdef-AMP* and antibiotic group

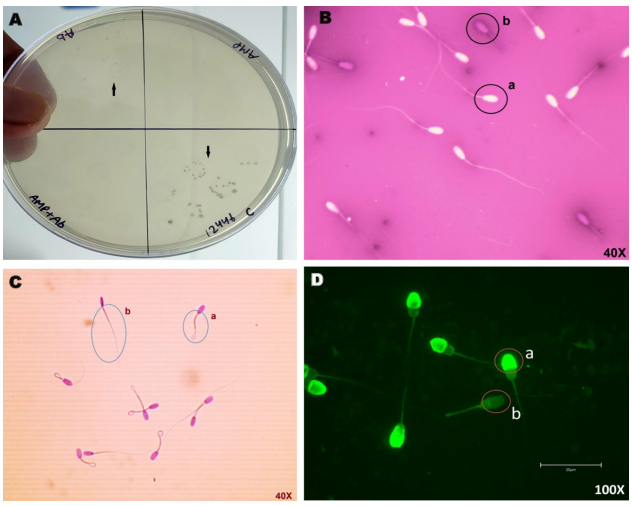

Fig. 1. A Microbial growth in goat semen with antibiotic and βdef-AMP, B-Live and dead sperm using eosin-nigrosin stain, a: live sperm, b: Dead sperm, C: Hypo Osmotic Swelling Test, a: hypo-osmotic swelled sperm, b: Abnormal sperm, D: FITC-PSA staining for acrosome integrity; a: Intact acrosome, b: Damaged acrosome

significantly (p<0.05) decrease (Table 1).

The progressive motile, live, HOS positive and acrosome intact sperm were 16.67%, 13.79%, 11.88% and 10.67% respectively more in 15µM *βdef-AMP* than control group. The post thaw semen qualities in terms of sperm motility, viability and membrane intactness were significantly higher (p<0.05) in 15µM *βdef-AMP* group than other groups (Table 2). The higher concentration of *βdef-AMP* did not show any significant difference from the control group. The post thaw semen qualities gradually improve up to 15µM of *βdef-AMP* and then decline in higher concentration tested.

In frozen thaw semen, the MDA concentration was significantly lower (p<0.05) when treated with 15 µM *βdef-AMP* compared to other concentrations (Table 2). Moreover, the addition of 15 µM *βdef-AMP* to the goat semen diluent led to a reduction of MDA by 42.00% compared to the control group.

Nowadays there is a pressing requirement to discover alternative methods for controlling bacterial growth in semen cryopreservation due to limitations of antibiotic use and the development of its resistance (Schulze *et al*. 2023). In both *in vivo* model systems and *in vitro* conditions, β-defensin1 forms a protective layer around sperm, providing defense against detection by immune-competent cells and safeguarding against antisperm antibodies (Ranjan *et al.* 2018). The incorporation of recombinant hBD-1 into spermatozoa with deficiencies has led to a significant enhancement in sperm motility, sperm viability, and bacterial activity (Khayamabed *et al.* 2020). Furthermore, the study by Speck *et al.* 2014 also suggested that the effects of AMPs were amplified when employed in conjunction with antibiotics.

The bacterial load at the concentration of 15 µM and 20µM of *βdef-AMP* showed distinct and significant (p<0.05) decrease than antibiotic and other treatment

Table 1. Effect of antibiotic and βdef-AMP on bacterial load in goat semen (n-30)

Concentration	Bacterial load
Control	6500.00±500.00^{a}
Antibiotic (Penicillin 1 lakh IU/mL and streptomycin 1 mg/mL)	2285.70±350.49^{c}
βdef-AMP (10 µM)	3678.60±404.67^{b}
βdef-AMP (15 µM)	1821.40±207.07^{c}
βdef-AMP (20 µM)	1250.00±208.01^{c}

Table 2. Effect of *βdef-AMP* on post thaw semen qualities (n-30)

βdef-AMP Conc. (µM)	Motility %	Live %	HOS %	Acrosome %	MDA (µmol/mL)
0	60.21±2.11^{c}	62.45±2.12^{c}	64.55±2.85^{c}	56.43±2.02^{b}	26.28±1.54^{a}
10	64.18±1.89^{b}	66.36±2.21^{b}	67.24±2.18^{b}	57.31±0.78^{b}	25.55±1.98^{a}
15	70.25±2.18^{a}	71.21±2.22^{a}	72.22±2.12^{a}	62.45±1.20^{a}	15.24±1.54^{c}
20	62.24±1.44^{c}	63.52±2.42^{c}	65.82±2.34^{c}	58.96±0.99^{b}	20.12±1.78^{b}

Values represent mean ± standard error from mean. Different letters (a, b, c) within each column indicate significant differences between groups (p<0.05).

group. Hence, 15 µM *βdef-AMP* is sufficient to the bacterial load and it can be used as a substitute in place of antibiotic penicillin and streptomycin. *βdef-AMP* into the goat semen diluter improve the post-thaw quality of Barbari bucks' semen. Furthermore, these findings imply that *βdef-AMP* could play a role in the development of male fertility and reducing the microbial load, while also leading to a reduction in MDA levels in both frozen thawed semen through antimicrobial and antioxidant properties.

The treatment with recombinant β-defensin 1 maintains sperm motility (Zupin *et al.* 2019). The process of exposing spermatozoa to recombinant human β-defensin 1 for 24 hours significantly increased sperm progressive motility. Furthermore, the addition of porcine β-defensin 1 and 2 to aseptic extenders for liquid-stored boar semen demonstrated some level of control over microbial growth without adversely affecting sperm viability and motility (Timonet *et al.* 2018). This finding aligns with a previous study by Diao *et al.* 2014, which demonstrated that treating spermatozoa from asthenozoospermic men with 500 ng/mL of recombinant β-defensin 1 resulted in increased sperm motility and viability.

In conclusion, *βdef-AMP* at 15µM exhibits potential antimicrobial agents and demonstrated no harmful impact on sperm viability and fertility. Therefore, this specific concentration is deemed safe and could potentially be incorporated into commercial semen extenders for buck semen cryopreservation.

SUMMARY

Cryoprotectant selection and their formulation for semen extenders are critical factors that significantly influence sperm survivability and fertility after cryopreservation. Antimicrobial peptides offer a promising alternative to antibiotics in semen cryopreservation. The objective of this study was to improve the post thaw sperm survivability and fertility by supplementation of synthesized β-defensin antimicrobial peptide (*βdef-AMP*). *βdef-AMP* was designed and synthesized from outsource supplemented in ejaculates (control, 10 µM, 15 µM and 20 µM). *βdef-AMP* exhibited significant efficacy in reducing the bacterial load in all tested concentrations (10, 15, and 20 µM). The post-thaw sperm motility, live sperm, sperm membrane and acrosome integrity were significantly higher ($p<0.05$) in 15 µM fortified diluent. Malondialdehyde production was significantly lower ($p<0.05$) in 15 µM fortified diluent showing low lipid peroxidation. So, the supplementation of 15 µM *βdef-AMP* is advantageous for the cryopreservation of Barbari buck semen to improve sperm fertility and to reduce microbial load in semen.

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