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Lowering the Level of Egg Yolk in Diluent or Removal of Seminal Plasma with Standard Egg Yolk Level do not Help in the Efficient Freezing of Buck Semen

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ABSTRACT

The aim of this investigation was to compare the sperm quality in removal or intact seminal plasma buck semen frozen in tris diluent containing 20% and 2.5% egg yolk and to test whether lowering egg yolk level of diluent or removal of seminal plasma with standard egg yolk level helps in efficient freezing of buck semen. A total of 18 fresh ejaculates were divided into three aliquots of 500µl each corresponding to three treatment groups designated as BT1 (tris with 20% egg yolk, washed), BT2 (tris with 20% egg yolk, non-washed) and BT3 (tris with 2.5% egg yolk, non-washed). Semen aliquots of BT1 (washed), BT2 (unwashed) and BT3 (unwashed) were diluted and frozen with standard procedures. Post thaw semen quality was carried out with phase contrast microscopy, fluorescent microscopy, Computer Assisted Sperm Analysis (CASA) and flow cytometry. Majority of fertility associated with post-thaw sperm quality parameters evaluated by CASA, Flow Cytometry and subjective methods were significantly ($p<0.05$) lower in BT2 compared to BT1 and BT3 counterparts. In conclusion, the removal of seminal plasma or reduction in egg yolk concentration did not result in any real advantage for efficient post-thaw sperm survival in goats. An alternate diluent needs to be explored for buck semen which would contain both seminal plasma and egg yolk factors for better cryo-survival of sperm.

Keywords: Buck, CASA, Egg yolk, Flow Cytometry, Seminal plasma, Washing

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INTRODUCTION

Goat breeding is an economically important enterprise in most parts of the country including UT of Jammu and Kashmir. Artificial insemination (AI) is first generation reproductive biotechnology that has brought tremendous genetic improvement in bovines; however, similar benefits have not been harvested in goats. Moreover, the success of this technology involves continuous efforts to improve semen quality and fertility (El Amiri and Rahim, 2024). In goats, besides the inability to deposit semen in the uterus due to the anatomical structure of the cervix (Leethongdee *et al.,* 2014), low post-thaw buck sperm quality negatively affects the pregnancy rate through AI (Galián *et al.,* 2023). The choice of a diluent for buck semen is very important because the traditional egg yolk or milk-based extenders are not suitable for buck semen cryopreservation due to detrimental effects on sperm motility and viability when such diluents are mixed with buck semen. This deleterious interaction is mainly due to the presence of phospholipase A_2 and yolk agglutinate in buck seminal plasma secreted from bulbourethral glands that hydrolyse lecithin in egg yolk into lysophosphatidic. Lysophosphatidic cause change in pH of diluent resulting in toxic effect on sperm cells (Zou *et al.,* 2022). In addition to this, another glycoprotein, BUSgp60, produced in goat bulbourethral gland, hydrolyses residual triglycerides in the egg yolk or milk-based diluent, producing fatty acids that are also toxic to spermatozoa (Pellicer-Rubio *et al.,* 1998). Such type of interaction decreases sperm motility and viability percentages and stimulates cryo-capacitation and acrosome damage (Morrell *et al.,* 2022). Therefore, cryostorage of buck semen in either egg yolk or milk-based extenders either requires almost complete removal of seminal plasma before dilution or alternatively a lower proportion of egg yolk to avoid or decrease the toxic interaction and damage to the sperm. Removal of seminal plasma is a routine in goat sperm cryopreservation protocols (Shah *et al.,* 2023). Numerous studies have confirmed its beneficial effect (Santiago-Moreno *et al.,* 2017), as the toxic interactions between seminal plasma and yolk components are avoided (Anand *et al.,* 2017). However, when eliminating the seminal plasma, the antioxidant systems present in it are also removed and the goat sperm becomes even more susceptible to reactive oxygen species during the cryopreservation process resulting in inferior post-thaw quality (Ferreira *et al.,* 2014). It also deprives sperm from some important seminal plasma proteins protecting them from thermal shock (Colas *et al.,* 2009) and maintains proper functionality of spermatozoa (Cardozo *et al.,* 2007). Egg yolk is a crucial component of the semen extenders, acting as a non-permeable cryoprotectant and inhibits plasma membrane destabilization. It contains low-density lipoprotein, cholesterol, and phospholipids, which protect the functional integrity of the sperm against cold shock and cryo injuries (Naz *et al.,* 2019). Therefore, higher concentration of egg yolk promotes cryo-survival of spermatozoa and reducing its concentration adversely affects post-thaw sperm quality (Tabarez *et al.,* 2017). After washing, a significantly higher progressive motility, plasma membrane and acrosome integrity in canary buck sperm has been reported with 16% egg yolk extender compared to the extender containing 0, 4, and 8% egg yolk (Kamal *et al.,* 2022).

Another aspect which limits efficiency of buck semen freezing is washing semen before freezing. Sarıozkan *et al.* (2010) strongly discouraged centrifugation and washing of Angora buck semen due to deterioration of post thaw semen quality and lower fertility, owing to centrifugation stress and elimination of important seminal plasma components. The seminal plasma is a complex biochemical mixture containing many factors which have an important impact on sperm cell function and its cryo-survival (Rodriguez-Martinez *et al.,* 2021, Dhara *et al.,* 2022b). Its components are believed to regulate the functions of several female reproductive organs to provide an ideal condition for effective embryo implantation and pregnancy (Schjenken and Robertson, 2020). In fact, the health state of the offspring is affected by exposure to seminal plasma (Ahmadi *et al.,* 2022). Although the removal of seminal plasma by centrifugation in buck makes the sperm deprived of many natural antioxidants and critical components, it is still carried out to avoid toxic interaction between egg yolk and phospholipase enzyme present in the buck seminal plasma.

Till date, researchers tried different egg yolk levels (ranging from 1.5 to 20%) for washed buck semen, but there is variability in the data of sperm quality obtained after storage of semen at refrigeration or by freezing of buck semen. Our experiment was aimed to investigate whether; there is any interaction effect of egg yolk at standard levels for unwashed buck semen in the animals of temperate Himalayas of Jammu and Kashmir.

MATERIALS AND METHODS

Place of investigation

The investigation was carried out at Mountain Research Centre for Sheep and Goats (MRCS and G), SKUAST-Kashmir, Frozen Semen Project, Rambirbag, Ganderbal, Department of Animal Husbandry, Govt of J and K and

Buck Semen Analytical Unit, Goat farm, Department of Veterinary Physiology, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura, Uttar Pradesh.

Experimental animals

After getting approval from institutional animal ethics committee vide No. 1809/GO/ReBiS/ReI/15/CPCSEA), three healthy Boer bucks aged between 2.0 and 3.0 years, weighing between 40 and 60 kg reared at MRCSG, were selected as semen donor during the entire study.

Semen processing

Eighteen semen ejaculates having mass activity ≥ 3 and progressive motility ≥ 70%, collected from the three Boer bucks (3 x 6 ejaculates each) using artificial vagina method were used. Ejaculates were pooled to generate a representative sample. The pooled sample was divided into 4 aliquots, designated as P (pre-freeze), BT1, BT2 and BT3, Semen sample aliquot P, was evaluated. BT1-containing 20%, BT2 – containing 20% and BT3- containing 2.5% egg yolk levels were prepared fresh by using tris buffer, fructose, citric acid, egg yolk, glycerol and Penicillin and streptomycin. The semen aliquots (100 µl each) were initially diluted 1:1 with respective warm diluent formulations then 1:10 dilution before washing. BT1 aliquots were subjected to centrifugation at 950 g for 10 minutes (15 °C) for washing, while BT2 and BT3 were not washed. All the three aliquots were finally diluted to a sperm concentration of 400 spermatozoa per ml. Diluted semen samples were transported at room temperature (20-22 °C) to Frozen Semen Project, Ranbir Bagh, Ganderbal for freezing, carried out as per the standard freezing protocol (Lone *et al.,* 2021). Post-thaw sperm quality was evaluated subjectively using phase contrast microscope (Nikon, Japan) and Fluorescent Microscope (EVOS M5000, Invitrogen, United States) 15 days post cryopreservation after thawing of semen dose at 37 °C for 40 seconds, using automatic electric thawing kit. Further the semen doses were transported in liquid nitrogen to buck semen analytical unit, Mathura for objective evaluation using CASA (IVOS II Hamilton Thorne, USA), Flow Cytometer (GUAVA easycyte, Instrument S. No. 8472050228, IMV Technologies, France).

Sperm motility

The mass motility of spermatozoa was graded on a scale of 0 to 5, as per the method of (Evans and Maxwell 1987) and progressive motility was estimated and expressed as a percentage, as per the method of Mortimer (Mortimer *et al.,* 1986)

Sperm concentration

The sperm concentration was estimated manually using the Neubauer haemocytometer method, as per the method of Bjorndahl (2013).

Hypo-osmotic swelling test

Hypo Osmotic Swelling Test (HOST) was performed to evaluate functional integrity of sperm plasma membrane as per the method described by (Vasquez *et al.,* 2013). Spermatozoa with visible coiling of the tail were considered as HOST reactive. More than 200 spermatozoa were counted to determine percent HOST reacted spermatozoa.

Spermatozoa viability

To determine the percentage of viable, moribund and dead spermatozoa in a semen sample, the physical integrity of the sperm plasma membrane was determined via florescent staining using CFDA and PI molecular probes, as described by Harrison and Vickers (1990), with some modifications. The sperm cells that appeared brilliant green under the blue filter and disappeared at the green filter were considered viable, and those appeared orange to red at the blue filter and green filters were considered dead (Fig. 1a). 400 total spermatozoa were counted in different fields.

Acrosomal Status

FITC-PNA and PI molecular probes, as described by Singh *et al.* (2016), with some modifications were used to differentiate between intact and damaged populations. Spermatozoa displaying bright green caps/green fluorescence at the head tip were considered to have acrosome reactions while those disappearing with uniformly green heads were considered to have intact acrosomes (Fig. 1b). The percentages of acrosome-intact, acrosome-reacted, dead acrosome-intact and dead acrosome-reacted sperm were determined by counting 400 total spermatozoa in different fields.

DNA integrity

DNA integrity was evaluated using acridine orange, as

described by Sadeghi *et al.* (2009), with some modifications. Sperm cells that showed orange to red tinge on the head were considered cells having compromised/damaged DNA, whereas the cells that showed green tinge on the head were considered to be DNA-intact sperm (Fig. 1c). Percentage was obtained by counting 400 total spermatozoa in different fields.

Mitochondrial activity

The mitochondrial activity of the sperm was evaluated by using a combination of rhodamine-123 and PI, as described by Zou *et al.* (1993), with some modifications. The sperm cells that showed a bright green beaded appearance at the midpiece were considered to have active mitochondria, whereas the cells that did not show distinct florescence were considered to have inactive mitochondria (Fig. 1d). The percentages were calculated by counting 400 total spermatozoa in different fields.

CASA and Flow Cytometry

CASA was used to observe the post thaw motility, morphology and sperm kinematics of frozen semen, using a sperm counting chamber (Leja slides) under negative phase contrast and 10x objective. Motility patterns and kinematic characteristic of spermatozoa were evaluated by total motility (TM %), progressive motility (PM %), average path velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s) straight line velocity (VSL; µm/s), amplitude of lateral head displacement (ALH; µm), the ratios STR (VSL/VAP), LIN (VSL/VCL) and WOB (VAP/VCL), amplitude of lateral head displacement (ALH; µm), and beat cross frequency (BCF; Hz). Besides DAP (Distance average path), DCL (Distance curved linear), DSL (Distance straight line) were observed. Flow Cytometry was used to evaluate viability, acrosome status and reactive oxygen species generation by easykit viability (IMV Technologies, France), easykit mitochondrial activity (IMV Technologies, France), easykit viability acrosome activity (IMV Technologies, France), easykit-3-oxidation (IMV Technologies, France). Intracellular Calcium (iCa^{2+}) measurement was recorded with the help of Fluo-4 NW Calcium Assay Kit (starter pack with buffer), Invitrogen by Thermo Fisher scientific. All the procedures were done as per manufacturer's instructions. Gating of cells was done for correct flow-cytometric evaluation.

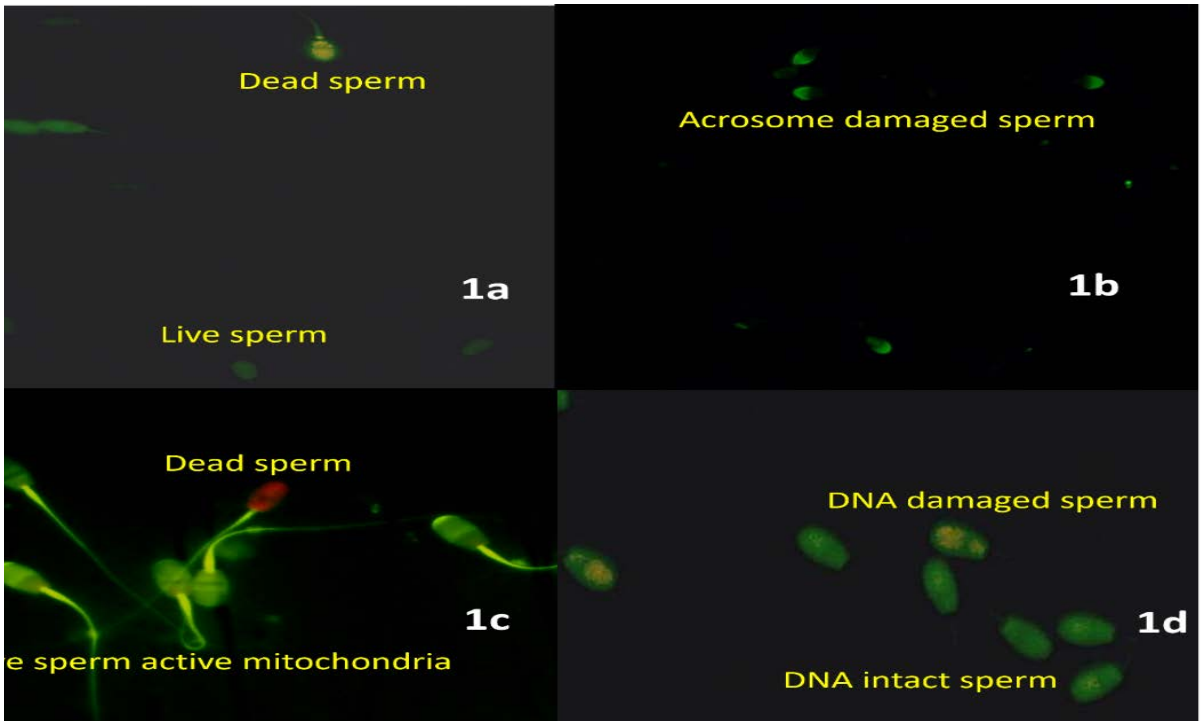

Fig. 1a, 1b, 1c, 1d: Sperm viability using CFDA+PI (1a), acrosome integrity using FITC-PNA+PI (1b), mitochondrial activity using Rhodamine 123+PI (1c) and DNA integrity using Acridine Orange fluorescent dyes

STATISTICAL ANALYSES

Statistical analyses were performed using the Statistical Package-IBM® SPSS® Version 20.0 for Windows®, SPSS Inc., and Chicago, USA to determine the difference in the post thaw semen quality of the three diluents variants. The means were tested using Tukey's b test. P values <0.05 were considered significant. The results are expressed as the means ± SEMs.

RESULTS AND DISCUSSION

In the present study, pre-freeze total and progressive motility percentages of spermatozoa were 88.70±0.50 and 80.16±0.60, respectively. Pre -freeze HOST reacted spermatozoa percentage was 76.21±0.32. Post-thaw sperm quality got significantly ($p<0.05$) reduced in BT2 (PM; 12.90±2.66%, VCL; 50.09±4.04 µm/sec, VSL; 20.52±1.64 µm/sec, ALH; 2.59±0.12 µm, BCF; 12.80±1.62 Hz, percent HOST+; 23.75±1.34%, viability; 41.00±1.39%, live sperm with intact acrosome-LSIA; 30.42±1.34%, viable sperm having low intracellular calcium- 20.28±0.88% and live sperm negative for reactive oxygen species-20.78±0.71%), compared to BT1 (PM;16.90±2.85%, VCL;58.36±2.06 µm/sec, VSL;31.21±2.28 µm/sec, ALH; 2.62±0.72µm, BCF; 10.70±1.62Hz, HOST+;33.57±2.92%, viability; 49.33±1.90, LS_lessiCa; 35.68±0.55%, LSIA; 33.25±1.54% and LSROS-;34.26±0.62%) and BT3 (PM; 24.91±2.52%, VCL;73.79±12.22 µm/sec, VSL; 38.17±5.22 µm/sec, ALH; 3.48±0.19 µm, BCF; 9.61±2.09 Hz, HOST+; 28.25±2.56%, Viability; 54.17±2.10%, LSIA; 40.75±1.68%, LS lessiCa; 44.58±0.47% and LSROS- 40.92±1.23%). The results are presented in fig. 2,3,4,5,6 and 7.

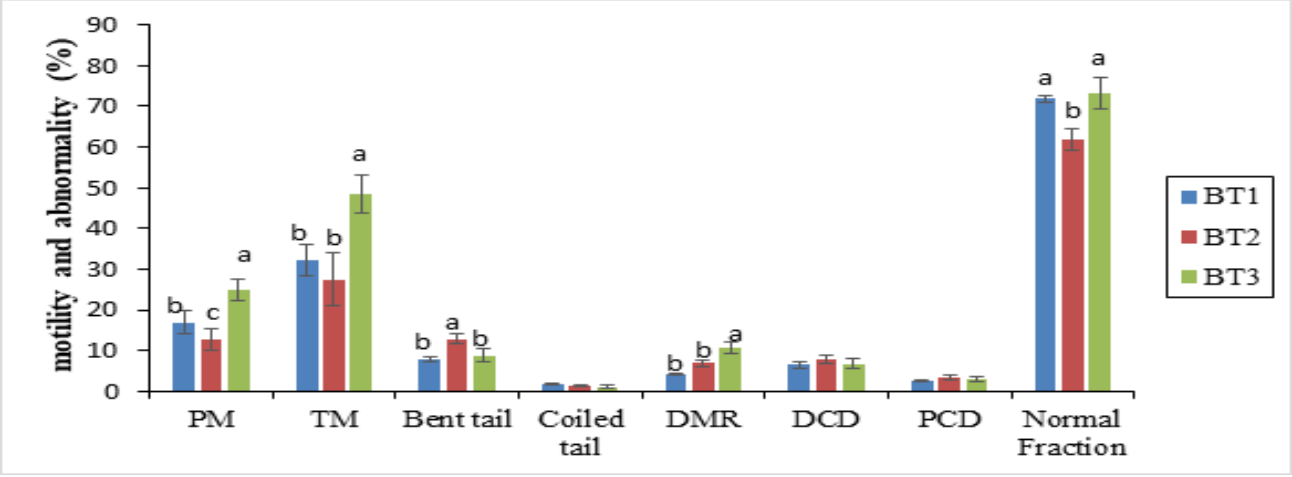

Fig. 2: Post thaw sperm motility and abnormality in three Tris diluent variants

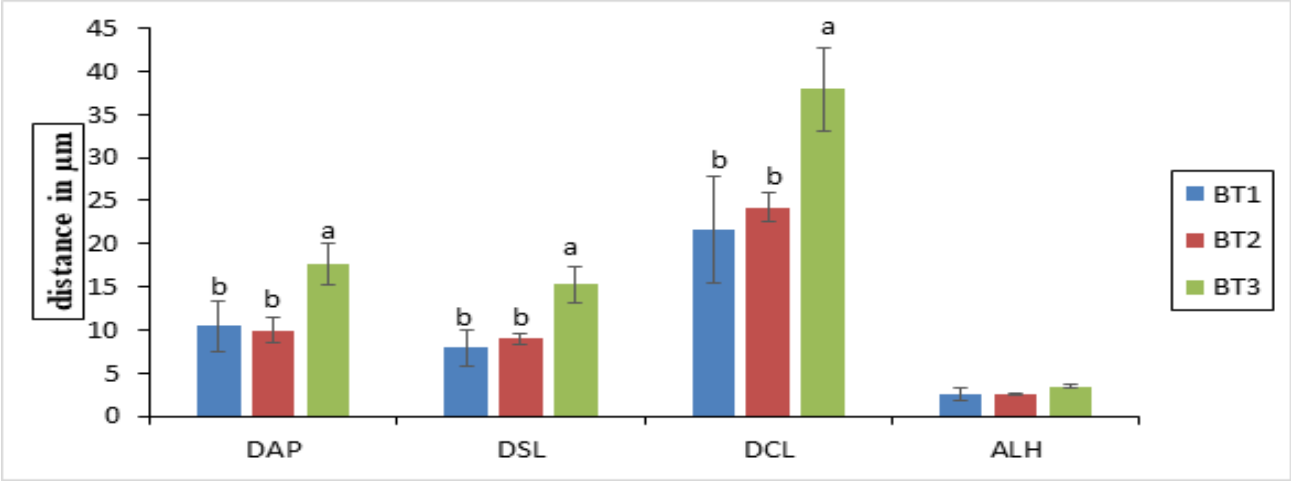

Fig. 3: Post thaw average path, straight line and curved line distances of spermatozoa frozen in three Tris diluent variants

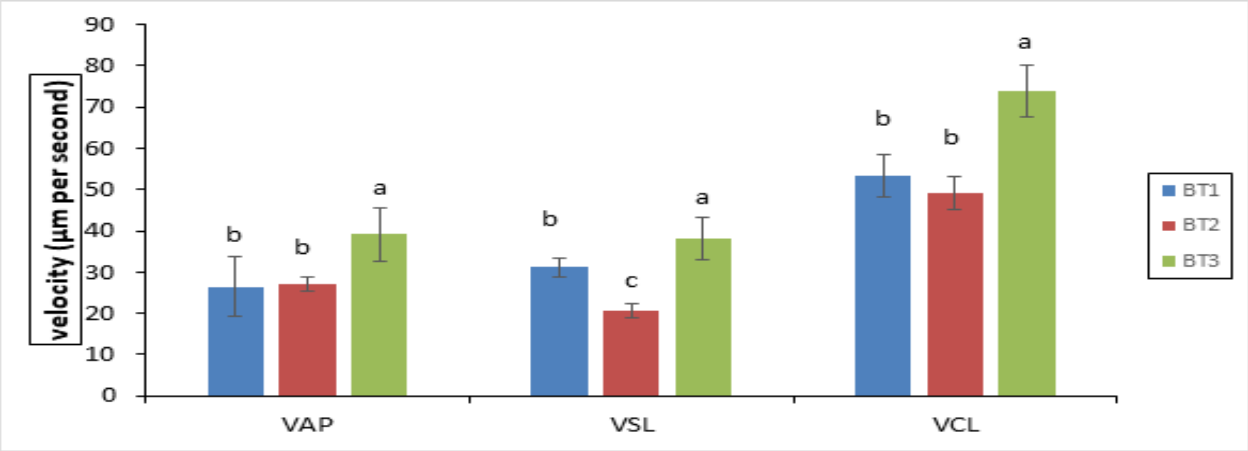

Fig. 4: Post thaw average path, straight line and curved line velocities of spermatozoa frozen in three Tris diluent variants

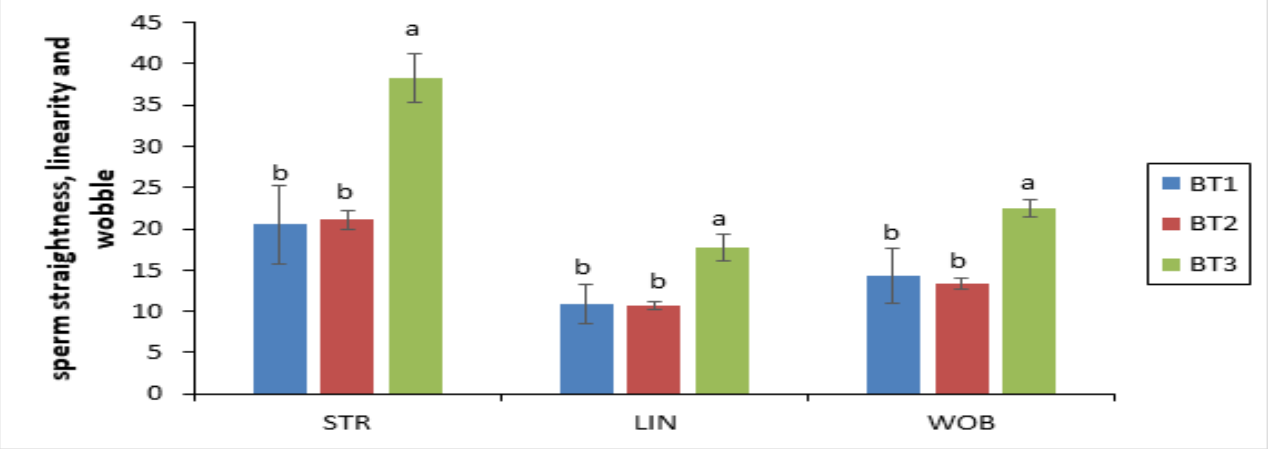

Fig. 5: Post thaw straightness, linearity index and wobble percentage of spermatozoa frozen in three Tris diluent variants

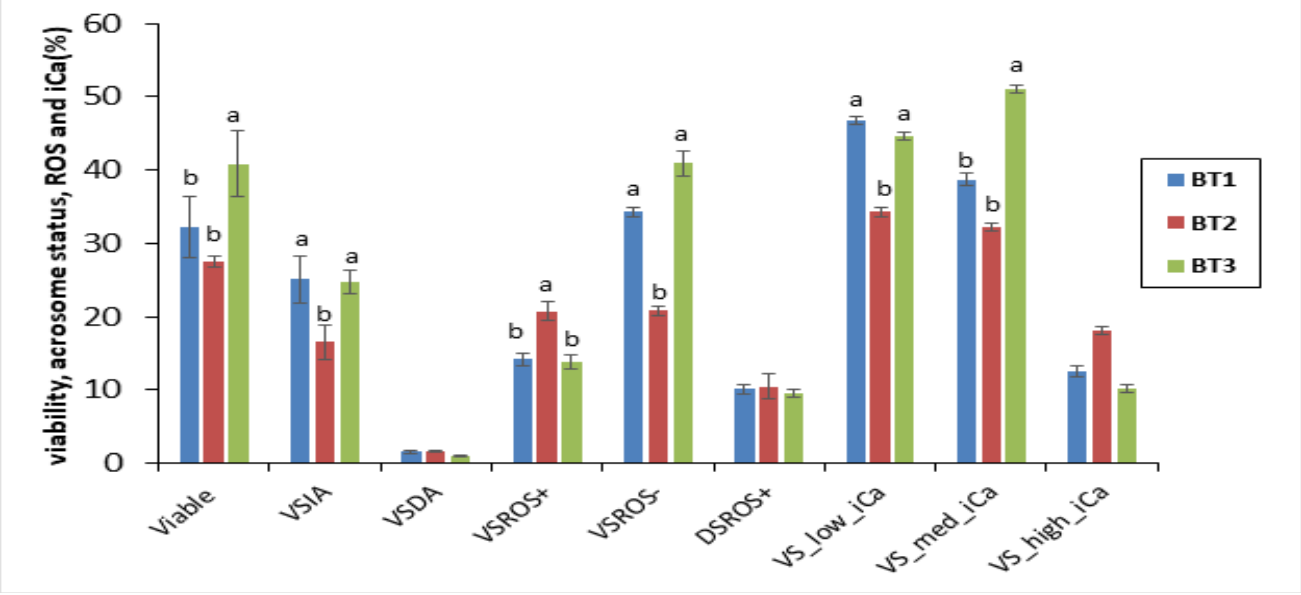

Fig. 6: Post thaw sperm viability and acrosome status by Flowcytometric evaluation

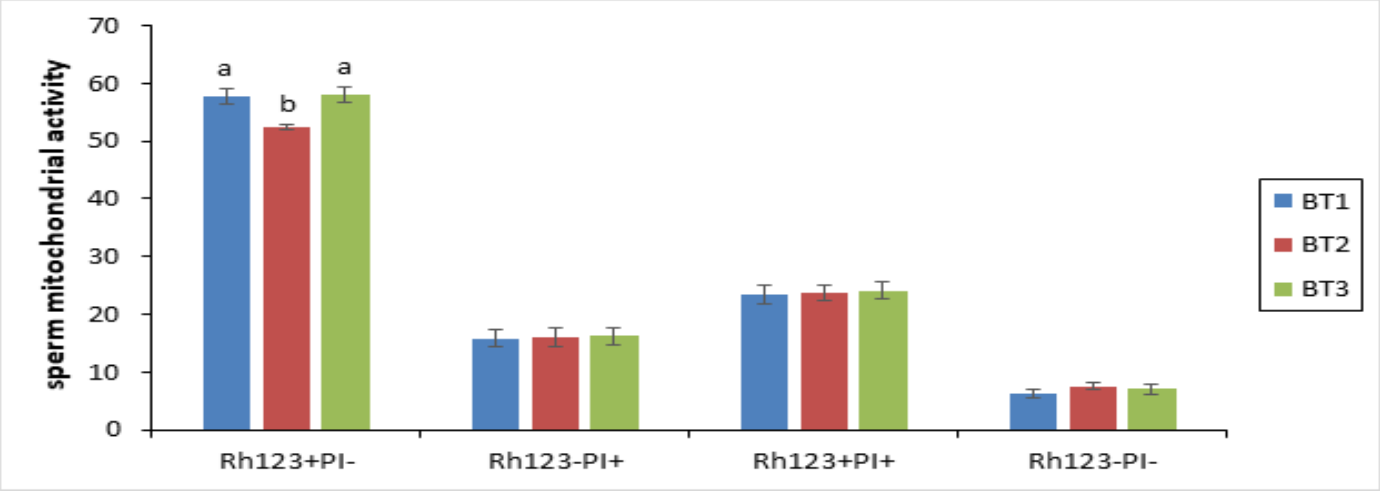

Fig. 7: Post thaw mitochondrial activity of spermatozoa frozen in three Tris diluent variants

PM, TM and normal fraction of spermatozoa were higher ($p<0.05$) in BT3 and lower ($p<0.05$) in BT2. The distances travelled by any sperm in linear and curved paths (DAP, DSL and DCL), ALH, STR, LIN, WOBBLE were higher for BT3 diluent variant ($P<0.05$). Among VAP, VCL and VSL, VSL is higher for BT3 ($p<0.05$). BCF although is more in BT3 but not statistically different between the variants. In flow cytometry, percentage of viable, acrosome intact, viable sperm which have not generated ROS and sperm with low intracellular calcium are lowest ($p<0.05$) for BT2 variant and do not differ in other two. Also, in subjective evaluation, motile, HOST+, viable, and active mitochondrial sperm populations were lowest in BT2 ($p<0.05$), while intact acrosome population was highest in BT3.

In BT2 variant, motility and other important quality parameters were decreased to greater extent, indicating a significant interaction between egg yolk component and buck seminal plasma. The results obtained in the present study are in agreement with the reports of interaction between egg yolk components and buck seminal plasma or toxic effects of egg yolk on the buck spermatozoa (Morrell et al., 2022). In the present findings the post thaw quality was improved in the diluent containing 2.5% egg yolk. This is supported by Bispo et al. (2011) where the authors reported that use of semen diluters containing 2.5% egg yolk reduce the morphologic injuries to the goat sperm during the cryopreservation process. And this was attributed to be due to the less depletory effect originating from the hydrolysis of egg yolk compounds. The authors suggested that egg yolk @ 2.5%, as a better option than its higher level (20%) for semen cryopreservation in Alpine and Saanen buck. Contrary to our findings, higher egg yolk levels (10-20%) provided significant protection from cryo-injury to spermatozoa in Jakhrana, Red Kalahari, Norduz, Jamunapari and Pantja bucks (Priyadharsini et al., 2011, Yimer et al., 2014, Sen et al., 2015, Ranjan et al., 2015, Kumar et al., 2024). Also, utilizing low egg yolk levels, a remarkable study by Cabrera et al., (2005) reported poor cryo-survival of buck sperm frozen in a diluent containing 1.5% egg yolk, instead 12% egg yolk was beneficial in improving post thaw quality. Further, incorporation of 20% egg yolk after washing in a diluent has been reported to offer better cryoprotection to Barbari buck spermatozoa (Anand et al., 2017). But, as per the data in most of these studies, post thaw semen quality especially progressive motility achieved does not seem to be enough to meet good fertility results. Therefore, washing or non-washing of buck semen for preservation is still a debate and controversial results are frequently encountered in the literature. Composition of diluent is still not explored for improving the cryo-survivability and fertility of frozen buck sperm. Decrease in post thaw semen quality in the present study irrespective of the diluent stands explained by the fact that freezing semen exposes the sperm to variable temperatures which inflict stress leading to its structural or functional alterations. There is considerable effect of this on sperm mitochondrial and membrane function and increases cell surface permeability to water, ions and cryoprotectants (Oldenhof *et al.,* 2010). This is reflected by decrease in sperm viability, progressive motility and other important quality parameters (Cormier and Bailey, 2003).

VCL, VAP and ALH of the sperm were not different between BT2 and BT3. These three kinematic parameters are reported to be associated with improved in-vitro fertilization rate (Robayo et al., 2008, Van de Hoek et al., 2022). This again indicates BT3 variant not meeting the expecta-

tions in buck sperm after freezing. Further, ALH aids the spermatozoa to penetrate cervical mucus and fuse with the oocyte effectively (Mortimer et al., 2015) and is positively correlated with motility (Patil et al., 2020). Moreover, the same authors reported that BCF, STR and LIN are negatively correlated with sperm motility and both are higher in BT3 variant of present study, indicating further investigations in this regard. Therefore, based on evaluation of these kinematic parameters in the present study, the diluent variant containing 2.5% egg yolk for unwashed semen of Boer bucks of temperate Himalayan region is also questionable.

Flow Cytometric evaluation in the present study showed that percentage viability and intact acrosome was improved and number of spermatozoa having low intracellular calcium was more in BT3 variant, while BT2 variant showed lowest percentage of such spermatozoa. This could be attributed to controlled ROS production in this variant. This is in agreement with earlier researcher (Dalal et al., 2020) wherein the authors observed that higher levels of ROS and intracellular calcium led to cholesterol influx, which initiated a cascade of reactions leading to an acrosome reaction. These findings again indicate that, despite comparatively better results obtained in BT3, removal of seminal plasma or reduction in egg yolk concentration do not offer any real advantage for efficient post-thaw sperm survival. Further, use of lower egg yolk concentration in unwashed samples may not be not be a worthwhile option.

In a nutshell, in the present investigation, we got advantage of decreasing egg yolk component and retaining seminal plasma of buck semen but still post thaw motility (25%) was not up to mark to achieve more fertility. Washing buck semen and diluting in standard tris diluent containing 20% egg yolk also did not yield results, probably due to loss of cryoprotection mechanisms conferred by seminal plasma components. Hence, the choice of a diluent for buck semen is very important because the traditional egg yolk or milk-based extenders are not suitable for its cryopreservation due to detrimental effect on sperm motility and viability when such diluents are mixed with buck semen. In goats, the semen quality is decreased to a greater extent than other species owing to seminal plasma and egg yolk interactions. Further, for effective survival of sperm during freezing, seminal plasma components offer enzyme and protein defense molecules. Washing of buck semen thus makes the sperm more compromised. Therefore, a semen diluent which meets all the above requirements needs to be standardized for better outcomes of buck semen freezing.

CONCLUSION

In conclusion, removal of seminal plasma or reduction in egg yolk concentration does not offer any real advantage for efficient post-thaw sperm survival in goats. An alternate diluent needs to be explored for buck semen which would contain both seminal plasma and adequate egg yolk factors for better cryo-survival of the sperm in this species.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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