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Research Article

Effect of clarified tris egg yolk citrate diluter and seminal plasma removal on plasma membrane integrity in cryopreserved Surti buck semen

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Abstract

The study was carried out on Surti bucks maintained under the All India Coordinated Research Project (AICRP) on goat at Livestock Research Station, Navsari Agricultural University, Navsari. A total of 32 ejaculates from two Surti bucks above one year of age (16 ejaculates/buck) were collected by artificial vagina method from November 2021 to March 2022. The objective of the present study was to evaluate the effect of clarified tris egg yolk diluter and/or seminal plasma removal on cryopreserved quality of Surti buck semen. In each replicate, the semen collected from both the bucks were pooled. Immediately after the evaluation of fresh semen, the semen was divided into four equal aliquots. Two aliquots were kept as such, while remaining two were washed by centrifugation to remove the seminal plasma. In group I and group II, non-washed semen was diluted with non-clarified and clarified tris egg yolk citrate diluter, respectively, whereas, in group III and group IV washed semen was diluted with non-clarified and clarified tris egg yolk citrate diluter, respectively. Thereafter, extended semen was packed in 0.5 mL French medium straws and stored in liquid nitrogen after equilibration and vapour freezing. At every stage of cryopreservation, *i.e.*, initial, pre-freezing, and post-thaw, the semen was evaluated for various seminal attributes. At the initial stage, the HOST reacted sperm count differed non-significantly between various groups. However, at pre-freezing level, it was significantly ($p<0.01$) higher in T4 (48.75±2.08) as compared to T1 (42.25±2.19) and T3 (39.19±1.81), whereas non-significantly higher as compared to T2 (47.88±2.05) group. However, the post-thaw HOST reacted sperm count (%) was significantly ($p<0.01$) higher in T4 (41.63±1.98) and T2 (36.5±1.64) as compared to T1 (29.56±1.85) and T3 (29.56±2.25) groups. It was concluded that the use of clarified tris egg yolk citrate diluter is more beneficial as compared to seminal plasma removal for cryopreservation of Surti buck semen.

Keywords: HOST, Plasma membrane integrity, Seminal plasma, Sperm, Surti buck

Highlights

- The higher post-thaw functional plasma membrane integrity recorded in both the clarified as compared to non-clarified egg yolk groups with or without removal of seminal plasma proposes the beneficial effect of clarified tris egg yolk citrate diluter as compared to seminal plasma removal for cryopreservation of Surti buck semen.
- The functional plasma membrane integrity recorded in the seminal plasma removal group was almost similar to the control non-washed group indicating no beneficial effect of seminal plasma removal for cryopreservation of buck semen, and it is also a very tedious job and badly affects the seminal attribute.

INTRODUCTION

Goats have significant economic importance and triggering impact on a wide scope of rural production systems, especially in developing countries. Furthermore, its cultural significance in some societies cannot be ignored by

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explaining its spreading on the earth. For India and other developing countries, goat (*Capra hircus)* is an important livestock species since it provides a good source of meat, milk, fiber and skin. It is popularly known as the "poor man's cow". Goat rearing is a good initiative to improve the economy of poor farmers. Goat husbandry provides a high socio-economic level for a better way of life. Gujarat State is rich in variety of goat breeds like Kutchi, Surti, Mehsani, Jhalawadi, Gohilwadi, Marwari etc. Surti goat is an important milk producing breed among the various goat breeds in Gujarat, India. The word "Surti" originated from the goat breed found in the surrounding areas of Surat, Baroda and Nasik of Maharashtra. Surti goat is also known for its kidding percentage.

Semen cryopreservation is the process to preserve male germplasm at ultra-low temperature to preserve biological nature of cells as close to its initial quality at fresh state as possible over a protracted period of time. Fertility of cryopreserved spermatozoa is influenced by a variety of parameters including storage temperature, chemical composition of extenders, cryopreservation process, quality of sperm, species to species variation, and individual differences.

Egg yolk is being routinely used in most cryopreservation protocols of many animal species (Priyadharsini *et al.*, 2011). Egg yolk is well known for its protective action on sperm membranes against shock during storage at 5ºC, which is due to the low-density lipoprotein fraction. It has been shown to have a beneficial effect on sperm cryopreservation as a protectant of the plasma membrane and acrosome against temperature related injury, in association with the other components. The non-penetrating cryoprotectants used are egg yolk (2–20%) (Ranjan *et al.*, 2015). The seminal plasma of buck semen contains Egg Yolk Coagulating Enzymes (EYCE; phospholipase A2) and bulbourethral secretion III (SBUIII) from the bulbourethral glands that interacts with the egg yolk and skimmed milk (one of essential and economical component of semen extender) thus producing toxic substances that affects sperm cells (Gangwar *et al.*, 2016) and reduces its freezing ability (Gangwar *et al.*, 2016). It is presumed that EYCE and SBUIII are the same molecules.

The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma causes harmful interactions between seminal plasma and egg yolk or milk, resulting into loss of motility, membrane integrity and low fertility rate. In addition, whole egg yolk has been reported to interfere with microscopic observation or biochemical assay, as it contains granular material of the same size and shape as spermatozoa and also reduces respiration and motility of sperm cells. By centrifugation, egg yolk can be separated into its two main fractions; plasma and granules. Therefore, it is essential to remove large particles in whole egg yolk by centrifugation, and only plasma (clarified) is used to obtain better post-cryopreserved sperm quality. Several researchers reported that the removal of seminal plasma had a favourable effect on semen freezing and thawing properties in buck (Kozdrowski *et al.*, 2007).

MATERIALS AND METHODS

Preparation of clarified Tris Egg Yolk Citrate (TEYC) diluter: The TEYC diluter was prepared on the day of experiment by adding 10% egg yolk in TRIS-citric acid-fructose buffer in sterile flask. The mixture was thoroughly mixed with the help of magnetic stirrer for five minutes. After preparation of TEYC diluter, half of the diluter was kept as a non-clarified TEYC diluter, whereas remaining half was centrifuged for 4 minutes at 3000 rpm in a centrifuge machine, and the supernatant from each tube was obtained carefully in a sterile glass bottle to use as a clarified TEYC diluter. Finally, 6% Glycerol was added to both non-clarified and clarified Tris Egg Yolk Citrate (TEYC) diluter and the mixture was thoroughly mixed by a magnetic stirrer for five minutes and stored at 37ºC.

Collection of semen and its processing: A total of 32 semen ejaculates (16 from each buck) were collected by artificial vagina twice a week

from two apparently healthy Surti bucks above 1 year of age maintained under the All India Coordinated Research Project (AICRP) on Goat at Livestock Farm Complex, College of Veterinary Science and Animal Husbandry, Navsari. The ejaculates were obtained in sterilized graduated glass tubes and transferred to the laboratory within 10 minutes. To eliminate individual buck variability and increase semen quantity, all the ejaculates were pooled during each semen collection.

Semen washing and removal of seminal plasma: The pooled semen was divided into four equal aliquots. Two aliquots were kept as such, while remaining two were washed by centrifugation in order to remove the seminal plasma as per following procedure. The semen was mixed with Ringer's solution in a ratio of 1:10, and the mixture was centrifuged at 2500 RPM for 7 minutes at room temperature. After centrifugation, supernatant was aspirated without disturbing the semen pellet. Again, same amount of Ringer's solution was added, and carefully re-suspend the pellet by slowly pipetting it back and forth. The procedure was repeated for centrifugation, and supernatant was aspirated and discarded without disturbing the semen pellet.

Experimental groups: In group I and group II, non-washed semen was diluted with non-clarified and clarified tris egg yolk citrate diluter, respectively, whereas in group III and group IV, washed semen was diluted with non-clarified and clarified tris egg yolk citrate diluter, respectively. After grouping, the semen samples were examined for initial evaluation of plasma membrane functional integrity for all the groups. According to different groups, extended semen was filled in previously marked 0.5 mL French medium straw (IMV Technologies, France) using a micropipette having final concentration of $50x10^6$ sperm/straw. Total four straws were prepared for each group.

Freezing of the semen: All the loaded straws were laid on a floating rack and placed in a refrigerator at 4ºC for equilibration about 4 hrs. Subsequently, the floating rack holding straws were placed in a manual vapour freezing unit, 4 cm above the liquid nitrogen in vaporous phase for 7 minutes. After the completion of freezing, the straws were directly and quickly plunged into a liquid nitrogen container. The pre-freezing and post thaw plasma functional membrane integrity was evaluated for all the groups.

Plasma membrane functional integrity: To evaluate the plasma membrane functional integrity of spermatozoa, Hypo Osmotic Swelling Test (HOST) was determined by mixing 0.1 mL of diluted semen with 1.0 mL of hypo-osmotic solution. The tube containing the mixture was incubated at 37ºC for 30 minutes. A drop of semen from the mixture was placed on a clean, dry glass slide and covered with a cover slip. The sperms characterized by varying degrees of coiling or swelling of tail were considered to have an intact plasma membrane (HOS reacted sperm), and the sperm without tail curling were considered to have damaged membrane (HOST non-reacted sperm). Total 200 spermatozoa were counted in different fields, and percentage of spermatozoa exhibiting tail curling (reacted) was calculated.

Statistical analysis: The data pertaining to various aspects was suitably tabulated and analyzed using R-3.3.2 software. The differences among the parameter means were performed using appropriate statistical methods, *viz.*, Two-Way ANOVA and DNMRT (Duncan's New Multiple Range Test). The mean differences were considered significant at $p<0.01$ and $p<0.05$.

RESULTS

The initial, pre-freezing and post-thaw functional plasma membrane integrity of spermatozoa in various groups was monitored in all the groups and presented in Table 1.

Table 1. Effect of clarified tris egg yolk citrate diluter and seminal plasma removal on HOST reacted sperm count (%) for cryopreserved Surti buck semen (Mean ± SE)

Groups N=16/group	HOST reacted sperm count (%)			Overall (n=48)	F value	P value
	Initial	Pre-freezing	Post thaw			
T1	57.06 ± 2.65^{a}	$42.25\pm2.19^{b}_{BC}$	$29.56\pm1.85^{c}_{B}$	$42.96\pm2.08_{BC}$	37.33**	0.00
T2	58.69 ± 2.79^{a}	$47.88\pm2.05^{b}_{AB}$	$36.5\pm1.64^{c}_{A}$	$47.69\pm1.82_{AB}$	25.16**	0.00
T3	54.13 ± 3.32^{a}	$39.19\pm1.81^{b}_{C}$	$29.56\pm2.25^{c}_{B}$	$40.96\pm2.06_{C}$	23.78**	0.00
T4	60.38 ± 3.04^{a}	$48.75\pm2.08^{b}_{A}$	$41.63\pm1.98^{c}_{A}$	$50.25\pm1.77_{A}$	15.37**	0.00
Overall (n=64)	57.56 ± 1.47^{a}	44.52 ± 1.11^{b}	34.31 ± 1.14^{c}	—	86.42**	0.00
F value	0.81	5.04**	9.14**	4.85**	—	—
P value	0.49	0.00	0.00	0.00	—	—

**p<0.01; $^{a-c}$Means with different superscripts between columns (between various stages of cryopreservation) at p<0.01; $_{A-C}$ Means with different subscripts within columns (between groups) differ significant at p<0.01, T1- Non clarified & non washed semen, T2- Clarified & non washed semen, T3- Non clarified & washed semen, T4- Clarified & washed semen

The initial HOST reacted sperm count (%) found just after dilution was differed non-significantly between T1 (57.06±2.65), T2 (58.69±2.79), T3 (54.13±3.32) and T4 (60.38±3.04) groups. However, HOST reacted sperm count (%) found at pre-freezing level was noted significantly (p<0.01) higher in T4 (48.75±2.08) as compared to T1 (42.25±2.19) and T3 (39.19±1.81), whereas, non-significantly higher as compared to T2 (47.88±2.05) groups with non-significant difference between T1 and T3 groups and highest value recorded in T4 group. Further, the value found in T2 was significantly (p<0.01) higher as compared to T3, while non-significantly higher as compared to T1 group. However, the post-thaw HOST reacted sperm count (%) found at 24 hrs after cryopreservation was found significantly (p<0.01) higher in T4 (41.63±1.98) and T2 (36.5±1.64) as compared to T1 (29.56±1.85) and T3 (29.56±2.25) groups with non-significant difference between T1 and T3 as well as T2 and T4 groups. The highest value for post-thaw HOST reacted sperm count was recorded in T4, whereas the lowest was in T1 and T3 groups. The corresponding overall mean values of HOST reacted sperm count (%) irrespective of various groups gradually declined with consecutive stages of cryopreservation as 57.56±1.47, 44.52±1.11 and 34.31±1.14 at initial, pre-freezing and post-thaw level, respectively.

Moreover, the HOST reacted sperm count (%) found in T1, T2, T3 and T4 groups at various stages of cryopreservation was significantly (p<0.01) different and recorded as 57.06±2.65, 58.69±2.79, 54.13±3.32 and 60.38±3.04, respectively at initial; 42.25±2.19, 47.88±2.05, 39.19±1.81 and 48.75±2.08, respectively at pre-freezing and 29.56±1.85, 36.5±1.64, 29.56±2.25 and 41.63±1.98, respectively at post-thaw stages. The HOST reacted sperm count found in all the groups gradually declined from one stage to another during cryopreservation. Further, the HOST reacted sperm count recorded at initial, pre-freezing, and post-thaw stages were highest in T4 while lowest in T3 group. The corresponding overall mean values of HOST reacted sperm count (%) irrespective of various stages of cryopreservation was found highest in T4 (50.25±1.77) followed by T2 (47.69±1.82), T1 (42.96±2.08) and T3 (40.96±2.06) groups.

In conclusion, significantly higher post-thaw functional plasma membrane integrity recorded in both the clarified as compared to non clarified egg yolk groups with or without removal of seminal plasma recommend the beneficial effect of clarified tris egg yolk citrate diluter as compared to seminal plasma removal on functional plasma membrane integrity for cryopreservation of Surti buck semen.

DISCUSSION

In the present study, a non-significant difference was observed between washed and non-washed groups for initial HOST reacted sperm count with apparently higher value in non-washed group in fresh semen. Similarly, Coloma *et al*. (2010) also reported a non-significantly

higher level of HOST reacted sperm count in fresh semen for non-washed (71.4±4.2%) as compared to either Tris citric acid glucose (66.2±4.5%) or Krebs ringer phosphate glucose (65.2±5.2%) washed semen in Spanish ibex (*Capra pyrenaica*) bucks. However, Sharma *et al*. (2018) reported non-significantly lower values for HOST reactive sperm count in non-washed (76.3±1.9%) as compared to washed (77.2±1%) fresh semen in Gaddi bucks. Furthermore, Sharma and Sood (2019) reported 64.07±1.75% HOST reactive sperm count in fresh diluted and washed semen extended with tris citrate egg yolk citrate diluter in Chegu bucks.

In the present study, almost similar values were observed between washed and non-washed groups for post-thaw HOST reacted sperm count. Similarly, Coloma *et al*. (2010) also reported a non-significant difference in post-thaw HOST reacted sperm count between either Tris citric acid glucose (25.9±3.1%) or Krebs ringer phosphate glucose (24.7±3.2%) washed and non-washed (19.7±4.4%) semen in Spanish ibex (*Capra pyrenaica*) bucks. Furthermore, Sharma and Sood (2019) reported 43.35±1.79% post-thaw HOST reactive sperm count in washed semen extended with tris citrate egg yolk citrate diluter in Chegu bucks.

However, Cabrera *et al*. (2005) reported significantly ($p<0.005$) higher level of post-thaw HOST reacted sperm count in washed (31.6%) compared to non-washed (14.5%) semen during the winter season in Canary Buck. Similarly, Sharma *et al*. (2018) too recorded significantly ($p<0.05$) higher post-thaw HOST reactive sperm count in washed (61.5±2.0%) as compared to non-washed (45.6±3.8%) semen diluted with tris egg yolk citrate extender in Gaddi bucks.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: KSK, NFC: Conception and design; KSK, NFC, CTK: Acquisition of data; LCM: Performed statistical analysis of data; KSK, NFC, LCM, SSC, DNS: Performed interpretation of the results.

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REFERENCES

Cabrera F, Gonzalez F, Batista M, Calero P, Medrano A *et al.*, 2005. The effect of removal seminal plasma, egg yolk level and season on sperm freezability of Canary buck (*Capra hircus*). Reprod Domest Anim, 40(3): 191-195, doi: 10.1111/j.1439-0531.2005.00544.x

Coloma MA, Toledano-Diaz A, Lopez-Sebastian A and Santiago-Moreno J, 2010. The influence of washing Spanish ibex (*Capra pyrenaica*) sperm on the effects of cryopreservation on dependency of the photoperiod. Theriogenology, 73: 900-908, doi: 10.1016/j.theriogenology.2009.11.014

Gangwar C, Kharche SD, Kumar S and Jindal SK, 2016. Cryopreservation of goat semen: status and prospects. Indian J Small Ruminants, 22(1): 1-10, doi: 10.5958/0973-9718.2016.00005.2

Kozdrowski R, Dubiel A, Bielas W and Dzieciol M, 2007. Two protocols of cryopreservation of goat semen with the use of computer-assisted semen analysis system. Acta Vet Brno, 76(4): 601-604, doi: 10.2754/avb200776040601

Priyadharsini R, Jindal SK, Sharma D, Ramachandran N, Karche SD *et al.*, 2011. Effect of different egg yolk level on the cryopreservation capability of Jakhrana goat semen. J Anim Sci Adv, 1(1): 28-37

Ranjan R, Goel AK, Ramachandran N, Kharche SD and Jindal SK, 2015. Effect of egg yolk levels and equilibration periods on freezibility of Jamunapari buck semen. Indian J Small Ruminants, 21(1): 32-36, doi: 10.5958/0973-9718.2015.00027.6

Sharma A and Sood P, 2019. Cryopreservation and fertility of frozen thawed Chegu goat semen. Indian J Anim Res, 53(11): 1414-1419, doi: 10.18805/ijar.B-3696

Sharma A, Sood P and Dogra PK, 2018. Seminal plasma removal improves cryopreserved semen quality in Gaddi bucks. Indian J Anim Reprod, 39(2): 25-28

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