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Goat semen cryopreservation using egg yolk and soya based extenders containing trehalose

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Frozen semen technology is gaining popularity worldwide to improve the genetic potential of livestock with success of artificial insemination (AI). There is increasing interest in the use of frozen-thawed buck semen for AI, as it can increase the number of offspring per sire, dissemination of superior germplasm over a wide geographical area, allows rapid and widespread diffusion of improved genotypes and exchange of genotypes without transmitting diseases. Egg yolk is most commonly used in extender for cryopreservation of mammalian semen (Forouzanfar *et al*. 2010). But in buck semen egg yolk coagulating enzyme is present and secreted from the bulbourethral gland and the interaction between egg yolk in semen extender and seminal plasma of buck has an adverse effect on sperm cells (Leboeuf *et al*. 2000, Purdy 2006). The interaction between egg yolk coagulating enzyme and lecithin does not produce any harmful effect on sperm, as in the case of egg yolk (Roof *et al.* 2012). Sperm cell maintained their motility after removal of seminal plasma but died in a case of addition of egg yolk in fresh semen (Purdy 2006). The conventional method of overcoming the harmful interaction of seminal plasma and egg yolk is to dilute the goat semen in buffered diluents and then separate the seminal plasma from the sperm by centrifugation. The low-density lipoprotein and phosphatidylcholine of egg yolk prevent cold shock and maintain sperm membrane during the freeze-thawing process. Soya lecithin could be an alternative of egg yolk in cryopreservation of spermatozoa (Bergeron and Manjunath 2006, Forouzanfar *et al*. 2010).

During the freeze-thawing process, trehalose protects the sperm membrane structure from oxidative stress and cold shock damage. The addition of trehalose into the extenders creates a difference of osmotic pressure that results in cell dehydration and lowers the incidence of intracellular ice crystal formation (Aisen *et al*. 2002, Purdy 2006). Various authors reported beneficial effects of supplementation of trehalose to the extender as a cryoprotectant in bull (Hu *et al*. 2010), buck (Aboagla and Terada 2003, Khalili *et al*.2009, Jafaroghli *et al*. 2011, Chelucci *et al*. 2015) and ram (Aisen *et al*. 2002, Bucak *et al*. 2007, Tonieto *et al.* 2010, Najafi *et al*. 2013) semen. Therefore, the present study was conducted to examine the influence of different dilutions having trehalose and soya based extender in different combination to replace the egg yolk and to assess the post-thaw quality of the buck semen in the winter season.

Experimental animals: The present investigation was conducted on 10 crossbred bucks (Alpine × Beetal and Sannen × Beetal) maintained at Goat farm of the Institute. Bucks were fed concentrate ration with 21% CP and 70% TDN @ 1.0 kg/buck during morning daily. Institute farm grown seasonal green fodder such as maize, cowpea, berseem, lucerne etc., depending on their availability, was fed *ad lib.* to the animals. The buck had free access to clean drinking water throughout the day. Bucks were trained to ejaculate in the artificial vagina on dummy buck before they were put in regular semen collection. Three to four ejaculates per week were collected during winter (January-February) from 7.00 to 8.00 AM and temperature varies from 2–10°C. Immediately after collection, the ejaculates were transferred into a water-bath at 30–32°C and evaluated for sperm motility and concentration as per standard protocols. Only the pooled ejaculates from the same buck with >80% initial motility were used for further processing and freezing.

Semen evaluation: Semen samples were collected in pre-warmed (37°C) 15 ml graduated tubes containing Tris buffer (TCG) and collected semen samples were pooled up to 10 ejaculates. Pooled semen samples were washed twice by centrifugation at 1,200 rpm for 10 min immediately after collection to remove seminal plasma and split into 4 equal parts and diluted with 4 different extenders. Semen quality parameters such as live percent, progressive motility, plasma

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membrane integrity (HOST), and intact acrosome were evaluated after washing, equilibration (pre-freeze) and thawing (post freeze) of semen samples.

Extenders preparation: Four different extenders were prepared for cryopreservation of buck semen such as (EXT1) Tris-citric acid-egg yolk (TCGEY) containing Tris (375mM), citrate (124mM), glucose(41mM) + 20% egg yolk (EY) (C1: TCG); (EXT 2) TCG containing 131.25mM trehalose + 20% EY (C2: TCG+T) ; (EXT3) TCG + 25% soya and (EXT4) TCG with 131.25mM trehalose 25% (TCG+T) + soya based and 54.83mM glycerol was added as cryoprotectant in each extender. pH of the buffer was adjusted to 7.0 before addition of egg yolk or soya. Prepared soya based milk was used at 25% of total volume of extender (Singh *et al*. 2012). The antibiotics i.e. gentamycin sulphate (500µg/ml), tylosin tartrate (100µg/ml), lincomycin hydrochloride (300µg/ml) and spectinomycin hydrochloride (600µg/ml) were added in the buffer (Akhter *et al*. 2008).

Cryopreservation of semen: The fresh semen and the extenders were kept together for 5 min in a water bath at 30–32°C. Semen was pre-diluted to a low volume of the extender and stored in a water bath till the final dilution rate was decided. Semen samples showing more than 80% progressive motility was used for further processing and freezing. 100 million motile sperm per dose were fixed before final volume of dilution had been determined. Four percent (54.83mM) glycerol was mixed gently with each type of extender and kept in water bath at 30–32°C. Automatic straw filling and sealing machine (MRS 3, IMV France) was used for filling of semen into the straws at room temperature (22°C). All straws were kept as a single layer horizontal position in the straw rack for 2.5 to 3 h at cold room (5°C) to give sufficient equilibration period. At the end of equilibration period, the pre-freeze motility, live percent, HOST and acrosome integrity were again recorded.

Only semen sample showing more than 70% motility during pre-freeze period was taken for “test freezing”. The straw racks were kept at 4 cm above the level of liquid nitrogen (LN_2), and exposing them to the LN_2 vapour in the wide mouthed LN_2 container for 5 min. The straws were collected into pre-cooled goblets with the help of pre-cooled forceps and finally immersed in LN_2 and stored in marked canisters. Straws were taken out 24 h after freezing from the LN_2 container and thawed at 37°C water bath for 30 sec. Post-thaw semen samples were further evaluated for live percent, progressive motility, HOST and intact acrosome percentage.

Statistical analysis: Data on semen quality parameters during fresh, pre- and post-freeze were statistically analyzed by one-way analysis of variance using SPSS statistical software package (Version 16.0). The pair wise difference of means between groups was compared by post hoc Tukey test.

The performance of different semen extenders in terms of sperm quality during different steps (initial, pre and post-thaw) of cryopreservation is presented in Table 1. Analysis revealed that there was no significant difference in initial semen quality parameters except HOST in all type of extenders (Table 1). HOST value was significantly (P<0.05) higher in soya-based extender compared to egg yolk extender in fresh semen. It is well known that the semen quality deteriorates during the process of cryopreservation which was also evident in our present observations as all the semen quality parameters decreased after freezing process as compared to fresh and pre-freeze semen in all extenders (Chelucci *et al*. 2015, Singh *et al.* 2015, Gangwar *et al*. 2016). Freezing and thawing process has a more deleterious effect on ultra structure, biochemistry and functional integrity of spermatozoa resulted in the reduction of sperm motility, membrane integrity and fertilizing ability (Purdy 2006). Increased production of reactive oxygen species (ROS) by sperm or by seminal leukocytes during

Table 1. Mean ± SE of seminal profiles of crossbred buck semen in different stages of cryopreservation in egg yolk and Soya based extenders containing trehalose

Parameters	Stage of cryopreservation	Types of extender			
		Ext. 1: TCG+20% EY	Ext. 2 : TCG+35% T+20% EY	Ext. 3: TCG + 25% Soya based	Ext. 4: TCG+35%T+25% Soya based
Live%	Initial	83.62±0.53	81.88±0.35	81.75±0.37	82.50±0.19
	Pre-freeze	78.50±0.82	79.25±1.25	76.75±1.18	77.25±1.05
	Post-thaw	58.88^{ad} ±1.52	65.75^{b}±1.70	55.25^{c}± 0.82	59.5^{d}±1.17
Progressive motility %	Initial	76.25±0.82	76.25±0.82	76.25±0.82	76.25±0.82
	Pre-freeze	70.63±1.13	71.88±1.32	70.63±1.13	71.25±0.81
	Post-thaw	48.1^{a}±2.10	56.25^{b}±1.83	45.00^{a}±1.64	50.63^{c}±1.75
HOST %	Initial	61.00^{a}±1.74	61.50^{a} ±1.82	64.63^{b} ±1.13	64.63^{b}±1.03
	Pre-freeze	60.13±1.81	62.38±1.91	59.88±1.73	58.13±1.63
	Post-thaw	45.5^{a}±1.35	51.00^{b}±1.44	43.38^{ac}±0.26	49.13^{d}±1.22
Intact acrosome %	Initial	83.00±0.38	81.75±0.25	81.5±0.19	81.63±0.18
	Pre-freeze	77.38±0.80	77.25±1.03	75.13±1.38	76.38±1.09
	Post-thaw	56.88^{a}±1.77	63.63^{b}±1.58	50.75^{c}±0.80	57.75^{ad}±1.26

Means bearing different superscripts in a row differ significantly (P<0.05); TCG, tris-citrate-glycerol; EY, egg yolk; T, trehalose; Ext.,extender.

cryopreservation reduces the sperm quality. Phospholipid plays an important physiological role in reducing the freezing point, thus avoiding the formation of large ice crystals, and also minimizes the replacement of plasmalogens to reduce the mechanical damage to the sperm membrane by large crystal formation (Giraud *et al*. 2000, Waterhouse *et al*. 2006). In the present study, elimination of the detrimental effect of goat seminal plasma by centrifugation from semen and diluted in different extenders resulted in better post-thaw semen quality.

In soya-based extender as compared to egg yolk based extender, there was significant ($P<0.05$) reduction of post-thaw semen quality parameters particularly live sperm and intact acrosome percent, but the reduction of progressive motility and HOST percent was not statistically significant. Present findings are in accordance with the earlier report of Akhter *et al.* (2010) who reported that egg yolk and Bioxcell® extender did not differ significantly for post-thaw sperm motility, viability, and HOST of buffalo semen. Yodmingkwan *et al*. (2016) reported nonsignificant differences of post-thaw sperm motility, viability and sperm abnormality among tris-fructose-citric acid, tris-fructose-citric acid + 1.5% soybean lecithin and tris fructose- citric acid + 2.5% egg yolk (TFCEY). In a similar line, various researchers reported that soya based extender was a suitable alternative to egg yolk extender for goat semen cryopreservation (Bergeron and Manjunath 2006, Forouzanfar *et al*. 2010, Roof *et al*. 2012, Salmani *et al.* 2013). Soya lecithin contains several fatty acids such as stearic, oleic, and palmitic acid and phosphatidyl choline, which plays an important role in structural stability to cells (Oke *et al*. 2010). Soya milk based phytoextender reduces the expression of apoptotic proteins, maintaining high mitochondrial membrane potential and can replace the conventional egg yolk based extenders as it gives better protection to sperms in buffalo (Mohan and Atreja 2014). Better performance of soya-based extender with trehalose may be due to heating and blenching of soya beans during soya extract preparation, which inactivates various lipoxygenase enzymes and trypsin inhibitors (Nelson *et al*. 1976) as well as containing natural soya-lecithin. Heating and blenching process also induces colloidal stability to soya extract and produce uniformity in protein and lipid molecule (Nelson *et al.* 1976).

The addition of trehalose in both egg yolk and soya based extenders, significantly ($P<0.05$) improved all the post-thaw semen quality parameters. Further, the study revealed that addition of trehalose improved all post-thaw semen quality parameters significantly ($P<0.05$) in egg yolk based extender as compared to soya based extender and gave better overall results. The results are in consonance with the findings of various researchers, who reported significant effect of trehalose on post-thaw semen quality parameters of goats (Aboagla and Terada 2003, Tuncer *et al*. 2013, Yodmingkwan *et al*. 2016) and rams (Aisen *et al.* 2000, Molinia *et al*. 1994, Bohlool *et al*. 2015) spermatozoa. Tris-based extender supplemented with soya lecithin show better functionality of cryopreserved spermatozoa and significantly higher fertilization rates as compared to commercial extender (Chelucci *et al*. 2015).

The better post-thaw results after addition of trehalose in goat semen may be due osmotic pressure difference that induces cell dehydration and lower the incidence of intracellular ice crystal formation (El-Sheshtawy *et al*. 2015). Trehalose is unable to penetrate the plasma membrane, consequently, more viable cells being recovered following cryopreservation as cell damage is minimum (Eiman *et al.* 2003). It may act as a universal stabilizer of protein conformation due to its exceptional effect on the structure and properties of solvent water compared with other sugars (Kaushik and Bhat 2003), which is a requirement for efficient cryopreservation of semen. Although the mechanism by which trehalose protects sperm during freeze thawing is not much clear.

Better results of membrane quality and acrosome status of trehalose in egg yolk or soya extract based extender as compared to TCG are in contrary to the findings of Salmani *et al*. (2013) and Vidal *et al.* (2013) who reported that, different concentration of soya lecithin was equally effective as skim milk during preservation of goat spermatozoa. The variation may be due to the difference in source and concentration of soya-lecithin and goat breed as in the present study we have used. Soya-lecithin is plant based suitable cryoprotectant for caprine sperms and can be used as a substitute for egg yolk during cryopreservation of goat spermatozoa (Jimenez-Rabadan *et al.* 2012, Roof *et al*. 2012, Salmani *et al*. 2013, Vidal *et al*. 2013, Salmani *et al.* 2014).

Egg yolk extenders along with trehalose performed best in terms of better cryo-preservability and maintenance of semen quality. But, as an alternative approach soya-based extender can also be used for goat semen cryo-preservation. Soya based extender showed a potential to maintain semen quality like individual motility, viability, membrane integrity acrosome integrity and incorporation of 35% trehalose improved the post-thaw quality to a significantly to a higher level. Further, studies are necessary to determine the effects of using these extenders on *in-vitro* and *in-vivo* fertility of the cryopreserved goat semen.

SUMMARY

To evaluate the effect of egg yolk and soybean based extenders containing trehalose on cryopreservation of goat spermatozoa. A total 100 ejaculates in winter were collected from 10 crossbred bucks. Ten pooled ejaculates from each buck were washed with phosphate buffer saline and splited into 4 different parts for dilution with 4 extenders (EXT): (EXT 1) tris (375mM), citrate (124mM), glucose (41mM) + 20% egg yolk (EY) (TCG); (EXT 2) TCG containing 131.25mM trehalose (TCG+T); (EXT 3) TCG + 25% soya and (EXT 4) TCG with 131.25mM trehalose + 25% soya. Analysis revealed that, the initial semen quality parameters except HOST did not differ significantly in all extenders. In soya based extender post-thaw live sperm and intact

acrosome were significantly reduced than egg yolk based extender. Further, the addition of trehalose to both egg yolk and soya based extender significantly improved all the post-thaw semen quality parameters and superiority of post-thaw semen quality parameters were significantly observed in egg yolk based extender containing trehalose as compared to other semen extenders. It can be concluded that TGC containing 20% egg yolk and 35% as well as TCG containing 131.25mM trehalose are suitable for crossbred buck semen cryopreservation.

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