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Effect of trehalose- an impermeant cryoprotectant on cryopreservation of Black Bengal buck semen

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ABSTRACT

The experiment was conducted to study the effect of trehalose an impermeant extra cellular cryoprotectant supplementation on cryopreservation of Black Bengal buck semen. Semen ejaculates (n = 24) were diluted in Tris- soybean lecithin - glycerol extender having trehalose @ 0, 50,100 and 150 mM. Samples were equilibrated at 5°C for 3 hrs, frozen and stored in liquid nitrogen. Semen samples were evaluated for sperm motility,functional membrane integrityand concentration of lipid peroxide compound malondialdehyde at three stages such as 10 minsafter dilution with extender, after completion of equilibration period and after freeze thawing. It was found that the control group (0mM trehalose) had significantly better *in vitro* sperm characters when compared to *extenders* supplemented with trehalose (50 mM , 100 mM and 150 mM) *during different* stages of cryopreservation. It can be concluded that supplementation of trehaloseas a cryoprotectant did not improve the cryopreservability of Black Bengal buck semen.

Key words: Black Bengal goat,Cryopreservation, Semen, Trehalose.

INTRODUCTION

Black Bengal goat is famous for its adaptability, fertility, prolificacy, delicacy of meat and superior skin quality. It can adapt well to harsh climatic conditions and generally produces twins and triplets. In recent times due to castration of male kids at an early age for meat purpose, availability of breeding bucks has reduced(Khandoker *et al.* 2011). Cryopreservation of semen is an efficient technique to save sperm cells, allowing the preservation of gene pools and expansion of desired merits through artificial insemination.However, despite years ofresearch, the cryopreservation of goat sperm still cannot be carried out efficiently (Purdy, 2006). The composition of the extender in which semen is diluted is one of the most important factors that influence the success of cryopreservation. The main cause of damage to cells undergoing cryopreservation is the formation of intracellular ice crystals. This damage may be reduced by the incorporation of cryoprotectants (Kumar *et al*. 2011).Glycerol is the most widely used cryoprotectant for bull sperm because it reduces the cryo-damage to sperm cells during the freezing process. When used in high concentrations, glycerol can cause great osmotic damage to spermatozoa because glycerol passes through the sperm membrane much slower than other cryoprotectants (Garner, 1991). When compare to bull semen, cryopreservation of buck semen in glycerol based extender results in low post thaw sperm motility (<40%) and studies are being carried out to improve the post thaw motility by incorporating semen additives to the extenders. Disaccharides are effective in stabilizing bio-membrane bi-layers and the sperm metabolism can be better sustained in diluents containing degradable sugar. Lactose, sucrose, raffinose, trehalose and dextrans are not able to diffuse across the plasma membrane, creating an osmotic pressure that induces cell dehydration and a lower incidence of intracellular ice formation. These sugars interact with phospholipids in the plasma membrane, increasing sperm survival to cryopreservation. Supplementation of trehalose helps to partially dehydrate spermatozoa before freezing which reduces the damaging effects of intracellular formation of ice crystals. Studies conducted on trehalose supplementation to the freezing extenders of semen in bovine (Chen *et al.* 1993), buffalo (Reddy *et al.* 2010), ram (Bucak *et al*. 2007), boar, dog and rabbit showed improved post-thaw semen quality. Keeping all these facts in view the present experiment was carried out to study the effect of supplementation of extra cellular, non-permeable cryoprotectant trehalose to Tris-soybean lecithin- glycerol based extender on cryopreservation of Black Bengal buck semen.

MATERIALS AND METHODS

The present study was carried out at ICAR- National Dairy Research Institute (NDRI), Eastern Regional Station, Kalyani, West Bengal, India.A total of 24 semen ejaculates were collected from adult Black Bengal bucksusing artificial vagina.The basic extender was prepared by mixing 300 mMTris, 28 mM glucose, 95 mM citric acid, soybean lecithin

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1% (w/v), 5% glycerol (v/v), and gentamicin 500 µg/ml in distilled water.Trehalose was added @ 0 (T0- control), 50mM (T50), 100mM (T100) and 150mM (T150) to the extender (Najafi *et al*. 2013). Each ejaculate wassplit into four equal aliquots and diluted at 37°C for extension in extenders containing different concentrations of trehalose. Filling and sealing of straws was done manually at room temperature (25°C to 27°C) in 0.25 ml French mini straws. The straws were cooled to 5°C slowly and equilibrated for 3hours. After completion of equilibration, straws were frozen in static liquid nitrogen vapor, 4 cm above the liquid nitrogen for 10 minutes. The straws were plunged into liquid nitrogen and stored at -196°C for at least 2 days prior to thawing.

The *in vitro* characters such as sperm motility, functional membrane integrity and concentration of lipid peroxide compound malondialdehyde (MDA) were assessed at three stages of preservation, such as after extension with semen extender, after completion of equilibration period and after freeze thawing. Sperm motility was assessed subjectively by using a phase contrast microscope at 400×magnifications. Plasma membrane integrity of sperm was determined by using Hypo-osmotic swelling test (HOST) in combination with eosin staining (HE-test) (Karunakaran *et al*. 2016a&b). Concentration of MDA was estimated using thiobarbituric acid and trichloroacetic acid (TBA-TCA) method as per Suleiman *et al*. (1996). The collected data were coded, compiled, tabulated and subjected to statistical analysis by using Least Square Analysis andTwo-way ANOVA for repeated measures.

RESULTS AND DISCUSSION

Glycerol has remained the cryoprotectant of choice for spermatozoa from all species and is commonly used at concentrations of 4% to 8%. The toxicity of glycerol limits the use of high concentration of glycerol in cryoprotective media (Hammerstedt *et al*. 1992). Sugars play an important role during the cryopreservation of biological material, not only due to their osmotic effects, but also to the interaction with the phospholipid bilayers at the low hydration conditions occurring during the freezing process, contributing to stabilize them. Sperm motility during different phase of semen preservation in trehalose supplemented extenders is presented in Table 1. There was significant reduction in sperm motility from initial dilution to equilibration and from equilibration time to post freeze thawing of semen samples in all the four extenders such as control (T0), T50, T100 and T150 groups.There was no significant difference in sperm motility observed in the semen samples mixed in T0 (77.95±1.13) and T50 extenders (77.73±0.52) after initial dilution. However, significant loss of sperm motility was observed as the concentration of trehalose increased with T100 (74.06±0.85) and T150 extender (56.09±1.13) during initial dilution. After completion of 3 hours equilibration at 5°C semen samples extended in T100 and T150 had significantly low motile cells than the control (T0) and T50 extenders. Post freeze thawing, sperm cells preserved in extender without trehalose (T0) had significantly ($P < 0.05$) more motile sperm cells (30.77±1.44), than the T50 (15.18±1.22), T100 (8.10±1.13) and T150 (1.45±0.67).

Aboagla and Terada (2003) reported that freezing of goat sperm at 375 mM trehalose resulted in significantly greater post-thaw total motility and progressive motility than in the absence of trehalose.Hu *et al.* (2010) did not found any difference between 50 and 100 mM trehalose supplementation on sperm motility of frozen–thawed ram and bull sperm. Woelders *et al*. (1997) demonstrated that an isotonic sugar medium in which Tris-citrate components were substituted with sucrose and trehalose was superior to a Tris-citrate-egg yolk extender for preserving the motility of bovine spermatozoa. Atessahin *et al*. (2008) reported that 75 mM rehalose significantly decreased the sperm motility in Angora goat semen. Further they inferred that high concentration of trehalose could have increased the osmolarity of the extender, which is deleterious to the sperm cells. High doses of trehalose could have destroyed the functional integrity of the axosome and mitochondria of the sperm cells, which are associated with motility (Atessahin *et al*., 2008).

Changes in the functional membrane integrity of buck semen during cryopreservation with trehalose are depicted in Table 2. No significant reduction in functional membrane integrity was observed in the sperm cells with intact functional membrane from initial dilution (62.13±3.24) to completion of equilibration (56.15±3.79) in the control group having only glycerol as cryoprotectant. However, the semen samples diluted with extenders supplemented with trehalose in addition to glycerol namely T50, T100 and T150 groups showed significant loss of functional membrane integrity from initial dilution to equilibration and to post freeze thawing of semen samples. No significant difference in sperm cells showing reaction to HOST was observed in

Table 1: Sperm motility during different stages of cryopreservation in trehalose supplemented extenders (%).

Treatment	Dilution	Equilibration	Freeze Thawing
Control(1% SL)	77.95±0.02 $^{a\,P}$	67.67±0.02 $^{b\,P}$	30.77±0.02 $^{c\,P}$
50mM (T50)	77.73±0.02 $^{a\,P}$	68.00±0.02 $^{b\,P}$	15.18±0.02 $^{c\,Q}$
100mM (T100)	74.06±0.02 $^{a\,Q}$	59.88±0.02 $^{b\,Q}$	8.10±0.02 $^{c\,R}$
150mM (T150)	56.09±0.02 $^{a\,R}$	41.61±0.02 $^{b\,R}$	1.45±0.02 $^{c\,S}$

Data shown all mean ± SEM (n = 24)

Means with different superscripts a, b, c in a row differ significantly ($P<0.05$).

Means with different superscripts P, Q, R, in a column differ significantly ($P<0.05$).

Table 2: Functional membrane integrity during different stages of cryopreservation in trehalose supplemented extenders (%).

Treatment	Dilution	Equilibration	Freeze Thawing
Control(1%SL)	62.13±0.07 $^{a\,P}$	56.15±0.07 $^{a\,P}$	27.33±0.07 $^{b\,P}$
50mM (T50)	57.56±0.07 $^{a\,P\,Q}$	47.99±0.07 $^{b\,Q}$	3.89±0.07 $^{c\,Q}$
100mM (T100)	52.69±0.07 $^{a\,Q}$	41.87±0.07 $^{b\,Q}$	2.29±0.07 $^{c\,Q\,R}$
150mM (T150)	40.25±0.07 $^{a\,R}$	25.08±0.07 $^{b\,R}$	0.67±0.07 $^{c\,R}$

Data shown all mean ± SEM (n = 24)

Means with different superscripts a, b, c in a row differ significantly (P<0.05).

Means with different superscripts P, Q, R, in a column differ significantly (P<0.05).

semen samples diluted in control (62.13±3.24) and T50 extenders (57.56±0.79) but as the concentration of trehalose increased, significantly (p<0.05) low per cent of sperm cells showing reaction to HOST was observed in T100 (52.69±1.22) and T150 (40.25±1.67) as compare to semen samples diluted with control (T0) and T50 extenders after extension with buffers. After completion of equilibration period semen samples extended in control group (56.15±3.79) had significantly more intact cells than the T100 (41.87±2.32) and T150 (25.08±1.17) groups. After post freeze thawing, samples preserved in extender having only glycerol (27.33±1.68) had significantly more number of HOST reacted cells than those samples preserved in trehalose supplemented extenders T50 (3.89±1.29), T100 (2.29±0.98) and T150 (0.67±0.56). Uysal*et al*. (2009) found that supplementation of semen extender with 50 and 100 mM concentrations of trehalose improved the post thawing results of ram spermatozoa, and 100 trehalose exerted more cryoprotective effect on sperm characteristics compared to the other and control groups significantly (p<0.05). Najafi *et al*. (2013) reported that Trehalose 100mM with 5% glycerol significantly reduced membrane damage in sperm cells after thawing, while 100mM trehalose with 7% glycerol did not produce better results. In ram (Jafaroghli *et al*. 2011), goat (Khalili *et al*. 2009) and buffalo, membrane integrity was better preserved when trehalose was used at 100 mM. Khalili *et al*. (2009) obtained the highest post-thawing quality when combining nearly 200 mM of trehalose (198.24 mM) and 8% glycerol. Tuncer, *et al*. (2013) reported that trehalose when added more than 100 mM, the membrane integrity was affected in a negative way.

Concentration of lipid peroxide compound in semen samples during different stages of preservation is presented in the Fig 1. Semen samples when extended in control buffer having only glycerol, it was observed that the MDA concentration (µmol/ml) did not increase significantly from initial dilution (0.16±0.02) level until the completion of equilibration period (0.17±0.01), but the MDA concentration significantly increased to 0.27±0.02 after freezing and thawing. Semen samples preserved in extenders containing 50, 100 and 150mM trehalose showed significant difference in the MDA concentration level which increased significantly (p<0.05) at every level from initial dilution to equilibration and to freeze thawing. Post freeze thawing estimation of MDA showed that all the four semen samples preserved in different extenders differed significantly with each other in terms of level of MDA concentration with the control group had the lowest level of MDA (0.27±0.04) while T150 group had the highest concentration of MDA (0.65±0.03). Tuncer *et al*. (2013) reported that the extender supplemented with trehalose did not achieve significant improvement in GPx, LPO, GSH, CAT and total antioxidant levels in comparison with the control. Aisen *et al*. (2002) reported that the extender containing trehalose enhanced the level of GSH and decreased the oxidative stress provoked by the freeze–thaw process in ram sperm. Atessahin *et al*. (2008) found that an extender supplemented with trehalose increased the GSH-Px and CAT activity of frozen–thawed goat semen.

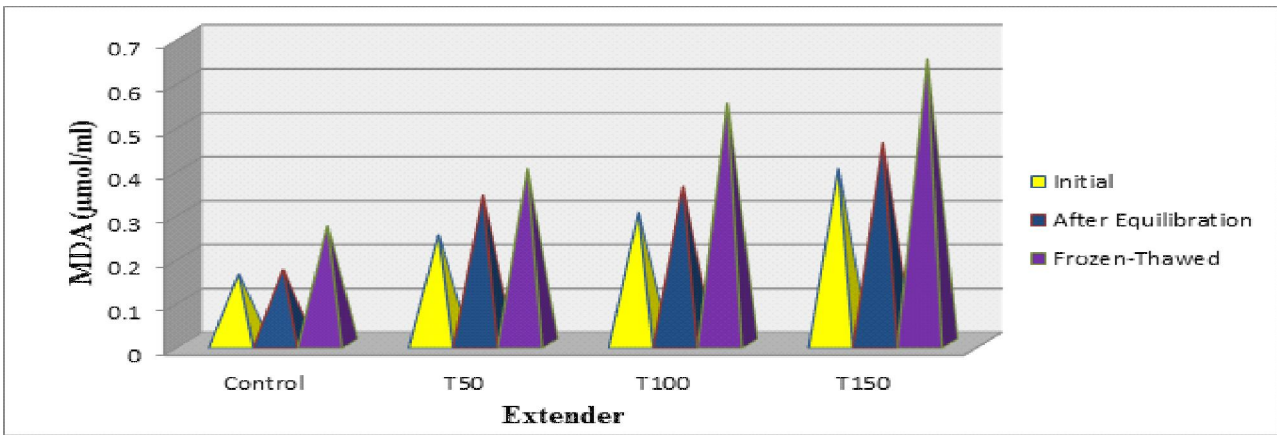

Fig 1: Concentration of malondialdehyde during different stages of cryopreservation in trehalose supplemented extenders (µmol/ml).

In the present study it was observed that supplementation of disaccharide trehalose a non-permeable cryoprotectant to Tris- soybean lecithin and glycerol based extender for preservation of Black Bengal buck semen did not have any positive effects on the post thaw sperm recovery. This suggests that there may be important differences between species regarding the optimal trehalose/glycerol concentration for better cryopreservability and this could be also influenced by many factors such as processing temperature, composition of extender and the rate of freezing. Further studies may be carried out with different combinations of glycerol and trehalose to optimize the cryosurvivabilityof Black Bengal buck semen.

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