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Cryopreservation of Ram Cauda Epididymal Spermatozoa Using Different Buffers and Sugar Combinations

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ABSTRACT

The aim of this experiment was to study the effect of different sugars and buffers combinations in the extenders *viz.* Tris citric acid fructose (TCF), Tris citric acid glucose (TCG), Sodium citrate fructose (SCF) and Sodium citrate glucose (SCG) on the quality of Cauda epididymal spermatozoa of ram during cryopreservation and post thaw. Spermatozoa were recovered from Cauda epididymidis by incision method. Samples showing ≥70 % progressive sperm motility were pooled. Each pooled cauda epididymal sperm sample was divided into four aliquots and spermatozoa in each aliquot were washed using isotonic buffer by double centrifugation. Washed spermatozoa in each aliquot were extended separately in the four different extenders using 20% egg yolk and 8% glycerol as cryoprotectant. The quality of spermatozoa was evaluated immediately after extension in the particular extenders (pre-freeze) and at post thaw. The percent sperm motility was significantly ($p<0.05$) higher for TCF (45.00±4.47) than TCG (27.50±6.55) and SCG (20.83±5.39) extenders at post thaw. The percentage of HOST reacted spermatozoa was significantly higher ($P<0.05$) for TCF (61.05±2.60) than SCF (45.81±4.90) and SCG (46.41±4.16) at post thaw. The percent intact acrosome was also significantly higher ($P<0.05$) in TCF (79.39±2.16), SCF (80.74±1.38) and SCG (78.34±2.94) than TCG (71.32±2.47) at post thaw. In conclusion, the use of fructose as energy source in the Tris extender (TCF) was found the best combination of buffer and sugar for maintaining higher sperm quality during cryopreservation of ram caudaepididymal spermatozoa.

Keywords: Epididymal Spermatozoa, Ram, Sugars, Extenders, Cryopreservation, Post thaw Sperm quality

The conservation of endangered species has now become mandatory to evade the threat of extinction. In the last decade the conservation of rare breeds of domesticated species has been very difficult and many of them have disappeared or are going to become extinct (Canali, 2006). Kashmir Stag (*Cervus elaphus hanglu*), a red deer species in J&K and 34 sheep breeds in Spain have been declared endangered (IUCN census, 2008, http://www.iucnredlist.org; Canul *et al*., 2011). The conservation of these animals by preservation of their gametes appears to be a plausible strategy. However, the spermatozoa cannot be obtained by ejaculation from wild animals due to their ferocious behaviour and thus the possible recovery of cauda epididymal spermatozoa has led to the increased attention towards conservation of the species (Foote, 2000). Appropriate retrieval and optimal cryopreservation of epididymal spermatozoa following accidental deaths of genetically meritorious animals and other endangered species would also greatly help in preserving the biodiversity (Hishinuma *et al*., 2003; Fernandez-Santos *et al*., 2006; Lone *et al*., 2011a).

The Cauda epididymal spermatozoa are capable of fertilization (Toshimori, 2003) and in fact have resulted in normal birth. The birth of a single Spanish ibex offspring

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(*Capra pyrenaicahispanica*) by artificial intrauterine insemination with Cauda epididymal spermatozoa serves a good example of their fertilizing capacity (Santiago-Moreno *et al*., 2006). The transportation of testicles from the place of slaughter of ram to the laboratory for retrieval of spermatozoa from cauda epididymis and subsequent cold storage of spermatozoa upto 72 hours at 4ºC has already been standardised (Lone *et al*., 2011a). However, cryopreservation will allow us to establish genome banks or frozen semen banks, thereby offering great opportunities for use of the germplasm for specific uses including revival of endangered species. Artificial insemination (AI) allows for the rapid dissemination of genetic material from a small number of superior sires to a large number of females (Vishwanath and Shannon, 1997). The successful storage of ram semen in liquid and frozen forms depends on the composition of the extender used (Kulaksiz *et al*., 2012). Research efforts are required to focus on the cryopreservation of cauda epididymal spermatozoa using different extenders to determine which extender is best in maintaining the quality during their storage. Short term preservation of ejaculated spermatozoa of rams (Paulenz *et al*., 2002; George *et al*., 2004; Islam and Khan, 2010) and goats (Misra *et al*., 1993; Islam *et al*., 2006) using various extenders has been reported. Similarly, short term preservation of recovered epididymal spermatozoa from slaughtered rams (Ganaie *et al*., 2009; Lone *et al*., 2011a, b; 2012) at 4°C has already been standardized in our laboratory. The effect of cooling and freezing rates on cryopreservation of ram ejaculated spermatozoa using modified Tris extender has been investigated (Ashrafi *et al*., 2011. Similarly, using *Tes Tris* Fructose solution, effect of epididymis handling conditions on the post thaw quality of ram spermatozoa is studied (Kaabi *et al*., 2003). However, the information on the cryopreservation of ram cauda epididymal spermatozoa using combinations of sugars and buffers is not available. Hence, the present study was undertaken with the objective to determine the best suitable sugar and buffer combination to obtain the ideal extender for cryopreservation of ram cauda epididymal spermatozoa retrieved from dead or slaughtered animals for long term storage.

MATERIALS AND METHODS

Collection of testicles

A total of 18 pairs of testicles along with epididymis were collected from 18 adult healthy rams after slaughter at the local abattoirs in and around Srinagar city. Six epididymides obtained from 3 adult rams were used for sperm recovery for making each pool sample. A total of six pool sperm samples were used for this study (n=6). The testicles were then transported in an ice chest to the semen processing laboratory at Sperm station, Frozen semen project, Ranbirbagh, Ganderbal, where they were further processed and spermatozoa were recovered.

Processing of testicles

Upon reaching the laboratory, the testicles were cleaned by removing the additional tissues with the help of scissors. Then the testicles were put to biometric measurements including testicular weight, epididymal weight and caudaepididymal weight. These biometric measurements were determined on an electronic balance. After determining the testicular weight, the epididymis was separated from the testicles weighing more than 100 gms (Ganaie *et al*., 2009). Finally the cauda epididymis was excised from the whole epididymis and similarly weighed.

Collection of spermatozoa

The recovery of spermatozoa from the cauda epididymis was done in Tris buffer using incision method as described previously (Lone *et al.,* 2011a,b; Lone *et al.,* 2012).

Incision method

Briefly, the cauda epididymis was aseptically excised from the testicles and various longitudinal incisions (6-8) were made on the ventral surface of the cauda epididymis to expose the spermatozoa to the outer environment. The incised cauda epididymis was dipped in 3ml tris bufferin a 35 mm petridish for 30 min to allow the spermatozoa to swim out into the buffer. Finally additional 1ml of the buffer was used to rinse spermatozoa from the incised cauda epididymis.

Initial evaluation of spermatozoa

After recovery of the spermatozoa from the cauda epididymis in petri dish the concentration and progressive motility of each sample were determined. The concentration of the sample was determined by using photometer. Then progressive motility of the samples

was determined (Zemjanis, 1970) and samples showing motility ≥70 percent were pooled for six occasions (n=6) and subsequently equally divided into four aliquots on each occasion to extend with four different extenders.

Washing of spermatozoa

Each aliquot was taken into a centrifuge tube, and centrifugation was carried out at 855 g for 10 min in centrifuge machine (REMI laboratory R4C, Maharashtra, India). After centrifugation the supernatant was discarded and the remaining spermatozoa were diluted with the particular buffer (4 ml) of the extender to be used and samples were centrifuged again. After discarding the supernatant the spermatozoa pellet was extended using 5 ml of respective extenders.

Extension of the sample

Each spermatozoa pellet obtained from the aliquot was extended using one of the four different extenders.

Composition of Extenders

1. Tris–citric acid–fructose (TCF) extender

TCF buffer: Tris (hydroxymethylamino methane) 3.028% (w/v) + citric acid monohydrate 1.70% (w/v) + fructose 1.25% (w/v) + in distilled water.

TCF Extender: TCF buffer 72% (v/v) + glycerol (v / v) 8% + egg yolk 20% (v/v) + penicillin G sodium 800 i.u./ml + streptomycin sulphate 1 mg/ml.

2. Tris–citric acid–glucose (TCG) extender

TCG buffer: Tris (hydroxymethylamino methane) 3.028% (w/v) + citric acid monohydrate 1.70% (w/v) + glucose 1.25% (w/v) + in distilled water.

TCG Extender: TCG buffer 72% (v/v) + glycerol (v / v) 8% + egg yolk 20% (v/v) + penicillin G sodium 800 i.u./ml + streptomycin sulphate 1 mg/ml.

3. Sodium citrate–fructose (SCF) extender

SCF buffer: Sodium citrate dihydrate 2.9% (w / v) + fructose 1.25% (w/v) in distilled water.

SCF extender: SCF buffer 80% (v/v) + glycerol (v / v) 8% + egg yolk 20% (v/v) + penicillin G sodium 800 i.u./ml + streptomycin sulphate 1 mg/ml.

4. Sodium citrate–glucose (SCG) extender

SCG buffer: Sodium citrate dihydrate 2.9% (w / v) + glucose 1.25% (w/v) in distilled water.

SCG extender: SCG buffer 80% (v/v) + glycerol (v / v) 8% + egg yolk 20% (v/v) + penicillin G sodium 800 i.u./ml + streptomycin sulphate 1 mg/ml.

Quality of extended spermatozoa

The quality of the extended caudaepididymal sperm samples was then evaluated for percentage of progressive motility, live sperm (Zemjanis, 1970), intact acrosome (Watson, 1975) and hypo osmotic swelling test (HOST) (Vasquez *et al*., 2013).

Percent progressive motility of the spermatozoa

A drop of sperm sample was placed on a grease free slide maintained at 37 ºC on an electric slide warmer and a cover slip (37 ºC) was put over the drop of spermatozoa and examined under high power objective of a microscope (40x). The percentage of spermatozoa that moved in forward direction was estimated (Zemjanis, 1970).

Percent live spermatozoa

The percentage of live spermatozoa was determined with a stain mixture of Nigrosin-Eosin (Blom, 1977) and descried briefly elsewhere (Lone *et al*., 2012)

Percent intact acrosome

The morphological changes of acrosome were studied using Giemsa stain (Watson, 1975) and the procedure has been described elsewhere (Lone*et al*., 2011a; 2012).

Hypoosmotic swelling test (HOST)

The positive response to HOST to determine the membrane integrity of spermatozoa was determined following the method described by Vasquez *et al*. (2013).

Preservation of the sample

The extended semen (described under 2.6) was taken to the automatic filling sealing and printing machine, where it was filled and sealed in French mini straws and subsequently printed. A total of twelve straws were filled for each extender on an occasion. The filled straws were then equilibrated at 4ºC for 4 h. The equilibrated straws were subjected to rapid vapour freezing in Biological Programmable Freezer and finally the frozen straw were placed into liquid nitrogen and stored in cryocans till further post thaw analysis.

Post thaw analysis

The frozen semen straws were thawed in warm water at 37ºC for 15 sec and the post thaw quality of each sample was determined on the basis of percent progressive motility (as already described under sub section 2.7.1), percent live spermatozoa (as already described under sub section 2.7.2), percent intact acrosome (as already described under sub section 2.7.3) and hypoosmotic swelling test (as already described under sub section 2.7.4).

STATISTICAL ANALYSIS

The data obtained in the study were analyzed statistically by using one-way analysis of variance (ANOVA) for comparison between the different extenders. The data pertaining to sperm quality between pre-freeze and post thaw were compared by Paired samples T-test with the help of statistical software SPSS version 16. The level of significance was set at $P<0.05$. The data are presented in the tables as mean ± SEM.

RESULTS AND DISCUSSION

Sperm motility

The percent sperm motility did not differ significantly ($P>0.05$) amongst the extenders at pre-freeze. The percent sperm motility was significantly ($p<0.05$) higher for TCF (45.00±4.47) than TCG (27.50±6.55) and SCG (20.83±5.39) extenders at post thaw. The percent sperm motility declined significantly ($P<0.01$) from pre-freeze to post thaw (Table 1).

Live sperm percentage

The percent live sperm did not differ significantly between the extenders both at pre-freeze and post thaw. However, the percentage of live spermatozoa declined significantly ($P<0.01$) from pre-freeze to post thaw for all the extenders (Table 1).

Percent intact acrosome

The percent intact acrosome was significantly higher ($P<0.01$) for SCF (94.80±0.69) than TCF (91.82±0.76) and TCG (90.37±0.67) at pre-freeze. However, at post thaw the percent intact acrosome was significantly higher ($P<0.05$) in TCF (79.39±2.16), SCF (80.74±1.38) and SCG (78.34±2.94) than TCG (71.32±2.47). The percent intact acrosome declined significantly ($P<0.01$) from pre-freeze to post thaw in all the extenders (Table 2).

Hypoosmotic swelling test (HOST) reacted spermatozoa

The percent HOST reacted spermatozoa was significantly higher ($P<0.01$) for TCF and TCG than SCF and SCG at pre-freeze. The percentage of HOST reacted spermatozoa was also significantly higher ($P<0.05$) for TCF (61.05±2.60) than SCF (45.81±4.90) and SCG (46.41±4.16) at post thaw. The percentage of HOST reacted spermatozoa declined significantly ($P<0.01$) from pre-freeze to post thaw in all extenders (Table 2).

Collection and cryopreservation of epididymal spermatozoa is a useful method to rescue germ plasm of dead animals that would otherwise be lost and these cryopreserved epididymal spermatozoa can be used to preserve the endangered breeds. Suitable extender is an essential component of the effective preservation methods of spermatozoa for their future use in artificial insemination and assisted reproductive technologies. In this study, effect of fructose and glucose as energy source in tris and sodium citrate based extenders was observed to find out the best extender for cryopreservation of ram cauda epididymal spermatozoa on the basis of the sperm quality parameters such as motility, viability, membrane integrity and acrosomal that were also considered to be the most valuable and useful tool in assessing the spermatozoa quality (Amann, 1999).

No variation in sperm motility amongst the extenders was observed at pre-freeze. However, TCF extender

Table 1: Effect of different sugars in the extenders on the motility and viability of ram caudaepididymal spermatozoa during cryopreservation (mean±SEM)

Extenders	Sperm motility (%)		Live sperm (%)	
	Pre-freeze	Post thaw *	Pre-freeze	Post thaw
TCF**	75.83±1.54^{A}	45.00±4.47^{bB}	81.98±1.04^{A}	61.56±6.31^{B}
TCG**	72.50±1.71^{A}	27.50±6.55^{aB}	79.93±1.18^{A}	45.22±9.93^{B}
SCF**	73.33±1.05^{A}	31.67±4.59^{abB}	80.51±0.85^{A}	55.40±8.01^{B}
SCG**	71.67±1.67^{A}	20.83±5.39^{aB}	79.64±1.19^{A}	43.34±10.73^{B}
Overall**	**73.33±0.78^{A}**	**31.25±3.09^{B}**	**80.51±0.53^{A}**	**51.38±4.44^{B}**

Means with different superscripts in a column (a, b) and row (A, B) within an evaluation parameter differ significantly; *P<0.05, **P<0.01

Table 2: Effect of different sugars in the extenders on the percentage of intact acrosome and HOST reacted caudaepididymal spermatozoa during cryopreservation (mean±SEM)

Extender	Intact acrosome (%)		HOST reacted spermatozoa (%)	
	Pre-freeze**	Post thaw*	Pre-freeze**	Post thaw*
TCF**	91.82±0.76^{abA}	79.39±2.16^{bB}	85.30±0.91^{bA}	61.05±2.60^{bB}
TCG**	90.37±0.67^{aA}	71.32±2.47^{aB}	84.27±0.61^{bA}	56.42±3.74^{abB}
SCF**	94.80±0.69^{cA}	80.74±1.38^{bB}	81.59±0.71^{aA}	45.81±4.90^{aB}
SCG**	93.58±0.48^{bcA}	78.34±2.94^{bB}	81.34±0.81^{aA}	46.41±4.16^{aB}
Overall**	**92.64±0.47^{A}**	**77.44±1.32^{B}**	**83.13±0.50^{A}**	**52.42±2.28^{B}**

Means with different superscripts in a column (a, b, c) and row (A, B) within an evaluation parameter differ significantly; *P<0.05; **P<0.01

maintained higher sperm motility at post thaw. The significantly higher post thaw sperm motility obtained using TCF extender was in concurrence with the previous reports in ram (Kaabi *et al*., 2003; Alvarez *et al*., 2012) and goat (Blash *et al*., 2000). Similarly Lone *et al*. (2012) also reported higher sperm motility in epididymal ram spermatozoa extended in Tris citric acid fructose egg yolk extender up to 72h at 4°C. However, the sperm motility both at pre-freeze and post thaw did not differ significantly between TCF and SCF. The reason might be attributed to the fructose being the common energy source in both the extenders. The present finding is in close agreement with the report of Lone *et al*. (2012) in ram epididymal spermatozoa stored at 4°C. The higher sperm motility observed for TCF compared to TCG and SCG might also be due to the use of fructose as energy source in the TCF extender. It is evident that the combination of fructose with the Tris buffer yielded better results that might be reason that the sperm motility did not differ significantly between the extenders SCF, TCG and SCG.

The sperm viability in our study did not vary significantly amongst the extenders either at pre-freeze or post thaw. The SCF extender showed live sperm percentage next to TCF. The higher sperm quality observed for TCF followed by SCF might be ascribed to the readily available fructose in the extenders – as fructose is the natural source of energy in the seminal plasma (Lone *et al*., 2012). Tris buffer with glucose (TCG) couldn't yield better result compared to Tris buffer with fructose (TCF). The reason for higher sperm quality observed in TCF extender might be attributed to the better capability of the extender to support the spermatozoa passing through the various changes and physical stress like centrifugation during the processing of the spermatozoa for subsequent preservation (Lone *et al*., 2012). Our results revealed that Trisextender with fructose as energy source (TCF) was best for extension and cryopreservation of the ram caudaepididymal spermatozoa. However, SCF also has potential for maintaining better live spermatozoa percentage next to TCF.

Fructose containing extenders (TCF and SCF) maintained comparatively higher intact acrosome than glucose containing extender (TCG). Similarly higher intact acrosome was also reported in fructose containing extenders (SCF, TCF) during preservation of ram caudaepididymal spermatozoa at 4°C (Lone *et al*., 2012). However, the intact acrosome percentage was within the acceptable limit for all the extenders both at pre-freeze and post thaw. Fructose has been used as energy source in Tes-Tris-based extender for cryopreservation of epididymal ram spermatozoa (Kaabi *et al*., 2003). Fructose is also found beneficial as energy source in Tris extender for cryopreservation of goat spermatozoa (Blash *et al*., 2000) and also for liquid preservation of cauda epididymal (Lone *et al*., 2012) and ejaculated ram (Paulenz *et al*., 2002) and goat (Misra *et al*., 1993; Islam *et al*., 2006) spermatozoa. Paulenz *et al*. (2002) found Tris extender to be best than milk and sodium citrate–based extender during the preservation of liquid ram semen at 5°C up to 30 h. Egg yolk citrate (EYC) extender could be successfully used to preserve ram ejaculated (Islam and Khan, 2010) and epididymal spermatozoa (Lone *et al*., 2011a) up to 72h at 4°C. However, Kakadiya and Kavani (1995) could obtain 50-55% sperm motility over 72h in Tris citric acid fructose yolk (TCFY) and up to 48h in egg yolk citrate glucose (EYCG) extender stored at 5°C.

Tris based extenders (TCF and TCG) maintained better membrane integrity ($P<0.05$) than Sodium citrate based extenders (SCF and SCG) at pre-freeze ($P<0.01$) and at post thaw TCF maintained greater membrane integrity ($P<0.05$) than the others (SCF, TCG and SCG). The percentage of HOST reacted epididymal spermatozoa obtained at pre-freeze in this study wasin agreement with the report of Vasquez *et al*. (2013). The significantly higher HOST reacted spermatozoa obtained at pre-freeze for TCF and TCG and for TCF extender at post thaw indicated the superiority of Tris extender over Sodium citrate based extenders (SCF and SCG) in maintaining membrane integrity. Considering the membrane integrity based on the HOST and other quality parameters at post thaw, it is revealed from the study that fructose as energy source in Tris extender(TCF) preserved better the ram cauda epididymal spermatozoa during cryopreservation than the glucose containing extenders (TCG and SCG). However, addition of fructose as energy source in Sodium citrate extender (SCF) also showed better results next to TCF for cryopreservation of ram cauda epididymal spermatozoa.

CONCLUSION

The use of fructose as energy source in the Tris extender (TCF) was found the best combination of buffer and sugar for maintaining higher sperm quality during cryopreservation of ram caudaepididymal spermatozoa. However, addition of fructose in Sodium citrate extender (SCF) also has better potential next to TCF for cryopreservation of ram caudaepididymal spermatozoa.

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