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Full Length Research Paper

A case study on cryopreservation of African sheep semen for the Red Maasai, Dorper breeds and their crosses

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The objective of this study was to examine the effect of different concentrations of trehalose and raffinose in Tris-citric acid-glucose (TCG) egg yolk extender for the sperm freezability of Red Maasai sheep, another African breed- the Dorper and their crosses. The results showed that Red Maasai, Dorper and their crosses sperm could be cryopreserved by extending with 0.075 to 0.01 M trehalose or raffinose-TCG egg yolk extender. We conclude that the cryopreservation of currently under threat Red Maasai sheep due to uncontrolled exotic breeds sperm makes it possible to establish a gene bank for the storage of animal genetic resources for endangered species, under the management of the International Livestock Research Institute for future genetic options in an uncertain world.

Key words: Red Maasai sheep sperm, freezability, raffinose, trehalose.

INTRODUCTION

Rams are one of the key resources for improving sheep production and financial profitability in East Africa, and are usually shared among several village flocks. There is a need to develop reproductive technology for rural sheep such as artificial insemination (AI), *in vitro* fertilization and embryo transfer. AI would promote rapid spread of identified superior genetic stocks by enabling a single ram to simultaneously sire progeny in many flocks of nearby villages and beyond, thereby improving mutton production. However, the sheep industry has not been able to utilize many of the assisted reproductive technologies in general and AI in particular, as other livestock industries, due to inefficiencies in collecting, freezing and inseminating frozen ram semen (Purdy et al., 2010).

We have previously studied the ability of trehalose to freeze goat sperm, and results showed that frozen semen and acrosome integrity (Yamashiro et al., 2006a,b). Use with trehalose-egg yolk extender provided higher viability of extender with trehalose for dog semen also improved motility and acrosome integrity after freeze-thawing (Yamashiro et al., 2007a). Aisen et al. (2000) showed that the combination with trehalose and in Tris-citric acid-fructose egg-yolk extender confers the highest sheep sperm cryopreservation effect on freezing and thawing. Molinia et al. (1994) examined the cryoprotective influence of different sugars such as fructose, glucose, lactose, sucrose, raffinose and trehalose in Tris-glucose egg yolk extender on the post-thawing motility of ram spermas diluent components.

Sugars of high molecular weight, have also been reported to provide a better protection to ram sperm during freezing than those of low molecular weight (Salamon, 1968). These researchers also suggested that low levels of sugars in a Tris based extender incorporated into ram semen freezing improved their cryopreservation. Furthermore, raffinose in Tris-citric acid-glucose (TCG) solution could be used as a cryopreservation for dog sperm (Yamashiro et al., 2009). We have previously also

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indicated that when rat epididymal sperm are frozen in an extender of raffinose and dissolved in modified Krebs-Ringer Bicarbonate solution containing egg yolk, the freezability of sperm is improved (Yamashiro et al., 2007b, 2010a, b). This also indicates that supplementation of trehalose or raffinose to the Tris based extender improves the characteristics and freezability of sheep semen. However, ram sperm are more sensitive to cold shock stress than other livestock animal sperm (Fiser and Fairfull, 1989). Furthermore, effective and efficient method of cryopreserving sheep semen under East African rural conditions remains undetermined.

The present study investigated the performance of trehalose and raffinose in TCG egg yolk solution as a component of a semen extender, and comparing its ability in a Kenyan indigenous Red Maasai breed with another African composite breed- the Dorper and their crosses.

MATERIALS AND METHODS

Animals

Semen from 10 rams each of Red Maasai, Dorper and their crosses, aged between 1 and 6 years, were collected twice by electro-ejaculation at experimental farm of International Livestock Research Institute (ILRI) in Kapiti, Kenya (30 rams in total) (Figures 1A and B).

Media

The basic semen extender was as described by Yamashiro et al. (2006b), which was composed of Tris(hydroxymethyl-aminomethane)-citric acid-glucose (TCG) solution containing 375 mM Tris (Calbiochem, Darmstadt, Germany), 124 mM citric acid (BDH Chemicals, Poole, England), and 41 mM glucose (BDH Chemicals). This solution was adjusted to pH 7.0 with 375 mM Tris solution and an osmolarity of 350 mOsm. Trehalose (Nakarai chemicals, Tokyo, Japan) was added at concentrations of 0, 0.025, 0.05, 0.075, 0.1 and M in TCG solution.

The osmotic pressures of these extenders were 350, 385, 415, 450 and 490 mOsm respectively, at pH 7.0. Raffinose (Sigma, St. Louis, MO, USA) was added at concentrations of 0, 0.025, 0.05, 0.075 and 0.1 M in TCG solution. The osmotic pressures of these extenders were 350, 370, 400, 425 and 455 mOsm respectively, at pH 7.0. The extenders were supplemented with 0.02% sodium dodecyl sulfate (SDS; BDH Chemicals) (w/v), followed by 20% (v/v) egg yolk, and the extender was stirred for 30 min in a mechanical stirrer. The solutions were then centrifuged at 9,000 rpm for 20 min. The supernatants were filtered through a 0.45 µm membrane filter (Sartorias, Goettingen, Germany). Finally, 3% bovine serum albumin (w/v) (BDH Chemicals) was added, completing the TCG egg yolk extender solutions.

Freezing and thawing procedure

The freezing protocol was performed by the two-step dilution according to the method described by Aboagla and Terada (2003). In experiment 1, after collection of semen by the electro-ejaculation method, the semen was diluted with a TCG solution. Diluted semen was divided into five aliquots, and each suspended in the trehalose with TCG-egg yolk extender solutions at 27°C. The tubes containing ejaculates were kept in room temperature at 27°C and transferred to the ILRI laboratory in Nairobi within 3 h after collection. Semen samples were cooled to 10°C within 2 h after arrival in Nairobi. They were then further diluted with equal volume of the different trehalose solutions with TCG-egg yolk extender containing 8% glycerol. The diluted semen was then equilibrated at 10°C within 30 min to 2 h before freezing.

Aliquots of 0.25 ml of sperm suspension were placed in straws and their ends were sealed. The straws were then placed in liquid nitrogen vapour for 10 min and then plunged directly into liquid nitrogen. In experiment 2, the semen collection and freezing protocol as described previously was modified. Briefly, care was taken during the semen collection method by electro-ejaculation to avoid urine contamination as much as possible. Urine is the course for decreased sperm characteristics such as motility, freezability or fertility. Collected and diluted semen with TCG solution was suspended in the five different concentration of raffinose with TCG-egg yolk extender solutions at 27°C. The samples were then cooled for 4 h in a vehicle refrigerator at 10°C and then transferred to the laboratory. Equal volumes of raffinose with TCG-egg yolk extender containing 8% glycerol were added to those of extended sperm. The diluted semen was then equilibrated at 10°C within 30 min to 2 h before freezing. Finally, the 0.25 ml straw were placed in the liquid nitrogen vapour for 10 min and then directly plunged into liquid nitrogen.

Frozen sperm were thawed by placing the straws for 10 s in a water bath at 37°C. The straws containing the thawed semen were then placed at room temperature (27°C) for 5 min before being subjected to post-thaw analyses. Post thaw motility was evaluated.

Evaluation of sperm motility characteristics

Ejaculates from different rams having thick consistency were estimated for individual motility of sperm immediately after dilution with a TCG solution using a subjective assessment under phase contrast microscope as described by Comizzol et al. (2001). Sperm motility was also evaluated, after freezing, thawing and incubation and after incubation at 37°C for 1, 2 and 3 h.

Statistical analysis

Results were analyzed using analysis of variance (ANOVA) and Fisher's protected least-significant difference post-hoc test using Statview Software (Abacus Concepts, Inc., Berkeley, CA). All percentages were subjected to arcsine transformation before statistical analysis and expressed as the means ± standard error. Differences in sperm quality were considered significant when P-values were lower than 0.05.

RESULTS

Freezing of sperm in various concentration of trehalose with TCG-egg yolk extender generally resulted in an increase in post-thaw motility (Table 1). The sperm that were extended with 0.075 M trehalose exhibited significantly ($P<0.05$) higher motility as compared to those that were extended with trehalose-free extender. There was no significant difference for post-thaw motility of sperm for extenders that were supplemented with concentrations of trehalose after 3 h incubation. However,

A B

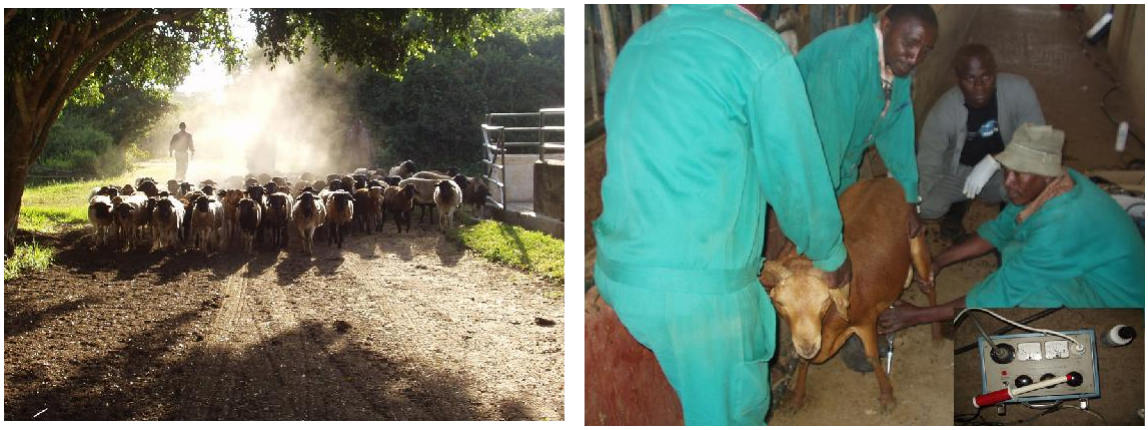

Figure 1. A) Red Maasai, Dorper and their crosses sheep. B) Illustration showing semen collection using an electro-ejaculation.

Table 1. Effect of different concentration of trehalose in the TCG-egg yolk extender on the semen from ten rams each of Red Maasai, Dorper and their crosses, after collection and post-thawing sperm motility during the thermal resistant test in experiment 1.

Trehalose concentration (M)	Sperm motility (%)[1)]				
	After collection	Post-thawing			
		0 h	1 h	2 h	3 h
0	58.1±7.2	14.0±2.4	10.5±1.9 [a]	8.7±1.9	6.2±1.4
0.025		16.8±2.6	12.5±2.2 [ab]	10.8±2.0	6.5±1.4
0.05		20.2±3.2	17.1±2.8 [ab]	13.8±2.5	10.8±2.0
0.075		21.9±3.6	18.6±2.9 [b]	14.5±2.6	11.5±2.3
0.1		21.7±3.5	17.4±2.8 [ab]	13.4±2.4	11.6±2.1

Values are the mean percentages ± SEM (n=30). [a-b] Different superscripts within the same column denote significant differences (P<0.05).

Table 2. Effect of different concentration of raffinose in the TCG-egg yolk extender on the semen from ten rams each of Red Maasai, Dorper and their crosses after collection and post-thawing sperm motility during the thermal resistant test in experiment 2.

Raffinose concentration (M)	Sperm motility (%)[1)]				
	After collection	Post-thawing			
		0 h	1 h	2 h	3 h
0	84.1±3.6	24.8±3.6	26.1±3.9	22.5±3.1	19.8±3.2
0.025		26.0±3.3	28.4±4.1	25.0±3.6	24.4±3.8
0.05		22.1±3.2	23.1±3.5	22.3±3.6	20.8±3.7
0.075		25.6±3.6	25.1±3.6	24.3±3.6	23.1±3.5
0.1		22.2±3.2	26.1±3.5	22.7±3.5	21.9±3.6

[1)]Sperm motility is expressed as an average of the percentages of motile spermatozoa and forward progressive motility.

when sperm were frozen in higher concentration of trehalose, they maintained higher post-thaw motility throughout a 3 h thermal resistance test, than the ones frozen in extender supplemented with lower concentration of trehalose.

When the sperm were in raffinose, there was no significant difference in the percentage of motility regardless of the concentration of raffinose (Table 2).

DISCUSSION

The Red Maasai sheep (Figure 2A) is an indigenous breed of Kenya and Tanzania, characterized by short

A B

Figure 2. A) Currently under threat Red Maasai sheep due to uncontrolled exotic breeds. B) South African Dorper sheep which are an important meat sire breed in Africa.

reddish brown to almost black coarse hair and in general is fat-tailed. This breed is known as genetically resistant and resilient to infectious endoparasites in East Africa (Preston and Allonby, 1979). While the Dorper sheep (Figure 2B) is a synthetic breed developed in South Africa and an important terminal meat sire breed all over Africa (Snowders and Ducktt, 2003). Mugambi et al. (1997) and Maweru et al. (1999) have cross-bred the worm-resistant Red Maasai with Dorper sheep that are susceptible to worm infections.

Fresh and frozen semen from genetically superior rams can be used through AI to serve many ewes than is possible with natural mating. AI in sheep therefore has the potential of delivering change faster and in larger sheep populations. In addition, frozen semen can aid semen banking for breeds such as the Red Maasai which are currently under threat from uncontrolled with exotic breeds. However, in East Africa, application of AI, including semen banking of valuable sheep genetic recourses has not yet been established. Here, we have examined the effect of trehalose and raffinose in TCG egg yolk extender on the freezability of sheep sperm.

Our results show that Red Maasai, Dorper and their crosses ram sperm could be cryopreserved with 0.075 to 0.1 M trehalose or raffinose-TCG egg yolk extender. In conclusion, experiments such as these could be used in future studies of sperm cryopreservation, to preserve rare genetic resources of endangered species. Further experiments are needed for the establishment of reproductive technology approaches for sheep in East Africa. Therefore, the establishment of gene banks to preserve rare genetic species in Africa must be encouraged under the leadership of ILRI, in order to safeguard the existence of endangered species.

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