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Cryoprotective effect of EDTA, lactose, ascorbic acid and L-cysteine as additives on garole ram (*Ovis aries*) semen

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Abstract

Forty eight pooled ejaculates collected from 24 adult Garole rams were cryopreserved in Liquid Nitrogen with Tris - Egg yolk - Citric Acid - Fructose - Glycerol (TEYCAFG) extender as control. EDTA (0.1% w/v), lactose (2% w/v), ascorbic acid (0.02% w/v) and L-cysteine (0.1% w/v) as additives in control medium were evaluated independently for their efficacy on different post thaw seminal attributes, viz, motility, viability, morphological abnormalities, acrosomal and plasma membrane integrity (HOST). All the seminal characters were compared with TEYCAFG. The seminal attributes were significantly ($P<0.05$) higher when the semen samples were treated with EDTA and lactose, whereas ascorbic acid and L-cysteine treated samples exhibited significantly ($P<0.05$) lower impact on different seminal characters except for morphological abnormalities with ascorbic acid which is least among the additives. The experiment thus demonstrate that EDTA and lactose as additive, had better cryoprotectivity. This new finding could have a better prospective to improve the quality of garole ram semen during cryopreservation to save the genetic material for improving this breed as well as for other native breeds too.

Keywords: Garole ram, semen additives, semen cryopreservation.

Introduction

Garole (*Ovis aries*), a non-descript breed of sheep, is recognized as prolific micro sheep of West Bengal, India. The animal is of short stature with a light brown coarse texture coat and is generally reared for meat purpose. Besides, it is also well known for the quality of felt produced from their fleece (Banerjee, 2009).

The interesting feature of this breed is its prolificacy (Sharma et al., 1999) and habitat. This breed has gained much attention for its biannual lambing with multiple births. Historical evidences favored the Bengal sheep as the origin of fecundity gene (F-gene) to the Booroola- Merino Australian because it was

imported to Australia from Bengal in late 18th century (Turner, 1982) and were crossed with Spanish-Merino to evolve Booroola- Merinos. The habitat of the breed is also quiet unique. It can thrive in the hot humid climate of the coastal saline belt of Sunderban area (N 21°–23° and E 87°–89°) of West Bengal, India which is not ideal for sheep production, so a genetic wonder gifted by nature.

The study on Garole sheep opens newer scopes of genetic improvement of the existing local breeds that are less prolific, less adaptable to harsh climatic conditions and are more susceptible to diseases. To achieve this, organized breeding programme through selection of superior individuals of the breed, followed by systemic cross-breeding with the preserved superior germplasm through Artificial Insemination (AI) are the essential paths to be traversed in succession.

The use of chilled-stored semen is limited by its relatively short time fertilizing capacity (Kheradmand and Babae, 2006). Oxidative damage during refrigerated storage of liquid semen is a potential cause of decline in motility and fertility of the male gamete (Ball et al., 2001). Hence, cryopreservation of semen is the only method for long time preservation of semen which will allow specific opportunities for the conservation of genetic resources through sperm bank.

There are several limiting factors of semen cryopreservation. In ram spermatozoa, there is labile interaction between membrane lipids and lipids from egg yolk and other components employed in extenders (Watson, 1981). Quin, (1980) reported that in ram, the difficulties found in cryopreservation of semen might be related with the higher unsaturated to saturated fatty acids ratio in the spermatic plasma membrane together with its cholesterol content which makes it more susceptible to oxidation process. Moreover, it has also been demonstrated that freezing induces early capacitation of ram spermatozoa (Grade, 1993). Thus, cervical AI, with frozen semen, is limited by low fertility rates in sheep. The application of laparoscopic techniques in sheep insemination has allowed the use of frozen semen in spite of cryopreservation problems (Eppleston, 1993). However, economic and geographic problems make it difficult to use these techniques widely. Moreover, this technique is also not easy to perform under field conditions. Thus, the development of better and improved extenders has been crucial challenge for enhancing pregnancies following AI in sheep.

A wide variety of semen additives, have been tested to minimize the damage caused by cooling and freezing-thawing on bull (Stradaioli et al., 2007), water bufallo (Ghosh and Datta, 2003), goat (Salvador et al., 2006) and ram (Paulenzen et al., 2002) semen. Fukui (1979), observed that post thawing sperm survival was the best when ram semen was frozen in Tris – glucose – yolk – glycerol extender. Choudhury (2003) reported that addition of Ascorbic acid (0.02%w/v) and EDTA (0.15 %w/v) and 0.1% (w/v) cysteine hydrochloride (Moulik, 2003) in the diluents showed better preservability of bull semen.

With the aim of genetic improvement of local breeds through AI, especially cryopreservation of Garole ram semen has been performed for the first time with TEYCAFG (Tris-Egg yolk-citric acid-Fructose-Glycerol) extender. Further, the effect of EDTA, lactose, ascorbic acid and L-cysteine as additives for improving the cryopreservability of garole ram semen were also studied.

Materials and Methods

Collection of semen from experimental animals

Twenty four healthy and adult Garole rams of 1.5 to 4.5 years of age were used as semen donors, reared in sheep and goat breeding farm, WBUAFS, Mohanpur, Nadia, West Bengal, India,. A total number of 48 collections (2 from each ram) were collected by a specially designed Artificial Vagina (George et al., 2003) twice weekly. Any semen sample of ram with concentration less than 3000×10^6 spermatozoa/ml and mass activity less than 3+ was discarded.

Extension of semen samples

The semen samples were diluted in Tris-Egg yolk-citric acid-Fructose-Glycerol (TEYCAFG) extender (containing Tris-24.2g/L, Citric acid-13.6g/L, D-fructose-10g/L, 6% v/v glycerol and 20% v/v egg yolk) in 1:1 dilution (Deka, 1984) with a single step addition of glycerol into the extender. The initial motility of semen sample was assessed after the dilution with TEYCAFG each time under the microscope (x100; x200) (Nikon, Japan). Final dilution made so that each ml of extended semen contained at least 100×10^6 spermatozoa (Anel et al., 2003) and labeled accordingly. After dilution, the samples were divided in five equal parts. The first part was considered as control group i.e. only TEYCAFG and the four remaining parts i.e. treatment groups were mixed with four different additives separately, viz.

TEYCAFG + 0.1% w/v EDTA (Merck), TEYCAFG + 2% w/v Lactose (Merck), TEYCAFG + 0.02% w/v Ascorbic acid (Spectrochem) and TEYCAFG + 0.1% w/v L-Cysteine (Merck). The pH of all the prepared extenders were adjusted within the range of 7.2 to 7.4.

Cryopreservation of semen samples

Pre-freezing sperm progressive motility was examined using Trinocular microscope (Nikon, Japan) at 37 °C. The extended samples were filled in pre-printed 0.5 ml medium French straw using a filling machine (Instruments De Medicine Veterinaire, France) and the same machine was used for ultrasound sealing of straws. The extended semen then equilibrated at 4 °C for 5 hours in cold cabinet (Instruments De Medicine Veterinaire, France) and then frozen using moderate rate of freezing up to -140 °C in about 8 – 10 minutes in Bio-freezer LN_2 cabinet (Instruments De Medicine Veterinaire, France) with integrated system of step down of cooling rate (i.e. from + 20°C to 4°C at the rate of –10°C/min., then from +4°C to –10°C at the rate of –5°C/min., then from –10°C to –100°C at the rate of –40°C/min. and lastly from –100°C to –140°C at the rate of –20°C/min.). The straws were then transferred to Liquid Nitrogen (LN_2) container directly.

Post- thaw evaluation of semen samples

Thawing was performed at 37°C for 30 seconds. Each sample from all the treatment and control groups were evaluated for spermatozoan motility, viability by Eosin - Nigrosin staining (Rao, 1957), morphological abnormality as per Hancock (1951), acrosomal integrity by Giemsa staining (Sarma, 1995) and plasma membrane integrity by HOST (Jayendran et al., 1984).

Statistical analysis

The data was analysed using statistical software SPSS v.20 for Windows. Descriptive statistics was carried out to calculate the mean and standard deviation while the means were compared using ANOVA-Tukey as post-hoc. Values were considered significant at $P<0.05$.

Results

Pre-freeze progressive motility of ram spermatozoa diluted in TEYCAFG was 77.3% ± 1.6 which is significantly higher ($P<0.01$) than post-thawing motility i.e. 60.8% ± 0.7, primarily due to cryo-effect. The mean post-thawing motility in semen among the four treatment groups, was higher in EDTA i.e. 66.8% ± 0.9, followed by in lactose 65.1% ± 0.6, then in Ascorbic acid 55.6% ± 0.5 and lowest in L-Cysteine treated group 42.7% ± 0.5 (Table 1).

Table 1. Comparison of post-thawing seminal characteristics of garole ram with Lactose, EDTA, L-Cysteine and Ascorbic acid treated groups with control group

Seminal characteristics (%)	TEYCAFG Control	Treatment groups			
		Lactose (2%)	EDTA (0.1%)	L-Cysteine (0.1%)	Ascorbic Acid (0.02%)
Motility	$60.80^{b} \pm 0.7$	$65.10^{a} \pm 0.6$	$66.80^{a} \pm 0.9$	$42.70^{d} \pm 0.5$	$55.60^{c} \pm 0.5$
Viability	$65.10^{c} \pm 0.6$	$69.60^{b} \pm 0.9$	$73.30^{a} \pm 1.2$	$52.40^{e} \pm 0.8$	$61.50^{d} \pm 0.6$
Morphological Abnormality	$8.08^{b} \pm 0.1$	$7.38^{a} \pm 0.1$	$8.04^{b} \pm 0.8$	$9.17^{c} \pm 0.1$	$7.13^{a} \pm 0.1$
Intact Acrosome	$84.30^{b} \pm 0.8$	$87.90^{a} \pm 0.9$	$88.00^{a} \pm 1.0$	$80.40^{d} \pm 0.7$	$82.90^{c} \pm 0.7$
HOST Reacted	$55.00^{b} \pm 0.5$	$58.70^{a} \pm 0.6$	$59.80^{a} \pm 0.9$	$37.70^{d} \pm 1.2$	$51.00^{c} \pm 0.5$

Values expressed as Mean ±SE; n=10;
[a-e]Same superscripts indicate groups of treatments with no significant differences within the same step (ANOVA-Tukey test at $P<0.05$).

The mean percent viability was highest with EDTA treated group i.e. 73.3% ± 1.2 followed by in lactose 69.6% ± 1.0, then in Ascorbic acid 61.5% ± 0.6 and the least with L-Cysteine treated group i.e. 52.4% ± 0.8 (Table 1).

In Ascorbic acid treated group the mean percent morphological abnormality was the least 7.1% ± 0.1 followed by lactose treated group 7.3% ± 0.1, then follows EDTA 8.0% ± 0.8 and the most with L-Cysteine treated group 9.2% ± 0.1 (Table 1).

Among the four treatment groups, the mean post-thawing acrosomal integrity in semen was again the best in EDTA i.e. 88.0% ± 1.0, followed by lactose 87.9% ± 0.9, then Ascorbic acid 82.9% ± 0.7 and lowest in L-Cysteine treated group i.e. 80.4% ± 0.7 (Table 1).

The results of mean percentage HOST reacted spermatozoa of ram semen was also the best in EDTA i.e. 59.8% ± 0.9, followed by lactose 58.7% ± 0.7, then ascorbic acid 51.0% ± 0.5 and lowest in L-Cysteine treated group 37.7% ± 1.2 (Table 1).

Experiment revealed that post thaw motility, viability, acrosomal integrity and HOST positivity of spermatozoa were better in the EDTA treated group followed by lactose treated group, although there is no significant difference with lactose treated group except for viability, followed by control, ascorbic acid and the poorest with L-Cysteine treated group respectively (Table-1). Whereas the incident of highest post – thaw morphological abnormality was noticed in L-Cysteine treated group followed by the control group, EDTA treated group and lactose treated groups respectively. The lowest incident of morphologically abnormal spermatozoa was observed in the Ascorbic acid treated group though there is no significant difference with lactose treated group. The differences between the treatment groups and control are statistically significant ($P<0.05$) for all the seminal parameters.

Discussion

Present observation corroborated with the findings of Singh et al., 2000 who observed higher percentage of motility in TEYFC extender containing EDTA but lower than containing cysteine HCl in Beetle buck

The cryoprotective effect of EDTA in Garole ram is evident from the present work and can be correlated with the results obtained by Aisen et.al., 2000, who described that the addition of trehalose and EDTA to base extender provides a better thermo protection for ram semen as evaluated in vitro by the percentages of sperm motility and acrosome integrity in the various processing steps.

Singh et al., (2000) and Sinha et al., (2002), also indicated favorable effect of EDTA on frozen buck spermatozoa in case of refrigerant temperature preservation. Choudhury (2003), reported beneficial effects of EDTA (0.15% w/v) on frozen bovine spermatozoa. But no literature was available in rams in this regard to compare the results.

It has been shown that Ca^{2+} plays a role in signaling pathways in acrosomal reaction, thus reduces sperm viability and affects acrosome morphology during cooling (Bailey et al., 1995), which is reduced in the presence of EDTA (Roldan et al., 1994), may be due to its chelating property as reported by Sinha et al., (2002). It could form stable chelates with heavy metals, which may cause damage to the live spermatozoa (Abdu, 1977). EDTA protects live spermatozoa against the amino acid oxidase, released by dead sperm that is harmful to surviving cells through the production of hydrogen peroxide (Shannon and Gurson, 1983). Also EDTA may reduce cold shock (Abdu, 1968).

The effect of Lactose as crpyoprotectant in ram semen is evident from the finding which can be corroborated with the findings of Kalev et al., (1971). The inclusion of Lactose (2% w/v) yields better results, in terms of post–thawing seminal attributes, which may be due to the better cryoprotective property of Lactose (Singh et al., 2003). Lactose, being a disaccharide, could not be metabolized by spermatozoa, provides cryoprotective benefits as it could not pass across the sperm cell membrane during freezing or thawing and they probably diminish the effects of solute concentration. Thus, it acts to contribute, to the osmotic balance of the extender (Nagase et al., 1964). Also Lactose in combination with Glycerol restricts the leakage of acrosin and hyaluronidase (Singh et al., 2003) the enzymes principally control the fertilizing potential of spermatozoa.

In rams, Uysal et al., (2000) reported lower mean percent post-thaw motility (48.30% ± 2.13) than that obtained in the present finding (55.63% ± 0.839) using Ascorbic acid as an additive. No more comparable result was available in rams. However, Choudhury (2003), reported higher post-thawing survival of spermatozoa while cryopreservation of bovine spermatozoa, using Ascorbic acid (0.02% w/v) as an

additive. This is probably due to its antioxidative property. Ascorbic acid prevents lipid peroxidation of the phospholipid bilayer of spermatozoa by reducing the production of free radical due to oxidation during sperm metabolism thus indirectly prove useful in maintaining structural integrity of plasma membrane. Thus it has a significant protective effect against sperm peroxidative damage (Singh et al., 1989). Though Ascorbic acid has been used as a cryoprotectant of semen in many species but the result could not be replicated and are poor in extension with this additive in the Garole semen. In spite of the poor performance in the species, but in regards to morphological abnormality, it has been the best to keep the abnormality count lowest and this could be due to the protective action against membrane damage.

The beneficial effect of L-Cysteine (0.1% w/v) as cryoprotectant in the diluent as an additive in bull semen showed significant ($P < 0.01$) improvement in sperm motility, viability, acrosomal integrity and HOS positivity (Moulik, 2003). The Cryoprotectant effect of Cysteine hydrochloride may be due to the fact that it restricts the aerobic metabolism of spermatozoa and stimulates the anaerobic metabolism. Besides, Cysteine have reducing property, which may help to decrease the oxydative damage to spermatozoa (Arora et al., 1996). However, cryoprotective action was poorest in the Garole semen when compared to other species.

In the present investigation as evident from the results, Ascorbic acid (2%) and L-Cysteine (0.1%) treatment groups could not be corroborated and failed to yield any beneficial effect, which may be due to species variation and breed variation.

Based on the above findings it can be concluded that, addition of EDTA (0.1% w/v) and lactose (2% w/v) as additives to the basic extender (TEYCAFG) improved the preservability of Garole ram semen significantly ($P<0.05$) than TEYCAFG (control). However, incorporation of ascorbic acid (0.02% w/v) significantly ($P<0.05$) lowered the post-thawing seminal attributes compared to Control group whereas, L-cysteine (0.1% w/v) had yielded most unfavorable results in terms of seminal attributes compared to all other treatments in this investigation which may be due to the breed and species variation.

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Conflict of interest statement and grant

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