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EFFECTS OF SOME ADDITIVES ON CRYOPRESERVATION OF BLACK BENGAL BUCK (*CAPRA HIRCUSBENGALENSIS*) SEMEN

[1]Krishna Prasad Mukherjee, [*,2]Siddhartha Basu, [3]Ajit Kumar Sahoo, [2]Uttam Datta and [2]Somraj Chattaraj

[1]Veterinary Officer, Department Animal Resource Development, Govt. of West Bengal, Kolkata, India
[2]Department of Veterinary Gynaecology and Obstetrics, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata-37, India
[3]Department of Animal Genetics and Breeding, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata-37, India

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ABSTRACT

Forty eight pooled ejaculates collected from 24 adult Black Bengal bucks were cryopreserved in Liquid Nitrogen with Tris - Egg yolk - Citric Acid - Fructose - Glycerol (TEYCAFG) extender as control. Efficacy of EDTA (0.1% w/v), lactose (2% w/v), ascorbic acid (0.02% w/v) and L-cysteine (0.1% w/v), added individually as additives in control medium, were evaluated independently 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 (control). Seminal attributes were significantly ($P<0.05$) higher when the semen samples were treated with EDTA and lactose, whereas L-cysteine treated samples exhibited significant ($P<0.05$) lower impact on different seminal characters. However, incorporation of ascorbic acid did not have any significant ($P<0.05$) cryoprotective effect. The experiment thus demonstrate that EDTA and lactose as additive, had better cryoprotectivity on Black Bengal buck semen. This new findings could have better prospective to improve the quality of Black Bengal buck semen during crypopreservation to save the genetic material for improving the breed.

Key words: Black Bengal buck, Semen cryopreservation, Semen additives.

INTRODUCTION

Black Bengal breed of goat is widely found throughout eastern regions of India and in the northern parts of Bangladesh. It is most popular due to its high prolificacy, excellent quality of skin and meat. Besides, the breed is has a superior germplasm with higher fecundity and early sexual maturity (Deb, 2003). The breed has suffered an extensive inbreeding due to negligence of the owners, which lead to the dilution of the high merit of the breed and loss of gens related to adoption resulting in loss of various economic traits. This native breed thus needs to be conserved by selective breeding of pure Black-Bengal goats in their home tract. The conservation of germplasm could be achieved through storage of semen at refrigerated temperature, but chilled-stored semen possess relatively short time fertilizing potential (Kheradmand and Babae, 2006). Oxidative damage during refrigerated storage of liquid semen is one of the potential cause of decline in motility and fertility of male gamete (Ball *et al.,* 2001). The process of freezing and thawing of buck semen reduces the percentage of viable sperm cells as well as acrosomal integrity (Blash *et al.,* 2000). Again, exposure of buck semen to light during manipulation before storage leads to formation of reactive oxygen species that adversely affect sperm cell motility and genomic integrity (Foote, 1967). Hence, cryopreservation of semen is the only method for long time preservation of the superior germplasm which will allow specific opportunities for the conservation of genetic resources through sperm bank. There are several limiting factors of buck semen cryopreservation. For cryopreservation, egg yolk-based extenders have been widely utilized for goat semen. However, freezing of buck semen with egg yolk based extender poses some difficulties due to the presence of seminal plasma (SP). Therefore, the use of egg-yolk containing extenders requires removal of most of the seminal plasma by washing before the dilution of spermatozoa (Leboeuf *et al.,* 2000). However, washing is a complex and time consuming process, and it also causes damage and some loss of spermatozoa (Corteel, 1981). Deka and Rao (1984), reported that post thaw motility did not vary significantly between semen processing with or without seminal plasma in egg-yolk-citrate-fructose-glycerol and Tris-egg-yolk-citric acid-glycerol based extenders. Thus, the development of better and improved extenders is a crucial challenge for enhancing pregnancies following AI in buck. A wide variety of semen additives, have been tested to minimize

Corresponding author: Siddhartha Basu,
Department of Veterinay Gynaecology and Obstetrics, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata-37, India

the damage caused by cooling and freezing-thawing on goat semen (Salvador *et al*., 2006). Singh *et al.* (2000) and Sinha *et al.* (2002) obtained better seminal attributes with cystein-Hcl and EDTA as additives in Tris-Egg-Yolk-Fructose-Citrate (TEYFC) extender in beetle buck. Lactose added to egg yolk extender for freezing of buck semen into pellets obtained better motility (Zheltobryukh and Ashurbego, 1982). Again, (Singh *et al.,* 2003) also observed that the inclusion of lactose (@ 2%) yields best results. Mukherjee *et al.* 2016, reported addition of lactose (2% w/v) and EDTA (0.1% w/v) yielded better cryoprotective effect on garole ram semen. Addition of Ascorbic acid (0.02%w/v) and EDTA (0.15 %w/v) (Choudhury, 2003) and 0.1% (w/v) cysteine hydrochloride (Moulik, 2003) in the diluents showed better preservability of bull semen. Ascorbic acid as additive to extenders could reduce the oxidative stress in bovine (Jian *et al.,* 2010). Hence with an aim to prevent gene loss and reduction of inbreeding of the breed, cryopreservation of black Bengal buck 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 Black Bengal buck semen were also studied.

MATERIALS AND METHODS

Collection of semen from experimental animals

Twenty four healthy and adult Black Bengal bucks of 2.5 to 4.5 years of age were selected as semen donor, reared in sheep and goat farm, WBUAFS, Mohanpur, Nadia, West Bengal, India,. A total number of 48 ejaculates from 24 bucks (i.e. two ejaculate per buck) were collected by specially designed Artificial Vagina (AV) (George *et al*., 2002 and Mukherjee *et al.* 2016). Any semen sample of buck with concentration less than 2000 X 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 LN2 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 (LN2) 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 group were evaluated after thawing for Motility by conventional method, 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 the buck spermatozoa diluted in TEYCAFG was 78. 04% ± 1.66 and the post-thawing motility recorded as 59.8% ± 0.61. The pre-freeze progressive motility was significantly higher ($P < 0.01$) than post-thawing motility, which is primarily due to the cryo-effect and may be due to dilution in egg yolk-based extender. The mean post-thawing motility in semen among the four treatment groups, was higher in EDTA i.e. 69.8% ± 0.95, followed by in lactose 68.1% ± 0.73, then in Ascorbic acid 61.0% ± 1.01and lowest in L-Cysteine treated group 39.1% ± 1.02 (Table 1). The mean percent viability was highest with EDTA treated group i.e. 74.2% ± 1.01 followed by in lactose 72.9% ± 1.24, then in Ascorbic acid 66% ± 1.11, control group 65.5% ± 1.01 and the least with L-Cysteine treated group i.e. 48.8%± 1.04 (Table 1). In lactose treated group the mean percent morphological abnormality was the least 5.9% ± 0.13 followed by EDTA treated group 6.4% ± 0.11, then follows the control group 8.6% ± 0.08 and 8.7% ± 0.12 L-Cysteine treated group and the most with ascorbic acid treated group 9.2% ± 0.14 (Table 1). Among the four treatment groups, the mean post-thawing acrosomal integrity in semen was again the best in EDTA i.e. 88.5% ± 0.94, followed by lactose 87.2% ± 1.08, then the control group with 84.5% ± 0.95 Ascorbic acid 84.1% ± 1.04 and lowest in L-Cysteine treated group i.e. 78% ± 1.84 (Table 1). The results of mean percentage HOST reacted spermatozoa of ram semen was also the best in EDTA i.e. 63.1% ± 0.84 followed by lactose 63% ± 1.07, then the control group 54% ± 0.84 ascorbic acid 52.9%

± 0.97 and lowest in L-Cysteine treated group 33.7% ± 1.31 (Table 1). Experiment revealed that post thaw seminal characters viz. motility, viability of spermatozoa were better in the EDTA treated group followed by lactose, ascorbic acid, control treated group and the poorest with L-Cysteine treated group respectively (Table-1). For post thaw acrosomal integrity and HOST positivity again EDTA treated group yielded better results followed by lactose, control, ascorbic acid treated group, and the poorest with L-Cysteine treated group respectively (Table-1). Though EDTA yielded better results than lactose but there was no significant difference ($P<0.05$) between EDTA and lactose and also between ascorbic acid and control. Post-thaw morphological abnormality was noticed higher in ascorbic treated group (significant $P<0.05$) followed by L-Cysteine and control (with no significant difference). Lactose yielded lower morphological abnormality (significant $P<0.05$) than EDTA treated group.

Table 1. Post-thaw seminal attributes of Balck Bengal buck with Lactose (2%), EDTA (0.1%), L-Cysteine (0.1%) and Ascorbic acid (0.02%) treated group

Seminal characteristics (%)	Treatment groups (Mean ± S.E.)				
	TEYCAFG (Control)	Lactose (2%)	EDTA (0.1%)	L-Cysteine (0.1%)	Ascorbic Acid (0.02%)
Motility	$59.80^{b} \pm 0.61$	$68.10^{a} \pm 0.73$	$69.80^{a} \pm 0.95$	$39.10^{c} \pm 1.02$	$61.00^{b} \pm 1.01$
Viability	$65.50^{b} \pm 1.01$	$72.90^{a} \pm 1.24$	$74.20^{a} \pm 1.01$	$48.80^{c} \pm 1.04$	$66.00^{b} \pm 1.11$
Morphological Abnormality	$8.67^{c} \pm 0.08$	$5.90^{a} \pm 0.13$	$6.42^{b} \pm 0.11$	$8.78^{c} \pm 0.12$	$9.29^{d} \pm 0.14$
Intact Acrosome	$84.50^{b} \pm 0.95$	$87.20^{ab} \pm 1.08$	$88.50^{a} \pm 0.94$	$78.00^{c} \pm 1.84$	$84.10^{b} \pm 1.04$
HOST Reacted	$54.00^{b} \pm 0.84$	$63.00^{a} \pm 1.07$	$63.10^{a} \pm 0.84$	$33.80^{c} \pm 1.31$	$52.90^{b} \pm 0.97$

Values expressed as Mean ±SE; n=10;

[a-c]Same superscripts indicate groups of treatments with no significant differences within the same step (ANOVA-Tukey test at ($P<0.05$).

DISCUSSION

Present observations of pre-freeze motility was higher than that reported by Sundaram and Edwin (2003). The result of post-thawing motility was in accordance with the findings of Haranath *et al.,* (1982)and better results were obtained than those obtained by Sundaram and Edwin (2003). The cryoprotective effect of EDTA in the Black Bengal buck semen was evident from the present work which can be correlated with the findings of Singh *et al.* (2000) who observed higher percentage of motility in TEYFC extender containing EDTA in Beetle buck. He also reported that incorporation of EDTA showed significant ($P< 0.01$) improvement of post-preservation seminal quality like motility, percentage of live sperm and percentage of acrosomal abnormality. Sinha *et al.* (2002), also indicated favorable effect of EDTA on frozen buck spermatozoa in case of refrigerant temperature preservation. Mukherjee *et al.* (2016), also reported EDTA (0.1% w/v) showed better cryopretective effect on garole ram semen. But no literature was available in Black Bengal bucks in this regard to compare the results. Ca^{2+} plays a role in signaling pathways in acrosomal reaction, thus during cooling it reduces sperm viability and affects acrosome morphology (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 Black Bengal buck semen was also evident in the present study. Mukherjee *et al.* (2016), reported lactose (2% w/v) as additive to TEYCAFG extender improved post-thaw seminal character of garole ram semen. The motility obtained with lactose obtained was better than of Zheltobryukh and Ashurbego (1982), but other attributes could not be compared as no data in this regards were available in bucks. 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, and in this way, it acts to contribute, to the osmotic balance of the extender (Nagase, 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.

Sinha *et al.*, (2002) while studying the effect of Ascorbic acid on preservation of caprine semen at refrigerant temperature got non-significant effect, which is to some extent can be corroborated with the present study. In garole ram ascorbic acid as additive could not yield any better effect (Mukherjee *et al.* 2016). In bovine spermatozoa, however, significant improvement was reported by Raina *et al.*, (2002), Srivastava and Kumar (2002) and Choudhury (2003). But no such comapartive data were available for the buck semen. This is probably due to its favourable effects of the antioxidation property of ascorbic acid. 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 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 in the Black Bengal buck semen as it resulted in maximum morphologically abnormal spermatozoa. The beneficial effect of L-cysteine as cryoprotectant in bull semen as described by Moulik (2003), was may be due to the fact that it restricts the aerobic metabolism of spermatozoa and stimulates the anaerobic metabolism. Besides, Cysteine also have reducing property, which may help to decrease the oxygen damage to spermatozoa (Arora *et al.,* 1996). However, the cryoprotective action was poorest when extented with this additive in the Black Bengal buck semen when compared to other species which could be corroborated with findings of Mukherjee *et al.* (2016), in garole ram senem. The reason of poor cryopetective action of L-cysteine however could not be ascertained. In the

present investigation it is evident that EDTA and lactose yielded better post-thaw seminal attributes, though there was no significant difference except for morphological abnormalities where lactose was better than EDTA. 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 was concluded that, Black Bengal buck semen was best preserved when EDTA (0.1%) and LACTOSE (2%) were added as additive to the basic extender (TEYCAFG) than all other additives. But Ascorbic acid (0.02%) failed to impart any significant favorable effect on post-thawing seminal characteristics compared to control group and L-Cysteine (0.1%) 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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