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Research Article

L-Carnitine improves the sperm attributes during cryopreservation of Black Bengal buck semen

R. Lalramchansangi[1], S. Singha[2], S. Das[3], D. Sarkar[4], M. Rajendar[5], M. Mondal[6], A. Mandal[7], C. Bhakat[8] and M. Karunakaran[9*]

[1]Junior Research fellow, College of Veterinary Sciences & Animal Husbandry, Selesi, North Aizawl - 796 014, Mizoram, India; [2]Veterinary Officer, Block Animal Health Centre, Chopra Block, Uttar Dinajpur - 733 207, West Bengal, India; [3]Veterinary Officer, Additional Block Animal Health Centre, Piplon, Manteswar, Purba Burdaman- 713 422, West Bengal, India; [4]Veterinary Officer, Veterinary dispensary, Kakraban, Gomati District, Udaipur- 799 105, Tripura, India; [5]Animal Biotechnology, ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia-741 235, West Bengal, India; [6]Animal Physiology, ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia-741 235, West Bengal, India; [7]Animal Genetics and Breeding, ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia-741 235, West Bengal, India; [8]Livestock Production and Management, ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia-741 235, West Bengal, India; [9]Animal Reproduction and Gynaecology, ICAR-National Dairy Research Institute, Eastern Regional Station, Kalyani, Nadia-741 235, West Bengal, India

Abstract

The demand for preserved buck semen has increased due to inadequate availability of quality breeding bucks. The present study had evaluated the effect of L- carnitine, a mitochondria targeted antioxidant on cryopreservation of Black Bengal buck semen. Semen samples were collected from five matured bucks by artificial vagina method and 20 pooled ejaculates were used for cryopreservation. Semen extender was supplemented with L- Carnitine at two different concentrations: 1 mM (LC-1) 2 mM (LC-2), and cryopreserved. It was found that, at both post equilibration and freeze-thaw stages, the semen samples supplemented with L- Carnitine @ 2 mM had significantly higher ($p<0.05$) progressive motility, functional membrane integrity and acrosome integrity when compared to the control group, though there was no significant reduction in the lipid peroxide compound malondialdehyde level. It could be concluded that supplementation of mitochondria targeted antioxidant L- carnitine @ 2 mM could improve post thaw sperm recovery during cryopreservation of Black Bengal buck semen.

Keywords: Antioxidant, Black Bengal buck, Cryopreservation, L-Carnitine, Sperm attributes

Highlights

- 2 mM Carnitine addition improved motility, membrane integrity during buck semen cryopreservation
- Carnitine @ 1.2 mM did not have effect on lipid peroxide production during buck semen preservation

INTRODUCTION

The Black Bengal goat (*Capra hircus bengalensis*) is known for its prolificacy, fertility, excellent quality of meat, and skin (Hussain et al., 1996). The breed is natively found all over West Bengal and in the conjoining states viz., Jharkhand, Bihar, Orissa, Tripura, and Assam, in India and it is also predominant in Bangladesh (Kumari et al., 2015). There has been a drastic reduction in the availability of breeding bucks due to slaughtering of male kids for meat purpose, resulting in a 1:88 buck-doe ratio in against the recommended 1:20 (Khandoker et al., 2011; Nandi et al., 2011). Further, it leads to inbreeding depression and the loss of valuable genetic material due to the frequent and unselective mating with other larger breeds. Hence these concerns could be countered through the widespread use of artificial insemination (AI). Since, cryopreservation of buck semen is not well established as like that of bull semen, the method for cryopreservation of buck semen requires improvement to get optimal post thaw sperm recovery and conception.

Several conventional, non-targeted antioxidants have been used in buck semen to counter lipid peroxidation (LPO) and oxidative stress, but results using these antioxidants have generally been

*Corresponding Author, E-mail: drmkarunakaran@gmail.com

inconsistent because the majority of the antioxidants could not penetrate the mitochondrial membranes to regulate the reactive oxygen species (ROS) generation (Bahmyari et al., 2020). Inclusion of mitochondria-targeted antioxidants (MTA) in the semen extender could better ameliorate the ROS-induced peroxidative damage due to their higher concentrations at ROS generation site i.e., mitochondria. Studies in large ruminants with supplementation of MTA had shown promising results (Younus et al., 2024), however not much information on the effects of mitochondria-targeted antioxidants in small ruminants, especially in bucks is available and the present study was carried out to study the effect of MTA, L-Carnitine on cryopreservation of Black Bengal buck semen.

MATERIALS AND METHODS

Experimental animals and semen collection: Five Black Bengal bucks of 2 to 2.5 years of age, weighing between 15 and 30 kg were used in the study. Bucks were fed with a concentrate mixture, chopped green fodder thrice daily and drinking water *ad libitum*. A total of 100 ejaculates were collected from selected bucks by artificial vagina. Semen samples with at least +3 mass activity score, concentration >2500 million/mL, volume >0.5 mL and individual motility >70%, were selected for further processing. Semen ejaculates that have passed the initial quality check were then pooled to avoid individual variation and 20 pooled semen samples were used for the study.

Preparation of extender and semen processing: The extender was prepared by mixing 300 mM Tris, 28 mM glucose, 95 mM citric acid, egg yolk 20% (v/v), glycerol 5% (v/v) by single step addition and 500 µg/mL gentamicin in distilled water and maintained at 37°C until final dilution (Konyak et al., 2018). The volume of extender to be added was calculated so that the final extended semen contains 80×10^6 sperms per straw (0.25 mL). L- Carnitine (Sigma Aldrich) was used for the supplementation study. Extended semen was divided into three groups: Control (Tris extender), LC-1 (Tris extender + L-Carnitine 1 mM) and LC-2 (Tris extender + L-Carnitine 2 mM).

Evaluation of *in vitro* sperm characters: Semen samples were evaluated for *in vitro* sperm characters viz. individual motility, viability, functional membrane integrity and acrosomal integrity, along with lipid peroxidation status after 3 hrs of equilibration and after 48 hrs of cryopreservation. The motility of sperm was assessed under a phase contrast microscope at 40X magnification. Sperm viability was assessed by Eosin-Nigrosin staining procedure. The plasma membrane integrity of sperm was determined by using the Hypo-osmotic swelling test (HOST) in combination with eosin staining (Konyak et al., 2018). Acrosome integrity was evaluated using Giemsa staining technique as per Watson and Martin (1975) with slight modifications. Smear was fixed in Hancock's fixative for 15-20 minutes, washed in slow-running water, air-dried, and stained with freshly prepared Giemsa stain for 3 hours. After staining, the slides were rinsed with distilled water, air-dried and examined under oil immersion (100X magnification). Sperm cells with intact acrosomes appeared as dark pink/purple, and a total of 200 spermatozoa were counted per smear to determine the percentage of intact acrosomes. Lipid peroxidation status in the sperm cells were estimated by measuring malondialdehyde (MDA) level using thiobarbituric acid (TBA), as described by Suleiman et al. (1996), with slight modifications. Semen samples were thawed, washed twice in Tris buffer via centrifugation (500 g, 5 minutes), and the sperm pellet resuspended in 1 mL of PBS (pH 7.2). Lipid peroxide levels were measured after adding 2 mL of TBA-TCA reagent (15% w/v TCA, 0.375% w/v TBA in 0.25N HCl) to 1 mL of sperm suspension, followed by incubation in a boiling water bath for 45 minutes. After cooling and centrifugation (500 g, 15 minutes), the supernatant's absorbance was measured at 535 nm using a UV-Vis spectrophotometer. MDA concentration was calculated using the specific absorbance coefficient ($1.56 \times 10\text{^}5$ /mol cm^-3).

Data analysis

All the data were analysed by using SPSS software (22.0 version). The statistical method of one-way ANOVA was employed and mean values of percentages of mass activity, sperm mortality, viability, membrane integrity, acrosome integrity and lipid peroxidation status were compared using post-hoc comparisons (Duncan's multiple range test) procedure, when F-value was significant ($p<0.05$ and $p<0.01$).

RESULTS

Neat semen characters: Semen ejaculates collected in the present study had an average of 0.59±0.13 mL volume, 4286.40±45.64 million sperms per mL sperm cell concentration, 4.54±0.10 mass motility, and 93.33±1.05% individual sperm motility. The mean values of different sperm characteristics observed in the extended semen samples before adding

Table 1. *In vitro* sperm characters of Black Bengal buck semen immediately after dilution with Tris extender (n=20)

Parameters	Mean±SEM
Individual motility (%)	92.50±0.57
Sperm viability (%)	95.77±3.69
Functional membrane integrity (%)	82.38±3.30
Acrosomal integrity (%)	90.33±1.55
MDA level (µmol/mL)	1.50±0.25

antioxidants (Control) are presented in Table 1. The extended semen had an average of 92.50±0.57% motile cells, 95.77±3.69 viable cells, 82.38±3.30% functional membrane intact cells, 90.33±1.55% and acrosome intact cells. The level of MDA in pre-equilibration stage was 1.50±0.25 (µmol/mL).

Effect of antioxidants on *in vitro* sperm characters after equilibration: After completion of equilibration period, the mean percentage of 60.83±1.54, 66.67±3.33 and 73.33±2.47% progressively motile cells; 58.50±7.02, 63.33±6.25, 68.01±6.45% viable cells; 42.73±4.53, 55.09±3.21 and 61.17±4.81%functional membrane intact cells; 53.55±4.20, 60.16±3.46, and 67.62±3.22% acrosome intact cells were recorded in the control, LC-1 and LC-2 groups, respectively (Fig. 1). Significantly higher individual motile cells ($p<0.01$), acrosomal intact cells ($p<0.05$) and functional membrane intact cells ($p<0.05$) were recorded in the equilibrated samples of LC-2 group when compared to the control group. The overall values of MDA level are given in Fig. 1. The mean values observed were 1.94±0.08, 1.82±0.12, 1.77±0.10 µmol/mL for control, LC-1 and LC-2 groups respectively. No significant difference in lipid peroxidation level was observed, however LC-2 group had the lowest value (1.77±0.10).

Effect of antioxidants on *in vitro* sperm characters after freeze-thaw: Percentages of progressively motile sperms in cryopreserved samples (Fig. 1) were significantly ($p<0.01$) higher in LC-1 (35.25±1.83%) and LC-2 (38.25±1.32%) groups when compared to the control (25.25±1.64%). Percentages of viable sperm cells for freeze thawed samples were 33.22±1.61, 34.02±1.89, and 37.76±1.80% in control, LC-1 and LC-2 groups, respectively (Fig. 1) and there was no significant difference observed between the groups. Percentage of sperm cells with functional membrane integrity in LC-2 (37.84±1.59) group was significantly higher than the LC-1 (32.76±2.29) and control group (31.69±1.71). The percentage of sperm cells with intact acrosome 45.06±2.27, 48.54±2.32 and 53.80±1.92% for control, LC-1 and LC-2 respectively (Fig. 1). The percentages of sperm bearing intact acrosome were found to be significantly higher ($p<0.01$) in LC-2 group when compared to the control and there was no significant difference observed between LC-1 and LC-2. No significant differences ($p>0.05$) in lipid peroxidation level were observed between control and treatment groups and also between the treatment groups even though LC-2 (1.97±0.11) showed the lowest value numerically followed by LC-1 (2.05±0.10), and control (2.52±0.41 µmol/mL).

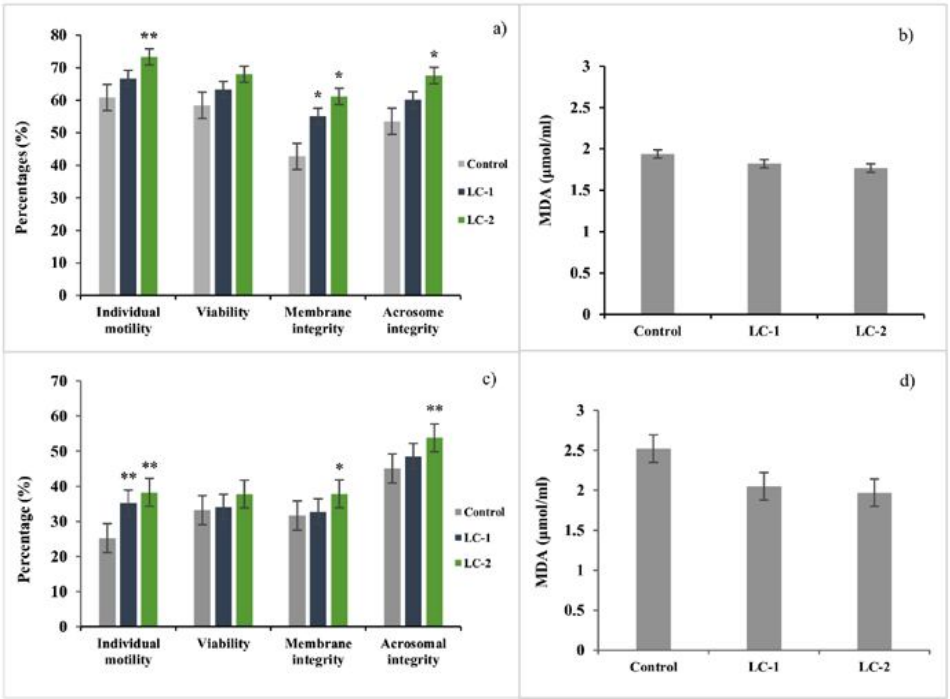

Fig. 1. *In vitro* sperm characters and LPO status in Black Bengal buck semen supplemented with antioxidants after completion of equilibration (a, b) and after freeze thawing (c, d)

DISCUSSION

Neat semen characters: The average volume of the semen ejaculates collected in the present

study was similar with the findings of Atara et al. (2018). Sperm cell concentration, mass activity and individual motility of sperm cells recorded in the present study was higher than the report of Das et al. (2021) in Black Bengal bucks. Ejaculates characters were influenced by the season of the year, frequency of semen collection, preparation of the bucks for semen collection, and nutritional status etc. The extended semen had an average progressive motility of 92.50±0.57%, which is consistent with Atara et al. (2018) for Surti buck, whilst slightly higher than Swarna et al. (2022) for Black Bengal bucks and Susilowati et al. (2021) for Kacang goat. The sperm viability percentage was 95.77±3.69% which is in agreement with Sultana et al. (2013) and Susilowati et al. (2021) but higher than Atara et al. (2018), Heydari et al. (2021).

Effect of antioxidants on in vitro sperm characters after equilibration: In the equilibrated samples supplementation of 2 mM L-carnitine significantly improved individual motility, functional membrane integrity and acrosomal integrity when compared to the control group. Heydari et al. (2021) observed that buck semen supplemented with 5 mM LC outperformed other concentrations (0, 1, 10 mM) and had a higher number of motile, membrane intact and live sperms comparatively at 24 and 48 hours of liquid storage Likewise, supplementation of bull semen with 1 and 2 mM of L-carnitine significantly improved *in vitro* sperm characters evaluated at 72 hours of chilling (Darussalam et al., 2020). Membrane intactness (plasma, acrosome and mitochondrial membrane) when accessed objectively by Computer-assisted semen analysis method was also found to be greater in all LC treated groups ($p<0.001$) of chilled ram (Galarza et al., 2020) and stallion semen (Lisboa et al., 2022). These improvements in function could be due to the promotion of ATP production through β-oxidation and reduction in ATP depletion due to the osmolytic property of L-carnitine (Heydari et al., 2021). Another reason may be due to the antioxidative property of L-carnitine that replaces the fatty acids in the membranes hence protecting the sperm membrane from deleterious ROS attack (Galarza et al., 2020). A recent study proved the cryoprotective and antioxidative properties of L-carnitine which was evident by the betterment in various *in vitro* sperm attributes and protects sperm through interaction with membrane phospholipids and modulates plasma membrane fluidity allowing sperm to resist cryo-damages (Heidari et al., 2022). However, a decreasing trend on sperm kinematics was observed when the concentration of LC was increased in the freeze-thawed buck (10mM) and in rooster sperm (Fattah et al., 2017), hence optimal dosing is critical for each species. Based on the present results of lipid peroxidation status, L-carnitine supplementation did not appear to have a significant effect on reducing LPO although they have numerically lower MDA levels as compared to control. In accordance with present findings, L-carnitine (2.5, 5, 10 mM) did not show any significant effect on MDA elimination and activity of Glutathione and Superoxide dismutase in cryopreserved goat semen (Bucak et al., 2010). Similarly, ram and stallion semen supplemented with different LC concentrations also did not provide any significant protection against lipid peroxidation before freezing, immediately after freezing and even after hours of incubation (Lisboa et al., 2022).

Effect of antioxidants on *in vitro* sperm characters after freeze-thaw: L-carnitine through facilitation of β-oxidation plays an important part in enhancing ATP production, thus providing a better supply of energy to increase sperm motility (Yang et al., 2020). The effects of supplementing 1 and 2 mM L-carnitine on sperm motility in the present study were significantly higher as compared to Control. In freeze thawed Angora goat semen, 2.5 mM LC showed an increase in both subjective motility percentage and CASA progressive motilities, albeit insignificant (Bucak et al., 2010) and a decreasing trend on sperm kinematics was observed when the concentration of LC was increased in the species under study (Bucak et al., 2010) as well as in rooster sperm freeze thaw (Fattah et al., 2017). On the contrary, 5 mM supplemented freeze thawed buck semen was superior to 1 and 10 mM comparatively (Heidari et al., 2022). There was a significant ($p<0.05$) increase in post thaw motility of buffalo semen treated by L-carnitine which also resulted in an improvement in conception rate as compared to control (El-Raey et al., 2016). Significantly higher progressive motilities and motility characteristics (VSL, VCL, LIN, VAP) were also observed in freeze thawed human sperm (Ghorbani et al., 2021). Similarly, in frozen thawed human sperm undergoing IVF/ICSI treatments, TM and PM increased significantly ($p=0.009$) by 26.2% and 42.8%, respectively as compared to control groups (Gonzalez et al., 2022). Though there was no significant difference in the viable count, percentage of sperm cells with functional membrane integrity and acrosomal integrity were significantly higher in LC-2 group than the control in the present study. According to Fattah et al.

(2017), LC protects sperm through interaction with membrane phospholipids and modulates plasma membrane fluidity allowing sperm to resist cryodamages. Higher percentage of sperm with lower apoptotic changes and membrane reorganization was found in 1 mM LC added frozen chicken semen (Partyka et al., 2017). Membrane integrity (plasma and mitochondrial membrane) was found to be significantly higher ($p<0.05$) in the presence of 1 and 2 mM LC in freeze thawed rooster sperm (Fattah et al., 2017). A well-defined intact plasma and acrosomal membrane along with a homogenous mitochondrial content was observed for LC supplemented buffalo semen (un-supplemented sample show swollen and vacuolated membranes) when evaluated by transmission electron microscopy which is attributed to its primary and secondary antioxidant activity i.e., lowering the synthesis of free radicals and strengthening intrinsic ROS scavengers respectively (El-Raey et al., 2016). L-carnitine treatment did not appear to have significant effect on reducing lipid peroxidation although 2 mM L-carnitine (1.97±0.11 μmol/mL) showed the lowest MDA level numerically as compared to Control and LC-1. In accord with this, ram semen supplemented with different LC concentrations did not provide any significant protection against lipid peroxidation before freezing, immediately after freezing and even after hours of incubation (de Souza et al., 2019). In contrary to this, 7.5 mM (Altyeb et al., 2022) and 5 mM (Heidari et al., 2022) LC were best in reducing LPO in post thaw buck semen as compared to their lower counterparts. One and 2mM decreased LPO significantly in freeze thawed rooster sperm while higher concentrations result in higher level of MDA when compare to control (Fattah et al., 2017). LC has shuttle capacity for fatty acid transport, excess of which can act as oxidative substrate hence optimal dosing is necessary for different species (Heidari et al., 2022). Further, in the current study LPO was measured by estimating MDA which is an indirect method, however direct measurement of ROS level might give an accurate picture of the LPO status.

The beneficial effects of supplementing membrane targeted antioxidant L-Carnitine in the Black Bengal buck semen cryopreservation program could be noticed from the improvement in different sperm traits observed in the present study such as sperm motility, functional membrane and acrosome integrity. It can be concluded that supplementation mitochondria targeted antioxidant L- Carnitine @ 2 mM could improve the post-thaw sperm recovery of Black Bengal buck semen and future studies may focus on investigating the antioxidant effects of L- Carnitine through direct measurement of ROS level and *in vivo* pregnancy studies to get an explicit understanding of their beneficial effects on cryopreservation of Black Bengal buck semen.

Conflict of interest: The authors declare that there are no conflicts of interest in the present study.

Author's contribution: MK: Conceptualized and designed the study; RL, DS, SS, SD: Carried out different parameters of the work; AM, MM: Helped in preparing the manuscript, tables and graphs; MK, RM: Drafted the manuscript; CB, MM: Reviewed the manuscript critically and given important suggestions. All the authors approved the final manuscript.

Data availability statement: The authors confirm that all data necessary for confirming the conclusions of the article are present within the article and tables. Data will be provided on specific request.

Ethical approval: This experiment was a part of the research project "Screening the role of seminal proteins and antioxidants on cryopreservation of buck semen" (Project No. A 61) approved by IRC, ICAR-NDRI, Karnal. The manuscript does not contain clinical studies or patient data.

Use of artificial intelligence tools: During the preparation of this work, the author(s) have not used artificial intelligence tools/services and authors take full responsibility for the content of the publication.

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