RESEARCH ARTICLE

Effect of Carnitine on Physico-Morphological Properties of Cryopreserved Hariana Bull Spermatozoa

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Abstract

The goal of current investigation was to ascertain the impact of carnitine as an additive in tris-egg yolk-based extender on Hariana bull semen (32 ejaculates) chosen for cryopreservation. Progressive motility, viability, HOS response, and acrosomal integrity were estimated after equilibration (pre-freeze) and after thawing. The activity of enzymes malondialdehyde (MDA), glutathione-S-transferase (GST), and superoxide dismutase (SOD) were quantified in post-thaw seminal plasma. Semen ejaculates from Hariana bulls (n=4) were diluted in tris egg yolk glycerol (TEYG) and were divided into four equal groups. Aliquot/Group I was processed without carnitine and served as control, while Groups II, III and IV received carnitine at concentration of 2.5 mM, 5 mM and 10 mM per 80×10^6 spermatozoa, respectively. Carnitine at 5 mM concentration significantly ($p<0.05$) enhanced sperm viability, acrosomal integrity, and intact plasma membranes both at pre-freeze and post-thaw stage. The addition of 5 mM and 2.5 mM carnitine reduced oxidative stress considerably ($p<0.05$). The results of this investigation revealed that carnitine has cryoprotective properties on bull spermatozoa via reducing oxidative stress and free radicals. Hence to improve post-thaw sperm functional dynamics, it was suggested that 5 mM of carnitine be supplemented as an additive to semen extender.

Key words: Bull semen, Carnitine, Cryopreservation, Hariana, Oxidative stress.

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Introduction

The foundation of the rural Indian economy is animal husbandry. One of the most effective and widely used methods for increasing and maintaining male fertility is sperm cryopreservation. One of the main factors for low-quality sperm has been found to be oxidative stress, which includes lipid peroxidation (LPO). Sperm motility and viability have been connected to reduced glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) as a protective mechanism against lipid peroxidation of sperm. ROS-induced damage may be diminished using antioxidant compounds (Donnelly *et al.,* 2000). Effective antioxidant systems or materials can shield sperm from harm and dysfunction during handling and increase their viability *in vitro* for artificial insemination (Sarica *et al.,* 2007). There are two isomer forms of the quaternary ammonium chemical carnitine: D-carnitine, which is inert, and L-carnitine, which is active. Water-soluble and naturally occurring, L-carnitine (3-hydroxy-4-N-trimethyl amino butyrate, C7H15NO3, M.W. 161.2) is an amino acid that resembles a vitamin. In the liver, it is mostly made from the amino acids methionine and lysine. In order to move fatty acids from the cytosol to the mitochondria during the breakdown of lipids (fats) to produce metabolic energy, L-carnitine is necessary. As an antioxidant, carnitine counteracts free radicals, particularly superoxide anion, and shields cells from oxidative damage-induced apoptosis (Ye *et al.,* 2010). In *in vitro* and animal studies, L-carnitine has been used as a free radical scavenger to protect antioxidant enzymes from oxidative damage (Sener *et al.,* 2004; Gulcin, 2006; Kolodziejczyk *et al.,* 2011). Given these details, the goal of current investigation was to determine how carnitine affects the physico-morphological characteristics of cryopreserved Hariana bull spermatozoa.

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Materials and Methods

The semen ejaculates of four Hariana bulls, 5 to 9 years old, weighing between 450 and 600 kg were included in this Study. The bulls were raised at the Semen Biology Lab of the Department of Gynaecology and Obstetrics at ILFC, DUVASU, Mathura (UP), India.

Semen ejaculates were collected weekly twice from each bull by using artificial vagina in the early morning. Following collection, the tube containing semen was soon put into a water bath maintained at 37°C in the lab. For the present investigation of pre-freeze and post-thaw evaluation, thirty-two ejaculates (n=32, 8 per bull) with acceptable semen quality were used. Each ejaculate, following routine evaluation, was extended with tris egg yolk glycerol (TEYG) extender at room temperature keeping 80 million sperm per mL, and divided into four equal aliquots in separate test tubes (Groups I to IV), and L carnitine was incorporated in such a way that final concentrations of 0.0, 2.5, 5.0, and 10 mM, respectively, were achieved in these tubes.

For this study, a 142.72 mM L carnitine stock solution was made by dissolving 28.21 mg of L carnitine in 1 mL of Millipore water. Four separate test tubes, each holding 5 mL of the extended semen, were added with 0.000, 0.143, 0.285, and 0.571 mL of L carnitine stock solution (142.72 mM), and the extended semen was added further to increase the total volume to 10 mL each, resulting in an effective final concentration of 0.0, 2.5, 5, and 10 mM of carnitine, respectively. After being diluted, these samples were utilised for filling straws. The French mini straws of capacity 0.25 mL were meticulously filled and sealed using filling and sealing machine. The straws were then equilibrated at 4°C for 4 h. Further the straws were evaluated for pre-freeze physico-morphological parameters. After thorough examination the straws were frozen in the presence of LN_2 vapour using a programmable biological freezer in controlled manner. The semen samples that had been vapour frozen were thereafter placed in LN_2 container and subjected to thawing for 45 seconds at 37°C following a 24-h of storage.

The pre-freeze (after equilibration) and post-thawed samples were evaluated for various sperm quality parameters and post-thawed oxidative enzyme profile using standard procedures. The progressive sperm motility and live sperm percentage were evaluated according to Salisbury *et al*. (1985). The functional integrity of the sperm tail membrane (HOST) was evaluated by performing the hypo-osmotic swelling test in accordance with the protocol outlined by Jayendran *et al.* (1984). Acrosomal integrity was assessed by Giemsa staining as per Watson (1975).

For oxidative status in the post-thawed seminal plasma, the MDA assay was performed by the TBARS (Thio-barbituric acid reactive substance) method of Ohkawa *et al.* (1979). Glutathione S-transferase was computed using the methodology outlined by Habig *et al.* (1974) with minor modifications, and superoxide dismutase (SOD) activity was determined with slight adjustments in the procedure outlined by Madesh and Balasubramanian (1997).

SPSS Software (version 20) was used to analyze the data adopting one-way ANOVA and Duncan's post hoc test. The differences in mean values were considered significant at $p<0.05$.

Results and Discussion

There were significant differences ($p<0.01$) in the percentages of progressively motile sperm, live spermatozoa, spermatozoa with intact acrosome, and HOST reactive spermatozoa between four groups at both the pre-freeze and post-thaw stages. The results were the best in group III (5.0 mM carnitine), followed by group II (2.5 mM carnitine), and group I (control, no cartinine) compared to group IV (10 mM carnitine), the higher level of carnitine being found toxic to sperm activity (Table 1). These findings agreed with reports of Badr *et al.* (2010) and Darussalam *et al*. (2020), as L-carnitine may have a positive impact on sperm cell quality because of its important function in enhancing energy metabolism. Sperm naturally

Table 1: Effect of L carnitine on sperm progressive motility**,** viability, acrosome integrity and HOST on pre-freeze and post-thaw stages of cryopreservation of bovine semen (Mean ± SEM, n=32)

Sperm quality parameters	Freezing stages	Groups and L Cartinine concentration in TFYG extender			
		Group I (0 mM carnitine)	**Group II** (2.5 mM carnitine)	**Group III** (5 mM carnitine)	**Group IV** (10 mM carnitine)
Progressive motility (%)	Pre-freeze	53.30^{a}±1.03	63.52^{b}±1.46	71.16^{c}±1.50	52.28^{a}±1.37
	Post-thaw	42.62^{q}±1.05	47.90^{r}±1.16	50.35^{r}±1.23	38.18^{p}±1.12
Sperm viability (%)	Pre-freeze	71.56^{b} ±0.58	76.47^{c}±0.75	81.11^{d} ±0.69	56.83^{a}±0.75
	Post-thaw	61.77^{q}±0.72	68.17^{r}±0.85	72.12^{s}±0.74	47.31^{p}±0.69
Intact acrosome (%)	Pre-freeze	66.36^{b}±0.69	73.38^{c}±0.88	76.91^{d}±0.70	54.27^{a}±0.76
	Post-thaw	54.96^{q}±0.75	64.15^{r}±0.88	68.54^{s}±0.89	45.81^{p}±0.78
HOS response (%)	Pre-freeze	59.05^{b}±0.88	69.21^{c}±0.69	72.51^{d}±0.61	49.54^{a}±0.91
	Post-thaw	50.15^{q}±0.88	60.87^{r}±0.76	65.19^{s}±0.64	40.20^{p}±0.88

Means bearing different superscripts in a row differ significantly ($p<0.05$).

Table 2: Effect of L carnitine on oxidative enzymes of Hariana bull spermatozoa at post-thaw stage (Mean ± SE, n=32)

Oxidative enzymes	Groups and L Cartinine concentration in TFYG extender			
	Group I (0 mM carnitine)	**Group II** (2.5 mM carnitine)	**Group III** (5 mM carnitine)	**Group IV** (10 mM carnitine)
MDA (nM/µL)	0.164^{b}±0.009	0.133^{a}±0.007	0.128^{a}±0.008	0.195^{c}±0.008
GST (nM/min/mL)	37.75^{a}±2.85	48.95^{b}±2.90	58.31^{c}±2.80	57.79^{c}±3.33
SOD (U/mL)	476.05^{b}±5.48	510.71^{c}±5.50	540.34^{d}±5.29	422.62^{a}±8.26

Means bearing different superscripts in a row differ significantly (p<0.05).

create trace levels of reactive oxygen species (ROS), which are essential for acrosomal response and capacitation. Due to the high concentration of polyunsaturated fatty acids in the cytoplasm and plasma membrane of mammalian sperm cells, these cells are susceptible to excessive levels of reactive oxygen species (ROS) resulting from the presence of leukocytes and dead or aberrant sperm cells (Darussalam *et al.,* 2020).

At post-thaw stage, the activities of seminal plasma enzymes malondialdehyde (MDA), glutathione-S-transferase (GST), and superoxide dismutase (SOD) were quantified (Table 2). Malondialdehyde (MDA) activity was observed to be significantly (p<0.01) lower in groups II and III than in groups I and IV. When compared to groups II, III, and IV, glutathione-S-Transferase (GST) activity of group I was found to be significantly (p<0.01) lower. Superoxide dismutase (SOD) activity in group III was significantly (p<0.01) higher than in the control and other treatment groups. These findings agreed with Bucak *et al.* (2010). L-carnitine's antioxidant properties and antiapoptotic actions help to prevent the detrimental effects of ROS and may also help to stabilise DNA structure and the mitochondrial membrane. The activities and levels of antioxidant enzymes like glutathione peroxidase and superoxide dismutase were found higher when semen was supplemented with L-carnitine (Neuman *et al.,* 2002). Because of the higher antioxidant levels, there are fewer free radicals available for lipid peroxidation, which protects the spermatozoon membranes and increases the vitality of sperm cells (Badr *et al.,* 2010). supplementation with L-carnitine may promote the transport of fatty acids into mitochondria for energy production, leading to an increase in antioxidant enzymes (Dayanandan *et al.,* 2001).

Conclusion

Addition of carnitine at 5 mM concentration to tris egg yolk-based extender improved the cryopreservation of Hariana bull spermatozoa in terms of motility, viability, intact acrosome, and intact plasma membrane, and reduced oxidative stress in post-thawed sperm compared to concentration taken in other groups. Carnitine at higher dose (≥10 mM) caused deleterious effect on bovine spermatozoa.

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