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L-Carnitine Improves Cytoprotection during Cryopreservation: A case study on Nili-Ravi Buffalo Sperm

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Abstract

The current study was aimed to evaluate the antioxidative effect of L-Carnitine at post thawing following cryopreservation of Nili-Ravi buffalo sperm. For the purpose, semen from three buffalo bulls were collected for 3 weeks using artificial vagina (N=18; replicates). The qualified ejaculates were diluted employing *tris*-citric acid extender i.e., control did not receive any L-Carnitine and experimental groups having 0.5, 1.0, and 1.5 ng/mL of L-carnitine at 37°C with approximately 50×10^6 sperm/mL. The semen was cooled at 4°C and then equilibrated (4 hours), filled in straws (0.5 mL) at 4°C, placed on LN_2 vapours for 10 min and kept into an LN_2 container. The thawed semen was evaluated for post-thaw quality. The integrity of the sperm plasma membrane and motility ($P<0.05$) was highest in the extenders having 1.0 ng/mL of L-carnitine as compared to the control (received no L-Carnitine). However, sperm chromatin integrity and viability (live sperm with intact acrosome) remained similar. It was concluded that supplementing 1.0 ng/mL L-Carnitine of extender can improve the post-thaw quality of cryopreserved sperm. Based on the results of the current experiments it is recommended to include L-carnitine extender to improve post-thaw quality of buffalo sperm in terms of its motility and integrity of its plasma membrane.

Key words: Buffalo, Sperm, Cryopreservation, Extender, L-Carnitine, Artificial insemination

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1. INTRODUCTION

Semen is naturally equipped with antioxidants that protect it against osmotic and thermal shocks mediated by reactive oxygen species (ROS) during cryopreservation[1]. The semen antioxidant system comprising of enzymatic and non-enzymatic antioxidants[2]. However, these indigenous antioxidants are insufficient to

protect cryopreserved semen because during cryopreservation activity of catalase and superoxide dismutase reduced to 50%, whereas, glutathione activity is reduced to 78% [1]. Along with these lower antioxidant level during the freeze-thaw cycle of cryopreservation, ROS production leads to induction of oxidative stress in the seminal plasma and extender, which consequently leads to lipid peroxidation and ultimately results in deterioration or reduction of semen quality, elevated permeability, and leakage of intracellular enzymes, lowered penetration capacity[2]. So, exogenous supplementation of suitable antioxidant in extender is required to prevent the oxidative stress to sperm and to enhance the quality of cryopreserved buffalo semen.

Beta-hydroxy-gamma-N-trimethylaminobutyric acid, namely L-carnitine (LC), naturally vitamin-like amino-acid, is synthesized from methionine and lysine[3]. It deals with the energy (ATP) production in cells via beta-oxidation (transfer of long chain fatty acids to mitochondria) [4]. LC protects sperm membranes due to its antioxidant properties. It reduces oxidative stress by interfering the integration of arachidonic acid into protein kinase C and phospholipids mediated NADPH oxidase system[5]. Previously, dietary supplementation of LC was reported to improve sperm motility and concentration of boar[6], buffalo[7], and human sperm[8]. However, information on the use of LC in extender for Nili-Ravi buffalo semen is not available. Therefore, this study was designed that aims to appraise the impact of L-carnitine on the post-thaw quality of cryopreserved semen.

2. MATERIALS AND METHODS

2.1 Extender preparation

Four extenders were made by use of *tris* citric acid as a buffer (osmotic pressure 320 mOsmol/Kg; pH 7.0) which is composed of citric acid (1.56 g) and *tris*–(hydroxymethyl) aminomethane (3.0g) dissolved in sterile water (74 mL). Egg yolk 20% v/v, glycerol 7%, fructose 0.2% w/v, streptomycin sulfate (1000 ug/mL), antibiotics, and benzylpenicillin (1000 IU/mL) were added to each extender. Four different extenders were prepared by adding LC at 0, 0.5, 1.0, and 1.5 ng/mL. The extender without LC was in control.

2.2 Collection of semen and cryopreservation

The two consecutive semen ejaculates collected from 3 bulls per week by artificial vagina for 3 weeks (N=18). Semen was quickly transported to the laboratory for assessment. Qualified ejaculates (>60% motility, >0.5×109 sperm/mL concentration, >0.5 mL volume) were divided into five parts. Semen was diluted with one of the five extenders at 50×10^6 sperm/mL approximately at 37°C. Semen was kept for 2 h at 4°C, equilibrated at 4°C for 4h and was then filled in 0.5mL French straws via suction pump (4°C) and straws were exposed to LN_2 vapors for 10 min. Semen straws were kept in an LN_2 container at -196°C. For post-thaw sperm functional assays, the semen was thawed in water bath (at 37°C for 30 sec) after 24 hours.

2.3 Post-thaw sperm functional assay

2.3.1 Sperm motility

A drop (5 µL) of semen was put on a slide (37°C). The slide was concealed with a coverslip and then appraised via phase contrast microscope (37°C; ×400)[9].

2.3.2 Sperm plasma membrane integrity

Sperm plasma membrane integrity (PMI) was determined by HOS (hypo-osmotic swelling assay) as practiced by Qadeer et al.[9]. HOS solution was prepared by adding fructose (1.35 g) and sodium citrate (0.73 g) in distilled water (100 mL of distilled water; ~190 mOsmol/Kg osmotic pressure). A total of 50 µL semen was mixed in 500 µL HOS solution and kept at 3°C for 30 to 40 minutes. Semen sample (10 µL) was put on a slide and assessed using a phase-contrast microscope (×400). Sperms with swollen tails have functional and intact membranes while sperms with unswollen tails have non-functional sperm membranes[10].

2.3.3 Sperm viability

Sperm viability (live sperm with intact acrosome) was determined by the dual staining process[11]. An equal volume of semen and trypan blue (Eschwege, Germany) was put on the slide. The solution was mixed and dried. Samples were placed in formaldehyde neutral red (5 minutes) and washed in sterile water. Samples

were placed for 4 h in 7.5% Giemsa solution (Sigma). Samples were placed in distilled water and dried at 37˚C. The samples were mounted by Canada balsam. Sperm with light blue or transparent color were live while sperm with dark blue color were dead. Light blue or transparent sperm with apparent acrosome end were viable (live sperm with intact acrosome) while sperm with blue colored discrimination and acrosome with blur ends were nonviable (dead sperm having disrupted acrosome). The sperm were assessed in each sample under a phase-contrast microscope (×1000) individually for sperm viability (live sperm with intact acrosome) and liveability.

2.3.4 Sperm chromatin integrity

Chromatin integrity of sperm was evaluated as practiced by Ejaz et al.[12]. Semen smears were prepared and air-dried. Semen samples were placed in ethanol acetone (96%; 1:1) for 30 min at 4°C and in HCl solution (4 N) for 10-30 min (25°C). Samples were placed in water thrice (2 minutes) in each and placed in toluidine blue solution for 10 min. Semen was dried and fixed via Canada Balsam (Sigma-Aldrich), and observed under a light microscope (×1000). Sperms with dark blue or purple color were having damaged chromatin and sperm with light blue color were having intact chromatin.

2.4 Data analysis

Results of the current study are shown as means ± SE. The effect of varying level of L-carnitine on post-thaw quality of buffalo semen was analyzed using analysis of variance (MSTAT-C; Ver. 1.42) In case of a significant F ratio (P<0.05), the least significant difference for comparison (means of treatment) was employed.

3. RESULTS

Figures 1 to 4 shows the effects of varying levels of L-Carnitine on post-thaw sperm quality. The integrity of plasma membrane of the sperm and progressive motility improved significantly (P<0.05) in extender having 1.0 ng/mL of LC compared to control. However, sperm chromatin integrity and viability (live sperm with intact acrosome) remained the same (P>0.05) in all extenders having 0.5 ng/mL, 1 ng/mL, and 1.5 ng/mL LC and control.

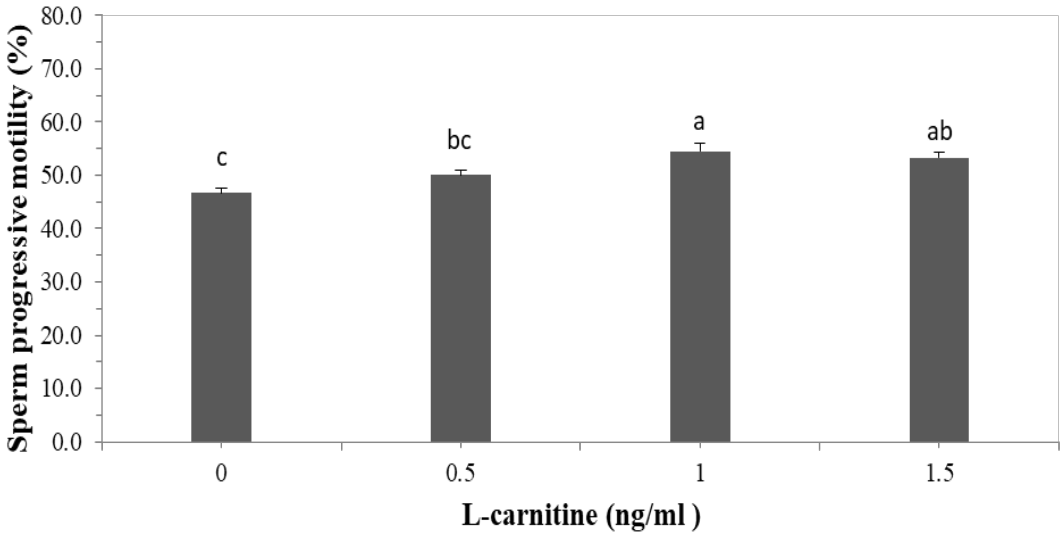

Fig. 1. Effect of L-carnitine addition in extender on sperm progressive motility of cryopreserved buffalo sperm (N=18)

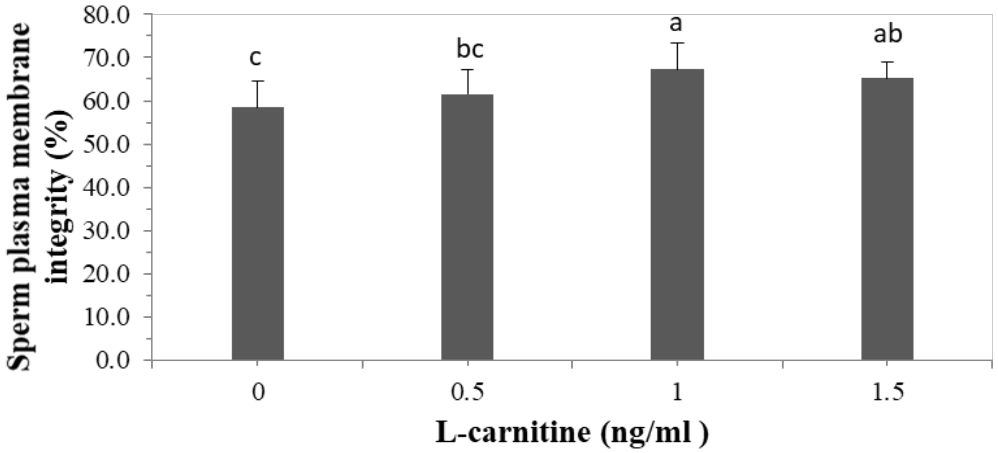

Fig. 2. Effect of L-carnitine addition in extender on sperm plasma membrane integrity of buffalo sperm

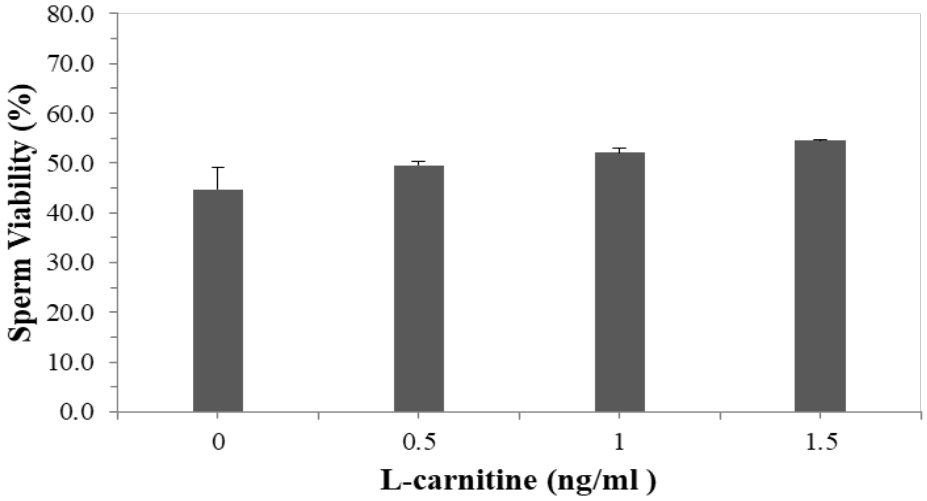

Fig. 3. Effect of L-carnitine addition in extender on viability (live sperm with intact acrosome) of cryopreserved buffalo sperm (*P*>0.05)

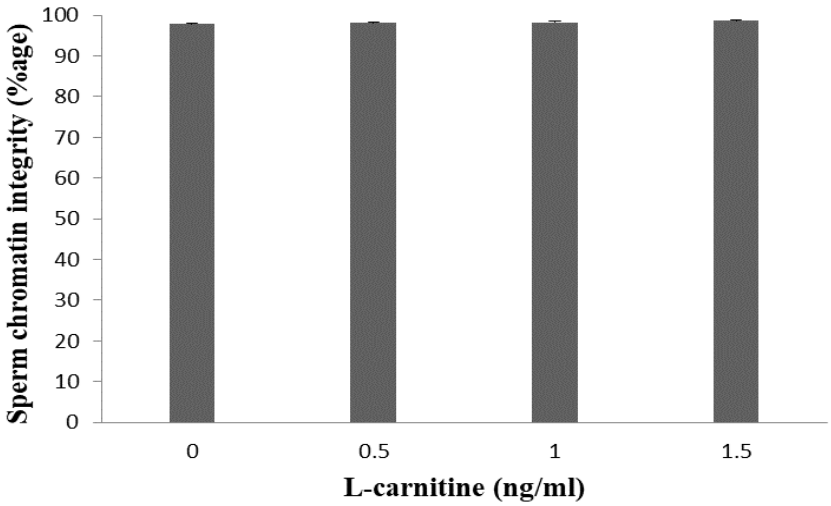

Fig. 4. Effect of L-carnitine addition in extender on chromatin integrity of cryopreserved buffalo sperm (*P*>0.05)

4. DISCUSSIONS

Seminal plasma and sperm have antioxidant properties to control intracellular redox state especially during stress conditions like cold shock during cryopreservation. Sperm viability and fertilizing ability are affected during cryo-damages. The oxidative stress because of ROS production is the main cause of adverse changes occurring during cryopreservation. During cryopreservation, the dilution of seminal plasma, which is enriched with enzymatic (catalase, peroxidase, superoxide-dismutase, etc.) and non-enzymatic (glutathione) antioxidants, causes a reduction of antioxidants. LC is a powerful antioxidant. It is a crucial cofactor reducing the lipids' availability for peroxidation via transporting fatty acid into the mitochondria to generate ATP through β-oxidation; an important source of fuel that aid sperm motility[8]. Moreover, LC increases the level and activities of various antioxidant enzymes including superoxide dismutase and glutathione peroxidase, among others, on account of its antioxidant potential[13]. LC also prevents protein oxidation, protects sperm DNA, and inhibits lactate oxidative damage[14].

In the present study, sperm motility was increased by supplementing the extender with LC up to 1 ng/mL in a dose-dependent manner after which it decreased with the increase in the concentration of LC. These findings are in line with previous studies[7, 15-17] that suggested LC can improve sperm motility and viability. LC potentiating role on buffalo sperm cell motility might be attributed to its critical role in promoting the cellular energetics 16 by the transportation of long-chain fatty acid into the mitochondria for consumption in metabolism by β-oxidation[8], thereby improving flagella movement. The improvement in plasmalemma integrity at 1 ng/mL of LC might be due to the protective role of LC against ROS by exerting antioxidant properties. The decrease in sperm motility by increasing concentration of LC may be due to exhaustion of sperm as LC is a bio-energetic and might have been due to adenyl cyclase-activating polypeptide that decreased the cAMP concentration which reduced Ca^{+2} influx and ultimately the sperm motility[18]. The improvement in plasmalemma integrity at 1 ng/mL of LC might be due to the protective role of LC against ROS by exerting antioxidant properties. PUFAs (polyunsaturated fatty acids) are the basic constituent of mammalian sperm cells including buffalo sperm. PUFAs make the major skeleton of sperms' membranes and contribute in their integrity, metabolism, and make them capable of penetrating and fertilizing oocytes via different physico-chemical alterations[19] that are vulnerable to peroxidative damage, which shows the main effect stimulated by ROS after cryopreservation. LC integration in feed elevates the level of antioxidant enzymes and their activities such as superoxide dismutase and glutathione peroxidase because of its antioxidant properties[19], which in turn protect the plasma membrane from peroxidative damage. In the present study, sperm viability and DNA integrity remained the same in an extender containing LC and control. In contrary to our findings, Longobardi et al.[7], Jeulin and Lewin[15], Lenzi et al.[16], and El-Raey et al.[17] reported that LC can improve sperm viability. This difference can be attributed to species-specific resistance to cryoinjuries and the requirement of a particular concentration of LC to compensate ROS mediated loss of sperm viability.

5. CONCLUSIONS

This study concluded that the addition of 1.0 ng/mL of extender improved the post-thaw quality of bull sperm in terms of their plasma membrane integrity and motility during cryopreservation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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