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Carnitine supplementation decreases capacitation-like changes of frozen-thawed buffalo spermatozoa

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ABSTRACT

The aim of this study was to evaluate the effect of carnitine supplementation of semen extender on fertility parameters of frozen-thawed buffalo sperm. Buffalo semen was cryopreserved in BioXcell containing 0 (control group), 2.5 and 7.5-mM carnitine. After thawing, viability, motility, membrane integrity and capacitation status (assessed by localization of phosphotyrosine-containing proteins and chlortetracycline, chlortetracycline assay) were evaluated. Furthermore, total antioxidant capacity, reactive oxygen species (ROS) and lipid peroxidation levels, as well as adenosine triphosphate (ATP) content and phospholipids concentration were assessed. Finally, *in vitro*–fertilizing ability was evaluated after heterologous IVF. An increased post-thawing sperm motility and membrane integrity were recorded in both treated groups compared with the control (44.4 ± 3.5, 53.1 ± 3.9, and $52.5 \pm 3.6\%$; $P < 0.05$ and 48.44 ± 0.69, 55.19 ± 0.54, $59.63 \pm 0.30\%$; $P < 0.01$ with 0, 2.5-mM, and 7.5-mM carnitine, respectively). Supplementation of carnitine to the freezing extender decreased ($P < 0.01$) the percentage of sperm displaying fluorescence at both equatorial and anterior acrosomal regions (pattern EA), corresponding to high capacitation level, compared with the control (30.3 ± 3.8, 18.8 ± 2.8, and $7.2 \pm 2.9\%$, respectively, with 0, 2.5-mM, and 7.5-mM carnitine). In agreement with this, carnitine also decreased ($P < 0.01$) the percentage of sperm displaying chlortetracycline pattern B (capacitated sperm) (63.8 ± 1.8, 46.8 ± 2.2, and $37.2 \pm 1.8\%$, respectively with 0, 2.5-, and 7.5-mM carnitine). Interestingly, carnitine increased total antioxidant capacity and ATP content of buffalo frozen-thawed sperm (1.32 ± 0.02, 1.34 ± 0.01, 1.37 ± 0.01 mM/L and 4.1 ± 0.1, 5.3 ± 0.1 and 8.2 ± 0.4 nM $\times 10^8$ sperm; $P < 0.01$, respectively, with 0, 2.5- and 7.5-mM carnitine). Intracellular ROS decreased in carnitine-treated sperm compared with the control, as indicated by dihydroethidium (DHE) values (0.22 ± 0.01, 0.18 ± 0.01, and 0.14 ± 0.0 μM/100 μL dihydroethidium, respectively, with 0, 2.5-, and 7.5-mM carnitine; $P < 0.01$), whereas lipid peroxidation levels (on average 30.5 ± 0.3 nmol/mL MDA) and phospholipids concentration (on average 0.14 ± 0.00 μg/120×10^6 sperm) were unaffected. Despite the improved sperm quality, the percentage of normospermic penetration after IVF was not influenced (on average 53.5 ± 1.8). In conclusion, enrichment of extender with carnitine improved buffalo sperm quality by increasing ATP generation and modulating ROS production, without affecting *in vitro* fertilizing ability.

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1. Introduction

Breeding of water buffalo (*Bubalus bubalis*) has been steadily increasing worldwide over the years, as this species plays a critical role as a protein producer in tropical countries [1]. The utilization of advanced reproductive technologies is hence fundamental to increase genetic improvement and grading up of native nonproductive populations bred in these countries. For a wide application of both artificial insemination and *in vitro* embryo production, semen cryopreservation plays a critical role [2]. However, buffalo spermatozoa are more susceptible to hazards during freezing and thawing than cattle spermatozoa, thus resulting in lower fertilizing potential [3,4]. Freezing–thawing of buffalo spermatozoa causes considerable damage to motility apparatus, plasma membrane, and acrosomal cap [5], as well as leakage of intracellular enzymes [6]. Furthermore, Elkhawagah et al. [7] recently reported that a very high incidence of capacitation-like changes was induced by cryopreservation in buffalo sperm Moreover, the high concentration of long chain polyunsaturated fatty acids in buffalo sperm membrane [8] makes them very susceptible to peroxidation damages. The lipid composition of the sperm membrane is in fact, a major determinant of the cold sensitivity, motility, and overall viability of spermatozoa [9]. Similar to capacitated spermatozoa, cryopreserved sperm display some alterations of lipid membrane, such as higher membrane fluidity, partial phospholipid scrambling [10] and loss of polyunsaturated fatty acids and cholesterol [11,12].

There is evidence that cryocapacitation is at least in part induced by increased generation of reactive oxygen species (ROS) during sperm processing [2]. Antioxidants in the ejaculate protect spermatozoa from free radicals produced by leukocytes, prevent DNA fragmentation, improve semen quality, reduce cryodamage to spermatozoa, block premature sperm maturation, and provide an overall stimulation to the sperm cells [13]. In buffalo, the semen extender has been supplemented with antioxidants such as cysteine and glutamine [14], as well as sericin [15], to decrease intracellular ROS and increase motility and membrane integrity of frozen-thawed spermatozoa. Moreover, taurine or trehalose supplementation improved buffalo frozen-thawed sperm quality, reducing capacitation-like changes [16].

Carnitine is a quaternary ammonium compound biosynthesized in the kidneys and liver from lysine and methionine [17]. It is a powerful antioxidant [18] able to reduce the availability of lipids for peroxidation by transporting fatty acids into the mitochondria for β-oxidation to generate ATP energy [19,20]. Moreover, it is also known to fulfill important roles in mammalian sperm maturation and metabolism because epididymal cells and spermatozoa derive energy from carnitine that is present in epididymal fluid [21]. It has been suggested that the high concentrations of carnitine in the epididymal fluid serve to stabilize the sperm plasma membrane [22], guarantee functional metabolic pathways, and increase the motility [23]. In humans, rams, and stallions, seminal carnitine is indeed correlated with sperm concentration and progressive motility [24–26]. Cattle supplementation of semen extender with carnitine improves sperm motility and DNA integrity, while reducing anomalies [27]. It is known that the cryopreservation processes, as well as the cryoprotectants used, decrease the intracellular concentration of carnitine in spermatozoa [28–30]. We hypothesized that the enrichment of semen extender with carnitine before cryopreservation, stabilizing the sperm membrane, and reducing lipids availability for peroxidation would improve the quality of buffalo sperm, by reducing capacitation-like changes. Therefore, this work was undertaken to evaluate the effects of carnitine supplementation of buffalo semen extender on postthawing sperm motility, viability, membrane integrity, and capacitation status. Furthermore, total antioxidant capacity (TAC), ROS, and lipid peroxidation (LPO) levels, as well as (adenosine triphosphate) ATP content, phospholipids concentration and *in vitro* fertilizing ability were also investigated.

2. Materials and methods

Unless otherwise stated, reagents were purchased from Sigma–Aldrich (Milan, Italy).

2.1. Experimental design

The study was carried out after approval of Animal Ethics Committee of the Institute. Four healthy Italian Mediterranean buffalo (*Bubalus bubalis*) bulls (4–6 years age) maintained at an authorized National Semen Collection Center (Centro Tori Chiacchierini, Civitella D'Arna, Italy) under uniform management conditions, routinely used for semen collection twice per week (to ensure homogeneous sperm quality), were selected for the trial. Eight ejaculates per bull ($n = 32$) were collected once per week by artificial vagina (IMV, L'Aigle Cedex, France). On fresh semen motility was evaluated by phase contrast microscopy, viability by Trypan Blue-Giemsa staining whereas the capacitation status was assessed by an indirect immunofluorescence assay to localize phosphotyrosine-containing protein and by chlortetracycline, CTC assay. Only ejaculates containing greater than 80% motile spermatozoa were used in the study. After the initial semen assessment, each ejaculate was split in three aliquots that were diluted at 37 °C with BioXcell (IMV-technologies, France), containing 0 (control group), 2.5-, and 7.5-mM carnitine (Sigma, Cat no: C9500) to a final concentration of 30×10^6 spermatozoa per mL. The aliquots were frozen according to standard procedures. After thawing at 37 °C for 40 seconds in a water bath sperm motility, viability, membrane integrity and capacitation status were assessed. Furthermore, TAC, ROS, and LPO levels, as well as ATP content and phospholipid concentrations were evaluated as previously described. Moreover, sperm *in vitro* fertilizing capability was assessed by evaluating cleavage, penetration, and polyspermy rates after heterologous IVF.

2.2. Sperm motility

Sperm motility was examined by phase-contrast microscopy (Nikon Diaphot 300 inverted microscope equipped with phase contrast and fluorescence filters) at × 40 magnification on a clean and dry glass slide overlaid

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