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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, CTC assay) were evaluated. Furthermore, total antioxidant capacity (TAC), reactive oxygen species (ROS) and lipid peroxidation (LPO) 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 to the control (44.4 ± 3.5, 53.1 ± 3.9 and 52.5 ± 3.6 %; P<0.05 and 48.44 ± 0.69, 55.19 ± 0.54, 59.63 ± 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 pattern EA sperm, corresponding to high capacitation level, compared to the control (30.3 ± 3.8, 18.8 ± 2.8 and 7.2 ± 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 CTC pattern B (capacitated sperm) (63.8 ± 1.8, 46.8 ± 2.2 and 37.2 ± 1.8 %, respectively with 0, 2.5 and 7.5 mM carnitine). Interestingly, carnitine increased TAC 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 x 10^8 sperm; P< 0.01 respectively with 0, 2.5 and 7.5 mM carnitine). Intracellular ROS decreased in carnitine treated sperm compared to the control, as indicated by Dihydroethidium (DHE) values (0.22 ± 0.01, 0.18 ± 0.01 and 0.14 ±0.0 µM/100 µL DHE respectively with 0, 2.5 and 7.5 mM carnitine; P<0.01), whereas LPO levels (on average 30.5 ±0.3 nmol/mL MDA) and phospholipids concentration (on average 0.14 ± 0.00 µg/120 x 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.

Keywords

Carnitine, buffalo sperm, capacitation-like changes , oxidative stress fertilizing ability, ATP content

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 non productive 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 motility [23]. In humans, rams and stallions, seminal carnitine is indeed correlated with sperm concentration and progressive motility [24; 25; 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; 29; 30]. We hypothesized that the enrichment of semen extender with carnitine prior to cryopreservation, stabilizing the sperm membrane and reducing lipids availability for peroxidation, would improve 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 post-thawing sperm motility, viability, membrane integrity and capacitation status. Furthermore, total antioxidant capacity (TAC), reactive oxygen species (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 weekby artificial vagina (IMV, L'Aigle Cedex, France). On fresh semen motility was evaluated by phase contrast microscopy, viability by Trypan Blue-Giemsa staining while the capacitation status was assessed by an indirect immunofluorescence assay to localize phosphotyrosine-containing protein and by chlortetracycline, CTC assay. Only ejaculates containing >80% motile spermatozoa were used in the study. After the initial semen assessment, each ejaculate was split in 3 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 sec 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 described below. 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 x magnification on a clean and dry glass slide overlaid with a coverslip and maintained on thermo-regulated stage at 37°C. Any drifting of the specimen was permitted to stop and the percentage of motile spermatozoa was subjectively determined to the nearest 5% by analyzing four to five fields of view [31].

2.3. Sperm viability by Trypan Blue/Giemsa technique

Sperm viability was assessed by Trypan Blue/Giemsa technique as reported by Boccia et al. [32]. Briefly, 5 µl of semen and 5 µl of 0.27% Trypan blue were spread on a clean slide that wasplunged in a fixative solution (86 mL 1N HCl, 14 mL 37% formaldehyde solution and 0.2 g neutral red) for 2 min and stained with 7.5% Giemsa overnight. Sperm cells were microscopically evaluated at 40 x magnification (Nikon Diaphot 300). A total of 100 spermatozoa were analyzed per slide and differentiated as: live with acrosome intact, dead with acrosome intact, live with acrosome reacted, or dead with acrosome reacted. To assess sperm viability, the percentage of live sperm with an intact acrosome was recorded.

2.4 *Sperm membrane integrity*

Sperm membrane integrity was assessed after thawing by the hypo-osmotic swelling (HOS) test, as described by Jeyendran *et al*. [33]. Fifty µL of semen were mixed with 500 µL of an hypo-osmotic solution (0.73 g sodium citrate and 1.35 g fructose in 100 mL of distilled water, 150 mOsm) and

144 incubated at 37 ˚C for 45 min. A drop of diluted semen was placed on a clean slide and covered
145 with a cover slip. A total of 200 spermatozoa were counted in different fields at 400 X under phase
146 contrast microscope (Nikon E200) and the percentage of spermatozoa positive to HOS test (having
147 coiled tails) was determined.

148

149 *2.5. Localization of tyrosine phosphorylated protein assay*

150

151 Localization of phosphotyrosine containing protein was detected using an indirect
152 immunofluorescence assay as described by Tardif et al. [34]. Frozen-thawed sperm were selected
153 by centrifugation (25 min at 300 × g) on a Percoll discontinuous gradient (45 and 80%) and washed
154 twice, at 160 and then at 108 x g for 10 min each in mPBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM
155 Na_2HPO_4, 137 mM NaCl, 5.5 mM glucose and 1.0 mM pyruvate, pH 7.4) containing 2% (w/v)
156 BSA. Sperm pellets were fixed in formaldehyde for 1 h at 4 °C, centrifuged at 300 g for 10 min
157 andincubated overnight at 4 °C in mPBS.Twenty µL of sperm suspension were smeared,
158 permeabilized in an absolute ethanol solution for 5 min. and incubated with anti-phosphotyrosine
159 primary antibody produced in rabbit (Sigma, Cat no: T1325) for 1 h at room temperature. The slides
160 were then incubated with secondary antibody, FITC-conjugated goat anti-rabbit IgG (Sigma, Cat
161 no: F0382) for 1 h in the dark at room temperature and the slides were mounted with 90% (v/v)
162 glycerol. Green fluorescence was observed by epifluorescent microscope (Nikon Diaphot 300)
163 using FITC filter (B2-A, 520 nm wave length). A total of 100 spermatozoa were screened per slide
164 and classified according to one of the four fluorescence patterns described by Cormier and Bailey
165 [35]: Pattern NF, i.e. no fluorescence over the entire spermatozoa (non capacitated sperm) Pattern
166 A, i.e. uniform fluorescence over the entire acrosome (low capacitation level); Pattern E, i.e. a short

line or triangle of fluorescence in the equatorial segment (medium capacitation level) and Pattern EA, i.e. fluorescence at both equatorial and anterior acrosomal regions (high capacitation level).

2.6. Chlortetracycline (CTC) fluorescent assay

The capacitation status of frozen-thawed buffalo spermatozoa was assessed by CTC fluorescent staining as described by Fraser et al. [36]. Briefly, 15µL of CTC staining solution (750 mM CTC, 5 mM cysteine in 130 mM NaCl, and 20 mM Tris HCl, pH 7.4) were gently mixed with 15 µL of Percoll separated spermatozoa and fixed with the addition of glutaraldehyde (12.5% v/v). Five µL aliquot of fixed spermatozoa was placed on a microscope slide, mixed with 5 µl of mounting medium and overlaid with a coverslip. At least 100 spermatozoa per slide were analyzed and classified into one of three CTC staining patterns as described by Fraser et al. [34]: 1) Uniform bright fluorescence over the whole head (uncapacitated spermatozoa, pattern F); 2) fluorescence-free band in the post-acrosomal region (capacitated spermatozoa, pattern B); 3) Dull fluorescence over the whole head except for a thin punctuate band of fluorescence along the equatorial segment (acrosome reacted spermatozoa, pattern AR).

2.7. *Indicators of oxidative stress*

2.7.1. Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) was estimated using a commercial kit (Antioxidant Capacity Assay Kit, Cayman Chemical Co. Ann Arbor, MI, USA) following the manufacturer's instructions. Briefly, Percoll separated spermatozoa were homogenized on ice in 1 mL of cold buffer (5 mM potassium phosphate pH 7.4, containing 0.9% sodium chloride and 0.1% glucose). Samples were centrifuged at 10.000 g x 15 min at 4°C and the supernatant was used for the assay. The standard curve was prepared using the Trolox standards. After the plate configuration, 10 µL of Trolox standards and samples were loaded in duplicate on the corresponding wells of a 96-well plate. Then 10 µL of metmyoglobin and 150 µL of chromogen were added to all standard/sample wells. The reaction was initiated by adding 40 µL of hydrogen peroxide as quickly as possible. The plate was covered and incubated for 5 min on a shaker at room temperature. Absorbance was monitored at 405 nm using a plate reader (GloMax®-Multi Detection System – Promega, Milano) and the values were expressed in mmol/L.

2.7.2 Superoxide levels by Dihydroethidium (DHE) Assay

Superoxide levels were measured by DHE Assay, previously described [37]. Dihydroethidium exhibits a weak blue fluorescence; however, once this probe is oxidized by superoxide anion, it intercalates within DNA, staining the cell nucleus or mitochondria with a red fluorescence. Dihydroethidium (2 µM) were added to sperm samples and incubated in the dark at room temperature for 20 min. A standard curve was prepared using DHE standards. Absorbance was monitored at 570 nm using a plate reader (GloMax®-Multi Detection System – Promega, Milano). The standard curve was prepared using the DHE standards, and the value for each sample was calculated from standard curve and expressed as µM/µL DHE.

2.7.3. Lipid peroxidation (LPO) levels

Percoll separated spermatozoa were rewashed twice with PBS at 800 x g for 20 min at 4 °C. Sperm cells suspended in PBS were sonicated and the supernatant was used to determine LPO levels by the estimation of MDA concentration using the TBARS assay kit (Cayman Chemical Company, Ann Arbor, U.S.A.). Briefly, to each tube 100µL of sample/standard, 100µL of SDS solution and 4 mL color reagent were added. The mixture was boiled in a water bath for 1 h, after which the samples and standards were removed and placed in an ice bath for 10 min to stop the reaction. After cooling, the suspension was centrifuged at 4°C for 10 min at 1600 x g. The 150µL suspensions were loaded into the colorimetric plate and absorbance was measured at 535 nm. The standard curve was prepared using the MDA standards, and the value of MDA for each sample was calculated from standard curve and expressed as nmol/mL.

2.8. ATP assay

ATP content was measured using a Colorimetric ATP Assay Kit (Biovision, Milpitas, USA) following the manufacturer's instructions. Briefly, Percoll separated spermatozoa were homogenized in 100 µl ATP Assay Buffer and deproteinized using 10 kDa spin columns. Samples were incubated at room temperature for 30 min, and the absorbance were measured at 570 nm in a micro-plate reader (Bio-Rad Model 680). The standard curve was prepared using the ATP standards, and the value of ATP for each sample was calculated from standard curve and expressed as nmol/10^8.

235 2.9. Phospholipids assay

236

237 Phospholipids was estimated in the sperm lipid extract as described by Bartlett [38]. A standard
238 phosphorous solution (8 µg/5 mL) was prepared by dissolving 3.5 mg KH_2PO_4 in 10 mL of 10 N
239 H_2SO_4 in 100 mL double-distilled water (DDW). For the estimation of phospholipids content, 0.5
240 mL of chloroform and 1 mL of perchloric acid (70%) were added to each tube containing 60 x 10^6
241 of Percoll washed spermatozoa and the mixture was digested in a sand bath at 150-160 ◦C until it
242 became clear. The samples were then removed and cooled to room temperature. Subsequently, 6
243 mL of DDW and 0.8 mL of ammonium molybdate (2.5%) were added, followed by 0.2 mL of
244 Fiske-Subba Row reagent (1.2 g of sodium metabisulfite, 20 mg of 1-amino-2-nephthol-4-sulphonic
245 acid and 120 mg of anhydrous sodium sulfite in 10 mL of DDW).The samples were heated for 7
246 min in a boiling water bath, cooled to room temperature and absorbance at 660 nm was recorded
247 (Perkin Elmer PTP-1). Simultaneously, the standard phosphorus solution and a blank were also run
248 in the same manner. Phospholipids concentration was expressed in µg/120 x 10^6 sperm.

249

250 *2.10. In vitro sperm fertilizing ability*

251 Abattoir-derived bovine cumulus-oocyte complexes (COCs) with uniform cytoplasm and
252 multilayered cumulus cells were matured in TCM 199 supplemented with 15% bovine serum (BS),
253 0.5 µg/mL FSH, 5 µg/mL LH, 0.8 mM L-glutamine and 50 µg/mL gentamycin for 22 h at 39 °C,
254 and 5% CO_2 in air. In vitro matured COCs were fertilized in TALP buffered with 25 mM sodium
255 bicarbonate and supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine and 10 µg/mL
256 heparin (IVF medium) with sperm treated with 0 (control; n=429), 2.5 mM (n=430) and 7.5 mM
257 carnitine (n=403), over 10 replicates. Percoll separated spermatozoa were diluted with IVF medium
258 and added in the fertilization wells at the concentration of 2 × 10^6 sperm/mL. Gametes were co-

incubated for 20 h at 39 °C, in 5% CO_2 in air, after which presumptive zygotes were vortexed for 2 min to remove cumulus cells, and incubated in synthetic oviduct fluid modified medium [39] in a humidified mixture of 5% CO_2, 7% O_2 and 88% N_2 in air at a temperature of 39 °C. After 24 h of culture, the cleavage rate was assessed and confirmed by fixation of zygotes with absolute ethanol overnight and staining with DAPI for nuclei examination under epi-fluorescence microscope (Nikon Diaphot 300) after zona removal by protease (2 mg/mL) digestion. The penetration, normal fertilization and polyspermy rates were assessed by examining both uncleaved and cleaved embryos. Normal fertilization included uncleaved embryos with two synchronous pronuclei (2PN) and cleaved embryos displaying a normal nucleus per cell. Polyspermic penetration included uncleaved embryos with >2PN or sperm heads and cleaved embryos with higher numbers of nuclei or sperm heads per cell. In addition, the proportion of fast (> 4 cells) and slow cleaving (2 cells) embryos were recorded.

3. Statistical analysis

Differences in sperm motility and viability, CTC and tyrosine phosphorylation patterns of fresh semen among bulls were analyzed by ANOVA. The same parameters and membrane integrity, TAC, ROS and LPO levels, ATP content and phospholipids concentration were analyzed in frozen-thawed semen by a linear mixed model with the bull as repeated effect. The Bonferroni method was used to evaluate the differences among groups. The percentages of cleavage, total, normospermic and polyspermic penetration, as well as the proportion of fast and slow cleaving embryos were analyzed by Chi Square test. The level of significance was set at $P<0.05$.

4. Results

4.1. Viability, motility and capacitation status of fresh semen

No differences among bulls were found in sperm motility (on average 82.5 ± 0.8 %), viability (on average 92.6 ± 1.0 %) and capacitation status, evaluated by CTC (on average 86.1 ± 0.7, 13.8 ± 0.7 and 0.2 ± 0.8 % of patterns F, B and AR, respectively). With regard to the immune-localization of tyrosine phosphorylated proteins, no sperm displayed the patterns NF and E, whereas the percentages of sperm showing patterns A and EA were 92.2 ± 0.9, and 7.8 ± 0.9, respectively. No differences were recorded in tyrosine phosphorylated proteins patterns among bulls, indicating the homogeneity of the samples at the beginning of the trial.

4.2. Post-thawing sperm motility, viability and membrane integrity

An increase in sperm motility and membrane integrity was recorded in both treated groups compared to the control, whereas sperm viability was not affected (Table 1).

Table 1.

4.3. Capacitation status of frozen-thawed sperm: localization of tyrosine phosphorylated proteins

Supplementation of carnitine to the freezing extender decreased the percentage of sperm displaying pattern EA compared to the control, with a greater effect ($P<0.01$) at the higher concentration tested

(Table 2). Furthermore, when the extender was supplemented with carnitine a higher ($P<0.05$) percentage of sperm showing pattern A was observed compared to the control. Interestingly, the percentage of sperm exhibiting no fluorescence also increased ($P<0.01$) when sperm were treated with 7.5 mM carnitine. No differences in sperm displaying pattern E, however, were detected among groups.

Table 2.

4.4. Capacitation status of frozen-thawed sperm: CTC assay

The results regarding the CTC patterns showed that supplementation of the freezing extender with carnitine , prior to cryopreservation, decreased the level of capacitation, in a dose-dependent manner (Table 3). In fact, the percentage of sperm displaying pattern F increased ($P<0.01$), while that of sperm displaying pattern B decreased ($P<0.01$) in both treated groups compared to the control (Table 3). Within treatment groups, the highest concentration was the most effective in reducing capacitation-like changes, as indicated by higher ($P<0.01$) percentages of pattern F and lower ($P<0.01$) percentages of pattern B sperm. However, no differences were detected in pattern AR among groups, as shown in Table 3.

Table 3.

4.5. Indicators of oxidative stress, ATP content and phospholipids concentration

328 The total antioxidant capacity increased (P<0.01) when sperm were treated with 7.5 mM carnitine
329 (Table 1). Both carnitine concentrations decreased (P<0.01) ROS levels, as indicated by reduced
330 DHE values (Table 1). However, supplementation of the semen extender with carnitine did not
331 affect LPO levels and phospholipids concentrations (Table 1). Interestingly, carnitine
332 supplementation increased the ATP content of buffalo frozen-thawed sperm in a dose-dependent
333 manner, as shown in Table 1.

334

335 *4.6. Sperm fertilizing ability*

336

337 Cleavage, total penetration, normospermic and polyspermic penetration rates were similar among
338 groups, as shown in Table 4. Likewise, no differences among groups were observed in the
339 percentage of slow cleaving, i.e. 2 cells-embryos (20.8 ± 5.3, 32.8 ±6.5 and 23.2 ± 4.1, respectively
340 in the control, 2.5 mM and 7.5 mM carnitine groups) and fast cleaving, i.e. > 4 cells-embryos (79.2
341 ± 5.3, 67.2 ± 6.5 and 76.8 ± 4.1, respectively in the control, 2.5 mM and 7.5 mM carnitine groups).

342

343 **Table 4.**

344

345 **5. Discussion**

346

347 The results of this study demonstrated that the supplementation of the freezing extender with
348 carnitine significantly improved post-thawing sperm motility and decreased capacitation-like
349 damages in buffalo sperm. It was also observed that the beneficial effects of carnitine on buffalo

sperm are due to reduced oxidative stress and increased ATP generation, resulting in improved membrane stability.

The improved post-thawing motility here recorded, when carnitine was supplemented prior to freezing, is in agreement with previous studies carried out in other species, such as human [40], bovine [27] and boar [41]. In contrast to these works, however, sperm viability was not improved in buffalo, remaining high in all groups (> 80%). On the other hand, carnitine did not influence fertility parameters, such as sperm motility and the incidence of sperm anomalies, in Angora goat [42]. The improved sperm motility recorded in this study may be due to the antioxidant activity of carnitine, as indicated by increased TAC and reduced ROS levels in treated sperm. These results confirm that buffalo sperm motility is negatively correlated with ROS levels [43].. However, it seems that carnitine is effective in reducing ROS concentration without affecting lipid peroxidation. In agreement with this, equine sperm challenged with ROS showed a decreased motility before any measurable increase in lipid peroxidation [44]. In addition, the dose dependent increase of ATP content in buffalo sperm treated with carnitine indicates that the enhanced post-thawing sperm motility is related to improved mitochondria function and ATP generation. It was previously suggested that the decline in motility after sperm incubation with ROS may be due to ATP depletion [45]. Sperm motility is normally ensured by the complex structure of the axoneme associated with the dense fibers in the mid-piece, surrounded by mitochondria, which are involved in energy generation through oxidative phosphorylation. It is known that carnitine shuttles acetyl and acyl groups across the mitochondrial inner membrane playing a buffering role, trapping excess mitochondrial acetyl-CoA as acetyl-L-carnitine and in turn protecting the activity of pyruvate dehydrogenase, a key enzyme for mitochondrial respiration [23]. The results of this study showed a dose dependent effect of carnitine on sperm capacitation status, assessed by both CTC and tyrosine phosphorylation proteins assays, widely used methods that detect capacitation at different levels. In fact, although both the concentrations tested significantly decreased the capacitation level, the effect was greater at the highest concentration (7.5 mM). The fluorescent antibiotic CTC was used to

assess the destabilization of sperm membrane [36] based on its ability to cross over the cell membrane, enter intracellular compartments and bind to free calcium ions. This method has been used to assess sperm capacitation in most domestic species [46; 47], including buffalo [48; 49]. Moreover, as it is well established that tyrosine phosphorylation of sperm proteins is a key event of sperm capacitation, several studies have correlated the degree of tyrosine phosphorylation with the capacitative state of spermatozoa [48; 34]. In this study carnitine supplementation significantly decreased the incidence of sperm displaying the CTC pattern B (capacitated sperm) and the tyrosine phosphorylated pattern EA (high capacitation level), while increasing the percentage of both tyrosine phosphorylated pattern A (low capacitation level) and non-fluorescent (non capacitated) sperm. Taken together, these results highlight a remarkable reduction of the cryopreservation-induced modifications to sperm membranes, indicating improved sperm quality. This finding is particularly important because in frozen-thawed buffalo sperm the proportion of capacitation-like changes is much higher than in other domestic species [17; 35; 10]. Furthermore, it is known that premature capacitation reduces the reproductive lifespan of the male gamete [35; 2]. What is unequivocal is that the pre-treatment with carnitine reduced capacitation-like changes by stabilizing the sperm membrane, as indicated by the results of CTC staining and HOS test]. In fact, in addition to the increased percentage of sperm displaying CTC pattern F, indicating membrane stability, the percentage of HOS positive sperm also significantly increased after carnitine treatment. The HOS test is a valuable tool to assess the functional integrity of sperm membrane [50], by evaluating the proportion of biochemically active sperm, after exposure to an hypo-osmotic extracellular solution. The mechanism by which carnitine stabilizes sperm membrane is not completely elucidated. The increased TAC and reduced ROS levels recorded in the presence of carnitine suggest that the beneficial effect is due to its protecting role of plasma membrane against ROS damages. An excess of ROS in fact, results in membrane damages through the initiation of lipid peroxidation [51]. However, unexpectedly LPO was not affected by carnitine treatment. It is worth noting that LPO and membrane damage are relatively independent processes [52] and that membrane stress

contributes more than LPO to the cryodamages [53]. Therefore, it is not possible to rule out that carnitine acts on membrane stability by reducing membrane stress during cryoconservation. Cryopreservation-induced membrane stress involves embrittlement of plasma membrane during phospholipid transition from fluid to glassy state. It is well known that membrane stability is directly associated to the membrane cholesterol:phospholipids ratio [54]. In this study, however, although the phospholipid concentration tended to decrease, the difference was not significantly different among groups The beneficial effects on frozen-thawed sperm quality suggested to evaluate whether carnitine supplementation would also affect the fertilizing ability of buffalo sperm that was here assessed by heterologous IVF. However, despite the increased sperm quality, the in vitro fertilization rate was not enhanced: cleavage rate, as well as total penetration and polyspermy were indeed unaffected. Therefore, the enrichment of the extender with carnitine prior to freezing improves post-thawing motility and prevents capacitation-like changes, without improving in vitro fertility. This may be accounted for by the artificial environment of the IVF system, where there is an abnormally high sperm-oocytes ratio in very small volumes and sperm encounter the oocytes directly at co-incubation. In addition, it is not possible to rule out that carnitine-treated sperm that are less capacitated may require more time to penetrate the oocytes. However, the percentages of fast cleaving embryos were also similar among groups, indirectly suggesting that this is not the case. It is indeed known that the chronology of development is correlated with first cleavage division that is in turn associated with sperm penetration time [55]. It seems that the presence of a capacitating agent such as heparin in the IVF medium counteracts the possible differences in penetration rate related to the capacitation status. It follows that it would be worth investigating in future studies the in vivo fertility after AI that is likely affected to a greater extent by the premature capacitation occurring after cryopreservation, because of the longer time required for sperm to reach the site of fertilization.

In conclusion, the supplementation of semen extender with carnitine significantly increased post-thawing motility and membrane integrity, reducing capacitation-like changes of buffalo sperm in a

dose dependent manner, with the 7.5 mM concentration being the most effective. It was also demonstrated that carnitine improved buffalo sperm quality by boosting mitochondrial ATP generation and decreasing ROS production. Nevertheless, in vitro fertilizing capability was not affected. The results of this study strongly suggest to investigate the effect of carnitine supplementation of buffalo semen on in vivo fertility in future studies. This assessment is fundamental to consider the utilization of carnitine-enriched extender for commercial purposes.

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Table 1. Effect of carnitine on characteristics of buffalo frozen-thawed semen.

Carnitine concentrations (mM)	0 (Control)	2.5	7.5
	Mean ± SE	Mean ± SE	Mean ± SE
Motility (%)	44.4 ± 3.53[a]	53.1 ± 3.95[b]	52.5 ± 3.59b
Viability (%)	80.7 ± 2.41	84.2 ± 2.12	84.6 ± 2.02
Hos positive (%)	48.44 ± 0.69[A]	55.19 ± 0.54[B]	59.63 ± 0.30[C]
TAC (mM/L)	1.32 ± 0.02[A]	1.34 ± 0.01[AB]	1.37 ± 0.01[B]
DHE (µM/µL)	0.22 ± 0.01[A]	0.18 ± 0.01[B]	0.14 ± 0.0[C]
LPO (nmol/mL MDA)	30.6 ± 0.43	30.4 ± 0.26	30.4 ± 0.26
ATP (nM x 10^8 sperm)	4.06 ± 0.06[A]	5.27 ± 0.14[B]	8.23 ± 0.37[C]
Phospholipid (µg/120 x 10^6sperm)	94.91 ± 3.64	91.23 ± 5.89	88.12 ± 7.60

[A, B] Values with different superscripts within columns are different; P< 0.01

[a, b] Values with different superscripts within columns are different; P< 0.05

Table 2. Effect of carnitine on the percentages of tyrosine phosphorylated proteins patterns of buffalo frozen-thawed semen.

Carnitine concentrations (mM)	NF- pattern Mean ± SE	A- pattern Mean ± SE	E- pattern Mean ± SE	EA- pattern Mean ± SE
0	2.8 ± 1.0^{A}	65.8 ± 3.6^{a}	1.1 ± 0.8	30.3 ± 3.8^{aA}
2.5	5.1 ± 1.9^{A}	75.8 ± 2.7^{b}	0.4 ± 0.3	18.8 ± 2.8^{bA}
7.5	16.5 ± 3.4^{B}	76.3 ± 2.8^{b}	0.0 ± 0.0	7.2 ± 1.9^{B}

$^{A, B}$ Values with different superscripts within columns are different; $P < 0.01$

$^{a, b}$ Values with different superscripts within columns are different; $P < 0.05$

Table 3. Effect of carnitine on the percentages of F-pattern, B-pattern and AR-pattern in buffalo frozen-thawed semen.

Carnitine concentrations (mM)	F- pattern Mean ± SE	B- pattern Mean ± SE	AR- pattern Mean ± SE
0	31.3 ± 2.1^{A}	63.8 ± 1.8^{A}	4.9 ± 0.9
2.5	49.4 ± 2.2^{B}	46.8 ± 2.2^{B}	3.8 ± 0.6
7.5	60.3 ± 3.6^{C}	37.2 ± 1.8^{C}	2.6 ± 1.9

$^{A, B, C}$ Values with different superscripts within columns are different; $P < 0.01$

Table 4. Effect of carnitine on the percentages of cleavage, total penetration, normospermic penetration and polyspermyafter heterologous IVF.

Carnitine concentrations (mM)	N.	Cleavage	Total penetration	Normospermic penetration	Polyspermy
		n (%)	n (%)	n (%)	n (%)
0	429	207(48.4)	239 (55.1)	234 (53.6)	3 (0.9)
2.5	430	228(51.3)	253 (56.9)	252 (56.6)	1 (0.3)
7.5	403	198(45.2)	233 (53.5)	218 (50.4)	2 (0.6)

Highlights

- Carnitine supplementation increases post-thawing motility in buffalo sperm
- Carnitine supplementation reduced the cryocapacitation damages of buffalo sperm
- Carnitine increases ATP generation modulating ROS production in buffalo sperm