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# EFFECTS OF L-CARNITINE ON MORPHOLOGY AND ANTIOXIDANT ENZYMES AND DNA INTEGRITY OF CRYOPRESERVED BUFFALO SPERMATOZOA

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## ABSTRACT

Cryopreservation induces sub lethal damage to the spermatozoa, thereby reduce their fertile life. There are some biochemical additives that may enhance buffalo semen freezability; L-carnitine is one of these biochemical semen additives. Till now, the exact effects of L-carnitine on buffalo semen processing outcomes haven't been discovered. The current study aimed to clarify L-carnitine roles during buffalo semen cryopreservation. Semen was cryopreserved in tris-based extender supplemented with different concentrations of L- carnitine (0.01, 0.05 and 0.1mg/ml)*Vs.*Tris-based extender only (control). Then they were processed to cryopreservation and thawing to assess different semen characteristics. Cryopreserved semen was assessed for percentage post- thawing motility, acrosomal and plasma membrane integrity, viability, DNA damage, antioxidant enzymes concentration, lipid peroxidation, in vitro fertilizing potentials and conception rate. Current results indicated that addition of 0.05 mg/ml L- carnitine to semen extender significantly (P<0.05) improved post-thawing motility, viability and acrosomal integrity (63.33±9.28 %, 133.33±9.40 and 12.33± 2.02 %, respectively) compared with control (43.33±6.01 %, 74.16± 10.93 and 24.67±2.03 %, respectively). Moreover, 0.05mg/ml L- carnitine significantly increased (P<0.05) total antioxidant capacity (TAC), superoxide dismutase (SOD) and glutathione peroxidase (GPx) concentrations (0.50±0.07 mμ/ml, 73.67±5.37 U/ml and 106.66±12.03 U/L, respectively) with respect to the control (0.19±0.01 mμ/ml, 27.33±3.49 U/ml and52.33±4.09 U/L, respectively). Furthermore, 0.05mg/ml L-carnitine significantly decreased (P<0.05) lipid peroxidation of the cryopreserved spermatozoa compared with the control semen (11.00±1.73 *vs* 24.82±4.90nmol/ml). Likewise, at this concentration sperm DNA damage, tail length and tail moment of the cryopreserved semen significantly (P<0.05) reduced (1.87±0.36 %, 1.83±0.33 μm and 3.72±1.44, respectively) compared with control (3.47±0.13 %, 3.48±0.17 μm and 11.91±0.87, respectively). Additionally, 0.05 mg/ml L- carnitine significantly (P<0.05) improved in vitro fertilization rate (57.45%) and conception rate (61.76%) compared with the control (33.33 and 37.93%, respectively). In conclusion the use of 0.05 mg/ml L-carnitine in the freezing extender improves DNA integrity through enhancing the antioxidant defense of buffalo sperm cells and decreasing the rate of lipid peroxidation. Therefore, L-carnitine may improve sperm cryopreservation quality, reduce cryodamage and improve sperm fertilizing potential.

***Key words:*** *L- carnitine, cryopreservation, buffalo semen, DNA integrity, antioxidant activity*

## INTRODUCTION

Infertility and reduced fertility are one of the major problems in veterinary practices especially for valuable animals. Male infertility represented about 30% to 50% of infertility cases (Aliabadi*et al.*, 2013).

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Semen cryopreservation is one of the most effective and acceptable methods to maintain male fertility potential. Long-term sperm storage, sperm banking and the possibility of storing the sperm of proven bull are the main objectives of using cryopreservation method. Unfortunately, despite many advantages of sperm cryopreservation, this methodentries some hazardous that reduces sperm fertilizing capacity through morphological damage, DNA damage and generally impaired sperm motility and viability (Singer *et al.*, 1980). Several researchers found that cryopreservation decreases sperm motility parameters, normal morphology and increases

programmed sperm death (Badr *et al.,* 2010). Poly unsaturated fatty acids (PUFA) are a basic component of mammalian sperm cells, which contributing the major skeleton of its membrane structure, integrity, metabolism, and their ability to penetrate and fertilize the oocytes through many of physicochemical modifications (Guthrie and Welch, 2012). In this respect, it had been found that buffalo sperms were prone more to lipid peroxidation compared with bull sperm because it is rich in PUFA (Garg *et al.,* 2009). Lipid peroxidation during in-vitro handling and sperm storage, are probably the primary causes of this fertility dysfunction (Cecil and Bakst, 1993).

It is extremely necessary to optimize cryopreservation method since it is an effective step in improving male infertility. Therefore, adding substances that prevent damage to sperm, to cryopreservation extender can improve sperm cryopreservation condition. These substances include antioxidants that are capable of preventing sperm damage and destruction through removing oxygen free radicals (Sarica *et al.,* 2007).

L-carnitine (LC) is one of these antioxidants, which plays an important role in increasing vital capabilities of the sperm. Free LC is a high polarized and water-soluble substance being first isolated from bovine muscle in 1905, and its chemical structure was identified in 1948. LC is the only carnitine isomer, which is biologically active (Zare *et al.,* 2009). LC is derived from lysine and methionine (amino acids) and is a foodstuff being found in meat and dairy products (Lenzi *et al.,* 2004). Free LC is essential for beta-oxidation (B-Oxidation) of long-chain fatty acids in mitochondrion. Fatty acids must be activated before entering the mitochondrion (making a connection with coenzyme A to form acetyl-CoA). Long-chain acetyl-CoA molecules cannot cross the inner mitochondrial membrane in the absence of LC. When fatty acids are entered the mitochondrion, beta-oxidation and adenosine triphosphate formation (ATP) will begin (Peyvandi *et al.,* 2009). ATP is an important fuel source for sperm motility (Stradaioli *et al.,* 2004). Furthermore, LC through its antioxidant properties had been shown to increase the activity and levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Sarica *et al.,* 2007). Additionally, LC was to fulfill vital roles in sperm maturation and metabolism when passing through the epididymis (Yakushiji *et al.,* 2006). The epididymis has the highest LC concentration found in body. LC concentration in epididymis is 2000 times higher than that in blood.

Therefore, we decided to investigate the effect of LCon morphology, the biochemical activities, sperm nuclear integrity and in vitro fertilizing potentials of the cryopreserved buffalo spermatozoain order to provide suitable strategies for improving the quality of cryopreservation, and preventing the risks of cryopreservation.

## MATERIALS AND METHODS

**Diluent Preparation**

The cryoprotective extender used in the current study was composed of 2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.6 ml glycerol, 20 mL egg yolk, 25 mg gentamicin and 50,000 IU penicillin; all of these components were dissolved in 100 mL deionized water and supplemented with different concentrations of L- carnitine.

**Semen Collection**

Semen samples were obtained randomly from six evaluated buffalo bulls (aged 3 to 5 y) kept at the Animal Reproduction Research Institute farm (Cairo, Egypt). Two consecutive ejaculates were collected from each bull weekly for successive eight weeks using an artificial vagina. The ejaculates were pooled to eliminate variability between the evaluated samples. The semen samples were assessed for volume, sperm concentration and percentage of motile spermatozoa. The ejaculates with at least 70% motility, $800\times10^6$ sperm cells/ml and>85% normal sperm morphology were used for the present study. All experiments were done with at least 3 replicates for each group.

**Semen Processing**

After the evaluation of semen quality, the fresh semen samples were pooled and then split into 4 equal portions and diluted at 30°C with Tris-based extender supplemented with different concentrations of L-carnitine (0.01, 0.05 and 0.1 mg/ml) Vs. Tris-based extender only (control) to obtain $120\times10^6$ sperm/ml. The fresh semen samples were transferred to pre warmed tubes. Semen was cooled from 37 to 5°C throughout 60 min in a cold cabinet. The cooled semen was loaded into 0.25 ml polyvinyl chloride straws (IMV, L'Aigle, France), horizontally placed in a refrigerator and kept at 4°C for 1 h. These straws were then placed 6 cm above the liquid nitrogen surface where the temperature was approximately −120°C. After 15 min, they were immersed directly into liquid nitrogen (−196°C) for storage. The straws were stored at least for24 h before evaluation (Badr *et al.,* 2010). Frozen semen straws were thawed in water bath at 37°C for 30 sec. Post-thawing sperm motility; viability and acrosomal integrity were assessed.

**I- Assessing of sperm quality post-thawing**

**I- a- Analysis of Sperm Motility**

The percentage of linear motile sperm was examined visually. For each treatment, 3 straws were thawed separately by immersion in a water bath at 37°C for 30 sec. The sperm samples were evaluated at 37°C by phase contrast microscope equipped with a warm stage at 200× magnifications.

**I- b- Assess of Acrosomal Membrane Integrity**
Acrosomal integrity was assessed using silver nitrate stain in a procedure as described by Chinoy *et al.* (1992).

**I- c- Assess of plasma membrane integrity**
Plasma membrane integrity (PMI) of sperm was assessed using the hypo-osmotic swelling (HOS) test (Correa and Zavos, 1994). Fifty µl of each semen sample was mixed with 500 µl of HOS solution and incubated at 37 ºC for 30 min. After incubation, a 5 µl semen sample drop was examined under a phase-contrast microscope (400 x). A minimum of 100 sperm were counted for their swelling ability in HOS solution. The sperm characterized by coiling or swelling of the tail of varying degrees were considered to have an intact plasma membrane.

**II- Assessing of biochemical activity of the frozen semen:**
**II- a- Estimating Transaminases Enzymes**
Semen straws from each group were centrifuged at 800 ×g for 10 min and supernatant was collected for estimating the Aspartate-Aminotransferase (AST); Alanine-Aminotransferase (ALT) and Alkaline Phosphatase (AKP) enzymes spectrophotometrically in all groups as described by Reitman and Frankel (1957).

**II-b-Estimating antioxidant activity:**
**II-b-1-Total Antioxidant Capacity (TAC)**
TAC of the frozen-thawed semen was estimated using a commercial kit (Antioxidant Capacity Assay Kit, Cayman Chemical Co. Ann Arbor, MI, USA) according to Cortassa *et al.* (2004). The reaction was measured spectrophotometrically at 532 nm.

**II- b-2-Superoxide Dismutase (SOD)**
The SOD activity was measured according to Flohe and Otting (1984). Briefly, each semen sample was diluted 1:5 with phosphate buffer saline (PBS) (pH 7.0). The SOD activity was measured at 560 nm on a spectrophotometer and expressed as units per milliliter.

**II-b-3-Glutathione peroxidase (GPx)**
The GPx content of sperm was measured using the method of Sedlak and Lindsay (1968). The semen samples were precipitated with 50% trichloracetic acid (vol/vol) and then centrifuged at 1,000 ×g at 22°C for 5 min. The GPx activity was measured at 412 nm on a spectrophotometer. The values of GPx were expressed as units per liter.

**II-b-4-Lipid Peroxidation (LPO)**
The concentrations of malondialdehyde (MDA), as indices of the LPO in the sperm samples were estimated. An aliquot (500 µl) of semen from each sample was centrifuged at 800 ×g for 10 min, sperm pellets were separated and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation,1 ml of deionized water was added to spermatozoa and they were snap-frozen and stored at -70°C till further analysis. The samples were thawed before the lipid peroxidation assay. The concentrations of Malondialdehyde (MDA), as indices of the LPO in the sperm samples, were measured using the thiobarbituric acid reaction according to the method of Placer *et al.* (1966). The MDA concentrations were expressed in nmol/ml.

**III-Assessment of Sperm DNA Integrity:**
DNA integrity and the incidence of DNA strand breaks or fragmentation were detected using the alkaline single cell gel electrophoresis (comet) assay according to Boe-Hansen (2005). Briefly, frozen-thawed spermatozoa were diluted in PBS, embedded in agarose, followed by cell lysis, DNA decondensation, electrophoresis and DNA staining with 50 µL of 20 µg mL −1 ethidium bromide (Sigma, St. Louis, MO). The cells were then visualized by fluorescent microscopy. Intact nuclei in the comet assay appeared to have compact and brightly fluorescent heads; in contrast, strand breaks in damaged cells allow DNA migration during electrophoresis and a tail of DNA could be seen behind the head, giving the appearance of a comet (Hughes *et al.,* 1996). After subjecting the spermatozoa to the comet assay, sperm nuclei were analyzed by computer software program (comet IV, 2001).

**IV- Evaluation of in vitro Fertilizing Potential:**
In vitro oocyte fertilization rate was examined on the base of the pronuclei number found in the oocyte after fertilization. In brief, the oocytes were matured in vitro according to Totey *et al.* (1992) and the nincubated with the capacitated sperm cells for 6 h at 5% $CO_2$inhumid incubator according to Parrish *et al.* (1988). The ooocytes were fixed for 24~48 h in ethanol: acetic acid (3:1 v/v), then stained with 1% Orcein dissolved in 45% aceticacid. The oocytes were evaluated under a phase-contrast microscope (Nikon, Tokyo, Japan). Oocytes were evaluated for normal fertilization according to Martino *et al.* (1994), on basis of oocyte that had set of male and female pronuclei in the ooplasm were considered to be fertilized normally.

**V- Evaluation of fertilizing potentials of treated semen (pregnancy rate).**
A preliminary fertility trial was performed to compare between control semen and the best L-carnitine concentration treated spermatozoa. Buffalo cows were randomly assigned to two groups: group 1 (29 buffaloes) was inseminated using control semen; group (2) (34 buffaloes) was inseminated using 0.05mg/ml L-carnitine treated semen. Pregnancy diagnosis was performed at 45 days post-insemination by rectal palpation.

**Statistical Analysis:**
All data were analyzed by using Costat Computer Program (1986), Version 3.03 copyright Cottort Soft ware and were compared by the Least Significant Difference least (LSD) at 5% levels of probability. The results were expressed as means ±SE. The mean values of the percentages of motile sperm, acrosome-intactsperm, enzyme activity and embryo development were compared using Duncan's multiple range test by one way ANOVA procedure, when the F-value was significant (P <0.05). Sperm fertilizing capacity were assessed using Chi-square at (P<0.01 and 0.05, respectively).

## RESULTS

The results presented in Table 1 revealed that, addition of L-carnitine to the freezing extender improved the freezability of buffalo bull spermatozoa compared with the control semen, in a dose-dependent trend. Addition of 0.05 mg/mlL-carnitine to semen extender, appeared to be the best concentration that increased significantly (P<0.05) the post-thawing sperm motility, viability index and maintained the plasma membrane and acrosomal integrity (63.33±9.28 %, 133.33±9.40, 53.33±7.27% and 12.33± 2.02 %, respectively) compared with the control semen (43.33±6.01 %, 74.16± 10.93 , 31.66±3.33 % and 24.67±2.03 %, respectively). However, high concentration of L-carnitine (0.1 mg/ml) significantly reduced (P<0.05) post-thawing sperm motility, viability index and increased the plasma membrane and acrosomal abnormalities (41.66± 6.01 %, 78.5±8.65, 33.33±7.28% and 19.00±2.08 %, respectively).

**Table (1):** Effect of L-carnitine addition to freezing extender on post- thawed sperm characteristics.

| Treatment | Post-thawing motility (%) | Viability index | HOST (%) | Acrosomal integrity (%) |
|---|---|---|---|---|
| Control | 43.33±6.01 $^{b}$ | 74.16± 10.93 $^{b}$ | 31.66±3.33 $^{b}$ | 24.67±2.03 $^{a}$ |
| LC 0.01 mg/ml | 48.33± 4.40 $^{ab}$ | 121.66±13.42 $^{a}$ | 45.00±5.01$^{ab}$ | 15.33±2.27 $^{b}$ |
| LC 0.05 mg/ml | 63.33±9.28$^{a}$ | 133.33±9.40 $^{a}$ | 53.33±7.27$^{a}$ | 12.33± 2.02$^{b}$ |
| LC 0. 1 mg/ml | 41.66± 6.01 $^{b}$ | 78.5±8.65$^{b}$ | 33.33±7.28$^{ab}$ | 19.00±2.08 $^{ab}$ |

Three replications of the experiment were conducted. Results are presented as mean ± SEM
Values with different superscripts in the same columns are significantly different at least (P<0.05).
- LC :L-carnitine HOST : hypoosmotic swelling test

Moreover, data presented in Table 2 showed the effect of L-carnitine supplementation to buffalo semen extender on its enzymatic activity. The current data clarified that, addition of 0.05 mg/mL L-carnitine to the semen extender maintained sperm cell membrane integrity and this appeared through reduction of AST, ALT and ALP enzymes leakage (55.75±1.75, 17.25±1.75 and16.13±1.65 U/L, respectively) compared with the control extender (100.25±7.73, 26.5±2.10 and 26.40±2.89U/L, respectively).

**Table 2:** Effect of different concentrations of L-carnitine on biochemical activity of buffalo spermatozoa.

| Treatment | AST (U/L) | ALT (U/L) | ALP (U/L) |
|---|---|---|---|
| Control | 100.25±7.73$^{a}$ | 26.5±2.10 $^{a}$ | 26.40±2.89 $^{a}$ |
| LC 0.01 mg/mL | 65.00±8.92 $^{bc}$ | 19.50±2.48$^{b}$ | 20.38±2.74 $^{b}$ |
| LC 0.05 mg/mL | 55.75±1.75 $^{c}$ | 17.25±1.75$^{b}$ | 16.13±1.65$^{b}$ |
| LC 0. 1 mg/mL | 82.25±9.29$^{ab}$ | 21.50±1.34 $^{ab}$ | 21.88±4.17 $^{ab}$ |

Three replications of the experiment were conducted. Results are presented as mean ± SEM
Values with different superscripts in the same columns are significantly different at least (P<0.05).
AST: Aspartate-aminotransferase ALT: Alanine-aminotransferase ALP: Alkaline phosphatase

Data regarding the effect of L-carnitine addition to the freezing extender on the total antioxidant capacity (TAC), antioxidant enzymes and lipid peroxidation of the cryopreserved semen are presented in Table 3. In vitro provision of semen extender with 0.05mg/ml L-carnitine significantly augmented (P<0.05) the total antioxidant, SOD and GPx concentrations (0.50±0.07 mμ/ml, 73.67±5.37U/ml and 106.66±12.03U/L, respectively) compared with the control extender (0.19±0.01 mμ/ml, 27.33±3.49 U/ml and 52.33±4.09 U/L, respectively). Furthermore, data presented in Table 3 revealed that addition of 0.05mg/ml L-carnitine to the freezing extender significantly (P<0.05) diminished lipid peroxidation of the frozen-thawed semen (11.00±1.73 nmol/ml) compared with the control extender (24.82±4.90 nmol/ml).

**Table 3:** Effect of L-carnitine addition to freezing extender on buffalo bull semen antioxidant activity.

| Treatment | TAC (mμ/mL ) | SOD (U/mL ) | GPx (U/L) | MDA (nmol/ml) |
|---|---|---|---|---|
| Control | 0.19±0.01$^{b}$ | 27.33±3.49$^{c}$ | 52.33±4.09$^{a}$ | 24.82±4.90 $^{a}$ |
| LC 0.01 mg/mL | 0.36±0.05 $^{a}$ | 52.66±6.07$^{b}$ | 83.33±4.48a$^{b}$ | 17.35±0.94 $^{ab}$ |
| LC 0.05 mg/mL | 0.50±0.07 $^{a}$ | 73.67±5.37$^{a}$ | 106.66±12.03$^{a}$ | 11.00±1.73$^{b}$ |
| LC 0. 1 mg/mL | 0.18±0.04 $^{b}$ | 31.00±5.51$^{c}$ | 59.67±10.67$^{bc}$ | 20.75±2.92 $^{a}$ |

Three replications of the experiment were conducted. Results are presented as mean ± SEM
Values with different superscript in the same column are significantly different (p<0.05).
LC: L-carnitine; TAC: Total Antioxidant Capacity; SOD: Superoxide Dismutase; GPx: glutathione peroxidase; MDA:Malondialdyhide

With respect to the effect of L-carnitine addition to the freezing extender on the DNA integrity of the frozen buffalo spermatozoa are demonstrated in Table 4 and Fig (1). The present data indicated that, in vitro provision of semen extender with 0.05 mg/ml L-carnitine significantly decreased (P<0.05) the DNA fragmentation, tail length and tail moment of the frozen-thawed buffalo semen (1.87±0.36%, 1.83±0.33 μm and 3.72±1.44, respectively) as compared with the control extender (3.47±0.13 %, 3.48±0.17 μm and 11.91±0.87, respectively).

**Table 4:** Effect of different concentrations of L-carnitine on DNA integrity of the cryopreserved buffalo spermatozoa.

| **Treatment** | **DNA integrity (%)** | **Tail length (μm)** | ***Tail moment** |
|---|---|---|---|
| Control | 3.47±0.13$^{a}$ | 3.48±0.17$^{a}$ | 11.91±0.87$^{a}$ |
| LC 0.01 mg/ml | 2.75±0.06$^{b}$ | 2.71±0.03$^{b}$ | 7.46±0.24 $^{b}$ |
| LC 0.05 mg/ml | 1.87±0.36$^{c}$ | 1.83±0.33$^{c}$ | 3.72±1.44$^{c}$ |
| LC 0. 1 mg/ml | 3.05±0.10$^{ab}$ | 2.98±0.11$^{a}$ | 9.12±0.65$^{ab}$ |

Three replications of the experiment were conducted. Results are presented as mean ± SEM
Values with different superscripts in the same column are significantly different (p<0.05).
*tail moment is the result of multiplying the percent of DNA fragmentation by Tail length.

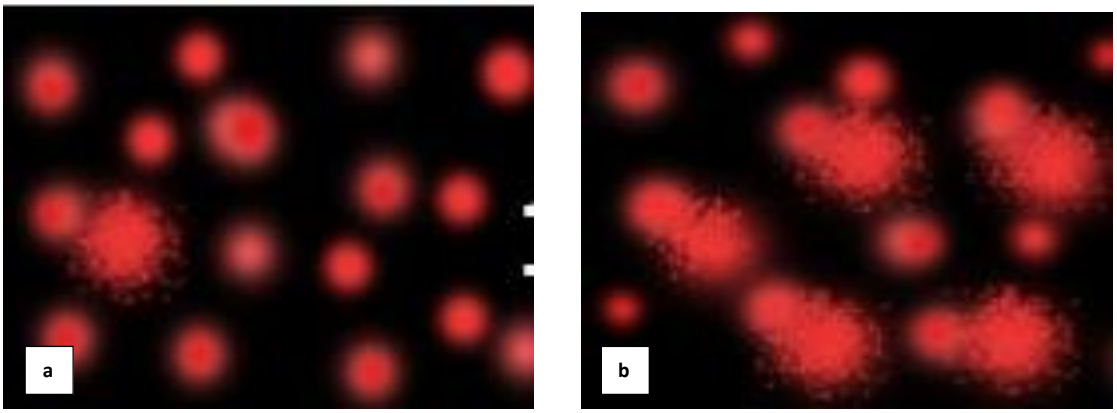


**Fig. 1 (a):** The single cell gel electrophoresis (comet) assay showed reduction in the DNA fragmentation as represented by a limited amount of DNA present in the comet tail of buffalo spermatozoa cryopreserved in freezing extender suplemented with 0.05 l- carnitine. **(b):** The single cell gel electrophoresis (comet) assay showed increased in the DNA fragmentation of the buffalo spermatozoa cryopreserved in control extender.

Data concerning the effect of replenishing of semen extender with L-carnitine on the in vitro fertilizing potentials of buffalo oocytes are presented in Table 5. The current results revealed that, addition of 0.05 mg/ml L-carnitine to the freezing extender resulted in a significant increase (P<0.05) in the in vitro fertilization rate (57.45%) compared with the control treated group (33.33 %, respectively).

**Table (5):** Effect of L-carnitine supplementation to buffalo semen diluent on the in vitro fertilizing capacity

| Treatment | No. oocytes | Penetration rate (%) | Fertilization rate (%) |
|---|---|---|---|
| Control | 39 | 27 (69.23) [a] | 13 (33.33) [a] |
| LC 0.01 mg/ml | 42 | 29 (69.05)[a] | 19 (45.24)[ab] |
| LC 0.05 mg/ml | 47 | 33 (70.21)[a] | 27 (57.45)[b] |
| LC 0. 1 mg/ml | 43 | 30 (64.76)[a] | 17 (39.53)[a] |

Five replications of the experiment were conducted. Percentages are based on the number of oocytes examined. Values with different superscripts in the same column are significantly different (*P<0.05*).
LC :L-carnitine

Table (6) demonstrated that there was significant increase in (P<0.05) in the conception rate of buffalo cows inseminated with frozen semen treated with 0.05 mg/ml ofL-carnitine (61.76%) compared with the control group (37.93%). Furthermore the conception rate after first insemination was high increased (P<0.01) in the 0.05mg/ml L-carnitine treated semen (47.06%) compared with the control semen (13.79%).

**Table (6)**: Effect of L-carnitine addition to buffalo semen diluent on the pregnancy rate

| Treatment | No. of Inseminated buffaloes | First insemination/ conception (%) | Second insemination /conception (%) | Pregnancy rate (%) |
|---|---|---|---|---|
| Control | 29 | 4 (13.79)[a] | 7(24.14)[a] | 11 (37.93) [a] |
| LC 0.05 mg/ml | 34 | 16 (47.06)[b] | 5 (14.71)[b] | 21 (61.77)[b] |

Values with different superscripts in the same column are significantly different (*P<0.05*).
L.C means L-carnitine

## DISCUSSION

L-carnitine is a well-known cellular antioxidant, but there is a paucity of exploration about its protective role during buffalo semen cryopreservation. The present study revealed that addition of 0.05mg/ml L-canrnitine to the semen extender enhanced post-thawing motility, viability of the cryopreserved spermatozoa and significantly reduced acrosomal deterioration. These results are in agreement with Lenzi *et al.* (2003) and Agarwal and Said (2004) who reported that L-carnitine can improve sperm motility and viability.

The beneficial effect of L-carnitine on buffalo sperm cell quality might be attributing to its critical role in improving the energy metabolism. The current results indicated that L-carnitine resulted in a significant increase in ATP concentration. This result coincided with the interpretation that mentioned byKeskin *et al.* (2015) who reported that L-carnitine is a natural nutrient related to B vitamins that is essential for β-oxidation of fatty acids in mitochondria to generate adenosine triphosphate via mitochondrial β-oxidation and subsequent oxidative phosphorylation (Perchec *et al.,* 1995, Parikh *et al.,* 2009 and Flanagan *et al.,* 2010). Furthermore, Liu *et al.* (2002) reported that L-carnitine acted by stabilizing the mitochondrial membrane, protecting the cell from apoptosis and markedly enhanced mitochondrial functions and its general metabolic activity by reducing oxidative stress pathways.

Moreover L-carnitine appears to have other functions which go beyond improving the energy metabolism. The beneficial effects of L- carnitine appear to arise, at least partially, from its antioxidant properties, which include the upregulation of the level and the elevation of the activities of antioxidant enzymes that may protect sperm membranes against toxic reactive oxygen species. Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosomal reaction (Agarwal *et al.,* 2003).Since buffalo spermatozoa contain large amounts of polyunsaturated fatty acids, it is extremely sensitive to lipid peroxidation (Agarwal and Prabakaran 2005). Normally, antioxidants such as superoxide dismutase, glutathione peroxidase, glucose-6- phosphate dehydrogenase enzymes prevent damage to sperm membrane through super oxide and hydrogen peroxide anions breakdown.

In the current study, L-carnitine supplementation to tris extender significantly increased TAC, GPx and SOD levels and reduced MDA concentration in 0.05mg/mlL-carnitine treated group compared with the control group, suggesting that L-carnitine has an antioxidant effect. These increased levels of antioxidants lead to reduced levels of free radicals available for lipid peroxidation, which protect the spermatozoon membranes resulting in an increase in sperm cell viability. These results are in accordance with Steinberg (1995) and Sayed-Ahmed et al. (2001) who reported that L-carnitine prevented the increase in MDA and the decrease in GSH. Dayanandan *et al.* (2001) noted elevation in GSH and GPx levels with carnitine supplementation in rats. Moreover, Rajasekar*et al.* (2005) found significantly increases antioxidant enzyme levels (GSH, SOD, CAT and GPx) of rats treated with L-carnitine compared with untreated rats. In the present study, increase antioxidant enzymes with L-carnitine treatment may be due to enhancement of transport of fatty acids by carnitine into mitochondria for energy production (Dayanandan *et al.,* 2001). In addition to its energy production and antioxidant effects, it has reported that carnitine inhibits the microsomal peroxidation and it has a role in chelating free $Fe_2$+ ions and by this way it reduces free radical generation and improves the overall level and activity of antioxidant enzymes in the cell (Sushama Kumari *et al.,* 1989). Moreover, Pignatelli *et al.* (2003) and Vicari and Calogero (2001) demonstrated that L-carnitine can reduces oxidative stress via interfering with arachidonic acid incorporation into phospholipids and protein kinase *C* mediated NADPH oxidase system as well as potentiate the repairing mechanism.

The current study clarified that L- carnitine has fantastic protecting functions on the DNA integrity of buffalo spermatozoa in a dose dependent trend. The current results indicated that addition of 0.05 mg/ml L-carnitine to sperm freeze extender significantly decreased the rate of DNA damage. These results are in consistent with Abdelrazik *et al.* (2009) who found that; L-carnitine protects the sperm DNA and prevents protein oxidation and lactate oxidative damage. This result may be attributed to the antioxidant effect of l- carnitine, where, oxidative damage can cause base degradation, DNA fragmentation and cross-linking of proteins (Arabi, 2004 and Sharma *et al.,* 2004). In molecular structure of DNA, free radicals can lead to the oxidation of purine and pyrimidine bases, breakage in one or two chromosome strands, formation of positions lacking base, formation of cross-bridges between DNA and protein, and change in deoxyribose sugar. Free radicals can oxidatively attack critical bio-molecules such as DNA and change DNA structure causing infertility (Rehman *et al.,* 2000). Guanine (G) is the most common organic base attacked oxidatively by free radicals and is converted to 8-hydroxyguanin (8-OHG) (Hammadeh *et al.,* 2001).Adding L-carnitine to sperm freeze extender can lead to the protection of DNA and cell membrane against damages caused by oxygen free radicals (Blokhina *et al.,* 2003).

The improvementin the *in vitro* fertilization and conception ratesrecorded in the 0.05mg/ml L-carnitine treated semen might be emphasized by

increased ATP production and antioxidant effect of l-carnitine. These results are consistent with Rizzo *et al*. (2010) and Manzano *et al.* (2015) who reported that LC have a beneficial roles on mammalian embryos metabolism and development; through its contribution in lipid and energy metabolism, as well as its antioxidant effects by enhancing the activity of numerous antioxidant enzymes in addition to its antiapoptotic effect.

However, the current study demonstrated that high LC concentration 0.1mg/ml LC resulted in a drastic effect on semen function and fertilizing potential. This may be due to the cryopreserved spermatozoa at this concentration become more susceptible to the cryotoxic effect of $H_2O_2$ or due to the removal of all oxygen free radicals which is an important mediatorof sperm function (Agarwaland Prabakaran, 2005).

In conclusion the use of 0.05 mg/ml L-carnitine in the freezing extender improves DNA integrity through enhancing the antioxidant defense of buffalo sperm cells and decreasing the rate of lipid peroxidation. Therefore, L-carnitine may improve sperm cryopreservation quality, reduce cryodamage and improve sperm fertilizing potential.

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## دراسة تأثير ل- كارنتين علي قدرة حيامن الجاموس للتجميد ، انزيمات الاكسدة والتغيرات في تركيب المحتوي النووي

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أجري هذا البحث لدراسة تأثير اضافة الكارنتين- ل الي ممدد السائل المنوى علي قابلية حيامن الجاموس للتجميد وعلي الخصائص الكميائية والمحتوي النووي لحيامن الجاموس المجمدة وكذلك اثره علي قدرتها الاخصابية معمليا وحقليا. تم تجميع عينات السائل المنوي من ستة طلائق وبعد تقييم السائل المنوي معمليا تم تمديده في ممدد التريس المضاف اليه تركيزات مختلفة من الكارنتين- ل(٠.٠٥،٠.٠١، ٠.١ ملليجرام/مللي علي التوالي) أو مخفف التريس فقط (المجموعة الضابطة). وبعد تبريد وتجميد الحيامن بالنظام الفرنسي تم تقيمه من حيث نسبه الحركه الأماميه والحيويه وتشوهات القلنسوه وسلامة الغشاء البلازمي. وتم قياس مستوي انزيمات الاكسدة ومعدل اكسدة الدهون وكذا اثر التجميد علي سلامة المحتوي النووي للحيامن وكذلك قدرته الاخصابيه في المعمل والحقل. ولقد أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى الجاموسي في ممدد مضاف اليه الكارنتين- لبتركيز ٠.٠٥ ملليمول نتج عنة زيادة معنوية كبيرة في نسبة الحركة الأمامية بعد الإسالة ومعدل الحيوية ونسبة سلامة القلنسوة (٦٣.٣٣% و١٣٣.٣٣و١٢.٣٣% علي التوالي) مقارنة بالمجموعة الضابطة (٤٣.٣٣% و ٧٤.١٦و٢٤.٦٧% علي التوالي). كما أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى الجاموسي في ممدد مضاف اليه الكارنتين- لبتركيز ٠.٠٥ ملليمول نتج عنة زيادة معنوية كبيرة في مستوي مضادات الاكسدة الكلية وانزيم سوبر اوكسيد ديسميوتيزو انزيم الجلوتاثيون بيروكسيداز(٠.٥٠ mµ/mL و٧٣.٦٧ U/mLو١٠٦.٦٦ U/L علي التوالي) مقارنة بالمجموعة الضابطة (٠.١٩ mµ/mL و ٢٧.٣٣U/mLو٥٢.٣٣U/L علي التوالي) كما نتج انخفاض معنوي كبيرة في معدل أكسده الدهون (١١.٠٠nmol/mL) مقارنة بالمجموعة الضابطة (٢٤.٨٢ nmol/mL). كما حافظ علي سلامة الحامض النووي للحيامن حيث قلل من تشظي الحامض النووي وطول ذيل المذنب وكثافته (١.٨٧% و١.٨٣ µm و٣.٧٢علي التوالي) بالمقارنة بالمجموعة الضابطة (٣.٤٧% و٣.٤٨ µm و١١.٩١علي التوالي). كما اوضحت النتائج ارتفاع نسبة الاخصاب المعملي (٥٧.٤٥) ونسبة الاخصاب في الحقل(٦١.٧٦) باستخدام السائل المنوي المجمد باضافة الكارنتين- ل بتركيز ٠.٠٥ملليجرام/ملليمقارنة بالمجموعة الضابطة (٣٣.٣٣و ٣٧.٩٣% علي التوالي). ومن خلال نتائج الدراسة الحالية يمكن أن نستنتج أن اضافة الكارنتين- ل بتركيز ٠.٠٥ ملليجرام/ملليلعب دوراهاما وكبيرا في تحسين وظائف السائل المنوي الجاموسي المجمد ويبدو ذلك من خلال قدرتها علي المحافظة علي سلامة تركيب المحتوي النووي لحيامن الجاموس المجمد من خلال التقليل من معدل اكسدة الدهون وارتفاع تركيز انزيمات الاكسدة.