Assiut University web-site: www.aun.edu.eg

L-CARNITINE ENHANCING ROLES ON BUFFALO SEMEN FREEZABILITY, ULTRA STRUCTURE AND FERTILIZING POTENTIALS

MOHAMED EL-RAEY [1]; BADR MR [2]; ASSI MM[2] and RAWASH ZM [2]
[1] Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, P.O. 13736, Tokh, Kaliobia.
[2] Artificial Insemination and Embryo Transfer Department, Animal Reproduction Research Institute, Al Haram (P. O. 12556), Giza, Egypt.

Received: 31 March 2016; **Accepted:** 30 April 2016

ABSTRACT

Recently, male impact has been recognized as a momentous cause of infertility. Male infertility isn't an entity but it evokes a variety of mechanisms and explanations to understand it. In between, a biochemical semen additive that improves or overcome certain semen situations so can enhance or treat semen fertility and infertility, respectively. 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 samples were obtained from six fertile buffalo bulls (aged 3 to 5 years). Weekly, two consecutive ejaculates were collected from each bull for successive six weeks using artificial vagina. The ejaculates were pooled to eliminate samples variability. Semen samples were extended 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). Then they were processed to cryopreservation and thawing to assess different semen characteristics. It had been found that L-carnitine (0.05mg/ml) significantly ($p<0.05$) improved post thawing motility, viability index, acrosomal integrity, *in vitro* fertilization, blastocyst and conception rates of treated buffalo semen (57.00±2.55%, 126.5±8.33, 14.40 ± 1.43%, 56.45%, 15.79% and 64.71%, respectively), compared with control (42.00±2.56%, 87.00±10.43, 23.80±1.86%, 36.53%, 2.13% and 42.86%, respectively). *Conclusion:* L-carnitine supplementation to buffalo semen extender significantly enhanced its characteristics and protecting its plasma membrane and mitochondrial functional integrity. Moreover, the preceding results focusing more light on the potential roles of L-carnitine in regulating male fertility and infertility.

Key words: *L-Carnitine, buffalo, frozen, semen, quality, Conception rate*

INTRODUCTION

Infertility is a significant and worrisome problem in veterinary practices especially for valuable animals. Male infertility represented about 30% to 50% of infertility cases (Aliabadi *et al.*, 2013). Less than a decade ago, treatment for severe male factors was limited to artificial inseminations or IVF using donor sperm. Today, promising advances in male infertility handling have been introduced to innovative new therapeutic options that offer males a greatly improved chance to conceive their own biological offspring.

Semen cryopreservation is one of the most effective and acceptable methods to enhance and maintain male fertility potential. But unfortunately, despite its long-proven value for the optimization of male genetic potential, the *in-vitro* semen preservation in domestic buffalo bulls entries some hazardous that affects its breeding potentials. Where, *In-vitro* procedures are known to decrease the sperm quality. Particularly, sperm motility and viability were decreased over time after ejaculation (Singer *et al.*, 1980). Moreover, it had been reported that rapid sperms cooling resulted in loss of energy supply and motility due to the reduction in the rate fructose breakdown, ATP synthesis and oxygen uptake (Blackshaw and Salisbury 1957 and Wales and White 1959); resulting in reduction in the sperm fertilizing capacity (Arabi, 2004).

Poly unsaturated fatty acids (PUFA) are a basic component of buffalo and 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 (Kothari *et al.*, 2010; Guthrie and Welch, 2012 and Sarica *et al.*, 2007). Additionally, there is considerable evidence that the lipid composition of sperm membrane is a major determinant to cold sensitivity, motility, and

Corresponding author: Dr. BADR MR
E-mail address: magdybadr69@yahoo.com
Present address: Artificial Insemination and Embryo Transfer Department, Animal Reproduction Research Institute, Al Haram (P. O. 12556), Giza, Egypt.

overall viability of spermatozoa (Kelso *et al.,* 1997). In this respect, it had been found that buffalo sperms were prone more to lipid peroxidation if compared with bull sperm because it was rich in the amount of polyunsaturated fatty acids (Garg *et al.,* 2009 and Nair *et al.,* 2006). Lipid peroxidation during *in-vitro* handling and sperm storage, are probably the primary causes of this fertility dysfunction (Aitken *et al.,* 1989 and Cecil and Bakst 1993).

Exposure of buffalo sperm cells membrane to the reactive oxygen species (ROS) generated during the sperm processing techniques showed decreased membrane integrity, reduced motility, abnormal morphology, increased permeability and leakage of intra cellular enzymes, and lowered penetration capacity mainly due to lipid peroxidation (Aitken 1995; Wang *et al.,* 1997 and Potts *et al.,* 1999); leading to relatively higher freeze-thaw associated damage, lower post thaw motility and conception rate (Kadirvel *et al.,* 2009). The redox system in the spermatozoa can regulate the level of these ROS; so in turn, improve fertilization process (Leclerc *et al.,* 1997). In other words, the presence of efficient antioxidant system or materials during the process of semen handling can protect it against this peroxidative damage and dysfunction; so increasing its lifespan *in- vitro* for artificial insemination (Cecil and Bakst 1993 and Sarica *et al.,* 2007).

Active L isomer of carnitine (β-OH-γ-N-trimethylaminobutyric acid) is a water-soluble, highly polarized vitamin like amino acid (Bremer 1983 and Vav and Wanders 2002). L-Carnitine (LC) is one of powerful antioxidants. LC is an essential cofactor or stimulator that functions by reducing the availability of lipids for peroxidation by accelerating the transportation of fatty acids into the mitochondria for β-oxidation to generate ATP which is an important fuel source for sperm motility (Kalaiselvi and Panneerselvam 1998; Matalliotakis *et al.,* 2000; Rani and Panneerselvam 2002 and 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 (Neuman *et al.,* 2002 and Sarica *et al.* 2007). Moreover, carnitine protects the sperm DNA and prevents protein oxidation and lactate oxidative damage (Arduini, 1992). Additionally, LC was to fulfill vital roles in sperm maturation and metabolism when passing through the epididymis (Jeulin and Lewin 1996 and Yakushiji *et al.* 2006). Where, L-carnitine was found to be contributed directly in ram and stallion sperm cells motility, so might be involved in the successful sperm maturation (Goa and Brodgen, 1987 and Lenzi *et al.,* 1992).

On the level of semen processing it had been reported that LC addition in extended semen of boar and chicken can improve the sperm cell quality including motility, viability, and morphological defect rates after semen incubation at different periods (Tabatabaei and Aghaei, 2012 and Kozink *et al.,* 2004).

Finally, considering the harmful effects of semen processing technology through ROS production; the present study was applied to standardize the LC doses that can be used safely in buffalo semen extenders during its processing for combating the processing hazards as well as enhancing its fertilizing potentials.

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 (C-0283, Sigma Co.).

Semen Collection

Semen samples were obtained randomly from six fertile Egyptian 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 six 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, 800×10^6 sperm cells/mL and >85% normal sperm morphology were used for the present study. All experiments were done with at least 5 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 120×10^6 sperm/mL. The required L-carnitine concentrations (0.01, 0.05 and 0.1 mg/mL) were achieved using serial dilution manner. The current work used a broad range of L-carnitine concentrations to determine the ideal dose that should be used for buffalo semen extension. 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 for 24h before evaluation. Frozen semen straws were thawed in water bath at 37°C for 30 sec. Post-thawing sperm motility; viability and acrosomal integrity were assessed according to Mohammed *et al.* (1998).

Analysis of Sperm Motility

The percentage of linear motile sperm was examined visually. For each extender, 3 straws were thawed separately by immersion in a water bath at 37°C for 30 sec. The sperm samples were placed on glass slides, covered with a glass cover and then estimated at 37°C by phase contrast microscope equipped with a warm stage at 200× magnifications. Sperm motility estimations were performed in 3 different microscopic fields and the mean of the 3 successive estimations was recorded as the final motility score.

Analysis of Acrosomal Membrane Integrity

Acrosomal integrity was assessed using silver nitrate stain in a procedure slightly modified from the method described by Chinoy *et al.* (1992). The sperm suspension was spread over slides and dried at room temperature. The preparations were fixed in ethyl alcohol 70% for 2 min and in ethyl alcohol 95% for another 2 min. The preparations were stained with the solution (AgNO3) for 2 h in an incubator at 65°C, in complete humidity. After the preparations turned gold in colour, the chemical reaction was interrupted and the preparations were rinsed several times with distilled water. The preparations were dried at room temperature. The dried preparations were analyzed for acrosomal integrity using the Olympus BX50 light microscope with a 100-fold magnification. At least 300 sperm cells were counted per slide and the percent of acrosome intact spermatozoa was calculated.

Ultra structure analysis of the cryopreserved spermatozoa

The ultra-structure changes occurred for the cryopreserved buffalo spermatozoa were evaluated by transmission electron microscopy (TEM) at VACSERA- Electron Microscopy Unit (Dokki, Egypt). Straws from each treatment were washed three times by centrifugation at 1000 rpm for 5 min using PBS (Phosphate Buffered Saline). The frozen-thawed semen was prefixed for 2-3 h with PBS containing 2% glutaraldehyde, washed three times by centrifugation at 1000 rpm with PBS (pH 7.4) for 5 min at 4°C and post-fixed in 1% osmium tetroxide for 1-2 h at 4°C (Boonkusol *et al*, 2010). Spermatozoa were dehydrated in propylene oxide and embedded in epon resin. Ultrathin sections were cut using the Leica EM UC6 ultra microtome and stained with uranylacetate and lead citrate. Randomly fields were examined by a transmission electronic microscope (JEOL-EM-100 S at 80 Kv at VACSERA- Electron Microscopy Unit) (Dokki, Egypt) and photographed for further analysis.

In-vitro Maturation

Immature buffalo oocytes were collected from fresh ovaries just after slaughter at a local abattoir. Cumulus-Oocyte Complexes (COCs) were collected by aspiration of medium-sized (2-8 mm) ovarian follicles using 18-gauge needle attached to a disposable 10 mL syringe. Evenly granulated oocytes surrounded with multi-layered compact cumulus cells were selected for the experiments. Selected COCs were firstly washed three times in sterile D-PBS and then washed three times in IVM medium; IVM was performed in TCM-199 medium (Earl's salt, Sigma Chemical CO., St. Louis, Mo., USA) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco, 30 K- 0351), 10 μg mL^{-1} Luteinizing hormone, 5 μg mL^{-1} follicle stimulating hormone and 1 μg mL^{-1} estradiol-17β. The oocytes were cultured for 24 h at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity (El-Raey *et al.*, 2014).

In-vitro Fertilization

Three straws from each L-carnitine treatment were thawed in a water bath at 37°C for 30 sec. The most motile spermatozoa were separated by swim up technique in the fertilization medium, modified Tyrode's Albumin-Lactate- Pyruvate (TALP) containing 6 mg mL^{-1} Bovine Serum Albumin (BSA), for 1 h as recorded by Parrish *et al.* (1988). The uppermost layer of the medium containing the most motile spermatozoa was collected and washed twice by centrifugation at 800 xg for 10 min. The sperm pellet was re suspended in the fertilization TALP medium containing 10 μg mL^{-1} heparin. After appropriate dilution, 2 μL (final concentration 2×106 sperm cell/mL) of sperm suspension was added to the fertilization drops, containing in vitro matured oocytes. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 h at 39°C in an atmosphere of 5% CO_2 in air with complete humidity.

Evaluation of in vitro Fertilizing Potentials

The oocyte fertilization rate was examined on the base of presence of male and female pronuclei in the oocyte after fertilization. In brief, *in vitro* oocytes were incubated with the sperm for 18 h at 5% CO_2 and 20% O_2 in humid incubator; after that the oocytes were subjected to denudation with perfect handmade pipette. Then they were cultured for 10~12 h in CR1aa medium. They were fixed for 24~48 h in ethanol: acetic acid (3:1 v/v), then stained with 1% Orcein dissolved in 45% acetic acid according to Marei *et al.* (2009). 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, while the penetration rate was evaluated by the presence of sperm tail only in the ooplasm.

In-vitro Culture

Denuded zygotes were transferred into SOFaa culture medium (SOF with 1 mM glutamine, 1% MEM nonessential amino acids and 1% MEM essential amino acids) according to Badr (2009) and then covered with mineral oil for 7days at 38.5°C in an atmosphere of 5% CO_2 in air with maximum humidity. The cleaved oocytes were recorded after 48 h of insemination and those developed to the morula and blastocyst stages were recorded at 5-7 days post-insemination according to Totey *et al.* (1992).

Evaluation of fertilizing potentials of treated semen (field study).

A preliminary fertility trial was performed to compare between control semen and 0.05mg/ml L-carnitine treated semen. Buffalo cows were randomly assigned to one of the chosen groups: group 1 (42 buffaloes) was inseminated using control semen; group (2) (51 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 Software 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 sperm motility, viability index, acrosomal integrity, 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 and zygotes developmental competences were assessed using Chi-square at ($p<0.01$ *and* 0.05, respectively).

RESULTS

As shown in Table (1), there was a significant ($P<0.05$) increase in post thawing motility of buffalo semen treated by 0.01 and 0.05 mg /ml of L-carnitine (50.00±3.54 and 57.00 ±2.55%, respectively), additionally the viability index of the same previous concentrations showing a significant ($P<0.05$) increase (107.00±11.74 and 126.5±8.33, respectively), compared with the control group (42.00±2.56 % and 87.00± 10.43, respectively). Moreover, there was significant ($P<0.05$) reduction in the acrosomal abnormalities of buffalo semen treated 0.01 and 0.05 mg /ml of L-carnitine (19.80±2.08 and 14.40± 1.43%, respectively) compared with the control group (23.80±1.86 %); reflecting that both those concentration have beneficial protecting effect on buffalo semen post thawing characteristics than other concentrations (control and 0.1 mg/ml L-carnitine). Where, high concentration of L-carnitine (0.1 mg/ml) significantly ($P<0.05$) decrease in the post-thawing motility, viability index and acrosomal abnormalities (39.00± 4.01%, 77.5±9.77 and 22.40±2.11 %, respectively).

Table 1: Effect of L-carnitine supplementation to buffalo semen diluent on sperm characteristics.

Treatment	Dilution motility	Cooling motility	Post-thawing motility (%)	Viability index*	Acrosomal integrity (%)
Control	78.00±2.59^{a}	74.00±3.32^{a}	42.00±2.56 ^{bc}	87.00± 10.43 ^{b}	23.80±1.86 ^{a}
LC 0.01 mg/ml	78.00±3.01^{a}	74.00±1.84^{a}	50.00± 3.54 ^{ab}	107.00±11.74 ^{ab}	19.80±2.08 ^{ab}
LC 0.05 mg/ml	82.00±2.00^{a}	75.00±1.58^{a}	57.00±2.55^{a}	126.5±8.33 ^{a}	14.40± 1.43 ^{b}
LC 0. 1 mg/ml	76.00±2.91^{a}	70.00±3.54^{a}	39.00± 4.01 ^{c}	77.5±9.77^{b}	22.40±2.11 ^{a}

- Five replications of the experiment were conducted.
- Results are presented as mean ± SEM
- Values with different superscripts in the same column are significantly different at ($P<0.05$).
- LC means L-carnitine

*Viability index = post-thawing motility/2 + motility after 1 h +motility after 2 h + motility after 3 h

Looking through Table (2) of the effect of L-carnitine supplementation to buffalo semen diluent on the *in vitro* fertilizing capacity, founding that 0.05 mg/ml of L-carnitine treatment resulted in a significant increase ($P<0.05$) in the *in vitro* fertilization rate (56.45%) compared with the control and 0.1mg/ml L-carnitine treated groups(36.53 and38.59%, respectively) . Indicating that, 0.05 mg/ml of L-carnitine is an ideal concentration enhancing both sperm penetration and fertilization rate.

Table 2: Effect of L-carnitine supplementation to buffalo semen diluent on the in vitro fertilizing capacity.

Treatment	No. oocytes	Penetration rate (%)	Fertilization rate (%)
Control	52	32 (61.53) [a]	19 (36.53) [a]
LC 0.01 mg/ml	56	34 (60.71)[a]	24 (42.86)[ab]
LC 0.05 mg/ml	62	40 (64.52)[a]	35 (56.45)[b]
LC 0. 1 mg/ml	57	33 (57.89)[a]	22 (38.59)[a]

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

Table (3) displaying that addition of 0.05 mg/mL LC to the semen extender resulted in significant increase (*P<0.05*) in the morula and blastocyst rate (26.32 and 15.79%, respectively) compared with control (8.51 and 2.13%, respectively) and 0.1mg/ml LC treated group (6.97 and 2.32%). In brief, buffalo semen treatment with L-carnitine during preparation for cryopreservation process resulting in potent enhancement in most of the sperm characteristics that finally improving the rate of blastocyst harvesting.

Table 3: Effect of L-carnitine supplementation to buffalo semen diluent on resultant zygotes developmental competences.

Treatment	No. oocytes	Cleavage Rate (%)	Morula stage (%)	Blastocyst stage (%)
Control	47	15 (31.91) [a]	4 (8.51) [a]	1 (2.13) [a]
LC 0.01 mg/ml	41	14 (34.15)[a]	8 (19.51)[ab]	4 (9.75)[ab]
LC 0.05 mg/ml	38	17 (44.73)[a]	10 (26.32) [b]	6 (15.79)[b]
LC 0. 1 mg/ml	43	13 (30.23)[a]	3 (6.97)[a]	1 (2.32)[a]

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

Table (4) 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 of L-carnitine (64.71%) compared with the control group (42.86%). In brief, buffalo semen treatment with L-carnitine (0.05 mg/ml) during cryopreservation process resulting in potent enhancement in the progeny yield.

Table 4: Effect of L-carnitine supplementation to buffalo semen diluent on the conception rate.

Treatment	No. of Inseminated buffaloes	Conception rate (%)
Control	42	18 (42.86) [a]
LC 0.05 mg/ml	51	33 (64.71)[b]

- [a,b]Values with different letters superscripts in the same column are significantly different (*P<0.05*).
- L.C means L-carnitine

Effect of L-carnitine supplementation during buffalo semen extension on its ultra structure properties

Electron microscopic images of sagital sections of the frozen thawed buffalo sperm cells in the control group showed, swollen plasma membrane segmentation of the outer acrosomal membrane and swollen acrosome (Fig, 1and 2), severe degeneration and marked vacuolation in the mitochondria with complete absence of the transverse cristae (Fig,5 and 6). Mean while, the frozen thawed buffalo semen treated with 0.05 mg/ml L-carnitine illustrated a well defined , intact plasma membrane and intact outer and inner acrosomal membranes (Fg. 3 and 4), homogenous mitochondria content and high-quality mitochondrial dense electron spaces with appeared transverse cristae (Fig, 7 and 8).

Fig. (1) **Fig. (2)**

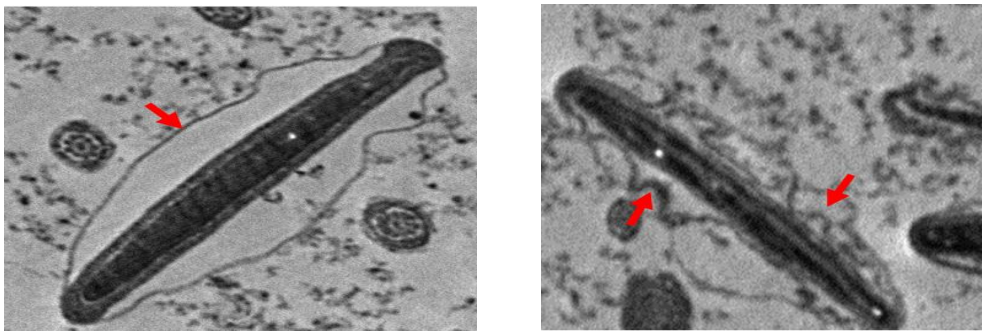

Fig. 1 & 2: Electron micrograph for a sagital section in the sperm head from a frozen-thawed semen sample of control group illustrating swollen, degenerated and vacuolated plasma membrane, segmentation of the outer acrosomal membrane (OAM) and swollen acrosome (× 14000).

Fig. (3) **Fig. (4)**

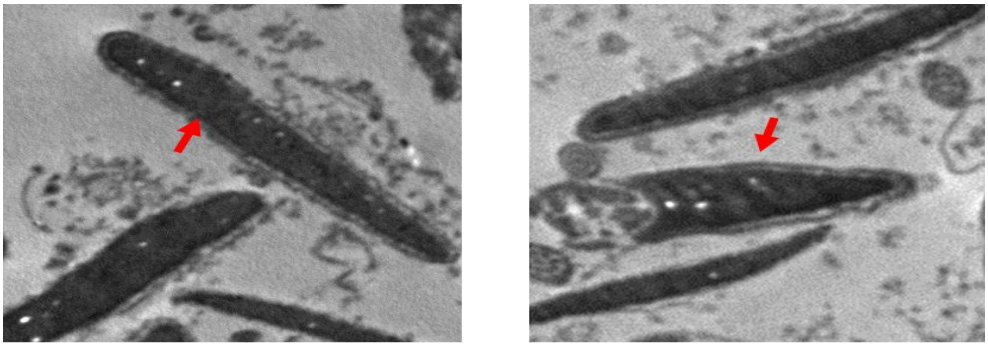

Fig. 3&4: Electron micrograph for a sagital section in the sperm head from frozen-thawed semen sample treated L-Carnitine (0.05 mg/ml) illustrating intact plasma membrane (PM) and the nucleus content (N) is homogenous in the electron density. Also, outer and inner acrosomal membranes are intact and the subacrosomal space is evident (× 2000). Also electron micrograph for a sagittal section in the sperm from a frozen-thawed semen sample illustrating intact outer acrosomal membrane (OAM) inner acrosomal membrane (IAM) in the electron density (×10000).

Fig. (5) **Fig. (6**

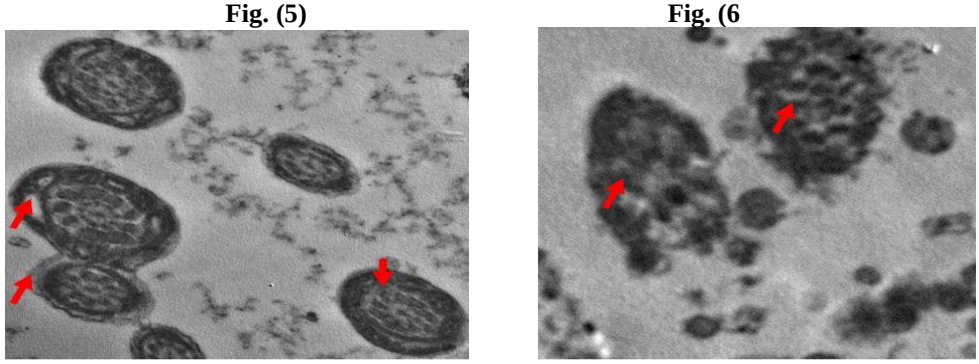

Fig: 5&6: Electron micrograph of a cross section in the neck region (note the presence of mitochondria in different orientation) of sperm from a frozen-thawed semen sample of control group showing severe degeneration (marked vacuolation) in the mitochondria that contained electron-translucent spaces with complete absence of the transverse cristae and some mitochondria are completely disappeared (× 20000).

Fig. (7)

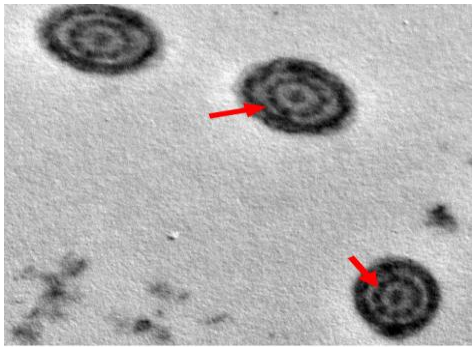

Fig. (8)

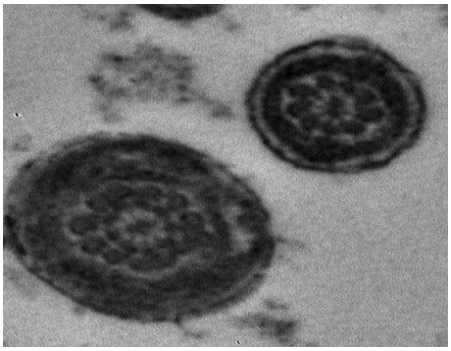

Fig: 7&8: Electron micrograph of a cross section in the mid-piece region and the tail from a frozen-thawed semen sample treated with 0.05 mg/ml L-Carnitine, illustrating good mitochondrial dense electron spaces with appeared transverse cristae (×25000).

DISCUSSION

L-carnitine is a well-known cellular antioxidant, but there is scarcity of information about its protective effects on buffalo semen quality parameters. Where, by looking through Table (1) it had been found that L-canrnitine (0.05mg/ml) supplement to buffalo semen extender can enhance its post-thawing motility (57.00±2.55%). This result came in agreement with Juelin and Lewin (1996); Vicari and Calogero (2001); Lenzi *et al.* (2003), who reported that L-carnitine can improve sperm motility and viability. In fact, this L-carnitine potentiating role on buffalo sperm cell motility might be attributing to its critical role in the process of mitochondrial β-oxidation and the esterification of free fatty acids; these esters are oxidized to acetyl CoA, which enters the Krebs cycle resulting in ATP generation via oxidative phosphorylation (Parikh *et al.,* 2009). It known well since many years that, ATP generated by mitochondrial respiration is the main the sperm movement energizer (Perchec *et al.,* 1995). Furthermore, Mazzilli *et al.* (1999) established strict correlation between intra sperm L-carnitine content and sperm motility; thereby L-carnitine content can be considered as an indicator of sperm motility life span.

Moreover, Table (1) showed that L-canrnitine (0.05mg/ml) supplements to buffalo semen extender can improve its viability index (126.5±8.33), and preserve its acrosomal integrity (14.40± 1.43%). These results came in harmony with Agarwal and Said (2004) who stated that carnitine promotes membrane stability. In fact, this vital role might be attributed to its antioxidant properties that protect sperm membranes against toxic reactive oxygen species.

In general, ROS have beneficial or detrimental effects on sperm functions depending on nature, concentration, location as well as length of exposure to ROS (Agarwal and Saleh 2002).

Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosomal reaction (Agarwal *et al.,* 2003). Since plasma membrane and cytoplasm contain large amounts of polyunsaturated fatty acids; mammalian spermatozoa are vulnerable to excessive amount of ROS that produced from dead, abnormal sperm cell as well as leukocytes content (Agarwal and Prabakaran 2005). Pignatelli *et al.* (2003) and Vicari and Calogero (2001) demonstrated that 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.

Table (2) demonstrating that L-canrnitine (0.05mg/ml) supplements to buffalo semen extender can improve its *in vitro* penetration rate and fertilization rate (64.52 and 56.45%, respectively). These results might be attributed again to enhanced ATP production beside its role as antioxidant (discussed above). Where, Oxidative damage can cause base degradation, DNA fragmentation and cross-linking of proteins (Sharma *et al.,* 2004). Spermatozoa with damaged DNA lose their ability to penetrate and fertilize the oocyte (Sun *et al.,* 1997).

Table (3) illustrating that L-canrnitine (0.05mg/ml) supplements to buffalo semen extender can significantly improve cleavage rate, morula, and blastocyst stage (44.73, 26.32 and 15.79 %, respectively). These results came in disagreement with Phongnimitr *et al.* (2013) who reported that L-carnitine supplementation to IVF media had no effect on development to the blastocyst stage of IVM oocytes treated with 0.6 mg/mL L-carnitine. This disagreement might be attributed to dose effect as well as the difference in the system of L-carnitine supplementation. However, Ferguson and Leese (2006); Abdelrazik *et al.* (2009); Rizzo *et al.* (2010) and Manzano *et al.* (2015) 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. So, and in accordance with the current study results; treatment of buffalo semen extender with L-carnitine (0.05 mg/ml) not only preserve the sperm cell quality parameters but also it potentiates developmental potential of resultant blastocysts and this clearly reflected in Table (3 and 4).

Figures (1to 8) for the effect of L-canrnitine (0.05mg/ml) supplements to buffalo semen extender on its elemental structural stability, showing that L-carnitine protecting buffalo sperm cell plasma and acrosomal membranes form degenerative changes that noted clearly with non L-carnitine treated semen samples that showing swollen, degenerated and vacuolated plasma membrane, outer acrosomal membrane segmentation, as well as acrosomal swelling. These results could be attributed depending on its primary antioxidant capacity (prevent formation of ROS and scavenging them) of L-carnitine as well as its secondary antioxidant capability by repairing oxidized polyunsaturated fatty acids esterified in membrane phospholipids so protecting living cells plasma membranes (Liu *et al*., 2004). Moreover, L-carnitine buffalo semen treatment provides stabling and protection for buffalo sperm mitochondria from degenerative changes such as vacuolation and absence of its transverse cristae. These results came in harmony with Hagen *et al.* (1998) and Liu *et al.* (2002) who 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. Furthermore L-carnitine facilitates entry of long-chain fatty acids into mitochondria for utilization as energy and facilitates removal of short and medium chains of fatty acids accumulate in the mitochondria as a result of normal and abnormal metabolism and inducing mitochondrial aging (Arduini, 1992).

CONCLUSION

The present data demonstrated that the presences of L-carnitine (0.05 mg/ml) in the extension medium not only preserve the sperm cell quality parameters but also it potentiates its fertilizing capacity, and resultant blastocysts developmental potential. The current study results suggested that this enhancing role of L-carnitine on the cryopreserved buffalo spermatozoa is mainly due to preserving its plasma membrane and mitochondrial functional integrity.

REFERENCES

Abdelrazik, H.; Sharma, R.; Mahfouz, R. and Agarwal, A. (2009): L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. Fertil Steril; 91: 589-596.

Agarwal, A. and Prabakaran, SA. (2005): Oxidative stress and antioxidants in male infertility: a difficult balance. Iranian J. Reprod Med; 3 (1): 1-8.

Agarwal, A. and Said, TM. (2004): Carnitines and male infertility. Reprod Biomed Online; 8: 376-384.

Agarwal, A. and Saleh, RA. (2002): Role of oxidants in male infertility: rationale, significance, and treatment. Urol. Clin. North Am; 29: 817-827.

Agarwal, A.; Saleh, RA. and Bedaiwy, MA. (2003): Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril; 79: 829-843.

Aitken, RJ. (1995): Free radicals, lipid peroxidation and sperm function. Reprod Fertil Dev 7: 659-680.

Aitken, RJ.; Clarkson, JS.; Hargreave, TB.; Irvine, DS. and Wu, FC. (1989): Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoosperm. J. Androl 10: 214-220.

Aliabadi, E.; Karimi, F.; Rasti, M.; Akmali, M. and Esmaeilpour, T. (2013): Effects of L-carnitine and Pentoxifylline on the Activity of Lactate Dehydrogenase C4 isozyme and Motility of Testicular Spermatozoa in Mice. J Reprod Infertil; 14(2): 56-61.

Arabi, M. (2004): nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. J. andrologia; 36: 305-310.

Arduini, A. (1992): Carnitine and its acyl esters as secondary antioxidants? Am. Heart J.; 123: 1726-1727.

Badr, MR. (2009): Effects of supplementation with amino acids on in vitro buffalo embryo development in defined culture media. Global Veterinaria; 3: 407-413.

Blackshaw, AW. and Salisbury, GW. (1957): Factors influencing metabolic activity of bull spermatozoa 11: cold-shock and its prevention. J. Dairy Sci; 40: 1099-1106.

Boonkusol, D.; Saikhun, K. and Ratanaphumma, P. (2010): Effect of extender and storage time on motility and ultrastructure of cooled-preserved boar spermatozoa. Kasetsart J. Nat Sci; 44: 582-589.

Bremer, J. (1983): Carnitine metabolism and functions. Physiol Rev; 63: 1420-1480.

Cecil, HC. and Bakst, MR. (1993): In vitro peroxidation of turkey spermatozoa. Poult Sci; 72: 1370-1378.

Chinoy, NJ.; Ranga, GM.; Highland, HN.; D'Souza, KJ. and Sequeira, E. (1992): A modified method for the differential staining of spermatozoa using alcoholic acidic silver nitrate. Int J Fertil; 37: 232-236.

El-Raey, M.; Badr, MR.; Rawash, ZM. and Darwish, GM. (2014): Evidences for the role of melatonin as a protective additive during buffalo semen freezing. American Journal of Animal and Veterinary Sciences; 9 (4): 252-262

Ferguson, EM. and Leese, HJ. (2006): A potential role for triglyceride as an energy source during bovine oocyte maturation and early- embryo development. Mol Reprod Dev; 73: 1195-1201.

Garg, A.; Kumaresan, A. and Ansari, MR. (2009): Effects of hydrogen peroxide (H_2O_2) on fresh and cryopreserved buffalo sperm functions during incubation at 37 degrees C in vitro. Reprod in Domest Anim; 44(6): 907-912.

Goa, KL. and Brodgen, RN. (1987): L-carnitine preliminary review of its pharmacokinetics and its therapeutic use in ischemic cardiac disease and primary and secondary carnitine deficiencies in relationships to its role in fatty acid metabolism. Drug; 34: 1-24.

Guthrie, HD. and Welch, GR. (2012): Effects of reactive oxygen species on sperm function. Theriogenology; 78: 1700-1708.

Hagen, TM.; Ingersoll, RT.; Wehr, CM.; Lykkesfeldt, J.; Vinarsky, V.; Bartholomew, JC.; Song, MH. and Ames, BN. (1998): Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. Proc Natl Acad Sci; 95: 9562-9566.

Jeulin, C. and Lewin, LM. (1996): Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. Hum Reprod Update; 2: 87-102.

Kadirvel, G.; Kumar, S. and Kumaresan, A. (2009): Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Anim Reprod Sci; 114(1-3): 125-134.

Kalaiselvi, T. and Panneerselvam, C. (1998): Effect of L-carnitine on the status of lipid peroxidation and antioxidants in aging rats. J Nutr Biochem; 9: 575-581.

Kelso. KA.; Redpath, A.; Noble, RC. and Speake, BK. (1997): Lipid and antioxidant changes in spermatozoa and seminal plasma throughout the reproductive period of bulls. J. Reprod Fertil; 109: 1-6.

Kothari. S.; Thompson, A.; Agarwal, A. and Plessis, SSD. (2010): Free radicals: Their beneficial and detrimental effects on sperm function. Indian J. Exp Biol; 48(5): 526-435.

Kozink, DM.; Estienne, MJ.; Harper, AF. and Knight, JW. (2004): Effects of dietary L-carnitine supplementation on semen characteristics in boars. Theriogenology; 61: 1247-1258.

Leclerc, E.; de Lamirande, E. and Gagnon, C. (1997): Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free RadicBiol Med; 22: 643-656.

Lenzi, A.; Lombardo, F.; Gandini, L. and Dondero, F. (1992): Metabolism and action of L-carnitine: Its possible role in spem tail function. Arch Ital Urol Nefrol Androl; 64(2): 187-196.

Lenzi, A.; Lombardo, F.; Sgro, P.; Salacone, P.; Caponecchia, L.; Dondero, F. and Gandini, L. (2003): Use of carnitine therapy in selected cases of male factor infertility: a double-blind crossover trial. Fertil Steril; 79: 292-300.

Liu, J.; Head, E.; Gharib, AM.; Yuan, W.; Ingersoll, RT.; Hagen, TM.; Cotman, CW. and Ames, BN. (2002): Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: Partial reversal by feeding acetyl-L-carnitine and/or R-alpha lipoic acid. Proc Natl Acad Sci; 19, 99 (4): 2356-2361.

Liu, J.; Head, E.; Kuratsune, H.; Cotman, CW. and Ames, BN. (2004): Comparison of the Effects of L-Carnitine and Acetyl-L-Carnitine on Carnitine Levels, Ambulatory Activity, and Oxidative Stress Biomarkers in the Brain of Old Rats. Ann NY Acad Sci; 1033: 117-131.

Manzano, PCVJ.; Ocampo, MB.; Ocampo, LC.; Maylem, ERS. and Lazaro, JV. (2015): Improved Bovine Blastocyst Developmental Potential by L-carnitine Supplementation. IJSK, 3(1): 021-029.

Marei, WF.; Wathes, DC. and Fouladi-Nashta, AA. (2009): The Effect of linolenic acid on bovine oocyte maturation and development. Biol. Reprod; 81: 1064 -1072.

Martino, A.; Palomo, MJ.; Mogas, T. and Paramio, MT. (1994): Influence of the collection technique of prepubertal goat oocytes on in vitro maturation and fertilization. Theriogenology; 42: 859-873.

Matalliotakis, I.; Koumantaki, Y.; Evageliou, A.; Matalliotakis, G.; Goumenou, A. and Koumantakis, E. (2000): L-Carnitine levels in the seminal plasma of fertile and infertile men: correlation with sperm quality. Int J Fertil; 45: 236-240.

Mazzilli, F.; Rossi, T.; Ronconi, C.; Germini, B. and Dondero, F. (1999): Intra-spermatic L-carnitine and survival of sperm motility. Minerva Ginecol; 51: 129-134.

Mohammed, KM.; Ziada, MS. and Darwish, GM. (1998): Practical trials for freezing semen of buffalo and Friesian bulls: Effect of various regimens of freezing, different milk extenders and types of straws packages on post-thawing semen characters. Assiut Vet. Med. J.; 39 (77):70- 93.

Nair, JS.; Brar, AS.; Ahuja, CC.; Sangha, SPS. and Chaudhary, KC. (2006): A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo

bull spermatozoa during storage at refrigeration temperature. Anim Reprod Sci; 96: 21-29.

Neuman, SL.; Lin, TL. and Hester, PY. (2002): The effect of dietary carnitine on semen traits of white leghorn roosters. Poult Sci; 81: 495-503.

Parikh, S.; Saneto, R.; Falk, MJ.; Anselm, I.; Cohen, BH. and Haas, R. (2009): A Modern Approach to the Treatment of Mitochondrial Disease. Current Treatment Options in Neurology; 11, 414-430.

Parrish, JJ.; Susko-Parrish, J.; Winer, MA. and First, NL. (1988): Capacitation of bovine sperm by heparin. Biol. Reprod; 38:1171-1180.

Perchec, G.; Jeulin, C.; Cosson, J.; André, F. and Billard, R. (1995): Relationship between sperm ATP content and motility of carp spermatozoa. J Cell Sci; 108 (2): 747-753.

Phongnimitr, T.; Liang, Y.; Srirattana, K.; Panyawai, K.; Sripunya, N.; Treetampinich, C. and Parnpai, R. (2013): Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. Anim Sci J; 84(11): 719-725.

Pignatelli, P.; Lenti, L.; Sanguigni, V.; Frati, G.; Simeoni, I.; Gazzaniga, PP.; Pulcinelli, FM. and Violi, F. (2003): Carnitine inhibits arachidonic acid turnover, platelet function, and oxidative stress. Am J Physiol Heart Circ Physiol; 284 (1): 41-48.

Potts, RJ.; Jefferies, TM. and Notarianni, LJ. (1999): Antioxidant capacity of the epididymis. Hum Reprod; 14: 2513-2516.

Rani, PJA. and Panneerselvam, C. (2002): Effect of L-carnitine on brain lipid peroxidation and antioxidant enzymes in old rats. J Gerontology Biol Sci; 57: 134-137.

Rizzo, AM.; Berselli, P.; Zava, S.; Montorfano, G.; Negroni, M. and Corsetto, P. (2010): Endogenous antioxidants and radical scavenger. Adv Exp Med Bio; 698: 52-67.

Sarica, S.; Corduk, M.; Suicmez, M.; Cedden, F.; Yildirim, M. and Kilinc, K. (2007): The effects of dietary L-carnitine supplementation on semen traits, reproductive parameters, and testicular histology of Japanese quail breeders. J Appl Poult Res; 16: 178-186.

Sharma, RK.; Said, T. and Agarwal, A. (2004): Sperm DNA damage and its clinical relevance in assessing reproductive outcome. Asian J Androl; 6: 139-148.

Singer, R.; Sagiv, M.; Landau, B.; Allalouf, D.; Segenreich, E. and Servadio, C. (1980): Motility and vitality of human spermatozoa at various time intervals after ejaculation. Experientia; 36: 578-579.

Stradaioli, G.; Sylla, L.; Zelli, R.; Chiodi, P. and Monaci, M. (2004): Effect of Lcarnitine administration on the seminal characteristics of oligoasthenospermic stallions. Theriogenology; 62: 761-777.

Sun, JG; Jurisicova, A. and Casper, RF. (1997): Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod; 56: 602-607.

Tabatabaei, S. and Aghaei, A. (2012): Effect of L-carnitine on sperm quality during liquid storage of chicken semen. Comp Clin Pathol; 21: 711-717.

Totey, SM.; Singh, G.; Taneja, M.; Pawshe, CH. and Talwar, GP. (1992): In vitro maturation, fertilization and development of follicular oocytes from buffalo (Bubals bubalis). J Reprod Ferti; 95: 597-607.

Vav, FM. and Wanders, RJ. (2002): Carnitine biosynthesis in mammals. Biochem J; 361: 417-429.

Vicari, E. and Calogero, AE. (2001): Effects of treatment with carnitines in infertile patients with prostate-vesiculo-epididymitis. Hum Reprod; 16: 2338-2342.

Wales, RG. and White, IG. (1959): The susceptibility of spermatozoa to temperature shock. J Endocrinol; 19: 211-220.

Wang, Y.; Sharma, RK. and Agarwal, A. (1997): Effect of cryopreservation and sperm concentration on lipid peroxidation in human semen. Urology; 50: 409-413.

Yakushiji, K.; Kai, S.; Yamauchi, M.; Kuwajima, M.; Osada, Y. and Toshimori, K. (2006): Expression and distribution of OCTN2 in mouse epididymis and its association with obstructive azoospermia in juvenile visceral steatosis mice. Int. J. Urol; 13: 420-426.

دراسة تأثير الكارنتين- ل علي قدرة حيامن الجاموس للتجميد ، التغيرات في التركيب الدقيق و قدرتها الاخصابية

محمد الراعي ، مجدي رمضان بدر ، محمد محمد عاصي ، زاهر ابراهيم رواش

E-mail: magdybadr69@yahoo.com Assiut University web-site: www.aun.edu.eg

أجري هذا البحث لدراسة تأثير اضافة الكارنتين- ل الي ممدد السائل المنوى علي قابلية حيامن الجاموس للتجميد وكذا قدرتها الاخصابية معمليا و حقليا. تم تجميع عينات السائل المنوي من ستة طلائق وبعد تقييم السائل المنوي معمليا تم تمديده في ممدد التريس المضاف الية 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 وكذا اثر التجميد علي سلامة التركيب الدقيق وكذلك قدرته الاخصابيه في المعمل و الحقل. ولقد أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى الجاموسي في ممدد مضاف اليه الكارنتين- ل بتركيز ٠.٠٥ ملليمول نتج عنة زيادة معنوية كبيرة في نسبة الحركة الأمامية بعدالإسالة ومعدل الحيوية ونسبة سلامة القلنسوة (٥٧.٠٠% و١٢٦.٥و١٤.٤٠% علي التوالي) مقارنة بالمجموعة الضابطة (٤٢.٠٠% و ٨٧.٠٠ و٢٣.٨٠% علي التوالي) كما حافظ علي سلامة التركيب الدقيق للحيامن بالمقارنة بالمجموعة الضابطة. كما اوضحت النتائج ارتفاع نسبة الاخصاب المعملي ومعدل النمو الي طور البلاستوسيست ونسبة الاخصاب في الحقل باستخدام السائل المنوي المجمد باضافة الكارنتين- ل بتركيز ٠.٠٥ ملليجرام/مللي (٥٦.٤٥ و ١٥.٧٩% و ٦٤.٧١% علي التوالي) مقارنة بالمجموعة الضابطة (٣٦.٥٣و ٢.١٣ و٤٢.٨٦% علي التوالي). ومن خلال نتائج الدراسة الحالية يمكن أن نستنتج أن اضافة الكارنتين- ل بتركيز ٠.٠٥ ملليجرام/مللي يلعب دوراهاما وكبيرا في تحسين وظائف السائل المنوي الجاموسي المجمد ويبدو ذلك من خلال قدرتها علي المحافظة علي سلامة التراكيب الدقيقة للحيامن وزيادة نسبة الاخصاب معمليا وحقليا.