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MOTILITY AND MEMBRANE INTEGRITY OF EJACULATED BOVINE SPERMATOZOA EXTENDED AND CRYOPRESERVED IN *L*-CARNITINE TRIS-EGG YOLK EXTENDER

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ABSTRACT

This study was conducted to examine if *L*-carnitine supplementation in the Tris-egg-yolk extender can improve the motility characteristics, membrane integrity, and in vitro fertilization potential after cryopreservation of ejaculated bovine spermatozoa. It was carried out on January to May 2013 at the University of Wisconsin-Madison. *L*-carnitine at final concentrations of 0.5, 1, 10, and 30mM were added to Tris-egg yolk extender where semen was suspended at $100x10^6$ sperm cells/mL. Tris-egg yolk without carnitine served as control. Motility characteristics and functional integrity of membrane were examined by computer assisted sperm analysis and hypo-osmotic swelling test at 2, 6, and 24 hour at room temperature. The post-thaw effect of *L*-carnitine was likewise assessed. Each treatment suspension was cryopreserved in Tris-egg yolk with 7% glycerol, stored in liquid nitrogen and subjected to computer assisted sperm analysis and hypo-osmotic swelling test post-thawing. Lastly, the fertilization potential of frozen semen treated with *L*-carnitine was used for *in vitro* fertilization and cleavage and blastocyst development were assessed. *L*-carnitine was demonstrated to improve the motility characteristics in a concentration-depended manner; 1mM concentration is efficient but high concentration beyond 30mM had cytotoxic effect. A similar trend was observed on membrane integrity although significant difference was not evident. After IVF, the use of 1 mM *L*-carnitine resulted in significantly higher cleavage rate (92.4% vs. 82.8%) suggesting that *L*-carnitine supplementation at low concentration in Tris-egg yolk extender improves motility and fertilization potential of bovine sperm cells.

Key words*:* bovine semen, computer assisted sperm analysis, L-carnitine

INTRODUCTION

Semen is still the cheapest component of artificial breeding both in the Southeast Asian and other regions with artificial insemination (AI) remains the most implemented reproductive biotechnology and cryopreservation the most important procedure in order to ensure viability of male gamete and guarantee the success of AI. However, the current available methods to preserve semen as a genetic resource and its successful dissemination via AI and other assisted reproductive technologies (ARTs) are still sub-optimal (Rodriguez-Martinez, 2012a, b).

During cryopreservation, sperm cells are being subjected to stress and studies done by Chatterjee and Gagnon (2001) proved that an increase in lipid peroxidation levels during cryopreservation and thawing affects the motility of the frozen-thawed spermatozoa. Oxygen free

radicals are produced during freezing and thawing and these Reactive Oxygen Species (ROS) cause the decrease in sperm function following cryopreservation. In fact, the increased in ROS is responsible for the damage of the sperm (Li et al, 2012).

L-carnitine (levocarnitine) is an amino acid that plays a powerful role in transporting long-chain fatty acids into the mitochondria for β-oxidation (Coulter, 1995) which produces energy (ATP) needed by the cells for proper functioning (Ramsay et al., 2001; Hoppel 2003). It also facilitates the removal from mitochondria of short-chain and medium-chain fatty acids (acyl-CoA) that accumulate as a result of normal and abnormal metabolism (Rabie and Szilagyi, 1998; Arrigoni-Martelli and Caso 2001). It has antioxidant properties; it protects cellular membranes against oxidative damages resulting from peroxidation of polyunsaturated fatty acids that are component of membrane phospholipids by reducing the availability of lipids for peroxidation through transporting fatty acids into the mitochondria to generate ATP (Kalaiselvi and Panneerselvam, 1998). In processing sperm for cryopreservation, natural *L*-carnitine levels in the semen is diluted resulting to *L*-carnitine deficiency that may result to lipid peroxidation, formation of ROS, failure of fatty acid transport to the mitochondria of the sperm to produce energy, hence, depletion of normal sperm function. Since *L*-carnitine plays a vital role in sperm detoxification by maintaining the sperm membrane, in controlling lipid peroxidation and in the enhancing metabolic processing of endogenous fuel into energy (expressed as sperm motility), supplementation of it in the semen extender maybe beneficial to the quality of the frozen semen.

With the above considerations, it is worthy to examine the effect of *L*-carnitine in the semen extender before and after freezing. Though *L*-carnitine may be available in the seminal plasma, preparation and dilution for freezing must resulted to significant decrease in the level of L-carnitine necessary to support the energy production of the sperm cells and protect the plasma membrane by controlling the formation of ROS, thus, resulting to decreased motility after freezing. Further study to elucidate the effect of *L*-carnitine is needed, hence, this study.

MATERIALS AND METHODS

Semen Source

Ejaculated semen was provided by the ABS Global, Inc. USA. The study was conducted from January to May 2013 at the Laboratory of Reproduction, Department of Animal Science, University of Wisconsin-Madison. Two semen ejaculates collected a week apart from three different bulls were used. These bulls are used as semen donor for AI. Tris egg-yolk extender without glycerol containing 0, 0.5, 1, 10, 30 mM *L*-carnitine was added to ejaculated semen at least 30 to 120 minutes after collection. Semen from each bull and from each ejaculate was processed individually.

Experimental Design

Three studies were carried out; In Study-1, the desired concentration and effect of L-carnitine (0, 0.5, 1.0, 10, 30 mM) as supplement to Tris-egg yolk extender was examined. Ejaculated semen from each bull was diluted 1:1 (0 hour) with Tris-egg yolk extender containing 0, 0.5, 1, 10 and 30 mM *L*-carnitine during transport. In the lab, the semen suspensions were further diluted with the respective extenders to make 100 x 10^6 sperm cells/mL and kept at room temperature. After the 2, 6 and 24 hours from time of exposure to *L*-carnitine, sperm motility characteristics were assessed by computer assisted sperm analysis (CASA) while functional integrity of membrane was examined by hypo-osmotic swelling test (HOST). In Study-2, 5 mL of the treated sperm cells in Study-1 were cryopreserved in liquid nitrogen and post-thaw semen characteristics and functional integrity of membrane were examined after thawing. In Study-3, frozen semen treated with the effective concentration of *L*-carnitine was used for In Vitro Fertilization (IVF). Cleavage and blastocyst development of the fertilized eggs were assessed.

Semen Extender, Processing and Cryopreservation

The Tris-egg yolk extender was made 1 to 3 days before semen collection and stored in 3-5ºC. It contains 20% egg yolk, 2.42% Tris (tris(hydroxymethyl)aminomethane), 1.38% citric acid, 1 % fructose and protected with antibiotic mix containing 5.25 mg tylosin, 26.25 mg gentamycin, 15.75 mg lincomycin and 31.5 mg spectinomycin. On the day of semen collection, 10 mL of Tris-egg yolk extender were supplemented with *L*-carnitine HCl (Sigma Chemicals) at final concentration of 0.5, 1.0, 10, and 30 mM. Without *L* -carnitine served as control. Ejaculated semen was diluted 1:1 with the extender (0 hour) and transported to the laboratory at room temperature. At the laboratory, sperm concentration was determined and the sperm concentration in each treatment was adjusted to 100 x 10^6 sperm cells/mL with the respective extender and kept at room temperature. Sample of sperm from each treatment was taken at 2, 6, and 24 hour for sperm motility evaluation by CASA (IVOS, Hamiltorn Thorne) and functional integrity of the membrane by HOST. For Study 2, 5 mL of the treated semen was cooled for cryopreservation.

For cooling, semen for each treatment was kept in 15 mL centrifuge tubes submerged in water in 250 mL beaker and brought to cooled room. Slow cooling to 5°C was carried out for 2 to 2.5 hours. At 5°C, Tris-egg yolk extender containing 14% glycerol (without *L*-carnitine) was added in stepwise manner at 15 minutes interval until 1:1 ratio was attained shaking the semen suspension every after addition. This makes the sperm concentration 50 x 10^6 sperm cells/mL. Semen was then loaded on 0.5 mL straw, sealed and exposed to liquid nitrogen vapor (3 cm above LN_2) for 10 minutes then completely submerged in liquid nitrogen. Semen straws were stored in liquid nitrogen tank for storage and future evaluation.

Analysis of Sperm Motility Characteristics

Motility characteristics of the sperm cells were analyzed by Computer Assisted Sperm Analysis (CASA) using IVOS Hamilton Thorne with the following settings: Frame rate: 60 Hz; Frames required: 30 images; Minimum contrast: 50 pixels; Minimum cell size: 5 pixels; Threshold straightness: 80.0%; Medium VAP cut off (MVV): 60.0 microns/second; Low VAP cut off (LVV): 25.0 microns/second; (VSL) cut off (LVS): 10.0 micron/second; Non-motile head size: 5 micron; Non-motile head intensity: 90; Static size limit: 0.69 to 2.75 pixels; Static intensity limit: 0.28 to 1.50; Static elongation limit: 5 to 73%.

Tris-egg yolk extender provided granules that interfere with the accurate motility analysis by CASA. To avoid this, semen sample from each treatment group were diluted with pre-warmed TL-hepes (Lonza, Cambrex Bio-Sciences, Walkersville, MD) containing 22 ug/mL Na-pyruvate and 3 mg/mL BSA Fraction V to make a sperm concentration of 20,000,000/mL. Ten microliter of the sperm suspension was subjected to sperm motion characteristics by CASA and parameters observed are detailed here. 1. Overall motility (MOT, %; is the population of cells that are moving at or above a minimum speed as determined by values defined under CASA setup above. 2. Progressive motility (PROG, %; is the number of cells moving in a straight line with both path velocity greater than medium Average path velocity (VAP) cut off of 60% (VAP>MVV) and straightness is greater than threshold straightness of 80% (STR>S_0). 3. Average path velocity (VAP, µm/s; the point to point velocity on a path constructed using a roaming average. The number of points in the roaming average is 1/6th of the frame rate of video used. Cells must not be SLOW in order to be included in the average). 4. Progressive velocity (VSL, µm/s; measured in the straight line from the beginning to the end of tract. 5. Track speed or curvilinear velocity (VCL, µm/s; total distance traveled by a sperm per second. It is measured over the point to point track followed by the cell. 6. Amplitude of lateral head displacement (ALH, µm; deviation of sperm head from the average path calculated from all cell tracks that have a straightness greater than the threshold straightness (S_0=80.0%) and are not measured as SLOW. 7. Beat cross frequency (BCF, Hz; frequency with which the sperm head moves back and forth in its track across the cell path. 8. Straightness (STR, %; measures the departure of the cell path from a straight line. It is the ratio of VSL/VAP). 9. Linearity (LIN, %; measures the linearity of the

cell track. It is the ratio of VSL/VCL). 10. Rapid (%) is the fraction of all cells moving with path velocity greater than the medium VAP cut off (VAP>MVV). In all parameters examined, cells must not be SLOW to be included in the average as indicated in the CASA settings. Five views were used per slide to gather the motility characteristics.

Analysis of Plasma Membrane Integrity

The plasma membrane integrity was evaluated using the HOST described by Brito et al (2003) with few modifications. The assay was performed by incubating 0.1 ml of semen with 1.0 ml of 100 milliosmole (mOsm) hypo-osmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) at 37ºC for 60 min. After incubation, 10 µL of the sperm suspension was loaded on a glass slide and covered with coverslip. Two hundred sperm cells were evaluated under magnification 400X with Phase contrast microscopy. Sperm with swollen or coiled tails were considered viable.

Statistical Analysis.

Statistical analysis was performed using one way analysis of variance (ANOVA) to compare the sperm parameters across treatments and t-tests (LSD) was done to compare the statistical difference between treatment means. A p-value less than 0.05 were accepted as a statistically significant difference.

RESULTS AND DISCUSSION

Prominently improved sperm motility characteristic was observed at 2 hour of *L*-carnitine treatment and this was further improved until 6-hour of exposure at room temperature (Fig.1). After that, decline in motility were observed in all treatments. Interestingly, all motility parameters were improved with *L*-carnitine treatment except the amplitude of lateral head (ALH) (Table 1). Overall motility was significantly (P=<0.007) improved in 0.5 to 1 mM *L*-carnitine containing extender as compared to the control group (77.7% and 80.50% vs. 71.45%) but progressive motility was significantly increased irrespective of the *L*-carnitine concentration.

Average path velocity, progressive velocity, culvilinear velocity, and proportion of rapid spermatozoa were significantly improved by 1 mM *L*-carnitine but Straightness and Linearity was improved by 10 and 30 mM concentrations. At extended period of *L*-carnitine treatment, results remained higher with differences noted on progressive motility, Average path velocity (VAP), Progressive velocity (VSL), Curvilinear velocity (VCL), Amplitude of lateral head displacement (ALH), Linearity (LIN) and spermatozoa moving rapidly at 6 hours (Table 2) and on overall motility, LIN and spermatozoa moving rapidly on 12 hours (Table 3) of *L*-carnitine exposure. Motility characteristics in *L*-carnitine treatment were still superior in 0.5 to 10 mM *L*-carnitine at 24 hour treatment but 30 mM exhibited negative effects.

Table 1. Motility characteristics of fresh ejaculated bovine sperm cells 1 to 2 hours after exposure in *L*-carnitine supplemented Tris-egg yolk extender.

LC (mM)	Mot (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	71.45[b]	20.50[c]	104.01[b]	70.86[bc]	186.17[b]	13.86[a]	28.59[bc]	66.80[c]	38.40[c]	53.90[b]
0.5	77.70[a]	24.65[b]	107.86[b]	75.95[ab]	193.29[ab]	7.69[b]	29.74[abc]	69.50[bc]	40.45[c]	60.30[ab]
1	80.50[a]	26,20[b]	118.49[a]	82.22[a]	211.67a	8.27[ab]	27.91[c]	68.85[c]	39.80[c]	61.25[a]
10	76.35[ab]	31.65[a]	102.72[b]	83.29[a]	174.37[b]	8.78[ab]	32.73[a]	72.35[b]	43.75[b]	60.25[ab]
30	71.95[b]	33.84[a]	83.84[c]	66.36[c]	144.82[c]	5.94[b]	31.05[ab]	77.95[a]	47.53[a]	55.84[ab]
p- value	<.0070	<.0001	<.0001	0.0005	0.0915	0.0203	<0.0001	<.0001	<.0001	0.01449

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different (P<0.05).

Table 2. Motility characteristics of sperm cells at 6 hours storage at room temperature in a *L*-carnitine supplemented Tris-egg yolk extender.

LC	Motility (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	76.80[ab]	21.47[c]	109.14[b]	74.21[b]	190.71[b]	7.41[b]	24.16[b]	70.40[b]	43.47[a]	56.87[c]
0.5	82.60[ab]	26.52[bc]	127.05[a]	88.17[a]	232.33[a]	8.82[a]	26.26[b]	67.80[b]	38.40[b]	74.20[ab]
1	79.80[ab]	26.13[bc]	116.04[ab]	81.62[ab]	203.06[ab]	7.92[b]	26.02[b]	70.27[b]	42.93[a]	78.86[a]
10	84.26[a]	32.40[ab]	110.75[ab]	80.33[ab]	199.47[ab]	8.01[ab]	27.54[b]	71.40[b]	41.46[ab]	73.73[ab]
30	74.27[b]	35.47[a]	105.61[b]	82.01[ab]	187.81[b]	7.63[b]	38.16[a]	75.46[a]	44.13[a]	67.73[b]
p-value	0.159	0.0004	0.117	0.235	0.074	0.016	0.001	0.002	0.071	<0.0001

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different (P<0.05).

Table 3. Motility characteristics of sperm cells at 24 hours storage at room temperature in *L*-carnitine supplemented Tris-egg yolk extender.

LC	Motility (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	67.60[b]	27.40[a]	93.91[a]	70.70[a]	175.62[a]	7.29[a]	26.85[a]	73.80[ab]	42.35[b]	59.85[b]
0.5	73.40[ab]	32.80[a]	96.97[a]	74.75[a]	174.35[a]	7.28[a]	26.78[a]	76.25[ab]	45.70[ab]	62.25[a]
1	78.80[a]	35.45[a]	99.02[a]	75.36[a]	176.03[a]	7.11[a]	27.94[a]	75.40[ab]	44.20[ab]	72.40[a]
10	76.10[ab]	37.55[a]	93.74[a]	73.25[a]	164.31[a]	6.97[a]	27.58[a]	76.95[a]	46.95[a]	73.70[a]
30	67.65[b]	29.05[a]	97.51[a]	72.47[a]	173.30[a]	7.36[a]	26.44[a]	73.45[b]	43.55[ab]	58.35[b]
p-value	0.0393	0.2627	0.8311	0.8952	0.7632	0.650	0.8022	0.1443	0.0914	0.0028

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different ($P<0.05$).

Table 4. Motility characteristics post-thawing of bovine sperm cells treated with *L*-carnitine in Tris-egg yolk extender.

LC (mM)	Mot (%)	Prog (%)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
0	71.45[b]	20.50[c]	104.01[b]	70.86[bc]	186.17[b]	13.86[a]	28.59[bc]	66.80[c]	38.40[c]	53.90[b]
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p-value	<.0070	<.0001	<.0001	0.0005	0.0915	0.0203	<0.0001	<.0001	<.0001	0.01449

LC: L-carnitine; Mot: motility; Prog: Progressive motility; VAP: average path velocity; VSL: average progressive velocity; VCL: average track speed; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity; Rapid: the fraction of all cells moving with VAP>Minimum VAP cut-off.
Figures in the same column with different superscript are significantly different ($P<0.05$).

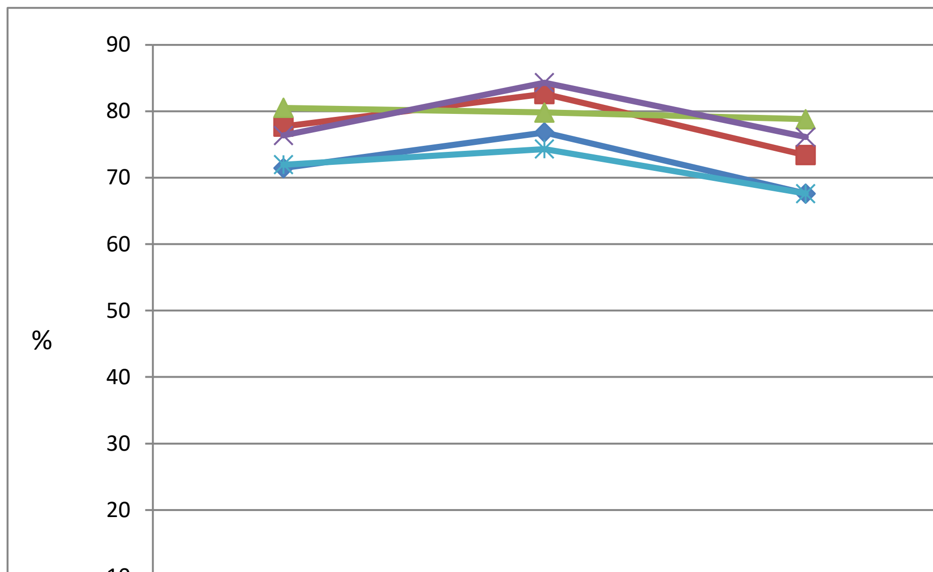

Fig. 1. Percent motility of fresh ejaculated bovine sperm cells after exposure in different concentration of *L*-carnitine in Tris-egg yolk extender and at extended time.

At post-thawing (Fig. 2), all motility characteristics were found of the same trend in all treatments but significant difference were observed on STR and LIN (Table 4) with 0.5 mM L-carnitine group superior to the Control group (80.33% vs. 77.95% and 49.60% vs. 46.56%, respectively). A high concentration of 30 mM consistently exhibited cytotoxic effect.

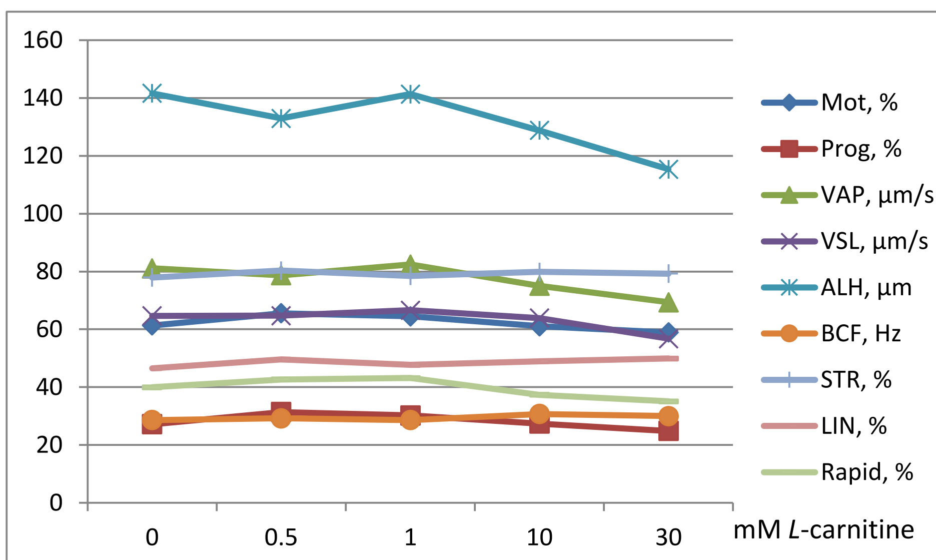

Fig. 2. Motility characteristics post-thawing of bovine sperm cells treated with different concentration of *L*-carnitine in Tris-egg yolk extender.

Functional integrity of the membrane was also improved with *L*-carnitine (Table 5) but significant difference with the Control group was not evident. Throughout the observation, lower concentration of *L*-carnitine, 1 mM, was beneficial but higher concentration, especially 30 mM, displayed negative effect at long time exposure and post-thawing.

Table 5. HOST reaction of sperm cells treated with or without *L*-carnitine in Tris-egg yolk extender before, after freezing, and at extended time.

Treatment	Pre-freezing		Post-freezing		Extended at RT, 24 h	
	N	HOST+ (%±SE)	N	HOST+ (%±SE)	N	HOST+ (%±SE)
0	1279	79.5±0.48	2013	49.5±0.30	1538	63.6±0.28
0.5	1206	81.9±0.37	1865	55.2±0.23	1149	69.1±0.30
1	1372	84.1±0.25	1781	56.9±0.30	1574	69.0±0.21
10	1338	84.2±0.20	1776	51.6±0.15	1469	70.8±0.23
30	1316	74.0±0.37	1721	53.8±0.24	1717	59.6±0.26

N: number of sperm cells; HOST +: oocytes that reacted to hypo-osmotic swelling test; RT: room temperature (P>0.05)

When sperm cells processed in the presence (1 mM) or absence (0 mM) of *L*-carnitine were used for IVF of *in vitro* matured oocytes, cleavage rate was significantly higher (P<0.05) in 1 mM group (92.44 vs. 83.74% and 82.81%, Fig. 3) but no significant difference was observed on blastocysts development and hatching rate (Table 6).

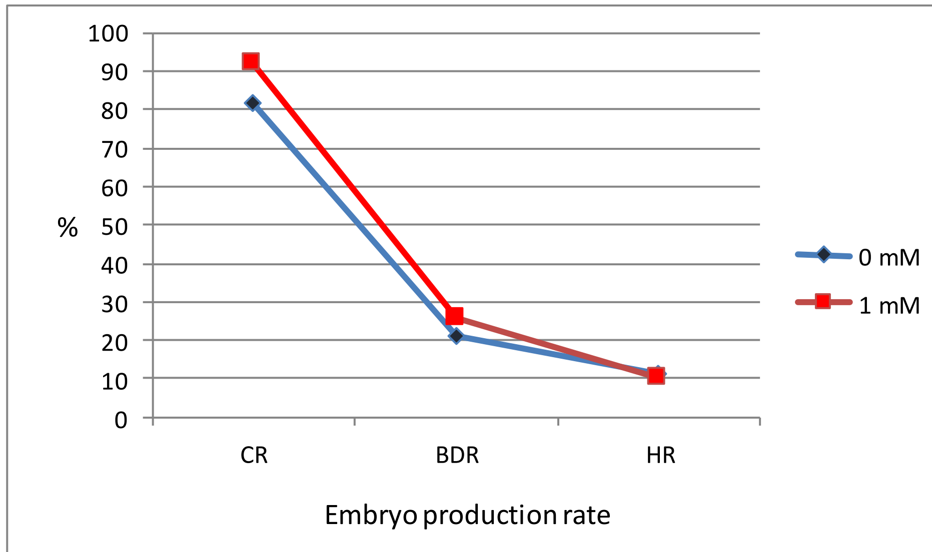

Fig. 3. Embryo development rate of *in vitro* matured bovine oocytes fertilized with semen cryopreserved with or without 1 mM *L*-carnitine in Tris-egg yolk extender

Table 6. Cleavage and blastocyst development of *in vitro* matured bovine oocytes fertilized with semen cryopreserved with or without 1 mM *L*-carnitine

L-carnitine	N	Cleavage Rate, CR (%±SE)	Blastocysts development rate, BDR (%±SE)	BDR/CR (%±SE)	Hatched/CR (%±SE)
0 mM	193	82.8±0.5[a]	21.4±0.3 [a]	26.0±0.7 [a]	10.6±0.9 [a]
1 mM	199	92.4±0.1[b]	25.8±0.2 [a]	27.9±0.6 [a]	9.9±0.9 [a]

N: number of *in vitro* matured oocytes used for IVF, BDR/CR: developed to blastocysts out of cleaved eggs; Hatched/CR: hatched blastocysts out of cleaved eggs.
Figures with different superscript are significantly different ($P<0.05$) .

With the above results, it was noted that a low concentration (0.5 to 1 mM) of *L*-carnitine in Tris-egg yolk extender exerts positive effect both on motility characteristics, membrane integrity and cleavage rate of *in vitro* matured oocytes *in vitro* fertilized with *L*-carnitine treated spermatozoa. Higher concentration of 10 mM and 30 mM also had positive effects but negative effect manifest at extended condition especially in 30 mM suggesting cytotoxic effect which supports earlier observations in water buffalo frozen-thawed sperm cells (Hufana-Duran et al., 2012) and in *in vitro* maturation of pig oocytes (Wu et al., 2011). These findings are also in agreement with that of Daena et al. (1984) indicating that addition of 20 mM *L*-carnitine to suspensions of ejaculated bovine spermatozoa resulted in an increase of cellular calcium transport, which inhibited the progressive motility, oxygen consumption as well as the release of the enzymes hyaloronidase and glutamate-oxaloacetate transaminase from spermatozoa but not at 2 mM concentration. High concentration of *L*-carnitine resulted to depressed motility especially at extended condition which could be explained by the earlier observations (Hamilton and Olson, 1976; Bohmer and Johansen, 1978) that *L*-carnitine inhibits respiration of ejaculated bovine spermatozoa.

The improved motility characteristics of spermatozoa observed in the present study could be due to the addition of L-carnitine which concentration in the seminal fluid was diluted during the process, into the Tris-egg yolk extender where *L*-carnitine played a key role in sperm metabolism by providing readily available energy for use by spermatozoa. Earlier reports (Matalliotakis et al., 2000) showed that *L*-carnitine mediates the transport of long chain fatty acids across the inner membrane of the mitochondria for utilization in metabolism through β-oxidation and exerts protective role against reactive oxygen species (ROS) by repairing mechanism via removing elevated intracellular toxic acetyl-coenzyme A (acetyl-CoA) and/or replacing fatty acids in membrane phospholipids (Vicari and Calagero, 2001). These properties and function of the *L*-carnitine together with its osmotic balance function (Brooks et al., 1974) and stabilization of the membrane (Daena et al., 1984) may contribute in the improvement of motility characteristics of the treated spermatozoa resulting to higher fertilization rate when used for IVF post-thawing. Tanphaichitr (1977) observed that carnitine potentiates the motility regardless of the sample's initial motility pattern. Earlier, Daena et al. (1984) reported a positive correlation between the fertility of bovine spermatozoa and the concentration of seminal carnitine which supports the results in Study 3 demonstrating higher cleavage in oocytes *in vitro* fertilized with sperm cells treated with 1 mM *L*-carnitine prior to cryopreservation. In humans, Lay et al. (2001) did not find significant difference in the concentration of carnitine between those who achieved and did not achieve pregnancy after IVF treatment. This positive effect of *L*-carnitine on cleavage rate could also be due to the *L*-carnitine's beneficial effect on DNA repair and proliferation of regenerating germ cells (Amendola et al., 1989) which may enhance better quality spermatozoa that fertilized the matured oocytes.

The observation that *L*-carnitine fails to stimulate sperm motility in washed spermatozoa and succeeds with raw semen samples suggest that it needs to be further metabolized and transported by or with factor(s) in the seminal plasma and this are present in the Tris-egg yolk extender. In ejaculated seminal fluid, most *L*-carnitine are found in the seminal plasma; very little are found in the spermatozoon itself (Bohmer et al., 1978). But washing the sperm cells from the seminal plasma would mean removal of *L*-carnitine. Similarly, dilution with extender means tremendous decrease on *L*-carnitine concentration, thus, supplementation is needed. In humans, however, Duru et al. (2000) found no improvement in motility and membrane damage after addition of *L*-carnitine to Tris-yolk buffer containing glycerol on post-thawed semen of male with primary infertility problems. This observation was in agreement to the present results showing no significant difference on the motility parameters and membrane integrity between *L*-carnitine and no carnitine treated groups after cryopreservation except on STR and LIN that may contribute in the significantly higher cleavage rate after *in vitro* fertilization. It is to be noted that the semen used in this study were from bulls used for AI and are proven fertile, hence, the semen quality is high. The beneficial effect of the *L*-carnitine could be on the provision of the needed concentration that was otherwise lost during the semen dilution, and needed by a fraction of the sperm population. This was evident by the constantly higher percentage of spermatozoa reacted in HOST than the Control group suggesting improvement on the functional integrity of the membrane in the *L*-carnitine treatment. This supports the earlier claim that *L*-carnitine has a stabilizing effect on plasma membranes (Deana et al., 1984). HOST positive sperm cells were reported to have higher fertilizing capacity (Brito et al., 2003). The significantly improved motility characteristics and better functional integrity of the plasma membrane after *L*-carnitine treatment supports the possibility that the action of *L*-carnitine is also due to osmolarity changes (Quinn and White 1969; Drevius, 1972) which *per se* could affect sperm functions (Deana et al., 1989).

The negative result observed in 30 mM *L*-carnitine is associated to earlier report indicating that *L*-carnitine at high concentration inhibits fatty acid oxidation (Hamilton and Olson, 1976) and oxygen consumption (Deana et al. 1989) by ejaculated bovine spermatozoa. In fact, higher concentrations of 60 mM have resulted to completely immobilized sperm cells after exposure (data not shown). These results suggest that *L*-carnitine is involved in the complex regulatory mechanism of metabolic pathways following cellular stimulation in bovine spermatozoa and its presence could rescue some sperm cells that require it for better functioning.

CONCLUSION

It was demonstrated that *L*-carnitine has beneficial effects on the sperm motility characteristics at lower concentration (≤10 mM) but high concentration (≥30 mM) exhibits negative effect. The exposure of bovine sperm cells in Tris-egg yolk extender containing *L*-carnitine improves the sperm motility post-thaw and resulted in improved *in vitro* fertilization of *in vitro* matured oocytes resulting in higher production of embryos *in vitro*.

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