

L-carnitine improved bovine blastocyst rate and quality when supplemented at different preimplantation stages

N. Ghanem

Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt.

SUMMARY

The present study investigated the effect of L-carnitine supplementation during different pre-implantation stages on bovine embryo development rate and quality. Good morphologically cumulus-oocyte complexes ($n=2126$) recovered from slaughterhouse ovaries were matured, fertilized *in vitro* and presumed zygotes were further cultured *in vitro* (IVC) in CR1-aa media till day 8. For the setup of the study, L-carnitine (1.5 mM) was added at different technological steps (IVM, IVF, IVC and all stages) and different developmental stages (early IVC: 1- to 4-cell vs. late IVC: 8-cell embryos). Moreover, untreated (control) group was also included in this experiment. Quality of embryos developed from all experimental groups were evaluated using terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay which assesses total normal and apoptotic cell numbers of embryos. Real-time PCR was used to profile 5 embryo marker genes (SOD2, CDX2, IFN γ , Glut8 and TFAM) in addition; GAPDH was used as housekeeping gene.

Results of the current study showed a significant increase in blastocyst rate at day 8 of culture when L-carnitine was added at IVM (34.1 ± 2.6), early IVC (34.6 ± 1.7), late IVC (38.4 ± 2.8), and all stages (36.4 ± 3.5) compared to control (22.5 ± 1.4) and IVF (26.0 ± 1.1) groups. The blastocyst total number of cells was greater ($P < 0.05$) in groups that L-carnitine was added at IVM (134.1 ± 4.9), IVF (125.7 ± 2.8), early IVC (133.8 ± 5.7), late IVC (139.2 ± 3.5) and all stages (137.0 ± 4.1) compared to control (115.3 ± 3.6). On the other hand, control embryo group has more apoptotic cells (10.4 ± 1.9) than L-carnitine treated groups at IVM (4.1 ± 1.2), IVF (5.5 ± 1.6), early IVC (3.9 ± 1.4), late IVC (4.0 ± 1.7) and all stages (2.8 ± 2.0). The expression profile of SOD2, IFN γ , Glut8 and TFAM genes was increased in embryos treated with L-carnitine at IVM, early IVC, late IVC and all stages compared to IVF and control groups. In addition, CDX2 transcript was more abundant in IVM, late IVC and all stages treated groups than in any other embryo group. Overall, the findings of the present study support the notion that L-carnitine could be a potential antioxidant substance that has a positive effect on embryo development rate and quality when supplemented to all culture media throughout bovine preimplantation.

Keywords: *in vitro*, embryo, development, antioxidant, gene expression

INTRODUCTION

Recovery of cumulus-oocyte complexes from slaughterhouse ovine ovaries or via transvaginal ultrasound-guided follicular puncture followed by *in vitro* maturation, fertilization and embryo culture is world-wide used technique in bovine assisted reproductive technology. During the last decades, *in vitro* embryo production was used as an efficient tool for producing embryos at low cost with substantial increase in the number of calves born with a specific genetic makeup from elite cows, irrespective of their physiologic status (Kruip *et al.*, 1994; Scott *et al.*, 1994; Meintjet *et al.*, 1995 and Machatkova *et al.*, 2008).

Despite the desire to expand the field application of this technology, the development rate and quality of *in vitro* produced embryos are lower than those of *in vivo* produced embryos. Moreover, *in vitro* derived embryos have frequently been associated with fetal and neonatal abnormalities after transfer (Farin *et al.*, 2006), in addition only half of these being able to initiate a successful pregnancy following transfer (Trownson *et al.*, 1994 and Hasler, 1998). Therefore, tremendous efforts were given to improve the efficiency of this technique by providing new tools of

cumulus-oocyte complexes selection and optimizing both *in vitro* maturation (Choi *et al.*, 2013) and embryo culture (Yuan *et al.*, 2003 and Sudano *et al.*, 2010) to be more similar to *in vivo* conditions.

Bovine embryos cultured *in vitro* are prone to oxidative stress (Lequarre *et al.*, 1997). The embryo undergoes oxidative stress when the production of reactive oxygen species (ROS) is higher than the cells capacity to produce antioxidants (Droge, 2002). Therefore, the balance between ROS and the presence of antioxidants is a key factor in the success of embryonic development (de Lamirande *et al.*, 1997). The oxidative stress reduces embryo quality and viability because of the peroxidation of membrane lipids and the modification of important molecules, such as proteins and nucleic acids, which results in cellular death through apoptosis (Nasr-Esfahani *et al.*, 1990 and Van Soom *et al.*, 2002).

L-carnitine is a powerful antioxidant (Gülçin, 2006), reducing the accumulation of ROS and lowering the frequency of apoptosis in animal cells (Pillich *et al.*, 2005 and Ye *et al.*, 2010). L-carnitine supplementation during mice *in vitro* follicle culture increased oocyte developmental competence (Dunning *et al.*, 2011). L-carnitine acted as

antioxidant by reducing the levels of ROS during *in vitro* maturation of porcine oocytes (Somfai *et al.*, 2011). Recently, L-carnitine supplementation in IVM medium has effectively maintained embryo developmental competence of IVM oocytes after vitrification (Chankitisakul *et al.*, 2013). In addition, L-carnitine added to *in vitro* culture medium has a positive impact on the quality and development of bovine embryos (Takahashi *et al.*, 2013 and Ghanem *et al.*, 2014). However, all previous research were performed at one specific stage either during IVM (Somfai *et al.*, 2011 and Chankitisakul *et al.*, 2013), IVC (Takahashi *et al.*, 2013 and Ghanem *et al.*, 2014) or different technological steps (IVM, all pre-implantation stages) (Phongnimitr *et al.*, 2013).

Therefore, the current investigation was performed to find out the possible effect of L-carnitine when supplemented at different technological steps (IVM, IVF, IVC and all stages) and different developmental stages (early IVC: 1- to 4-cell vs. late IVC: 8-cell embryos) on embryo development rate and quality. In addition, gene expression profile of some embryo candidate genes was analyzed using quantitative real-time PCR.

MATERIALS AND METHODS

Unless stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design

L-carnitine (1.5 mM) was added at different preimplantation stages (IVM, IVF, early IVC, late IVC, all stages) in addition; a control group (untreated) was also included in this experiment. The concentration of L-carnitine (L-Carnitine hydrochloride, Sigma-Aldrich, Product Number: C0283) was selected according to our previous publication (Ghanem *et al.*, 2014). Cumulus-oocyte complexes were randomly allocated into six treatment groups: IVM, IVF, early IVC (from zygote till 8-cell stage), late IVC (from 8-cell stage till day 8 blastocyst), all stages and no addition (control). All groups were evaluated on day 8 (day 0=IVF) to determine the proportion of embryos that had reached the blastocyst stage. Number of total, normal and apoptotic cells was used to assess embryo quality. Finally, expression of selected candidate genes was profiled using real-time PCR.

Cumulus-oocyte complexes recovery

Bovine ovaries were collected from a local abattoir, placed in physiological saline (0.9% NaCl) at 35–37 °C and transported to the laboratory within 2 h after death. Ovaries were washed in fresh Dulbecco's PBS and cumulus-oocyte complexes (COCs) were recovered as described Ghanem *et al.* (2014). In brief, COCs were aspirated from 2- to 8-mm diameter follicles using an 18-G needle attached to a vacuum pump. Aspirated fluid was expelled into dishes containing TL-HEPES medium (114 mM sodium chloride, 3.2 mM potassium chloride, 2 mM

sodium bicarbonate, 0.34 mM sodium biphosphate, 10 mM sodium lactate, 0.5 mM magnesium chloride, 2.0 mM calcium chloride, 10 mM HEPES, 1 µL/mL phenol red, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin), COCs were searched and selected under a stereomicroscope. After collection, only morphologically good-quality COCs (n=2126) with more than three layers of compact cumulus cells and homogenous cytoplasm were used in this experiment. The selected COCs were washed three times in TL-HEPES medium.

In vitro maturation (IVM)

Cumulus-oocyte complexes were cultured in maturation medium, as described by Ghanem *et al.* (2014). In brief, COCs (n=40–50 per each group) were washed three-times in maturation medium (TCM-199) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 µg/mL of estradiol-17β, 10 µg/mL of FSH, 0.6 mM of cysteine, and 0.2 mM of sodium pyruvate and transferred into a well of a 4-well dish containing 700 µL of IVM medium for 23 to 24 h at 38.5 °C in a humidified atmosphere of 5% CO₂ in air.

In vitro fertilization (IVF) and in vitro culture (IVC)

In vitro-matured COCs were fertilized with frozen-thawed bovine sperm from a freezing batch that had previously been successfully used in our laboratory. Semen was thawed at 36 °C for 1 min. and spermatozoa were washed and pelleted in Dulbecco's PBS (D-PBS) by centrifugation at 750 x g for 5 min at room temperature. The pellet was diluted with 500 µL of heparin (20 µg/mL) in *in vitro* fertilization (IVF) medium (Tyrodes lactate solution supplemented with 6 mg/mL of BSA, 22 µg/mL of sodium pyruvate, 100 IU/mL of penicillin, and 0.1 mg/mL of streptomycin) and incubated at 38.5 °C in a humidified atmosphere of 5% CO₂ in air for 15 min (to facilitate capacitation). Then, spermatozoa were diluted in IVF medium (final concentration, 1×10^6 sperm/mL). Mature COCs were transferred into IVF medium (700 µL) containing spermatozoa for 18 to 20 h at 38.5 °C in a humidified atmosphere of 5% CO₂ in air.

After IVF, cumulus cells were removed by pipetting and the presumed denuded zygotes were placed in a well of a 4-well dish containing 700 µL of CR1-aa medium (Rosenkrans *et al.*, 1993) supplemented with 44 µg/mL of sodium pyruvate, 14.6 µg/mL of glutamine, 10 µL/mL of penicillin/streptomycin, 3 mg/mL of BSA, and 310 µg/mL of glutathione for 3 days (IVC-I). Embryos (8-cell stage) of day 3 were cultured in CR1-aa medium of the same composition (IVC-I), except that the BSA was replaced with 2.5% (v/v) FCS (IVC-II). The day 8 blastocysts were washed three times in TL-HEPES, fixed in 4% (v/v) paraformaldehyde in 1 M PBS, and stored at 4 °C pending TUNEL assay. For gene expression analysis, blastocysts were transferred to a 1.5-mL Eppendorf tube, directly snap frozen in liquid nitrogen, and stored at -80 °C.

Terminal Deoxynucleotidyl Transferased UTP Nick End Labeling Staining (TUNEL)

The terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) assay was performed according to the manufacturer's protocol using the In Situ Cell Death Detection Kit (Fluorescein, Roche Diagnostics Corp., Indianapolis, IN, USA). Briefly, fixed embryos (n=67) were washed twice with 0.3% (w/v) PVP in 1 M PBS (PVP-PBS) before being incubated in a permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature. After permeabilization, embryos were washed twice in PVP-PBS and incubated in the dark with fluorescence-conjugated dUTP and terminal deoxynucleotide transferase for 1 h at room temperature. TUNEL-stained embryos were then washed in PVP-PBS and incubated in PVP-PBS containing 10 µg/ml of Hoechst 33342 for 10 min. The washing was then done twice in PVP-PBS to remove excess Hoechst 33342, and blastocysts were mounted on glass slides under coverslips. The number of cells in each blastocyst was counted using an epifluorescence microscope equipped with a mercury lamp. The TUNEL-positive cells were evident on the basis of their bright red fluorescence (apoptosis), whereas the total number of cells was determined by the extent of blue fluorescence. All blastocysts from five culture sessions of each treatment group were used for the TUNEL assay.

RNA isolation and cDNA synthesis

Isolation of mRNA was done on five biological replicates, each containing six embryos, using Dynabeadoligo (dT)25; Dynal Biotech, Oslo, Norway), according to manufacturer's instructions. Briefly, embryos were lysed in 100 µl lysis buffer at room temperature for 2 min by vortexing. The lysate was mixed with 20 µl of prewashed Dynabeadsoligo (dT) and annealed by rotating for 3 min at room temperature. The mRNA attached to Dynabead was washed twice with 300 µl washing buffer 300 A and twice with 150 µl of washing buffer B. Then, 8 µL of 10 mM Tris-HCl were added, denatured at 65 °C for 5 min, and then placed on ice for 3 min. The eluted mRNA was reverse-transcribed to cDNA using the SuperScript III first strand reverse transcriptase kit (catalog number 18080051, Invitrogen). The mRNA samples were transferred to a 200-µL Eppendorf tube containing 1 µl of Olgo (dT), 1 µl of dNTP mix (10 mM) and incubated at 65 °C for 5 min, then placed on ice for 2 min. Then, 10 µl of cDNA synthesis mix and 1 µl of superscript III reverse transcriptase (200 U/µl) were added to a 200-µL tube containing RNA mix and incubated at 50 °C for 50 min. The reaction was terminated by heating at 85 °C for 5 min and then chilled on ice for 2 min. Finally, 1 µl of RNase H was added per tube and incubated at 37 °C for 20 min. The final volume of cDNA was increased to 80 µl by adding RNase-free water.

Table 1: List of primers used for quantitative real-time PCR analysis

Gene Name	Gene bank accession number	Primer sequence	Fragment size (bp)
<i>GAPDH</i>	NM_001034034.2	F: 5'- AGGTCGGAGTGAACGGATTC -3' R: 5'- GGAAGATGGTGATGGCCTTT -3'	219
<i>SOD2</i>	NM_201527	F: 5'-GTGATCAACTGGGAGAATGT-3' R: 5'-AAGCCACACTCAGAAACACT-3'	163
<i>IFNt</i>	NM_001015511	F: 5'-CTGGGAAATCATCAGAGTGGAG-3' R: 5'-TAAGGACTCATGCCCTACAG -3'	279
<i>CDX2</i>	DQ126146	F: 5'-GCCACCATGTACGTGAGCTAC-3' R: 5'-ACATGGTATCCGCCGTAGTC-3'	140
<i>TFAM</i>	NM_001034016	F: 5'-CTGGTCAGTGCTTTGTCTGC-3' R: 5'-CTAAAGGGATAGCGCAGTCG-3'	128
<i>Glut8</i>	AF321324	F: 5'-GCTCCTCATGTCAGAGATCTTC-3' R: 5'-GGCTGTGATTTGTTCCAGAGTC-3'	135

Abbreviations: PCR, polymerase chain reaction; bp, base pair.

Quantitative real-time PCR analysis

Real-time PCR was used to profile 5 embryo marker genes regulating metabolism (glucose transporter 8: *Glut8*), mitochondrial activity (transcription factor A, mitochondrial: *TFAM*), cellular defense (superoxide dismutase 2: *SOD2*), trophoectoderm marker (caudal type homeobox 2: *CDX2*) and pregnancy recognition (interferon tau: *IFNt*). In addition, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. Real-time PCR primers were designed based on the mRNA sequences of selected genes available in GenBank (Table 1) using Primer3

Express version 4.0 software (<http://primer3.wi.mit.edu/>) developed by the Whitehead Institute for Biomedical Research, the University of Massachusetts Medical School (Massachusetts, MA, USA). Quantitative analysis of cDNA samples was performed using a CFX98 instrument (Bio-Rad Laboratories, Hercules, CA, USA). Prior to quantification, primer optimization was performed for both forward and reverse primers. Specific primer combinations with lower threshold cycle (CT) value and without primer-dimer formation were selected for subsequent PCRs. The PCR reactions were performed in a 12-µL reaction volume containing 8 µl of 1X iQ SYBR Green

Supermix (Bio-Rad Laboratories). During each PCR reaction, samples from the same cDNA source were run in duplicate (to improve reproducibility of real-time results). A universal thermal cycling parameter (3 min at 95 °C followed by 44 cycles of 15 s at 95 °C, 20 s at 57 °C, and 30 s at 72 °C, then a final extension of 5 min at 72 °C) was used to quantify each gene of interest. After the end of the last cycle, a dissociation curve was generated by starting fluorescence acquisition at 65 °C and making measurements every 7 s until the temperature reached 95 °C. Final quantitative analysis was done using the $\Delta\Delta C(t)$ method (Ghanem *et al.*, 2014) and results were reported as the relative expression or n-fold difference to the calibrator (control group) after normalization of the transcript amount relative to the value of the endogenous control gene (*GAPDH*).

Statistical analyses

All data were analyzed using the Statistical Analysis System (SAS) software package for Windows (Release 8.02, SAS Institute Inc., Cary, NC, USA). Data of at least five replicates of each treatment were analyzed with ANOVA, using the general linear model (GLM) procedure. Results were expressed as mean $\pm$ SEM. All percentage data were arcsine-transformed before analysis. Data on embryo development rate, normal and apoptotic cell number were analyzed by one-way ANOVA followed by multiple pair wise comparison (Tukey's Test). Relative expression data were analyzed using GLM. Differences in mean values were tested using ANOVA, followed by a multiple pair wise comparison using a Student's t-test. In all cases, $P < 0.05$ was considered significant.

RESULTS

Embryo development rate

Percentages of cleaved embryos were not ($P > 0.05$) affected by L-carnitine supplementation at any stage of preimplantation (Table 2). The percentage of cleaved embryos at 48 hpi was 77.03%, 75.9%, 77.1%, 77.4% and 77.3% in groups treated with L-carnitine at IVM, IVF, early IVC, late IVC and all stages while it was 76.2 in control group. The percentage of cleaved embryos at 96 hpi was 56.1%,

55.9%, 56.2%, 55.8% and 57.0% in groups treated with L-carnitine at IVM, IVF, early IVC, late IVC and all stages in addition, it was 55.6% in control group.

Blastocyst development rate after addition of L-carnitine at IVM, IVF, early IVC, late IVC, all stages and control group (no L-carnitine addition) are shown in Table 2. The blastocyst development rate was increased significantly ($P < 0.05$) when L-carnitine was added at IVM (34.1%), early IVC (34.6%), late IVC (38.4%), and all stages (36.4%) compared to control (22.5%) and IVF (26.0%) groups.

Blastocyst quality assessment

The blastocyst total number of cells was significantly greater ($P < 0.05$) in groups supplemented with L-carnitine at IVM (134.1), IVF (125.7), early IVC (133.8), late IVC (139.2) and all stages (137.0) compared to control (115.3) but there were no significant differences among the IVM, early IVC, late IVC and all stages groups (Table 3 and Fig. 2). Moreover, embryos exposed to L-carnitine during IVF showed lower number of total cells than IVM, early IVC, late IVC and all stages treated groups.

On the other hand, control embryo group has significantly ($P < 0.05$) more apoptotic cells (10.4) than those embryos developed from L-carnitine treated groups at IVM (4.1), IVF (5.5), early IVC (3.9), late IVC (4.0) and all stages (2.8) as shown in (Table 3 and Fig. 2). Cells from the total cell number per blastocyst was also increased ($P < 0.05$) in control group (9.0%) compared to L-carnitine supplemented groups at IVM (3.1%), IVF (4.4%), early IVC (2.9%), late IVC (2.9%) and all stages (2.0%) as shown in Table (3).

Gene expression profile

The expression profile of *SOD2*, *IFNt*, *Glut8* and *TFAM* genes was significantly increased ($P < 0.05$) in embryos treated with L-carnitine at IVM, late IVC and IVC2 and all stages compared to IVF and control groups. In addition, *CDX2* transcript was more abundant in IVM, late IVC and all stages than any other experimental groups.

Table 2. Effect of L-carnitine treatment during bovine preimplantation on cleavage and blastocyst development rate at Day 8 post-insemination (196 hpi) (Mean $\pm$ SEM)

Treatment group	Total No. COCs	Cleavage 48hpi (%)	8-cell stage 96 hpi (%)	Blastocyst 192 hpi/oocyte (%)
Control	371	76.2 $\pm$ 5.7 ^a	55.6 $\pm$ 3.9 ^a	22.5 $\pm$ 1.4 ^a
LC-IVM	326	77.0 $\pm$ 7.3 ^a	56.1 $\pm$ 2.5 ^a	34.1 $\pm$ 2.6 ^b
LC-IVF	380	75.9 $\pm$ 6.1 ^a	55.9 $\pm$ 4.1 ^a	26.0 $\pm$ 1.1 ^a
LC-IVC1	347	77.1 $\pm$ 8.2 ^a	56.2 $\pm$ 3.8 ^a	34.6 $\pm$ 1.7 ^b
LC-IVC2	353	77.4 $\pm$ 5.7 ^a	55.8 $\pm$ 3.0 ^a	38.4 $\pm$ 2.8 ^b
All-LC	349	77.3 $\pm$ 6.4 ^a	57.0 $\pm$ 5.3 ^a	36.4 $\pm$ 3.5 ^b

^{a, b} Within a column, means without a common superscript differed ($P < 0.05$).

Abbreviations: hpi, hours post insemination; LC, L-carnitine; SEM, standard error of the mean.

Table 3. Effect of L-carnitine treatment during bovine preimplantation on total number of normal and apoptotic cells per blastocyst at Day 8 post-insemination (196 hpi) (Mean $\pm$ SEM)

Treatment group	Total No. blastocysts examined	Total number of cells per blastocyst	Total number of normal cells per blastocyst	Total number of apoptotic cells per blastocyst	Percentage of apoptotic cells per blastocyst (%)
Control	13	115.3 $\pm$ 3.6 ^a	104.9 $\pm$ 2.5 ^a	10.4 $\pm$ 1.9 ^a	9.0 $\pm$ 1.7 ^a
LC-IVM	15	134.1 $\pm$ 2.9 ^b	130 $\pm$ 2.0 ^b	4.1 $\pm$ 1.2 ^b	3.1 $\pm$ 0.9 ^b
LC-IVF	14	125.7 $\pm$ 2.8 ^c	120.2 $\pm$ 3.3 ^c	5.5 $\pm$ 1.6 ^b	4.4 $\pm$ 1.1 ^b
LC-IVC1	16	133.8 $\pm$ 3.7 ^b	129.9 $\pm$ 2.8 ^b	3.9 $\pm$ 1.4 ^b	2.9 $\pm$ 0.5 ^b
LC-IVC2	14	139.2 $\pm$ 3.5 ^b	135.2 $\pm$ 3.1 ^b	4.0 $\pm$ 1.7 ^b	2.9 $\pm$ 0.8 ^b
All-LC	17	137.0 $\pm$ 4.1 ^b	134.2 $\pm$ 2.2 ^b	2.8 $\pm$ 2.0 ^b	2.0 $\pm$ 0.6 ^b

^{a, b} Within a column, means without a common superscript differed ($P < 0.05$).

Abbreviations: LC, L-carnitine; SEM, standard error of the mean.

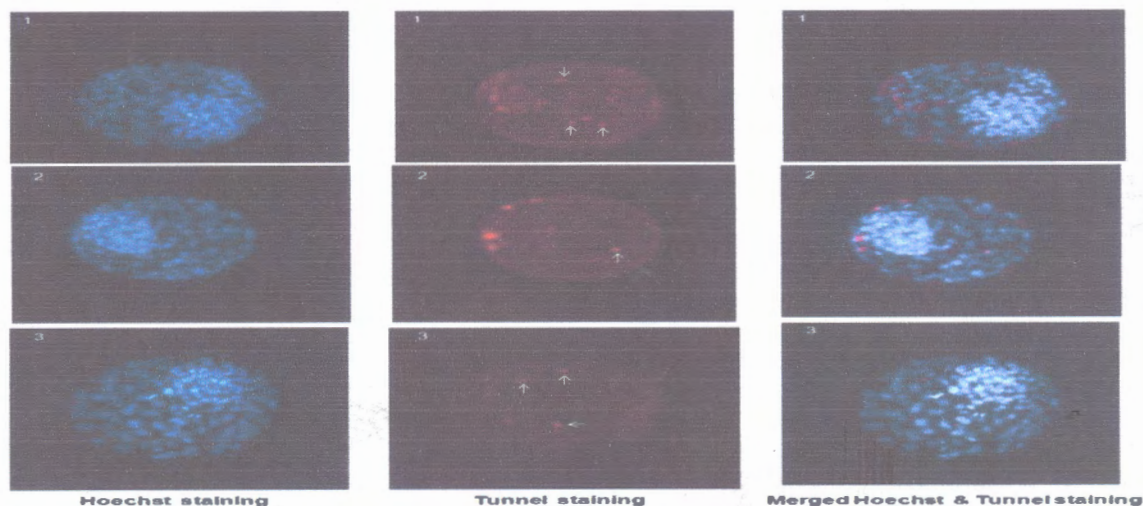


Fig. 1. Hoechst 33342 staining (blue color) identifying nuclei in bovine embryos (100X); TUNEL staining for identifying apoptotic cells (small bright red color as indicated by arrows); merged photographs of Hoechst 33342 and TUNEL staining of embryo in the control (1), LC-IVM (2) and LC-IVF (3) groups, respectively.

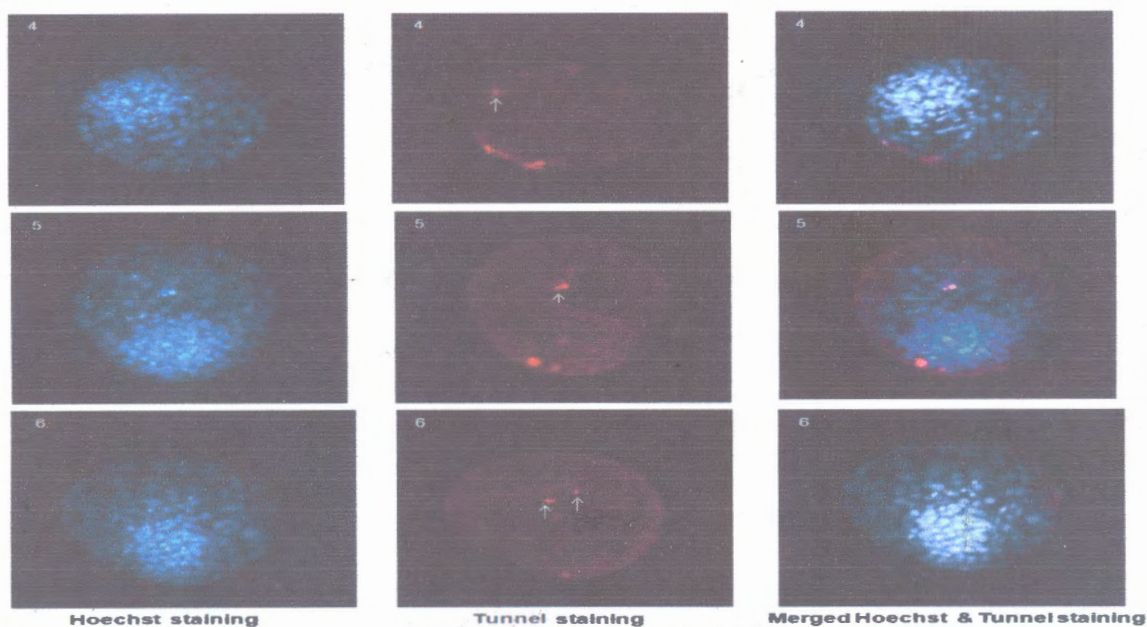


Fig. 2. Hoechst 33342 staining (blue color) identifying nuclei in bovine embryos (100X); TUNEL staining for identifying apoptotic cells (small bright red color as indicated by arrows); merged photographs of Hoechst 33342 and TUNEL staining of embryo in the LC-early IVC (4), LC-late IVC (5) and LC-all stages (6) groups, respectively.

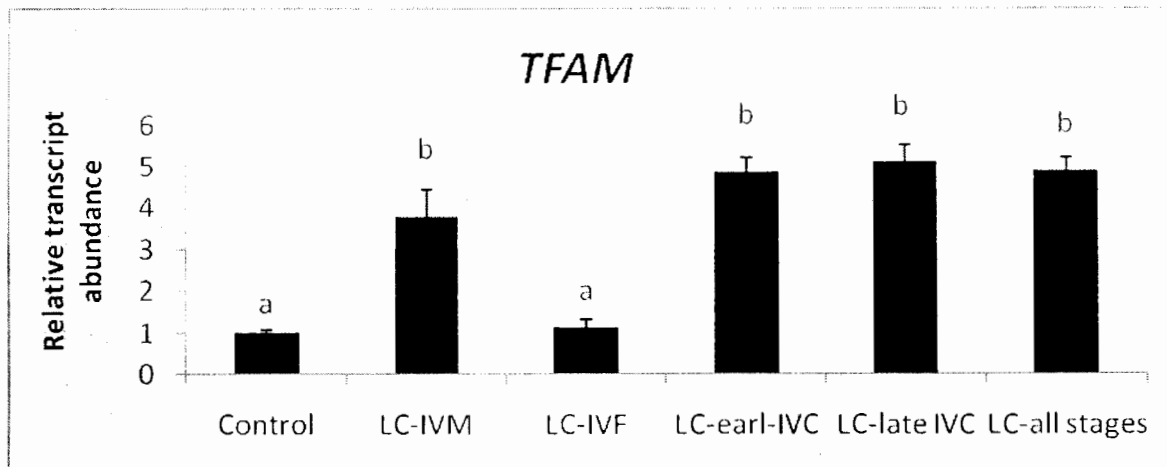


Fig. 3. Relative transcript abundance of *TFAM* gene on bovine embryos cultured with L-carnitine at different pre-implantation stages using real-time PCR (mean ± SEM). Means without a common superscript differed ($P < 0.05$).

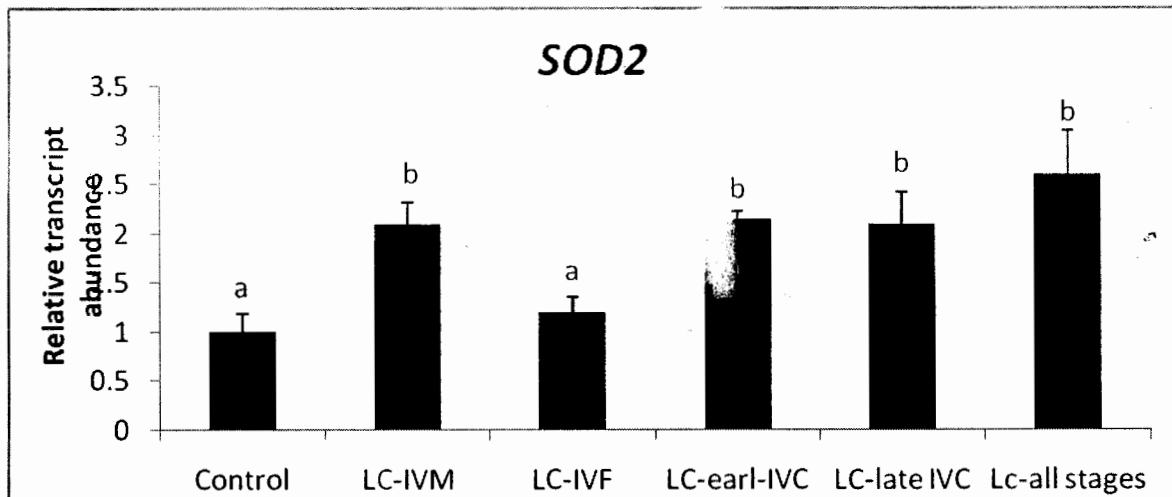


Fig. 4. Relative transcript abundance of *SOD2* gene on bovine embryos cultured with L-carnitine at different pre-implantation stages using real-time PCR (mean ± SEM). Means without a common superscript differed ($P < 0.05$).

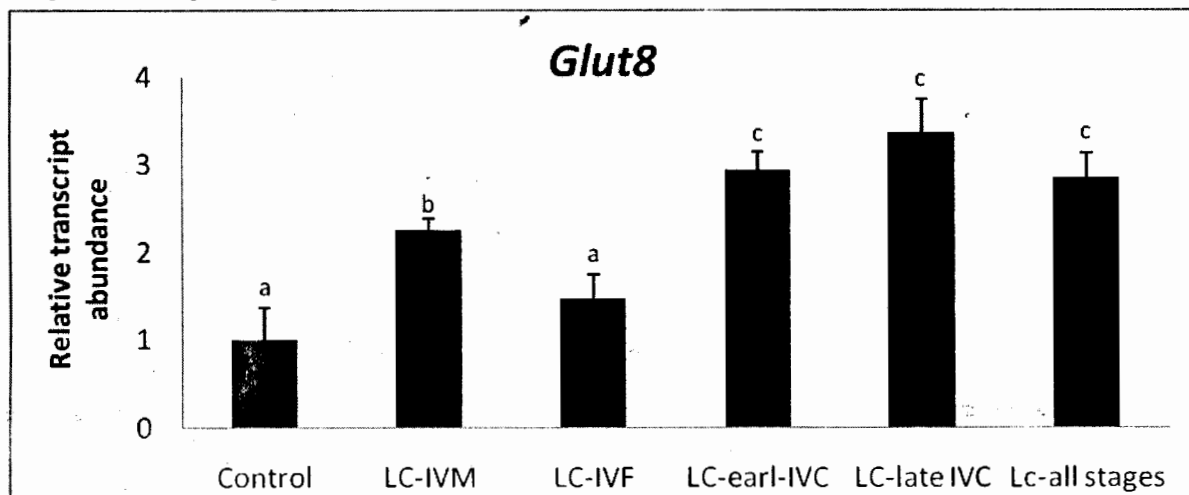


Fig. 5. Relative transcript abundance of *Glut8* gene on bovine embryos cultured with L-carnitine at different pre-implantation stages using real-time PCR (mean ± SEM). Means without a common superscript differed ($P < 0.05$).

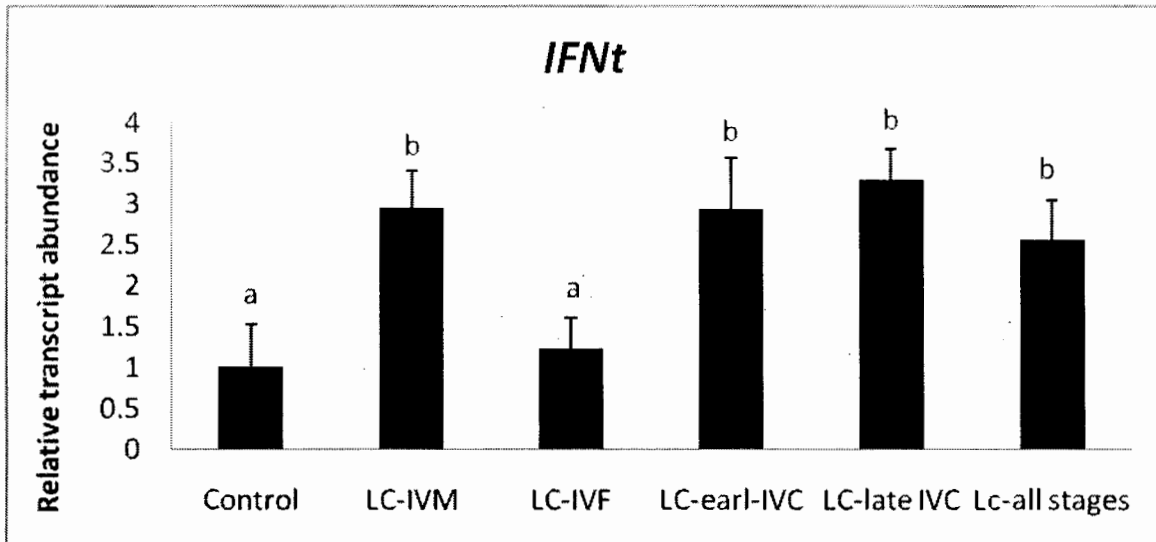


Fig. 6. Relative transcript abundance of *IFNt* gene on bovine embryos cultured with L-carnitine at different pre-implantation stages using real-time PCR (mean ± SEM). Means without a common superscript differed ($P < 0.05$).

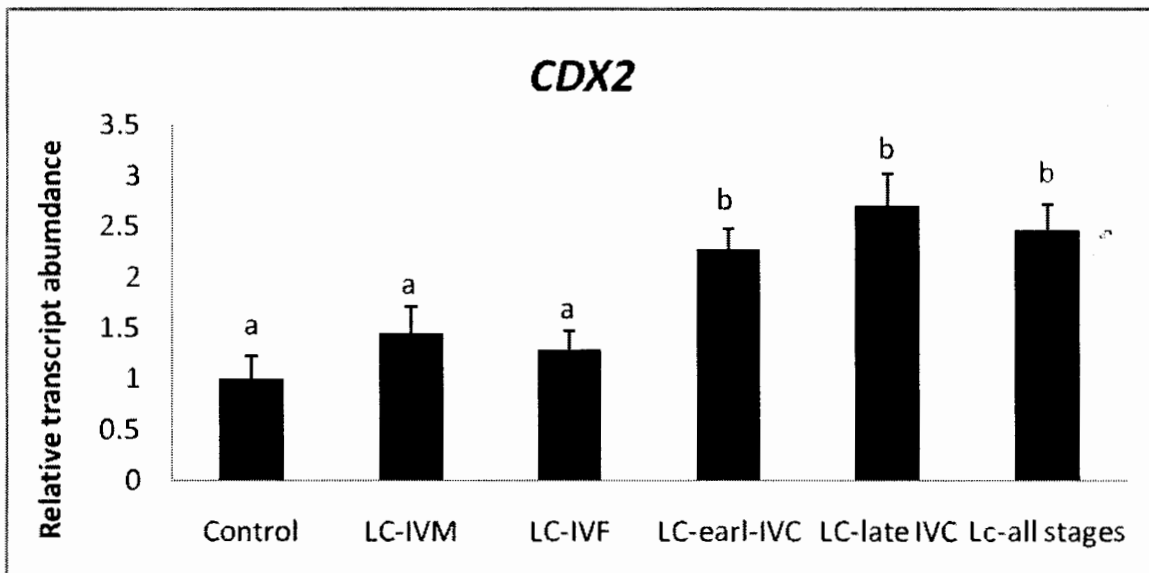


Fig. 7. Relative transcript abundance of *CDX2* gene on bovine embryos cultured with L-carnitine at different pre-implantation stages using real-time PCR (mean ± SEM). Means without a common superscript differed ($P < 0.05$).

DISCUSSION

Collection of cumulus-oocyte complexes from heterogeneous follicular population of slaughterhouse ovaries has resulted in a wide variation in bovine *in vitro* embryo production rate and quality (Gordon, 2003 and Krisher, 2004). As well-established, the quality of the oocyte is the main determinant of blastocyst rate, while the culture environment affects embryo quality (Rizos *et al.*, 2002 and Lonergan *et al.*, 2003). Therefore, many efforts have been made to either select competent oocytes based on morphology (Gordon, 2003), the content of G6PDH enzyme, (brilliant cresyl blue staining; Alm *et al.*, 2005) molecular profiling (gene expression; Ghanem *et al.*, 2007) or combining different approaches (Torner *et al.*, 2008). In parallel, many efforts have been done to improve preimplantation culture

environment especially during IVM (Choi *et al.*, 2013) and IVC (Ghanem *et al.*, 2014).

Results of the current study showed no significant differences in cleavage rate at either 48 or 96 hpi when L-carnitine was added at IVM, IVF, early IVC, late IVC, and all stages compared to control group. However, blastocyst development rate was increased when L-carnitine was added at IVM, early IVC, late IVC, and all stages compared to IVF and control groups. Phongnimitr *et al.* (2013) reported an increase in morula and blastocyst rates when cumulus-oocyte complexes were *in vitro* matured in presence of L-carnitine however. When oocytes were exposed to L-carnitine at all stages (IVM, IVF and IVC), there was no significant difference in rate of morula or blastocyst formation compared with the non-treated group. One possible reason to explain why L-carnitine does not improve embryo development when added at IVF could be that L-

carnitine is antagonizing the action of ROS at this stage. It is noteworthy that ROS might play a positive role during IVF and antioxidant chemicals supplemented at this stage could antagonize the beneficial effect on the developing embryos (Ali *et al.*, 2003). The balance between ROS and the presence of an antioxidant is a key factor in the success of embryonic development (de Lamirande *et al.*, 1997). However, L-carnitine added to *in vitro* culture medium has a positive impact on the development of bovine (Takahashi *et al.*, 2013 and Ghanem *et al.*, 2014) as well as mice embryos (Abdelrazik *et al.*, 2009). In porcine, COCs treated with L-carnitine during *in vitro* maturation showed higher rate of blastocyst formation after parthenogenetic activation (39.4% vs. 27.1%) and somatic cell nuclear transfer (23.2% vs. 14.9%) compared with untreated counterparts (You *et al.*, 2012). Moreover, supplementation of L-carnitine during IVM or IVC improved developmental potential of porcine oocytes after parthenogenetic activation (Wu *et al.*, 2011). This increase in the blastocyst development rate could be attributed to the positive metabolic effect of this chemical, as reported earlier by Sutton-McDowall *et al.* (2012). The metabolically activity of L-carnitine treated embryos was increased which subsequently reflected in higher rates of ATP-ADP conversion via increased β -oxidation within embryos. Therefore, the developing embryos that have been treated with L-carnitine are more likely to be able meet their increasing energy requirement than their untreated counterparts.

The results of the current study showed also improvement of blastocyst quality, based on more total cell number and fewer TUNEL-positive cells in embryos that developed from all treatment groups than untreated group. These results are in accordance with two previous studies that showed an increase in the number of total cells and reduction in apoptotic cells when bovine embryos cultured in presence of L-carnitine (Takahashi *et al.*, 2013 and Ghanem *et al.*, 2014). Similarly, L-carnitine added to the IVM or IVC media decreased the number of apoptotic cells and reduced ROS level in porcine embryos (Wu *et al.*, 2011). Additionally, L-carnitine has been shown to protect mice embryos from the negative effects of tumor necrosis factor α , oxidative stress, and apoptosis (Abdelrazik *et al.*, 2009). Collectively, the potentiality of L-carnitine in improving embryo quality could be due to its high ability in reducing oxidative stress by enhancing the activity of numerous antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) (Rizzo *et al.*, 2010) and antagonizing ROS formation (Abdelrazik *et al.*, 2009).

In order to understand the changes observed in embryo development and quality, some selected candidate genes were profiled as an indicator of the molecular characteristics of treated embryos. The expression profile of two genes regulating metabolism (*Glut8* and *TFAM*) was increased in embryos treated with L-carnitine at IVM, early IVC,

late IVC and all stages compared to IVF and control groups. This is consistent with the observation that L-carnitine enhances metabolism through increased β -oxidation within embryos to meet the energy requirement (Sutton-McDowall *et al.*, 2012). In addition, Ghanem *et al.* (2014) have also reported increase in the expression of genes related to metabolism *NADH*, *CPT1B*, *CPT2*, *SLC27A1* and *SLC22A5* in L-carnitine treated embryos compared with control group. Moreover, Takahashi *et al.*, (2013) reported increases in ATP concentration and expression of two metabolism-related genes (*ATP6* and *COX1*) in bovine embryos treated with L-carnitine. One possible mechanism of L-carnitine action to increase embryo metabolism could be through enhancing mitochondrial activity as previously reported for *in vitro* matured porcine oocytes (Somfai *et al.*, 2011) and bovine embryos (Ghanem *et al.*, 2014).

In accordance with the previous reports in regard to the anti-apoptotic and anti-oxidative stress potentiality of L-carnitine, the expression profile of gene regulating cellular defense (*SOD2*) was increased in embryos treated with L-carnitine at IVM, early IVC, late IVC and all stages compared to IVF and control groups. Indeed, up-regulation of *SOD2* which located in the mitochondrial matrix could improve the ability of embryos with increased metabolism to neutralize reactive oxygen species (Holley *et al.*, 2010). Nevertheless, increased expression of *SOD2* in embryos was linked with enhancing mitochondrial activity (Rizos *et al.*, 2002) and improving embryos quality (Ramalho-Santos *et al.*, 2009).

Two markers of trophoectoderm (TE) cells (*IFNt* and *CDX2*) were profiled on all treatment groups. Interferon tau (*IFNt*) is the maternal recognition signal required for successful pregnancy (Hernandez-Ledezma *et al.*, 1992 and Bazer *et al.*, 1997). Results of the present work observed higher expression of *IFNt* gene in embryos treated with L-carnitine at IVM, early IVC, late IVC and all stages compared to IVF and control groups. Rizos *et al.* (2003) have observed a significantly higher level of expression of *IFNt* in blastocysts produced in serum-free medium, which would be consistent with the notion that mRNA levels for this transcript are higher in good-quality embryos. In accordance with this idea, a greater abundance of *IFNt* transcript was detected in embryos developed in the presence of LC only (Ghanem *et al.*, 2014). Accordingly, the previous authors inferred that blastocysts developed in culture supplemented with LC were most likely to survive after transfer. On the other hand, the transcript abundance of *CDX2* was greater in IVM, late IVC and all stages than any other experimental groups. *CDX2* expression in porcine and bovine embryos resembled that of mice, suggesting a conserved role for *CDX2* in the formation of the TE between mammals (Kuijk *et al.*, 2008). Bovine biopsies derived from blastocysts resulting in calf delivery showed higher level of *CDX2* expression compared

to those terminated with resorption (El-Sayed *et al.*, 2006). In buffalo, *in vitro* produced blastocysts have shown higher transcript abundance of *CDX2* mRNA expression compared to parthenogenetic derived counterparts (Abdoon *et al.*, 2012).

In conclusion, the data of the current study have showed an improvement in embryo development and quality when L-carnitine added to IVM, early IVC, late IVC and all pre-implantation stages. This beneficial effect of this chemical was mainly due to metabolic enhancement of cleaving embryos and anti-apoptotic potentiality as indicated by increase of total normal cells and reduction in apoptotic cells which was linked with increase expression of genes involved in metabolism and cellular defense (*TFAM*, *Glut8* and *SOD2*). The up-regulation of genes related with embryo-maternal recognition and trophoectoderm (*IFNt* and *CDX2*) in embryos treated with L-carnitine support the notion that these embryos are most likely to survive after transfer to recipients.

REFERENCES

- Abdelrazik, H., R. Sharma, R. Mahfouz, and A. Agarwal, 2009. L carnitine decreases DNA damage and improves the *in vitro* blastocyst development rate in mouse embryos *Fertil. Steril.*, 91: 589-596.
- Abdoon, A.S., N. Ghanem, O.M. Kandil, A. Gad, K. Schellander, and D. Tesfaye, 2012. cDNA microarray analysis of gene expression in parthenotes and in vitro produced buffalo embryos. *Theriogenology*, 77: 1240-1251.
- Ali, A.A., J.F. Bilodeau, M.A. Sirard, 2003. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology*, 59: 939-949.
- Alm, H., H. Torner, B. Löhrke, T. Viergutz, I.M. Ghoneim, and W. Kanitz, 2005. Bovine blastocyst development rate in vitro is influenced by selection of oocytes by brilliantcresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology*, 63: 2194-2205.
- Bazer, F.W., T.E. Spencer, and T.L. Ott, 1997. Interferon tau: a novel pregnancy recognition signal. *Am. J. Reprod. Immunol.*, 37: 412-420.
- Chankitisakul, V., T. Somfai, Y. Inaba, M. Techakumphu, and T. Nagai, 2013. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes. *Theriogenology*, 79: 590-598.
- Choi, B.H., J.I. Bang, J.I. Jin, S.S. Kim, H.T. Jo, G.K. Deb, N. Ghanem, K.W. Cho, and I.K. Kong, 2013. Coculturing cumulus oocyte complexes with denuded oocytes alters zonapellucida ultrastructure in *in vitro* matured bovine oocytes. *Theriogenology*, 80: 1117-1123.
- deLamirande, E., H. Jiang, A. Zini, H. Kodama and C. Gagnon, 1997. Reactive oxygen species and sperm physiology. *Rev. Reprod.*, 2: 48-54.
- Droge, W., 2002. Free radicals in the physiological control of cell function. *Physiol. Rev.*, 82: 47-95.
- Dunning, K.R., L.K. Akison, D.L. Russell, R.J. Norman, and R.L. Robker, 2011. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. *Biol. Reprod.*, 85: 548-555.
- El-Sayed, A., M. Hoelker, F. Rings, D. Salilew, D. Jennen, E. Tholen, M.A. Sirard, K. Schellander, D. Tesfaye, 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiol. Genomics*, 28: 84-96.
- Farin, P.W., J.A. Piedrahita, and C.E. Farin, 2006. Errors in development of fetuses and placentas from *in vitro* - produced bovine embryos. *Theriogenology*, 65: 178-191.
- Ghanem, N., A.N. Ha, M. Fakruzzaman, J.I. Bang, S.C. Lee, and I.K. Kong, 2014. Differential expression of selected candidate genes in bovine embryos produced in vitro and cultured with chemicals modulating lipid metabolism. *Theriogenology*, 82: 238-250.
- Ghanem, N., M. Hölker, F. Rings, D. Jennen, E. Tholen, M.A. Sirard, H. Torner, W. Kanitz, K. Schellander, and D. Tesfaye, 2007. Alterations in transcript abundance of bovine oocytes recovered at growth and dominance phases of the first follicular wave. *BMC Dev. Biol.*, 7:90.
- Gordon, I., 2003. Recovering the bovine oocyte. Gordon I: In laboratory production of cattle embryos, Cambridge: CAB International/Cambridge University Press:79-111.
- Gülçin, I., 2006. Antioxidant and antiradical activities of L-carnitine. *Life Sci.*, 78: 803-811.
- Hasler, J.F., 1998. The current status of oocyte recovery, in vitro embryo production, and embryo transfer in domestic animals with an emphasis on the bovine. *J. Anim. Sci.*, 76: 52-74.
- Hernandez-Ledezma, J.J., J.D. Sikes, C.N. Murphy, A.J. Watson, G.A. Schultz, R.M. Roberts, 1992. Expression of bovine trophoblast interferon in conceptuses derived by in vitro techniques. *Biol. Reprod.*, 47: 374-380.
- Holley, A.K., Dhar, S.K., and D.K. St Clair, 2010. Manganese superoxide dismutase vs. p53: regulation of mitochondrial ROS. *Mitochondrion*, 10: 649-661.
- Krisher, R.L., 2004. The effect of oocyte quality on development. *J. Anim. Sci.*, 8: 214-223.
- Kruip, T.A., R. Boni, Y.A. Wurth, M.W. Roelofsen, and M.C. Pieterse, 1994. Potential use of ovum pick-up for embryo production and breeding in cattle. *Theriogenology*, 42: 675-684.

- Kuijk, E.W., L. Du Puy, H.T. Van Tol, C.H. Oei, H.P. Haagsman, B. Colenbrander, B.A. Roelen, 2008. Differences in early lineage segregation between mammals. *Dev. Dyn.* 237: 918-927.
- Lequarre, A.S., B. Grisart, B. Moreau, N. Schuurbiens, A. Massip, and F. Dessy, 1997. Glucose metabolism during bovine preimplantation development: analysis of gene expression in single oocytes and embryos. *Mol. Reprod. Dev.* 48: 216-226.
- Loneragan, P., D. Rizos, A. Gutierrez-Adan, T. Fair, and M.P. Boland, 2003. Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reprod. Dom. Anim.*, 38: 259-267.
- Machatkova, M., P. Hulinska, Z. Reckova, K. Hanzalova, J. Spanihelova, and R. Pospisil, 2008. In vitro production of embryos from high performance cows and the development of frozen-thawed embryos after transfer: a field study. *Veterinari Medicina*, 53: 358-364.
- Meintjet, M., M.S. Bellow, J.R. Broussard, J.B. Paul, and R.A. Godke, 1995. Transvaginal aspiration of oocytes from hormone-treated pregnant beef cattle for in vitro fertilization. *J. Anim. Sci.*, 73: 967-974.
- Nasr-Esfahani, M.H., J.R. Aitken, and M.H. Johnson, 1990. Hydrogen peroxide levels in mouse oocytes and early cleavage stages embryos developed in vitro or in vivo. *Development*, 109: 501-507.
- Phongnimitr, T., Y. Liang, K. Srirattana, K. Panyawai, N. Sripunya, C. Treetampinich, and R. Parnpai, 2013. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. *Anim. Sci. J.* 84: 719-725.
- Pillich, R.T., G. Scarsella, and G. Risuleo, 2005. Reduction of apoptosis through the mitochondrial pathway by the administration of acetyl-L-carnitine to mouse fibroblasts in culture. *Exp. Cell Res.*, 306: 1-8.
- Ramalho-Santos, J., S. Varum, S. Amaral, P.C. Mota, A.P. Sousa, A. Amaral, 2009. Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum. Reprod. Update*, 15: 553-572.
- Rizos, D., A. Gutiérrez-Adán, S. Pérez-Garnelo, J. De La Fuente, M.P. Boland, P. Lonergan, 2003. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biol. Reprod.*, 68: 236-243.
- Rizos, D., P. Lonergan, M.P. Boland, R. Arroyo-Garcia, B. Pintado, J. de la Fuente, and A. Gutiérrez-Adán, 2002. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biol. Reprod.* 66: 589-595.
- Rizzo, A.M., P. Berselli, S. Zava, G. Montorfano, M. Negroni, P. Corsetto, and B. Berra, 2010. Endogenous antioxidants and radical scavengers. *Adv. Exp. Med. Biol.*, 698: 52-67.
- Rosenkrans, C.F. Jr., G.Q. Zeng, G.T. McNamara, P.K. Schoff, and N.L. First, 1993. Development of bovine embryos in vitro as affected by energy substrates. *Biol. Reprod.*, 49: 459-462.
- Scott, C.A., L. Robertson, R.T. de Moura, C. Paterson, and J.S. Boyd, 1994. Technical aspects of transvaginal ultrasound-guide follicular aspiration in cows. *Vet. Rec.*, 134: 440-443.
- Somfai, T., M. Kaneda, S. Akagi, S. Watanabe, S. Haraguchi, E. Mizutani, T.Q. Dang-Nguyen, M. Geshi, K. Kikuchi, and T. Nagai, 2011. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod. Fertil. Dev.*, 23: 912-920.
- Sudano, M.J., M.C.C. Mattos, C.B. Fernandes, R.R. Mazieiro, and F.C. Landim-Alvarenga, 2010. In vitro production of bovine embryos using Sigma antioxidant supplement®, α -tocopherol and L-ascorbic acid. *Anim. Reprod.*, 7: 42-48.
- Sutton-McDowall, M.L., D. Feil, R.L. Robker, J.G. Thompson, and K.R. Dunning, 2012. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. *Theriogenology*, 77: 1632-1641.
- Takahashi, T., Y. Inaba, T. Somfai, M. Kaneda, M. Geshi, T. Nagai, and N. Manabe, 2013. Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro. *Reprod. Fertil. Dev.*, 25: 589-599.
- Turner, H., N. Ghanem, C. Ambros, M. Hölker, W. Tomek, C. Phatsara, H. Alm, M.A. Sirard, W. Kanitz, K. Schellander, and D. Tesfaye, 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction*, 135: 197-212.
- Trounson, A.O., D. Pushett, L.J. MacLellan, and J. Lewis, 1994. Current status of IVM/IVF and embryo culture in humans and farm animals. *Theriogenology*, 41: 57-66.
- Van Soom, A., Y.Q. Yuan, L.J. Peelman, D.G. Matos, J. Dewulf, H. Laevens, and A. Kruif, 2002. Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. *Theriogenology*, 57: 1453-1465.
- Wu, G.Q., B.Y. Jia, J.J. Li, X.W. Fu, G.B. Zhou, Y.P. Hou, and S.E. Zhu, 2011. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. *Theriogenology*, 76: 785-793.
- Ye, J., J. Li, Y. Yu, Q. Wei, W. Deng, and L. Yu, 2010. L-carnitine attenuates oxidant injury in HK-2 cells via ROS-mitochondria pathway. *Regul. Pept.* 161: 58-66.

You, J., J. Lee, S.H. Hyun, and E. Lee, 2012. L-carnitine treatment during oocyte maturation improves in vitro development of cloned pig embryos by influencing intracellular glutathione synthesis and embryonic gene expression. *Theriogenology*, 78: 235-243.

Yuan, Y.Q., A. Van Soom, F.J. Coopman, K. Mintiens, M.L. Boerjan, A. Van Zeveren, A. De

Kruif, and L.J. Peelman, 2003. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. *Theriogenology*, 59: 1585-1596.

إضافة الكارنتين خلال مراحل تطور أجنة الأبقار له تأثير محفز لزيادة تطور وتحسين جودة الأجنة

ناصر غانم

قسم الإنتاج الحيواني، كلية الزراعة، جامعة القاهرة، مصر.

أجريت هذه التجربة بهدف التعرف على تأثير إضافة مادة الكارنتين (مضادة للأكسدة) خلال مراحل تطور أجنة الأبقار على معدل تطورها وجودتها عند مرحلة البلاستوسيس. لذا تم تجميع بويضات الأبقار المذبوحة في المجزر وتقييمها مورفولوجياً واختبار عالية الجودة منها (ن = 2126).

عملية الإنضاج تمت معملياً وكذلك إخصابها وزراعة الأجنة الناتجة في بيئة نمو CR1-aa لإستكمال النمو والتطور في مرحلة البلاستوسيس. تم إضافة الكارنتين بتركيز 1.5 ملليمول في مراحل (الإنضاج IVM، الإخصاب IVF، المراحل الأولى للتطور الجنيني early IVC، المراحل الأخيرة للتطور الجنيني late IVC، وجميع مراحل التطور all stages). بالإضافة الى ذلك تم وضع مجموعة مقارنة (control). قيمت جودة الأجنة باستخدام اختبار (TUNEL) والذي يحدد عدد الخلايا الجنينية الطبيعية والميتة. وتم دراسة التعبير الجيني لخمس جينات (TFAM، SOD2، CDX2، GLUT8) مرتبطة بجودة الأجنة باستخدام تقنية البلمرة المتزامن Real-Time PCR.

أظهرت نتائج الدراسة، زيادة في معدل تطور الاجنة (الوصول لمرحلة البلاستوسيس) عند إضافة الكارنتين خلال مراحل الإنضاج (20.6 ± 3.1)، المراحل الأولى للتطور الجنيني (1.6 ± 3.4)، المراحل الأخيرة للتطور الجنيني (1.4 ± 22.1) والمجموعة المعاملة في مرحلة الإخصاب (1.1 ± 26.0).

وأظهرت أيضاً النتائج أن العدد الكلي للخلايا الطبيعية للأجنة كانت أكثر عند إضافة الكارنتين خلال مراحل الإنضاج (9.1 ± 13.4)، الإخصاب (2.8 ± 120.7)، المراحل الأولى للتطور الجنيني (3.5 ± 139.2) وجميع مراحل التطور (1.1 ± 137.0) وذلك مقارنة بالمجموعة الغير معاملة (3.6 ± 110.3). وعلى الجانب الآخر، فقد زاد عدد الخلايا الميتة في الأجنة الغير معاملة (9.9 ± 1.4) مقارنة بالمجموعات التي عوملت بالكارنتين خلال مراحل الإنضاج (1.2 ± 4.1)، الإخصاب (1.6 ± 0.5)، المراحل الأولى للتطور الجنيني (1.4 ± 30.9) والمراحل الأخيرة من التطور الجنيني (1.7 ± 4.0) وجميع مراحل التطور الجنيني (2.0 ± 2.8).

التعبير الجيني لجينات (TFAM، SOD2، IFNT، Glut8) قد زاد في الاجنة التي عوملت بالكارنتين خلال مراحل الإنضاج، المراحل الأولى من التطور الجنيني مقارنة بكلاً من المجموعة الغير معاملة والمجموعة التي عوملت بالكارنتين خلال مرحلة الإخصاب.

التعبير الجيني ل CDX2 قد زاد فقط في المجموعات التي عوملت بالكارنتين خلال المراحل الأولى للتطور الجنيني والمراحل الأخيرة وجميع مراحل التطور الجنيني مقارنة بباقي المجموعات.

نستخلص من هذه الدراسة أن إضافة المادة المضادة للأكسدة (الكارنتين) خلال مراحل التطور الجنيني له تأثير إيجابي على معدل نمو وتطور وكذلك جودة الأجنة الناتجة.