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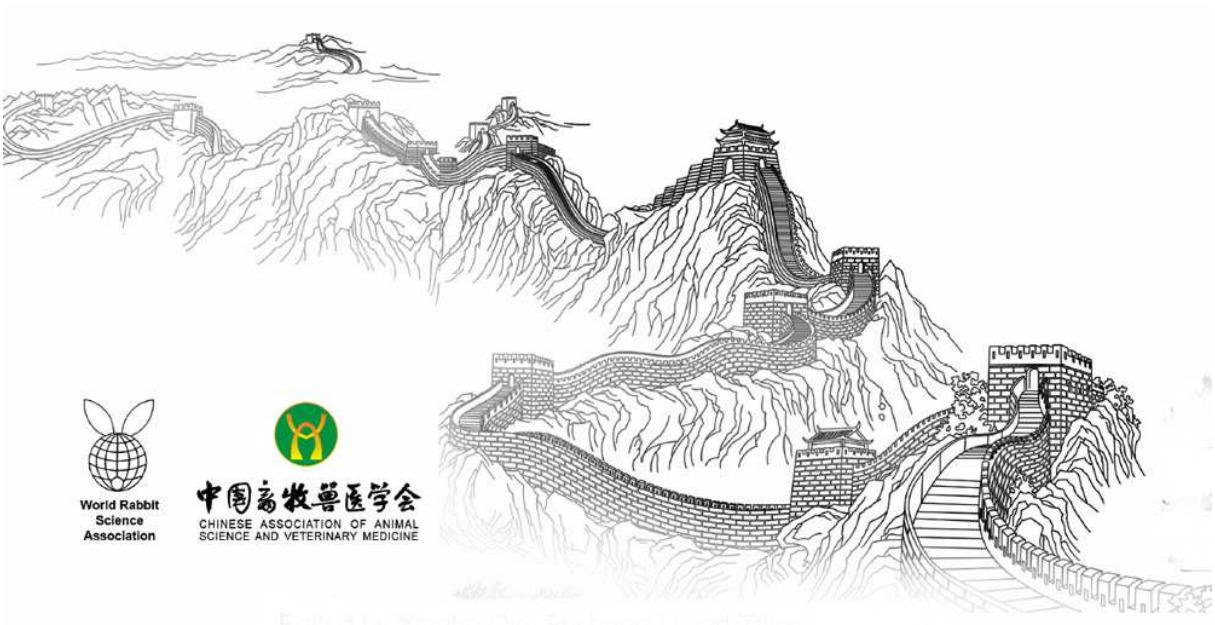

EFFECT OF FREE L-CARNITINE ADDED TO MATURATION MEDIUM ON *IN VITRO* MATURATION, FERTILIZATION AND CULTURE OF RABBIT OOCYTES

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ABSTRACT

To study the effect of supplementing maturation medium with L-carnitine (LC) at level of 0.0, 0.6 and 0.9 mg/ml on *in vitro* maturation (IVM) of immature rabbit oocytes, and consequently in their fertilization (IVF) and embryo development, oocytes were harvested by slicing from 32 mature rabbit does after slaughter, and only compact-cumulus oocytes (COCs) were used in this study. COCs were *in vitro* matured in CO_2 incubator (5% CO_2, at 38°C with high humidity, HH) for 20 h. Maturation rate (percentage of oocytes at metaphase II, MII) was determined. Fertilization rate (FR) of COCs matured with different levels of LC was determined and fertilized ova were cultured for 5 days at 38°C with 5% CO_2 with HH for determining morula (MPR) and blastocyst (BPR) production rates. Results showed that adding LC to maturation medium at levels of 0.6 and 0.9 mg/ml increased ($P \leq 0.05$) the percentage of oocytes arrested at MII (86.79 and 80.77%) as compared to free medium (74.72%). Percentage of MII oocytes was higher ($P<0.05$) for 0.6 than 0.9 mg LC/ml. *In vitro* maturation of rabbit oocytes with TCM-199 supplemented with LC at 0.9 and 0.6 mg/ml improved ($P<0.05$) the fertilization rate (68.89 and 67.94, vs. 50.98%) and production rate of embryos at morula (34.78 and 38.06 vs. 23.52%) and blastocyst stages (15.89 and 17.78 vs. 7.84%) as compared to control medium, respectively. Adding 0.6 mg LC per ml of maturation medium (TCM) improved nuclear maturation and consequently their cleavage rate and morula/blastocyst production of follicular rabbit oocytes.

Key words: Rabbit, oocytes, L-carnitine, metaphase II, *in vitro* culture.

INTRODUCTION

Most improvements in the success rate of *in vitro* embryo production were attributed to the development of media based on metabolic requirements of the oocytes and embryos (Manzano *et al.*, 2015). During *in vitro* maturation (IVM), lipid droplets in the oocyte cytoplasm (Dunning *et al.*, 2014) increased in number (Aardema *et al.*, 2011) and undergo structural reorganization, aggregating centrally (Yang *et al.*, 2010) as compared to oocytes at a maturation stage. High levels of functional mitochondria (MIT) are required for energy production needed to oocyte meiotic process (May-Panloup *et al*., 2007), leading to oocyte development (May-Panloup *et al*., 2005) and increasing fertilization rate (Reynier *et al*., 2001). Also, the process of aerobic metabolism produces reactive oxygen species (ROS), which are a natural and important part of many physiological processes. MIT are particularly susceptible to damage, in part due to their proximity to the source of ROS generation. If MIT become damaged, it can create a self-perpetuating cycle as defective respiration leads to a further increase in ROS. With damaged MIT, the energy-intensive processes of meiosis, fertilization, and blastocyst formation can become impaired (Pritchard *et al*., 2015).During IVM and fertilization (IVF), L-carnitine (LC) has a role in reducing ROS production and lipid droplets in oocytes or embryos during these processes. The beneficial effects of LC on IVM, IVF and embryo development were indicated by Somfai *et al.* (2011) and Krisher *et al.* (2007). Adding LC as a component of lipid

metabolism to IVM medium reduced lipid content in porcine oocytes (Somfai *et al.*, 2011), and increased potentiality of mitochondria in oocytes to produce energy needed for meiotic processes and fertilization supporting further development of resulting embryos to the blastocyst stage (Krisher *et al*., 2007). Adding L-carnitine (LC) as a component of lipid metabolism to maturation medium improved *in vitro* maturation (IVM) and cleavage rate of porcine oocytes (Somfai *et al.*, 2011; Krisher *et al*., 2007). However, Manzano *et al.* (2015) showed marked effect of LC on embryos rather than bovine oocytes. Therefore, the aim of this study was to investigate the effect of addition of L-carnitine at levels of 0.6 and 0.9 mg/ml to maturation medium to increase the successful of embryo production from follicular oocytes in rabbits.

MATERIALS AND METHODS

Animals

A total of 32 mature rabbit does of New Zealand white (NZW, 5-6 mo of age, 3-3.5 kg LBW) as oocyte donors and 6 NZW bucks (7.5-8 mo of age and 3.5-4.0 kg LBW) for semen collection were taken from Sakha Experimental Station, Agricultural Research Center, Egypt. Immediately after slaughtering, ovaries were removed, washed by 0.9% NaCl and dried.

Oocytes were collected by slicing technique into tissue culture dishes containing 4 ml of harvesting medium (Dulbecco's Phosphate buffer saline, PBS, Sigma. Co.), supplemented with 3 mg bovine serum albumin (BSA)/ml, 0.03 % sodium pyruvate and 50 µg/ml gentamycin sulfates. Oocytes were examined and evaluated. Only compact-cumulus oocytes (COCs) were used in this study.

In vitro Maturation and Fertilization

Tissue Culture Medium (TCM-199) supplemented with 10% BSA (w/v), 10 IU/ml PMSG, 10 IU/ml hCG, 1 µg/ml estradiol, 0.03 % sodium pyruvate and 50 µg/ml gentamycin sulfates was used as *IVM* medium. Three types of *IVM* medium were supplemented with 0.0, 0.6 or 0.9 mg/ml of LC. The pH value and osmolarity of the medium were adjusted to 7.2-7.4 and 280-300 mOsmol/kg, respectively. Filtered *IVM* medium (100 µl) was placed into well dishes and covered by sterile mineral oil and incubated in 5%CO_2, at 38°C with saturated humidity for at least 60 min. COCs were washed three times with *IVM* medium, cultured and incubated under the same conditions for 20 h. After *IVM* period, COCs were washed using DPBS containing 1 mg /ml hyaluronidase, washed two times with PBS plus 2% BSA, and loaded into fixation solution (3 ethanol: 1 glacial acetic acid) overnight and stained with 1 % orcein in 45% acetic acid to determine the proportion of oocytes at metaphase II (MII).

Semen from 15 ejaculates was capacitated in DPBS medium supplemented with 3 mg/ml BSA 50 µg/ml gentamycin sulfates and 35 µg/ml heparin. Maturated COCs by both levels of LC were *in vitro* fertilized by pipetting 50 µl of fertilization medium under sterile liquid paraffin oil and incubated at 38°C for 2 h in 5% Co_2 and high humidity. About 50 µl washing media was added to each droplet with 10-15 oocytes following by adding 2 µl of prepared semen and then incubated together at 38°C for 24 h in 5% Co_2.

After IVF, cumulus cells were separated from presumptive zygotes by pipetting and were washed twice in culture medium comprising SOF medium supplemented 10% FCS, 0.03 % sodium pyruvate and 50 µg/ml gentamycin sulfates. To determining the rate of cleavage, 10 zygotes were cultured in each four-well Petri dish containing 100 µl of culture medium under mineral oil for 5 days at 38 °C with 5 % CO_2 in air.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using computer program of SAS (1998). ANOVA was performed after arcsine transformation of original data expressed as proportions. The significant differences among group means were preformed using Duncan Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Results in Table 1 showed that adding LC to maturation medium at levels of 0.6 and 0.9 mg/ml increased (P≤0.05) the percentage of oocytes arrested at MII as compared to free medium, however, the percentage of MII oocytes was higher (P<0.05) for 0.6 than 0.9 mg LC/ml. Contrary, both LC levels reduced (P<0.05) the percentages of GVBD and degenerated oocytes as compared to control medium.

Table 1: *In vitro* maturation of rabbit oocytes in maturation medium supplemented with L-carnitine (LC).

Maturation medium	Oocytes	GV	GVBD	M I	M II	Degen.
Control	87	4.59±0.22[a]	9.20±1.32[a]	4.59±1.09	74.72±0.68[c]	6.90±0.45[a]
LC (0.6 mg/ml)	91	0.00±0.00[b]	2.15±1.08[b]	7.75±1.30	86.79±0.38[a]	3.30±0.09[b]
LC (0.9 mg/ml)	88	4.49±0.98[a]	4.61±1.27[b]	5.64±1.04	80.77±1.69[b]	4.49±0.98[b]

Means with different letters on the same column differ significantly at P<0.05.
GV: Germinal vesicle. GVBD: Germinal vesicle breakdown. MI: Metaphase I. MII: Metaphase II.

Results in Table 2 revealed that *in vitro* maturation of rabbit oocytes with TCM-199 supplemented with LC at 0.9 or 0.6 mg/ml improved (P<0.05) the fertilization rate and production rate of embryos at morula and blastocyst stages as compared to control medium, respectively. However, the differences between both LC levels were not significant.

Table 2: *In vitro* Fertilization rate and developmental competence of rabbit oocytes matured in maturation medium supplemented with L-carnitine (LC).

Maturation medium	Mature oocytes (n)	Fertilized ova (n)	Fertilization rate (%)	Morula (%)	Blastocyst (%)
Control	102	52	50.98±0.72[b]	23.52±1.89[b]	07.84±1.43[b]
LC (0.6 mg/ml)	100	69	68.89±1.38[a]	34.78±0.78[a]	15.89±0.48[a]
LC (0.9 mg/ml)	100	68	67.94±0.63[a]	38.06±1.94[a]	17.78±2.65[a]

Means with different letters on the same column differ significantly at P<0.05.

The current study indicated beneficial effects of LC supplementation in maturation medium (TCM) at levels of 0.6 and 0.9 mg/ml on IVM, IVF and the developmental competence of follicular rabbit oocytes. Adding LC as a component of lipid metabolism to maturation medium improved IVM and cleavage rate of porcine oocytes (Somfai *et al.*, 2011; Krisher *et al.*, 2007). However, Manzano *et al.* (2015) showed that no significant differences were noticed between the control (84.0%) and LC supplemented groups (0.1, 0.5 and 1.0 mg/ml, 83.1%–87.1%) in terms of nuclear maturation. However, they found an impact on the cellular metabolism, cellular antioxidant and prevention of lipid peroxidation in bovine embryos. Also, Moawad *et al.* (2014) found that LC supplementation in the IVM medium alone did not affect nuclear maturation. In mice, LC has been reported to improve maturation rate of oocytes and embryo developmental competence (Moawad *et al.*, 2013) due to its dual effects by enhancing mitochondrial lipid metabolism (Dunning *et al.*, 2010) and acting as an antioxidant to reduce the effects of ROS that induce DNA fragmentation (Abdelrazik *et al.*, 2009).

In accordance with the present results, supplementation of medium with 10 mM LC showed higher rates of blastocyst formation and improves its developmental competence in porcine. Such effect has been assumed to be due to increased intracellular gluthathione synthesis which reduces the ROS levels (You *et al*., 2012). ATP is synthesized by β-oxidation of fatty acids within mitochondria, which requires carnitine (Dunning *et al.*, 2010, 2011) has an essential role in determining oocyte quality and its ability to support embryo (Van Stojkovic *et al*., 2001; Ferguson and Leese, 2006). In two separate studies, it was shown that the *in vitro* addition of LC to the culture media not only improved oocyte chromosomal structure and reduced embryo apoptosis (Mansour *et al*., 2009), but also improved blastocyst development rate (Abdelrazik *et al*., 2009).

CONCLUSION

Based on the foregoing results adding 0.6 mg LC per ml of maturation medium (TCM) improved nuclear maturation and consequently their cleavage rate and morula/blastocyst production of follicular rabbit oocytes.

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