ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of DEVELOPMENT RESEARCH

International Journal of Development Research
Vol. 5, Issue, 07, pp. 5029-5033, July, 2015

Full Length Research Article

IMPROVED DEVELOPMENTAL POTENTIAL OF PORCINE BLASTOCYST BY L-CARNITINE SUPPLEMENTATION

***,1,2Marlon B. Ocampo and 2Lerma C. Ocampo**

1Department of Animal Science, Central Luzon State University
2Philippine Carabao Center, Reproductive Biotechnology Unit, Science City of Munoz, Nueva Ecija, Philippines 3120

ARTICLE INFO

Article History:
Received 10th April, 2015
Received in revised form
29th May, 2015
Accepted 25th June, 2015
Published online 30th July, 2015

Key words:
Blastocyst,
Cell count,
Embryos, Oocytes.

ABSTRACT

Objective: In this study, the potential role of L-carnitine supplementation in the maturation and preimplantation development was investigated using porcine as a model.
Materials and Methods: In Expt. 1, porcine oocytes were matured in the presence of L-carnitine (1.0, 0.5. 0.25 mg/ml) or absence (control), subjected to in vitro fertilization and assessed on their developmental potential up to the blastocyst stage. In Expt. 2, zygotes derived from in vitro fertilization were cultured in NCSU-23 medium with or without L-carnitine supplementation.
Results: L-carnitine addition had no significant influence on the maturation, cleavage and development to the blastocyst stage versus the control. However, the total cell count of blastocyst from L-carnitine groups were higher (45.0 ± 3.1, 46.4 ± 4.9, 44.6 ± 2.7 for 1.0, 0.5 and 0.25 mg/ml, respectively) than the control (34.5 ± 1.6). Also, zygotes development to the blastocyst stage had no difference, but the total cell count of blastocyst derived from L-carnitine groups (46.5 ± 12.7, 42.2 ± 14.2, 45.5 ± 9.1 for 1.0, 0.5 1nd 0.25 mg/ml, respectively) were higher than the control (38.6 ± 13.2).
Conclusion: The results demonstrated the efficiency of the procedures used in the maturation, fertilization and culture of porcine oocytes and early- stage embryos. That, L-carnitine supplementation regardless of the concentrations used in the maturation and culture media improved the developmental potential of blastocyst stage embryos as indicated by a higher total cell count (improved cell activity).

INTRODUCTION

In many mammalian species, the critical factors of utmost importance in the development of in vitro produced embryos are the culture conditions for the maturation of immature oocytes and early- stage embryos. Most improvements in the success rate of in vitro embryo production was attributed to the development of media based on metabolic requirements of the oocytes and embryos and the composition of oviductal and uterine fluids. For instance, the energy requirement needed by the oocytes and embryos for the acquisition of developmental competence differ (Biggers *et al*., 1967; Thompson *et al*., 2000). Cumulus-oocyte complexes (COCs) preferentially utilize glucose (Sutton *et al*., 2003; Harris *et al*., 2007), whereas pre-compaction embryos use pyruvate and lactate (Thompson *et al*., 1996) and return to glucose after compaction (Krisher *et al*., 1999; Purcel and Moley, 2009).

Corresponding author: Marlon B. Ocampo
Department of Animal Science, Central Luzon State University

Additionally, COCs derived in the ovaries collected from the slaughterhouse matured "spontaneously" in vitro. Such in vitro maturation (IVM) of the oocyte occurs in the absence of certain crucial cytoplasmic events and components that are required for complete developmental competence of the oocyte (Gilchrist and Thompson, 2007). Hence, the purpose of many researchers dealing on reproduction is to improve the oocyte and pre-implantation embryos developmental competence by delaying or temporarily preventing spontaneous maturation while at the same time promoting ooplasm development. In this study, two approaches have been tried to determine the possible role of L-carnitine in a dose-dependent manner on the acquisition of developmental competence of immature porcine COCs and early- stage embryos resulting from in vitro fertilization. First is on the incorporation of L-carnitine in the maturation medium, since it is during maturation that oocytes produce ATP, mostly by mitochondrial metabolism to fuel the energy needed for meiotic processes and fertilization supporting further

development of resulting embryos to the blastocyst stage (Hashimoto, 2009). Secondly, is on the inclusion of L-carnitine in the culture medium for early- stage embryos, since it is during this period that the "cell-block" phenomenon inhibit further development to the blastocyst stage of most mammalian embryos when cultured in vitro. L-carnitine has been claimed to have a beneficial role in the cellular metabolism and embryonic development of some mammalian species (Kruip *et al*., 1983; Ferguson and Leese, 2006; Abdelrazik *et al*., 2009) but got limited information available in porcine, hence this study.

MATERIALS AND METHODS

Media

The basic media for maturation of oocytes is a defined tissue culture medium (TCM-199; Gibco Co., Grand Island, N.Y., USA, Cat. No. 31100-035, Lot No. 1137722) with Earle's salts and L-glutamine (Krisher *et al*.,1999; 2007), for fertilization is a modified Tris-buffered medium (mTBM; Berger, 1990) and for culture is the NCSU-23 medium (Petters and Wells, 1993).

Oocyte collection and maturation

Porcine ovaries were collected immediately postmortem at local abattoirs and transported to the laboratory in 0.9 % NaCl solution at 30 - 35º C within 4 - 6 hr. The ovaries were pooled irrespective of the donors estrus cycle. COCs were aspirated from antral follicles (3 - 5 mm in diameter) by using an 18-gauge needle attached to a 10- ml sterile plastic syringe, washed three times in the maturation medium before selecting using a stereomicroscope based on the criteria described by Ocampo *et al*., (1993). A group of 10 - 15 COCs were transferred into 50 µl droplets of maturation medium under mineral oil (Sigma Chem Co., St. Louis, USA) in a 35 x 10 mm Falcon polysterene culture dish (Becton and Dickinson Labware, N.J., USA) which had been previously pre-incubated to equilibrate for at least 2 hr in a CO_2 incubator. COCs were cultured at 39º C under an atmosphere of 5 % CO_2 and 95 % air with high humidity. After culture for 42 hr, the cumulus and corona cells were removed by vortexing.

Sperm preparation

The sperm-rich fractions of ejaculate from a boar of known fertility was collected by the glove-hand method. Semen samples were washed three times with phosphate buffered saline solution containing 1 mg/ml bovine serum albumin (BSA) and 0.1 mg/ml penicillin/streptomycin sulphate. After the final was, the sperm was resuspended at 1 x 10^6 cells/ml in mTBM + 2mM caffeine + antibiotic + 2 mg/ml Fraction V pig BSA.

In vitro fertilization and culture

Selected oocytes were transferred to the fertilization droplets (50 µl) with 15 oocytes per drop under mineral oil. A portion of sperm suspension was added to the droplets giving a final sperm concentration of 5 x 10^5 cells/ml. The motility rate of sperm during insemination was more than 70%. After sperm-oocyte coculture for 6 hr, the extra sperm cells attached to the oocytes were removed by pipetting using a finely drawn glass pipette. Subsequently, the oocytes were washed twice with NCSU-23 medium + 4 mg/ml BSA. Groups of 10 - 15 oocytes were then transferred to 50 µl drops of NCSU-23 medium and cultured for 6 days.

Cell counting

Blastocyst appearing on the 6th day were collected and subjected to a staining protocol (Thouas *et al*., 2001) with modification. Briefly, blastocyst were washed in PBS-PVP, then placed in 1 ml of Hoechts working solution (0.75 ml of 2.3% Na citrate dehydrate solution; 0.25 ml of ethanol; 10 µl of Hoechts 33342 stock solution of 1 mg/ml concentration dissolved in ethanol) in an Effendorf tube, wrapped in aluminum foil and stored in the refrigerator for at least 24 hr. Subsequently, the blastocyst were recovered and washed in glycerol, mounted on a glass slide, flattened in glycerol by a cover slip to a level where all nuclei appeared at the same focal plane and examined by using a fluorescent microscope (Eclipse E-600; Nikon) under ultraviolet light. A digital image of each embryo was taken and the total cells (both inner cell mass and trophectoderm) counted.

Experimental design

Effect of L-carnitine supplementation in the IVM medium on nuclear maturation, fertilization and subsequent development to the blastocytst stage. COCs were matured in IVM medium supplemented with L-carnitine (1.0 mg, 0.50 mg and 0.25 mg/ml) or without (control) for 42 hr. Nuclear maturation of the oocytes was based on the presence of 1st polar body. Selected matured oocytes were subjected for fertilization and the cleavage and blastocyst formation rate analyzed followed by staining for cell counting. The experiment was replicated five times. *Effect of L-carnitine supplementation in the IVC medium on the blastocyst formation of cleaved embryos following fertilization*. Early stage embryos (2- to 4- cell stage) found 32 hr post insemination were randomly assigned to droplets of in vitro culture medium supplemented with L-carnitine (1.0 mg, 0.50 mg and 0.25 mg/ml) or without (control) for 5 days. Resulting blastocyst were counted and their cell count analyzed. The experiment was replicated four times.

Statistical analysis

The nuclear maturation, cleavage and blastocyst formation rate was analyzed by using ANOVA and the total cell count (mean ± SEM) by Student's t-test with $P < 0.05$ considered significant

RESULTS

In Expt. 1, the completion of 1st meiosis (nuclear maturation) as indicated by the emission of 1st polar body of immature COCs in maturation medium with or without L-carnitine supplementation showed no significant differences after 42 hr of culture. The high maturation rate obtained was attributed to the strict selection of COCs for maturation using only the good quality oocytes from a pool of hundreds of oocytes aspirated

during each collection. Subsequently, selection of matured oocytes for fertilization resulted to more than 70% cleavage rate in all treatment groups. The blastocyst formation rate similarly showed no difference, though highest in treatment with 0.5 mg/ml L-carnitine supplementation. Total cell count of blastocyst embryos derived from treatment groups with L-carnitine were higher versus the control (Table 1).

Table 1. Dose-related response of porcine oocytes matured with or without L-carnitine supplementation

Treatment mg/ml	No. of oocytes (%)					Cell count (mean±SEM)
	Cultured	Matured	Inseminated	Cleaved	Blastocyst	
Control	200	182 (91.0)	182	139 (76.4)	86 (47.3)	34.5 ± 11.6[a]
1.0	141	137 (97.1)	125	99 (79.2)	57 (45.6)	45.0 ± 13.1[b]
0.5	142	137 (96.4)	132	105 (79.5)	72 (54.5)	46.4 ± 14.9[b]
0.25	151	139 (92.0)	139	105 (75.5)	65 (46.8)	44.6 ± 7.2[b]

Table 2. Dose-related response of porcine early- stage embryos cultured with or without L-carnitine supplementation

Treatment mg/ml	No. of embryos (%)		Total cell count (mean±SEM)
	Cultured	Blastocyst	
Control	67	44 (65.7)	38.6 ± 13.2
1.0	58	36 (62.1)	46.5 ± 12.7
0.5	54	43 (79.6)	42.4 ± 14.2
0.25	46	33 (71.7)	45.5 ± 9.1

In Expt. 2, cleaved embryos resulting from COCs matured in maturation medium alone were assigned randomly in droplets of IVC medium with or without L-carnitine supplementation. The blastocyst formation rate was highest in 0.5 mg/ml L-carnitine supplemented group but did not differ with other groups. Also, the total cell count of blastocyst from treatment groups with L-carnitine supplementation were higher versus the control (Table 2).

DISCUSSION

The cytoplasm of ruminants and porcine oocytes and preimplantation embryos are rich with lipids which are associated with the endoplasmic reticulum and mitochondria and are degraded to some extent during maturation (Kruip *et al*., 1983; McEvoy *et al*., 2000), and has been shown to play a role as an energy source and potentially during embryo development (Ferguson and Leese, 2006). Apparently the ß-oxidation pathway that metabolizes lipids/fatty acids within the mitochondria to generate cellular ATP has an essential role in determining oocyte quality and its ability to support embryo development (Stojkovic *et al*., 2001). The initial, and rate limiting step in ß-oxidation is the entry of activated fatty acids into the mitochondrion, which is catalyzed by carnitine palmitoyl transferase (CPT1B). CPT1B attaches carnitine, enabling the entry of the fatty acid into the mitochondrial matrix where carnitine is removed by CPT2 and the fatty acid enters the ß-oxidation spiral producing multiple Acetyl-CoA molecules from which ATP is generated via the TCA cycle and the electron transport chain. Therefore, more studies to fully understand on how the oocytes and embryos utilize lipid substrates for energy production and whether this form of metabolism is developmentally beneficial is required. In this study, we tried to determine the efficient L-carnitine concentration as a supplement in the maturation and culture of porcine preimplantation embryos since excessive concentration may result to depletion of lipid density which may be detrimental to development per se (Sutton *et al*., 2012).

In porcine, treatment of immature oocytes with 10 mM (1.98 mg/ml) L-carnitine during IVM resulted to improved preimplantation development of parthenogenetically activated and somatic cell nuclear transferred embryos.

In contrast, L-carnitine supplementation at 2 mg/ml concentration during IVM inhibited nuclear maturation of porcine oocytes (Wu *et al*., 2011). Whereas, using L-carnitine as a supplement at 0.6 mg/ml concentration on oocytes and 8-cell embryos significantly improved the integrity of microtubule and chromosome structure aside from decreasing the level of apoptosis (Mansour *et al*., 2009). Also, supplementation of culture medium with 0.3 mg/ml L-carnitine improved blastocyst formation in mice by reducing tumor necrosis factor-α, blocking effects of actinomycin-D and hydrogen peroxide plus decreasing levels of DNA damage (Abdelrazik *et al*., 2009). By considering the range of L-carnitine concentration used, we tried 0.25 mg/ml – 1.0 mg/ml concentration for our experiment. Data gathered showed that neither L-carnitine concentration used in the maturation medium did not enhance the nuclear maturation, cleavage and subsequent blastocyst formation of porcine oocytes after IVF but was effective at improving the quality of resulting blastocyst as evidenced by having a higher cell count. These findings indicated that the beneficial effect of L-carnitine was prominent on improvement of embryonic development rather than completion of 1st meiosis. Moreover, the high nuclear maturation rate we obtained even in untreated control (91.0 %) indicates that the current IVM system utilized is effective in supporting the nuclear (spontaneous) maturation of immature porcine oocytes and that the addition of L-carnitine is less than necessary in terms of completion of 1st meiosis. The results were similar with other studies in porcine that L-carnitine treatment reduced reactive oxygen species level in IVM oocytes and help improved embryonic development of parthenogically activated and IVF embryos (Wu *et al*., 2011). Also, immature porcine COCs matured with L-carnitine was found to have improved glutathione level during maturation (You *et al*., 2012), indicating that its influence is more prominent on the cytoplasmic maturation rather than nuclear maturation. Nonetheless, it has been shown that L-carnitine improve the meiotic competence of porcine oocytes by

holding back the apoptosis of granulosa cells and enhancing its mitochondrial activity (Hashimoto, 2009; Somfai *et al*., 2011). Also, the cleavage and blastocyst formation rate obtained when L-carnitine was added either in the IVM or IVC of preimplantation embryos produced in vitro showed no significant difference with the untreated control, indicating that the current culture medium utilized is effective in overcoming the "cell-block" phenomenon in porcine and its subsequent development to the blastocyst stage. Interestingly, the total cell number of blastocyst embryos produced in L-carnitine treated groups was found higher than the untreated control. We hypothesized that some factors that promote embryo development could be related in one way or another to lipid metabolism, such as increased production of ATP and conversion of lipids to steroids and hormones, that serve as a major contributing factors for embryo development during in vitro culture. Endogenous lipids are a potential source of energy within the oocytes and embryos via β-oxidation but require transportation of fatty acids into the mitochondria by CPT1, thus the addition of L-carnitine, a cofactor of CPT1 has become necessary (Thompson *et al*., 2000b; Sutton *et al*., 2012; Yamada *et al*., 2006).

Conclusion

In this study, we have demonstrated that L-carnitine supplementation in the maturation and culture media had no significant effect on the completion of 1st meiosis (nuclear maturation) and embryo development to the blastocyst stage respectively, but maintained the developmental potential of porcine blastocyst by having a higher cell count, an indication of improved cell activity.

Conflict of interest statement

We declare that conflict of interest or whatsoever does not exist among authors that would prejudice the impartiality of this research work.

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