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Effects and pregnancy outcomes of L-carnitine supplementation in culture media for human embryo development from *in vitro* fertilization

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Abstract

Aim: Antioxidants have been studied to be effective in improving embryo qualities from *in vitro* fertilization. L-carnitine (LC) has been known to reduce reactive oxygen species and enhance adenosine triphosphate production, which contribute to the development of a high-quality embryo. This is the first study to include both mouse and human subjects and aimed to evaluate whether LC supplementation in culture media has any beneficial effect on the development of the embryos, as well as its clinical outcomes.

Methods: Mouse embryos were used as models in the animal studies for cell immunofluorescent staining evaluation. Inner cell mass and trophectoderm (TE) cells were counted and statistically analyzed between LC and control groups. For human studies, medical records of patients with infertility undergoing *in vitro* fertilization procedures from January to May 2017 were included and the embryos were divided into two groups at the two pronuclear stage. Statistical analysis was performed to compare the embryo status and clinical outcomes of the two groups.

Results: In the animal study, the LC group showed significantly higher numbers of cells in the inner cell mass and trophectoderm, indicating better development. In the human studies, there were significantly higher numbers of good-quality embryos on days 2, 3 and 5 in the LC group than in the control. The clinical outcomes, such as implantation, clinical pregnancy and ongoing pregnancy rates, were also higher in the LC group than in the control.

Conclusion: LC supplementation in culture media improved human embryo quality and eventually achieved better pregnancy outcomes.

Key words: antioxidant, embryo quality, *in vitro* fertilization, L-carnitine, pregnancy rate.

Introduction

In vitro fertilization (IVF) methods have been developed for over four decades, but the resultant pregnancy success rates are still relatively low, with live births per transfer at ~30%.[1] Several efforts have been made to develop better quality embryos, one of which is making the culture environment as close to the *in vivo* environment as possible, with less oxidative stress. It is known that only 2–8% of low oxygen tension is available *in vivo* for fertilization and embryo development.[2] Physiological oxygen concentration (usually 5%) has been shown to be beneficial not only for laboratory embryos or domestic animals[3,4] but also for humans.[5] It is proposed that high levels of oxygen compromise embryo development because of

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reactive oxygen species (ROS).[6] Fragmentation in human embryos, which adversely affects their quality and viability, is mediated by ROS-induced apoptosis.[7] Therefore, reducing ROS stress to embryos during their culture period has been an emerging issue, and antioxidant use has been a frequent source for research.

Among the antioxidants, L-carnitine (LC) has been mostly studied for improving male infertility[8] and, recently, for improving oocyte or embryo quality.[9] LC is a highly polar, small, water-soluble quaternary amine that is crucial to fat metabolism. LC is essential for the normal mitochondrial oxidation of fatty acids and excretion of acyl-coenzyme A (acyl-CoA) esters, and it affects adenosine triphosphate (ATP) levels.[10] LC protects the cell membrane and DNA from damage induced by ROS and is important in mitochondrial β-oxidation of long-chain free fatty acids, which increase energy supply to the cell.[11]

Beneficial effects of LC on embryonic development in culture have been reported in many mammalian species. In juvenile lamb models, LC increased the *in vitro* blastocyst rate of juvenile oocytes and, therefore, improved juvenile *in vitro* embryo transfer methods.[12] In sheep models, LC supplementation during *in vitro* maturation (IVM) reduced oxidative stress-induced embryo toxicity by decreasing intracellular ROS and increasing intracellular glutathione, which in turn improved the developmental potential of oocytes and embryos and altered the transcript level of antioxidant enzymes.[13] Supplementation with LC during IVM of bovine oocytes improved their nuclear maturation and subsequent embryo development after IVF.[14] However, to our knowledge, no human embryo study has yet been published. Therefore, the aim of this study was to demonstrate LC effectiveness in improving both animal and human embryo development and ultimately the clinical pregnancy outcomes.

Methods

Animal studies

Mouse embryo

Animal experiments were approved by the Animal Care and Use Committee of CHA University (Institutional Review Board [IRB] approval number: IACUC170177), and mice were kept under controlled temperature and humidity conditions, with 12-h light/dark conditions and free access to water and food. The 4–5-week-old female mice (ICR strain, Orient Bio) were superovulated via intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (Sigma-Aldrich), followed by injection with 5 IU human chorionic gonadotropin (hCG; Intervet) 48 h later. Study subjects were mated with 8–10-week-old male mice (ICR strain, Orient Bio) and their vaginal plugs were checked 13–15 h after mating. Female mice were sacrificed by cervical dislocation 18–20 h after hCG injection. Two pronuclear (2PN) stage embryos were recovered by flushing the ampulla of the oviducts with a 1-mL (29 gauge needle) insulin syringe, into a two-well dish (3260, Corning Costa), and examining under light microscopy; the removed oviducts were suspended in Quinn's advantage medium with HEPES (Quinn's-HEPES; Sage, in vitro Fertilization), containing 10% substitute protein serum (Sage). All zygotes were washed twice in Quinn's-HEPES, once in cleavage medium (COOK), and then divided into control and experimental groups for culture. Control groups were cultured in commercial media and experimental groups in commercial media with 1 mM of LC (Sigma Aldrich) based on previous studies.[15,16]

Immunofluorescence staining

Blastocysts were prepared for immunofluorescence by removing the zona pellucida (acid Tyrode solution, pH = 2.5), followed by washes in 0.1% bovine serum albumin (BSA) supplemented Dulbecco's phosphate-buffered saline (DPBS-BSA). They were then fixed in 3.7% paraformaldehyde with 0.02% Triton X-100 in 0.1% DPBS-BSA for 30 min at 4°C, permeabilized with 0.1% Triton X-100 DPBS-BSA for 1~2 min at room temperature, and washed three times with 0.1% DPBS-BSA for 5 min. Nonspecific binding sites were blocked with blocking buffer (Protein Block Serum Free) for 2 h at 4°C. They were then incubated with anti-Oct-4 antibodies (Oct-4 antibody; 1:100, mouse monoclonal antibody) contained in blocking buffer overnight at 4°C to stain the inner cell mass (ICM). The blastocysts were washed three times in 0.1% DPBS-BSA for 10 min. Next, embryos were stained with secondary antibodies (Molecular Probes) diluted to 1:200 and phalloidin with 3% DPBS-BSA for staining the cell walls. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 555 goat anti-rabbit IgG (Molecular Probes) were incubated with the blastocysts for 1 h at room temperature. All of the samples were counterstained with 1 μg/mL 4,6-diamidino-2-phenyindiol (DAPI, Sigma Aldrich) diluted to 1:500

with 0.1% DPBS-BSA for 15 min at room temperature for staining the nucleus of ICM and trophectoderm (TE). Then, the samples were mounted with VECTASHIELD Mounting Medium (Vector Laboratories) and observed with a ×40 objective lens in oil immersion, using an Axiovert 200 M microscope with a charge-coupled device camera controlled with the Z-stack (Carl Zeiss). Z-stack images (15–20) of individual blastocysts were obtained and analyzed using Axiovision software 4.8.1 (Carl Zeiss). Oct-4- and DAPI-positive cells were counted as ICM; DAPI-only positive cells were counted as the total cell number. The number of TE was calculated as total cell number minus ICM number. ICM and TE cell number was counted manually by two trained embryologists.

Human studies

Study subjects

This was a retrospective study reviewing medical records of patients with infertility undergoing IVF procedures from January to May 2017. The study was approved by the IRB of CHA Gangnam Medical Center (IRB approval number: GCI-17-32). Among the patients, those who responded normally to ovarian stimulation were recruited. The normal responders were selected according to age, anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH) levels and number of aspirated oocytes. The exclusion criteria were as follows: age > 40 years; AMH < 1 ng/mL; FSH ≥ 15 mIU/mL; and aspirated oocytes <2. For embryo quality evaluation, the embryos from each participant were divided into two groups at 2PN embryo stage after IVF: control group was cultured in commercial media and LC group in commercial media with 1 mM of LC (Sigma Aldrich) based on previous studies.[15–17] To evaluate the clinical outcomes, the patients who had control embryos transferred (n = 31 patients) and those who had LC embryos transferred (n = 78 patients) were compared.

Clinical protocols

Gonadotropin-releasing hormone (GnRH) antagonist and agonist protocols were mostly used in this study. The medications used in these protocols were as follows: recombinant FSH (Gonal-F, Merck; Pergoveris, Merck), GnRH antagonist (Cetrotide, Merck) and GnRH agonist (Lorelin, Dongkook Pharmaceutical). Recombinant FSH doses were formulated according to the patient's antral follicular counts, AMH and previous response to stimulation.

Transvaginal ultrasound tests were performed to monitor follicular response to stimulation, and gonadotropin doses were adjusted accordingly. Final oocyte maturation was triggered with recombinant hCG (Ovidrel, Merck) when the mean diameter of two or more follicles was 17 mm or more. Ultrasound-guided oocyte-retrieval under conscious sedation was performed 34–36 h after the trigger. Luteal phase support was provided with progesterone vaginal suppositories (Utrogestan, Han Wha Pharma Co., Ltd.) starting on the day of ovum pick-up. The β-hCG level was checked 10–12 days after embryo transfer.

Embryo culture and grading procedures

Conventional IVF or intracytoplasmic sperm insemination was used for fertilization of embryos. Fertilization was confirmed when 2PN stage was observed after 16–20 h. Fertilized oocytes were divided into two groups (control and LC) and cultured. During the cleavage stage, embryos were cultured in cleavage medium (COOK); for blastocysts, blastocyst medium (COOK).

Embryos were cultured in incubators (HERA cell 240, Thermo Fisher Scientific) under 5% O_2, 6% CO_2 and 37°C. The oil-drop culture method was applied, using a four-well dish (NUNC, Thermo Fisher Scientific). At 10 μL drops, the embryos were cultured singly to facilitate observing their development. Light paraffin oil (Ovoil, Vitrolife AB) was dropped onto the media to prevent it from drying and undergoing fast pH change.

The embryos from each group were morphologically graded at 2, 3 and 5 days of culture after fertilization. On day 2, four-cell stage embryos with no fragmentation as well as blastomeres were selected as high-quality embryos.[18,19] Day 3 embryos were graded according to the following parameters: number of blastomeres, rate of fragmentation and multinucleation of the blastomeres. Day 3 embryos were considered as good quality when they had a minimum of six blastomeres of normal size, a maximum of 20% of anucleated fragments and no multinucleated blastomeres. Embryo quality on day 5 was assessed according to the criteria of Gardner and Schoolcraft.[20] Good-quality embryos on day 5 were defined as having blastocoels being equal to or greater than half the volume of the embryo and good ICM and TE.

Embryo transfer was performed on day 3–5 under transabdominal sonographic guidance. Surplus embryos that were left after transfer were cultured, and the morphologically good blastocysts on day 5–6 were cryopreserved.

Outcome measures and statistical analysis

Clinical pregnancy was defined as the presence of one or more gestational sacs and fetal heartbeat at week 7 after transfer. Women over 16 weeks of gestation were defined as ongoing pregnancies. Continuous data are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise, and categorical variables are in absolute numbers and percentages. Clinical outcomes were analyzed using Student's *t*-test for parametric analysis, Mann–Whitney test for nonparametric analysis, and chi-squared test. The statistical analysis for comparing two groups of mouse embryos was performed using Student's *t*-test. All statistical analyses were performed using SPSS ver.23 (IBM). *P* value less than 0.05 was considered statistically significant.

Results

The mouse study revealed beneficial effects of LC supplementation in embryo culture media. The

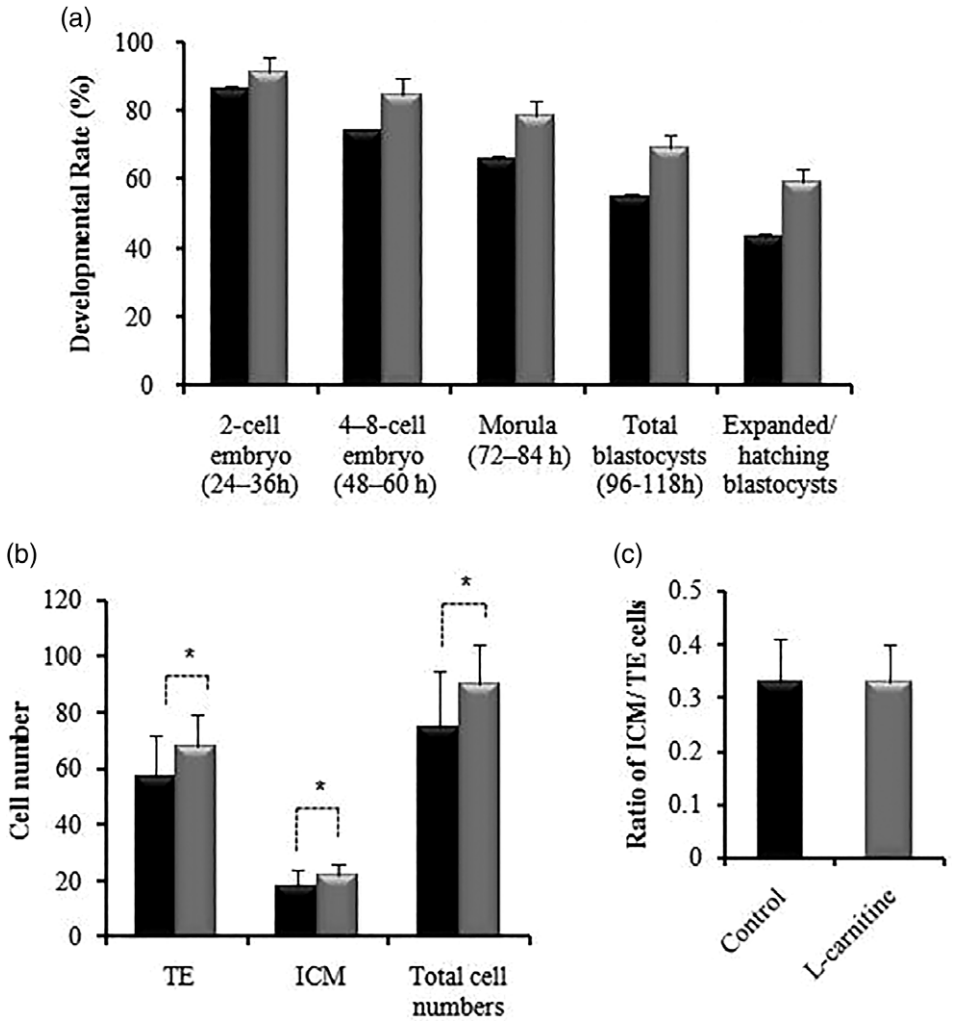

Figure 1 Comparisons of *in vitro* development of mouse embryos between L-carnitine (LC) and control groups. (a) Effect of LC supplementation during postfertilization on the developmental potential of embryos. The experiment was performed 10 times. (■) Control (n = 153), (■) L-carnitine (n = 161). (b) Differential staining in the inner cell mass (ICM) and trophectoderm (TE) of the blastocysts. Cell numbers in the ICM were 18.1 ± 5.6 for the control and 22.0 ± 4.1 for LC; cell numbers in the TE were 56.8 ± 15.3 vs 67.8 ± 11.7; and total cell numbers were 74.8 ± 19.8 vs 89.9 ± 14.2; all values significantly different between the two groups. The experiment was performed three times. (■) Control (n = 35), (■) L-carnitine (n = 47). (c) Ratio of ICM/TE cells. *P < 0.05, compared to control group.

Table 1 Comparisons of human embryonic development between control and L-carnitine groups

	Control	L-carnitine	*P* value
Number of embryos	166	326	
High-quality embryo culture rate (%)			
Number of good-quality embryos on day 2	60/166 (36.14)	156/326 (47.85)	0.01*
Number of good-quality embryos on day 3	66/166 (39.76)	190/326 (58.28)	<0.01*
Number of good-quality embryo on day 5	27/133 (20.30)	81/252 (32.14)	0.01*

*Statistically significant results.

graph in Figure 1a shows that more embryos developed in the LC group than in the control (2 cell: 91.3% vs 86.3%; 4–8 cell: 84.5% vs 73.9%; morula: 78.3% vs 66.0%; total blastocysts: 68.9% vs 54.9%; expanded/hatching blastocysts: 59.0% vs 43.1%), but the differences lacked statistical significance. On the other hand, when the mouse blastocysts were stained, significantly higher numbers of cells in the ICM and TE were noted from the LC group (Figure 1b); 67.8 vs 56.8 for TE ($P < 0.05$) and 22.0 versus 18.1 for ICM ($P < 0.05$). Total cell numbers (89.9 vs 74.8) were also significantly greater for the LC group ($P < 0.05$), indicating better embryo development. The ICM : TE ratio was calculated, and the results of LC groups versus control were 0.33 ± 0.07 versus 0.33 ± 0.08 which showed no significant difference (Figure 1c).

To compare the qualities of human embryos, 166 embryos were categorized as the control group and 326 embryos as the LC group at the 2PN embryo stage. The embryos were morphologically graded, and there were significant differences in the two groups' number of good-quality embryos for every embryo development stages (Table 1; day 2: 47.9% vs 36.1%, $P = 0.01$; day 3: 58.3% vs 39.8%, $P < 0.01$; day 5: 32.1% vs 20.3%, $P = 0.01$).

Table 2 The characteristics of the patients and *in vitro* fertilization (IVF) protocols

	Control	L-carnitine	*P* value
Number of total cycles (*n*)	31	78	
Maternal age (year)	35.13 ±2.63	34.67 ±2.98	0.45
Paternal age (year)	37.68 ±4.36	36.99 ±4.06	0.44
BMI (kg/m^2)	22.09 ± 3.60	21.79 ±3.07	0.28
Number of total IVF attempts	1.55 ± 0.89	1.60 ±0.73	0.47
AMH (ng/mL)	3.88 ± 2.01	3.78 ±2.98	0.67
Basal E2 (pg/mL)	50.48 ± 17.95	45.61 ± 21.90	0.23
Basal LH (mIU/mL)	5.60 ± 2.62	5.20 ± 2.24	0.20
Basal FSH (mIU/mL)	7.37 ± 2.14	7.42 ± 2.13	0.80
Antral follicle count (*n*)	14.77 ± 5.50	13.66 ± 5.93	0.46
Etiology of infertility (%)			
Female factor	38.71	48.72	0.10
Male factor	9.68	7.69	
Combined (female + male)	32.26	11.54	
Unexplained	12.90	15.38	
Others	6.45	16.67	
Ovarian stimulation protocol			
GnRH-agonist (%)	1 (3.23)	9 (12)	0.10
GnRH-antagonist (%)	30 (96.77)	67 (85.90)	0.10
Duration of ovarian stimulation (days)	7.52 ± 1.70	7.52 ± 1.63	0.99
Total dosage of gonadotropins (IU)	1462.93 ± 359.04	1465.27 ± 431.03	0.98
E2 on hCG day (pg/mL)	2364.85 ± 1500.75	2246.93 ± 1390.11	0.57
Progesterone on hCG day (ng/mL)	0.86 ± 0.34	0.84 ± 0.41	0.67
Endometrial thickness (mm)	11.2 ± 2.4	10.9 ± 2.2	0.62
Number of retrieved oocytes (*n*)	414	954	
Fertilization rate (%)	272†/414 (65.70)	598†/954 (62.68)	0.29

†The total number of embryos includes the ones fertilized on day 2 after oocyte retrieval due to immature oocytes, which were excluded from this study. AMH, anti-Müllerian hormone; BMI, body mass index; E2, estradiol; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone.

Clinical outcomes were investigated with 109 patients having embryos transferred. Among them, 31 had control embryos transferred and 78 had the LC embryos. The patient characteristics were similar between the two groups (Table 2). Although it was statistically not significant, the LC group showed increasing trend to have more number of patients with positive results on their first β-hCG tests (59.0% vs 38.7%, $P = 0.056$) (Table 3). Also, significantly better implantation rates (40.6% vs 21.8%, $P = 0.01$), clinical pregnancy rates (55.1% vs 32.3%, $P = 0.03$) and ongoing pregnancy rates (53.9% vs 32.3%, $P = 0.04$) were noted in the LC group. Multiple pregnancy rates (17.4% vs 8.3%) showed increased trend in the LC group, but without statistical significance. Extrauterine pregnancy (2.2% vs 8.3%) and chemical pregnancy rates (4.4% vs 8.3%) were less in the LC group, but were not significantly different.

Discussion

The present study is the first to describe the effects of LC in culture media on human embryo development. Mouse embryos were used for staining analysis, and the results revealed a significantly higher number of cells developed in LC embryos than in control embryos. Human studies have also shown promising results. The LC treatment group showed an increased number of morphologically good-quality embryos. The clinical outcomes such as implantation rates and ongoing pregnancy rates were also higher in the LC group compared with those in the control. The study results demonstrated that LC supplementation in embryo culture media improved embryo quality and pregnancy outcomes.

High levels of ROS increase the risk of both oocytes and embryos having severe damages to mitochondrial DNA. This causes a defect in generating enough ATP for the oocyte or embryo to develop.[21] Oocytes or embryos are exposed to various environmental conditions such as manipulation, temperature, medium constituents, sperm and O_2 concentration that likely increase ROS and may lead to downregulation of their defense mechanism when cultured *in vitro*.[22] Thus, it is imperative that the developing embryo has less contact with ROS as much as possible.

LC has antioxidant effects that reduce ROS level and oxidative stress in both oocytes and embryos.[15,16] Previous reports support this: when LC was supplemented in the culture media of MII oocytes, the intracellular H_2O_2 level significantly lowered compared with that in the control group.[9,23] For mouse embryos, LC reduced DNA damage and improved oocyte chromosomal structure and blastocyst development.[24] LC also plays an essential role in energy production, which is important for developing embryos.[25,26] LC is known to be involved in fatty acid oxidation and glucose metabolism needed for ATP generation.[27] Supplementation of LC in culture medium significantly increased β-oxidation in mice oocytes growing *in vitro* and *in vivo*, and this led to significant improvement in oocyte quality and its ability to reach the blastocyst stage.[15,16] Previous reports show that when there are no other energy substrates, LC stimulates the metabolism of intracellular lipid stores, given that it significantly improved embryo development in mice[16] and bovines.[28]

The present mouse study revealed promising results of combining LC in embryo culture media. Figure 1a showed increasing developmental rate in LC group, and the differences between control and LC group seemed to increase as the embryo stage was more advanced. Long-chain free fatty acids are known to contribute as energy source to early embryonic development.[25,26] Long-chain fatty acids need a carnitine transport system to reach the mitochondrial matrix and generate ATP via β-oxidation.[29] Therefore,

Table 3 Comparisons of the clinical outcomes of embryos from control and L-carnitine groups

	Control	L-carnitine	*P* value
Number of cycles with ET	31	78	
Number of average transferred embryos	1.77 (55 embryos/31 cycles)	1.71 (133 embryos/78 cycles)	
β-hCG positive (%)	12/31 (38.71)	46/78 (58.97)	0.06
Implantation (%)	12/55 (21.82)	54/133 (40.60)	**0.01**
Clinical pregnancy (%)	10/31 (32.26)	43/78 (55.13)	**0.03**
Ongoing pregnancy (%)	10/31 (32.26)	42/78 (53.85)	**0.04**
Multiple pregnancy (%)	1/12 (8.33)	8/46 (17.39)	0.67
Extrauterine pregnancy (%)	1/12 (8.33)	1/46 (2.17)	0.37
Chemical pregnancy (%)	1/12 (8.33)	2/46 (4.35)	0.51

Bold numbers mean statistically significant results.

LC has an important role as energy source to support preimplantation development. This role of LC, however, might be limited to late stages of mouse preimplantation development, because molecules which catalyze long-chain fatty acids in β-oxidation (e.g. long-chain fatty-acid acyl-CoA dehydrogenases) are not yet expressed at cleavage stages.[16] Therefore, LC likely has a significant role as an anti-oxidant rather than as an energy source at the cleavage stages of preimplantation development.

Compared with the control group, the LC group had more ICM, TE and total cell numbers, indicating better embryo development and potentiating more embryos to succeed in clinical pregnancy if transferred. Another previous study using bovine oocytes demonstrated no specific difference in total, ICM and TE cell numbers of blastocysts in the LC group.[30] This conflicting result is due to the difference in study methods, i.e., the previous authors used LC only during maturation of the oocytes and not during the embryo culture period. The mammalian preimplantation embryo is most sensitive to its environment during the cleavage stages[31] and, therefore, improvements were noted in embryo quality when antioxidants such as LC were supplemented during this stage.[32]

Our human embryo study demonstrated improved embryo quality and significantly better implantation, clinical pregnancy and ongoing pregnancy rates. For every embryo development stage, LC in culture media proved to be beneficial in producing more number of good-quality embryos than control. Although the results of this study showed no evident disadvantages in using 1 mM of LC in embryo culture media, further larger studies will be needed to identify any unfavorable outcomes in using LC and the most effective dose for human embryo culture.

The limitation of this study would be mainly owing to the retrospective design. The numbers of cycles enrolled in each group were different, and because of the short follow-up period, the total number of participants was not large enough to draw a significant conclusion. However, emphasis should be made on the fact that this is the first research to demonstrate the effectiveness of LC supplementation in human embryo culture media. Future prospective studies will ameliorate the methodological limitations of this study.

This study was undertaken during the postfertilization period, but we expect better results when LC is supplemented in the culture media of immature oocytes during the prefertilization state. For the immature oocytes to mature, a large amount of ATP is necessary, and LC, which is known for being an effective agent of the lipid pathway, will greatly aid the maturation process. Future studies regarding this hypothesis will be needed to further improve clinical outcomes. Additionally, a previous study reported that mouse embryos developed faster when LC was supplemented in the early cleavage stages of embryo development.[32] Time-lapse embryoscope imaging will be required to observe whether human embryos cultured in LC develop at better rates than those in the control group.

In conclusion, LC has great potential to improve human embryo quality during embryo culture and positively affects clinical outcomes such as inducing higher pregnancy rates. Further prospective randomized control trials with careful planning are needed to confirm the LC supplementation effect on human embryos.

Disclosure

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 (a, b) Oct-4 protein expression and localization in mouse embryo blastocysts, which were fixed and labeled with phalloidin (red) along with DAPI (blue) labeling of the cell nuclei. The inner cell mass (ICM) is identified by the expression of Oct-4 (green). (magnification ×200). (c) Control group, some of the blastocysts have grown and some have degenerated (arrow) (magnification ×40). (d) L-carnitine treatment group, some of the blastocysts have grown and some have undergone a developmental delay (magnification ×40).