

L-Carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos

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Objective: To optimize the L-carnitine (LC) concentration as a supplement in embryo culture medium and to investigate the effect of LC on developing embryos.

Design: Experimental study.

Setting: Reproductive research center at a tertiary hospital.

Intervention(s): To optimize the LC concentration, 420 mouse embryos were divided into seven groups and incubated with different LC concentrations (0, 0.3, 0.6, 1.2, 2.5, 5.0, and 10 mg/mL). To investigate the effect of LC on the developing embryos, 500 mouse embryos were divided into three groups and incubated with either actinomycin-D (AD; 0.005 $\mu\text{g/mL}$), hydrogen peroxide (H_2O_2 ; 500 $\mu\text{mol/L}$), or tumor necrosis factor α (TNF- α ; 500ng) with and without LC 0.3 or 0.6 mg/mL. Blastocyst development rate (%BDR) and DNA damage were examined for all groups.

Main Outcome Measure(s): Effect of LC on embryogenesis.

Result(s): Significant improvement in %BDR was seen at LC 0.3 mg/mL compared with the control ($p = 0.006$). L-Carnitine at 0.3 and 0.6 mg/mL significantly reduced the blocking effect of AD, H_2O_2 , and TNF- α and significantly decreased the level of DNA damage.

Conclusion(s): Embryo culture medium supplementation with LC may offer a novel and a cost-effective technique to improve the embryogenesis of cultured embryos. This may be beneficial in improving IVF outcomes. (Fertil Steril® 2009;91:589–96. ©2009 by American Society for Reproductive Medicine.)

Key Words: L-Carnitine, antioxidant, actinomycin-D, blastocyst development rate, TNF- α

L-Carnitine (LC) is a small water-soluble molecule which plays an important role in fat metabolism. It is essential for the normal mitochondrial oxidation of fatty acids and excretion of acyl-coenzyme A (acyl-CoA) esters and affects adenosine triphosphate (ATP) levels (1). Free carnitine was first isolated from bovine muscle in 1905, and only the L-isomer was found to be bioactive (2). L-Carnitine protects cell membrane and DNA against damage induced by free oxygen radicals and has a pivotal role in mitochondrial oxidation of long-chain fatty acids which increase energy supply to the cell (2). Mitochondrial dysfunction may lead to incomplete detoxification of the free radicals, which may lead to oxidative damage to macromolecules such as lipids, proteins,

and DNA. L-Carnitine has free radical-scavenging activity and ability to scavenge superoxide anion and inhibit lipid peroxidation, thereby conferring protection against damage induced by hydrogen peroxide (H_2O_2) (3).

The LC levels may also affect ATP levels. L-Carnitine has a role in intramitochondrial fatty acid oxidation. Acyl-CoA esters cannot directly cross the mitochondrial inner membrane, and their entry into the mitochondrion is a major point for control and regulation of the β -oxidation of fatty acids (4). Expression of fatty acid oxidation enzymes such as carnitine palmitoyl transferase 1 and medium-chain acyl-CoA dehydrogenase has been demonstrated in embryonic and fetal tissues and human placenta (5). In addition to glucose production, fatty acid oxidation may be important for generation of ATP necessary to meet the energy requirement of developing embryos (6, 7).

Fertilization and embryo development in vivo takes place in a low oxygen tension environment (8). During culture, low oxygen tension improves implantation and pregnancy rates (9). Also, in vitro blastocyst formation during assisted reproductive techniques (ART) is suboptimal, and supplementation with antioxidants may improve blastocyst

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development (10). Similarly, higher implantation and clinical pregnancy rates have been reported when antioxidant-supplemented medium was used for ART compared with standard medium lacking antioxidants (11). Therefore, antioxidants are likely to play a significant role not only during ART procedures but also in preventing subsequent loss or damage to the embryo.

L-Carnitine has antioxidant activity that combines both free radical scavenging and metal-chelating properties. L-Carnitine was found to be effective in reversing age-related trends and improved the mitochondrial function during the ageing process in rat skeletal muscle mitochondria (3). Human embryos generated from IVF exhibit varying degrees of cytoplasmic fragmentation indicative of apoptosis (12). Stabilization of the mitochondrial membrane leads to increase in the supply of energy to the organelle and protect the cell from apoptotic death.

Antiapoptotic effect of LC has also been demonstrated in human lymphoma cells treated with apoptosis-inducing agents (13). Higher concentrations of tumor necrosis factor α (TNF- α) have been reported in the serum and follicular fluids of infertile women with polycystic ovary syndrome (14). In various tumors and inflammatory diseases, elevated serum level of TNF- α decreased after treatment with LC (15).

The objectives of the present study were to: 1) determine the optimum concentration of LC as a supplement in mouse embryo culture medium; and 2) investigate the role of LC in antagonizing the harmful effect of TNF- α , apoptosis, and oxidative stress on embryo development. This may offer a novel approach to improve embryogenesis and ART outcome.

MATERIALS AND METHODS

Frozen straws with two-cell mouse embryos (Embryotech Laboratories, Wilmington, MA) were exposed to room temperature for 2 min. Each straw was bisected between the lower heat seal and the column of medium. By using the syringe, the contents of the straw are flushed as a single drop into a sterile culture dish (Allegiance Health Care, McGaw Park, IL). We conducted a pilot study to establish the nontoxic embryonic concentration of LC in mouse embryo culture medium. The second, third, and fourth experiments were designed to evaluate the antiapoptotic, antioxidant, and the anti-TNF- α effects of LC on the mouse embryo culture medium by assessing the blastocyst development rate (%BDR) and level of DNA damage.

Experiment 1: Pilot Study

L-Carnitine was dissolved in human tubal fluid (HTF) culture medium, and 6 concentrations were prepared: 0.3, 0.6, 1.2, 2.5, 5, and 10 mg/mL. A total of 420 two-cell embryos were used in this study. Thawed mouse embryos were pooled and randomly distributed among the six concentrations of LC. These concentrations were based on the earlier concentrations of LC (10 mmol/L, 2.39 mg/mL) which was used in

culture media with other tissues, such as lymphoma cells and skeletal muscle (13, 16). At this concentration (10 mmol/L, 2.39 mg/mL) L-carnitine showed potent antiapoptotic agent to human lymphoma cells in vitro. The same concentration of LC decreased the level of apoptosis in skeletal muscle cells cultures treated with the apoptosis-inducing factor staurosporine. No literature examining the use of LC on embryo cultures is reported. Therefore, we selected this concentration and two higher and three lower concentrations. All groups were incubated at 37°C in 5% CO₂ for 72 h until the blastocyst stage. The percentage of %BDR was calculated in each group by dividing the total number of blastocysts formed at 72 h by the total number of embryos incubated.

Apoptosis was estimated by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche, Indianapolis, IN) for the highest concentration of LC which did not show significant decrease in %BDR, and these concentrations were used in experiments 2–4.

Experiment 2: Antiapoptotic Effect of LC

To evaluate the antiapoptotic effect of LC, eight-cell mouse embryos were incubated with 0.005 μ g/mL actinomycin in culture medium. Actinomycin concentration was derived from previous studies investigating the effect of actinomycin-D on mouse embryo in vitro (17). A total of 240 thawed mouse embryos were randomly distributed among the following four groups: group 1: HTF medium only, control group; group 2: HTF medium + 0.005 μ g/mL actinomycin-D; group 3: HTF medium + 0.005 μ g/mL actinomycin-D + 0.3 mg/mL LC; and group 4: HTF medium + 0.005 μ g/mL actinomycin-D + 0.6 mg/mL LC. All groups were incubated at 37°C in 5% CO₂ for 4 h and transferred to HTF medium and incubated until 48 h for formation of the blastocyst stage.

Experiment 3: Antioxidant Effect of LC

To evaluate the antioxidant effect of LC, two- to four-cell mouse embryos were exposed to toxic concentrations of H₂O₂ (500 μ mol/L) in the culture medium for 72 h. Hydrogen peroxide at a concentration of 50 μ mol/L is reported to significantly decrease BDR in two-cell mouse embryos (18). In our experiment we used tenfold higher concentrations recorded in the previous literature to examine the extent to which level LC can neutralize the oxidant effect of H₂O₂. A total of 160 two-cell mouse embryos were thawed and randomly distributed among the following four groups: HTF medium alone (control); HTF medium + H₂O₂ (500 μ mol/L); HTF medium + H₂O₂ (500 μ mol/L) + 0.3 mg/mL LC; and HTF medium + H₂O₂ (500 μ mol/L) + 0.6 mg/mL LC. All groups were incubated at 37°C in 5% CO₂ for 72 h until the formation of blastocyst stage.

Experiment 4: Antiproliferative Effect of LC on TNF- α

Treatment with 500 ng/mL TNF- α treatment inhibited the development of two-cell mouse embryos to the expanded and hatched blastocyst stages evaluated after 72 and 96 h of

culture, respectively, compared with a control group ($P < .05$) (19). In our experiment we used a tenfold higher concentration of TNF- α to investigate the concentration of LC that was effective in neutralizing the antiproliferative effect of TNF- α . A total of 100 thawed mouse embryos were randomly distributed among the following four groups: HTF medium alone (control); HTF medium + TNF- α (500 ng/mL); HTF medium + TNF- α (500 ng/mL) + 0.3 mg/mL LC; and HTF medium + TNF- α (500 ng/mL) + 0.6 mg/mL LC). All groups were incubated at 37°C in 5% CO₂ for 72 h until the blastocyst stage. The number of embryos progressing to the blastocyst stage was recorded, and %BDR was calculated in each group by dividing the total number of blastocysts by the total number of embryos incubated. Apoptosis was estimated by the TUNEL assay.

Embryo staining by TUNEL for detection of blastomere DNA damage

Individual embryos were stained with the TUNEL technique (in situ cell death detection system; Roche Diagnostic, Indianapolis, IN). After washing the embryos in phosphate-buffered saline (PBS), they were fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. Embryos were washed at least three times in PBS containing 0.3% polyvinylpyrrolidone (PBS/PVP) and permeabilized in 0.5% Triton X-100 on ice for 2 min. The embryos were then washed three times in PBS/PVP and incubated in TUNEL reaction cocktail at 37°C for 1 h in the dark. The embryos were washed extensively and mounted with slight coverslip compression in Vectashield, 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) antibleaching solution (Vector Labs, Burlingame, CA). The slides were sealed with clear nail polish and stored at -20°C in the dark for analysis by confocal microscope.

Confocal Microscopy

Images were collected with a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Lasertechnik, Heidelberg, Germany). The specimen was excited at 364 nm (UV) for DAPI and at 488 nm for visualization of TUNEL staining. Images were collected sequentially at each level of the specimen to prevent crosstalk of the fluorophores and then collected along the z-axis of the sample with a step size of 1–3 μ m. Each optical section of the blastocyst was analyzed for TUNEL-negative nuclei stained with DAPI and TUNEL-positive nuclei stained with DAPI and TUNEL.

The percentage of apoptotic cells in each embryo was calculated by using the projection of the three-dimensional stack of images that was created with the Leica software. The original stack of embryo images was transferred to Velocity software (Improvision, Lexington, MA) for analysis and counting of both damaged and intact individual blastomere DNA. This software enables going through the depth of the scanned blastocyst to observe the TUNEL staining and verify if each blastomere is independently stained or not. The apoptotic blastomeres in each blastocyst were counted and the level of apoptosis in each group was calculated.

Statistical Methodology

Post hoc and Fisher exact tests were used for %BDR differences, and Kruskal-Wallis or Wilcoxon test was used to analyze the level of apoptosis differences between the groups. Statistical analyses were performed using R version 2.3.1 (<http://www.R-project.org>).

RESULTS

In the pilot study, a significant improvement in %BDR was seen at LC 0.3 mg/mL compared with control (100% vs. 83.3%; $P = .006$). No significant difference was seen in %BDR using 0.6 mg/mL LC compared with control (81.6% vs. 83.3%). Decrease in %BDR was seen at 1.25, 2.5, and 5 mg/mL concentration of LC (68.3%, 70%, and 71.6%, respectively, vs. control 83.3%). However, LC at 10 mg/mL was embryotoxic (%BDR 35% vs. control 83.3%; $P < .001$).

The TUNEL assay was done for LC 0.3 and 0.6 mg/mL. The %BDR for LC 0.3 mg/mL was 100% ($P < .006$) and for 0.6 mg/mL was 80% ($P < .75$) versus 85% for control. Similarly, the level of apoptosis (median [25th, 75th percentiles]) for LC 0.3 mg/mL was 0 (0, 5.8) ($P < .13$) and for 0.6 mg/mL was 3 (5.57, 9.1) ($P < .42$) versus 2.2 (3.9, 6.1) for control (Figs. 1A–1C).

Antiapoptotic Effect of LC

Incubation of the eight-cell mouse embryos with 0.005 μ g/mL actinomycin-D for 4 h significantly reduced the %BDR from 80% to 40% ($P < .001$) and increased the level of apoptosis (median [25th, 75th percentiles]) from 0 (0, 8.7) to 41.3 (49.7, 58.4) ($P < .001$). Addition of LC 0.3 mg/mL improved the %BDR (from 40% to 70%; $P < .002$) and reduced the level of apoptosis (1.8 [13.6, 15.4] vs. 41.3 [49.7, 58.4]; $P < .001$) compared with the actinomycin-D only-treated group. L-Carnitine 0.6 mg/mL in combination with actinomycin was less effective in improving the %BDR (58.3% vs. 40%; $P < .002$) and level of apoptosis (10.7 [16.6, 23] vs. 41.3 [49.7, 58.4]; $P < .001$; Figs. 1D–1F).

Antioxidant Effect of LC

L-Carnitine demonstrated a strong antioxidant effect on mouse embryo development at 0.3 and 0.6 mg/mL concentrations. It was able to neutralize the embryotoxic effects of exogenous induction of oxidative stress by 500 μ mol/L H₂O₂ (Table 1; Figs. 2A–2C).

Antiproliferative Effect of LC on TNF- α

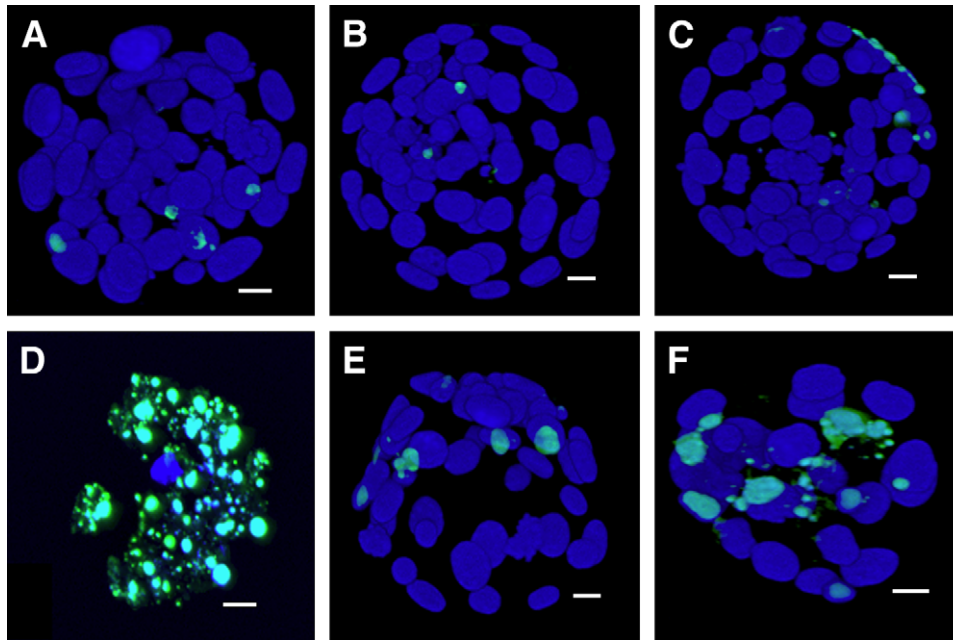
The TNF- α reduced the blastocyst development rate in mouse embryos. L-Carnitine was effective in reversing the TNF- α -induced inhibition of %BDR (Table 2; Figs. 2D–2F).

DISCUSSION

Reactive oxygen species (ROS) are involved in the etiopathogenesis of defective embryo development (20). An increase in ROS production leads to arrest of embryo development.

FIGURE 1

Confocal photomicrographs of mouse blastocyst stained for apoptosis. All nuclei of the blastocyst are labeled with 4',6'-diamidino-2-phenylindole hydrochloride (*blue channel*) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (*green channel*). (A) Control group; (B) human tubal fluid (HTF) medium + 0.3 mg/mL L-carnitine (LC); (C) HTF medium + 0.6 mg/mL LC. Significant improvement in blastocyst development rate (%BDR) was seen at LC 0.3 mg/mL compared with control (100% vs. 83.3%; $P = .006$). No significant change was seen in the level of apoptosis in embryos cultured in 0.3 and 0.6 mg/mL LC ($P = .13$ and $P = .42$, respectively). (D) Actinomycin-D 0.005 $\mu\text{g/mL}$; (E) actinomycin-D + 0.3 mg/mL LC; (F) actinomycin-D + 0.6 mg/mL LC. Treatment of the embryos with actinomycin-D at 0.005 $\mu\text{g/mL}$ for 4 h significantly increased the level of apoptosis ($P < .001$) compared with control. A significant decrease in the level of apoptosis induced by actinomycin-D also was seen in embryos treated with 0.3 and 0.6 mg/mL LC (both $P < .001$). Scale bar = 10 μm .



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TABLE 1

Comparison of %BDR and apoptosis between control, H₂O₂ alone, and H₂O₂ + LC.

Treatment	%BDR	Apoptosis (%), median (25th, 75th percentiles)	%BDR		Apoptosis	
			<i>P</i> value ^a	<i>P</i> value ^b	<i>P</i> value ^c	<i>P</i> value ^d
Control (n = 20)	80.0	4.4 (10.8, 14.6)	—	—	—	—
H ₂ O ₂ 500 $\mu\text{mol/L}$ (n = 40)	7.5	39.2 (42.3, 55.5)	<.001	—	<.01	—
H ₂ O ₂ + LC 0.3 mg/mL (n = 40)	100.0	2.8 (7.1, 13.4)	.001	<.001	.34	<.006
H ₂ O ₂ + LC 0.6 mg/mL (n = 40)	100.0	8.7 (14.6, 15.9)	.001	<.001	.37	<.007

Note: %BDR = percentage blastocyst development rate; LC = L-carnitine.

^a $P < .05$ was considered to be significant using Fisher exact test for %BDR differences between H₂O₂ with and without LC compared with control.

^b $P < .05$ was considered to be significant for %BDR differences between H₂O₂ and H₂O₂ + LC groups.

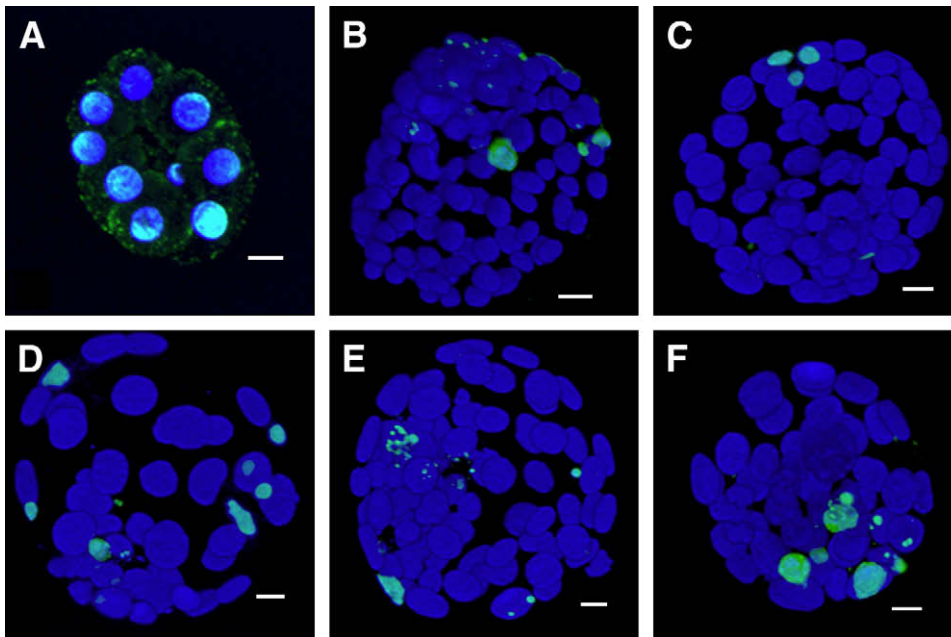
^c $P < .05$ was considered to be significant for differences in apoptosis using Kruskal-Wallis or Wilcoxon rank sum test between H₂O₂ with and without LC compared with control.

^d $P < .05$ was considered to be significant for apoptosis differences between H₂O₂ and H₂O₂ + LC groups.

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FIGURE 2

Confocal photomicrographs of mouse blastocyst stained for apoptosis. All nuclei of the blastocyst are labeled with 4',6'-diamidino-2-phenylindole hydrochloride (*blue channel*) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (*green channel*). **(A)** Hydrogen peroxide (H_2O_2) 500 $\mu\text{mol/L}$; H_2O_2 at this concentration arrested more than 92% of the embryos. **(B)** H_2O_2 + 0.3 mg/mL L-carnitine (LC); **(C)** H_2O_2 + 0.6 mg/mL LC. Incubation of mouse embryos in 500 $\mu\text{mol/L}$ H_2O_2 significantly increased the level of apoptosis ($P < .001$) compared with control. There was no significant difference in apoptosis level in H_2O_2 + 0.3 or 0.6 mg/mL LC compared with control ($P = .34$ and $P = .37$, respectively). L-Carnitine at 0.3 and 0.6 mg/mL concentration significantly decreased the level of apoptosis ($P < .006$ and $P < .007$, respectively) compared with the group treated with H_2O_2 alone. **(D)** Tumor necrosis factor α ($\text{TNF-}\alpha$) 500 ng/mL; **(E)** $\text{TNF-}\alpha$ + 0.3 mg/mL LC; **(F)** $\text{TNF-}\alpha$ + 0.6 mg/mL LC. Incubation of two-cell mouse embryos with 500 ng $\text{TNF-}\alpha$ significantly decreased the %BDR ($P < .001$) but did not increase the level of apoptosis ($P = .48$) compared with control or with 0.3 and 0.6 mg/mL LC groups ($P = .59$ and $P = .62$, respectively). Scale bar = 10 μm .



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The ROS may originate in the embryo or from the extraneous factors. In the IVF setting, strategies to reduce ROS production, such as addition of free radical scavengers and lowering the oxygen tension, are important for improving the fertility potential in ART (11). The ROS are involved in defective embryo development and retardation of embryo growth (10, 20) and induces cell membrane damage, DNA damage, and apoptosis. Apoptosis results in fragmented embryos, which have limited potential to implant and therefore result in poor fertility outcomes (12).

A study evaluating the possible beneficial effects of L-carnitine on tissue injury and oxidative stress in acetic acid-induced colitis in rats showed that acetic acid administration significantly decreased reduced glutathione, superoxide dismutase, and catalase levels in colonic homogenate. Supplementation of L-carnitine prevented the depletion of free radical scavengers, such as reduced glutathione, and significantly increased superoxide dismutase levels (21).

The present study showed that supplementation of the embryo culture medium with certain concentrations of LC (0.3 and 0.6 mg/mL) improves the embryo development. Treatment of the two-cell mouse embryos with 0.3 mg/mL LC significantly improved the %BDR ($P = .006$), whereas no significant change was seen in %BDR at 0.6 mg/mL LC, compared with the control group. Similarly, no significant change was seen in the level of apoptosis in embryos cultured in 0.3 and 0.6 mg/mL LC ($P = .13$ and $P = .42$) compared with the control group, which indicates that LC in these specific concentrations was not embryotoxic (Figs. 1A–1C).

Incubation of mouse embryos in 500 $\mu\text{mol/L}$ H_2O_2 significantly decreased the %BDR and increased the level of apoptosis ($P < .001$ and $P < .01$) compared with the control group (Figs. 2A–2C). L-Carnitine has a very potent antioxidant effect in the mouse embryo culture medium, because it is able to neutralize the embryotoxic effects of exogenous induction of oxidative stress by H_2O_2 . L-Carnitine at 0.3

TABLE 2

Comparison of %BDR and apoptosis between control, TNF- α alone, and TNF- α + LC.

Treatment (n = 25)	%BDR	Apoptosis (%), median (25th, 75th percentiles)	%BDR		Apoptosis (%)	
			P value ^a	P value ^b	P value ^c	P value ^d
Control	84	2.3 (6.8, 10.7)	—	—	—	—
TNF- α 500 ng	24	4.6 (8.9, 13.7)	<.001	—	.48	—
TNF- α 500 ng + LC 0.3 mg/mL	80	4 (9.6, 10)	1	<.001	.88	.59
TNF- α 500 ng + LC 0.6 mg/mL	68	1 (7.3, 12.1)	.32	.004	1	.62

Note: TNF- α = tumor necrosis factor α ; other abbreviations as in Table 1.

^a $P < .05$ was considered to be significant using Fisher exact test for %BDR differences between TNF- α with and without LC compared with control.

^b $P < .05$ was considered to be significant for %BDR differences between TNF- α and TNF- α + LC groups.

^c $P < .05$ was considered to be significant for differences in apoptosis using Kruskal-Wallis or Wilcoxon rank sum test between TNF- α with and without LC compared with control.

^d $P < .05$ was considered to be significant for apoptosis differences between TNF- α and TNF- α + LC groups.

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and 0.6 mg/mL significantly improved the %BDR ($P < .001$ and $P < .001$, respectively) and decreased the level of apoptosis ($P < .006$ and $P < .007$, respectively) compared with the group treated with H₂O₂ alone (500 μ mol/L).

Studies have suggested that blastomere fragmentation in mouse and human preimplantation embryos may be indicative of apoptosis (22). Human embryos generated from IVF also exhibit varying degrees of cytoplasmic fragmentation (23). L-Carnitine is able to stabilize mitochondrial membranes and increase the supply of energy to the organelle and protect the cell from apoptotic death (24). Reduction of apoptosis through the mitochondrial pathway by the administration of LC to mouse fibroblasts in culture medium has been demonstrated (24). The present study demonstrates that treatment of the embryos with actinomycin-D at 0.005 μ g/mL for 4 h significantly decreases %BDR and increases the level of apoptosis ($P < .001$ and $P < .001$, respectively), compared with the control group (Figs. 1D–1F). L-Carnitine (0.3 mg/mL) significantly improved the %BDR ($P < .002$) compared with the groups treated with actinomycin-D (0.005 μ g/mL) alone. A significant decrease in the level of apoptosis induced by actinomycin-D was also seen in embryos treated with both 0.3 and 0.6 mg/mL of LC ($P < .001$ and $P < .001$, respectively).

Significantly higher concentration of basal levels of TNF- α from the granulosa cells have been reported from women with endometriosis (25). Granulosa, cumulus, and sperm cells are potential sources of cytokine production, especially during the first 24 h (26). TNF- α can inhibit cell proliferation and glucose consumption in the rat blastocyst (27). Elevated TNF- α has been reported to restrict inner cell mass and trophoblast proliferation in the mouse blastocyst. This leads to retardation of embryo development, reduced viability of embryos, and even embryonic death (28, 29). Exposure of mouse embryos to TNF- α (500 ng/mL) affects their protein synthesis both quantitatively and qualitatively in the morula

and blastocyst stage (30). Dietary supplementation with LC has been reported to significantly reduce TNF- α levels in rats with alcoholic liver damage (31).

Incubation of two-cell mouse embryos with 500 ng/mL TNF- α significantly decreased the %BDR ($P < .001$) but did not increase the level of apoptosis compared with the control group ($P = .48$) or the 0.3 and 0.6 mg/mL LC groups ($P = 0.59$ and $P = .62$, respectively; Figs. 2D–2F). The lack of any significant effect on the level of apoptosis suggests that TNF- α may have an antiproliferative effect on the developing mouse embryos which may not necessarily be through the induction of apoptosis. This finding coincides with the reports of Pampfer et al., who showed that incubation of mouse embryos with 50 ng/mL TNF- α did not increase the incidence of apoptosis compared to the control group, although it decreased the number of nuclei in the developing blastocyst (32). Lalitkumar et al. showed that exposure of mouse embryos to TNF- α (50 ng/mL) affected their protein synthesis both quantitatively and qualitatively in the morula and blastocyst stage (32). L-Carnitine at 0.3 and 0.6 mg/mL was able to neutralize this antiproliferative effect and improve the %BDR ($P < .001$ and $P = .004$, respectively) compared with the group treated with TNF- α alone.

Oxygen-controlled incubators have been introduced recently in clinical embryology procedures, and these may help reduce the harmful effects of free radicals and thereby improve blastocyst rates. However LC works with multiple mechanisms, one of them is by antagonizing ROS formation through its antioxidant effect. In addition, LC can also decrease the level of apoptosis induced by an apoptotic inducer and decrease the antiproliferative effect induced by presence of an interleukin such as TNF- α .

Although the blastocyst formation rate in humans is around 50%–60% depending on both patient and laboratory,

it is extremely difficult from an ethical standpoint to conduct a similar experiment using human embryos. Therefore, the next best solution would be to use animal embryos, and mouse embryos have been extensively studied. The present study also involves detection of the level of DNA damage by TUNEL assay and this required the embryos to be fixed for use with the TUNEL assay. The results of these experiments confirm that the addition of LC to the embryo culture medium can improve both %BDR and the quality of the developed embryos. Similar studies using human embryos may be possible to conduct only after obtaining the informed consent from patients to use those embryos which are not selected for transfer where the patient is not interested in cryopreservation of the excess embryos. The %BDR could then be compared with or without addition of L-carnitine to the culture medium. In the present study, we did not examine the percentage of chromosomally abnormal embryos in the culture medium and how LC may affect their development. It would be interesting to investigate if LC can improve the %BDR in chromosomally abnormal embryos.

In conclusion, the success rates of ART are still unsatisfactory. Relatively few in vitro cultured embryos reach the blastocyst stage. Mouse embryos are an excellent research model, because preimplantation development is fairly similar to that of human embryos. Tumor necrosis factor α , oxidative stress, and apoptosis significantly affect the embryogenesis in different studies. Improvement in embryo developmental competence may be accomplished by LC supplementation through its potent antioxidant effect, its ability to reduce DNA damage, and by protecting the cells from the harmful effect of TNF- α . Improvement in the %BDR and reduction in the level of DNA damage at low LC concentrations (0.3 and 0.6 mg/mL) is a novel approach, because it has a combination of beneficial effects on embryos and may result in a higher yield of good-quality embryos. This approach can have significant clinical applications in the ART setting, and its use may improve the fertility outcomes and prove to be cost-effective.

REFERENCES

- Vanella A, Russo A, Acquaviva R, Campisi A, Di Giacomo C, Sorrenti V, et al. L-Propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector. *Cell Biol Toxicol* 2000;16:99–104.
- Zhou X, Liu F, Zhai S. Effect of L-carnitine and/or L-acetyl-carnitine in nutrition treatment for male infertility: a systematic review. *Asia Pac J Clin Nutr* 2007;16(Suppl 1):383–90.
- Panneerselvam KS, Kumaran S. L-Carnitine and alpha-lipoic acid improves mitochondrial function during ageing process. *Clin Nutr* 2006.
- Eaton S. Control of mitochondrial beta-oxidation flux. *Prog Lipid Res* 2002;41:197–239.
- Oey NA, Ruitter JP, Attie-Bitach T, Ijlst L, Wanders RJ, Wijburg FA. Fatty acid oxidation in the human fetus: implications for fetal and adult disease. *J Inherit Metab Dis* 2006;29:71–5.
- Berger PS, Wood PA. Disrupted blastocoele formation reveals a critical developmental role for long-chain acyl-CoA dehydrogenase. *Mol Genet Metab* 2004;82:266–72.
- Oey NA, Ijlst L, van Roermund CW, Wijburg FA, Wanders RJ. Dif-1 and colt, both implicated in early embryonic development, encode carnitine acylcarnitine translocase. *Mol Genet Metab* 2005;85:121–4.
- Burton GJ, Hempstock J, Jauniaux E. Oxygen, early embryonic metabolism and free radical-mediated embryopathies. *Reprod Biomed Online* 2003;6:84–96.
- Catt JW, Henman M. Toxic effects of oxygen on human embryo development. *Hum Reprod* 2000;15(Suppl 2):199–206.
- Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003;79:829–43.
- Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005;3:28.
- Juriscova A, Varmuza S, Casper RF. Programmed cell death and human embryo fragmentation. *Mol Hum Reprod* 1996;2:93–8.
- Qi SN, Zhang ZF, Wang ZY, Yoshida A, Ueda T. L-Carnitine inhibits apoptotic DNA fragmentation induced by a new spin-labeled derivative of podophyllotoxin via caspase-3 in Raji cells. *Oncol Rep* 2006;15:119–22.
- Amato G, Conte M, Mazziotti G, Lalli E, Vitolo G, Tucker AT, et al. Serum and follicular fluid cytokines in polycystic ovary syndrome during stimulated cycles. *Obstet Gynecol* 2003;101:1177–82.
- Sener G, Eksioglu-Demiralp E, Cetiner M, Ercan F, Sirvanci S, Gedik N, et al. L-Carnitine ameliorates methotrexate-induced oxidative organ injury and inhibits leukocyte death. *Cell Biol Toxicol* 2006;22:47–60.
- Vescovo G, Ravara B, Gobbo V, Sandri M, Angelini A, Della Barbera M, et al. L-Carnitine: a potential treatment for blocking apoptosis and preventing skeletal muscle myopathy in heart failure. *Am J Physiol Cell Physiol* 2002;283:C802–10.
- Fabian D, Rehak P, Czikkova S, Il'kova G, Baran V, Koppel J. Induced cell death of preimplantation mouse embryos cultured in vitro evaluated by comet assay. *Theriogenology* 2003;60:691–706.
- Zhang X, Sharma RK, Agarwal A, Falcone T. Effect of pentoxifylline in reducing oxidative stress-induced embryotoxicity. *J Assist Reprod Genet* 2005;22:415–7.
- Wuu YD, Pampfer S, Becquet P, Vanderheyden I, Lee KH, De Hertogh R. Tumor necrosis factor alpha decreases the viability of mouse blastocysts in vitro and in vivo. *Biol Reprod* 1999;60:479–83.
- Guerin P, El Moutassim S, Menezes Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;7:175–89.
- Cetinkaya A, Bulbuloglu E, Kantarceken B, Ciralik H, Kurutas EB, Buyukbese MA, et al. Effects of L-carnitine on oxidant/antioxidant status in acetic acid-induced colitis. *Dig Dis Sci* 2006;51:488–94.
- Juriscova A, Latham KE, Casper RF, Casper RF, Varmuza SL. Expression and regulation of genes associated with cell death during murine preimplantation embryo development. *Mol Reprod Dev* 1998;51:243–53.
- Juriscova A, Antenos M, Varmuza S, Tilly JL, Casper RF. Expression of apoptosis-related genes during human preimplantation embryo development: potential roles for the Harakiri gene product and caspase-3 in blastomere fragmentation. *Mol Hum Reprod* 2003;9:133–41.
- Pillich RT, Scarsella G, Risuleo G. Reduction of apoptosis through the mitochondrial pathway by the administration of acetyl-L-carnitine to mouse fibroblasts in culture. *Exp Cell Res* 2005;306:1–8.
- Carlberg M, Nejaty J, Froysa B, Guan Y, Soder O, Bergqvist A. Elevated expression of tumour necrosis factor alpha in cultured granulosa cells from women with endometriosis. *Hum Reprod* 2000;15:1250–5.
- Austgulen R, Arntzen KJ, Vatten LJ, Kahn J, Sunde A. Detection of cytokines (interleukin-1, interleukin-6, transforming growth factor-beta) and soluble tumour necrosis factor receptors in embryo culture fluids during in-vitro fertilization. *Hum Reprod* 1995;10:171–6.
- Pampfer S, Moulart B, Vanderheyden I, Wu YD, De Hertogh R. Effect of tumour necrosis factor alpha on rat blastocyst growth and glucose metabolism. *J Reprod Fertil* 1994;101:199–206.
- Glabowski W, Kurzawa R, Wiszniewska B, Baczkowski T, Marchlewicz M, Brelik P. Growth factors effects on preimplantation development of mouse embryos exposed to tumor necrosis factor alpha. *Reprod Biol* 2005;5:83–99.
- Whiteside EJ, Boucaut KJ, Teh A, Garcia-Aragon J, Harvey MB, Herington AC. Elevated concentration of TNF-alpha induces trophoblast

- differentiation in mouse blastocyst outgrowths. *Cell Tissue Res* 2003;314:275–80.
30. Lalitkumar PG, Sengupta J, Ghosh D. Endometrial tumor necrosis factor alpha (TNFalpha) is a likely mediator of early luteal phase mifepristone-mediated negative effector action on the preimplantation embryo. *Reproduction* 2005;129:323–35.
31. Bykov I, Jarvelainen H, Lindros K. L-carnitine alleviates alcohol-induced liver damage in rats: role of tumour necrosis factor-alpha. *Alcohol Alcohol* 2003;38:400–6.
32. Lalitkumar PG, Sengupta J, Ghosh D. Effect of tumour necrosis factor-alpha (TNF-alpha) on protein synthesis by mouse preimplantation stage embryos in vitro. *Indian J Physiol Pharmacol* 2005;49:139–47.