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—Original Article—

L-carnitine prevents bovine oocyte aging and promotes subsequent embryonic development

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Abstract. L-carnitine (LC) is well known for its antioxidant activity. In this study, we explored the potential mechanistic effects of LC supplementation on aged bovine oocytes *in vitro*. We showed that *in-vitro* maturation could enhance the subsequent developmental capacity of aging oocytes, when supplemented with LC. After *in vitro* fertilization, the blastocyst formation rate in the aged oocytes post-LC treatment significantly increased compared to that in untreated aged oocytes (29.23 ± 2.20% *vs*. 20.90 ± 3.05%). Furthermore, after LC treatment, the level of intracellular reactive oxygen species in aged oocytes significantly decreased, and glutathione levels significantly increased, compared to those in untreated aged oocytes. Mitochondrial membrane potential, the percentage of early apoptotic oocytes, and caspase-3 activity were significantly reduced in LC-treated aged oocytes compared to those in untreated aged oocytes. Furthermore, during *in vitro* aging, the mRNA levels of the anti-apoptotic genes, *Bcl-xl* and *survivin* in LC-treated aged oocytes were significantly higher than those in untreated aged oocytes. Overall, these results indicate that at least in *in vitro* conditions, LC can prevent the aging of bovine oocytes and improve the developmental capacity of bovine embryo.

Key words: Bovine, Embryo development, L-carnitine, Oocyte aging

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Mammalian oocytes are arrested in the metaphase of the second meiosis (MII) phase, where they await fertilization. If no fertilization occurs within an appropriate time, the quality of oocytes gradually deteriorates, a process termed as "postovulatory aging" [1]. In humans and livestock, it is well known that postovulatory aging of oocytes may affect the results of assisted reproductive technologies (ARTs), such as artificial insemination [2], *in vitro* fertilization (IVF) [3, 4], and intracytoplasmic sperm injection [5, 6]. In bovine, both *in vivo* and *in vitro* aging of oocytes can result in reduced fertilization and embryonic development [2, 3, 7–10]. Extensive research on aged bovine oocytes may help in the development of a method to prevent aging in matured bovine oocytes, resulting in improved efficacy of ARTs.

It has been demonstrated that, following ovulation, intracellular reactive oxygen species (ROS) accumulation increases in oocytes with time [11, 12]. Oocytes exhibit an intracellular defense [(via the antioxidant glutathione (GSH)] mechanism against an oxidative attack. However, this defense response decreases with aging after ovulation [13]. Thus, aging oocytes after ovulation undergo oxidative stress due to an increase in ROS level, and a decrease in antioxidant defenses, causing multiple oxidative damages in cell structures, including lipid peroxidation of membranes, enzyme inactivation, protein oxidation, and DNA damage [14, 15]. The imbalance between ROS and their normal scavenger antioxidants leads to oxidative stress, which adversely affects embryonic development through structural and functional alterations. Increased production of ROS in aging oocytes reduces intracellular ATP concentration [16] and glutathione disulfide ratio [17–19]. This outcome adversely affects fertilization and subsequent embryonic development, thereby increasing the risk of an early miscarriage and abnormal development of offspring [20, 21].

L-carnitine (LC), the biologically active form of carnitine (3-hydroxy-4-N-trimethyl amino butyrate, $C_7H_{15}NO_3$), is a naturally occurring, vitamin-like water-soluble quaternary ammonium compound. It is mainly synthesized from the amino acids lysine and methionine, in the liver. LC is required to transport fatty acids from the cytosol to the mitochondria during the breakdown of lipids (fats), to generate metabolic energy. As an antioxidant, LC neutralizes free radicals, especially superoxide anions, and protects cells from oxidative damage-induced apoptosis [22]. Although the effects of LC on the *in vitro* development of bovine embryos [23], pig embryos [24], and mouse embryos [25] have been previously reported, there are no reports regarding the effects of LC on aging bovine oocytes.

The best mature culture period for bovine embryo production is 20–22 h. Upon extension of this period, the blastocyst formation rate relatively decreases [3]. Previous studies have considered bovine oocytes at about 30 h after *in-vitro* maturation (IVM), as aged or slightly aged and used them to investigate age-related changes [8, 26, 27]. Oocytes after 30 h of IVM showed a low blastocyst development rate [8].

In the present study, aging bovine oocytes treated with LC were

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evaluated for ROS and GSH levels, mitochondrial membrane potential (ΔΨm), early apoptosis levels, and caspase-3 activity indicators in order to identify whether LC treatment improved the performance of oocytes. The aim of this study was to investigate the potential of LC in delaying aging via reducing oxidative stress in bovine oocytes.

Materials and Methods

All the chemicals and reagents used for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

IVM and aging of bovine oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory within 2 h at 38ºC in phosphate buffered saline (PBS). Bovine cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (2–8 mm in diameter). Oocytes surrounded by intact cumulus layers were washed 5 times in IVM medium composed of TCM199 (Earle's salts; 11150-59, Gibco, NY, USA), 0.57 mmol/l cysteine, 10% fetal bovine serum (FBS), 10 μg/ml follicle-stimulating hormone, 0.04 mg/ml pyruvate, 1 μg/ml estradiol, 10 ng/ml epidermal growth factor, and 1% penicillin-streptomycin solution. The COCs were randomly divided into the following three groups: fresh, aged, and aged + LC. The fresh group was cultured in IVM medium (200 μl) for 24 h, the aged group was cultured in IVM medium (200 μl) for 30 h, and the aged + LC group was cultured with 2.5 mM LC (L-carnitine hydrochloride; C0283) [28] during the entire 30 h duration of IVM (200 μl) in a humidified atmosphere of 5% CO_2 at 38.5°C. The IVM dishes had 4 wells per plate (10034; SPL Lifesciences, Pocheon, Korea) and all the oocytes were covered with mineral oil. A frozen stock solution of LC was used.

IVF and embryo culture

After the fresh, aged, and aged + L-carnitine groups were cultured for 24, 30, and 30 h, respectively, the matured oocytes were washed and cultured in fertilization medium (100 μl) (IVF100; Research Institute for the Functional Peptides, Higashine, Japan), overlaid with mineral oil, and incubated in a humidified atmosphere of 5% CO_2 at 38.5°C. Frozen bull semen straws were thawed by immersing them in a water bath at 37.5°C for 30 sec. The sperm was then centrifuged twice with Brackett Oliphant (BO) medium at 25°C at 1000 rpm for 6 min. The BO medium comprised of 6.63 mg/ml NaCl, 0.299 mg/ml KCl, 0.25 mg/ml $CaCl_2$, 0.12 mg/ml NaH_2PO_4, 0.11 mg/ml $MgCl_2$, 2.1 mg/ml $NaHCO_3$, 2.5 mg/ml glucose, 2.98 mg/ml HEPES, 3.88 mg/ml caffeine, 0.01 mg/ml heparin, 0.13 mg/ml pyruvate, and 6.25 mg/ml bovine serum albumin (BSA; fatty acid-free BSA; A8806). The matured oocytes were co-incubated with spermatozoa in fertilization medium for 6 h in a humidified atmosphere of 5% CO_2 at 38.5°C. After fertilization (day 0), the presumptive zygotes of the three groups were washed three times with 0.4% BSA in Charles Rosenkrans medium (CRI) (fatty acid-free BSA; A8806; BSA-CRI), maintained in BSA-CRI medium (10 μl), overlaid with mineral oil, and cultured to the 8-cell stage (72 h). CRI medium comprised 6.7 mg/ml NaCl, 0.23 mg/ml KCl, 2.2 mg/ml $NaHCO_3$, 0.15 mg/ml L-glutamine, 0.05 mg/ml gentamycin, 0.01 ml Non-Essential Amino Acid (MEM), 0.02 ml Amino Acid (BME), 0.04 mg/ml pyruvate, and 0.55 mg/ml L (+)-Lactate. Subsequently, oocytes in the three groups were washed three times using 10% FBS in CRI (Gibco; 04-002-1B), placed in 10% FBS-CRI medium (10 μl), overlaid with mineral oil, and cultured to the blastocyst stage (96 h).

Terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assay

TUNEL assay was used to measure the intracellular apoptosis rates of blastocysts using the In Situ Cell Death Assay Kit (Cat #11684795910, Roche Diagnostics, Mannheim, Germany). The day-7 blastocysts were fixed in 3.7% paraformaldehyde for 30 min at 25°C and then permeabilized by incubating in 0.5% Triton X-100 at 37.5°C for 30 min. Following this, they were blocked in PBS containing 1% BSA (BSA-PBS) for 1 h. The embryos were then incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme for 1 h at 37.5°C, and subsequently washed three times with 0.1% BSA-PBS. Post end labeling, the embryos were treated with 10 μg/ml Hoechst 33342 for 20 min at 37.5°C, washed three times with 0.1% BSA-PBS, and mounted onto glass slides. Images were captured by fluorescence microscopy (Nikon, Tokyo, Japan) using the blue (for DNA) and green fluorescence filters (for apoptosis), and analyzed by ImageJ software [29]. The apoptosis index was denoted as the percentage of TUNEL-positive nuclei based on the total number of nuclei.

Measurement of ΔΨm, ROS, and GSH levels

To assess ΔΨm, denuded MII-stage oocytes were incubated with 2 μM JC-1 (Invitrogen, Waltham, MA, USA) for 1 h at 37.5°C in the dark. The ΔΨm of oocytes was then calculated as the ratio of red fluorescence intensity (J-aggregates; corresponding to activated mitochondria) to green fluorescence intensity (J-monomers; corresponding to inactive mitochondria) using ImageJ software. The fluorescence intensity of the resulting oocytes was analyzed using a fluorescence microscope (Nikon). ROS levels were measured by a 2′,7′-dichlorofluorescein assay (H2DCFDA; Thermo Fisher Scientific, Waltham, MA, USA). In brief, denuded MII-stage oocytes were cultured in 0.1% BSA-PBS containing 10 μM H2DCFDA for 15 min at 37.5°C in the dark, and then visualized at an excitation of 485 nm and emission of 535 nm. GSH levels were quantified with the CellTracker™ Blue dye (4-chloromethyl-6, 8-difluoro-7-hydroxycoumarin, CMF2HC; Invitrogen). In brief, denuded MII-stage oocytes were incubated in 0.1% BSA-PBS medium containing 10 μM CMF2HC for 15 min at 37.5°C in the dark, and then visualized at an excitation of 371 nm and emission of 464 nm. The fluorescence intensity (1 sec after the shutter opening with 10 msec exposure for H2DCFDA; 3 sec after the shutter opening with 100 msec exposure for CMF2HC) of the resulting oocytes was analyzed by fluorescence microscopy (Nikon) using ImageJ.

Immunofluorescence and Annexin V-FITC assay

Approximately 10 oocytes from each of the three (fresh, aged, and aged + LC) groups were washed in 0.1% BSA-PBS, fixed for 30 min in 3.7% formaldehyde in PBS with 1% Polyvinyl alcohol (PVA), and permeabilized with 0.5% Triton X-100 in 1% BSA-PBS for 30 min at room temperature. The oocytes were then blocked using 1% BSA-PBS. Next, the oocytes were incubated with rabbit anti-caspase-3 antibody (Sigma-Aldrich) at 4°C overnight, followed

by incubation with an Alexa Fluor 488-conjugated secondary antibody (1:200; Sigma-Aldrich) for 1–2 h at 25°C. Hoechst 33342 (10 µg/ml in PBS) was used for DNA counterstaining.

An Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Vazyme, Nanjing, China) was used to stain the oocytes with Annexin V- FITC to detect the externalization of phosphatidylserine in early apoptotic MII oocytes, according to the manufacturer's instructions. Briefly, 20–30 MII oocytes were washed three times in 0.1% BSA-PBS and then incubated for 30 min in the dark at room temperature in 100 µl binding buffer containing 5 µl Annexin V-FITC. The oocytes were again washed three times in 0.1% BSA-PBS, following which Hoechst 33342 (10 µg/ml in PBS) was used for DNA counterstaining. Oocytes were then mounted on a glass slide. Annexin-V-positive oocytes were identified using a confocal microscope (Zeiss LSM 710 META; Carl-Zeiss Jena, Germany). Specifically, a green circle observed on the cellular membrane indicated the presence of an Annexin-V-positive oocyte.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

MII oocytes were harvested, and mRNA was extracted from each of the 15 oocyte pools using the DynaBeads mRNA Direct Kit (Cat #61012; Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of mRNA using oligo(dT)12-18 primers and SuperScript III reverse transcriptase (Invitrogen). RT-PCR was performed using KAPA SYBR® FAST kit (KK4601; Kapa Biosystem, Salt River Cape Town, South Africa), wherein each reaction contained 10 µl SYBR Green, 1 µl of each forward and reverse primers, and 2 µl of cDNA template (10 ng/µl) in a final reaction volume of 20 µl. The amplification cycle was programmed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 3 sec, 60°C for 30 sec, and 72°C for 20 sec. The target genes were B-cell lymphoma-extra-large (*Bcl-xl*), Bcl-2-associated X (*Bax*), and *survivin*. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. The primer sequences used to amplify each gene are listed in Table 1. mRNA quantification data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical analyses

Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test, using SPSS software, version 19.0 (SPSS, Chicago, IL, USA). Figures were generated using the GraphPad Prism software package (version 6.01; GraphPad, La Jolla, CA, USA). Data are expressed as the mean ± standard deviation (SD). $P < 0.05$ was considered to be statistically significant. The total number (N) of oocytes/embryos used in each group are shown in the data columns and replicates (R) in each experiment are mentioned in the figure legends.

Table 1. Sequences of primers used for RT-PCR

Genes	Primer sequences	Product size (bp)
Bax	F: AGAAGGATGATCGCAGCTGTG	198
	R: AGTCCAATGTCCAGCCCATG	
Survivin	F: GCCAGATGACGACCCCATAG	199
	R: GGCACAGCGGACTTTCTTTG	
Bcl-xl	F: AGGCAGGCGATGAGTTTGAA	159
	R: AGAAAGAGGGCCAVAATGCGA	
Gapdh	F: ACAGTCAAGGCAGAGAACGG	235
	R: GGTTCACGCCCATCACAAAC	

The annealing temperature for all reactions was 60°C. F: forward primer, R: reverse primer.

Results

Effect of LC on the development and quality of aged bovine oocytes in vitro

The aim of our study was to determine whether LC supplementation could maintain the quality of aged oocytes, especially for subsequent embryo development after IVF (Fig. 1A). The blastocyst formation rate in the aged + LC group was comparable to that in the fresh group (29.23 ± 2.20% *vs*. 31.37 ± 2.93%; Fig. 1B), while the rate in the aged group was significantly lower than that in the aged + LC group (20.90 ± 3.05% *vs*. 29.23 ± 2.20%; Fig. 1B). Moreover, the number of cells per blastocyst in the aged + LC group was higher compared to that in the aged group (97.29 ± 17.70 *vs*. 72.10 ± 6.44; Fig. 1C), but similar to that in the fresh group (97.29 ± 17.70 *vs*. 103.3 ± 15.06; Fig. 1C). Furthermore, the apoptotic rate of blastocysts derived from the aged + LC group was lower than that of blastocysts derived from the aged group (1.08 ± 0.12 *vs*. 1.52 ± 0.14; Fig. 1D).

Effect of LC on ROS and GSH levels in aged bovine oocytes in vitro

Oxidative stress is a potential threat to developmental potential; therefore, we evaluated ROS levels, as shown in Fig. 2A. The ROS levels in aged oocytes were significantly higher than those in LC-treated aged oocytes (1.73 ± 0.31 *vs*. 1.07 ± 0.29; Fig. 2B), while the levels in LC-treated oocytes were similar to those in fresh oocytes (1.07 ± 0.29 *vs*. 1 ± 0.21; Fig. 2B). Though GSH levels vary among different cell types, it exerts a powerful antioxidant function in protecting cells against oxidative stress damage. Thus, quantification of intracellular GSH was performed, as shown in Fig. 2C. The GSH levels in aged oocytes were significantly lower than those in LC-treated oocytes (0.67 ± 0.19 *vs*. 0.81 ± 0.20; Fig. 2D), while the levels in LC-treated aged oocytes were significantly lower than those in fresh oocytes (0.81 ± 0.20 *vs*. 1 ± 0.13; Fig. 2D).

Effect of LC on the ΔΨm of aged bovine oocytes in vitro

In cells, the mitochondria play a crucial role in maintaining normal metabolic functions [30]. Thus, we evaluated mitochondrial function (as indicated by ΔΨm) during *in vitro* aging of oocytes, with and without LC treatment. Representative images of JC-1 staining are shown in Fig. 3A. The ΔΨm of the aged group was higher than that of the fresh group (3.49 ± 1.30 *vs*. 1.33 ± 0.41; Fig. 3B), while the ΔΨm of the aged + LC group was lower than that of the aged group (3.49 ± 1.30 *vs*. 1.69 ± 0.61; Fig. 3B).

Effect of LC on caspase-3 activity in aged bovine oocytes in vitro

As caspase-3 is an important apoptosis marker, we measured the caspase-3 activity in aging oocytes (Fig. 4A). The caspase-3 activity

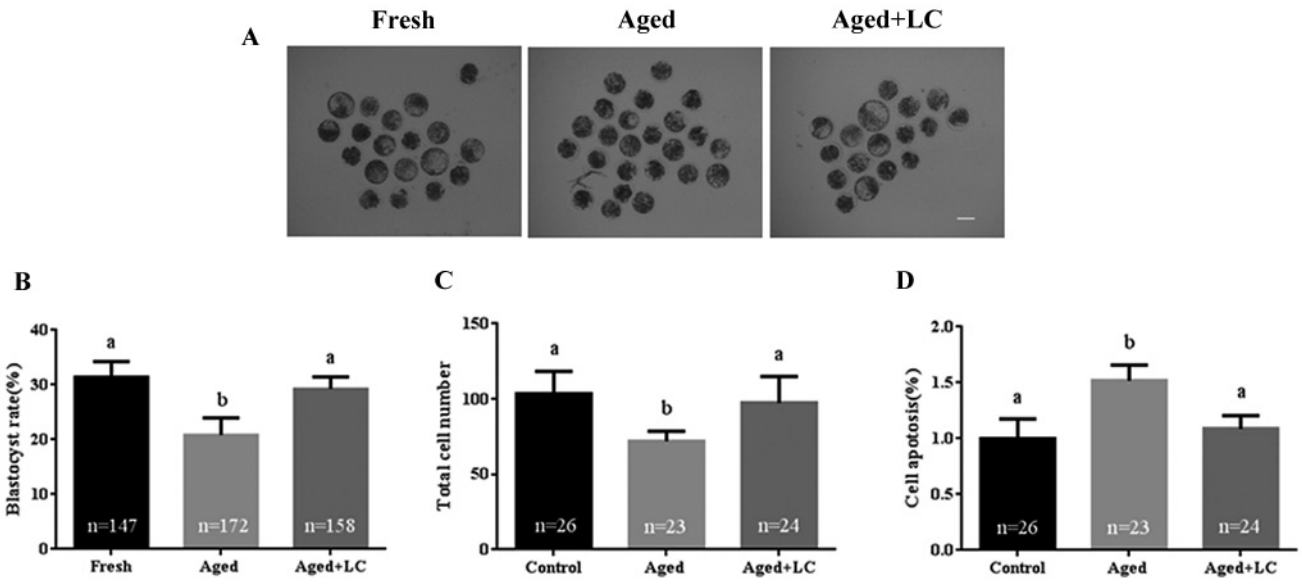

Fig. 1. Effect of L-carnitine (LC) on the development and quality of aged bovine oocytes *in vitro*. (A) Blastocyst formation on day 7. Scale bar: 100 μm. (B) Blastocyst rate. R = 5. (C) Total cell number in each day-7 blastocyst. R = 3. (D) The rate of cell apoptosis in the day-7 blastocysts, R = 3. Statistically significant differences are represented with different letters ($P < 0.05$).

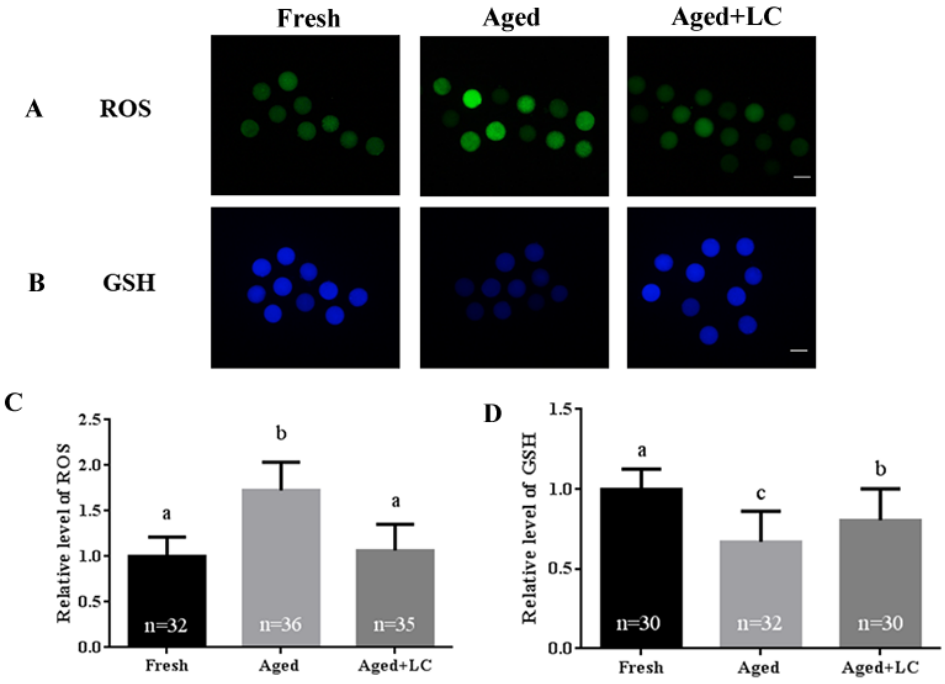

Fig. 2. Effect of L-carnitine (LC) on ROS and GSH levels in aged bovine oocytes *in vitro*. (A) Oocytes were stained with H2DCFDA to detect the intracellular levels of ROS. Scale bar: 100 μm, R = 3. (B) Oocytes were stained with Tracker Blue CMF2HC dye to detect the intracellular levels of GSH. Scale bar: 100 μm, R = 3. (C) and (D) The relative intracellular levels of ROS and GSH in bovine oocytes from the three groups (fresh, aged, and aged + LC). Statistically significant differences are represented with different letters ($P < 0.05$).

of LC-treated aged oocytes was significantly lower than that of untreated aged oocytes (1.08 ± 0.38 *vs*. 1.54 ± 0.78; Fig. 4B). The level of caspase-3 in fresh oocytes was significantly lower than that in aged oocytes (1.00 ± 0.30 *vs*. 1.54 ± 0.78; Fig. 4B), but similar to that in LC-treated aged oocytes.

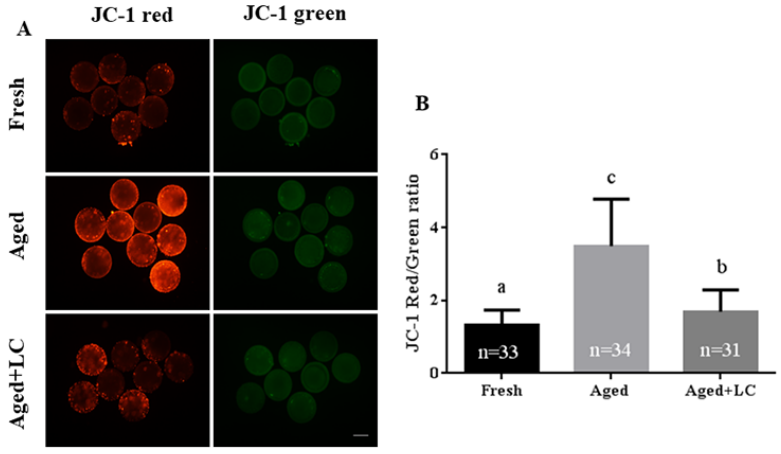

Fig. 3. Effect of L-carnitine (LC) on the mitochondrial membrane potential (ΔΨm) of aged bovine oocytes *in vitro*. (A) Representative fluorescent images of JC-1-stained oocytes after *in vitro* aging. Scale bar: 200 μm, R = 3. (B) Quantification of JC-1 fluorescence intensity. Statistically significant differences are represented with different letters ($P < 0.05$).

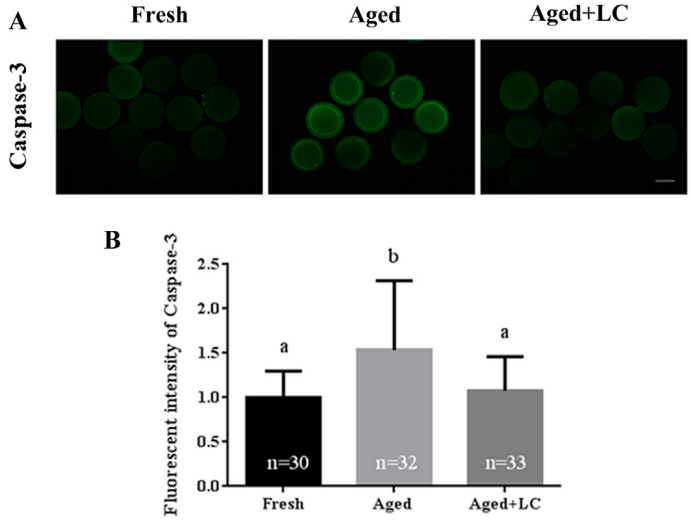

Fig. 4. Effect of L-carnitine (LC) on the caspase-3 activity of aged bovine oocytes *in vitro*. (A) Representative images showing caspase-3 activity in fresh, aged, and LC-treated aged MII oocytes. Scale bar: 200 μm, R = 3. (B) Quantified fluorescence intensity for caspase-3 in oocytes. Statistically significant differences are represented with different letters ($P < 0.05$).

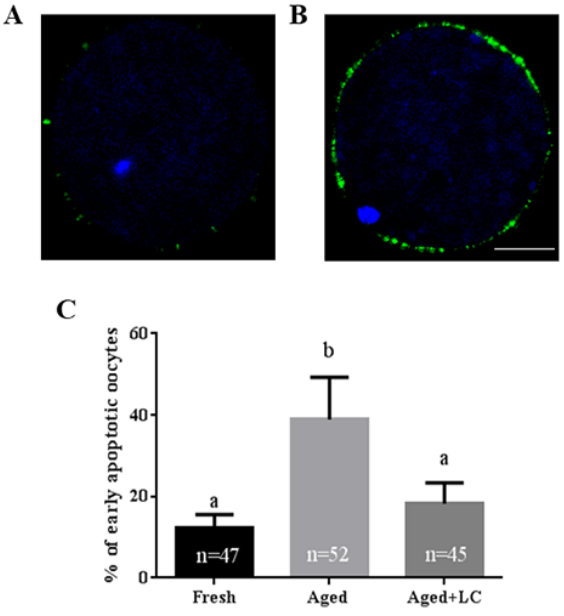

Fig. 5. Effect of L-carnitine (LC) on the percentage of early apoptotic aged bovine oocytes *in vitro*. (A) Negative control. (B) Annexin V-positive. (C) The percentage of Annexin V-positive oocytes in the fresh, aged, and aged + LC groups. Scale bar: 100 μm, R = 3. Statistically significant differences are represented with different letters ($P < 0.05$).

Effect of LC on the level of early apoptosis in aged bovine oocytes in vitro

Oocyte aging is accompanied by apoptosis. Therefore, we detected the proportion of aged oocytes undergoing early apoptosis by the Annexin V-FITC assay. In this assay, a green circle indicating the position of the oocyte on the outer cell membrane was defined as Annexin V-positive (Figs. 5A and 5B). The results showed that the percentage of oocytes undergoing early apoptosis in the fresh group was significantly lower than that in the aged group (12.43 ± 3.09% *vs*. 38.97 ± 10.38%; Fig. 5C), but was similar to that in the LC-treated aged group (12.43 ± 3.09% *vs*. 18.23 ± 5.09%; Fig. 5C).

Effect of LC on the mRNA levels of apoptosis-related genes in aged bovine oocytes in vitro

To determine the effect of LC on the mRNA levels of apoptosis-related genes, we measured the transcript levels of *Bcl-xl*, *Bax*, and

survivin in the oocytes from each group (Fig. 6). The mRNA levels of *Bcl-xl* and *survivin* were significantly lower in the aged oocytes than those in the fresh oocytes. However, the mRNA levels of *Bcl-xl* and *survivin* in LC-treated aged oocytes were higher than those in aged oocytes. No significant differences in *survivin* transcript levels were observed between the LC-treated aged oocytes and fresh oocytes. Moreover, the mRNA levels of *Bcl-xl* were significantly lower in the aged + LC group than those in the fresh group, while the mRNA levels of *Bax* were significantly lower in the aged + LC and aged groups than those in the fresh group. No significant difference in *Bax* transcript level was observed between the aged + LC and aged groups.

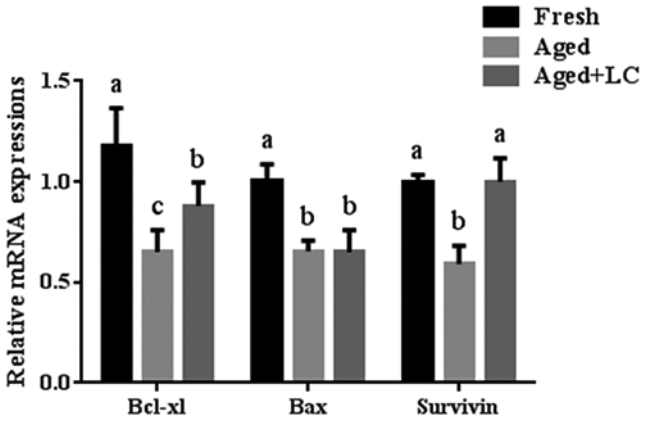

Fig. 6. Effect of LC on apoptosis-related gene expression in aged bovine oocytes *in vitro*. The relative mRNA levels of apoptosis-related genes encoding Bcl-xl, Bax, and survivin, as analyzed by RT-PCR; R = 3. Statistically significant differences are represented with different letters ($P < 0.05$).

Discussion

Oocyte aging is a complex and irreversible biological process that may lead to several changes in the structure and functional states of mammalian oocytes, including DNA damage, reduced fertilization rates, abnormal mitochondrial structure, oxidative damage, and early oocyte apoptosis [31, 32, 33]. Here, we demonstrated that LC treatment may effectively delay the aging of oocytes and enhance subsequent embryo development.

Oocyte quality is a major determinant of subsequent embryo development. Oocyte aging has been shown to severely reduce the quality of oocytes, significantly affecting embryo development before and after implantation [1, 34]. To demonstrate that LC can improve oocyte quality and delay oocyte aging, we investigated the *in vitro* developmental capacity of oocytes after IVF. We found that prolonged IVM significantly impaired blastocyst formation. A higher proportion of bovine embryos developed into blastocysts in LC-treated aged oocytes compared to that in untreated aged oocytes after IVF, following IVM treatment for 30 h. Previous studies have shown that LC supplementation can increase blastocyst formation rates [28, 35]. Furthermore, we determined the number of cells in day-7 blastocysts, and found that the number of cells per blastocyst in the aged + LC group was higher than that in the aged group. The results of TUNEL assay showed that the aged + LC group showed a smaller percentage of apoptotic cells in the blastocyst at 30 h than the aged group. Previous studies have shown that increased DNA fragmentation in developing aged oocytes has negative effects on subsequent embryonic development, resulting in developmental arrest and apoptosis [1, 12, 36]. Our results indicate that LC can maintain the capacity of aged oocytes to develop into the blastocyst stage and downregulate apoptosis in the resultant blastocysts.

Oocyte quality is usually affected by oxidative stress or oxidative damage [37, 38]. As the oocyte ages, ROS accumulate [39]. GSH levels are a key factor affecting the quality of oocytes. GSH is known to be present in varying amounts in a diverse range of cells and exerts strong antioxidant effects to protect cells against oxidative stress-induced damage [40]. It has been reported that ROS accumulate in oocytes during aging, and affect their subsequent fertilization capacity [41]. Previous studies have shown the beneficial effects of LC as an antioxidant that reduces ROS levels during oocyte maturation and increases ATP content and GSH levels [24, 42]. In our study, results showed that LC supplementation decreased ROS levels and increased GSH levels in aging oocytes, indicating that LC may improve the quality of aging oocytes via reducing oxidative stress.

Mitochondria play a crucial role in maintaining cellular metabolic functions [43]. As oocytes age after ovulation, the impaired mitochondrial function may seriously affect the quality of oocytes. Therefore, we carried out a mitochondrial membrane potential assay to evaluate the mitochondrial membrane potential of aged oocytes. A previous study reported a lower ΔΨm in bovine oocytes after an extended IVM time of up to 30 h [36]. Another study showed that the ΔΨm and ATP content of oocytes subjected to 40 h of IVM were higher than those in oocytes subjected to 20 h of IVM [44]. After 30 h of IVM, the oocytes presented an intermediate value [44]. In our study, the ΔΨm of the aged group rapidly increased, while that of the aged+LC group markedly decreased. However, we were unable to elucidate a direct relationship between the enhanced ΔΨm and low developmental competence of bovine oocytes. Thus, further research is required to investigate the ΔΨm of embryos (2-cell stage, 4-cell stage, 8-cell stage, and morula stage) derived from oocytes from extended IVM culture.

Previous studies have shown that *in vitro* aged oocytes are affected by oxidative stress, which can result in apoptosis [31, 45]. Further, previous reports also indicate that LC has antioxidant properties, whereby it reduces oxidative stress by enhancing the activity of several antioxidant enzymes like superoxide dismutase and glutathione peroxidase [42, 46]. Meanwhile, studies have shown that LC upregulates glutathione peroxidase (*GPx*) and downregulates superoxide dismutase 2 (*SOD2*) at mRNA level in oocytes and embryos [35]. We presumed that LC may prevent apoptosis due to its resistance to oxidation. Phosphatidylserine on the outer cell membrane is a marker of early apoptosis in mature oocytes. Annexin V has a high binding affinity to phosphatidylserine, therefore we used the Annexin V-FITC Apoptosis Detection Kit to detect early apoptosis in aged oocytes [47]. Our results showed that LC treatment decreased the percentage of early apoptotic cells, which is in accordance with a previous finding that antioxidants reduce the level of early apoptosis in aging oocytes [48]. Caspase-3 is a member of the cysteine-aspartic protease (caspase) family [49]. Caspases are

crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease that catalyzes the specific cleavage of many key cellular proteins [50]. The sequential activation of caspases plays an important role in apoptosis [51]. Previous research has shown that caspase-3 activity is associated with oocyte quality [52]. Bcl-xl, which belongs to the Bcl-2 family, prevents apoptosis by blocking the leakage of cytochrome c through the mitochondrial membrane pores [53]. Survivin is the smallest member of the inhibitor of apoptosis protein family, which regulates cell cycle/apoptosis balance [54]. A previous study showed that oocyte aging eventually leads to cell death via an apoptotic pathway characterized by phosphatidylserine externalization [47], caspase activation [55], accumulation of the apoptotic signaling protein Bax, suppression of Bcl-xl [56, 57], and DNA fragmentation [58]. In our study, the mRNA levels of *Bcl-xl* and *survivin* were significantly lower in the aged group than those in the fresh and aged + LC groups. No significant differences in *survivin* transcript levels were observed between the aged + LC and fresh groups. Moreover, the mRNA levels of *Bcl-xl* were significantly lower in the aged + LC group than those in fresh group. These results suggest that LC may affect the mRNA levels of apoptosis-related genes.

Finally, our results, as well as previous reports [28, 42] suggest that LC is not only likely to be involved in mitochondrial function and lipid metabolism, but is also involved in regulation of other vital cellular functions, such as apoptosis, which may enhance the developmental capacity of aged oocytes.

In conclusion, our results demonstrate that treating aging oocytes with LC may improve oocyte quality and maintain their developmental capacity. We thus propose LC as a suitable agent to delay oocyte aging *in vitro* and to prevent the developmental loss of bovine oocytes in ARTs.

Acknowledgements

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