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ORIGINAL ARTICLE

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Developmental regulation and modulation of apoptotic genes expression in sheep oocytes and embryos cultured in vitro with L-carnitine

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Contents

The objective of this study was to find out the impact of L-carnitine (10 mM) on developmental regulation of preimplantation sheep embryos cultured in vitro when supplemented in maturation medium and post-fertilization medium separately. Subsequent objective was to observe the L-carnitine-mediated alteration in expression of apoptotic genes (Bcl2, Bax, Casp3 and PCNA) in sheep oocytes and developing embryos produced in vitro. Oocytes matured with L-carnitine showed significantly ($p < .05$) higher cleavage (67.23% vs 43.12%), morula (47.65% vs 28.58%) and blastocysts (32.12% vs 13.24%) percentage as compared to presumptive zygotes cultured with L-carnitine during post-fertilization period. So it is suggested to use L-carnitine during maturation than post-fertilization period. Antiapoptotic and proliferative effects of L-carnitine were confirmed by inducing culture medium with actinomycin D (apoptotic agent) and TNFα (antiproliferative agent), respectively, with and without L-carnitine. Oocytes and embryos cultured with actinomycin D and TNFα showed developmental arrest with significant ($p < .05$) decrease in morula and blastocysts percentage but supplementation of L-carnitine to actinomycin D and TNFα induced culture medium showed similar result as that of control. L-carnitine supplementation during IVM significantly ($p < .05$) upregulated the expression of Bcl2 and PCNA genes in majority of the developmental stages. Although L-carnitine upregulated the expression of Bax in initial developmental stages but downregulated at latter part, whereas the expression of Casp3 was upregulated upto 16-cell stage but after that there was no difference in expression. Expression of GAPDH gene was not affected by L-carnitine supplementation. In conclusion, L-carnitine acted as an antiapoptotic and proliferative compound during embryo development and supplementation of L-carnitine during IVM altered the expression of apoptotic genes in the developmental stages of embryos.

1 | INTRODUCTION

In vitro embryo production (IVEP) is an important reproductive technique which has great potential for genetic improvement in animals. The success of IVEP has been improved over time, employing a variety of culture media (Abazari-Kia, Mohammadabadi, Sangcheshmeh, Zhandi, & Salehi, 2015; Bavister, 1995). IVEP technology is not only helping to produce high-genetic merit animals, but it also provides an excellent source of embryos for other emerging biotechniques. In spite of number of media used to produce in vitro embryos, but these embryos are still not at par to in vivo-derived embryos. The fertilized oocytes divide continuously and undergo a series of distinct morphological changes such as compaction, cavitation and blastocoel formation that require well-orchestrated expression of genes from

the maternal and embryonic genome (Kidder, 1992). During embryo development, there is production of unstable metabolites of oxygen (O_2^-, H_2O_2 and OH^-) called reactive oxygen species (ROS) from gametes, embryos and their surroundings. ROS have the ability to react with any molecule, resulting in structural and functional alterations and cause several pregnancy-related disorders, leading to pregnancy loss (Agarwal, Aponte-Mellado, Premkumar, Shaman, & Gupta, 2012). Imbalance between ROS and their normal scavengers' antioxidants results in oxidative stress (OS). There are multiple factors to increase OS those adversely affect embryo development and gene expression in preimplantation embryos, resulting suboptimal outcome for IVEP set-up (Elamaran et al., 2012). Inhibition of OS-induced apoptosis of developing embryos by means of antiapoptotic factors is a useful approach to decrease the fragmentation of embryos. Stability and turnover rate of mRNA is also affected by OS that modulates the expression of developmentally important genes in in vitro-produced embryos as compare to in vivo-derived embryos (Mishra, Sharma, & Kumar, 2010a; Wrenzycki et al., 2005).

For better embryo development and to prevent apoptosis of early embryos in vitro, it is necessary to ameliorate the state of OS in micromilieu. There are number of compounds used in different studies to reduce OS for better embryo development in vitro (Mishra, Chandra, & Sharma, 2010b; Mukherjee et al., 2014; Reader, Cox, Staton, & Juengel, 2015). Embryonic losses that interfere with the developmental potential of embryo due to various cellular and morphological abnormalities affect the outcome of IVEP procedure. There are many reasons of embryonic losses after fertilization, but the main reasons are defects in embryo, suboptimal uterine environment or improper interaction of the embryo with the endometrium (Hansen, 2002). Apoptosis and mitosis regulates early embryonic development and differentiation, whereas occurrence of apoptosis is the most common in the developing embryos (Mirkes, 2002). The balance between pro- and anti-apoptotic protein expression regulates the fate of any cell (Korsmeyer, Shutter, Veis, Merry, & Oltvai, 1993). Irregular expression patterns of these proteins cause apoptosis and embryo mortality during early stages of development (Hardy, 1997).

L-carnitine, the biologically active form of carnitine (3-hydroxy-4-*N*-trimethyl amino butyrate, $C_7H_5NO_3$, M.W.-161.2), is a water-soluble quaternary ammonium compound and vitamin-like naturally occurring substance (Blum, 1994). It is mainly synthesized from amino acids lysine and methionine in liver and present especially in muscle and liver tissues of all animals. In living cells, it is required to transport fatty acids from cytosol to mitochondria during breakdown of lipids to generate metabolic energy (Scholte et al., 1996). Carnitine acts as an antioxidant that neutralizes the free radicals, especially superoxide anion in the body and protects cell against oxidative damage-induced apoptosis (Ye et al., 2010). Effect of L-carnitine on in vitro sheep embryos (Reader et al., 2015), bovine embryos (Takahashi et al., 2013), pig embryos (You, Lee, Hyun, & Lee, 2012) and mouse embryos (Abdelrazik, Sharma, Mahfouz, & Agarwal, 2009) has already been reported. We have reported antioxidant effect of L-carnitine on sheep embryo development and L-carnitine-mediated alteration in transcript level of antioxidant enzymes in embryos produced in vitro (Mishra, Reddy, Gupta, & Mondal, 2016). There is no report available to find out antiapoptotic and proliferative effect of L-carnitine and L-carnitine-mediated alteration in the expression of apoptotic genes in sheep oocytes and embryos. Therefore, this experiment was designed in sheep model with the objectives to find out the impact of L-carnitine (10 mM) on developmental regulation of preimplantation sheep embryos when supplemented in maturation medium and post-fertilization medium separately as well as to observe the L-carnitine-mediated alteration any, in expression of apoptotic genes such as B-cell lymphoma protein 2 (Bcl2), Bcl2-associated protein (Bax), Caspase3 (Casp3) and proliferating cell nuclear antigen (PCNA) in oocytes and developing embryos produced in vitro.

2 | MATERIALS AND METHODS

2.1 | Reagents and media

All the chemicals used in this study were obtained from Sigma-Aldrich Chemical, St. Louis, MO, USA. Disposable 35-mm Petri dishes, 90-mm Petri dishes and 0.22-μm syringe filters were obtained from Himedia laboratories, Mumbai, India, whereas 15-ml and 50-ml centrifuge tubes were obtained from Tarsons products pvt. ltd., Kolkata, India. Chemicals and kit required for gene expression study were from Invitrogen, Life technologies, USA. Primers for gene expression study were procured from Sigma-Aldrich Chemical, St. Louis, MO, USA.

2.2 | In vitro embryo production

In vitro embryos were produced as per the protocol standardized in our laboratory (Mishra et al., 2016). Briefly, oocytes were aspirated from follicles (2–6 mm) of slaughter house ovaries (carried to the laboratory in normal saline solution fortified with antibiotics at 35–37°C) with the help of 20-gauge needle attached to 5-ml syringe containing oocyte collection medium (TCM-199 + BSA (3 mg/ml) + 5% FBS + heparin (10 μg/ml)). Cumulus oocyte complexes (COCs) (excellent and good quality) (15–20 numbers) were matured in 100 μl of maturation medium (TCM-199 + 10% FBS + BSA (3 mg/ml) + pyruvate (4 mM) + glutamine (0.68 mM) + gentamycin (50 μg/ml) + FSH (5 μg/ml) + LH (5 μg/ml) + estradiol (1 μg/ml) under paraffin oil for 27 h in 35-mm Petri dish in CO_2 incubator at 5% CO_2, 38.5°C and 95% RH. Maturation rate was assessed by the degree of cumulus expansion and extrusion of the first polar body by aceto-orcein staining method. In vitro fertilization was performed by fresh semen collected from the ram with the help of electro ejaculator. Semen was washed twice with washing medium {Fert-TALP + heparin (10 μg/ml) + pyruvate (1 mM)} by centrifuging at 400 × ***g*** for 5 min. Supernatant was removed and pellet was reconstituted in fertilization medium {Fert-TALP + fatty acid free BSA (4 mg/ml) + heparin (10 μg/ml) + pyruvate (1 mM) + BME (100X) (1%) + MEM (50X) (1%)}. Final sperm concentration was adjusted to 2–3 × 10^6 sperms/ml which was assessed through Neubauer chamber. The sperm suspension was kept in CO_2 incubator till matured oocytes were washed four to five times in fertilization medium. Finally, matured oocytes (15–20 numbers) were co-incubated with 100 μl of processed spermatozoa for 18 h.

Following 18-h co-incubation, presumptive zygotes were cultured in 100 µl of culture medium (TCM-199 + 20% FBS + BSA (3 mg/ml) + pyruvate (4 mM) + glutamine (0.68 mM) + gentamycin (50 µg/ml) + BME (100X) (1%) + MEM (50X) (1%)) to get embryos of different developmental stages (two cells to blastocyst). Both fertilization and culture were carried out in the same temperature and gaseous condition described for maturation. Cleavage rates were recorded on day 2 (48hpi) of culture, and stages of embryonic development were evaluated every 24 h. Blastocyst development was recorded on day 7 (day 0 = day of IVF). Every 48 h, medium was replaced with 50% of freshly prepared culture medium. Subsequent experiments were conducted with and without L-carnitine (10 mM) to assess the developmental effect, antiapoptotic effect and proliferative effect of L-carnitine on embryo development. It is to remember that to study the effect of L-carnitine on embryo development during post-fertilization period, IVM of oocytes was conducted without L-carnitine.

2.3 | Antiapoptotic effect of L-carnitine

To find out the antiapoptotic effect of L-carnitine, embryos (2–4 cells) were cultured with actinomycin D (0.005 µg/ml), an apoptotic agent for 4 h with and without L-carnitine (10 mM). After 4 h, embryos were washed and cultured in medium without L-carnitine and actinomycin D till blastocyst development. Concentration of actinomycin D and its exposure time to the embryos for this study was selected from the study of mice (Abdelrazik et al., 2009). In control group, embryos were cultured neither with actinomycin D nor with L-carnitine.

2.4 | Proliferative effect of L-carnitine

TNFα inhibits in vitro bovine embryo development (Jackson, Farin, & Whisnant, 2012). To find out the proliferative effect of L-carnitine, oocytes were matured with TNFα (25 ng/ml) with and without L-carnitine (10 mM), then fertilized and cultured without TNFα. In subsequent experiment, embryos (2–4 cells) were cultured with TNFα (25 ng/ml) upto blastocyst development with or without L-carnitine (10 mM). Concentration of TNFα and its exposure time to the oocytes and embryos for this study was selected from the study of bovine (Jackson et al., 2012). In control group, oocytes and embryos were cultured neither with TNFα nor with L-carnitine.

2.5 | Blastocyst staining for total cell number

Blastocysts produced from L-carnitine-treated and non-treated oocytes were stained to compare the total cell numbers between these groups. Oocytes and embryos exposed to actinomycin D and TNFα were not developed to blastocyst, so day 7 embryos were stained to compare the total cell numbers. Zona pellucida of the blastocysts and day 7 embryos were removed by 1% protease digestion, then washed three times in PBS + polyvinyl pyrrolidone (PVP) (0.5%) and were fixed in 4% paraformaldehyde for 30 min. Blastocysts were permeabilized in Triton X-100 (1%) for 10 min, washed thrice in PBS+PVP (0.5%) and finally stained with Bisbenzimide (Hoechst 33258) (10 µg/ml) for 20 min. Blastocysts were then mounted on slides and covered with a cover slip. The total cell numbers of blastocysts were determined by counting the number of nuclei under an epifluorescent microscope (Euromex, Holland) equipped with a digital camera.

2.6 | Expression profile of apoptotic genes

The transcript abundance of apoptotic genes Bcl2, Bax, Casp3 and PCNA was analysed by real-time quantitative PCR (RT-qPCR). For gene expression study, oocytes matured with L-carnitine followed by embryos cultured without L-carnitine were treated as treatment group, whereas in control group neither oocytes nor embryos were exposed to L-carnitine.

2.7 | RNA isolation

Immature oocytes, in vitro mature oocytes and developing embryos (zygote, 2–4 cells, 8–16 cells, morula and blastocysts) generated in vitro were used for RNA isolation. Before RNA isolation, immature and in vitro-matured oocytes were treated with 0.25% Trypsin – EDTA solution, vortexed and washed five to six times in the handling medium (TCM-199 + 5% FBS) to remove attached cumulus cells. Total RNA was isolated from pools of oocytes, immature (n = 20), in vitro-matured (n = 20) and embryos of zygote (n = 20), 2–4 cell (n = 20), 8–16 cell (n = 20), morula (n = 10) and blastocyst (n = 10) by Trizol (Invitrogen, Life Technologies, USA) method as per manufacturer's guidelines with some modifications. Briefly, 200 µl of trizol was added to the oocytes and embryos, mixed thoroughly by pipetting up and down, and the mixture was incubated at room temperature for 10 min. Fifty microlitres of chloroform was added to the tubes, mixed and incubated again at room temperature for 10 min. Sample mixture was centrifuged at 12,000 × ***g*** for 15 min at 4°C, upper aqueous phase was collected without touching interphase and transferred to a new RNAse free tube. Two microlitres (40 µg) of acrylamide (20 mg/ml stock) and 100 µl of isopropanol were added to the aqueous phase collected, mixed them well by inversion and incubated on ice for 30 min. The tubes were centrifuged at 12,000 × ***g*** for 10 min at 4°C after incubation and supernatant was discarded. The pellet was washed twice with 150 µl of 75% ethanol by centrifuging at 7,500 × ***g*** for 5 min at 4°C and supernatant was discarded. The pellet was dried in an incubator for 10 min at 37°C and was dissolved in 10 µl of DEPC water. The dissolved pellet was incubated for 10 min at 55–60°C with little shaking in between incubation. The genomic DNA contamination was removed using TURBO DNA-free™ kit (Ambion, Life technologies, Carlsbad, CA, USA).

2.8 | RNA integrity and cDNA synthesis

The integrity of total RNA was checked on 1.5% agarose gel electrophoresis using 1× TAE buffer. The bands of 28sRNA and 18sRNA reflected the quality of extracted total RNA. The purity of total RNA (free from protein and genomic DNA contamination) was checked using nanodrop by OD 260/280 values which was equal to or more than 1.8. About 150 ng RNA was used in the reverse transcription

(RT) as the template for first-strand synthesis using SuperScript III First-Strand Synthesis kit (Invitrogen, Life technologies, USA) as per manufacturer's guidelines using oligo dT (50 µm), dNTP (10 mM), RT buffer, mgCl2 (2.5 mM), reverse transcriptase (RT)(200U), RNAse inhibitor (40U), DTT (0.1 M) in volume of 20 µl. The synthesized cDNA was stored in −20°C until used for RT-qPCR.

2.9 | Real-time quantitative PCR (RT-qPCR)

The expression levels of specific genes in oocytes and embryos were quantified by RT-qPCR using step one plus qPCR system (Applied Biosystem, Carlsbad, CA, USA). Relative quantification method was used to analyze the changes in gene expression level. The gene-specific primer was designed from NCBI, Primer Blast software (Ye et al., 2012). The specificity of the primers was tested using a BLAST analysis against the genomic NCBI database. Primer for Bax was taken from article published before (Ebrahimi, Valojerdi, Yazdi, & Baharvand, 2010) (Table 1). cDNA of oocytes and different stages of embryos were used as template and GAPDH was used as the reference gene in this study. The qPCR reactions were performed using Maxima SYBR Green/Rox qPCR master mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA). Each run was performed in duplicate in a 10 µl reaction that contains 5 µl qPCR master mix, 5 pM of gene-specific forward and reverse primers, 2 µl of cDNA as template, and final volume of 10 µl was made up with nuclease-free water. The PCR condition used to amplify all genes were initial denaturation at 95°C for 10 min with 40 cycles of denaturation at 95°C for 15 s followed by annealing and extension at 60°C for 1 min. Expression of each targeted gene was quantified relative to GAPDH. The melting curve analysis was carried out to confirm the qPCR specificity. Ct (threshold cycle for target amplification) values were analysed using $2^{-\Delta\Delta Ct}$ (normalized expression ratio) method to determine the relative level of expression of each mRNA. ΔCt = Ct (target gene) − Ct (housekeeping gene) and ΔΔCt = ΔCt (target gene sample) − ΔCt (calibrator).

2.10 | Confirmation of qPCR amplicons

The qPCR amplicons of apoptotic genes were confirmed by ethidium bromide (0.5 µg/ml)-stained 2% agarose gel electrophoresis.

TABLE 1 Primers used for gene expression study

Sl No	Genes	Primer sequence	Product size (bp)
1	GAPDH	F-ATGGGCGTGAACCACGAGAA R-ATGGCGTGGACAGTGGTCAT	146
2	Bcl2	F-ATGACTTCTCTCGGCGCTAC R-CTCCACACACATGACCCCTC	176
3	Bax	F-CATGGAGCTGCAGAGGATGA R-GTTGAAGTTGCCGTCGGAAA	100
4	Casp3	F-ACCTCACGGAAACCTTCACGA R-ACCATGGCTTAGAAGCACGC	149
5	PCNA	F-AGCCACTCCACTGTCTCCTACA R-TCATCCTCGATCTTGGGAGCC	123

2.11 | Experimental design

2.11.1 | Experiment 1: Effect of L-carnitine (10 mM) on embryo development supplemented in maturation medium and post-fertilization medium, respectively. A comparative study

To find out the effect of L-carnitine (10 mM) on embryo development, immature oocytes were randomly divided into two groups and matured in vitro without and with L-carnitine (10 mM) for 27 h. Fertilization was performed post-maturation and presumptive zygotes were cultured without L-carnitine. In subsequent experiment, presumptive zygotes from oocytes matured without L-carnitine were cultured without and with L-carnitine (10 mM) to observe further development. The objective of the experiment was to compare the beneficial effect of L-carnitine supplemented in maturation medium and post-fertilization period, respectively. This experiment was repeated for five times.

2.11.2 | Experiment 2: Antiapoptotic effect of L-carnitine on embryo development

To find out the antiapoptotic effect of L-carnitine, embryos (two to four cells) were cultured with actinomycin D (0.005 µg/ml), an apoptotic agent for 4 h, with and without L-carnitine (10 mM). After 4 h, embryos were washed and cultured in the medium without L-carnitine and actinomycin D till blastocyst development. Embryos exposed to actinomycin D were not developed to blastocyst, so day 7 embryos were stained by Bisbenzimide (Hoechst 33258) to count the total cell numbers. The objective of the experiment was to prove whether L-carnitine acts as antiapoptotic or not on embryo development. This experiment was repeated for four times.

2.11.3 | Experiment 3: Proliferative effect of L-carnitine on oocytes and embryo development

To find out the proliferative effect of L-carnitine, oocytes were matured with TNFα (25 ng/ml) with and without L-carnitine (10 mM) then fertilized and cultured without L-carnitine and TNFα. In subsequent experiment, embryos (2–4 cells) were cultured with TNFα (25 ng/ml) upto blastocyst development with and without L-carnitine (10 mM). Oocytes and embryos exposed to TNFα were not developed to blastocyst, so day 7 embryos were stained by Bisbenzimide (Hoechst 33258) to count the total cell numbers. The objective of the experiment was to prove whether L-carnitine has proliferative effect or not on embryo development. This experiment was repeated for four times.

2.11.4 | Experiment 4: Effect of L-carnitine on total cell number (TCN)

To evaluate the effect of L-carnitine on total cell numbers, the blastocysts produced from L-carnitine-treated and non-treated oocytes in maturation medium were stained with Bisbenzimide (Hoechst 33258). Embryos exposed to actinomycin D and TNFα with and without

L-carnitine on day 7 were also stained with Bisbenzimide (Hoechst 33258) to count the total cell numbers. The total cell number was recorded by counting the cell numbers on photographs. This experiment was repeated for three times.

2.11.5 | Experiment 5: L-carnitine-mediated alteration in apoptotic genes expression in developmental stages of embryos

To find out the effect of L-carnitine on apoptotic genes expression in oocytes and developmental stages of embryos, immature oocytes, matured oocytes and embryos produced from L-carnitine (10 mM) treated and non-treated (control) oocytes in maturation medium were taken for RNA isolation and subsequent RT-qPCR analysis. RT-qPCR was conducted for three times for three different sets of oocytes and embryos.

2.12 | Statistical analysis

The results are expressed in mean ± SEM. Statistical analysis was carried out using GraphPad Prism5, San Diego, USA. The mean between groups for embryonic development and TCN were compared by analysis of variance (ANOVA). Embryo development data have been presented in the percentage form in relation with total oocytes cultured. Percentage values were arcsine-transformed before analysis. $p < .05$ was considered as significant. Relative gene expression level at particular stage between control and L-carnitine-treated groups were compared using students "T" test.

3 | RESULTS

3.1 | Experiment 1: Effect of L-carnitine on embryo development, when supplemented in maturation and post-fertilization medium, respectively

The result of in vitro maturation and embryo development in the absence and presence of L-carnitine in maturation medium and post-fertilization medium of this study is detailed in Table 2. It has been observed that significantly ($p < .05$) higher percentage of cleavage (67.23% vs 38.58%) followed by morula (47.65% vs 21.32%) and blastocyst (32.12% vs 8.12%) were observed in L-carnitine supplementation in maturation medium (group II) as compared to control (0 mM) (group I). When presumptive zygotes (oocytes matured without L-carnitine) were cultured with L-carnitine (10 mM) during post-fertilization period, (group III) showed no significant change in cleavage percentage (43.12% vs 38.58%) but resulted significantly higher morula (28.58% vs 21.32%) and blastocyst (13.24% vs 8.12%) percentage as compared to control (group I). It was observed from the study that the oocytes matured with L-carnitine (group II) showed significantly ($p < .05$) higher cleavage (67.23% vs 43.12%), morula (47.65% vs 28.58%) and blastocysts (32.12% vs 13.24%) percentage as compared to presumptive zygotes cultured with L-carnitine during post-fertilization period (group III).

3.2 | Experiment 2: Antiapoptotic effect of L-carnitine on embryo development

Embryos (2–4 cells) cultured with actinomycin D without L-carnitine showed significant ($p < .05$) decrease in morula (46.11% vs 8.11%) and blastocysts (17.15% vs 0%) percentage as compared to control. Supplementation of L-carnitine (10 mM)- to actinomycin D-induced culture medium significantly ($p < .05$) increased morula (48.24% vs 8.11%) and blastocysts (15.02% vs 0%) percentage and showed similar result as that of control (Fig. 1).

3.3 | Experiment 3: Proliferative effect of L-carnitine on oocytes and embryo development

Oocytes matured with TNFα (25 ng/ml) without L-carnitine showed significant ($p < .05$) decrease in maturation percentage and subsequent developmental arrest. Oocytes matured with TNFα did not develop to blastocyst with significant ($p < .05$) decrease in cleavage (38.86% vs 18.84%), morula (22.57% vs 2.36%) and blastocyst (6.98% vs 0). Supplementation of L-carnitine to TNFα-induced maturation medium significantly ($p < .05$) increased the cleavage (38.24% vs 18.84%), morula (7.38% vs 2.36%) and blastocyst (5.65% vs 0) percentage and showed similar result as that of control (Fig. 2). In subsequent experiment, embryos (2–4 cells) cultured with TNFα (25 ng/ml) upto blastocyst development without L-carnitine showed developmental arrest, and there was a significant ($p < .05$) decrease in morula (51.63% vs 8.11%) and blastocyst (18.30% vs 0) percentage. Supplementation of L-carnitine to TNFα-induced culture medium significantly ($p < .05$) increased the morula (49.38% vs 8.11%) and blastocyst (17.22% vs 0) percentage and showed similar result as that of control (Fig. 3).

TABLE 2 Effect of L-carnitine on developmental potential of oocytes and embryos supplemented during maturation and post-fertilization period separately

Groups	L-carnitine in maturation medium (mM)	L-carnitine in culture medium (mM)	Oocytes cultured (numbers)	Cleavage (%)	Morula (%)	Blastocyst (%)
I	0	0	238	38.58 ± 1.9^{a}	21.32 ± 1.8^{a}	8.12 ± 1.1^{a}
II	10	0	282	67.23 ± 2.6^{b}	47.65 ± 2.7^{b}	32.12 ± 1.9^{b}
III	0	10	244	43.12 ± 2.1^{a}	28.58 ± 1.9^{c}	13.24 ± 0.9^{c}

Percentage results are presented as mean + S.E.M. Different superscripts in the same column differ significantly at $p < .05$. Five experiments were performed.

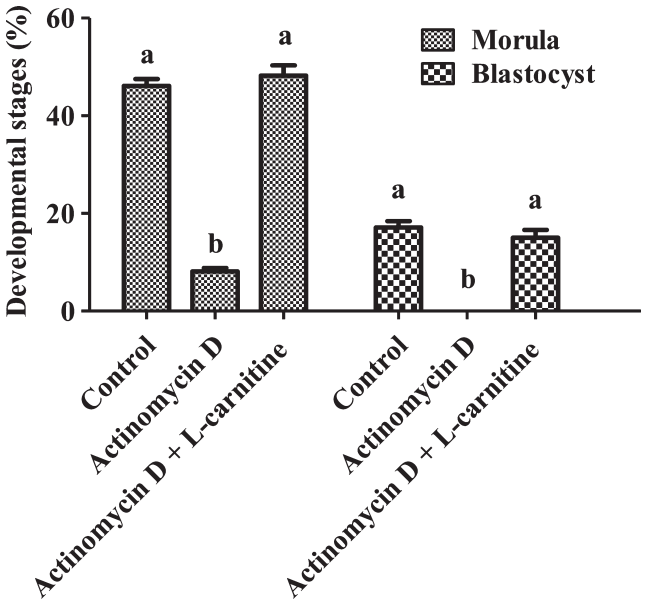

FIGURE 1 Effect of actinomycin D on embryo development supplemented in culture (post-fertilization) medium with and without L-carnitine. Percentage results are presented as mean + S.E.M. Different superscripts in the same group differ significantly at $p < .05$. Four experiments were performed

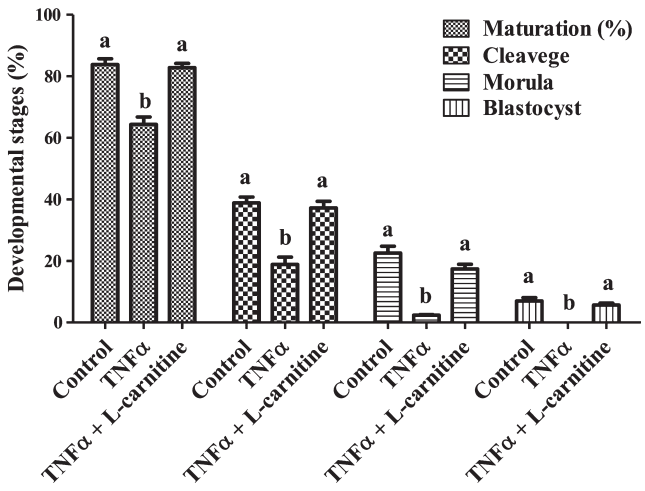

FIGURE 2 Effect of TNFα on embryo development supplemented in maturation medium with and without L-carnitine. Percentage results are presented as mean + S.E.M. Different superscripts in the same group differ significantly at $p < .05$. Four experiments were performed

3.4 | Experiment 4: Effect of different culture conditions on total cell numbers

Although blastocyst percentage (32.12% vs 8.12%) was significantly ($p < .05$) increased due to supplementation of L-carnitine in maturation medium, total cell numbers was not significantly ($p < .05$) different (188 ± 11.2 vs 182 ± 8.6) between L-carnitine-treated and L-carnitine non-treated groups. Embryos exposed to actinomycin D or TNFα did not develop to blastocyst and total cell numbers in day 7 were significantly ($p < .05$) reduced to 48 ± 6.4 and 42 ± 4.3, respectively (Figs. 4 and 5).

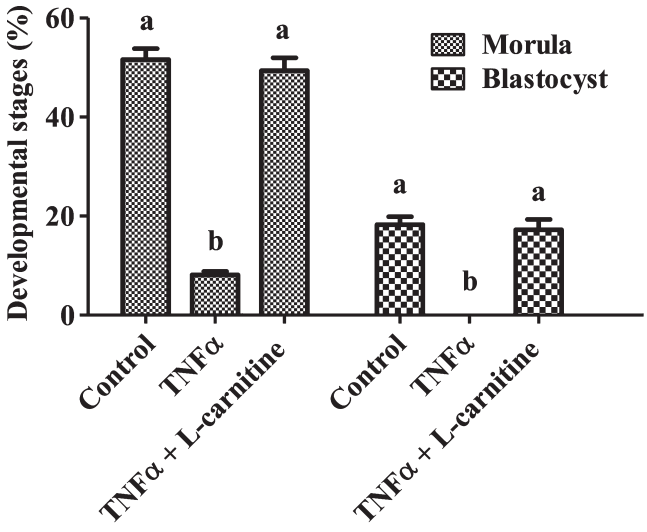

FIGURE 3 Effect of TNFα on embryo development supplemented in culture (post-fertilization) medium with and without L-carnitine. Percentage results are presented as mean + S.E.M. Different superscripts in the same group differ significantly at $p < .05$. Four experiments were performed

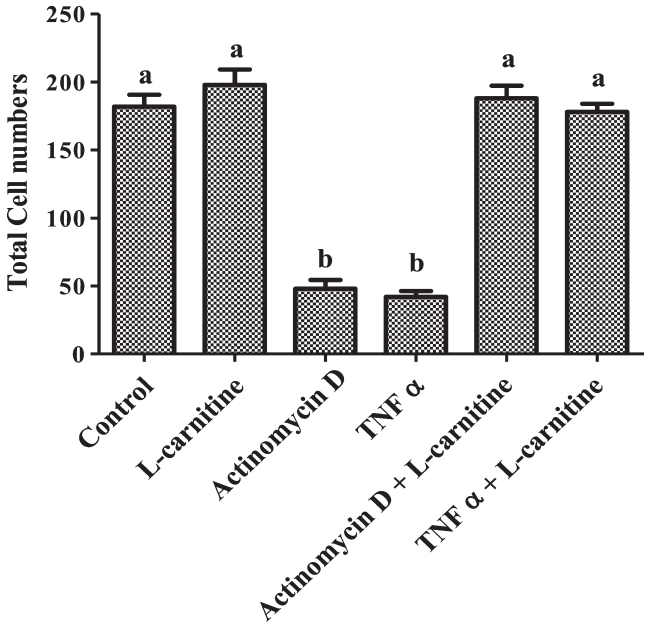

FIGURE 4 Effect of different culture conditions on total cell numbers of blastocyst and day 7 embryo. Results are presented as mean + S.E.M. Different superscripts differ significantly at $p < .05$. Three experiments were performed

Supplementation of L-carnitine to actinomycin D and TNFα-induced medium resulted similar cell numbers (188 ± 9.2, 178 ± 5.91) as that of control.

3.5 | Experiment 5: Effect of L-carnitine on expression profile of apoptotic genes

The relative expression of apoptotic genes in oocytes and different developmental stages of both control and L-carnitine supplemented

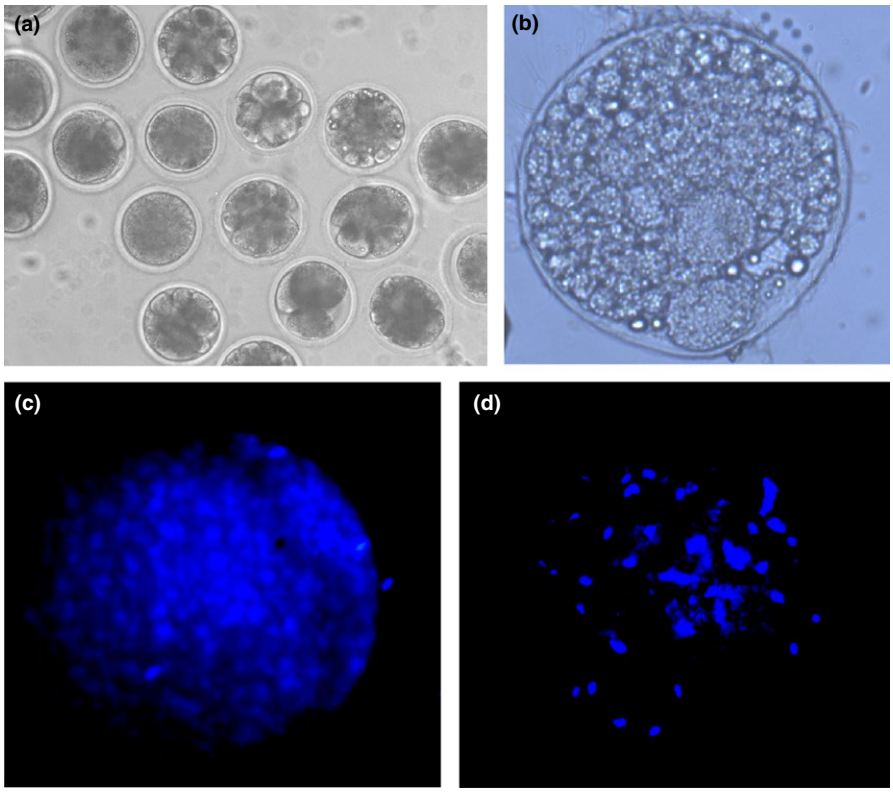

FIGURE 5 a: Different developmental stages of sheep embryos produced in vitro. b: Day 7 blastocyst fixed and coverslip mount. c: Hoechst-stained day 7 blastocyst cultured without apoptotic agent. d: Hoechst-stained day 7 embryo cultured with apoptotic agent.

groups in relation to expression level at immature oocytes is in Fig. 6. In this study, L-carnitine-mediated alteration in relative expression of apoptotic genes in oocytes and developmental stages has been compared in relation to particular stage of control group. L-carnitine treatment during maturation significantly ($p < .05$) upregulated the expression of Bcl2 in all stages of developing embryos except in morula where expression level was not affected. There was upregulated expression of Bax in matured oocytes and initial stages of developing embryos (upto four cells) followed by significantly ($p < .05$) downregulated expression from 8-cell stage to blastocyst in L-carnitine supplemented group as compared to control. L-carnitine supplementation upregulated the expression of Casp3 in matured oocytes and initial stages of developing embryos (upto 16 cells), but after that there was no significant difference in Casp3 expression. L-carnitine treatment during maturation significantly ($p < .05$) upregulated the expression of PCNA in all stages of developing embryos except in matured oocytes where expression level was not altered. GAPDH (housekeeping) gene was unaffected by L-carnitine treatment.

4 | DISCUSSION

Micromilieu surrounding preimplantation embryos is critical determinant of its developmental competence (Guerin, El Mouatassim, & Menezo, 2001). Although higher numbers of developmental stages of embryos were observed by the use of L-carnitine (10 mM) in maturation medium, maturation rate (MII stage) was not influenced. Embryos cultured with L-carnitine during post-fertilization period showed no significant change in cleavage percentage but showed significantly higher morula and blastocyst percentage. Improved numbers of developmental stages of embryos by L-carnitine supplementation during in vitro maturation and post-fertilization period might be due to its effects of providing more energy to the organelles of oocytes and embryos, increasing GSH and reducing intracellular ROS that protect cells from apoptosis (Mishra et al., 2016). Beneficial effects of L-carnitine supplementation on embryo development of this study support the finding of different studies (Takahashi et al., 2013; You et al., 2012). Increased lipid metabolism in oocytes and embryos cause more ROS production (Harvey, Kind, & Thompson, 2002). L-carnitine neutralizes the ROS-produced cellular organelles and protects cellular organelles from OS-induced damage or apoptosis including mitochondria the major source of ROS, proving L-carnitine as an antioxidant and antiapoptotic compound. The oocytes matured with L-carnitine showed significantly ($p < .05$) higher cleavage, morula and blastocysts percentage as compared to presumptive zygotes cultured with L-carnitine during post-fertilization period, which might be due to the ability of L-carnitine to reduce intracellular ROS from maturation stage than in the developmental stage. Therefore, this study concluded that it is better to use L-carnitine during maturation period than culturing embryos during post-fertilization period.

Antiapoptotic effect and proliferative effect of L-carnitine were further proved by culturing embryos (2–4 cells) with actinomycin D and oocytes and embryos with TNFα with or without L-carnitine, respectively. It was observed that embryos (2–4 cells) exposed to actinomycin D and TNFα showed developmental arrest and embryos

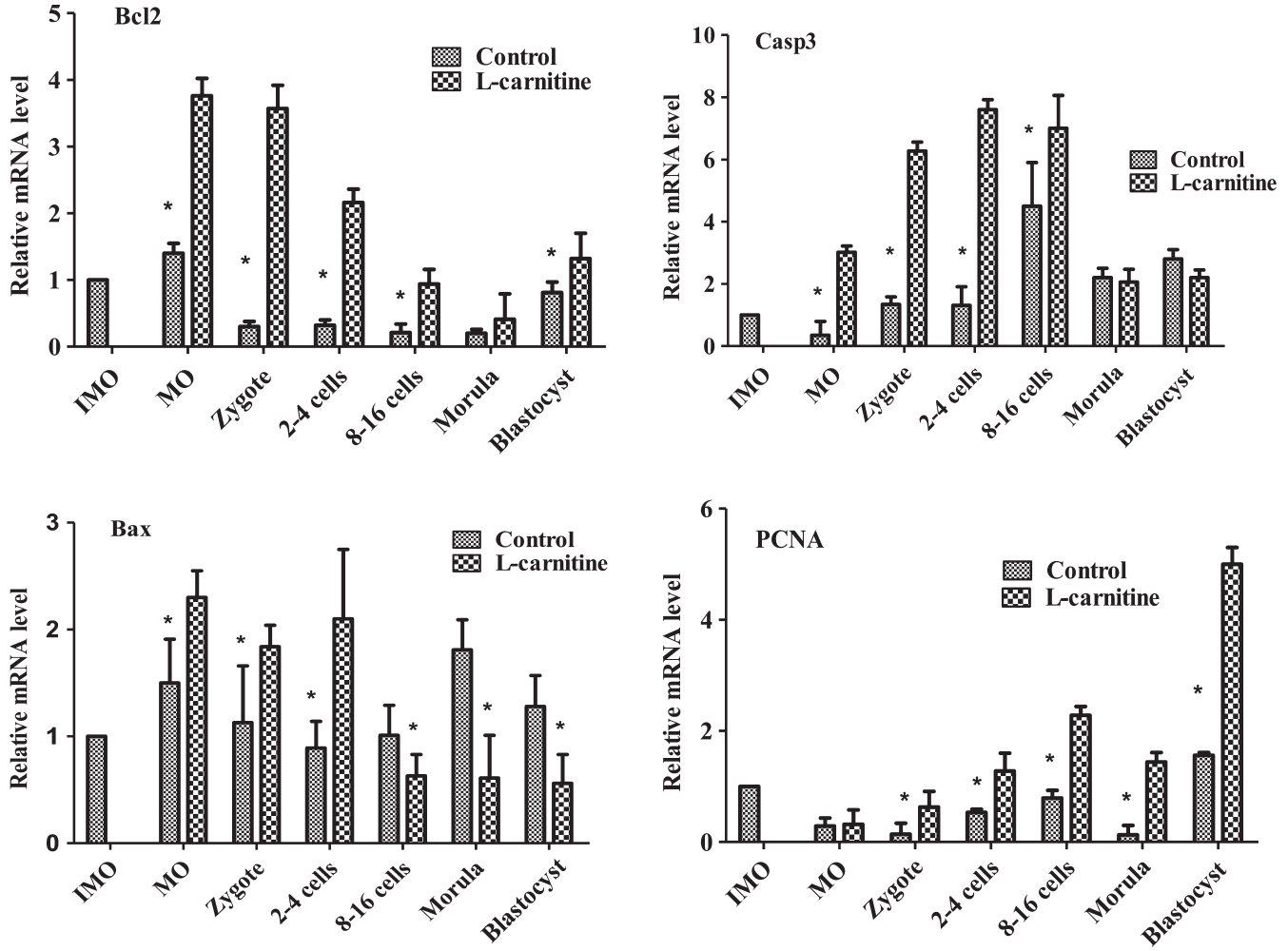

FIGURE 6 L-carnitine-mediated alteration in mRNA level of apoptotic genes in oocytes and embryos. *differs significantly at $p < .05$ in the same group, IMO: immature oocytes, MO: in vitro-matured oocytes

could not reach upto blastocyst and there was significant ($p < .05$) decrease in total cell numbers. When embryos (2–4 cells) exposed to actinomycin D or TNFα with L-carnitine showed further development to blastocysts with total cell numbers as control group proves L-carnitine as an antiapoptotic and proliferative compound. Similarly oocytes exposed to TNFα showed developmental arrest and embryos could not reach upto blastocyst but supplementation of L-carnitine improved the result as that of control. Reduction in apoptosis of embryos by supplementation of L-carnitine in culture medium of this study is in similar way with mouse study (Abdelrazik et al.,2009). Culture condition affects the gene expression level in oocytes and embryos (Mukherjee et al., 2014). The expression of many genes may be upregulated and downregulated by ROS. To the best of our knowledge, there is no such report available to find out the effect of L-carnitine on developmental potential and its effect on apoptotic genes expression in sheep embryos produced in vitro. L-carnitine supplementation during IVM upregulated the expression of Bcl2 in all stages of developing embryos except at morula. Since L-carnitine reduced ROS and increased GSH that protected oocytes and embryos from apoptosis, therefore expression level of Bcl2 was significantly ($p < .05$) upregulated by L-carnitine supplementation. Although there was upregulated expression of Bcl2 in morula of L-carnitine-treated group, it was non-significant as compared to control. In contrast, L-carnitine supplementation downregulated the expression of Bax in morula and blastocysts but upregulated in matured oocytes and initial stages of developing embryos (upto four cells). Downregulation of Bax in blastocysts of bovine embryo by L-carnitine has already been reported (Takahashi et al., 2013). The reason for upregulated expression of Bax in mature oocytes and initial stages of developing embryos in spite of L-carnitine's property as an antioxidant and antiapoptotic by reducing ROS and increasing GSH is not known, but it can be explained that in sheep, initial stages of developing embryos might be susceptible to self-induced apoptosis or culture-induced developmental arrest, showing upregulation of Bax gene. Although L-carnitine was able to reduce ROS and increase GSH in matured oocytes, there was upregulated expression of Bax in L-carnitine-treated oocytes. L-carnitine (10 mM) concentration used in the study might not be sufficient to neutralize the free radicals generated during maturation and subsequent initial developmental stages and that might be putting effect on upregulated expression of Bax. Culture-induced developmental arrest of sheep embryos is observed

at 8- to 16-cell stage (Gandolfi & Moor, 1987). At this stage, sheep embryos undergo transition from maternal to embryonic genome control (Telford, Watson, & Schultz, 1990). Perhaps due to L-carnitine supplementation, the culture-induced developmental arrest at 8- to 16-cell stage is improved, so the expression of Bax is downregulated at this stage and followed upto blastocyst. Therefore, it can be concluded that the mammalian embryos are highly sensitive to their environment around them which alters the gene expression level. It has already been proved before that in vitro culture increases the expression of Bax (Zhang et al., 2013).

Casp3 is an executor of apoptosis. L-carnitine supplementation upregulated the expression of Casp3 in matured oocytes and initial stages of developing embryos (upto 16 cells), but after that there was no significant difference in Casp3 expression in both the groups. In support to our finding, L-carnitine supplementation in culture medium during in vitro bovine embryo production has proved no difference in expression level of Casp3 at blastocyst stage (Takahashi et al., 2013). The only difference between our study and bovine study was the use of L-carnitine in maturation medium and culture medium, respectively. For gene expression study, the concentration of L-carnitine in our experiment was 10 mM and used in maturation medium, whereas the concentration of L-carnitine used in bovine study was of 3.03 mM in culture medium during post-fertilization period. mRNA expression of Casp3 cannot be used as a reliable detection method for apoptosis because "caspases" are secreted as inactive "procaspases," which are activated after further modification. Casp3 and Casp7 are executioner caspases which are activated only during the process of apoptosis by active precursor Casp8 and Casp9. So mRNA expression level of caspases reflects the amount of procaspases, but not the level of biologically active caspases (Huert et al., 2007). So it has been suggested that immunofluorescent detection of active caspase might be a better choice for apoptosis detection (Vandaele, Goossens, Peelman, & Soom, 2008). PCNA protein is one of the central molecule responsible for cell death or survival. If PCNA protein levels are high in cell in absence of P53, DNA replication occurs. On the other hand, if PCNA protein levels are high in presence of P53, DNA repair takes place. When not engaged in DNA replication, PCNA commits cell-to-cell cycle arrest and repairs DNA damage under the control of p53. When repair is not possible, PCNA drives cells into apoptosis (Paunesku et al., 2001). L-carnitine supplementation during maturation period in this study significantly ($p < .05$) upregulated the expression of PCNA in all stages of developing embryos supported to the finding of others, who have reported upregulated expression of PCNA in four-cell embryo due to L-carnitine supplementation in maturation medium (You et al., 2012). It can be explained from the result that L-carnitine treatment created beneficial microenvironment for nuclear programming and stimulated PCNA gene expression because of which there is upregulated expression of PCNA in L-carnitine-treated group. Expression of GAPDH (housekeeping gene) was not influenced by L-carnitine supplementation.

From the study, it has been concluded that L-carnitine supplementation during IVEP improved developmental potential of developing embryos and acted as an antiapoptotic and proliferative compound for embryo development. L-carnitine supplementation during maturation period improved significantly ($p < .05$) cleavage, morula and blastocysts percentage than supplementing L-carnitine during post-fertilization period. L-carnitine supplementation during IVM altered the expression of apoptotic genes in the developmental stages of embryo.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

AM contributed towards experimental design, embryo culture work and statistical analysis. IJR was involved in manuscript preparation and gene expression study. PSP was involved in embryo culture work. SM carried out the gene expression study.

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