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L-carnitine Mediated Reduction in Oxidative Stress and Alteration in Transcript Level of Antioxidant Enzymes in Sheep Embryos Produced *In Vitro*

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Contents
The objective of this study was to find out the effect of L-carnitine on oocyte maturation and subsequent embryo development, with L-carnitine-mediated alteration if any in transcript level of antioxidant enzymes (GPx, Cu/Zn-SOD (SOD1) and Mn-SOD (SOD2) in oocytes and developing sheep embryos produced *in vitro*. Different concentrations of L-carnitine (0 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM) were used in maturation medium. Oocytes matured with 10 mM L-carnitine showed significantly ($p < 0.05$) higher cleavage (66.80% vs 39.66, 41.76, 44.64, 64.31%), morula (48.50% vs 20.88, 26.01, 26.99, 44.72%) and blastocyst (33.22% vs 7.66, 9.19, 10.71, 28.57%) percentage as compared to lower concentrations (0 mM, 2.5 mM, 5 mM and 7.5 mM). Cleavage percentage between 10 mM and 7.5 mM L-carnitine were not significantly different. Maturation rate was not influenced by supplementation of any experimental concentration of L-carnitine. There was a significant ($p < 0.05$) decrease in intracellular ROS and increase in intracellular GSH in 10 mM L-carnitine-treated oocytes and embryos than control group. Antioxidant effect of L-carnitine was proved by culturing oocytes and embryos with H_2O_2 in the presence of L-carnitine which could be able to protect oocytes and embryos from H_2O_2-induced oxidative damage. L-carnitine supplementation significantly ($p < 0.05$) upregulated the expression of GPx and downregulated the expression of SOD2 genes, whereas the expression pattern of SOD1 and GAPDH (housekeeping gene) genes was unaffected in oocytes and embryos. It was concluded from the study that L-carnitine supplementation during *in vitro* maturation reduces oxidative stress-induced embryo toxicity by decreasing intracellular ROS and increasing intracellular GSH that in turn improved developmental potential of oocytes and embryos and alters transcript level of antioxidant enzymes.

Introduction

Biotechnologies have made great progress over decades. Numbers of reproductive technologies have been introduced to improve genetic progress of animals. Of all these technologies, *in vitro* embryo production (IVEP) is an important technique which has great potential for genetic improvement in animals. In spite of number of permutation and combinations in the media, embryos produced *in vitro* are not at par to *in vivo*-derived embryos because *in vitro* set-up can never mimic the exact physiological conditions of *in vivo* system (Agarwal et al. 2006). There are evidences showing differences in results between *in vivo*-derived and *in vitro*-produced embryos which involve morphological and molecular aspects that impair IVEP efficiency (Camargo et al. 2006). The micromilieu during *in vitro* culture of preimplantation embryos is important in terms of the developmental competence of the embryos. Proper selection of developmentally competent oocytes is crucial for successful IVEP. IVEP is highly affected by culture condition so better *in vitro* culture condition is required to produce quality embryos with repeatable results.

During embryo development, there is a production of unstable metabolites of oxygen called reactive oxygen species (ROS) from gametes, embryos and their surroundings. Like other living cells, the embryos and oocytes are major sources of ROS because they use oxygen to produce energy through mitochondrial oxidative phosphorylation (Guerin et al. 2001). Imbalance between ROS and their normal scavenger antioxidants results oxidative stress (OS) that adversely affects embryo development by structural and functional alteration resulting suboptimal outcome for IVEP set-up. Apoptosis of embryos is strongly correlated with OS that results in fragmented embryos with altered developmental gene expression and has limited potential to implant, hence results in poor fertility (Elamaran et al. 2012). Several pregnancy-related disorders, defective embryo development, pregnancy loss and infertility attributing to cell membrane damage, DNA damage and modulation of gene expression are OS induced (Aruoma et al. 2006). To ameliorate the state of OS in micromilieu, a number of free radical scavengers have been used in different studies for better embryo development (Mukherjee et al. 2014). Culture conditions affect transcript expression in oocytes and preimplantation embryos (Mishra et al. 2010). To circumvent the suboptimal outcome for IVEP set-up, attempts have been made still to develop media composition which take account of the maturation that attributes improving embryo development. The expression of many transcripts in oocytes and preimplantation embryos may be upregulated and downregulated by ROS that even affect the embryo viability and offspring survivability (Wrenzycki et al. 2007). *In vitro* maturation (IVM) medium influences the relative abundance of certain oocyte transcripts that relate to differences in developmental competence (Watson et al. 2000). In the cell antioxidant system, antioxidant enzymes remove various ROS produced during embryo development. Of these enzymes, superoxide dismutases (SODs) are the initial enzymes, to allow the conversion of superoxide (O_2^-) anion to H_2O_2 and oxygen that in turn is removed by catalase or glutathione peroxidase (GPx) (Maier and Chan 2002). SODs are characterized by their metal

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requirements and their subcellular localization. The copper/zinc-containing SOD (SOD1) is found in the cytoplasm, and the manganese-containing SOD (SOD2) is mitochondrial. Besides SODs, GPx also plays an important role in antioxidant system and catalyses the reduction of H_2O_2 to the corresponding alcohols at the expense of the reducing equivalent glutathione. Glutathione peroxidases depend on the availability of reduced glutathione (GSH) for their enzymatic activity. Glutathione (GSH) is the major cellular non-protein sulfhydryl compound and plays a major role in regulating intracellular ROS concentrations directly as a free radical scavenger and indirectly as a substrate with NADPH for detoxifying ROS. Expression pattern of antioxidant enzyme genes is reported in oocytes and embryos of different species such as bovine (Takahashi et al. 2013), human and mouse (El Mouatassim et al. 1999), but no literature is available on the expression pattern of these genes in sheep oocytes and developing embryos produced *in vitro*, although qualitative expression of antioxidant enzymes in *in vivo* sheep oocytes is reported (Livingston et al. 2009). The antioxidant enzyme genes are modulated by oxidative stress (Correa et al. 2008).

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. It is mainly synthesized from amino acids lysine and methionine in liver. It is required to transport fatty acids from cytosol to mitochondria during breakdown of lipids (fats) to generate metabolic energy. Carnitine acts as an antioxidant that neutralizes the free radicals especially superoxide anion and protects cell against oxidative damage-induced apoptosis (Ye et al. 2010). Effect of L-carnitine on *in vitro* bovine embryos (Takahashi et al. 2013), pig embryos (You et al. 2012) and mouse embryos (Abdelrazik et al. 2009) has already been reported, but its effect on sheep blastocyst development rate and its effect on mitochondrial DNA copy number have been reported recently (Reader et al. 2015). There is no report available to find out L-carnitine-mediated alteration in expression of antioxidant enzyme genes in sheep oocytes and embryos. Therefore, this experiment was designed in sheep model with the objective to find out the effect of L-carnitine supplementation on *in vitro* oocyte maturation and subsequent embryo development with L-carnitine-mediated reduction in oxidative stress and alteration any in transcript level of antioxidant enzymes [glutathione peroxidase (GPx), Cu/Zn-super oxide dismutase (SOD1) and Mn-super oxide dismutase (SOD2)].

Materials and Methods

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., Kolkota, India. Chemicals and kits required for gene expression study were from Invitrogen, Life Technologies, Carlsbad, CA, USA. Primers for gene expression study were procured from Sigma-Aldrich Chemical.

In vitro embryo production

Oocyte collection and IVM

Sheep ovaries were collected from local slaughter house in normal saline solution (NSS) fortified with antibiotics and carried to the laboratory within 3 h at 35–37°C. Ovaries were washed in NSS and rinsed in 70% ethyl alcohol for a few minutes to eliminate surface organisms. Oocytes were aspirated from follicles (2–6 mm) with the help of 20-gauge needle attached to 5-ml syringe containing oocyte collection medium (OCM) (TCM-199 + BSA (3 mg/ml) + 5% FBS + heparin (2 IU/ml). Aspirated oocytes (excellent and good quality) were selected for IVM. Cumulus oocyte complexes (COCs) (15–20 numbers) were matured in 35-mm Petri dish and incubated in CO_2 incubator at 5% CO_2, 38.5°C and 95% RH 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) with L-carnitine (0 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM) under paraffin oil for 27 h. Maturation rate was assessed based on the degree of cumulus expansion and extrusion of the first polar body (oocytes at MII stage) by aceto-orcein staining method. From the result of different concentration of L-carnitine, finally 10 mM L-carnitine was used for subsequent experiments.

In vitro fertilization (IVF)

In vitro fertilization was performed by collecting fresh semen from the ram with the help of electro-ejaculator. Semen after collection was washed twice with washing medium [Fert-TALP + heparin (10 μg/ml) + pyruvate (1 mM)] by centrifuging at 400 x ***g*** for 5 min. After washing, supernatant was removed, pellet was reconstituted in fertilization medium [Fert-TALP + fatty acid free BSA (4 mg/ml) + heparin (10 μg/ml) + pyruvate (1 mM) + BME (100×) (1%) + MEM (50×) (1%)], and final sperm concentration was adjusted to 2–3× 10^6 sperms/ml which was assessed through Neubauer's chamber. The sperm suspension after process was kept in 5% CO_2, 38.5°C and 95% RH till matured oocytes were washed 4–5 times in fertilization medium. Finally, *in vitro* matured oocytes (15–20 numbers) were inseminated with 100 μl of processed spermatozoa, and fertilization was carried out by co-incubation of sperm and oocytes for 18 h in fertilization medium in the same temperature and gaseous condition described for maturation.

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In vitro culture (IVC)

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 (100×) (1%) + MEM (50×) (1%)] in the same temperature and gaseous condition described for maturation and fertilization to get embryos of different developmental stages from two cells to blastocysts stage. Cleavage rates were recorded on day 2 (48 hpi) 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 IVC medium.

Intracellular ROS and GSH levels in oocytes and embryos

Intracellular ROS levels in oocytes and embryos were quantified using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) and detected as green fluorescence at excitation wave length of 495 nm and emission wavelength of 520 nm. ROS level was quantified by measuring 2′,7′-dichlorofluorescein (DCF) fluorescence generated from the reaction of the 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) with intracellular H_2O_2 (Nasr-Esfahani et al. 1990). GSH levels was quantified by using 4-chloromethy L-6.8-difluoro-7-hydroxycoumarin (CMF2HC) and detected as blue fluorescence at excitation wave length of 350 nm and emission wavelength of 450 nm as described by You et al. (2012). Briefly, DCHFDA (Sigma-Aldrich Chemical) and CMF2HC (Sigma-Aldrich Chemical) stock was prepared by dissolving with dimethyl sulfoxide (DMSO) at 1 mM concentration. Further stock solution was diluted in phosphate-buffered saline (PBS) to a working concentration of 10 μM. Oocytes and embryos were washed twice in PBS + polyvinyl pyrrolidone (PVP) (0.5%) (wt/vol) and fixed with 4% paraformaldehyde (PFA) and then placed in 50 μl of 10 μM DCHFDA and 10 μM of CMF2HC for 15 min at 5% CO_2, 38.5°C and 95% RH to detect ROS and GSH, respectively. Finally, the oocytes and embryos were washed three times by PBS + PVP (0.5%), carefully mounted on glass slide and covered with coverslip. The fluorescence intensity of oocytes and embryos in each group was observed under an epifluorescence microscope (Euromex, Arnhem, Holland) equipped with a digital camera. Fluorescence intensities of oocytes and embryos were analysed by grey pixel intensity using ImageJ software (NIH, USA) normalizing untreated control oocytes and embryos as 1.

Antioxidant effect of L-carnitine

To proof the antioxidant effect of L-carnitine, first oocytes were matured with different concentrations (0, 0.5, 1, 2.5, 5, 10, 20 μM) of hydrogen peroxide (H_2O_2) an oxidant supplemented to maturation medium to find out the concentration of H_2O_2 which arrests completely the developmental potential of oocytes to embryos after fertilization. From these concentrations of H_2O_2, it was observed that at concentration of 10 μM of H_2O_2 in maturation medium, there was significant ($p < 0.05$) decrease in cleavage and further development, whereas at 20 μM of H_2O_2 there was no cleavage at all. Therefore, to assess the antioxidant effect of L-carnitine, there were two studies conducted: in first study, oocytes were matured (27 h) with 20 μM H_2O_2 with and without 10 mM L-carnitine, and in second study, embryos (2–4 cells) (obtained from oocytes matured without H_2O_2 and L-carnitine) were cultured with 20 μM H_2O_2 for 48 h post-fertilization followed by replacing 50% fresh culture medium without H_2O_2 with and without 10 mM L-carnitine to find out developmental potential of embryos.

Commet assay to assess integrity of oocytes DNA

Integrity of oocytes DNA due to H_2O_2-induced oxidative damage was assessed by commet assay as described by Men et al. (2003) with modification for sheep oocytes. There were three groups of oocytes to be assessed such as (i) oocytes cultured neither with H_2O_2 (20 μM) nor L-carnitine (10 mM) (ii) oocytes cultured with H_2O_2 (20 μM) and (iii) oocytes cultured with both H_2O_2 (20 μM) and L-carnitine (10 mM). Zona pellucidae of the oocytes were removed by 1% pronase digestion. Microscopic slides pre-coated with 1% L-poly lysin were coated with 1% normal melting agarose and dried at room temperature. A drop of 10 μl 1% low melting agarose was quickly mixed with the oocytes, put onto microscope slide pre-coated with 1% agarose and quickly placed on ice to solidify the agarose. The oocytes were then lysed by immersing the slides into a pre-cooled (4°C) lysis buffer (1% sodium sarcosinate, 2.5 mM NaCl, 100 mM Na_2-EDTA, 1% Triton X-100 in 10 mM Tris buffer, pH 10) for 1 h. After 1 h, the slides were removed from lysing buffer and placed in a horizontal gel electrophoresis unit filled with pre-chilled (4°C) alkali electrophoresis buffer (1 mM Na_2-EDTA, 300 mM NaOH, pH > 13) and electrophoresis was conducted for 30 min at 25V, 300 mA. After electrophoresis, slides were neutralized by in 0.4 M Tris-HCl (pH 7.5) for 5 min at room temperature. The slides were stained with ethidium bromide (20 μg/ml) for 10 min. The integrity of oocyte DNA was examined under fluorescent microscopy (Euromax), and images were captured. A filter combination of 510- to 560-nm excitation filter and 590-nm barrier filter was used for the study. The integrity of oocyte DNA was determined by the migration of DNA. If no DNA migrated outside the oocyte, then DNA was considered as intact but DNA was considered as damaged if DNA was migrated outside the oocyte.

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Expression profile of antioxidant enzyme genes

The transcript abundance of antioxidant enzyme genes [glutathione peroxidase (GPx), Cu/Zn superoxide dismutase (SOD1) and Mn superoxide dismutase (SOD2)] was analysed by rea L-time quantitative PCR (qPCR). For gene expression study, oocytes were matured with and without 10 mM L-carnitine for treatment and control groups, respectively, but during post-fertilization period, presumptive zygotes were cultured without L-carnitine. The gene-specific primers used in this study were designed from NCBI, PRIMER BLAST (www.ncbi.nlm.nih.gov/BLAST) (Table 1). The specificity of the primers was tested using BLAST analysis against the genomic NCBI database.

RNA isolation

Immature oocytes, *in vitro* matured 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 5–6 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) method as per manufacturer's guide line 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. 50 µl 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. 2 µl (20 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 7500 × ***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 by using TURBO DNA-free™ kit (Ambion, Life Technologies, Carlsbad, CA, USA).

Table 1. Primers used for gene expression study

Sl No	Genes	Primer sequence	Product size (bp)
1	GAPDH	F-ATGGGCGTGAACCACGAGAA R-ATGGCGTGGACAGTGGTCAT	146
6	GPx	F-CGTGCAACCAGTTTGGGCAT R-GATGCGCCTTCTCGCCATTC	141
7	SOD1	F-CCACTTCGAGGCAAAGGGAGA R-CCTTTGGCCCACCGTGTTTT	167
8	SOD2	F-CCGTCAGCCTTACACCAAGT R-CAAGCCACGCTCAGAAACAC	112

RNA integrity and cDNA synthesis

The integrity of total RNA was checked on 1% 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:OD 280 values which was more than 1.8. 150 ng RNA was used in the Reverse Transcription (RT) as the template for first-strand synthesis by using SuperScript III First-Strand Synthesis kit (Invitrogen, Life Technologies) as per manufacturer's guide line using oligo dT (50 µm), dNTP (10 mM), RT buffer, mgCl2 (2.5 mM), reverse transcriptase (RT) (200 U), RNAse inhibitor (40 U), DTT (0.1 M) in volume of 20 µl. The synthesized cDNA was stored in −20°C until used for rea L-time quantitative PCR.

Real time quantitative PCR

The expression levels of specific genes in oocytes and embryos were quantified by qPCR using step one plus qPCR system (Applied Biosystem, Carlsbad, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene in this study. The qPCR reactions were performed using Maxima SYBR Green/Rox qPCR master mix (2×) (Fermentas, Waltham, MA, USA). Each run was performed in duplicate in a 10 µl reaction containing 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 was 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. 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. $\Delta C_t = C_t$ (target gene)-C_t (housekeeping gene) and $\Delta\Delta C_t = \Delta C_t$ (target gene sample) − ΔC_t (calibrator). qPCR was conducted for three times for three different sets of embryos.

Confirmation of qPCR amplicons

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

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Experimental design

Experiment 1

Immature oocytes were randomly divided into five groups and matured *in vitro* with different concentrations of L-carnitine (0 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM) supplemented to maturation medium (described in method). The objective of this experiment was to find out the effect of L-carnitine on maturation and subsequent embryo development as well as to select a suitable concentration of L-carnitine for subsequent experiment.

Experiment 2

From the result of experiment 1, finally 10 mM L-carnitine was used in subsequent experiments. Matured oocytes and embryos produced from 10 mM L-carnitine were taken to measure the intracellular ROS and GSH levels in oocytes and embryos. The objective of this experiment was to find out the effect of L-carnitine on intracellular ROS and GSH levels.

Experiment 3

Immature oocytes were matured *in vitro* with different concentrations (0, 0.5, 1, 2.5, 5, 10, 20 μM) of H_2O_2 supplemented to maturation medium. The objective of this experiment was to find out the concentration of H_2O_2 which arrests completely the developmental potential of oocytes to embryos after fertilization.

Experiment 4

From the result of experiment 3, 20 μM of H_2O_2 was used for subsequent study. Oocytes were randomly divided into three groups and matured for 27 h supplemented with 20 μM H_2O_2 with and without 10 mM L-carnitine. Control group oocytes were matured without H_2O_2 and L-carnitine. Group 1 oocytes were matured with H_2O_2, and Group 2 oocytes were matured with H_2O_2 and L-carnitine. Randomly oocytes were taken from the three groups, and commet assay was conducted. Objective of this experiment was to assess the antioxidant effect of L-carnitine on oocyte maturation and subsequent development.

Experiment 5

Embryos (2–4 cells) were cultured with 20 μM H_2O_2 with and without 10 mM L-carnitine for 48 h followed by replacing 50% fresh culture medium without H_2O_2 up to blastocyst development. Control group embryos were cultured without H_2O_2 and L-carnitine. Group 1 embryos were cultured with H_2O_2, and Group 2 embryos were cultured with H_2O_2 and L-carnitine. Objective of this experiment was to assess the antioxidant effect of L-carnitine on embryo development.

Experiment 6

Immature oocytes, matured oocytes and embryos produced from 10 mM L-carnitine in maturation medium were taken for RNA isolation and subsequent study of antioxidant enzyme genes expression. Objective of this experiment was to find out the L-carnitine-mediated alterations in antioxidant enzymes gene expression in developmental stages of embryos.

Statistical analysis

The results are expressed in mean ± SEM. Statistical analysis was carried out using GraphPad Prism 5, San Diego, CA, USA. The mean between groups for embryonic development, ROS and GSH level and gene expression level was compared by analysis of variance (ANOVA). Embryo development data have been presented in the percentage in relation with total oocytes cultured. Percentage values were arcsine-transformed before analysis. $p < 0.05$ values were considered as significant.

Results

Experiment 1

Effect of L-carnitine supplementation on IVM and subsequent embryo development

Supplementation of different concentrations (0 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM) of L-carnitine in the maturation medium did not influence the maturation rate (80.20–83.83%) (Fig. 1). The result of *in vitro* embryo development in the presence and absence of L-carnitine in maturation medium is detailed in Fig. 2. 10 mM L-carnitine during IVM resulted significantly ($p < 0.05$) higher percentage of cleavage (66.80% vs 39.66, 41.76, 44.64, 64.31%) followed by morula (48.50% vs 20.88, 26.01, 26.99, 44.72%) and blastocyst (33.22% vs 7.66, 9.19, 10.71, 28.57%) as compared other groups of lower concentration (0 mM, 2.5 mM, 5 mM and 7.5 mM). Cleavage percentage between 10 mM and 7.5 mM L-carnitine groups were not significantly different. For subsequent studies, 10 mM L-carnitine was used in maturation medium. In this study, cleavage was observed on day 2 (48 hpi) and blastocyst was observed on day 7 (day 0 = day of IVF).

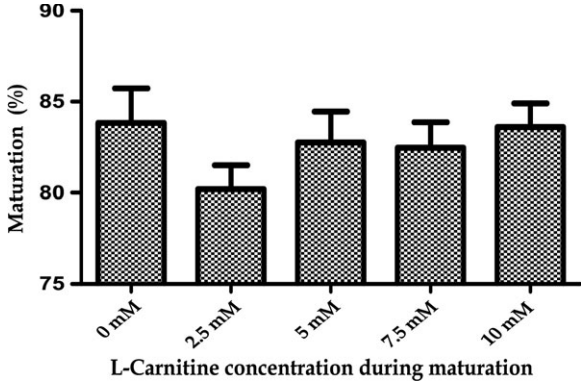

Fig. 1. Effect of L-carnitine on *in vitro* maturation of sheep oocytes ($p < 0.05$)

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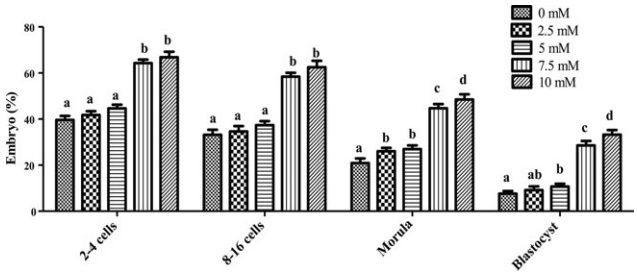

Fig. 2. Effect of L-carnitine during *in vitro* maturation on embryo development. Percentage results are presented as mean + SEM ($p < 0.05$). Different superscripts in the same group differ significantly at $p < 0.05$. Six experiments were performed for each group

Experiment 2

Effect of L-carnitine on intracellular ROS and GSH levels in oocytes and embryos

L-carnitine-treated oocytes with subsequent embryos showed significantly ($p < 0.05$) lower intensities for ROS indicating decreased intracellular ROS, and significantly ($p < 0.05$), higher intensities for GSH indicating increased intracellular GSH as compare to oocytes and embryos not treated with L-carnitine (Fig. 3A,B).

Experiment 3

Effect of H_2O_2 exposure during oocyte maturation for subsequent embryo development

The result (Fig. 4) of the experiment showed that up to 2.5 μM H_2O_2 in maturation medium, there was no significant ($p < 0.05$) change in cleavage (37.56–39.66%) percentage followed by further development to morula (16.66–20.88%), but the percentage of blastocysts (5.13–7.66%) was significantly decreased at 2.5 μM H_2O_2. At 5 μM H_2O_2, there was significant ($p < 0.05$) decrease in cleavage (29.43%), morula (6.93%) and blastocyst (2.15%) percentage. At 10 μM H_2O_2, there was further significant ($p < 0.05$) decrease in cleavage (18.12%) and morula (2.13%) but blastocyst was nil, whereas at 20 μM of H_2O_2 there was no cleavage at all.

Experiment 4

Antioxidant effect of L-carnitine on oocyte maturation and subsequent development

To assess the antioxidant effect of L-carnitine on oocyte maturation and subsequent development, oocytes were matured with 20 μM H_2O_2 with and without 10 mM L-carnitine. There was no cleavage at concentration of 20 μM H_2O_2 but when 10 mM

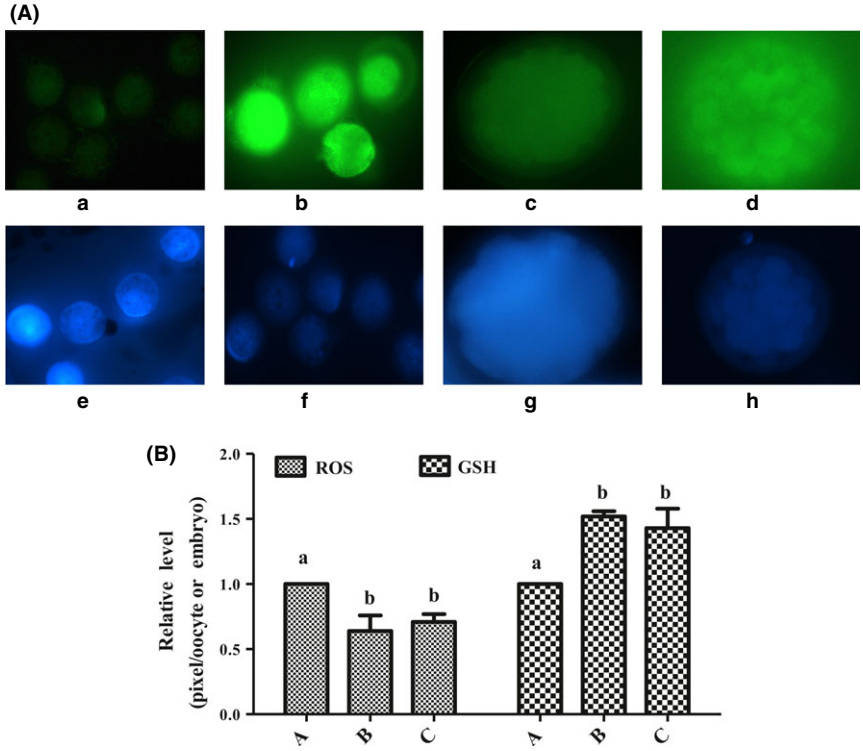

Fig. 3. (A) Fluorescent photograph of *in vitro* matured sheep oocytes and embryo for ROS and GSH level. (a) ROS in oocyte mature with 10 mM L-carnitine. (b) ROS in oocyte mature without 10 mM L-carnitine. (c) ROS in embryo developed from oocyte mature with 10 mM L-carnitine. (d) ROS in embryo developed from oocyte mature without 10 mM L-carnitine. (e) GSH in oocyte mature with 10 mM L-carnitine. (f) GSH in oocyte mature without 10 mM L-carnitine. (g) GSH in embryo developed from oocyte mature with 10 mM L-carnitine. (h) GSH in embryo developed from mature without 10 mM L-carnitine. (B) Effect of L-carnitine on intracellular ROS and GSH level in oocytes and embryos. A: Control oocytes/embryos; B: L-carnitine-treated oocytes; C: Embryos from L-carnitine-treated oocytes. Different superscripts in the same group differ significantly at $p < 0.05$. Three experiments were performed for each group

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L-carnitine was supplemented with 20 μM H_2O_2, the cleavage percentage (38.44%) was significantly ($p < 0.05$) increased followed by morula (19.31%) and blastocysts (6.58%) percentage and showed similar result as that of control. The integrity of oocyte DNA was determined by the migration of DNA and assessed by commet assay (Fig. 5). It has been observed that DNA of oocytes exposed to H_2O_2 migrated outside the oocyte, so DNA was considered as damaged, whereas oocytes exposed to H_2O_2 with L-carnitine showed no DNA migrating outside the oocyte so DNA was considered as intact.

Experiment 5

Antioxidant effect of L-carnitine on embryo development

To assess the antioxidant effect of L-carnitine on embryo development, embryos (2–4 cells) were cultured with 20 μM H_2O_2 with and without 10 mM L-carnitine (Fig. 6). There was significant ($p < 0.05$) decrease in morula (52.38% vs 15.92%) and blastocysts (18.69% vs 3.24%) percentage when embryos (2–4 cells) cultured with 20 μM H_2O_2 as compare to control. Supplementation of 10 mM L-carnitine to 20 μM H_2O_2-induced culture medium, there was significant ($p < 0.05$) increase in morula (47.23% vs 15.92%) and blastocysts (16.19% vs 3.24%) and showed similar result as that of control.

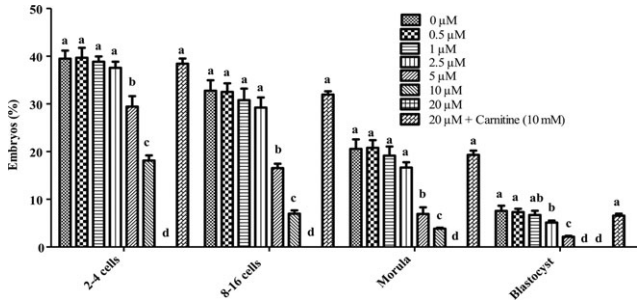

Fig. 4. Embryo development in the presence and absence of H_2O_2 during maturation with and without L-carnitine (10 mM). Percentage results are presented as mean + SEM. Different superscripts in the same group differ significantly at $p < 0.05$. Six experiments were performed for each group

Experiment 6

Effect of L-carnitine on expression profile of antioxidant enzyme genes

The relative expression of antioxidant enzyme genes in oocytes and different developmental stages of both control and L-carnitine supplemented groups in relation to expression level of gene in immature oocytes is in Fig. 7. GPx, SOD1 and SOD2 were expressed in all stages of developing embryos including immature and *in vitro* matured oocytes. Significantly ($p < 0.05$), maximum upregulated expression of GPx was in *in vitro* matured oocytes, and downregulated expression was at zygote. SOD1 was expressed significantly highest at 8–16 cells and rest of the stages were non-significantly different from each other, whereas expression of SOD2 was significantly least among all the antioxidant enzymes studied in this experiment. Highest upregulated expression of SOD2 ($p < 0.05$) was observed in immature oocytes, and downregulation was at 8–16 cells. L-carnitine-mediated alteration in transcript level of antioxidant enzymes in sheep oocytes and developing stages in relation to particular stage of control group is in Fig. 8. L-carnitine treatment during maturation significantly ($p < 0.05$) upregulated the expression of GPx and downregulated the expression of SOD2 gene in oocytes and embryos. The expression pattern of SOD1 and GAPDH (housekeeping) genes was unaffected by L-carnitine treatment.

Discussion

The micromilieu during *in vitro* culture of preimplantation embryos is a critical determinant to the developmental competence of the embryos. During embryo development, ROS generated from embryos and their surrounding cause defective embryo development (Guerin et al. 2001). The present study was undertaken to ameliorate OS in IVMFC protocol by L-carnitine supplementation for better embryo development. Subsequent studies were conducted to find out the antioxidant effect of L-carnitine followed by intracellular GSH and ROS level in oocytes and embryos due to L-carnitine supplementation. L-carnitine-mediated alterations any in transcript level of antioxidant enzymes (GPx, SOD1 and SOD2) were also analysed. To our knowledge, this is the first report describing L-carnitine-mediated alteration any in transcript level of antioxidant

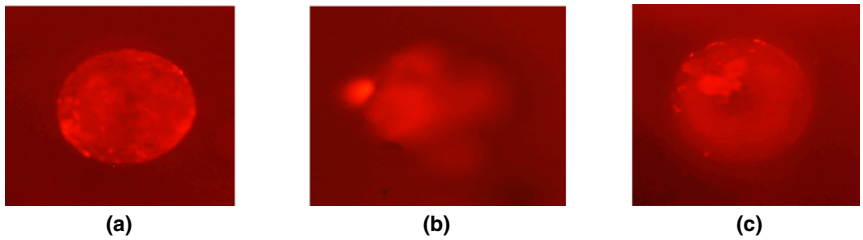

Fig. 5. Commet assay to check integrity of oocytes DNA due to H_2O_2 exposure. (a) Intact oocyte (Exposed to neither L-carnitine nor H_2O_2) (intact DNA). (b) Damaged oocyte (Exposed to H_2O_2) (Fragmented DNA). (c) Intact oocyte (Exposed to carnitine + H_2O_2) (intact DNA)

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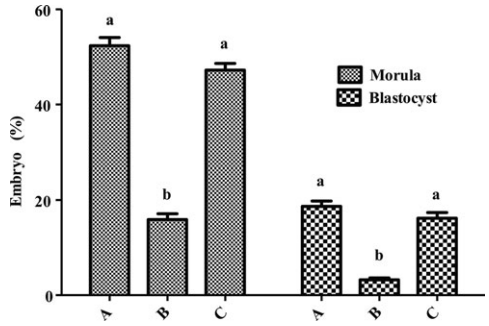

Fig. 6. Embryo development in the presence and absence of H_2O_2 during post-fertilization period with and without L-carnitine (10 mM). A: Embryo cultured with neither H_2O_2 nor L-carnitine; B: Embryo cultured with H_2O_2 (20 μM); C: Embryo cultured with H_2O_2 (20 μM) + L-carnitine (10 mM). Percentage results are presented as mean + SEM. Different superscripts in the same group differ significantly at $p < 0.05$. Six experiments were performed for each group

enzymes in sheep oocytes and embryos produced *in vitro*.

About 2.5 mM and 5 mM of L-carnitine supplementation during IVM showed no significant change in cleavage percentage, but morula and blastocyst percentage were significantly more as compared to control (0 mM) group. L-carnitine (7.5 mM and 10 mM) supplementation during IVM improved the developmental potential of sheep embryos from zygote to blastocyst stage, and a significantly higher number of developmental stages (morula and blastocysts) were observed at 10 mM L-carnitine as compared to 7.5 mM L-carnitine although cleavage percentage was non-significantly more at 10 mM L-carnitine. For the present study, 10 mM concentration of L-carnitine was chosen to get higher numbers of developmental stages of embryos for further gene study. Highest numbers of developmental stages of embryos were observed at 10 mM concentration of L-carnitine in maturation medium but maturation rate (MII stage) (80.20% to 83.83%) was not influenced by supplementation of any experimental concentration of L-carnitine as compared to control. In contrast to this finding, some reports suggested that L-carnitine supplementation improved nuclear maturation (Somfai et al. 2011; Wu et al. 2011). No significant difference in maturation percentage but significant increase in cleavage percentage at 7.5 mM and 10 mM L-carnitine as compared to other lower concentrations of this study may be suggestive of that L-carnitine has prominent effect on cytoplasmic maturation than nuclear maturation. Supplementation of 10 mM L-carnitine in this study significantly increased GSH level and decreased ROS level in oocytes and embryos that triggers the embryonic development, which supports the finding of studies in swine and bovine (You et al. 2012; Takahashi et al. 2013). Improved embryo development by L-carnitine supplementation during *in vitro* culture might be due to its effect of providing more energy through β oxidation to the organelles of oocytes and embryos, increasing GSH and reducing intracellular

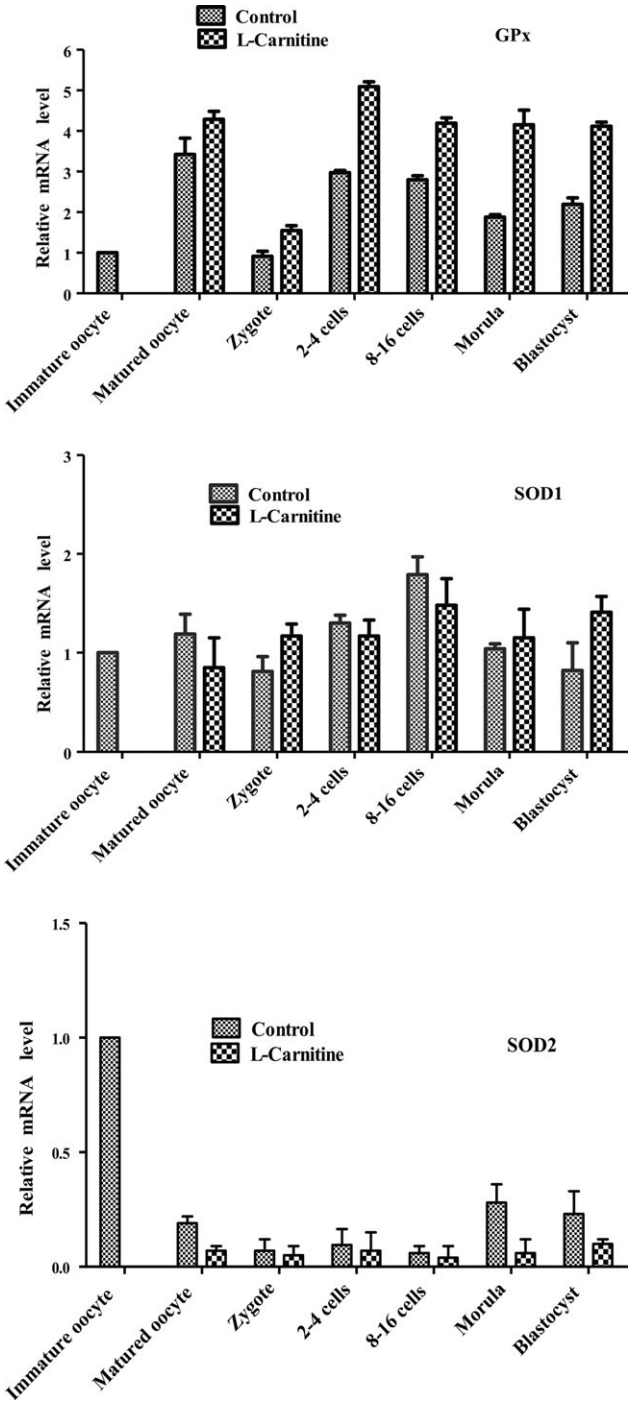

Fig. 7. Relative mRNA level of antioxidant enzymes in oocytes and developing embryos

ROS that protect cells from apoptosis. Increased lipid metabolism in oocytes and embryos causes more ROS production (Harvey et al. 2002), but the ROS produced is being neutralized by free radical scavenging effect of L-carnitine that protects cellular organelles including mitochondria the major source of ROS proving

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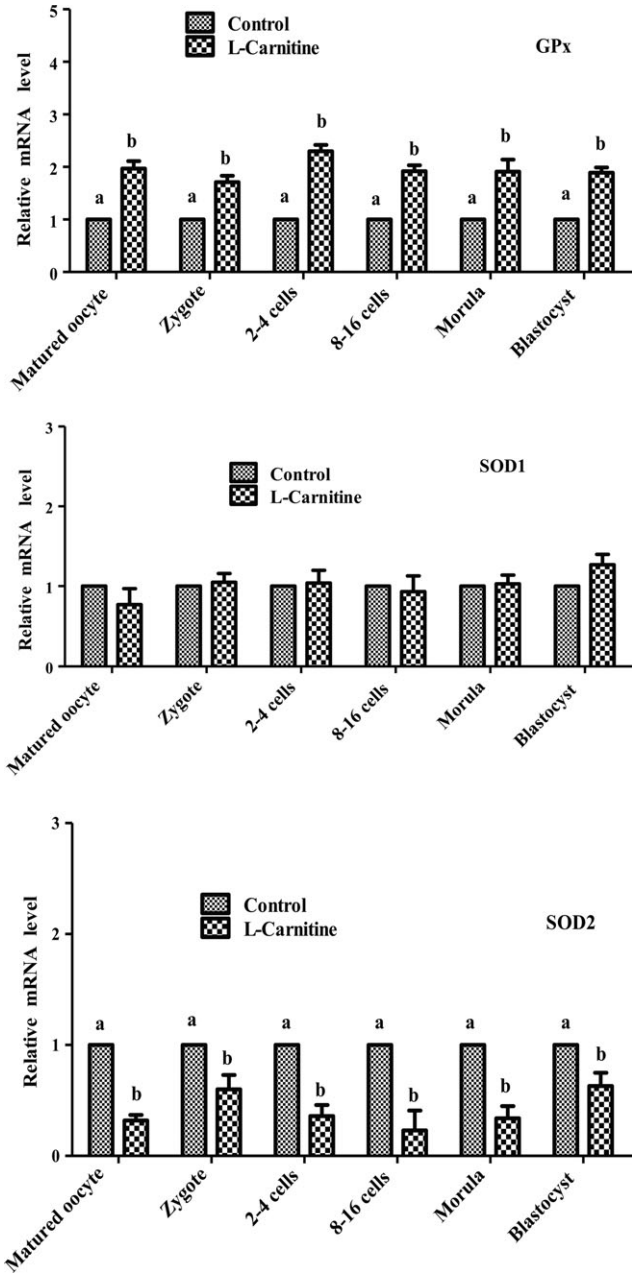

Fig. 8. L-carnitine-mediated alteration in mRNA level of antioxidant enzymes in oocytes and embryos. Different superscripts in the same group differ significantly at $p < 0.05$

L-carnitine as an antioxidant. Therefore, this study suggested that it is better to use L-carnitine during maturation of oocytes to get subsequent better number of developmental stages.

There were no significant changes in cleavage and morula up to 2.5 μM H_2O_2 in maturation medium but significant decrease in blastocysts at 2.5 μM as compared to other lower concentrations than 2.5 μM H_2O_2, whereas from 5 to 20 μM H_2O_2 there was significant decrease in developmental stages. No significant change in developmental stages except blastocyst up to 2.5 μM H_2O_2 might be due to the fact that these concentrations of H_2O_2 supplementation might not have crossed threshold oxidative stress-tolerant capacity of embryos to hinder their development. Normal H_2O_2 concentration in our culture condition throughout developmental stages of *in vitro* sheep embryo was <5 μM (unpublished data). Therefore, above 5 μM of H_2O_2 in maturation medium significantly reduces the developmental stages. Oxidant effect of H_2O_2 has already proved by culturing mouse embryos with 500 μM H_2O_2 (Abdelrazik et al. 2009) and bovine embryos with 100 μM H_2O_2 (Bain et al. 2011). In these studies, exposure timing of oocytes and embryos to H_2O_2 was different from our study. In mouse study, embryos were exposed to H_2O_2 for 72 h, and in bovine study, oocytes and embryos were exposed for 1 h. In our study, oocytes were exposed to H_2O_2 throughout the maturation (27 h) and embryos (2–4 cells) were exposed for first 48 h followed by change in fresh culture medium without H_2O_2 in every 48 h which implied that embryos were not completely devoid of H_2O_2 throughout post-fertilization period. 20 μM H_2O_2 in combination with 10 mM L-carnitine during maturation showed no significant difference in cleavage followed by morula and blastocyst percentage as compared to control. There was developmental arrest when embryos (2–4 cells) were cultured with 20 μM H_2O_2 but when using 20 μM H_2O_2 in combination with 10 mM L-carnitine, embryo development (from the 2–4 cells stage to the blastocyst stage) was significantly improved as compared to control proved L-carnitine has potent antioxidant and antiapoptotic effect. 50 μM H_2O_2 is reported to decrease significantly blastocyst development rate in two cell mouse embryos (Zhang et al. 2005). The difference in concentration of H_2O_2 affecting blastocysts development between our study and mouse study might be due to species specific oxidative stress tolerance capacity of developing embryos. Commet assay to proof protective ability of L-carnitine against oxidative damage showed that oocytes cultured with 20 μM H_2O_2 had fragmented DNA but oocytes cultured with 20 μM H_2O_2 in combination with 10 mM L-carnitine had no fragmented DNA.

Significantly, maximum upregulated expression of GPx was observed in *in vitro* matured oocytes and downregulated expression was observed at zygote. The relative expression level of GPx in control group was in a pattern of matured oocyte >2–4 cells >8–16 cells > blastocyst > morula > immature oocyte > zygote. L-carnitine supplemented group showed expression of GPx in same pattern as that of control, but in this group expression level of GPx was more in 2–4 cells stage which was non-significantly different from matured group. The relative expression level of SOD1 in both control and L-carnitine-treated groups were in a pattern of 8–16 cells >2–4 cells > matured oocytes > morula > immature oocytes > blastocyst > zygote.

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SOD1 was expressed significantly highest at 8–16 cells, and there were no significant differences in expression of SOD1 in rest of the developmental stages. Relative expression level of SOD2 in both control and treatment groups was significantly low among all the antioxidant enzymes studied in this experiment in a pattern of immature oocytes > morula > blastocyst > matured oocytes >2–4 cells > zygote >8–16 cells. Highest upregulated expression of SOD2 was observed in immature oocytes, and significant downregulation was at 8–16 cells as compared to all the rest of the developmental stages which were non-significantly different from each other. The antioxidant enzyme genes GPx, SOD1 and SOD2 in this experiment were expressed in all stages of developing embryos including immature and *in vitro* matured oocytes. Culture-induced developmental arrest of sheep embryos is normally observed at 8–16-cell (Gandolfi and Moor 1987). At this stage, sheep embryos undergo transition from maternal to embryonic genome control (Telford et al. 1990). So maternal SOD2 mRNA might have degraded progressively during embryo development because of which the level of transcript of SOD2 was significantly downregulated at 8–16 cells stage in our study.

To our knowledge, there is no such report available to find out the effect of L-carnitine on expression pattern of antioxidant enzyme genes in sheep oocytes and embryos. The antioxidant enzyme genes expression is modulated by oxidative stress (Correa et al. 2008). Expression of these antioxidant enzyme genes throughout the developmental stages indicates their inheritance from maternal pool mRNA. The intracellular redox potential can modulate the activity of some transcription factors, and ROS may activate the antioxidant defence genes (Schultz 1993). L-carnitine supplementation in maturation medium significantly upregulated the expression of GPx and downregulated the expression of SOD2 in all stages of developing embryos including matured oocytes, whereas expression of SOD1 and GAPDH (reference gene) was not altered in any stage. GPx is considered the major antioxidant enzyme within the glutathione peroxidase family and its deficiency renders cells more sensitive to stress that results in elevated induction of apoptosis (Flentjar et al. 2002). GPx depends on availability of reduced glutathione (GSH) and acts in conjunction with GSH a tripeptide cofactor for their enzymatic activity. GSH constitutes a vital component of the cellular antioxidant system (Mari et al. 2009). In our study, L-carnitine increased the GSH level in oocytes and embryos. Therefore, L-carnitine-mediated upregulation of GPx might be also due to increase in cofactor GSH concentration. There is no *in vitro* study to support this finding, but an *in vivo* study reported that L-carnitine supplementation increased glutathione and glutathione peroxidase activity (Fatouros et al. 2010). SOD2 is considered as an indicator of oxidative stress in cells and embryos (He et al. 2004). Downregulated expression of SOD2 due to L-carnitine supplementation might be due to the fact that L-carnitine helps in transportation of long-chain fatty acid to mitochondria and synthesize adenosine triphosphate (ATP). During ATP synthesis, a large quantity of oxygen is utilized causing decrease in concentration of oxygen and reduction in ROS formation that requires less SOD2 to neutralize free radicals causing downregulation of SOD2 gene (Gulcin 2006). L-carnitine might be acting as a buffer for excessive acetyl groups in mitochondria, so there may be decrease in mitochondrial superoxide production causing downregulation of SOD2 gene expression (Lee et al. 2014). Our finding on SOD2 expression due to L-carnitine supplementation is in contrast to the previous report that L-carnitine supplementation showed no alteration in expression of SOD2 (Takahashi et al. 2013), but Correa et al. (2008) has reported that high oxygen concentration during culture upregulated the expression of SOD2 which is indirectly in accordance to our finding because L-carnitine reduces oxidative stress in micromilieu causing decrease in expression of SOD2. As discussed earlier, expression of SOD2 (mitochondrial SOD) is culture condition dependant (Lequarre et al. 2001) and oxidative stress affects mainly to mitochondria, so expression of SOD2 is influenced. In contrast, the relative expression of SOD1 is not affected by L-carnitine supplementation which is might be due to the fact that oxidative stress during our *in vitro* culture condition might have not influenced so much to alter SOD1 (cytoplasmic SOD).

In conclusion, the results of the present study demonstrated that L-carnitine supplementation during *in vitro* sheep oocyte maturation improved developmental potential of embryos by reducing ROS and increasing GSH and alter the expression of antioxidant enzyme genes throughout the embryo development.

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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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