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L-Ergothioneine improves the developmental potential of *in vitro* sheep embryos without influencing OCTN1-mediated cross-membrane transcript expression

A. Mishra[1], *I.J. Reddy*[2], *A. Dhali*[3] *and P.K. Javvaji*[3]
Animal Biotechnology Laboratory and OMICS Laboratory, ICAR – National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore, India

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Summary

The objective of the study was to investigate the effect of L-ergothioneine (L-erg) (5 mM or 10 mM) supplementation in maturation medium on the developmental potential and OCTN1-dependant L-erg-mediated (10 mM) change in mRNA abundance of apoptotic (*Bcl2*, *Bax*, *Casp3* and *PCNA*) and antioxidant (*GPx*, *SOD1*, *SOD2* and *CAT*) genes in sheep oocytes and developmental stages of embryos produced *in vitro*. Oocytes matured with L-erg (10 mM) reduced their embryo toxicity by decreasing intracellular ROS and increasing intracellular GSH in matured oocytes that in turn improved developmental potential, resulting in significantly ($P < 0.05$) higher percentages of cleavage (53.72% vs 38.86, 46.56%), morulae (34.36% vs 20.62, 25.84%) and blastocysts (14.83% vs 6.98, 9.26%) compared with other lower concentrations (0 mM and 5 mM) of L-erg without change in maturation rate. L-Erg (10 mM) treatment did not influence the mRNA abundance of the majority of apoptotic and antioxidant genes studied in the matured oocytes and developmental stages of embryo. A gene expression study found that the *SLC22A4* gene that encodes OCTN1, an integral membrane protein and specific transporter of L-erg was not expressed in oocytes and developmental stages of embryos. Therefore it was concluded from the study that although there was improvement in the developmental potential of sheep embryos by L-erg supplementation in maturation medium, there was no change in the expression of the majority of the genes studied due to the absence of the *SLC22A4* gene in oocytes and embryos that encode OCTN1, which is responsible for transportation of L-erg across the membrane to alter gene expression.

Keywords: Antioxidant genes, Apoptotic genes, L-Ergothioneine, *In vitro* sheep embryos, OCTN1

Introduction

Reproductive failure is a significant animal health and productive performance concern. Successful implantation of embryo requires a good quality and endometrium receptive embryo. Interference with any of these two prerequisites leads to implantation failure. During embryo development there is production of unstable metabolites of oxygen called reactive oxygen species (ROS) from gametes, embryos and their surroundings. Like other living aerobic cells, embryos and oocytes are major sources of ROS because they use oxygen to produce energy through mitochondrial oxidative phosphorylation. Intracellular imbalance between ROS and their normal scavenger antioxidants results in oxidative stress (OS). Oxidative stress adversely affects embryo development and can jeopardize embryonic health and endometrial receptivity resulting in suboptimal outcome for *in vitro* embryo production (IVEP) (Agarwal *et al.*, 2012). Several pregnancy-related disorders, defective embryo development, pregnancy loss and infertility that are attributed to cell membrane damage, DNA damage and modulation of gene expression are OS induced

[1]All correspondence to: Ashish Mishra. ICAR-NIANP, Bangalore 560030, India. Tel: +91 80 25711304. E-mail: ashishvet1@gmail.com
[2]Animal Biotechnology Laboratory, ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore 560 030, India.
[3]OMICS Laboratory, ICAR-National Institute of Animal Nutrition and Physiology Adugodi, Bangalore 560 030, India.

(Aruoma *et al*., 2006). The effect of OS on reproduction is amplified because of the lack of physiological defence mechanisms against ROS. Early embryonic development is adversely affected by ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^-) that originate from embryo metabolism and their surrounding environment (Agarwal *et al*., 2005). So for better *in vitro* embryo development and to prevent the apoptosis of early embryos it is necessary to improve OS status in the microenvironment. Several studies have attempted to enhance *in vitro* embryo production (IVEP) by improving the techniques performed in different species (Sirard & Coenen, 2006; Abdelrazik *et al*., 2009; Mishra *et al*., 2010a, 2016a). To maximize the potential application of IVEP procedures, the effects of culture conditions on the developmental potential of oocytes and quality of embryos produced *in vitro* were assessed. The most important factor influencing the developmental potential of embryos produced *in vitro* is the oxygen concentration during culture that affects both oocyte maturation and embryo development (Kang *et al*., 2012). There are multiple factors that increase OS, these affect both embryo development and gene expression in preimplantation embryos resulting in suboptimal outcome of the IVEP set up (Elamaran *et al*., 2012). To improve the suboptimal outcome of IVEP, attempts have been made to develop the composition medium using free radical scavengers to improve OS status in the microenvironment for better embryo development (Mukherjee *et al*., 2014). Culture conditions affect transcript expression in oocytes and preimplantation embryos. The expression levels of many transcripts in oocytes and preimplantation embryos are upregulated and downregulated by ROS that even affect embryo viability and offspring survivability (Wrenzycki *et al*., 2007; Mishra *et al*., 2010b).

L-Ergothioneine (L-erg) is a dietary water-soluble naturally occurring amino acid mainly found in mushrooms and is a thiourea derivative of histidine, containing a sulphur atom at position 2 in the imidazole ring. This compound is made by a few organisms, notably actinobacteria and filamentous fungi. L-Erg has antioxidant properties and is a powerful scavenger of hydroxyl radicals, but its chemistry differs from conventional sulfur-containing antioxidants such as glutathione or lipoic acid. It reduces the damaging effects of environmental ultraviolet radiation and also neutralize OS by providing a reactive oxygen and nitrogen species (RONS) scavenging capacity (Akanmu *et al*., 1991). L-Erg inhibits hydroxyl radical formation, superoxide and singlet oxygen production and lipid peroxidation (Obayashi *et al*., 2005). It has already been shown that L-erg eliminates most active free radicals compared with some well known antioxidants such as glutathione, uric acid and trolox (Franzoni *et al*., 2006). The effects of L-erg on *in vitro* maturation (IVM) and embryo development of sheep and bovine have been reported previously. In the sheep model it was observed that addition of L-erg (10 mM) has a beneficial effect on *in vitro* maturation of oocytes and embryonic development, especially from cleavage to morulae (Ozturkler *et al*., 2010). The bovine model showed that enrichment of *in vitro* culture medium with L-erg (0.1 mM) improved overall embryo quality, the most important factor affecting post-implantation development (Zullo *et al*., 2016). However there is no such report available investigating the effect of L-erg on the intracellular oxidative status of oocytes and its antioxidant effect on subsequent embryo development *in vitro*. L-Erg is cell membrane impermeable and requires an organic cation transporter (OCTN1) for transport across the membrane; the gene *SLC22A4* encodes OCTN1 (Grundemann *et al*., 2005). The expression pattern of the *SLC22A4* gene in oocytes and embryos has not been reported with regard to L-erg-mediated change in apoptotic and antioxidant genes in oocytes and embryos. Therefore this experiment was designed in the sheep model to investigate the effect of L-erg (5 mM and 10 mM) supplementation in maturation medium on oocyte maturation and subsequent developmental potential of preimplantation embryos. The subsequent objective was to show L-erg-mediated (10 mM) change in oxidative status of oocytes and mRNA abundance of apoptotic [B-cell lymphoma protein 2 (*Bcl2*), Bcl2-associated protein (*Bax*), Caspase3 (*Casp3*) and proliferating cell nuclear antigen (*PCNA*)] and antioxidant [glutathione peroxidase (*GPx*), Cu/Zn cytoplasmic super oxide dismutase (*SOD1*), Mn mitochondrial super oxide dismutase (*SOD2*) and catalase (*CAT*)] genes as well as expression of the *SLC22A4* gene in matured oocytes and developing embryos (2–4-cell and blastocyst) produced *in vitro*.

Materials and methods

In vitro embryo production with or without L-erg

In vitro embryos were produced as per the protocol standardized in our laboratory (Mishra *et al*., 2016a). Briefly, oocytes were aspirated from follicles (2–6 mm) of slaughterhouse ovaries (carried to the laboratory in normal saline solution fortified with antibiotics at 37–39°C) with the help of a 20-gauge needle attached to a 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) (Singh *et al*., 2012) (n = 15–20) were matured in 100 μl maturation medium [TCM-199 (glutamine added) + 10% FBS + BSA (3 mg/ml) + pyruvate (4 mM) + gentamycin (50 μg/ml) + FSH

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(5 μg/ml) + LH (5 μg/ml) + estradiol (1 μg/ml)] with L-erg (Sigma code no: E7521,) control (0 mM) (oocytes: $n = 252$) and treatment (5 mM) (oocytes: $n = 268$) and 10 mM (oocytes: $n = 264$) under paraffin oil for 27 h in a 35 mm Petri dish at 5% CO_2, 38.5°C and 95% relative humidity (RH) in a CO_2 in air incubator. Maturation rate was assessed by the degree of cumulus expansion and extrusion of the first polar body by an aceto-orcein staining method (Sharma *et al*., 1996). *In vitro* fertilization was performed by fresh semen collected from the ram with the help of an electro ejaculator. Semen was washed twice with washing medium [Fert-TALP (Parrish *et al*., 1988) + heparin (10 μg/ml) + pyruvate (1 mM)] by centrifuging at 400 *g* for 5 min. Supernatant was removed and the pellet was reconstituted in fertilization medium [Fert-TALP + fatty acid free BSA (4 mg/ml) + heparin (10 μg/ml) + pyruvate (1 mM) + MEM non-essential amino acid solution (100×) (1%) + MEM amino acids solution (50×) (1%)]. Final sperm concentration was adjusted to 2–3 × 10^6 sperms/ml, which was assessed using a Neubauer chamber. The sperm suspension was kept in a CO_2 in air incubator until matured oocytes were washed 4–5 times in fertilization medium. Finally, matured oocytes ($n = 15$–20) were co-incubated with 100 μl processed spermatozoa for 18 h. Following the 18 h co-incubation, presumptive zygotes were cultured in 100 μl culture medium [(TCM-199 (glutamine added) + 20% FBS + BSA (3mg/ml) + pyruvate (4 mM) + gentamycin (50 μg/ml) + MEM non-essential amino acid solution (100×) (1%) + MEM amino acids solution (50×) (1%)] to produce embryos at different developmental stages (cleavage to blastocyst). Both fertilization and culture were carried out at the same temperature and gas conditions described for maturation. 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 culture medium. Finally L-erg (10 mM) was used in maturation medium to find out the oxidative status of matured oocytes, then the antioxidant effect of L-erg on embryo development was tested by partial enrichment of culture medium and L-erg-mediated change in transcript levels of apoptotic (*Bcl2*, *Bax*, *Casp3* and *PCNA*) and antioxidant (*GPx*, *SOD1*, *SOD2* and *CAT*) genes in oocytes and developing embryos.

Intracellular ROS and GSH levels in matured oocytes

Matured oocytes from L-erg (10 mM) treated or non-treated (0 mM) groups were taken to measure the intracellular ROS and GSH levels. Intracellular ROS and GSH levels were estimated as per the procedure described previously (Mishra *et al*., 2016b). ROS levels in *in vitro* matured oocytes were quantified using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) and detected as green fluorescence at an excitation wavelength of 495 nm and emission wavelength of 520 nm. GSH levels were quantified using 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CMF2HC) and detected as blue fluorescence at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Briefly, oocytes were washed twice in phosphate-buffered saline (PBS) + polyvinyl pyrollidone (PVP) (0.5%) (wt/vol) and fixed with 4% paraformaldehyde (PFA) and then placed in 50 μl of DCHFDA (10 μM) and CMF2HC (10 μM) for 15 min at 5% CO_2, 38.5°C and 95% RH to detect ROS and GSH respectively. Finally, the oocytes were washed three times with PBS + PVP (0.5%), and then carefully mounted on a glass slide and covered with a coverslip. The fluorescence intensity of oocytes in each group was observed under a fluorescence microscope (Euromex, The Netherlands) equipped with a digital camera. Fluorescence intensities of oocytes were analysed by grey pixel intensity using Image J software (NIH,USA) normalizing untreated control oocytes as 1.

Antioxidant effect of L-erg (10 mM) on embryo development

Interestingly, 10 μM concentrations of H_2O_2 significantly ($P < 0.05$) decreased cleavage and further development, whereas at 20 μM H_2O_2 there was no cleavage at all (Mishra *et al*., 2016b). Therefore to assess the antioxidant effect of L-erg (10 mM), embryos (2–4-cells) were cultured with H_2O_2 (20 μM) for 48 h followed by replacing 50% of the medium with fresh medium without H_2O_2 but with or without L-erg (10 mM) on every alternate day up to blastocyst development. The control group embryos ($n = 56$) were cultured without H_2O_2 and L-erg. Group 1 embryos ($n = 48$) were cultured with H_2O_2 but without L-erg and Group 2 embryos ($n = 54$) were cultured with H_2O_2 and L-erg. Embryonic development was observed in all groups.

Blastocyst staining for total cell numbers

Blastocysts produced from L-erg (10 mM) treated or non-treated oocytes and embryos exposed to H_2O_2 with or without L-erg were stained with bisbenzimide (Hoechst 33258) to compare the total cell numbers between the groups (Mishra *et al*., 2016c). Zona pellucida of the day 7 embryos/blastocysts 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 three times in PBS + PVP (0.5%) and finally stained with bisbenzimide (Hoechst 33258)

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Table 1 Primers used for gene expression analysis

Sl no.	Genes	Primer	Product size
1	*GAPDH*	Forward: ATGGGCGTGAACCACGAGAA; Reverse: ATGGCGTGGACAGTGGTCAT	146 bp
2	*Bcl2*	Forward: ATGACTTCTCTCGGCGCTAC; Reverse: CTCCACACACATGACCCCTC	176 bp
3	*Bax*	Forward: CATGGAGCTGCAGAGGATGA; Reverse: GTTGAAGTTGCCGTCGGAAA	100 bp
4	*Casp3*	Forward: ACCTCACGGAAACCTTCACGA; Reverse: ACCATGGCTTAGAAGCACGC	149 bp
5	*PCNA*	Forward: AGCCACTCCACTGTCTCCTACA; Reverse: TCATCCTCGATCTTGGGAGCC	123 bp
6	*GPx*	Forward: CGTGCAACCAGTTTGGGCAT; Reverse:GATGCGCCTTCTCGCCATTC	141 bp
7	*Cu/Zn SOD* (*SOD1*)	Forward: CACTTCGAGGCAAAGGGAGA; Reverse: CCTTTGGCCCACCGTGTTTT	167 bp
8	*Mn SOD* (*SOD2*)	Forward: CCGTCAGCCTTACACCAAGT; Reverse: CAAGCCACGCTCAGAAACAC	112 bp
9	*CAT*	Forward: GCCTGTGTGAGAACATTGCG; Reverse: TCCAAAAGAGCCTGGATGCG	121 bp
10	*SLC22A4* (*OCTN1*)	Forward: GTGGAGTCTGGTGTGTGAGG; Reverse: TGGTTGCGAAGAGGATGGTC	137 bp

(10 μg/ml) for 20 min. Blastocysts were then mounted on slides and covered with a coverslip. The total cell numbers (TCN) of blastocysts were determined by counting the number of nuclei detected as blue fluorescence at an excitation wavelength of 350 nm and an emission wavelength of 450 nm under a fluorescence microscope (Euromex, The Netherlands) equipped with a digital camera. All exposures were carried out at room temperature.

Expression levels of apoptotic, antioxidant and *SLC22A4* genes in oocytes and embryos

The transcript abundance of apoptotic genes (*Bcl2*, *Bax*, *Casp3* and *PCNA*), antioxidant genes (*GPx*, *SOD1*, *SOD2* and *CAT*) and *SLC22A4* gene (encodes the OCTN1 the transporter of L-erg) were analyzed in oocytes and embryos by real-time quantitative PCR (qPCR). For the gene expression study, oocytes matured with L-erg (10 mM) followed by embryos cultured without L-erg were treated as the treatment group, whereas in the control group neither oocytes nor embryos were exposed to L-erg. The gene-specific primers used in this study were designed using NCBI, Primer Blast software (Table 1). The specificities of the primers were tested using BLAST analysis against the genomic NCBI database.

qPCR analysis

Total RNA isolation, cDNA synthesis and qPCR were carried out by the procedure discussed previously (Mishra *et al*., 2017). Briefly, total RNA was isolated from equal numbers of oocytes, immature ($n = 20$), *in vitro* matured ($n = 20$) and embryos of zygote ($n = 20$), 2–4-cell ($n = 20$) and blastocysts ($n = 10$) from both the groups by TRIzol (Invitrogen, Life Technologies, USA). Genomic DNA contamination was removed using the TURBO DNA-free™ kit (Ambion, Life Technologies, USA). Total RNA isolated from both groups was used for reverse transcription (RT) as the template for first strand synthesis using the evertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) as per manufacturer's guidelines. The expression levels of specific genes in oocytes and embryos were quantified by qPCR using the Step One Plus qPCR system (Applied Biosystem, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene in this study. The qPCR reactions were performed using KAPA SYBR Fast 2× master mix (KAPA Biosystems, Wilmington, 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, 1 μl of cDNA as template and final volume was adjusted to 10 μl with nuclease-free water. The PCR conditions used to amplify all genes were initial denaturation at 95°C for 20 s with 40 cycles of denaturation at 95°C for 3 s followed by annealing and extension at 60°C for 30 s. The melting curve analysis was carried out to confirm the qPCR specificity. C_t (threshold cycle for target amplification) values were analysed using the $2^{-\Delta\Delta Ct}$ (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 $\Delta\Delta C_t = \Delta Ct$ (target gene sample) $- \Delta Ct$ (calibrator). qPCR was conducted three times for three different sets of matured oocytes and embryos. The qPCR amplicons were confirmed by ethidium bromide-stained (0.5 μg/ml) 2% agarose gel electrophoresis.

SLC22A4 gene expression in testes

SLC22A4 gene expression in testes was carried out to get a positive sample for the expression of SLC22A4 to confirm that the used primer is specific for the *SLC22A4* gene. A sheep testes sample was taken for RNA isolation with subsequent cDNA synthesis and qPCR analysis. Before RNA isolation, the testes (10 g) was exposed to liquid nitrogen and homogenized with an homogenizer. Total RNA was isolated by the

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conventional TRIzol (Invitrogen, Life Technologies, USA) protocol as per manufacturer's guidelines. Briefly 500 µl of TRIzol was added to the pre-homogenized testes and homogenized again, followed by the addition of another 500 µl of TRIzol and was mixed. The mixture was incubated for 5 min at room temperature. Next, 200 µl chloroform was added to the mixture, mixed and incubated for 10 min at room temperature. The whole mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The upper aqueous phase was collected without touching the interphase. 500 µl isopropanol was added to the supernatant, mixed by pipetting up and down and incubated for 30 min on ice. The tubes were centrifuged at 12,000 rpm for 10 min at 4°C after incubation and the supernatant was discarded. The pellet was washed with 1000 µl of 75% freshly prepared ethanol by centrifuging at 7500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was air dried at room temperature for 10 min. The RNA pellet was resuspended in 20 µl of diethyl pyrocarbonate (DEPC) water and mixed. The mixture was incubated at 60°C for 10 min with a little shaking in-between incubations. Genomic DNA contamination was removed using the TURBO DNA-free™ kit (Ambion, Life Technologies, USA). Subsequent cDNA synthesis, qPCR and confirmation of qPCR amplicons were carried out using same procedure described for oocytes and embryos.

Statistical analysis

The results were expressed in mean ± standard error of the mean (SEM). Statistical analyses were carried out using GraphPad Prism5 software, San Diego, USA. The means between groups for developmental stages (cleavage to blastocyst) and TCN were compared by analysis of variance (ANOVA). Embryo development data were presented in the percentage form in relation to total oocytes cultured. Percentage values were arcsine transformed before analysis. A P-value < 0.05 was considered as significant. ROS levels and GSH levels between matured oocytes of control and L-erg treated groups as well as relative gene expression level at particular developmental stage of embryo between control and L-erg-treated groups were compared by Student's t-test.

Results

Effect of L-erg (5mM and 10mM) supplementation on *in vitro* maturation and subsequent embryo development

The results of *in vitro* embryo development in the presence or absence of L-erg (5 mM and 10 mM) in maturation medium are detailed in Fig. 1. Supplementation of different concentrations of L-erg (5 mM and 10 mM) in the maturation medium did not influence the maturation percentage (82.96–84.11%). L-Erg (10 mM) supplementation during the maturation period resulted in a significantly ($P < 0.05$) higher percentage of cleavage (53.72 vs 38.86, 46.56%) followed by morulae (34.36 vs 20.62, 25.84%) and blastocysts (14.83 vs 6.98, 9.26%) as compared with other groups of lower concentration [0 mM (control) and 5 mM]. The cleavage percentage of the 10 mM L-erg group was not significantly more than for the 5 mM L-erg group.

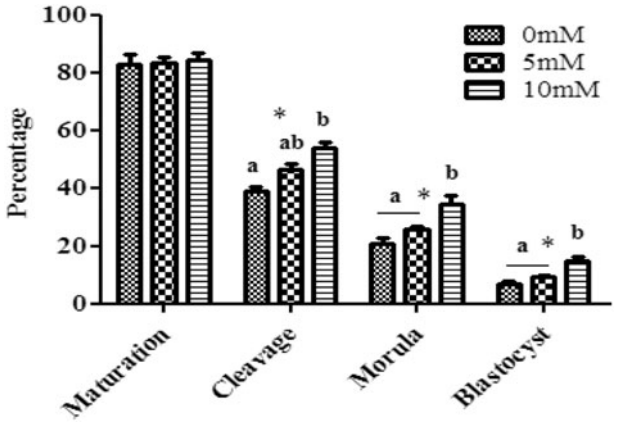

Figure 1 Effect of L-erg (5 mM and 10 mM) during *in vitro* maturation on maturation of oocytes and embryo development. Percentage results are presented as mean + standard error of the mean (SEM). [a,b]Different superscripts in the same group indicate values that differ significantly at $P < 0.05$. Six experiments were performed for each group. *Cleavage was calculated from total number of oocytes cultured, whereas morula and blastocysts percentage were calculated from the number of embryos cleaved.

Effect of L-erg (10 mM) on intracellular ROS and GSH levels of matured oocytes

L-Erg-treated (10 mM) matured oocytes 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 compared with matured oocytes in the control group without L-erg (Fig. 2).

Antioxidant effect of L-erg (10 mM) on embryo development

Embryos (2–4-cell) cultured with H_2O_2 (20 µM) but without L-erg (10 mM) (Fig. 3*B*) for 48 h showed a significant ($P < 0.05$) decrease in the percentage of morulae (13.14 vs 50.46%) and blastocysts (2.86 vs 17.54%) compared with the control group (embryos cultured with neither H_2O_2 nor L-erg) (Fig. 3*A*). Supplementation of L-erg (10 mM) to H_2O_2-induced

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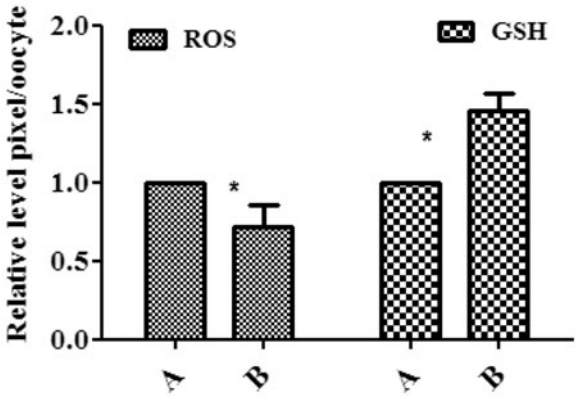

Figure 2 Intracellular ROS and GSH level in matured oocytes with L-erg (10 mM) during *in vitro* maturation. A, Control oocytes; B, L-erg treated oocytes. Asterisks indicate values that differ significantly at $P < 0.05$. Three experiments were performed for each group.

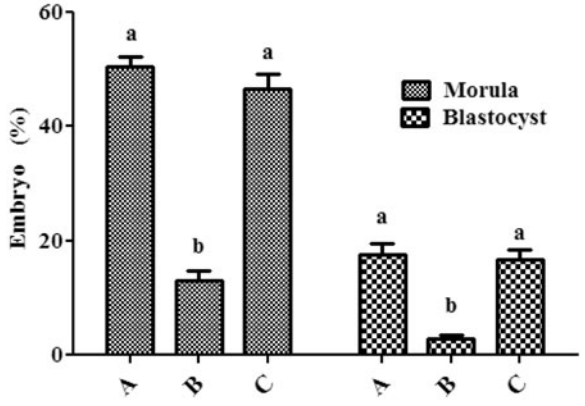

Figure 3 Antioxidant effect of L-erg (10 mM) on embryo development in the presence or absence of H_2O_2 during the post-fertilization period. A, Embryo cultured with neither H_2O_2 nor L-erg. B, Embryo cultured with H_2O_2 (20 μM). C, Embryo cultured with H_2O_2 (20 μM) + L-erg (10 mM). *Percentage results are presented as mean + standard error of the mean (SEM). [a,b]Different superscripts in the same group indicate embryo stages that differ significantly at $P < 0.05$. Four experiments were performed for each group.

(20 μM) culture medium, showed a significant ($P < 0.05$) increase in the percentage of morulae (46.58 vs 13.14%) and blastocysts (16.68 vs 2.86%), which was not significantly different to that of the control group (Fig. 3C).

Effect of L-erg (10 mM) on total cell numbers

Although the percentage of blastocysts (14.83 vs 6.98%) was significantly ($P < 0.05$) increased due to L-erg (10 mM) supplementation in the maturation medium, TCN was not significantly different (184 ± 7.8 vs 178 ± 9.2) between L-erg-treated (10 mM) and non-treated groups. The TCN of the embryos exposed

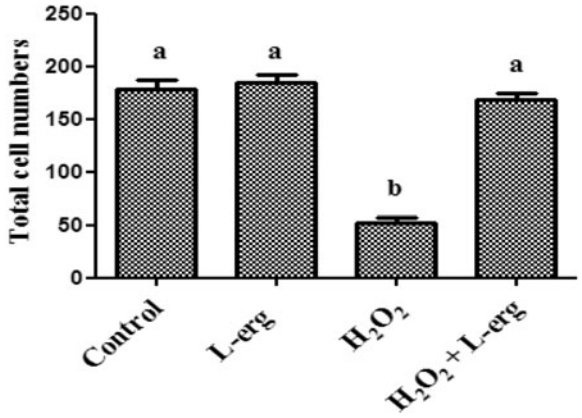

Figure 4 Effect of different culture conditions on total cell numbers of blastocyst and day 7 embryos. Results are presented as mean + standard error of the mean (SEM). [a,b]Different superscripts indicate groups that differ significantly at $P < 0.05$. Three experiments were performed.

to H_2O_2 were significantly ($P < 0.05$) reduced to 52 ± 4.6. Supplementation of L-erg (10 mM) to H_2O_2-induced medium resulted in significantly ($P < 0.05$) no difference in cell numbers (168 ± 6.7) compared with that of the control (Fig. 4).

Effect of L-erg (10 mM) on the relative expression levels of apoptotic and antioxidant genes in oocytes and embryos

The relative expression levels of apoptotic and antioxidant genes in matured oocytes and different developmental stages (zygote, 2–4-cell and blastocyst) of both control and L-erg-supplemented groups in relation to the expression level of immature oocytes is detailed in Fig. 5. In this study L-erg-mediated change in the relative expression level of genes in matured oocytes and developmental stages were compared with the particular stage of the control group. It was observed that L-erg (10 mM) treatment during maturation did not influence the expression of the majority of the apoptotic and antioxidant genes in matured oocytes and all the developmental stages except for antioxidant genes i.e. *GPx* was upregulated significantly ($P < 0.05$) in 2–4-cell stage embryos and *SOD2* was significantly ($P < 0.05$) upregulated in all the developmental stages.

Expression profile of *SLC22A4* in oocytes, developmental stages of embryos and testes

The relative expression level of *SLC22A4* in oocytes (immature and matured) and developmental stages (zygotes, 2–4-cell and blastocysts) of embryos is shown in Fig. 6. It was observed that the *SLC22A4* gene was not expressed in any oocytes (immature and matured)

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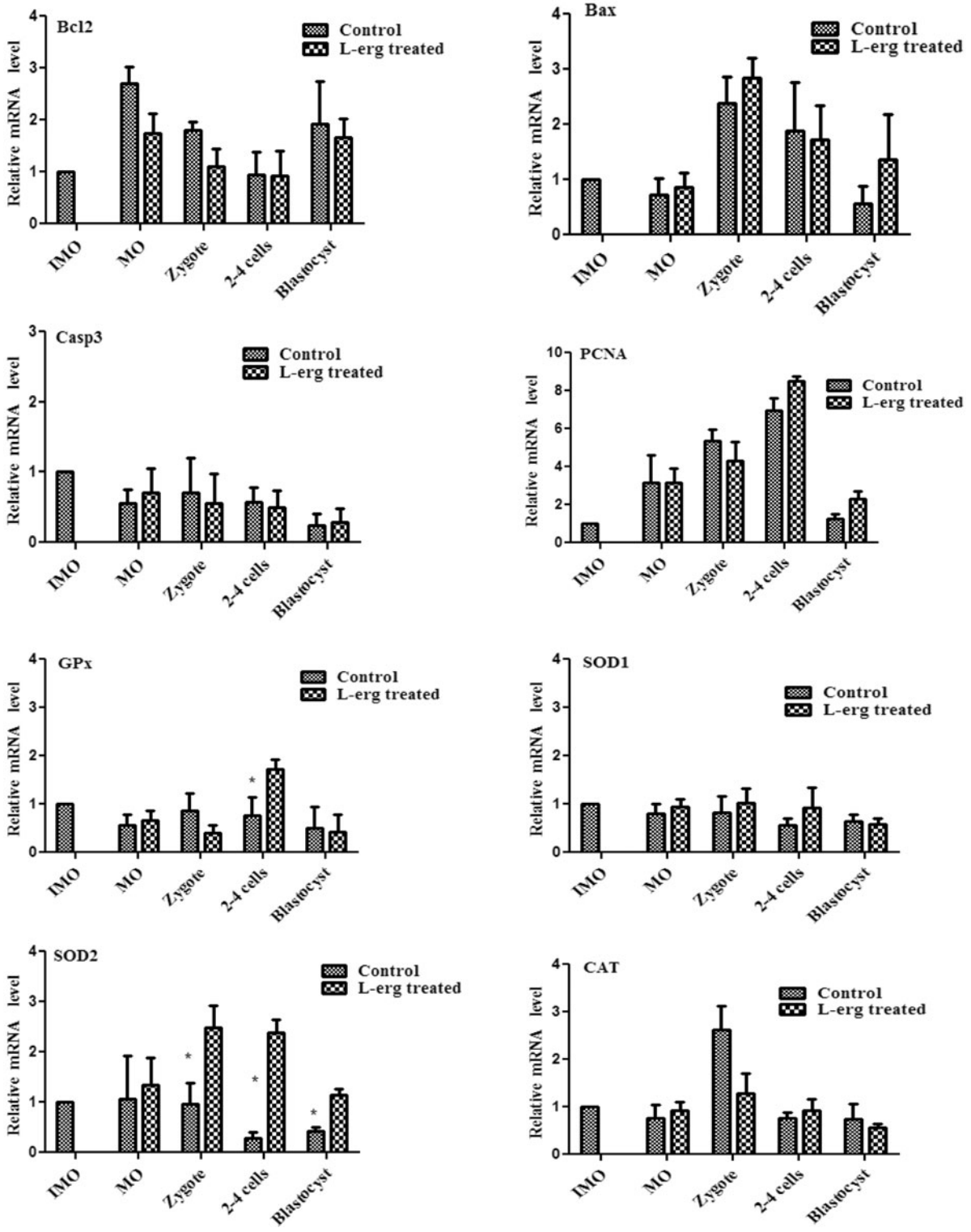

Figure 5 L-erg-mediated alteration in mRNA level of apoptotic and antioxidant genes in oocytes and embryos. Asterisk indicates the values that differ significantly at $P < 0.05$ in the same group, IMO: immature oocytes, MO: *in vitro* matured oocytes.

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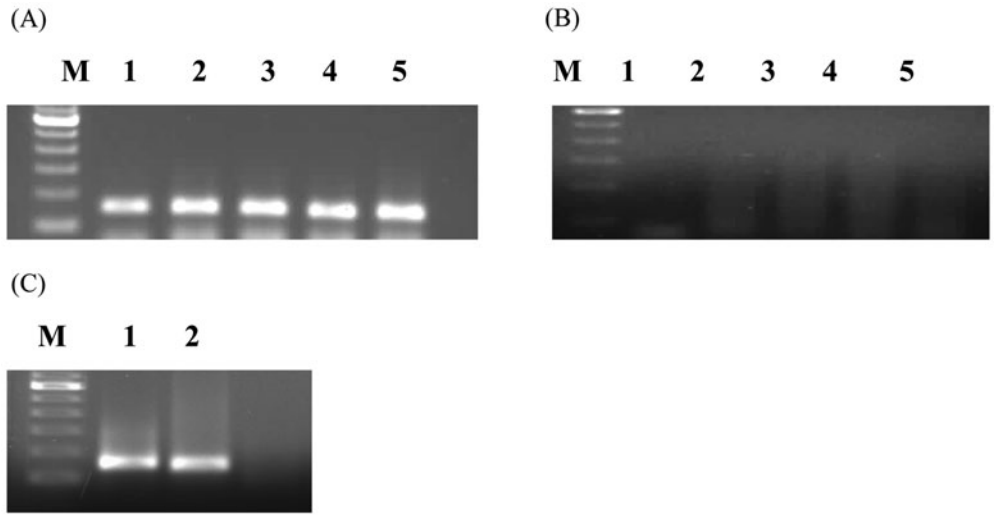

Figure 6 Expression of *SLC22A4* in oocytes, developmental stages of embryo and testes. (*A*) Expression of *GAPDH* (146 bp) in oocytes and developmental stages of embryo. (*B*) No expression of *SLC22A4* (137 bp) in oocytes and developmental stages of embryo. M: Marker (DNA ladder 100 bp); 1: Immature oocytes; 2: *In vitro* matured oocytes; 3: Zygote; 4: 2–4-cell; 5: Blastocyst. (*C*) Expression of *GAPDH* (146 bp) and *SLC22A4* (137 bp) in testes. M: Marker (DNA ladder 100 bp); 1: GAPDH; 2: SLC22A4.

and developmental stages of embryos. In testes the *SLC22A4* gene was expressed and it was confirmed that the primer used in this study was specific for *SLC22A4*.

Discussion

Studies are needed to minimize the OS during *in vitro* embryo culture to increase the developmental potential of oocytes and embryos. The present study was undertaken to ameliorate OS in the IVEP protocol in a sheep model by L-erg (5 mM and 10 mM) supplementation during the maturation period for better embryo development. Subsequent studies were conducted to find out the L-erg-mediated (10 mM) changes in expression level of apoptotic and antioxidant genes. To our current knowledge, there is no report in the literature on L-erg-mediated reduction in oxidative status of oocytes and change in the relative expression levels of apoptotic and antioxidant genes in oocytes and embryos of any species. Different concentrations of L-erg (5 mM and 10 mM) supplementation during IVM in this study showed that at 10 mM L-erg there was significant ($P < 0.05$) increase in the percentages of cleavage, morula and blastocyst as compared with other lower concentrations [0 mM (control) and 5 mM]. There was a beneficial effect on embryo development from zygote to blastocyst by supplementing L-erg (5 mM and 10 mM) during IVM. Supplementation of L-erg (10 mM) in maturation medium significantly ($P < 0.05$) increased GSH levels and decreased ROS levels in matured oocytes. To our knowledge, this report is the first to reflect L-erg-mediated change in GSH and ROS levels in matured oocytes. Improvement in developmental stages of embryos by L-erg supplementation during IVM could be due to its effects of increasing GSH and reducing intracellular ROS that protect cells from apoptosis, reported by our group previously (Mishra *et al.*, 2016b). The present findings of ROS and GSH levels in response to L-erg supplementation during maturation were similar to other previous reports of a reduction in ROS and an increase in GSH due to free radical scavenger supplementation during IVEP (You *et al.*, 2012; Takahashi *et al.*, 2013). GSH is a substrate for GSH peroxidase and it is well known that L-erg increases GSH peroxidase enzyme activity, which might influence the significant increase in GSH in oocytes (Kawano *et al.*, 1983). ROS produced from oocytes during the maturation period are neutralized due to the free radical scavenging effect of L-erg that protects cellular organelles including mitochondria, the major source of ROS for further subsequent developmental stages demonstrating L-erg as an antioxidant and anti-apoptotic compound. Subsequent L-erg supplementation (10 mM) of H_2O_2-induced (20 μM) culture medium resulted in a significant ($P < 0.05$) increase in the percentages of morulae and blastocysts and TCN compared with H_2O_2-induced culture medium and confirmed that L-erg neutralized ROS generated by H_2O_2 and supported the antioxidant properties of L-erg (Akanmu *et al.*, 1991). Therefore this study suggested that it is better to use L-erg during IVM of oocytes to achieve an improved number of developmental stage embryos.

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Although there was improvement in the developmental potential of sheep embryos by the addition of L-erg in maturation medium, the maturation rate (82.96% to 84.11%) was not influenced by any experimental L-erg (5 mM and 10 mM) concentration. In contrast with our findings, another study reported that the addition of L-erg (10 mM) to IVM, IVF and IVC medium showed a beneficial effect on IVM of oocytes and embryonic development in sheep, especially from cleavage to morulae stages (Ozturkler *et al*., 2010). Bovine presumptive zygotes when cultured with L-erg (0.5 mM) during the post-fertilization period showed a marked decrease in both cleavage and blastocyst development and evidence of a toxic effect was observed with no embryo production following culture with 1 mM L-erg. There was improvement in embryo quality, as indicated by improved cryotolerance, lower apoptotic rate and a higher percentage of blastocysts with the most physiological ICM:total cells ratio (Zullo *et al*., 2016). These studies were the only two that discussed the effect of L-erg on the developmental potential of oocytes and embryos. It should be noted that the concentration of L-erg that was beneficial on sheep embryos including our study was 10 times higher than the concentration used in the bovine study and was toxic to bovine embryos. Because of the limited information available, differences in the effect of L-erg on developmental potential of oocytes and embryos might be due to species-specific differences or concentration of L-erg used in the experiments. The concentration of L-erg in mammalian tissue is 1–2 mM, which may act as a non-toxic thiol buffering antioxidant (Grigat *et al*., 2007). The concentration of L-erg used for the bovine study was lower than the physiological range and might have not given a significant embryo developmental potential effect, whereas in the sheep study the concentration used was 10 times more and showed a significant result in terms of embryo developmental stages.

The present study is the first to determine the effects of L-erg (10 mM) on the relative expression levels of apoptotic (*Bcl2*, *Bax*, *Casp3* and *PCNA*) and antioxidant (*GPx*, *SOD1*, *SOD2* and *CAT*) genes in oocytes and developing embryos produced *in vitro*, therefore we cannot compare our results with other similar studies. The only studies available in the literature in sheep and bovine models were discussed above, but these only assessed the qualitative and quantitative effect of L-erg on *in vitro* embryo development, not at the transcript level. Although in our previous studies (Mishra *et al*., 2016b, c) the use of L-carnitine altered the expression levels of apoptotic and antioxidant genes in oocytes and embryos, it was surprising to observe in the present study that L-erg (10 mM) treatment during maturation did not influence the expression levels of all apoptotic genes studied for the oocytes and developmental stages. Among the antioxidant genes studied, L-erg (10 mM) treatment significantly ($P < 0.05$) upregulated the expression of *GPx* in the 2–4-cell stage but not in matured oocytes and blastocyst stages, whereas expression of *SOD2* was significantly ($P < 0.05$) upregulated both in 2–4-cell and blastocyst stages, but not in matured oocytes. The expression of other antioxidant transcripts (*SOD1* and *CAT*) was not altered at both the matured oocytes and developmental stages by L-erg (10 mM) supplementation. Modifications in the microenvironment modulate gene expression in developmental stages of embryos (Zhou *et al*., 2008; Mishra *et al*., 2016b, c). Antioxidant gene expression is modulated by OS (Correa *et al*., 2008). Change in the expression in *GPx* and *SOD2* in the 2–4-cell stage and blastocyst might be explained by the fact that ROS produced by the embryo diffuses to the microenvironment and is neutralized by L-erg, as ROS are able to diffuse through the plasma membrane (Guerin *et al*., 2001). GPx is considered as the major antioxidant enzyme within the glutathione peroxidase family and its deficiency renders cells more sensitive to stress (Flentjar *et al*., 2002). GPx levels depend on the availability of reduced GSH and it 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-erg (10 mM) increased the GSH level in mature oocytes. Therefore L-erg-mediated upregulation of GPx in initial developmental stages (2–4-cell) was due to increased cofactor GSH concentration, whereas in the blastocyst stage GSH levels may be reduced compared with the initial developmental stages because of ROS production by embryos themselves. SOD2 is considered an indicator of OS in cells and embryos (He *et al*., 2004). L-Erg is cell membrane impermeable and requires a specific carrier to be transported across the membrane. Its function is restricted only to cells and tissues in which the ergothioneine transporter OCTN1 is produced or expressed (Grundemann *et al*., 2005). Upregulated expression of *SOD2* due to L-erg (10 mM) supplementation may be due to the fact that L-erg cannot permeate across the plasma membrane of oocytes and embryos and that the oxidative status inside mitochondria is not reduced. Because of this intracellular OS, ATP synthesis is reduced, causing an increase in ROS formation that requires more SOD2 to neutralize the free radicals with an upregulated expression of the *SOD2* gene. It has been reported previously that during ATP synthesis a large quantity of oxygen is utilized, causing a decrease in oxygen concentration and reduction in ROS formation (Gulcin, 2006). In the present study, L-erg mediated the upregulated of *GPx* and *SOD2* expression in the developmental stages with no effect on SOD1, similar to findings in an *in vitro* study of mouse liver

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that showed an inhibitory effect of ergothioneine on lipid peroxide formation (Kawano *et al*., 1983). The CO_2 incubator used for the present experiment had no controlled oxygen, so it was assumed that the oxygen tension inside incubator was at maximum tension of atmospheric oxygen. It has already been reported that high oxygen tension during *in vitro* embryo culture upregulated *SOD2* expression due to more ROS production (Correa *et al*., 2008). In the present experiment, L-erg supplementation decreased the ROS concentration in oocytes, and this decrease was expected to be carried forward in embryos compared with the control. Therefore downregulation of *SOD2* expression in developmental stages of embryos was expected due to L-erg supplementation during the maturation period, but in contrast there was upregulation in *SOD2* expression. The L-erg-mediated upregulated expression of *SOD2* in developmental stages might also be explained by reaction of cellular stressors with L-erg that may occur outside the cell, giving a false impression of intracellular effects as seen in many cell culture assays (Cheah & Halliwell, 2012). So a complete understanding of the action of L-erg in the body is still limited and much more remains to be learned. It can also be explained that, as *SOD2* expression is culture condition dependant (Lequarre *et al*., 2001) and OS affects mainly the mitochondria, this influences SOD2 expression. In contrast, the relative expression of SOD1 is not affected by L-erg supplementation because OS during our *in vitro* culture conditions might have not been influenced enough to alter *SOD1* expression. L-Erg can chelate metalloenzymes and has affinity for metals with a strong affinity for Cu/Zn in the order Cu > Hg > Zn > Cd > Co > Ni. Because of the strong affinity of L-erg for Cu/Zn, deactivating Cu/Zn would have no effect on Cu–Zn SOD (SOD1), cytoplasmic SOD, but might stimulate Mn SOD (SOD2), mitochondrial SOD (Motohashi *et al*., 1974). CAT is an enzymatic antioxidant that neutralizes H_2O_2 molecules by converting them into oxygen and water (Kobayashi et al. 1991), and which is stimulated by OS (Guerin *et al*., 2001). Use of antioxidants such as L-carnitine upregulated the expression of CAT in patients with coronary artery disease (Lee *et al*., 2014). The lack of change in CAT expression due to L-erg supplementation might be due to the fact that OS during culture may not be influenced enough to alter CAT expression.

Although microenvironment modification by L-erg (10 mM) treatment created beneficial microenvironments by increasing GSH level and reducing ROS in oocytes, it is still not clear how L-erg alters the expression of the majority of apoptotic and a few antioxidant genes studied in matured oocytes and in developmental stages. L-Erg has already been proved to be anti-apoptotic for a higher percentage of blastocysts with a lower apoptotic proportion, as assessed by TUNEL staining (Zullo *et al*., 2016). But in the present study the expression of apoptotic genes was not altered in matured oocytes and in developmental stages. The ergothioneine transporter (ETT) has a beneficial role for L-erg activity. Cells with high expression of ETT can accumulate ergothioneine to high levels (Grundemann *et al*., 2005). The gene *SLC22A4* encodes an integral membrane protein, OCTN1 which is a powerful and highly specific transporter for the uptake of L-erg that facilitates pH-dependent transport of ergothioneine across the plasma membrane (Lamhonwah & Tein, 2006). Cells lacking ETT do not accumulate ergothioneine, because the plasma membrane is virtually impermeable to ergothioneine (Cheah & Halliwell, 2012). Cellular uptake of L-erg exhibits significant temperature and Na^+ dependence and is saturable, further supporting the involvement of carrier-mediated transport (Nakamura *et al*., 2007). Polymorphisms in the *OCTN1* gene are involved in inflammatory and autoimmune diseases and show abnormal levels of ROS (Urban *et al*., 2007). Although studies indicated the expression of the *SLC22A4* gene that encodes OCTN1 for ergothioneine transport inside cells, expression of *SLC22A4* on oocytes and embryos has not been reported (Lamhonwah & Tein, 2006; Wu *et al*., 2000; Markova *et al*., 2009). In this study, *SLC22A4* gene expression was not observed in matured oocytes and in any of the developmental stages of the embryo. We tried to determine *SLC22A4* expression in different tissues to confirm that the primer used was specific for *SLC22A4*. It was observed that *SLC22A4* was not expressed in all the tissues studied except in the testes (unpublished data). Therefore L-erg mediated no change in expression of the majority of the apoptotic and antioxidant genes. From this study it can be speculated that L-erg is not permeable to oocyte and embryo plasma membranes due to the absence of OCTN1, the transporter for L-erg across the membrane. The cellular uptake of L-erg is temperature and Na^+ dependent and OCTN1 facilitates pH-dependent transport of L-erg across the membrane, so the *in vitro* culture medium and study conditions would not have influenced proper transportation of L-erg across the membrane to alter the gene expression. OCTN1 has 11 predicted trans-membrane domains and can variously function as an organic cation/proton exchanger, or a Na^+-dependent or Na^+-independent zwitterion transporter. Its physiological substrates are carnitine and ergothioneine, but ergothioneine is a superior substrate. OCTN1 specifically and very efficiently transports ergothioneine and OCTN2 is responsible for carnitine transport. Although transport of L-carnitine by OCTN1 is measurable, it is negligible compared with transport of L-erg (Grundemann *et al*., 2005). OCTN2 is predominantly involved in L-carnitine transport and OCTN1

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has poor L-carnitine transport activity, suggesting that there may be another physiological role for transport (Lamhonwah and Tein, 2006). There are reports of OCTN1 localization in mitochondria, and significant species differences in both localization and transport mechanisms among species (Wu *et al*., 2000). Therefore further investigation of L-erg transport and action is required. The differences in the role of both L-erg and L-carnitine towards altering gene expression were due to absence of OCTN1 (responsible for L-erg transport) across the membrane of oocytes and embryos, whereas OCTN2 (responsible for carnitine transport) might be present in oocytes and embryos to help transportation of carnitine across the membrane to alter embryonic genes expression. It was concluded from the study that L-erg supplementation during *in vitro* maturation reduces OS-induced embryo toxicity by decreasing intracellular ROS and increasing intracellular GSH, that in turn improved the developmental potential of oocytes and embryos. Although L-erg altered the mRNA abundance of a few antioxidant genes, the mRNA abundance of the majority of the apoptotic and antioxidant genes studied in oocytes and embryos was not altered, due to the absence of the *SLC22A4* gene in oocytes and embryos that encodes OCTN1, an integral membrane protein and highly specific transporter for the uptake of L-erg across the membrane to alter gene expression. It can also be speculated that the change in the relative expression of only a few antioxidant genes in this study was not L-erg mediated, but rather microenvironment dependant and cell function mediated.

Conflict of interest

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

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