Kafkas Univ Vet Fak Derg
16 (5): 757-763, 2010
DOI:10.9775/kvfd.2009.1646

RESEARCH ARTICLE

The Effects of L-Ergothioneine and L-Ascorbic Acid on the *In Vitro* Maturation (IVM) and Embryonic Development (IVC) of Sheep Oocytes [1] [2]

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[1] *This study was supported by TÜBİTAK (The Scientific and Technological Research Council of Turkey), Research Grand: TÜBİTAK-TOVAG 104O335*
[2] *This study was presented in 12th ESDAR Conference and Annual Conference of EU AI-Vets 2008, 19th-23rd November, Utrecht, Netherlands*
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Makale Kodu (Article Code): KVFD-2009-1646

Summary

The aim of this study was to compare the effects of L-ergothioneine (LE) and L-ascorbic acid (LAA) on the IVM of immature oocytes and embryonic development of *in vitro* matured oocytes in sheep. The ovaries were collected from slaughterhouse, then they were transferred (within 1-2 hs) to the laboratory at warm (30-35°C) 0.9% saline solution. Cumulus Oocyte Complexes (COCs) were obtained by incision of follicles. Oocytes covered with at least three compact layers of cumulus oophorus cell were *in vitro* matured in TCM 199 with 10% Foetal Calf Serum (FCS) (v/v), Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Na Pyruvate with supplemented of 10 mM of LE (Group 1), 10 mM LAA (Group 2) or without any antioxidant (Group 3: Control) at 24 h. COCs with expanded cumulus layers following IVM were transferred to IVF TALP micro-drops with or without antioxidants (LE or LAA). Fresh semen from ram were separated by Percoll-Gradient and capacitated with Hepes TALP with heparine (30 IU/ml). Then, separated and capacitated spermatozoa were transferred to IVF TALP micro-drops containing matured oocytes and incubated for 20 hs. Presumptive zygotes were *in vitro* cultured in TCM 199 with or without antioxidants (LE or LAA) in 7-8 days. The results showed that, in LE group, the percentages of maturation, 8 -cell and morula stages were significantly higher than those of LAA and control groups ($P<0.01$-0.05) except for cleavage rate which had a numerically higher ($P\geq0.05$). In conclusion, addition of L-ergothionine into IVM, IVF and IVC medium had a beneficial effect on *in vitro* maturation of oocytes and embryonic development, especially from cleavage to morula stages compared to LAA and Control groups.

Keywords: *L-ergothioneine, Ascorbic acid, Antioxidant, In vitro maturation, Fertilization and culture, Sheep, Embryo, Oocyte*

Koyun Oositlerinin *İn vitro* Maturasyonu (IVM) ve Embriyonal Gelişimi (IVC) Üzerine L-Ergotiyonin ve L-Askorbik Asit'in Etkileri

Özet

Bu çalışmanın amacı koyun oositlerinin *in vitro* olgunlaştırılması ve olgunlaşmış oositlerin embriyonal gelişimi üzerine L-Ergothiyonin (LE) ve L-askorbik asit (LAA)'in etkilerini karşılaştırmaktı. Mezbahaneden toplanan ovaryumlar % 0.9'luk ılık (30-35°C) fizyolojik tuzlu suda (1-2 saat içerisinde) labaratuvara sevkedildi. Kumulus Oosit Kompleksleri folliküllerin ensizyonu ile elde edildi. Sadece 3 katlı cumulus hücre tabakasına sahip Kumulus Oosit Kompleksleri TCM199 [%10 Fötal Buzağı Serumu (FCS) (v/v), Follikül stimüle edici hormone (FSH), Luteinleştirici Hormon (LH) ve Sodyum Piruvat içeren] içinde 24 saat süreyle *in vitro* maturasyona (IVM) alındı. IVM ardından, genişlemiş cumulus tabakasına sahip Kumulus Oosit Kompleksleri IVF TALP mikro-droplarına aktarıldı. Koçlardan elde edilen taze sperma Percoll-Gradientle separe edildi ve heparin (30 IU/ml) içeren Hepes TALP ile kapasite edildi. Separe ve kapasite edilen spermatozoonlar, olgunlaşmış ositleri içeren IVF TALP mikro-droplarına aktarıldı ve 20 saat süreyle inkübe edildi. Olası zigotlar TCM 199'a aktarılarak 7-8 gün *in vitro* kültüre edildi. Çalışmanın sonuçları, LE eklenmiş grupta, sayısal olarak daha yüksek olan yarıklanma oranı hariç ($P\geq0.05$), 8-hücreli ve morula oranları açısından LAA ve kontrol gruplarından önemli derecede yüksekti ($P<0.01$-0.05). Sonuç olarak, IVM, IVF ve IVC medyumlarına LE ilavesinin oositlerin *in vitro* olgunlaştırılması ve özellikle yarıklanma-morula arası aşamalarda embriyonal gelişim üzerine yararlı etkileri görüldü.

Anahtar sözcükler: *L-ergotiyonin, L-askorbik asit, Antioksidan, İn vitro olgunlaşma, Fertilizasyon ve kültür, Embriyo, Oosit*

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INTRODUCTION

Oxidative stress has a negative effect on *in vitro* maturation and embryonic development of oocytes, and several studies have been conducted in this subject [1-4]. Various studies were conducted to measure the effects of antioxidants on oocyte maturation and early embryo development [1,5-14].

L-ergothionine (LE) with substantial antioxidant properties, has not been used in in-vitro fertilization (IVF) studies. In addition, oxidative stress has been considered in the aetiology of defective embryo development. Reactive oxygen species (ROS) are originated from embryo metabolism (enzymatic mechanism) and/or surrounded environment. The relative contribution of each ROS source differs according to developmental stages, species and culture conditions. It is also well known that several exogen factors and culture conditions could increase ROS production by embryos. ROS activity could change most types of cellular molecules and pathways, inducing block and regression of cellular development; as a result, embryos have multiple protective mechanisms against ROS as complementary systems. The extrinsic protection of embryo presented in follicular and oviductal fluids mainly involves antioxidants such as hypotaurine, taurine and ascorbic acid. Embryo instrinsic protection involves enzymes including superoxide dismutase, glutathione peroxidase and gamma glutamylcysteine synthetase. The transcripts for these enzymes were described in oviduct. These important transcripts are saved in order to get to capability of embryonal development. Considering these points, antioxidants are widely used in embryo culture medium [1].

LE is a powerful scavenger of hydroxyl radicals (OH) and an inhibitor of iron or copper ion-dependent generation of OH from hydrogen peroxide (H_2O_2) [15].

In a study, LE was tested for its ability to inhibit cell death caused by H_2O_2 and to inhibit DNA oxidation by peroxynitrite in a human neuronal hybridoma cell line in culture and it was suggested that LE could have a role as an antioxidant, *in vivo* non-toxic thiol compound and obtain the oxidative stability in pharmacological preparations [16]. The concentration of ergothionine in human and mammalian tissue was estimated in 1-2 mM, which suggests that the compound may act as a non-toxic thiol buffering antioxidant *in vivo,* and it could be used in pharmaceutical preparations where oxidative stability is desired. Intracellular ergothionine was stable to depletion of glutathione (GSH) by N-ethylmaleimide and more severe oxidant stress induced by hydrogen peroxide in the presence of catalase [17]. LE is also an inhibitor of copper ion-dependent oxidation of oxyhaemoglobin, and of arachidonic acid peroxidation promoted by combination of myoglobin (or haemoglobin) and H_2O_2 [15]. The absence of toxicity, the broad spectrum of its antioxidant properties, its high water solubility, and its unusual stability at physiologic pH make EGT a very attractive component of the antioxidant defence system. However, unlike a number of water-soluble antioxidants (e.g., flavonoids, phenolic acid, and polyphenols), EGT is cell membrane impermeative and requires a specific carrier to be internalized [18]. LE is determined in erythrocytes of human, rat, swine, cattle, cat, guinea pig, dog, poultry and pigeon and existed in all the species is shown as a unique and strong antioxidant that neutralises the hydroxyl radicals, hypochloric acid and peroxynitrites [18]. It has been shown that LE inhibits the peroxynitrite-mediated oxidation of amino acids such as the nitration of tyrosine and protects against the peroxynitrite-induced inactivation of alpha-1-antiproteinase [19].

Recently, it has been shown that antioxidant activity of LE is the highest, and it eliminates the most active free radicals compared to some classic well-known antioxidants such as glutathion, uric acid and trolox [20].

Studies have shown that LE has a critical protective role in seminal fluid [21-24]. LE is the predominant sulfhydryl in human, horse and pig semen. It has been clearly demonstrated that LE protects the spermatozoa from oxidative stress caused by the extraordinary high metabolic rate in these cells. LE, as consequence of its antioxidant properties, counteracts the effects of hydrogen peroxide on spermatozoa viability and survival while also enhancing the viability of sperm during storage [21,22]. Yıldız and Daşkın [23] reported that ram semen extenders supplemented with LE or combination of other antioxidants resulted in more successful in short term semen storage.

It has been known that the constant maintenance of the prooxidant balance in embryos is difficult and complicated during the addition of antioxidants. There is no study on the effects of an antioxidant with strong and unique features, such as LE. Thus, further studies are needed to limit and minimize the oxidative stress during embryo culture [1].

It was aimed to investigate the effects of LE on the IVM and IVC compared to control and LAA groups in this study.

MATERIAL and METHODS

COCs Collections and IVM

Sheep ovaries (different aged, fat tailed ewes) were collected from Municipality Slaughterhouse at Kars Province, according to the methods used by Birler et al.[5] and Guerin et al.[1] with slight modification, and kept in a filtered warm (30-35°C) phosphate buffer solution, then transferred to the laboratory within 1-2 h. Cumulus oocyte complexes (COCs) were obtained by incision and washing [(with Dulbecco's Phosphate Buffered Solution (PBS)] of 1-6 mm follicles. Only oocytes surrounded with at least three layers of compact cumulus cells were considered suitable for IVM. COCs were *in vitro* matured in TCM 199 (M5017, medium 199 with Earl's salt, Sigma Co Ltd., Germany) supplemented with 10% FCS (v/v), 25 mM $NaHO_3$ (S4019, Sigma), 25 mM Hepes, 2 mM Na Pyruvate (P3662, Sigma), 50 µM EGF, 10 µg/ml FSH, 10 µg/ml LH and 1 µg/ml estradiol, 50 µg/ml gentamicin (Basic media) [25,26]. COCs were cultured for 24 h, in three different media: (1) basic media with 10 mM of LE; (2) basic media with 10 mM of LAA and; (3) basic media (control). COCs were incubated in 0.5 ml medium drops covered with 0.3 ml mineral oil and kept at the incubator (38.5°C, 5% CO_2 in air) for 24 h.

Sperm Collection and Preparation

Fresh semen was collected by electro ejaculator from a proven fertility Tujin breed ram and prepared by percoll-gradient method [27]. Briefly, two layered Percoll (P1644, Sigma) (45%-90%) were used. Fresh semen (0.2 ml) was layered on discontinuous gradient of 45 and 90% (v/v) Percoll. To prepare 90% Percoll solution, Percoll was mixed 9:1 with a concentrated solution containing 31 mM KCl (P5405, Sigma), 800 mM NaCL (S5886, Sigma), 3 mM NaH_2PO_4 (S5011, Sigma) and 100 mM Hepes (H6147, Sigma), 2 mM $CaCl_2$ (C7902, Sigma), 0.4 mM $MgCl_2$, (M2393, Sigma) 21,6 mM lactic acid (L7900, Sigma) and 25 mM $NaHCO_3$. To prepare 45% Percoll solution, the 90% percoll solution was mixed 1:1 with SP-TL. The gradient consisted of 0.2 ml of semen layered over 1 ml layer of 45% and 1 ml of 90% Percoll in 15 ml conical tube. The gradient and spermatozoa were centrifuged at 700 g for 20 min. The sperm pellet was isolated, washed twice and kept for one hour at the incubator 39°C in TALP medium containing 100 µg/ml heparin and HEPES (SP-TALP) {modified TL-HEPES-PVA medium composed of 114 mmol NaCl/l, 3.2 mmol KCl /l, 2 mmol $NaHCO_3$/l, 0.34 mmol KH_2PO_4/l (P5655, Sigma), 10 mmol sodium lactate/l, 0.5 mmol $MgCl_2 \bullet 6H_2O$/l, 2 mmol $CaCl_2 \bullet 2H_2O$/l, 10 mmol HEPES/l, 0.2 mmol sodium pyruvate/l (P4562, Sigma), 12 mmol sorbitol/l, 0.1% (w/v) polyvinylalcohol, 25 µg gentamicin/ml and 65 µg potassium penicillin G/ml (P4687, Sigma) } for capacitation [28]. The concentration of spermatozoa was determined and then diluted in the equilibrated fertilization medium, SP-TL, supplemented with 6 mg/ml BSA (A3311, Sigma), 0.2 mM Na pyruvat, 100 IU/ml heparin (H3149, Sigma) and 10 µg/ml gentamicin to obtain final concentration of $0.1x10^6$ spermatozoa in 3 µl.

In Vitro Fertilization

After 24 h of the IVM, the oocytes showing cumulus expansion and having homogen ooplasm were partially denuded of granulose cells in TCM-199 (M5017, Sigma) with 300 IU/ml hyaluronidase for 3-5 min. Maximum fifteen oocytes with expanded cumulus layers were transferred to IVF TALP medium micro drops (97 µl) covered by mineral oil and then sperm ($0.1x10^6$ spermatozoa/3 µl) was added to all the groups of IVF medium containing oocytes. Oocytes and sperm were incubated for 20 h [28,29].

The Culture of Presumed Zygotes

After a 24 h incubation period, presumptive zygotes were denuded by successive pipetting and transferred to IVC medium composed by TCM-199 supplemented with FSH, LH, sodium pyruvate and 10% FCS. Embryo development was compared every day, for seven days, in three different media: (1) basic media with 10 mM of LE; (2) basic media with 10 mM of LAA and; (3) basic media at 38.5°C, 5% CO_2 and maximum humidity conditions. The rates of cleavage, 4-cell, 8-cell, morula, compact morula and blastocyst stages were determined.

Microscopic Evaluation

Oocyte maturation and embryonic development were observed by phase-contrast microscope and stereomicroscope (zoom). The oocytes with cumulus expanded were accepted as mature by stereo-microscope, followed by detecting of released first polar body by phase-contrast microscope *(Fig. 1-2),* then also confirmed the presence of first polar body by aceto-orcein (O7380, Sigma) fixation by light microscope.

Aceto-Orcein Staining

Preparation of Aceto-orcein staining solution (2%)

Orcein	1 g
Glacial acetic acid	55 ml
ddH_2O to	100 ml
Total volume	100 ml

Denuded oocytes were mounted on slides and fixed with 25% (v/v) glacial acetic acid in absolute ethanol for 24 h. After 24 h of fixation, the slides containing oocytes

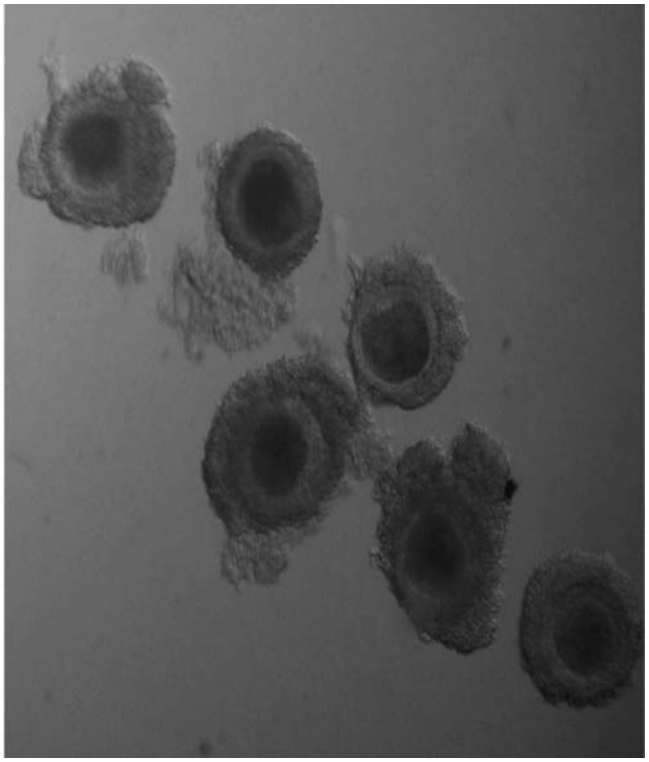

Fig 1. The cumulus oocyte complexes (COCs) taken for maturation (stereo-zoom)

Şekil 1. Olgunlaşma için işleme alınan kumulus oosit kompleksleri (stereo-zum)

were stained with 2% aceto-orcein and examined under microscope (40x - 400 x) [30].

Pour 55 ml boiling glacial acetic acid over 1 g orcein powder. Cool the solution, add 45 ml of distilled water and filter. Prepare fresh before use. All chemical additives came from Sigma Chemical Co., Germany, unless stated otherwise.

Statistical Analyses

Data were analyzed by using logistic regression procedure of SAS, and results were reported as odds ratios along with 95% confidence intervals [31].

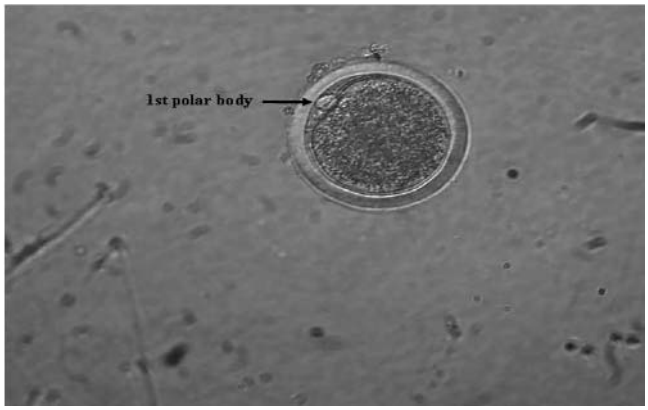

Fig 2. The observation of first polar body in oocytes with cumulus expansion at 24 h after IVM (stereo-zoom)

Şekil 2. Kumulus oosit genişlemesi gösteren oositlerde IVM'den 24 saat sonra birinci kutup cisimciğinin gözlenmesi (stereo-zum)

RESULTS

The results obtained are described in *Table 1*. No expanded and hatched blastocysts were observed. The percentages of oocyte maturation, cleavage, 8-cell and morula stage embryos in LE supplemented group (Group I) were significantly higher compared with the other groups (P<0.01-0.05). The percentages of oocyte maturation in LE Group (I) and LAA Group (II) were 2.67 and 1.22 times higher than control group respectively. The percentages of cleavage in LE Group (I) and LAA Group (II) were 2.01 and 1.34 times higher than control group respectively, which had a numerically higher tendency (P≥0.05). The percentages of 8-cell stage embryos in LE Group (I) and LAA Group (II) were 2.44 and 1.36 times higher than control group respectively (P=0.05). The percentage of morula in LE Group (I) and LAA Group (II) were 7.13 and 2.23 times higher than control group respectively (P<0.01).

Table 1. *The percentage of oocyte maturation and embryonic stages in experimental groups* [1]

Tablo 1. *Deneysel gruplarda oosit maturasyonu ve embriyonal aşamaların oranları* [1]

Developmental Stages (%)	L-Ergothionine n=184	Ascorbic Acid n=164	Control n=164
Maturation	80.4 (148/184) *	67.6 (111/164)	63.4 (104/164)
Cleavage	29.9 (55/184) **	25.0 (41/164)	21.9 (36/164)
4 - cell	19.6 (36/184)	12.8 (21/164)	6.7 (11/164)
8 - cell	14.1 (26/184) ***	9.1 (15/164)	5.4 (9/164)
Morula	6.5 (12/184) ****	3.0 (5/164)	1.8 (3/164)
Compact Morula	2.7 (5/184)	0.6 (1/164)	0.6 (1/164)
Early Blastocyst	2.7 (5/184)	0.6 (1/164)	0.6 (1/164)
Blastocyst	1.6 (3/184)	0.6 (1/164)	0.0 (0/0)

[1] *The percentages were calculated by based on the processed oocytes*
*** (P<0.01)* ** *Numerically higher* *** *(P=0.05)* **** *(P<0.01)*

DISCUSSION

The results of the present study indicate that LE has a significant effect on the oocyte maturation and embryonic development compared with the other groups, and this positive effect has been remarkably kept until morula stage. Among all significant variables, no differences were detected between control and L-ascorbic acid as a classical antioxidant. This result supports our hypothesis that L-ergothionine has more potential effect on embryo development compared to L-ascorbic acid.

No study focused on LE effects of IVM and IVC is available in the literature to compare the present results. Therefore, the effects of LE in the present study in the view of IVM and IVC could not be objectively discussed and compared to other investigations in details. Nevertheless, Dong et al.[32] showed that ergothionine is a natural skin antioxidant as evidenced by the presence of the ergothoneine transporters in fibroblasts. Ergothionine is a more powerful antioxidant than either coenzyme Q^{16} or idebenone due to its relatively greater efficiency in directly scavenging free radicals and in protecting cells from UV-induced ROS. However, there is only one study which is even indirectly interested in this subject was published by Guijarro et al.[33]. In this study, it was showed that the combination of ergothionine with vitamin E had an important role in part of prophylactic mechanism of diabetic embryopathy in rats. Furthermore, in the another study, it has been suggested that ergothionine may reduce skin anti-aging effects following UV irradiation by the scavenging of superoxide and singlet oxygen and reducing signals for protease and inflammatory activities [34].

In the present study, the percentages of embryonic stages mostly seem as lower than some studies' results [35,36]. It might be due to the some reasons such as composition of media, season (transition period), ram specific effect [37] or semen etc. In fact, it has been shown that the rates of *in vitro* fertilization and development of embryos are significantly affected by individual differences of ram semen. Fukui et al.[38] reported that results of unstable or unpredictable rates of *in vitro* fertilization, cleavage of embryos and oocyte maturation indicate that the development of sheep oocytes *in vitro* are differentially affected by different rams from which the spermatozoa are collected. Therefore, Madan et al.[39] showed that the frozen and thawed ram semen significantly affected the rates of *in vitro* fertilization. Low rates obtained in this study might be due to the period of season, as the present study was carried out in transition period of season.

In this study, merely it was hypothesized to investigate the effects of LE in comparison to a classic antioxidant and control. Morton et al.[40] have reported that the percentages of cleavage (in M 199 medium) at 24th hour and blastocyst (Sidney IVF blastocyst medium) at 7th day were 55.6% and 56.7%, respectively in sheep.

While the percentages of oocyte maturation in this study are similar to average of recent studies' results [40-43], the percentages of other developmental stages (cleavage, blastosyt etc.) are lower than those of them. O'Meara et al.[42] indicated that the percentages of cleavage and blastocyst were 72% and 41%, respectively in the presence of TCM 199 (as an IVM medium) and SOF (culture medium) in ewes .

Farin et al.[43] reported that the percentages of zygotes cultured with Menezo's B2 and TCM-199 media were 46% and 33% in bovine.

Cleavage rates obtained in this study are a little comparable, but the rates of blatocytes are low. Abdulhadi and Ahmed [44] recorded that the percentages of cleavage, 2-cell,4-cell and morula were 81%, 66%, 48 % and 14% in TCM medium; 62%, 25%, 40% and 7% in MCM medium, respectively. It seems as higher than the present study.

Henceforth, the success of the oocyte maturation and embryonic development may be increased with the combinations of another antioxidants, co-enzymes, amino acids or vitamins with LE [36,45,46]. In this regard, Minimum Essential Medium (MEM) with or without glutamine supplemented with or without oviduct epithelial cells were compared in a study performed on sheep oocytes, and it was concluded that the percentage of blastocysts was many times higher in group with glutamine than the group without glutamine (6%-35%).

On the other hand, addition of antioxidant alone is not enough to protect from ROS. Besides, the choose of antioxidant and its concentrations are very critical. The improving and increasing knowledge concerned with antioxidants and their mechanisms may contribute to development of embryos and evaluation methods of embryo/oocyte quality in *in vitro* culture systems. Nevertheless, Bucak et al.[47] revealed that different antioxidants had different effects in different culture medias. Similarly, different effects were determined between antioxidants in current study. In this context, it is needed to do more studies in order to reduce the oxidative stress [1]. Therefore, various aspects of studies are needed to investigate potential effects of LE (or antioxidants). In physiological concentrations, LE exhibits potent diffusion-controlled inactivation of hydroxyl radical and prevention of singlet oxygen production. It

does not function as a direct scavenger of superoxide anion, hydrogen peroxide or lipid peroxides [48-50]. Actually, it also differs significantly from natural thiol-containing antioxidants in that it does not stimulate lipid peroxidation in the presence of ferric ions [19].

In conclusion, addition of L-ergothionine into IVM, IVF and IVC medium had a beneficial effect on *in vitro* maturation of oocytes and embryonic development, especially from cleavage to morula stages in sheep. These positive effects of ergothionine could indicate potential applications for ergothionine in IVF and cryopreservation of oocytes and spermatozoa, and it warrants further research.

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