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The Effect of Adding L-Ergothioneine in Culture Medium on Blastocyst Development Rates

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ABSTRACT

***Background*:** Oocytes and embryos produce energy through mitochondrial oxidative phosphorylation by using oxygen. The membrane structure of the embryo is mostly composed of unsaturated fatty acids, for this reason DNA fragmentation, apoptosis, and abnormal gene expression are shaped as a result of the lipid peroxidation during culture. Oxidative stress (OS) is one of the most important problems affecting the *in vitro* embryo development. Antioxidant supplementation to the culture medium has been an alternative way to reduce cell damage caused by oxidative stress in *in vitro* embryo production systems. In this study, it was aimed to determine the effect of L-ergothioneine on blastocyst development when added to the culture medium.

***Materials, Methods & Results*:** The material of the study consisted of oocytes aspirated from the ovaries of Holstein cows which were collected from the local slaughterhouse. The ovaries were delivered to the laboratory within 2-3 h in a thermos which provided a constant temperature of 25-30ºC with physiological saline (0.9%) containing antibiotics. All follicles in the 3-8 mm range on the ovaries were aspirated using 20 G needle. The collected follicle fluid was filtered through filters with a pore diameter of 70 micrometers. Cells remaining in the filter were washed with OPU medium and transferred to the petri dishes. Fluids were examined under a stereomicroscope. The cumulus-oocyte complexes were classified, and A and B quality oocytes were included to the study (A, B, C, and D quality COC). Oocytes aspirated from the ovaries and collected later on were incubated in IVM medium for 22 h. After maturation, it was taken into IVF medium, semen was added and incubated for 20-22 h. Possible zygotes to be taken to the culture stage were transferred to culture (IVC) drops with (L-ergothioneine 100 µL/mL (n:121) added and without antioxidant (control (n:124)), and kept in the incubator for 6-7 days. Evaluated on the 7th day. differences in *in vitro* embryo production stages were evaluated with the Chi-square test. The study was run in 5 replicates each time, with at least 20 possible zygotes for per group being cultured. It was determined that 262 (87.33%) of a total of 300 oocytes undergoing *in vitro* maturation were matured. It was determined that 245 of the mature oocytes were fertilized (93.51%). The cleavage rates of the groups were determined as 87.60% and 86.29%, respectively. Eighty-two (33.47%) blastocysts were obtained from 245 zygotes taken into the culture stage, and the blastocyst rates in the groups were found to be 40.50% and 26.61%, respectively. After the study, it was determined that the statistical difference between L-ergothioneine and control in cleavage rates was insignificant ($P > 0.05$) and blastocyst rates was significant ($P < 0.05$)

***Discussion*:** Oxygen content above normal ratios can increase the formation of reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), and peroxyl radicals (ROO·). The increased rate of ROS negatively affects the success of IVP in mammalian embryos. It was observed that L-ergothioneine, which has high antioxidant activity, improved blastocyst development rates, and higher blastocyst rates could be achieved compared to the control group. By investigating the use of L-ergothioneine in different doses, it was thought that the dose with the highest antioxidant activity could be added to the culture medium in *in vitro* embryo production and more blastocysts could be produced.

Keywords: antioxidant, blastocyst, L-ergothioneine, *in vitro*.

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INTRODUCTION

Oocytes and embryos develop in the female reproductive system in an approximately 5-8% O_2-containing environment [3,4,22]. Excess oxygen in the environment in *in vitro* embryo production causes supraphysiological ROS levels which results in cell membrane damage, DNA fragmentation, increased inducing of apoptosis and abnormal gene expression [2,7,11-13], leading to problems such as deterioration in proteins and nucleic acids, suppression of embryo development and preservation of cellular integrity [16,19,25,30].

Oxidative stress is triggered by the fact that embryo culture media contains a lower concentration of antioxidants than needed to control ROS production [17]. By regulating oxidative stress, apoptosis can be prevented in the early periods of culture, and in vitro embryo development can be supported [1,27,29,35].

Antioxidants inhibit or delay the oxidation of other molecules [34]. They act as free radical scavengers, protecting cells or repairing damage caused by free radicals [36,37]. L-ergothioneine is a natural amino acid [26]. Although ergothioneine is synthesized by some fungi and bacteria [5], it cannot be synthesized in mammals [23]. It acts as an antioxidant and cellular protector against various types of ROS [26]. It has also been reported that L-ergothioneine can target mitochondria and reduce mitochondria-specific ROS (mROS) [28]. With the thought that L-ergothioneine, which is used for freezing sperm, can also be used for its antioxidant activity during embryo development; it was aimed to determine the effect of L-ergothioneine added to the culture medium on blastocyst development.

MATERIALS AND METHODS

Chemical materials

Bioscience bovine IVF (BO-IVM, BO-IVF, BO-IVC, Semenprep, BO-Wash, BO-Oil)[1] kit and L-Ergothioneine[2] were used in the study.

In vitro *embryo production (IVEP)*

The material of the study consisted of oocytes aspirated from the ovaries of Holstein cows collected from the local slaughterhouse. The ovaries were delivered to the laboratory within 2-3 h in a thermos that provided a constant temperature of 25-30°C with physiological saline (0.9%) containing antibiotics. All follicles in the 3-8 mm range determined on the ovaries were aspirated using a 20 G needle. The collected follicle fluid was filtered through filters with a pore diameter of 70 micrometers. Cells remaining in the filter were washed with OPU medium and transferred to petri dishes. Fluids were examined under a stereomicroscope[3]. The cumulus-oocyte complexes were classified, and A and B quality oocytes were included in the study (A, B, C, and D quality COC).

While evaluating the collected COCs, the following criteria were used;

A Grade: Has ≥ 5 compact cell layers and homogeneous cytoplasm,

B Grade: It has few inhomogeneous areas in the cytoplasm and 3-5 layers of compact cell layers or ≥ 5 layers of compact cells and dense inhomogeneous areas,

C Grade: Has few cell layers (> 3) and few inhomogeneous areas in the cytoplasm or localized loss of cumulus along with lesser homogeneous area.

D Grade: It has small, granular, inhomogeneous cytoplasm with complete loss of the cumulus layer [33].

The oocytes to be included in the study were collected in petri dishes containing BO WASH solution. Then washed 3 times within IVM medium and transferred to IVM (maturation) medium, which was equilibrated for 24 h. Oocytes were incubated in a mono gas incubator[4] (38.5°C, %6 CO_2, in Air, high humidity) for 22 h. The thawed (37°C) semen was taken into a heated 2 mL semenprep and centrifuged at 328 *g* for 5 min, the process was repeated after the supernatant was discarded. After the second supernatant was removed, it was diluted to contain 10,000 spermatozoa per oocyte (100,000 spermatozoa/30 µL). The semen, together with 10 mature oocytes, were placed in IVF (fertilization, 70 µL) medium which was equilibrated overnight and kept in the incubator for 20-22 h. Fertilization control was performed by observing the formation of a fertilization cavity or a second polar body in the cytoplasm of the oocyte. Before being taken into the culture medium, the cumulus cells were cleaned with the help of a pipette. The culture media[1] was prepared with antioxidants (L-Ergothioneine 100 µL/mL) and without antioxidants (control). Possible zygotes were transferred in the culture (IVC) media (L ergothioneine, n:121) [Control, n:124] and cultured in

the incubator[4] (38.5°C, %6 O_2, %6 CO_2, %88N, high humidity). Blastocyst rates and embryo quality were evaluated on the 6th and 7th days in the culture medium. The study was run in 5 replicates each time, with at least 20 possible zygotes per group being cultured.

Calculus equations

The following formulas were used in the calculations [21,24]:

$$\text{Maturation Rate} = \frac{\text{Number of oocytes with cumulus expansion or polar body detected (Mature oocyte)}}{\text{Number of oocytes left to maturation}}$$

$$\text{Cleavage Rate} = \frac{\text{Number of embryos divided into 2 or more cells}}{\text{Number of mature oocytes}}$$

$$\text{Blastocyst Rate} = \frac{\text{Blastocyst count}}{\text{Number of mature oocytes}}$$

Statistical analysis

SPSS-Statistics-22[5] package program was used for statistical analysis. Differences in *in vitro* embryo production stages were evaluated with the Chi-square test.

RESULTS

For each replication, minimum 10 ovaries were brought from the slaughterhouse. As a result of the aspiration made from the received ovaries, an average of 6.8 oocytes, and from these oocytes 5.9 A and B quality oocytes were collected per ovary. It was determined that 262 (87.33%) of the 300 oocytes taken into the maturation stage were matured and 245 of these oocytes were fertilized (93.51%) in the fertilization stage. It was determined that 15 of the possible zygotes cultured in the L-ergothioneine group together with 17 in the control group were not cleaved. It was determined that the cleavage rates of the groups were 87.60% and 86.29%, respectively, and the statistical difference was insignificant ($P > 0.05$).

Eighty-two (33.47%) blastocysts [Figure 1] were obtained from 245 oocytes which were taken into the culture stage and the blastocyst rates in the groups were 40.50% and 26.61%, respectively. After the study, it was determined that there was a statistically significant difference in blastocyst rates compared to the L-ergothioneine control group ($P < 0.05$) [Table 1].

Table 1. Comparison of maturation, cleavage and blastocyst rates between culture media.

Number of matured oocytes	300	
Maturation rate %	87.33 (262)	
Number of fertilized oocytes	262	
Fertilization rate %	93.51 (245)	
	L-ergothioneine	Control
Cleavage Rate %	87.60	86.29
Blastocyst Rate %	40.50[a]	26.61[b]

[a,b]There was a statistically significant difference ($P < 0.05$) in blastocyst rates compared to the L-ergothioneine control group.

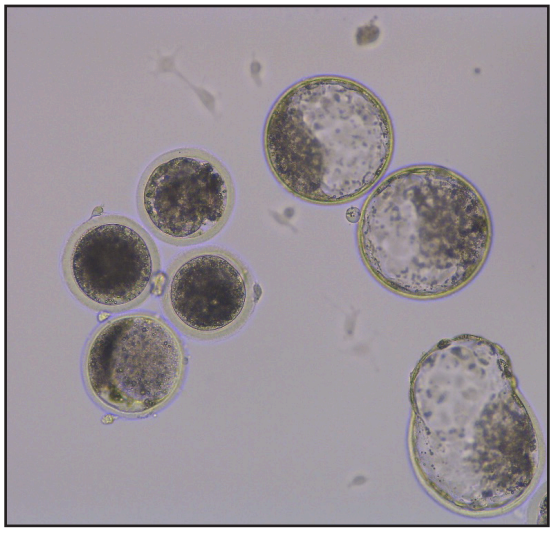

Figure 1. Expanded and hatched blastocysts and degenerated embryos at day 7 in culture medium.

DISCUSSION

To increase cellular protection against the harmful effects of ROS during *in vitro* embryo culture, O_2 levels can be reduced (5-7%) and antioxidants can be added [20,31]. However, it is still under investigation whether antioxidants are required during embryonic development under low O_2 levels, and whether this protection should be intracellular or extracellular. The balance between ROS production and scavenging appears to be a significant factor in the acquiring of *in vitro* fertilization ability [6].

As a result of the *in vitro* embryo production experiment conducted to compare the addition of 50 µM and 100 µM L-ergothioneine to the culture medium with the control group, it was reported that the cleavage rates were 78%, 78%, and 80.4%, respectively. At this study, blastocyst rates were reported as 41.5%, 43.5% and 46% respectively. It was reported by the researchers that enrichment of the *in vitro* culture medium with the L-ergothioneine increases the embryo quality which is also important for development of embryo after implantation [38]. In another study, it was reported that in the group which 50 µM L-ergothioneine was added to the culture medium the cleavage ratio was 92.55 and was 92.04 in control groups in respect. It has been reported that the blastocyst ratios in the groups were 45.74 and 18.58, and the blastocyst development rates were statistically significant [14].

It has been reported that L-ergothioneine scavenges hydroxyl radicals, hypochlorous acid, and peroxyl radicals, [9,10,32] and inhibits peroxynitrite-dependent nitration of proteins and DNA [8]. It is stated that the antioxidant activity of L-ergothioneine is quite high and it scavenges the most active free radicals compared to some classical well-known antioxidants such as glutathione (GSH), uric acid, and trolox [18]. In the presented study, it was observed that the blastocyst rate was similar to the results of L-ergothioneine in previous studies. Influencing the success of *in vitro* embryo production of blastocyst rates obtained in the control group; it was thought that it might be affected by factors such as age, race, body condition score (BCS), follicle diameter, cycle period, oocyte quality, oocyte diameter.

In a study which buffalo oocytes were used for IVF; result of the comparisons of the groups that were added 0.05 mM, 0.1 mM, and 1 mM L- ergothioneine to the culture medium with the control group, the cleavage rates were 71.4%, 66.8%, 68.7%, and 63%, respectively; blastocyst rates were reported to be 21.6%, 30.9%, 33.9% and 21.7% [39]. The fact that the reproductive parameters in buffaloes are quite low compared to the cattle and they are affected by many factors such as breed care and feeding was thought to be the reason for the difference in the results.

In the presented study, it was observed that blastocyst rates increased significantly in each replication as a result of the addition of L-ergothioneine to the culture medium and this addition supported blastocyst development. It was determined that the blastocyst rates obtained were consistent with the literature or even higher.

CONCLUSION

In the light of the information presented, it was observed that L-ergothioneine, which has high antioxidant activity, improved blastocyst development rates, and higher blastocyst rates could be achieved compared to the control group. By investigating the use of L-ergothioneine in different doses, it was thought that the dose with the highest antioxidant activity could be added to the culture medium in *in vitro* embryo production and more blastocysts could be produced.

MANUFACTURERS

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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