



## RESEARCH ARTICLE

### Effect of Ergothioneine and Quercetin Additions in to the *In Vitro* Embryo Development

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### *In Vitro* Embryo Gelişiminde Ergotiyonin ve Kuersetin İlavesinin Etkisi

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#### Öz

**Amaç:** Bu çalışmanın amacı, ergotiyonin ve kuersetinin sığır embriyolarının in vitro gelişimi üzerindeki etkilerini araştırmaktır

**Gereç ve Yöntem:** Oositler yerel bir kesimhaneden ovaryum aspirasyonu yoluyla elde edildi ve maturasyon için in vitro ortamda kültüre alındı. Maturasyon sonrası fertilizasyon işlemi gerçekleştirildi. Pronükleer embriyolar kontrol, 10 µM L-ergotiyonin ve 10 µM kuersetin ilave edilen olmak üzere üç gruba ayrıldı. Antioksidanların CR1aa besi ortamına eklenmesinin ardından embriyolar in vitro kültüre edildi. Bölünme ve morula oranları sırasıyla 2. ve 5. günlerde değerlendirildi. Blastosist oluşumu ve kalitesi 7-8. günlerde belirlendi.

**Bulgular:** İstatistiksel analizler ergotiyonin grubunda bölünme ve morula oranlarının kuersetin ve kontrol grubuna göre anlamlı olarak daha yüksek olduğunu gösterdi (P<0,05). Kuersetin grubunda hiç blastosist oluşmazken, ergotiyonin grubunda 8. günde %17,96'lık bir blastosist oranı saptandı.

**Öneri:** Sonuç olarak, 10 µM ergotiyonin sığır oositlerinin in vitro gelişimini olumlu yönde etkilediği; ancak 10 µM kuersetinin gelişimi olumsuz yönde etkilediği ve hiç blastosist oluşmadığı belirlendi. Farklı konsantrasyonların kullanılacağı ileri çalışmalara ihtiyaç vardır. Çalışma, in vitro embriyo üretimi sırasındaki oksidatif stresin kontrolünü sağlamaya ışık tutmaktadır.

**Anahtar kelimeler:** Embriyo, in vitro fertilizasyon, L-ergotiyonin, kuersetin

#### Abstract

**Aim:** This study aimed to investigate the effects of ergothioneine and quercetin on the in vitro development of bovine embryos

**Materials and Methods:** Cumulus-oocyte complexes (COCs) were collected via ovary aspiration from a local abattoir and cultured in vitro for maturation. After maturation, in vitro fertilization was performed. The pronuclear embryos were divided into three groups: control, 10 µM L-ergothioneine, and 10 µM quercetin supplemented. After the addition of antioxidants to the CR1aa medium, in vitro culture of embryos were performed. The cleavage and morula rates were assessed on days 2 and 5, respectively. Blastocyst formation and quality were assessed on days 7-8.

**Results:** Statistical analysis showed cleavage and morula rates were significantly higher in the ergothioneine group compared to the quercetin and control groups (P<0.05). While no blastocysts formed in the quercetin group, the blastocyst rate reached to 17.96% with ergothioneine supplementation on day 8.

**Conclusion:** In conclusion, supplementation with 10 µM ergothioneine enhanced the in vitro development of bovine oocytes. However, 10 µM quercetin supplementation impaired development, and no blastocyst formation observed. Further studies utilizing different concentrations are warranted to better understand the effects. This study provides insights into modulating oxidative stress during in vitro embryonic production.

**Keywords:** Embryo, in vitro fertilization, L-ergothioneine, quercetin

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## Introduction

Recent advances in the field of animal reproduction have facilitated the emergence of assisted reproduction techniques (ART). The goal of assisted reproductive technology (ART) is to maximize the production of offspring from animals with superior genetic traits and to disseminate their genetic material worldwide. In addition, these techniques strive to use donors with anatomical limitations, preserve species and contain the spread of disease (Berglund 2008). The global cattle population is approximately 1.5 billion animals (Mottet et al 2017). Cattle have a significant impact on the global production of meat and milk (Alexandratos and Bruinsma 2012). The introduction of Assisted Reproductive Technologies (ART) in cattle production aims to improve operational efficiency (Greenwood 2021). Techniques grouped under ART (Assisted Reproductive Technology) include artificial insemination, MOET (Multiple Ovulation and Embryo Transfer), in vitro production techniques, and semen sexing. The main goal of assisted reproductive technology (ART) is the successful birth of children in good health. This is achieved through the use of various techniques involving the modification of germ cells (sperm and eggs) and embryos (Galli et al 2003).

The Data Retrieval Committee of the International Embryo Technology Society (IETS) presented the 31st annual report on global embryo transfer (ET) activities in 2021. In 2021, around 20% of countries reported ET statistics. The consistency of the data is due to continuous data collection in the Americas and Europe. However, detailed information from Africa and Asia is missing. Embryo export statistics show that the technologies are used in 28.7% of countries worldwide. In 2021, a total of more than 2 million embryos were collected or produced from the four main livestock species, namely cattle, sheep, goats and horses. The number of cattle with in vitro produced (IVP) embryos exceeded 1.5 million, a significant increase of 31.5% compared to the previous year. In particular, the number of embryos harvested and transferred from living organisms (in vivo) increased compared to 2020. The number of cow embryos increased by 25.6% worldwide, which is the highest growth rate since 2003. In 2021, in vitro-produced (IVP) embryos accounted for 79.7% of all cow embryos suitable for transfer worldwide (International Embryo Technology Society by Viana 2022).

L-Ergothioneine (L-Erg) is a naturally occurring amino acid that has the ability to effectively remove OH<sup>-</sup> and prevent the formation of OH<sup>-</sup> from H<sub>2</sub>O<sub>2</sub> with the help of iron or copper ions (Akanmu et al 1991). Oxidative stress is characterized by an imbalance in the redox system in which there is a significant increase in free radicals that exceeds the body's ability to remove them using its own antioxidant system (Liochev 2013, Schieber and Chandel 2014). Reactive oxygen species (ROS)

account for 95% of free radicals (Halliwell and Cross 1994). ROS is an abbreviation for reactive oxygen species, which includes free oxygen radicals such as superoxide anions(O<sub>2</sub><sup>-</sup>), hydroxyl radicals (HO<sup>-</sup>), alkoxy (RO<sup>-</sup>), peroxy (RO<sub>2</sub><sup>-</sup>) and non-radicals such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Addabbo et al 2009). Intracellular ROS are mainly generated by the degradation of substrates of the mitochondrial respiratory chain, which is facilitated by NADPH oxidase (Agarwal et al 2012). The right amount of ROS is needed to help cells grow and break down food by starting up signaling pathways inside cells (Sies 1997). However, if the production of free radicals exceeds the body's ability to eliminate them, an excessive number of intracellular ROS would form and cause oxidative stress to DNA, proteins, lipids and carbohydrates, leading to various damages of biological macromolecules and organelles. L-Erg is an endogenous, hydrophilic amino acid that is mainly found in fungi and can be dissolved in water. It mitigates the harmful effects of UV radiation and counteracts oxidative stress by having the ability to scavenge reactive oxygen and nitrogen species (RONS) (Akanmu et al 1991). L-Erg suppresses the formation of hydroxyl radicals, superoxides and singlet oxygen as well as the oxidation of lipids (Obayashi et al 2005). Mammals ingest ergothioneine through their diet, which includes mushrooms, oats, corn and meat (Ey et al 2007). Previous studies have shown the effects of L-Erg on in vitro maturation and embryonic development in sheep and cattle (Ozturkler et al 2010, Zullo et al 2016).

Some flavonoids have polyphenolic structures with hydroxyl groups that are able to eliminate free radicals, which classifies them as free radical scavengers. These chemicals have an antioxidant effect. The antioxidants mentioned above are found in a variety of medicinal herbs, vegetables, fruit juices and beverages (Ciccoli et al 2008). Quercetin, specifically known as 3,3',4',5,7-pentahydroxyflavone, is the predominant flavonoid found in various foods such as leafy vegetables, fruits, tea, wine, comparable to forsythia (Boots et al 2008). Quercetin is believed to protect the human body from various diseases due to its ability to eliminate free radicals (Kim and Jang 2009). Recently, much research has been conducted on the consumption of quercetin in the diet, mainly due to its antioxidant properties (Robaszekiewicz et al 2007, Xu et al 2019). The research also found that fully developed porcine oocytes had a lot less ROS than the control group (Davis et al 2009, Kang et al 2016). Several studies suggest that quercetin may exhibit anti-inflammatory and antioxidant properties, due to its ability to scavenge free radicals and chelate metals (Naderi et al 2003). It also has strong antioxidant effects. However, it has also been hypothesized that quercetin may have pro-oxidant effects (Kim and Jang 2009). A link has been established between quercetin's ability to scavenge free radicals and its abilities as an anti-carcinogenic and anti-inflammatory agent (Jackson et al 2006, Yuan et al 2006). Studies have shown that the addition of quercetin affects both the maturation process



(Kang et al 2013, Kang et al 2016, Banihosseini et al 2018) and in vitro development rates (Yu et al 2014, Khadrawy et al 2020, Cerezetti et al 2021).

The addition of L-ergothioneine and quercetin to the media used for in vitro embryo formation has the potential to improve the quality of oocytes and embryos by mitigating oxidative damage. The aim is therefore to increase fertilization and implantation rates. However, further empirical studies are needed to substantiate this claim. The aim of this study was to investigate the effects of ergothioneine and quercetin on the in vitro development process.

## Material and Methods

### Reagents

Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Recovery of Cumulus-oocyte complex (COC) and In Vitro Maturation (IVM)

The procedure for obtaining embryos was identical to that reported by Bucak et al (2010). The maturation media was manually prepared 18 hours prior to oocyte aspiration. Following the filtration process, the produced medium was dispensed in drops measuring 100 micrometers onto petri plates. Subsequently, the drops were coated with mineral oil. The ovaries were obtained subsequent to the slaughtering process at the nearby slaughterhouse. They were then immersed in a solution of physiological saline containing 0.9% sodium chloride, and maintained at a temperature of around 37.5°C. Subsequently, the ovaries were delivered to the laboratory within a maximum time frame of 4h following the slaughtering process. Following the procedure outlined by Wydooghe et al (2011), the ovaries were subjected to a thorough washing using fresh Dulbecco's phosphate-buffered saline (D-PBS) in order to collect the cumulus-oocyte complexes (COCs). The cumulus oocyte complexes (COC) were obtained by aspiration from antral follicles measuring 2-8mm in diameter. This was achieved using an 18-gauge needle and a disposable 10mL syringe specifically designed for this purpose. The procedure was performed on the ovaries in question. Following this, the acquired COCs were examined using a stereomicroscope and assessed in accordance with the methods outlined by Gordon Ian (2004). The research only included oocytes of A-quality, which are characterized by their compactness, multilayered structure, and uniform ooplasm. These oocytes also include cumulus cells that cover the whole zona pellucida. The maturation process was carried out in an incubator at 5% CO<sub>2</sub>, over 95% humidity and 38.5°C for 22h.

### In Vitro Capacitation and Fertilization (IVC and IVF)

The capacitation and fertilization media was manually prepared 18h before using according to Bucak et al (2010).

The oocytes, which had undergone full maturation, were retrieved from the incubator, and the evaluation of cumulus expansions was conducted (Machado et al 2015). In this study, the growth of cumulus clouds was assessed using three different methodologies. Grade 1 exhibits minimal expansion and limited morphological alterations in comparison to the pre-maturation phase. Grade 2 displays partial cumulus expansion alongside areas that remain unexpanded. Grade 3, on the other hand, showcases complete or nearly complete cumulus expansion, accompanied by a uniform distribution of cumulus cells. Subsequently, the cumulus-oocyte complexes (COCs) exhibiting a cumulus expansion degree of 2 and 3 were relocated from the maturation medium to the in vitro fertilization medium and maintained inside the incubator until the semen was appropriately processed.

The oocytes underwent fertilization for a period of 5-6h through direct washing using Brackett Oliphant (BO) media, along with cumulus expansion. Cryopreserved bovine semen, contained in 0.25 milliliter straws, each containing 17.5 million spermatozoa, was utilized in the fertilization process. Modified Brackett and Oliphant medium was employed to cryoprotect, separate, reconstitute, and capacitate spermatozoa by removing seminal plasma. A concentration of 5 IU/mL heparin and 2mM caffeine was used for the capacitation process. To ensure optimal spermatozoa density and capacitation, the thawed semen (4-5 straws) was treated with a sperm washing solution (SWS) and subjected to centrifugation at 1800 rpm for 5 min. This centrifugation process was repeated, and the supernatant component was separated. The semen was initially thawed in a water bath set at 37°C (Bucak et al 2010).

To facilitate spermatozoa concentration, 1 ml of sperm diluting solution (SDS) was added to the sediment and the spermatozoa contained in the sediment were quantified by counting on the Thoma slide. The fertilization media, comprised of a final dilution with sperm diluent to achieve a concentration of 6.25x10<sup>6</sup> motile spermatozoa/ml, were then incubated in the incubator for 15-20 min for capacitation. Following this, fertilization procedures were carried out for a duration of 18h in 100µl fertilization medium drops, containing matured oocytes that had been washed with oocyte washing medium (Nagao et al 1995). Each drop typically contained 15-20 oocytes.

### Preparation of the quercetin and L-ergothioneine

A stock solution of quercetin was prepared by diluting it to a concentration of 1 mM with DMSO (D4540, Sigma-Aldrich Co, USA). From this stock solution, quercetin hydrate (Cat. No. 337951, Sigma-Aldrich Co, USA) was added at a rate of 10 micromolar in a 100 microliter culture (CR1aa) droplet (Yu et al 2014). A stock solution of L-ergothioneine (E7521, Sigma-Aldrich Co, USA) was prepared by diluting it to a concentration of 1 mM with DMSO. From this stock solution,



L-ergothioneine was added at a rate of 10 micromolar in a 100 microliter culture (CR1aa) droplet (Zullo et al 2016).

### *In Vitro Culture*

The culture medium was prepared manually 18 hours prior to use, following the protocol described by Bucak et al (2010). Pipetting was used to remove cumulus cells before in vitro culture. Following pipetting, doses of 10  $\mu$ M Ergothioneine and Quercetin were added to the CR1aa culture medium, resulting in the formation of three experimental groups, including a control group. Following the aforementioned technique, putative zygotes were subjected to regulation and then placed in an in vitro culture medium, which was then coated with mineral oil. The zygotes were cultured for a duration of 7 to 9 days at a temperature of 38.5°C, with an atmosphere consisting of 5% carbon dioxide and 20% oxygen. The evaluation of embryo quality and developmental stages achieved by culture was conducted in accordance with the standards established by the International Embryo Technology Society (IETS).

On the second day of the culture medium, after conducting cleavage controls, the development of the embryos in the culture medium was monitored. On the seventh day, the blastocyst rates and embryo quality were evaluated. The hatching rates were recorded by observing the embryo development on the eighth and ninth days.

### *Statistical Analysis*

The SPSS version 25.0 for windows program (IBM SPSS Statistics for Windows, Version 25.0. IBM Corp.) was used throughout all phases of the study's statistical analysis. The data from each treatment group were first subjected to a one-way analysis of variance (ANOVA), then subjected to Duncan's multiple range post hoc test. This was done so that we could make comparisons across the groups. The data are provided with the mean together with its associated standard error. In every instance,  $p < 0.05$  was regarded as statistically significant.

### **Results**

As shown in Table 1 embryos presented higher cleavage and morula rate in both the ergothioneine (E) group (respectively  $51.35 \pm 6.81$ ,  $32.26 \pm 6.26$ ), and the control (C) group (respectively  $45.27 \pm 6.60$ ,  $25.2 \pm 5.25$ ) when compared to the quercetin (Q) group (respectively  $6.08 \pm 2.53$ ,  $1.47 \pm 1.47$ ) ( $p < 0.05$ ).

The percentage of morula stage embryos on day 5 and day 6 was significantly higher in both group E and group C compared to the group Q ( $p < 0.05$ ).

Although the cleavage and morula stage embryos percentage

was higher in the group E (respectively  $51.35 \pm 6.81$ ,  $32.26 \pm 6.26$ ) compared to the group C (respectively  $45.27 \pm 6.60$ ,  $25.2 \pm 5.25$ ) the difference between the E and C groups was not statistically significant ( $p > 0.05$ ).

When embryos examined on day 8, blastocyst rate was higher in group E ( $17.96 \pm 4.13$ ) compared to group C ( $6.43 \pm 2.09$ ) and group Q (0) ( $p < 0.05$ ). However hatched blastocyst rates on day 9 was similar between groups.

### **Discussion**

The manipulation of oocyte and sperm in IVF typically results in oxidative stress, which causes an increased formation of ROS during the co-incubation period (Agarwal et al 2014). This can have harmful consequences on both gametes, ultimately resulting in decreased development of embryos in vitro. Utilizing targeted antioxidants, such as L-ergothioneine and Quercetin, to counteract ROS can safeguard gametes from harmful ROS effects and perhaps enhance the effectiveness of assisted reproductive technologies (ARTs) (Al-Gubory et al 2004, Al-Gubory et al 2012, Tamura et al 2014, Tamura et al 2020).

Ergothioneine is a derivative of histidine and thiourea that exists in two forms, thiol and thion (Sao Emani et al 2019). The ergothioneine used in our study was in the thione form. It has been reported that the thione form of ergothioneine provides ideal thermal and pH stability in solution (Fu and Shen 2022). It has also been reported that ergothioneine increases maturation and fertilization rates (Agustin et al 2023). In our research, the highest blastocyst rate was found to be 17.96% in the culture group to which ergothioneine was added, compared to the control group. Jeong et al (2023) found the highest blastocyst rates statistically in groups to which 10 and 50  $\mu$ M L-ergothioneine were added to pig culture media (49% and 44%, respectively). Cizmeci and Yusufu (2022) studied 100  $\mu$ L/mL L-ergothioneine in cattle and obtained a statistically higher blastocyst rate in the L-ergothioneine group (40.50%) compared to the control group (26.71%). On the similar study performed by Yusufu and Cizmeci (2022) determined the highest blastocyst rate statistically in cattle in vitro development media supplemented with 100  $\mu$ M L-ergothioneine, 100  $\mu$ M Vitamin E and 50  $\mu$ M Sisteamine (33.13%) compared to the control group (32.62%). Parallel to other studies, our study clearly shows L-ergothioneine increases blastocyst rates. However, the lower blastocyst rates we obtained may be due to characteristics of the incubator used (mono- or trigas), commercial solutions used in other studies, and differences in application. Further studies are needed using different ergothioneine concentrations and incubation conditions.

Quercetin, a prominent bioflavonoid present in fruits and vegetables, demonstrates distinctive biological



Table 1. Percentages of embryos examined during/reached different developmental stages in CR1aa culture medium containing various antioxidants

Groups	n	Cleavage (48th hours) %	Morula (day 5-6) %	Blastocyst (day 8 ) %	Hatched Blastocyst (day 9) %
Cr1aa (C)	122	45.27 ± 6.60 <sup>a</sup>	25.2 ± 5.25 <sup>a</sup>	6.43 ± 2.09 <sup>b</sup>	2.11 ± 1.04
Cr1aa+ Ergothioneine (E)	123	51.35 ± 6.81 <sup>a</sup>	32.26 ± 6.26 <sup>a</sup>	17.96 ± 4.13 <sup>a</sup>	4.37 ± 2.69
Cr1aa+ Quercetin (Q)	123	6.08 ± 2.53 <sup>b</sup>	1.47 ± 1.47 <sup>b</sup>	0 <sup>b</sup>	0
P-values		0,000	0,000	0,000	0,203

<sup>ab</sup> Values within a row with different superscripts differ significantly at P<0.05

characteristics (Abdelkawy et al 2017). The article describes a wide range of advantageous features of quercetin, such as its ability to prevent mutations, reduce fibrosis, decrease inflammation, lower the risk of atherosclerosis, and fight against germs. Additionally, quercetin has a strong capacity to prevent oxidative damage. Furthermore, the primary elements that appear to be significant are the antihypertensive effects on the human body and the enhancement of endothelial function (Boots et al 2008, Murakami et al 2008, D'Andrea 2015). Khadrawy et al (2020) added quercetin at doses of 5, 10 and 20 µM to in vitro culture media in cattle and obtained the highest blastocyst rate in the group with 10 µM quercetin added compared to the control. Maturana et al (2019) found the highest blastocyst rate statistically in cattle in vitro culture media supplemented with 1 and 5 µM quercetin compared to the control group when they added quercetin at doses of 1, 5, 10 and 15, 20 and 50 µM. Karimian et al (2018) obtained the highest blastocyst rates in sheep in vitro culture media supplemented with 5 and 15 µg/mL quercetin when they added quercetin at doses of 1, 5, 15, 25, 50, 100, 200, 400, 800, 1000 µg/mL. In our study, 10 µM quercetin was added to the in vitro development medium and a decrease in daily development rates was observed with no blastocyst formation. Similarly, in a study on mouse embryos (Yu et al 2014), 10 µM quercetin was reported to reduce in vitro development rates and cell numbers. Looking at other studies, higher blastocyst rates were obtained with quercetin doses below 10 µM. The lack of blastocyst formation in our study may be due to chelation complexes forming between quercetin and divalent cations (Na<sup>+2</sup> and K<sup>+2</sup>) in CR1aa, or a redox system causing quercetin loss. Complex formation with cations in certain solutions has also been reported for quercetin (Leopoldini et al 2006). Quercetin is also reported to induce apoptosis by disrupting glucose metabolism (Gao et al 2012, Yang et al 2015). It can be said that 10 µM quercetin application in our study may

have disrupted glucose metabolism in embryos. According to Muñoz-Reyes et al (2021), quercetin metabolites not quercetin itself, can enter in in vivo conditions. But it can be changed in in vitro condition. We think that it acts outside the cell since there is no enzyme that can break down quercetin in the in vitro environment. According to Gründemann et al (2022), ergothioneine can not penetrate the cell membrane.

While ergothioneine and quercetin have many beneficial effects, their effects can clearly differ in certain environments and solutions. In summary, supplementing in vitro culture media with 10 µM L-ergothioneine has a positive effect on embryo development in vitro. Quercetin at the same dose was found to have a negative effect. Therefore, more studies are needed on quercetin.

### Conclusion

The results of this study are important for animal husbandry and reproduction as they determine the effects of ergothioneine and quercetin on in vitro embryo development. Improving embryo quality and development, and increasing fertilization and implantation rates will positively affect reproductive efficiency. Based on the results, the widespread use of in vitro culture media containing ergothioneine can be recommended, while quercetin concentration should be tested at lower levels. The effects of ergothioneine and quercetin use at different species and concentrations can also be investigated. Additionally, the effects of different environmental factors can be examined. The findings indicate that ergothioneine use in in vitro embryo production by producers may become widespread, thus increasing productivity. Based on the initial results, new studies under different conditions can be planned and method recommendations can be developed.



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## Conflict of Interest

Authors declare that there are no conflicts of interest related to the publication of this article.

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### Author Contributions

Data Collection, Writing Article, Performing project: MB; Literature Review, Performing the Project: AEO; Design and Performing the Project: ME; Data Analysis: YEA; Literature Review: SN; Critical Review: MBA; Supervisor of the Project, Article control: MNB

### Ethical Approval

This study was approved by the Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (SÜDAVMEK) (Approval no: 2016/41).