Original Article

Gamma-oryzanol dose optimization in maturation or culture media for *in vitro* ovine oocyte and embryo development

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Abstract

Background: The efficiency of ovine *in vitro* embryo production remains low yet. **Aims:** The present study evaluated the effect of different concentrations of gamma (γ)-oryzanol in maturation or culture media on *in vitro* ovine oocytes and embryo developments. **Methods:** Morphologically normal COCs were aspirated from ovine ovaries, subjected to maturation media supplemented with 0, 2.5, 5, 10, 20, 50, and 100 µM γ-oryzanol, then processed for conventional *in vitro* fertilization and culture to assess their potential to cleave and develop to blastocyst. Another group of COCs was matured and fertilized. Presumptive zygotes were subjected to culture in drops of media supplemented with 0, 2.5, 10, 20, and 50 µM γ-oryzanol, and the developments of embryos were assessed under 7% and 20% O_2 levels. A control group of no supplementation was included in each experiment. **Results:** The expansion of cumulus cover and survival rate tended to decrease with concentrations of 20, 50, and 100 µM in maturation media, suggesting an overdose effect. The cleavage and total blastocyst rates were significantly higher for oocytes matured at 5 µM γ-oryzanol. The presumptive zygotes cultured in supplemented media showed significantly higher cleavage and total blastocyst rates with concentrations of 5 and 10 µM γ-oryzanol ($P<0.04$) in both 7% and 20% O_2 levels. **Conclusion:** These results represent the first study showing a significant positive effect of the γ-oryzanol supplement on *in vitro* ovine oocyte and embryo development, at optimal concentrations of 5 µM in maturation, and 5 and 10 µM in embryo culture media.

Key words: Antioxidant, Embryonic development, Gamma-oryzanol, Sheep

Introduction

Despite many efforts to improve the outcome of *in vitro* embryo production (IVP) in small ruminants, there is a long way to bring it nearly close to *in vivo* conditions. During the process of *in vitro* manipulation, oocytes and embryos are exposed to exogenous factors that affect the antioxidant defense mechanism resulting in oxidative stress which is recognized as the underlying driving factor for the suboptimal outcome of IVP (Cognie *et al*., 2003); It seems to be responsible for damage to cell membranes, structures and macromolecules in oocytes and embryos that compromises the development of oocyte and embryo (Noda *et al*., 1991; Agarwal *et al*., 2014; Agarwal *et al*., 2022). One way to improve oocyte quality and embryo growth in the laboratory is to supplement culture media with antioxidant compounds (Aruoma *et al*., 1989). To date, different antioxidants such as vitamin E, vitamin C, Quercetin, Melatonin, Resveratrol, and Nobiletin have been evaluated for their scavenging effect on free radicals during the development of oocytes and embryos (Wang *et al*., 2002; Abecia *et al*., 2019; Zabihi *et al*., 2019; Davoodian *et al*., 2021).

γ-oryzanol comprises a mixture of ferulic acid esters, sterols, and triterpene alcohols that is extracted from rice bran oil. It has been introduced as an anti-tumor (Yasukawa *et al*., 1998), anti-inflammatory (Akihisa *et al*., 2000), antioxidant agent (Juliano *et al*., 2005), and anti-diabetic (Jung *et al*., 2015). Recently, the mechanism underlying γ-oryzanol activity as an antioxidant has been well investigated. γ-oryzanol acts as a free radical scavenger improves endogenous antioxidant enzyme activities and modulates the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway which is considered as the main regulator of the defense mechanism of antioxidants (Yasukawa *et al*., 1998; Moon *et al*., 2017). Different experiments have indicated

the *in vitro* antioxidative effects of γ-oryzanol. This compound inhibited the production of ROS, improved the activity of SOD and glutathione peroxidase (GPx), and induced the expression of *Nrf2* and some of the related genes including *NQO1*, *HO-1*, and *GSS* in the culture of human embryonic kidney cells and hepatocyte (Rungratanawanich *et al*., 2018). γ-oryzanol blocked ROS activated mitochondrial apoptotic pathway, inhibited cell apoptosis, and alleviated cytotoxicity in H_2O_2-stimulated L02 cells (Huang *et al*., 2020). Moreover, frozen boar sperm with γ-oryzanol supplemented extender showed higher progressive motility, viability, and membrane integrity (Chanapiwat and Kaeoket, 2015). It's hypothesized that hydroxy and phenoxy groups of γ-oryzanol bind to free electrons, reduce the effect of reactive oxygen species, and stabilize lipids of the plasma membrane (Cicero and Gaddi, 2001).

Therefore, the present study was designed to investigate the effect of γ-oryzanol in a dose-response study, on the development of ovine oocyte and embryo to provide the first basic information for its role as a supplement of media during *in vitro* ovine embryo production.

Materials and Methods

All the chemicals used were purchased from Sigma Chemicals Co. (St. 85 Louis, MO, USA) except for those stated.

Recovery of oocytes

This experiment was performed between November and March, 2021. Ovine ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermos flask containing normal saline. Immature cumulus-oocyte complexes (COCs) were obtained from the follicles with a 2-6 mm diameter which was aspirated with 20-G needles using a vacuum pump into 50-ml conical tubes containing 10 ml aspiration media (HEPES-buffered media 199 supplemented with 5% FBS and 100 IU/ml heparin).

Maturation of oocytes

Recovered COCs were evaluated morphologically by a stereomicroscope. All the oocytes with evenly granulated cytoplasm and at least three surrounding layers of cumulus cells were selected. Every 10 COCs were matured in a 50 µL drop of media 199 containing (bicarbonate-buffered media 199 supplemented with 0.05 IU/ml FSH, and 10% FBS) under mineral oil at 38.5°C in a humidified atmosphere with 5% CO_2 for 24 h.

The cumulus-oocyte complexes were evaluated at the end of the maturation period to assess the expansion of the cumulus cover. The criteria used for describing cumulus expansion was a subjective scoring system in which COCs were divided into three groups:

a) With no detectable expansion

b) With minimum to moderate observable changes

c) With the maximum degree of expansion, where all layers of cumulus cells expanded, even those closest to the oocyte

Only group C was considered as expanded (Bonni *et al*., 2002).

Fertilization of oocytes and embryo culturing

Matured COCs were transferred to the droplets of IVF-TALP media (a modified Tyrode preparation containing 25 mM sodium bicarbonate, 0.6% bovine serum albumin, 10 mM lactate, 1.0 mM pyruvate, and 5.6 mM glucose), and incubated at 39°C with 5% CO_2 in a humidified atmosphere. Ovine fresh epididymal sperm were incubated in HEPES-buffered media 199 supplemented with 0.4% BSA for 1 h for capacitation, next in SOF media supplemented with 0.4% BSA for 20 min at 39°C. Then the media containing sperm was centrifuged at 200 ×g for 2 min for swim up, and highly motile spermatozoa were separated. Finally, separated spermatozoa co-incubated with mature oocytes (1×10^6 motile sperm/ml), under mineral oil at 39°C and 5% CO_2 in a humidified atmosphere for 24 h.

Presumptive zygotes on the 1st day (day 0= the day of fertilization) were denuded from the cumulus cover. Every five to six morphologically normal zygotes were cultured in a 20 µL droplet of IVC-SOF media (SOF supplemented with amino acids and 0.8% BSA), incubated in a humidified atmosphere containing 5% CO_2, and 7% or 20% O_2 at 39°C. Embryonic development was assessed morphologically under a stereomicroscope. Oocytes with morphologic evidence of shrinkage, cytoplasmic vacuolization, damaged membrane, or zona pellucida were considered degenerated and discarded. Cleaved embryos were separated and cultured in IVC-SOF media supplemented with 10% charcoal-stripped FBS on the 3rd day. The blastocyst development rate was assessed on the 7th day.

Experimental design

Experiment 1: The effect of γ-oryzanol on the ovine oocyte development

Eight experimental groups were included in experiment 1 to evaluate the effects of different doses of γ-oryzanol (Sigma-Aldrich-CDS021604) in maturation media, on ovine oocyte maturation and development. The concentration series of γ-oryzanol in the maturation media were 0, 2.5, 5, 10, 20, 50, or 100 µM, introduced as ctrl, O-0, O-2.5, O-5, O-10, O-20, O-50, and O-100 respectively. γ-oryzanol was dissolved in ethanol and the concentration of solvent remained lower than 1%. Four biological replications were included in this experiment.

Experiment 2: The effect of γ-oryzanol on the development of the ovine embryo

This experiment was performed to investigate the effects of supplementing culture media with γ-oryzanol, on ovine embryo development. Presumptive zygotes were divided into two parts, one of them included seven experimental groups identified as ctrl, O-0, O-2.5, O-5,

O-10, O-20, and O-50, incubated in a humidified atmosphere with 5% CO_2 and 7% O_2 levels. Another part of presumptive zygotes was included in seven experimental groups identified as ctrl, O-0, O-2.5, O-5, O-10, O-20, and O-50, incubated in a humidified atmosphere with 5% CO_2 and 20% O_2 levels. Four biological replications were included in this experiment.

Statistical analysis

The traits of interest were collected as binary (0 and 1) and affected by the levels of treatment described earlier. Thus, the one-way logit model was implemented in the R software environment (version 4.0.3) as follows (Kaps and Lamberson, 2017):

$$\log\left(\frac{Pi}{1-Pi}\right) = \mu + Ti$$

Where,
pi: The probability of success in the ith trial
μ: The overall mean of the proportion on the logit scale
t: The effect of ith treatment

Due to the unequal number of replications at each level, least-square means were employed to compare the treatments. The z-test was performed on the log odds ratio scale to determine significant differences (P<0.05) between the levels of treatments using the means package (Lenth *et al*., 2019).

Results

Experiment 1

The results of the oocyte development outcome are presented in Fig. 1 and Table 1. The pattern of cumulus-cover expansion (24 h after *in vitro* maturation (IVM)) in matured oocytes of ctrl, O-0, O-2.5, O-5, O-10, and O-20 groups seemed not to differ significantly. However, the proportion of expanded COCs decreased significantly in O-50 and O-100 and tended to be lowest in O-100 (P<0.05).

Supplementing the maturation media with γ-oryzanol had no significant effect on the proportion of oocytes surviving at the end of fertilization, but the proportion of survived zygotes significantly decreased in the groups O-20, O-50, and O-100 which tended to be lowest in the last (P<0.05).

Cleavage and total blastocyst rates were recorded on days 2nd and 7th, respectively. The supplementation of IVM media with O-5 μM improved the cleavage rate when compared to the control group (P<0.05) (Fig. 1). There was no significant difference between the cleavage rate in O-0, O-2.5, O-5, and O-50 groups with the control group (P<0.05). The total blastocyst rate was significantly higher in O-5 compared with the control (P<0.05). O-100 had the significantly lowest total blastocyst rate (P<0.05). Other groups were not significantly different from the control (Fig. 1).

Table 1: The number of ovine oocytes subjected to experimental groups for evaluation and the effect of γ-oryzanol on the development

Groups	Total number of oocytes	Expansion of cumulus cover	Survived zygotes	Cleaved embryos	Total blastocyst
ctrl	202	0.95 ± 0.02^{a}	0.92 ± 0.02^{a}	0.55 ± 0.03^{ab}	0.26 ± 0.03^{bc}
O-0	200	0.95 ± 0.02^{ab}	0.91 ± 0.02^{ab}	0.54 ± 0.05^{abc}	0.25 ± 0.04^{bc}
O-2.5	322	0.93 ± 0.02^{ab}	0.89 ± 0.02^{ab}	0.55 ± 0.04^{ab}	0.30 ± 0.04^{b}
O-5	324	0.94 ± 0.02^{a}	0.91 ± 0.02^{a}	0.69 ± 0.04^{a}	0.47 ± 0.04^{a}
O-10	326	0.92 ± 0.02^{ab}	0.89 ± 0.02^{ab}	0.38 ± 0.04^{cd}	0.20 ± 0.03^{bc}
O-20	326	0.88 ± 0.02^{ab}	0.77 ± 0.03^{bc}	0.36 ± 0.04^{cd}	0.13 ± 0.03^{cd}
O-50	332	0.81 ± 0.02^{bc}	0.64 ± 0.04^{c}	0.41 ± 0.04^{bcd}	0.16 ± 0.03^{bcd}
O-100	320	0.74 ± 0.03^{c}	0.34 ± 0.04^{d}	0.33 ± 0.04^{d}	0.08 ± 0.02^{d}

The concentration series of 0, 2.5, 5, 10, 20, 50, or 100 μM, γ-oryzanol in medium are introduced as O-0, O-2.5, O-5, O-10, O-20, O-50, and O-100, respectively; ctrl represents no supplementation. Data are mean±SEM from 4 replicates. Different superscripts in each columns indicates a significant difference between groups (P<0.05)

Table 2: The number of presumptive zygotes subjected to experimental groups for evaluation of ovine embryo development *in vitro*

Groups	Total number of zygotes	Cleaved embryos	Total blastocyst	Early blastocysts	Expanded blastocysts	Hatched blastocysts
ctrl	288	0.56 ± 0.04^{b}	0.31 ± 0.04^{b}	0.59 ± 0.07^{a}	0.32 ± 0.07^{a}	0.09 ± 0.04^{a}
O-0	288	0.51 ± 0.06^{bc}	0.32 ± 0.05^{abc}	0.52 ± 0.1^{a}	0.35 ± 0.07^{a}	0.13 ± 0.07^{a}
O-2.5	288	0.54 ± 0.04^{b}	0.31 ± 0.04^{b}	0.59 ± 0.07^{a}	0.30 ± 0.07^{a}	0.11 ± 0.05^{a}
O-5	288	0.73 ± 0.04^{a}	0.48 ± 0.04^{a}	0.43 ± 0.06^{a}	0.38 ± 0.06^{a}	0.19 ± 0.05^{a}
O-10	288	0.53 ± 0.04^{b}	0.28 ± 0.04^{bc}	0.49 ± 0.08^{a}	0.41 ± 0.08^{a}	0.10 ± 0.05^{a}
O-20	288	0.39 ± 0.04^{bc}	0.15 ± 0.03^{cd}	0.81 ± 0.09^{a}	0.14 ± 0.08^{a}	0.05 ± 0.05^{a}
O-50	288	0.33 ± 0.04^{c}	0.13 ± 0.03^{d}	0.50 ± 0.12^{a}	0.39 ± 0.11^{a}	0.11 ± 0.07^{a}
OS-trl	144	0.36 ± 0.04^{b}	0.15 ± 0.03^{bc}	0.95 ± 0.05^{a}	0.14 ± 0.08^{a}	0.0^{a}
OS-2.5	144	0.31 ± 0.04^{b}	0.13 ± 0.03^{c}	0.74 ± 0.10^{ab}	0.16 ± 0.08^{a}	0.11 ± 0.07^{a}
OS-5	144	0.58 ± 0.04^{a}	0.31 ± 0.04^{a}	0.42 ± 0.07^{b}	0.38 ± 0.07^{a}	0.20 ± 0.06^{a}
OS-10	144	0.56 ± 0.04^{a}	0.28 ± 0.04^{ab}	0.54 ± 0.08^{ab}	0.37 ± 0.08^{a}	0.10 ± 0.05^{a}
OS-20	144	0.37 ± 0.04^{b}	0.14 ± 0.03^{c}	0.85 ± 0.08^{a}	0.10 ± 0.07^{a}	0.05 ± 0.05^{a}
OS-50	144	0.22 ± 0.03^{b}	0.06 ± 0.02^{c}	0.78 ± 0.14^{ab}	0.22 ± 0.14^{a}	0.0^{a}

Data are mean±SEM from 4 replicates. Different superscripts in each column indicates a significant difference between groups (P<0.05)

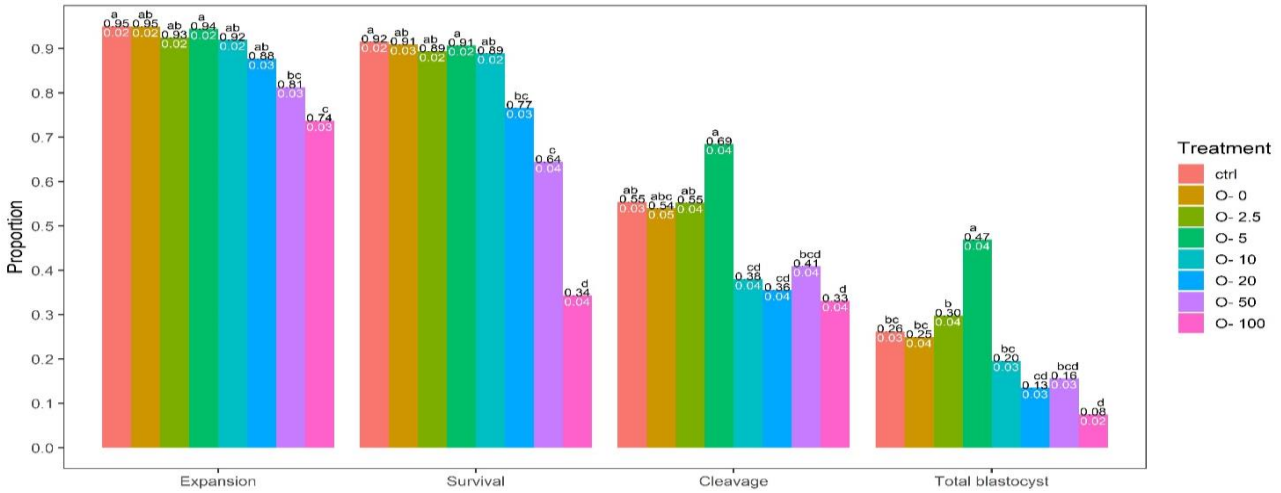

Fig. 1: The effect of γ-oryzanol on developmental competence of ovine oocytes. The expansion of cumulus cover, survival, cleavage, and blastocyst rates were recorded on days 0st, 2nd, and 6th, respectively (day 0: the day of IVF); The concentration series of 0, 2.5, 5, 10, 20, 50, or 100 µM, γ-oryzanol in medium are introduced as O-0, O-2.5, O-5, O-10, O-20, O-50, and O-100, respectively; ctrl represents no supplementation. Different letters show statistical differences. SE values are presented in each column in white color (results from 4 replicates)

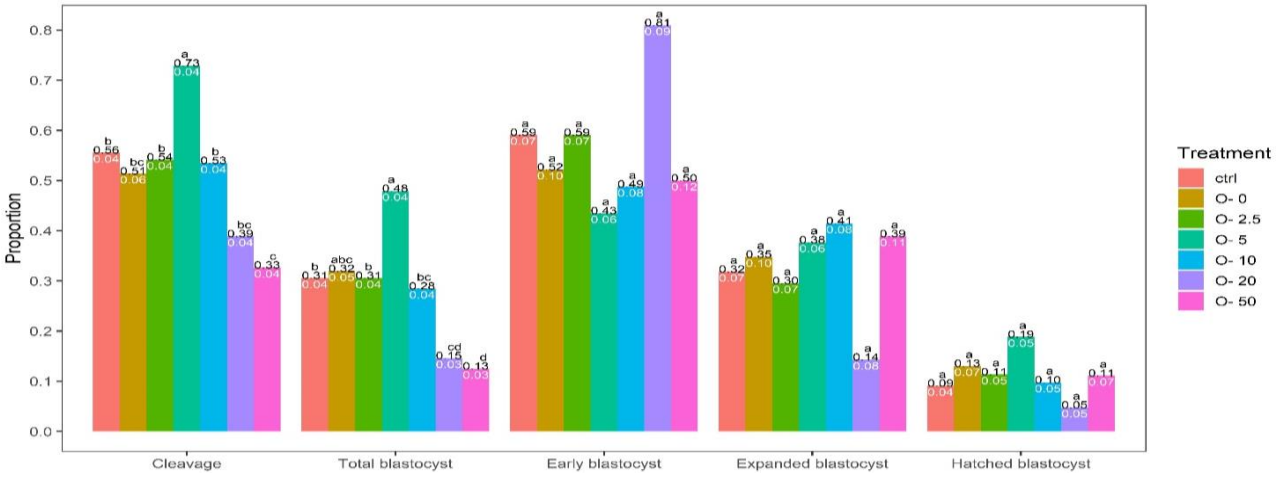

Fig. 2: Effects of γ-oryzanol supplementation during ovine embryo development under 7% O_2. Cleavage, total blastocyst rates, and the proportion of early, expanded and hatched blastocysts were recorded on days 2nd, and 7th, respectively; groups are described in Fig. 1. Different letters show statistical differences. SE values are presented in each column in white color (results from 4 replicates)

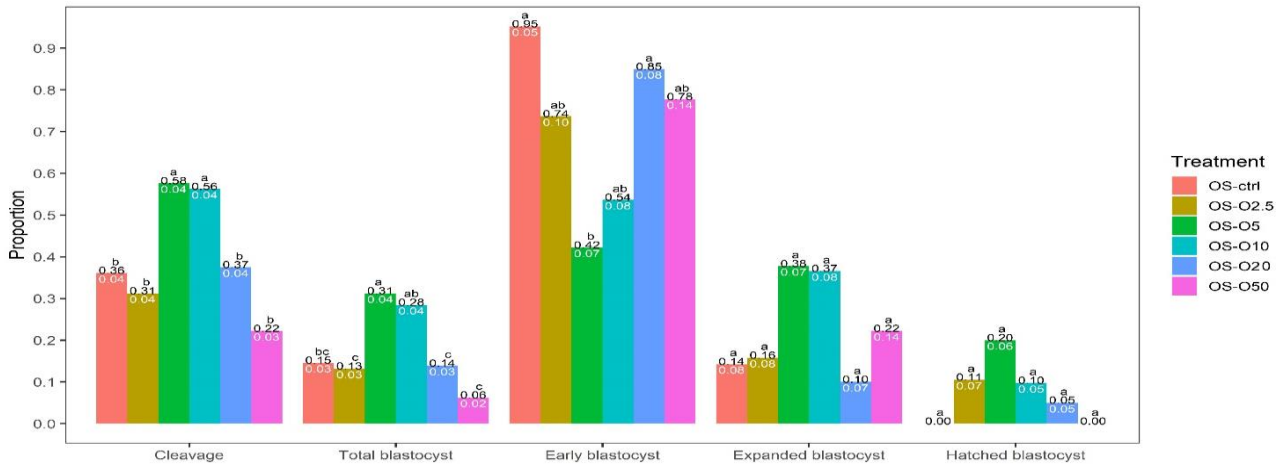

Fig. 3: Effects of γ-oryzanol supplementation of culture media on embryo development under oxygen tension (20% O_2). Cleavage, total blastocyst rates, and the proportion of early, expanded and hatched blastocysts were recorded on days 2nd, and 7th, respectively. Different letters show statistical differences. SE values are presented in each column in white color (results from 4 replicates)

Experiment 2

Embryo development was assessed visually under a stereomicroscope on days 2nd, and 7th (day 0 = the day of fertilization) to evaluate the proportion of cleaved embryos, and total blastocysts including early, expanded, and hatched blastocysts, respectively (Table 2).

The outcome of embryo culture under 7% O_2 is presented in Fig. 2. The proportion of cleaved embryos was significantly higher in group O-5 compared with all other groups ($P<0.05$). O-50 had the lowest cleavage rate ($P<0.05$). O-5 showed the highest total blastocyst rate compared to all other groups except for O-0 group. O-20 and O-50 groups had significantly the lowest rate of total blastocyst development. The proportion of early, expanded, and hatched blastocysts was not different between groups.

The outcome of embryo culture under oxygen tension (20% O_2) is presented in Fig. 3. O-5 and O-10 groups had the highest cleavage rates compared with ctrl and other groups ($P<0.05$). The total blastocyst rate was significantly higher in group O-5 compared to ctrl ($P<0.04$). The proportion of early blastocyst in the O-5 group was lower than in the control group but the difference in the proportion of expanded and hatched embryos was not statistically significant.

Discussion

The present study showed for the first time the positive effect of γ-oryzanol supplement in IVM and *in vitro* culture (IVC) media of ovine oocytes and determined the optimal concentration of γ-oryzanol based on cleavage and total blastocyst production rates.

Several factors may cause poor results in the process of IVP, including culture media (Sá *et al*., 2020), and overproduction of free radicals inside the oocyte (de Oliveira *et al*., 2021), which lead to oxidative stress due to an imbalance between the production and scavenging of ROS, and compromises the outcomes of embryo production *in vitro*. The supplementation of media with the antioxidants such as Resveratrol (Zabihi *et al*., 2019), Quercetin (Davoodian *et al*., 2021), has been extensively noticed as one of the effective strategies to ameliorate the impact of ROS during the development of an embryo. In this regard, γ-oryzanol, a mixture of ferulic acid esters and phytosterols, extracted from rice bran oil is well known for its antioxidant potential. Different *in vivo* and *in vitro* experiments have investigated the antioxidant properties of γ-oryzanol, demonstrating its ability in preventing and reducing ROS/RNS formation (Minatel *et al*., 2016). To the best of our knowledge, the present study is the first one aimed to investigate the effects of γ-oryzanol supplementation in IVM as well as IVC on the developmental capacity of embryos.

In the first experiment, the expansion of cumulus cover was not affected by supplementing the maturation media with γ-oryzanol, at 0, 2.5, 5, 10, and 20 μM concentrations but significantly decreased with 50, and 100 concentrations, suggesting an overdose effect. The survival rate was not compromised significantly by the supplementation except for 20, 50, and 100 μM γ-oryzanol groups which showed the worst outcome regarding cleavage and total blastocyst rates, too. Interestingly, cleavage and total blastocyst rates were the highest in the group supplemented with 5 μM γ-oryzanol. The second experiment evaluated the effect of γ-oryzanol supplement in culture media on the outcome of embryo development under low oxygen pressure as well as higher oxygen pressure to resemble oxygen tension. We demonstrated that the addition of 5 μM γ-oryzanol improved significantly cleavage and total blastocyst rates under 7% oxygen. A similar effect was observed under 20% oxygen, while 10 μM γ-oryzanol increased cleavage but not total blastocyst rate, although the differential counting of blastocysts was not different among groups.

The results of experiments 1 and 2 suggest that γ-oryzanol at these concentrations could promote a better outcome of embryo production. These positive effects might be related to the antioxidant properties of γ-oryzanol. *In vivo* experiments in rat and rabbit models have demonstrated that γ-oryzanol decreases ROS and MDA levels and increases antioxidant enzyme activities (Rao *et al*., 2016; Panchal *et al*., 2017; Francisqueti *et al*., 2018). On the other hand, anti-inflammatory properties are shown for γ-oryzanol too which are related to its antioxidant properties (Zolali *et al*., 2015).

It's well known that during *in vitro* development of an embryo, endogenous and exogenous factors cause an imbalance between ROS production and antioxidant defense mechanism, resulting in oxidative stress which leads to increased apoptosis, changes in gene expression, and the reduction of embryo quality (Guerin *et al*., 2001; Ullah *et al*., 2019). The primary response pathway to oxidative stress in cells is mediated by transcription factor Nrf2 which binds to ARE and induces antioxidant enzymes including GPx, SOD, and CAT, to detoxify ROS in culture media. In oocytes and embryos, most of the genes related to the production of antioxidant enzymes are controlled through this pathway (Gad *et al*., 2012). These enzymes can increase the expression of the anti-apoptotic gene, *Bcl-2*, and decrease the expression of proapoptotic genes such as *Bax* and *caspase-3* in blastocysts, indicating the protective effects of antioxidant defense mechanism on embryo growth (Mishra *et al*., 2018).

The mechanism of action of γ-oryzanol as an antioxidant has been well-established *in vitro*. γ-oryzanol eliminates free radicals and protects membranes against damage from oxidative stress and lipid peroxidation (Wilson *et al*., 2007). Pretreatment of L02 cells with γ-oryzanol exposed to H_2O_2 significantly decreased the levels of MDA and ROS, and increased GSH content, as well as the activity of SOD, and CAT. The results of gene expression analysis revealed that γ-oryzanol exerted its antioxidant effect through the Nrf2-ARE pathway (Huang *et al*., 2020). Another study demonstrated the antioxidant power of γ-oryzanol in human embryonic kidney cells (HEK-293) through activating intracellular antioxidant pathways to inhibit reactive oxygen species, increasing the activity of superoxide dismutase and

glutathione peroxidase and inducing the expression of nuclear factor *Nrf2* and defense genes *NQO1*, *HO-1*, and *GSS* (Rungratanawanich *et al*., 2018).

On the other hand, it's well known that TNF-α and its receptors promote cytoplasmic growth and maturation in oocytes (Lima *et al*., 2018), and IL-6 improves embryo quality (Wooldridge and Ealy, 2019). *In vivo* experiments have demonstrated that an overdose of γ-oryzanol suppresses the secretion of TNF-α and IL-6 (Panchal *et al*., 2017). Our experiments showed significant negative effects of high concentrations of γ-oryzanol on cumulus expansion, survival, cleavage, and blastocyst production rates. It might be related to the suppressing effect of γ-oryzanol on the expression of TNF-α and IL-6 that compromised the outcome of oocyte and embryo development.

To date, there are no studies available on γ-oryzanol effects on oocytes and embryos, while the beneficial effects of γ-oryzanol on cell protection have been demonstrated obviously due to its hydrophobic nature allowing it to incorporate into membranes protecting them against ROS and lipid peroxidation damage. Therefore, we attribute the positive effects of 5 μM γ-oryzanol in the present study to its antioxidant properties. Nonetheless, further studies are suggested to provide increased insight into the mechanism of its antioxidant action in oocytes and embryos.

In conclusion, supplementing γ-oryzanol to media during IVM and IVC improved the outcome of ovine oocyte and embryo development *in vitro* in terms of cleavage and total blastocyst rates. We recommend that supplementation of media with 5 μM γ-oryzanol is a promising strategy during ovine oocyte maturation and embryo culture *in vitro*.

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Conflict of interest

The authors have no conflicts of interest with respect to the manuscript to declare.

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