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Effect of different gonadotropins on in vitro maturation of sheep oocytes

Efeito de diferentes gonadotrofinas na maturação in vitro de oócitos ovinos

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Highlights

IVM of sheep oocytes.
Equine chorionic gonadotropin in IVM.
Replacement of FSH in IVM.

Abstract

The aim of this study was to examine the effect of replacing the use of follicle-stimulating hormone (FSH) with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) on the *in vitro* maturation (IVM) of sheep oocytes. After sheep ovaries were collected (n=300), the cumulus-oocyte complexes were aspirated, selected, and divided into four groups according to the IVM medium: CON group, in which the basic IVM medium was used; and eCG, hCG, and FSH groups, in which the oocytes were immersed in basic IVM medium with 10 IU/mL eCG, 10 IU/mL hCG, and 10 µg/mL FSH-p, respectively. In vitro maturation of the oocytes was performed at 38.5 °C, in a humidified atmosphere of 5% CO2 in air, for 24 h. Subsequently, the oocytes were evaluated for the degree of cumulus-cell expansion, chromatin configuration, GSH levels, and active mitochondria. There were no significant differences for the rate of cumulus cell expansion. The percentage of oocytes in MII was higher in the eCG group than in the CON and hCG groups ($P<0.05$) and similar to that of the FSH group. In conclusion, eCG can be used as a substitute for FSH in IVM of sheep oocytes.

Key words: Follicle-stimulating hormone. Metaphase II. Hoechst 33342. GSH.

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Resumo

O objetivo deste estudo foi avaliar o efeito da gonadotrofina coriônica equina (eCG) e da gonadotrofina coriônica humana (hCG), em substituição ao uso de hormônio folículo estimulante (FSH) na maturação *in vitro* (MIV) de oócitos ovinos. Após a coleta de ovários (n=300) ovinos, os complexos cúmulus-oócitos (CCOs) foram aspirados, selecionados e divididos em quatro grupos de acordo com o meio de MIV: grupo CON, em que foi utilizado o meio MIV base; e grupos ECG, HCG e FSH, em que os oócitos foram imersos em meio MIV base adicionado de 10 UI/mL de eCG, 10 UI/mL de hCG e 10 µg/mL de FSH-p, respectivamente. A MIV dos oócitos foi realizada a 38,5°C, em atmosfera umidificada de 5% de CO2 em ar, durante 24 horas. Posteriormente, os oócitos foram avaliados, quanto grau de expansão das células do cumulus, configuração da cromatina, níveis de GSH e mitocôndrias ativas. Não foram observadas diferenças significativas com relação à taxa de expansão de células do cumulus. A percentagem de oócitos em MII foi maior no grupo ECG do que no grupo CON e HCG (P<0,05) e semelhante ao grupo FSH. Em conclusão, a eCG pode ser utilizada em substituição ao FSH na MIV de oócitos ovinos.
Palavras-chave: Hormônio folículo estimulante. Metafase II. Hoechst 33342. GSH.

Introduction

Oocyte in vitro maturation (IVM) is a crucial step for the success of in vitro embryo production during which the oocyte changes necessary for embryonic development must occur (Rizos et al., 2008). One of these changes is nuclear maturation, i.e., the reversal of the first meiotic arrest of the oocyte in the germinal vesicle stage (GV) until the second meiotic arrest in metaphase II (MII) (Zhu et al., 2018). This process occurs simultaneously with cytoplasmic maturation, which is characterized by structural changes such as the distribution and redistribution of cortical granules, storage of proteins and RNA, development of the calcium regulatory mechanism, as well as migration of mitochondria to a perinuclear position (Del Collado et al., 2015).

These events start with the binding of gonadotropins to their receptors and consequent activation of the associated G protein, which triggers a phosphorylation cascade in cyclic adenosine monophosphate (cAMP)-dependent protein kinases (Russell et al., 2016). Physiologically, in the follicular environment, cAMP is one of the main agents responsible for controlling the meiotic maturation of oocytes, constituting the second messenger of the gonadotropin signaling pathway in ovarian tissue (Botigelli et al., 2017).

In this context, the addition of gonadotropins, such as follicle-stimulating hormone (FSH), to the maturation medium, can increase mRNA expression of FSH and luteinizing hormone (LH) receptors in cumulus cells, inducing their expansion and culminating in oocyte maturation (Lee et al., 2007; Xiao et al., 2014). Despite these benefits, porcine pituitary FSH, which is routinely used, has a high cost, highly variable drug purity, differences in commercial formulations, and is often contaminated with other gonadotropins, which can lead to non-standard or unsatisfactory results (Sha et al., 2010). Thus, other gonadotrophic sources

with similar function to FSH, such as equine chorionic gonadotropins (eCG) and human chorionic gonadotropins (hCG), have been used in the in vitro maturation of mammalian oocytes (Coelho et al., 2002 De los Reyes et al., 2005).

Equine chorionic gonadotropin acts as a FSH, which can stimulate the appearance of LH receptors in cumulus cells, allowing LH to activate intracellular messengers that regulate the cellular response (Mingoti et al., 2002).

The addition of eCG to maturation medium also improved MII oocyte rates in sheep (Wei et al., 2016). On the other hand, when added to the IVM medium, hCG, which has a predominant LH function, can alter the distribution of calcium in the ooplasm, in addition to increasing glutamine metabolism in the oocyte (Mingoti et al., 2002).

However, there are no reports comparing the isolated effects of FSH, eCG, and hCG on the IVM of the oocytes of Dorper crossbred sheep reared in the semi-arid region of Northeast Brazil. Therefore, the present study was undertaken to compare the effects of using different gonadotropins (eCG, hCG, and FSH) on the in vitro maturation of sheep oocytes.

Materials and Methods

Ethics

All protocols used in this study were approved by the Research and Study Ethics and Deontology Committee at the Federal University of Vale do São Francisco (approval no. 0003/121218. CEDEP / UNIVASF).

Chemical products

The chemicals used in this study were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) or another company specified in the text.

Oocyte aspiration

A total of 300 ovaries were collected from female sheep from a local slaughterhouse and transported to the laboratory, within 2 h after collection, in saline solution (0.9% NaCl, Fresenius®, Dismed, Brazil), at 30 °C (Souza-Fabjan et al., 2014). The ovaries were washed three times in pre-warmed fresh saline solution (30 °C) and maintained at this temperature in a water bath (Model 1102®, Fanem, Brazil). All visible follicles, between 2 and 6 mm in diameter, were aspirated with a short-bevel 18G needle (Solidor®, Lamedid Comercial & Serviços, Brazil) connected to a Falcon tube under controlled vacuum (30 mmHg or 6-8 mL/min). The collection tube was previously filled with 3 to 5 mL of collection medium composed of TCM 199, supplemented with 50 IU/mL heparin, 50 µg/mL gentamicin, and 10% (v/v) fetal bovine serum.

Evaluation and IVM of sheep oocytes

After collection, cumulus-oocyte complexes (COCs) were transferred to 60-mm Petri dishes (Petri Dish60®, Nutricell, Brazil), analyzed under a stereomicroscope (SMZ 645®, Nikon, Japan), and classified into different quality grades according to Avelar et al. (2012). Only COCs classified as Grade I (with more than three layers of compact

cumulus cells) and Grade II (with one to three layers of finely granulated cumulus cells) were indicated for in vitro maturation.

The selected oocytes were kept in collection medium until the maturation procedures. Subsequently, the oocytes were homogeneously divided into four maturation groups: in the control group (CON), they were immersed in basic medium composed of TCM-199, supplemented with epidermal growth factor (EGF; 20 ng/mL), estradiol (E2; 1 µg/mL), fetal bovine serum (10% v/v), L-glutamine (1 mM), sodium pyruvate (40 µg/mL), and cysteamine (100 µM/mL); in the FSH (n=128), eCG (n=142), and hCG (n=140) groups, the oocytes were matured in the control medium supplemented with 10 µg/mL FSH-p (Folltropin-V®, Vetoquinol, Brazil) (Ni et al., 2015), 10 IU/mL eCG (Sincro eCG®, Ouro Fino Saúde Animal, Brazil), and 10 IU/mL eCG (Vetecor®, CEVA, Brazil) (Coelho et al., 2002), respectively. The oocytes were kept in drops of 50 µL of IVM medium (10 oocytes/drop), under mineral oil, in an incubator in a humidified atmosphere with 5% CO2 for 24 h, at 38.5 °C.

Morphological evaluation and assessment of chromatin configuration

After IVM, the mature oocytes were evaluated under a stereomicroscope (SMZ 645®, Nikon, Japan) considering the presence or absence of cumulus-cell expansion, following the methodology of Avelar et al. (2012). The degree of cumulus-cell expansion was also evaluated and classified into Full (Grade I), Moderate (Grade II), and Mild (Grade III), according to Aghaz et al. (2015).

Additionally, the oocytes were denuded and incubated for 15 min in drops of PBS containing 10 mM Hoechst 33342, at room temperature, in the dark. Then, they were washed three times in drops of 50 µL of PBS/PVP and visualized under fluorescence microscopy (Nikon E200®, Tokyo, Japan). Chromatin configuration (blue fluorescence) was analyzed and classified into: intact germinal vesicle (GV), meiotic resumption (including germinal vesicle breakdown [GVBD] or metaphase I [MI]), or nuclear maturation (metaphase II [MII]) (Rajabi-Toustani et al., 2013).

Measurement of GSH levels and metabolically active mitochondria

Intracellular levels of glutathione (GSH) and mitochondrial activity were measured in mature oocytes as previously described by Silva et al. (2018) with minor modifications. Briefly, after IVM, oocytes (20 per treatment group) were incubated in the dark for 30 min, at 39 °C, in PBS supplemented with 10 mM 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker® Blue, CMF2HC; Invitrogen Corporation) and 100 nM MitoTracker Red (MitoTracker® Red, CMXRos, Molx, Melbourne, Victoria, Australia) to detect intracellular GSH levels and mitochondrial activity as blue and red fluorescence, respectively. After incubation, the oocytes were washed with PBS and fluorescence was observed under an epifluorescence microscope (Nikon E200®, Tokyo, Japan) with UV filters (370 nm for GSH and 579-599 nm for active mitochondria). Oocyte fluorescence intensities were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data on maturation percentage, rates of cumulus cell expansion, and rates of different degrees of cumulus-cell expansion were expressed as percentages and compared by the chi-square test using Epi Info statistical software (Epi Info 7.1.5.0, Atlanta, GA, USA, 2015). Data on GSH levels and mitochondrial activity were subjected to the Shapiro-Wilk test to check for data normality and variance homogeneity. Then, the non-parametric Kruskal-Wallis test was applied for comparisons between groups. When significant, means were compared using the Student-Newman-Keuls test. Differences were considered significant when $P<0.05$.

Results and Discussion

After IVM, the treatment with FSH showed a higher proportion ($P<0.05$) of Grade-1 expanded ('Full') oocytes than the other treatments, whereas the hCG and CON groups exhibited higher proportions ($P<0.05$) of Grade-3 expanded ('Mild') oocytes. Group eCG had the highest proportion of Grade-2 expended ('Moderate') oocytes ($P<0.05$) (Table 1).

Table 1
Degrees of cumulus cell expansion (%) of sheep oocytes matured *in vitro* in the absence of gonadotropins (CON) or in the presence of FSH, eCG, or hCG

Group	Expansion rate (%)	Degree of expansion		
		% D1 - Full (n)	% D2 - Moderate (n)	% D3 - Mild (n)
CON	90.07	4.23 (5/118) ^{Cc}	32.20 (38/118) ^{Bb}	63.55 (75/118) ^{Ba}
FSH	96.96	57.03 (73/128) ^{Aa}	35.15 (45/128) ^{Bb}	7.81 (10/128) ^{Dc}
eCG	97.26	16.90 (24/142) ^{Bb}	57.72 (82/142) ^{Aa}	25.35 (36/142) ^{Cb}
hCG	93.95	5 (7/140) ^{Cc}	12.14 (17/140) ^{Cb}	82.85 (116/140) ^{Aa}

$^{A-D}$ Different uppercase letters in the same column indicate a significant difference ($P < 0.05$). a-c Different lowercase letters in the same row indicate a significant difference ($P < 0.05$).

The evaluation of chromatin configuration revealed the presence of oocytes in GV (Figure 1A), GVBD (Figure 1B), MI (Figure 1C), and MII (Figure 1D). The use of gonadotropins (FSH, eCG, and hCG) reduced ($P<0.05$) the percentage of oocytes that were stationary at GVBD, while FSH and hCG increased ($P<0.05$) the oocytes in the MI stage, compared with the control group. Furthermore, the medium supplemented with 10 IU/mL of eCG showed a percentage of oocytes in MII similar ($P>0.05$) to that of the FSH group and higher ($P<0.05$) to those of the CON and hCG groups (Table 2).

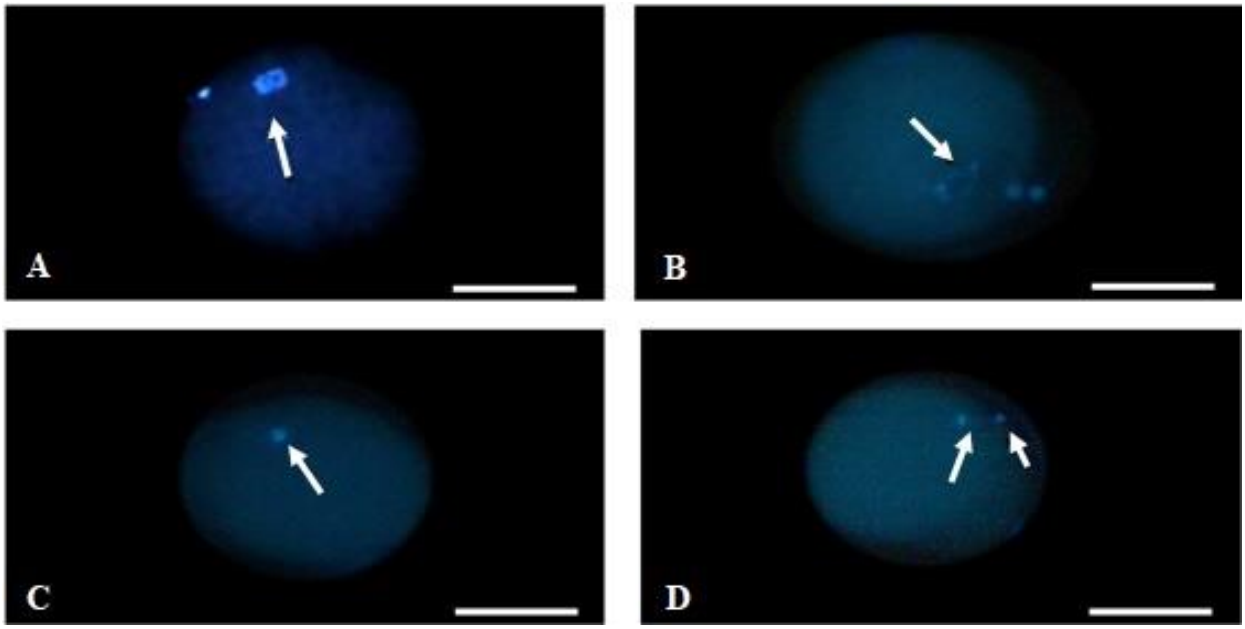

Figure 1. Ovine oocyte chromatin stained with Hoechst 33342 and subjected to epifluorescence microscopy. (A) Germinal vesicle after *in vitro* maturation (IVM) of sheep oocytes in the absence of gonadotropins (Control); (B) Germinal vesicle breakdown after IVM in the presence of FSH (FSH); (C) Nucleus in metaphase I (MI) after IVM in the presence of eCG (ECG); (D) Nucleus in metaphase II (MII) after IVM in the presence of hCG (HCG). White arrow indicates labeled chromatin after epifluorescence. Scale bar: 100 µm.

Table 2
Stages of nuclear maturation of sheep oocytes matured *in vitro* in the absence of gonadotropins (CON) or in the presence of FSH, eCG, or hCG

Group	% GV (n)	% GVBD (n)	% MI (n)	% MII (n)
CON	9.09 (3/33) ^{A}	30.30 (10/33) ^{B}	33.33 (11/33) ^{C}	27.27 (9/33) ^{B}
FSH	0.00 (0/42) ^{A}	14.29 (6/42) ^{A}	52.38 (22/42) ^{AB}	33.33 (14/42) ^{AB}
eCG	0.00 (0/40) ^{A}	15.00 (6/40) ^{A}	40.00 (16/40) ^{BC}	45.00 (18/40) ^{A}
hCG	0.00 (0/37) ^{A}	16.22 (6/37) ^{A}	56.76 (21/37) ^{A}	27.03 (10/37) ^{B}

$^{A-C}$ Superscript letters in the same column indicate a significant difference ($P < 0.05$). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II.

There was no difference ($P>0.05$) in GSH levels between treatment groups. However, oocytes that matured in the presence of gonadotropins showed higher ($P<0.05$) levels of active mitochondria than those in the CON group (Figure 2).

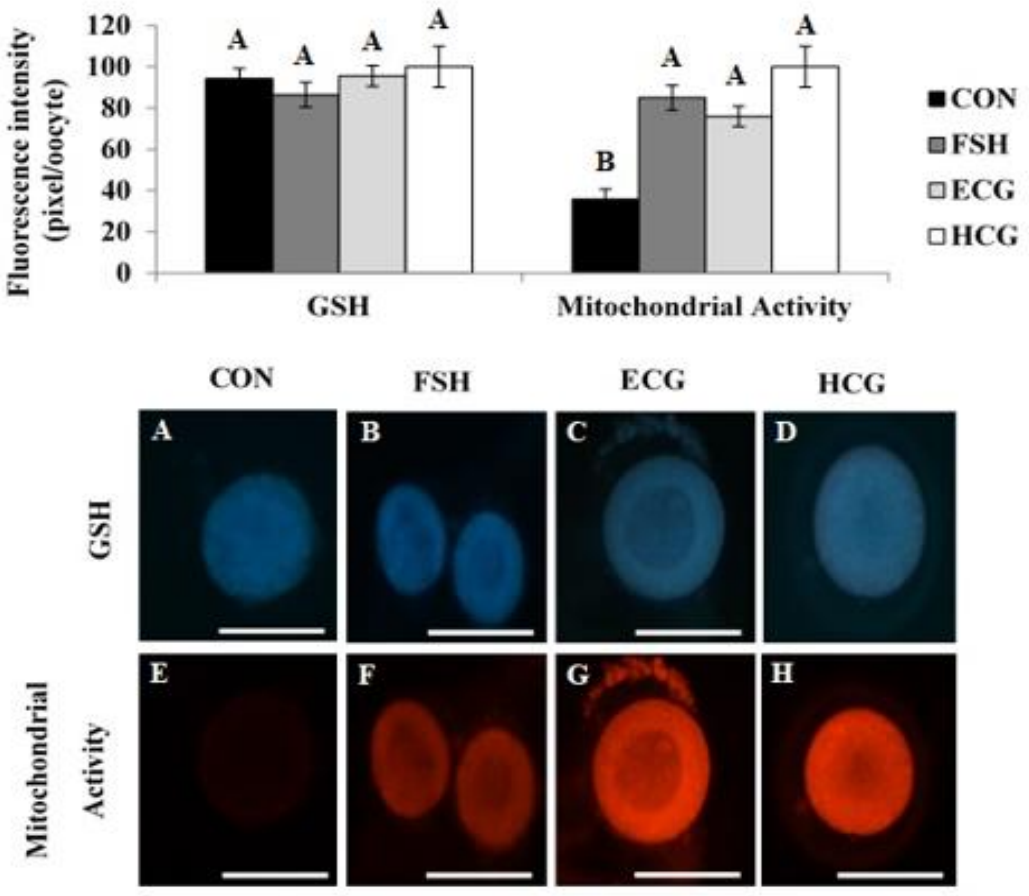

Figure 2. Intracellular GSH levels and mitochondrial activity (pixel/oocyte) of mature sheep oocytes in the absence (CON) or presence of FSH (FSH), eCG (ECG), and hCG (HCG). Different letters between columns, within each parameter, indicate a significant difference (P<0.05). Epifluorescence photomicrograph of sheep oocytes stained with Cell Tracker Blue, detecting intracellular levels of GSH (A, B, C, D); and MitoTracker Red, detecting levels of mitochondrial activity (E, F, G, H) in oocytes matured in the absence of gonadotropins (CON; A, E) and in media containing FSH (FSH; B, F), eCG (ECG; C, G), and hCG (HCG; D, H). A, B Different letters between columns, within each parameter, indicate a significant difference (P<0.05). Scale bar: 75 µm.

When we compared the degree of cumulus cell expansion, the FSH group had a higher proportion of oocytes with full cumulus cell expansion (57.03%), whereas moderate expansion predominated in the eCG group and mild expansion in the CON and hCG groups. Similar results were also found in cattle (Sugimura et al., 2018), humans (Pogrmic-Majkic et al., 2018), and pigs (Blaha et al., 2017). These findings corroborate Cantanhêde (2018), who stated that FSH is the main regulatory gonadotropin of the cumulus-cell expansion process.

In our experiment, the eCG group, in turn, showed a moderate expansion rate. This can be explained by the composition of this gonadotropin, whose formula involves the association of FSH and LH, which promoted balanced expansion in this group.

As mentioned by Turathum et al. (2021), cumulus-cell expansion is important for meiotic maturation for disrupting the communicating junction, which leads to a decrease in cAMP in the oocyte. The result of this phenomenon can be viewed in our oocyte maturation data, since a higher percentage of oocytes in MII was found in the eCG and FSH groups than in the CON and hCG groups, which, coincidentally, had a lower degree of cumulus-cell expansion.

According to Mingoti et al. (2002), FSH stimulates the appearance of LH receptors in cumulus cells, allowing LH to activate intracellular messengers. Equine chorionic gonadotropin has the ability to bind to FSH and LH receptors simultaneously.

In both situations, when gonadotropins bind to their receptors, the activation of the associated G protein is initiated, inducing the conversion of guanosine triphosphate into GDP (guanosine diphosphate). Guanosine diphosphate then connects to the α subunit of the G protein, stimulating adenylate cyclase to generate cAMP, one of the main responsible factors for controlling oocyte meiotic maturation (Botigelli et al., 2017).

In this way, the rates of oocytes in MII observed in the media supplemented with FSH (33.3%) and eCG (45%) may be due to the interaction between FSH and LH, present in both gonadotropins. This is because porcine FSH commercially purchased is contaminated with other hormones, mainly LH, whereas eCG has a mixed function of FSH and LH (Sha et al., 2010). It is possible that eCG led to an increased expression of FSH, LH, and gonadotropin-releasing hormone (GnRH) receptors in cumulus cells (Wei et al., 2016), which could explain our result.

However, our data (33.33% and 45% of oocytes in MII after IVM in the media containing FSH and eCG, respectively) are lower than the 48.4% sheep oocytes in MII found by Ni et al. (2015) when 10 µg/mL of FSH were added to the maturation medium; and the 51% described by Wei et al. (2016), who added 20 µg/mL of eCG. We believe that the inferiority of our data is related to heterogeneity in the stages of nuclear and cytoplasmic maturation of the oocytes taken to IVM, which remains a limiting factor for this technique in small ruminants (Souza-Fabjan et al., 2014). In addition, other aspects (e.g., source of ovaries and ovarian stimulation, age, and season of the year) also interfere with the results of nuclear maturation (Souza-Fabjan et al., 2014).

In our study, there was a significant difference in terms of GSH levels between the different experimental groups.

We believe that this result is related to the antioxidant components of the basic IVM medium used in this study: epidermal growth factor (EGF) and cysteamine. The main effect of EGF, for instance, is stimulating oocyte intracellular glutathione synthesis (Whitaker & Knight, 2004). Cysteamine, on the other hand, increased the synthesis of GSH in the bovine oocyte, supplying it with a large stock of GSH available to protect the embryo until the blastocyst stage (Gottardi et al., 2012).

Nonetheless, mitochondrial activity was higher in the gonadotropin-treated groups than in the control group. Once active, mitochondria ensure the functional competence of oocytes, especially during nuclear and/or cytoplasmic maturation (Moussa et al., 2015). This mitochondrial activation is also known to be always greater

in mature oocytes than in immature ones (Tarazona et al., 2006). These findings once again indicate the importance of adding gonadotropins to the IVM medium to ensure quality oocytes.

Conclusions

By comparing the effects of different gonadotropins, we observed that eCG can indeed be used as a substitute for FSH during the maturation of sheep oocytes, since both provided a good degree of expansion of cumulus cells as well as similar rates of metaphase II and active mitochondria. However, the use of hCG in the IVM medium of sheep oocytes did not yield satisfactory results.

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Statement of Conflicts of Interest

The authors have no conflict of interest to declare.

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