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Gonadotropin supplementation improved *in vitro* developmental capacity of Egyptian goat oocytes by modulating mitochondrial distribution and utilization

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ABSTRACT

Goat production in Mediterranean countries is economically and socially significant. The study investigated the impact of hCG supplementation on the *in vitro* maturation, fertilization, and mitochondrial activity of goat oocytes. In total, 2356 good cumulus-oocyte complexes (COCs) were recovered from 476 freshly collected goat ovaries. Selected oocytes were incubated in IVM medium and supplemented with hCG at varying concentrations depending on the experimental group for 24 hours at 38.5 °C, 5% CO_2 and 95% humidity. The first group (G1) was allocated as a control group, while the G2 and G3 groups were supplemented with 10 and 20 IU/mL hCG, respectively. The maturation rate was calculated using Hoechst 33342 staining in addition to cumulus expansion and the first polar body extrusion rates. The fertilization rate was evaluated. Moreover, mitochondrial activity was assessed using Mito-Tracker fluorescent staining. The results indicated a higher rate ($P \leq 0.05$) of nuclear maturation in G3 (82.5%) than in G2 (65.9%) and the control group (64.8%). Furthermore, fertilization rate was significantly improved in G3 (18.7%) compared to G2 (10.6%) and the control group (9.5%). Notably, the percentage of diffuse patterns of mitochondrial distribution increased in G3 (73.3%) compared to G2 (13.3%) and the control group (6.7%). In the same trend, the fluorescent mitochondrial intensity was higher in G3 (95%) and G2 (89.2%) compared to the control group (79.9%). In conclusion, supplementing the IVM medium with 20 IU/mL of hCG improved goat oocytes' maturation and fertilization rates by modulating cytoplasmic distribution and promoting the utilization of oocyte mitochondria.

Keywords: *Cytoplasmic maturation, Goats, hCG, IVF, IVM, Oocytes*

INTRODUCTION

The technique of *in vitro* embryo production (IVEP) is widely used to overcome problems associated with infertility in various mammalian species (Baldassarre, 2021; Wani, 2021). IVEP has been identified as a critical technique for reproducing large numbers of offspring from animals with superior genetic traits (Baldassarre, 2018). IVM is the first and most critical step in the IVEP process, in which oocytes acquire the potential to sustain further embryonic development. Therefore, identifying a suitable IVM environment is essential to applying an effective IVEP procedure (Hatırnaz *et al.*, 2018).

Goats are essential genetic resources and a good experimental model for assisted reproductive techniques such as cloning and transgenesis, which propagate breeds under endangerment or for conservation purposes (Mariano *et al.*, 2015). *In vitro* embryo production provides a crucial tool for the genetic improvement of livestock, such as goats. In addition, IVEP can be applied to enhance the productive and reproductive traits of goats (Fernandes *et al.*, 2018). *In vivo* maturation has considerably enhanced oocyte quality and post-fertilization development compared to *in vitro* systems. In this regard, the efficiency of goat IVEP is still low, despite the increasing interest in applying this reproductive technique worldwide (Saeedabadi *et al.*, 2018). Therefore, it is essential to continue research on optimizing the IVEP system in this species to reach the efficiency of the *in vivo* environment (Chang *et al.,* 2019).

Indeed, the improvement of the IVM system has resulted in substantial enhancement of preimplantation embryonic development in several mammalian species (Albuz *et al.*, 2010; Khattab *et al.*, 2020; Mishra *et al.*, 2016; Veshkini *et al.*, 2018). Supplementation of gonadotropins to IVM medium has enhanced the developmental potential of oocytes (Dinopoulou *et al.*, 2016; Bahrami and Cottee, 2022). However, the mechanism by which gonadotropins improve oocyte quality is not completely clarified. In this regard, cytoplasmic maturation is one of the key changes that are essential for the maturation process. The mitochondrial distribution and metabolic activity (mitochondrial intensity) impact oocyte cytoplasmic and nuclear maturation (Czernik *et al.*, 2022; Ghanem *et al.*, 2021a; Ghanem *et al.*, 2021b; Torner *et al.,* 2008). At the cellular level, it is known that the distribution of active mitochondria within the oocyte is indicative of energy and ion requirements necessary for various key events during oocyte maturation and fertilization (Reader *et al.*, 2017).

Therefore, to the best of our knowledge, this study is the first in Egypt to investigate the effect of hCG supplementation on IVM and IVF in goat oocytes in relation to mitochondrial distribution and fluorescent intensity.

MATERIALS AND METHODS

Experimental Site

The current work was carried out at the Embryology Manipulation Unit (EMU), Department of Animal and Poultry Physiology, Division of Animal and Poultry Production, Desert Research Center (DRC), Cairo, Egypt.

Ethical endorsement

The conduct of this study adhered to the code of ethics and animal rights standards (Desert Research Center). In addition, this work attempted to comply as closely as possible with the laws and standards outlined in the European Union directive for protecting experimental animals (2010/63/EU).

Chemicals and Media

Unless otherwise stated, all chemicals and media components were bought from Sigma-Aldrich Chemicals in Germany. Russo *et al.* (2014) clarified, all media were made the night before from a stock solution of each component and sterilized by passing through a 0.22 μm diameter Millipore filter fitted to a 10 ml syringe.

Biological Material

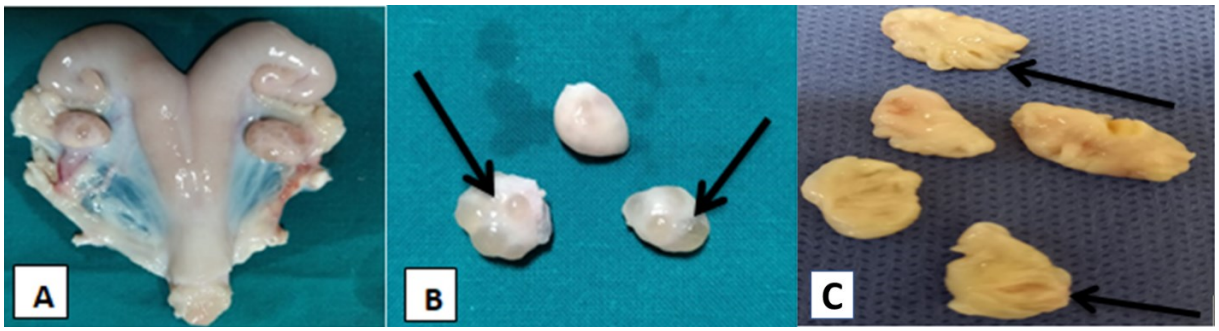

Figure 1. Genital system of female goats. A Complete uterus with both ovaries. B Ovaries show numerous graafian follicles (black arrows). C ovaries after slicing.

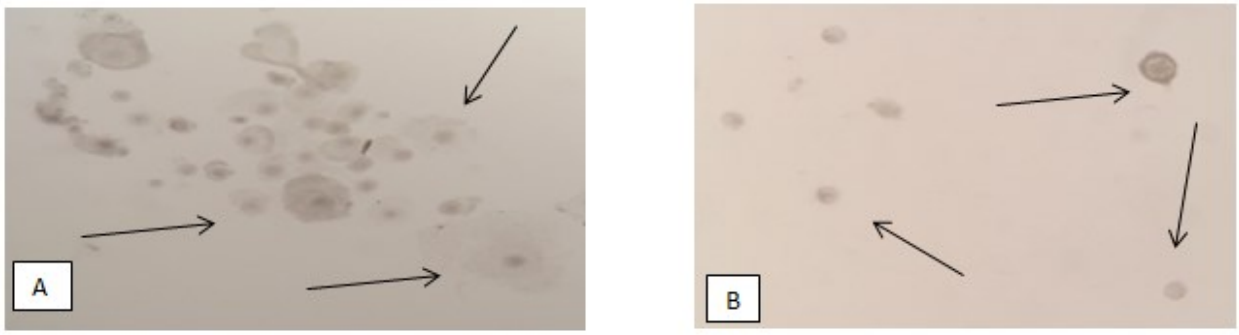

Figure 2. Different grades of retrieved oocytes by slicing technique. A good quality oocyte (a malty layer of cumulous cells and homogeneous cytoplasm (black arrow)). B Poor quality oocyte (denuded oocytes, heterogeneous cytoplasm) by stereo microscope (40x)

The whole uterus and ovaries of goats (Figure 1) with unknown reproductive histories were collected from a local slaughterhouse in Cairo, Egypt. The ovaries were delivered to the lab within two to three hours of slaughtering in a thermos flask filled with warm saline solution (NSS, 0.9% NaCl), held at 35 °C, and treated with antibiotic antimitotic (AA, 100 IU penicillin and 100 g streptomycin/mL). All extra tissues were discarded, and the ovaries were washed four times: once with warm (37 °C) phosphate-buffered saline (PBS), twice with 70% ethanol, and once more with NSS for 10 seconds each. After the final wash, the ovaries were kept in a water bath at 35 °C until the recovery of COCs. In total, 2356 good cumulus-oocyte complexes (COCs) were recovered from 476 freshly collected goat ovaries. In addition, a total of 16 testicles were taken from adult males slaughtered to obtain epididymal spermatozoa. They were brought to the lab in an ice box, which was kept at 20 to 25 °C (Ashour *et al.*, 2020; Khattab *et al.*, 2021).

In vitro Maturation

Cumulus-oocyte complexes were retrieved from the ovaries' surfaces using the slicing technique and placed in a 9-cm petri dish (Ashour *et al.*, 2020). The ovaries were rinsed in warm (37 °C) PBS that was fortified with 50 µg/mL gentamicin (Ashour *et al.*, 2020). The recovered COCs were morphologically assessed under the stereomicroscope (GX microscope, UK), according to Wieczorek *et al.* (2020). The morphological assessment of COCs was done as follows: Class I: those that had homogenous cytoplasm and with at least three layers of cumulus cells; class II: those that had homogenous cytoplasm and with one to two layers of cumulus cells; class III: those that had homogenous cytoplasm and without cumulus cells; and finally, class IV:

those that had heterogeneous cytoplasm and regardless of the number of cumulus cells. The classes I and II COCs were considered good-quality COCs that qualified for IVM, while classes III and IV were of poor quality and discarded (Figure 2).

In total, 3002 good COCs were recovered and allocated to the three experimental groups. Selected COCs were first washed twice with tissue culture medium 199 (TCM-199) supplemented with 25 mM HEPES and 5% fetal bovine serum (FBS), according to Ashour *et al.* (2020). The COCs were washed once in IVM medium consisting of TCM-199 supplemented with 10% (v/v) pre-heated FBS at 56 ºC for 30 min, 10 ng/mL epidermal growth factor (EGF), 1 µg/mL estradiol (E2), 0.25 mg/mL Na+ pyruvate, and 20 IU pregnant mare serum gonadotropin (PMSG) (Gonaser, 500 IU) according to previous publications (Soto-Heras *et al.*, 2019; Jose *et al.*, 2021; Maksura *et al.,* 2021). The hCG hormone (choriomon®, 5000 IU) was added to IVM medium at various doses (G1: IVM medium without hCG, which was allocated as a control group; G2: IVM medium supplemented with 10 IU of hCG; and G3: IVM medium supplemented with 20 IU of hCG). After that, COCs were incubated in drops of IVM medium (15 to 25 oocytes per 100 µl drop), then covered with mineral oil and kept in a CO_2 incubator for 24 hours at 38.5 °C with 5% CO_2 and 95% relative humidity in the air (Ramkumar and Ananthakumar, 2021).

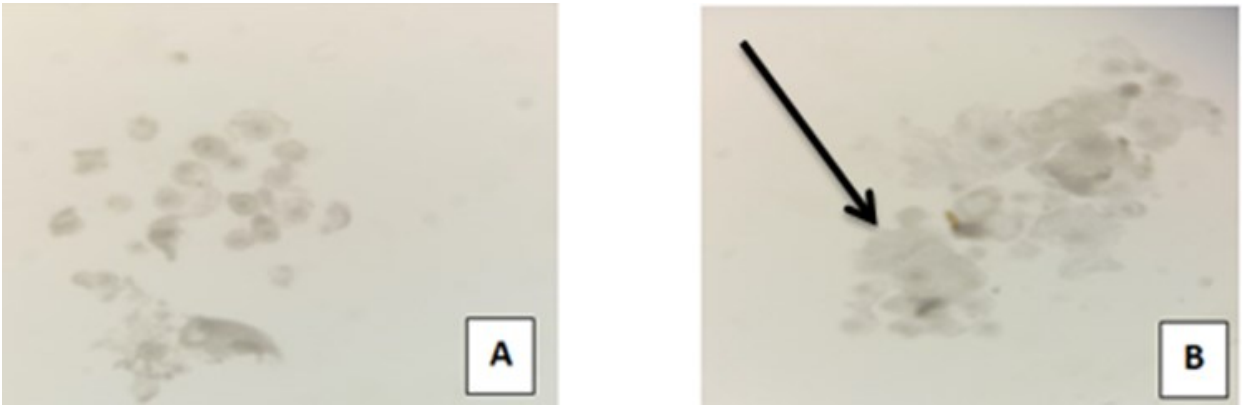

Figure 3. Maturation of oocytes according to cumulus expansion. A immature oocytes. B matured oocytes with expanded cumulus cells (black arrow) by stereo microscope (40x)

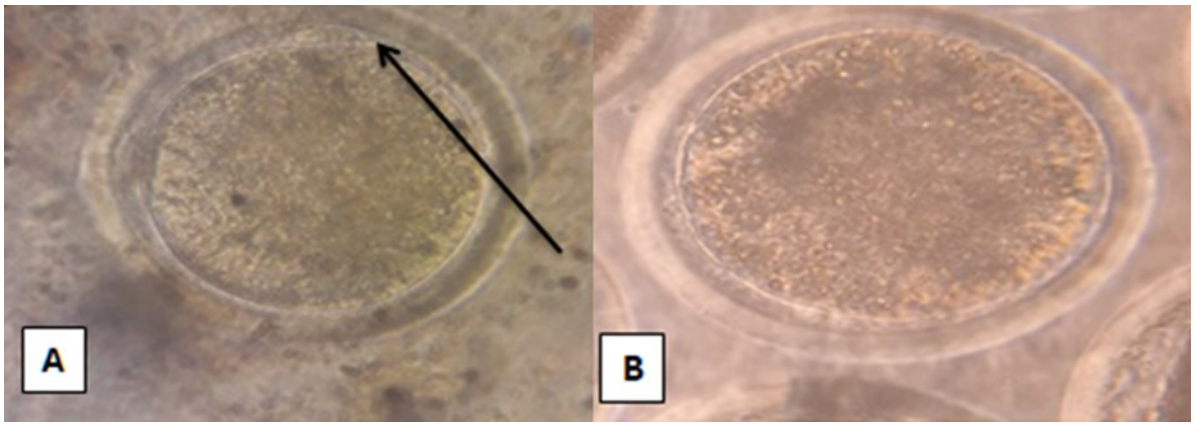

Figure 4. Developmental stages after IVM and denudation of oocytes. A Mature oocyte at MII stage (1st polar body black arrows). B Immature oocyte MI stage. Inverted microscope 400x.

Assessment of Oocyte Maturation

Oocyte maturation was assessed 24 hours after incubation based on the expansion of cumulus cells and the first polar body extrusion. Cumulus cell expansion was assessed under the stereomicroscope by evaluating the degree of expansion among cumulus cells (GX microscope, UK). Accordingly, expanded COCs were considered that clearly showed one or more expanded layers of cumulus cells and those that did not show any observable degree of expansion, as presented in Figure 3. The cumulus expansion rate was calculated as a percentage of total incubated COCs (Maksura *et al.*, 2021).

The observation of the first polar body was done after mechanical denudation of cumulus cells, followed by three washings with IVM medium (Figure 4). Finally, COCs were incubated for two minutes in a 200-µl drop of 80 IU/mL Hyaluronidase **(**de-Moura *et al.*, 2017) in prewarmed FertiCult flushing medium (Fertipro®, Beernerm, Belgium), followed by repeat pipetting in washing medium to remove any remaining cumulus cells. Denuded oocytes were examined under an inverted microscope (Leitz Fluovert FU Leica Microsystems, Wetzlar, Germany) to calculate the number of first polar bodies in each group.

Sperm Preparation for IVF

The IVF procedure was carried out according to Shakir and Adil Jebur (2022), with a few modifications. The sperm of adult male goats was recovered from the caudal epididymis into Tyrode's albumin lactate pyruvate (TALP), which consisted of 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.29 mM NaH2PO4, 21.6 mM Na lactate, 2.0 mM CaCl2, 1.5 mM MgCl2, 10 mM HEPES, and 10 mg/L phenol red. Supplemented with 0.6% BSA, 1 mM 33 sodium pyruvate, and 50 µg/mL gentamicin (Ashour *et al.,* 2021). This suspension was spun at 500 xg for 1 minute at room temperature. The spermatozoa pellet was incubated for 60 min at 38.5 °C before fertilization in 10 ml of capacitation medium, which was intended for use during *in vitro* procedures for insemination and incubation of oocytes. Consisted of sperm TALP media: supplemented with 4 mg/mL BSA, 50 IU/mL penicillin, and 10 µg/mL heparin (Ashour *et al.,* 2021). After centrifugation, the supernatant was carefully discarded. The pellet was re-suspended in the appropriate volume of pre-warmed fertilization medium (based on the sperm concentration assessed by a hemocytometer slide).

***In vitro* Fertilization**

Saini *et al.* (2022) reported, *in vitro* matured COCs were first washed twice with washing medium (TCM-199 supplemented with 25 mM HEPES and 5% FBS) and then washed three times with fertilization medium. After this, the mature COCs were co-cultured with the prepared sperms in a four-well plate (400 ml sperm suspension fertilization medium covered with 400 l mineral oil per well) in a CO_2 incubator. The sperm concentration was then adjusted to be $3x10^6$ sperm/mL (Khattab *et al.,* 2020).

Assessment of Fertilization

Presumptive zygotes were denuded as described above, and the first cleavage or second polar body extrusion was evaluated by visual observation using an inverted microscope (Leitz Fluovert FU Leica Microsystems, Wetzlar, Germany). Fertilization rate was calculated based on the number of cleaved embryos or zygotes that had a second polar body after 24 hours of fertilization process.

Evaluation of Mitochondrial Distribution

The *in vitro*-matured goat oocytes were fixed with 4% paraformaldehyde in a PVP-PBS solution. The fluorescent staining of intracellular goat oocyte mitochondria was performed using Mito-Tracker green® dye (Invirogen-M7514, USA) dissolved in DMSO (125 nm) and incubated for 5 minutes in the dark at room temperature according to Ghanem *et al.* (2021a). This was followed by three times washing in PVP-PBS solution for 15 minutes each. Thereafter, the goat oocytes were mounted on a clean glass slide with

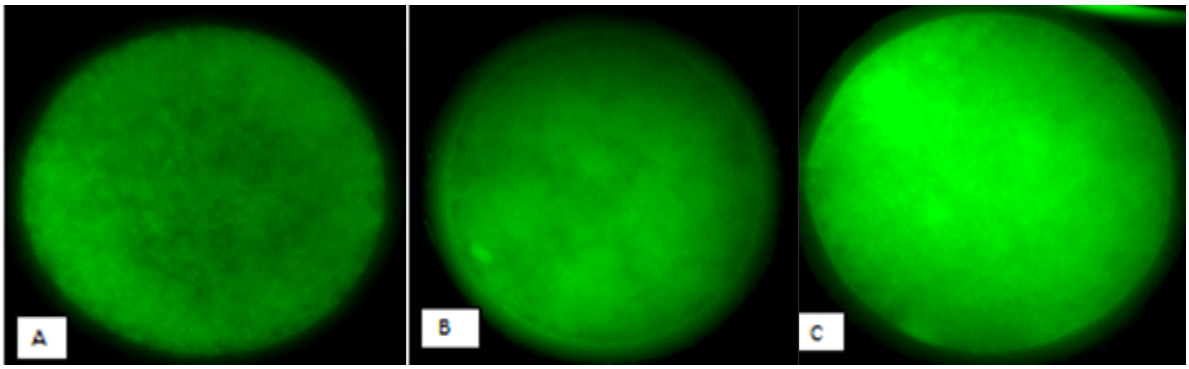

Figure 5. Representative fluorescence images of goat oocytes for (A): G1. (B): G2. (C): G3 Mito-Tracker-Green staining visualizes the mitochondrial distribution.

3 µl of glycerol, covered with glass slip, and a fluorescent image was captured by a fluorescent microscope (VE-146YT, Velab, Co. USA) at 580 –596 nm emission. Mitochondrial intensity and distribution (diffused, periphery, and semi-periphery) were evaluated (Figure 5) for each oocyte individually using Image J software (National Institutes of Health, USA).

Nuclear Maturation

Denuded oocytes were incubated in PVP-PBS containing 10 µg/mL Hoechst 33342 for 10 min. After washing in PVP-PBS, oocytes were mounted onto glass slides, and their nuclear configuration was analyzed. The meiotic stages of the oocytes were then evaluated using an inverted fluorescence microscope (Nikon, Tokyo, Japan), and each oocyte was classified according to the stage of nuclear configuration: the germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) stage (Satrio *et al.,* 2022).

Statistical Analysis

The data obtained in the current experiment were analyzed statistically by SAS Enterprise Guide 4. A chi-square test was applied for maturation and fertilization rates and staining results; in addition, one-way ANOVA was performed for the same parameters (after the angular transformation of the data) to distinguish the significant differences between groups' means. All statements of significance are based on a probability of less than 0.05.

RESULTS AND DISCUSSION

Recovery Rate of Goat's Oocytes

In the current study, 2356 goat oocytes were collected from 476 ovaries. As shown in Table (1), the total number of recovered oocytes per ovary was 6.31 oocytes per ovary. The average number of high-quality oocytes recovered from each ovary was 4.95 oocytes per ovary. The proportion of good-quality oocytes was 78.48%, while the percentage of bad-quality COCs was 21.52%.

***In vitro* Maturation Rate**

The cytoplasmic maturation rate as estimated by cumulus expansion was significantly higher in the G3 (82.53±0.81%) than the control (64.75±0.91%) and G2 (65.88±0.98%) groups, as shown in Table 2. Polar body extrusion was used to assess the maturation rate. Accordingly, the maturation medium rate was increased ($P \leq 0.01$) in G3 group (26.49±0.52%) compared to the G2 group (19.84±0.76%) and control group (G1:18.9±0.7%), as shown in Table 3.

Evaluation of Mitochondrial Distribution

The mitochondrial fluorescent intensity was higher ($P \leq 0.01$) in G3 (95.06±4.1) and G2 (89.21±2.13) than the control group

Table 1. Recovery Of Goats Cumulus-Oocyte Complexes Using Slicing Technique

Parameter	Values
Mean of oocyte recovery rate (total recovered oocytes/ovaries)	6.31 (3002/476)
Mean of good quality rate (good quality oocytes/ ovaries)	4.95 (2356/476)
Good oocytes % (No. of good oocytes /total no. of oocytes)	78.48 (2356/3002)
Bad oocytes% (No. of bad oocytes /total no. of oocytes)	21.52 (646/3002)

Table 2. Effect of hCG on Maturation Rate (%) of Goat oocytes according to cumulus layer expansion

Groups	Total COCs (n)	No. of COCs with Expanded Cumulus Layer	Cumulus Expansion Rate Mean ±SE	Chi Square Value (P value)	P Value of F-test
G1	882	570	64.75±0.91[b]	77.2898 (0.0001**)	P<0.0001**
G2	692	456	65.88±0.98[b]		
G3	782	646	82.53±0.81[a]		

Values within the same column with different letter superscripts are significantly different (P≤0.01). *P value < 0.05 means significant. **P value < 0.01 means highly significant. G1 = group 1 (free hCG). G2 = group 2 (+10 IU/mL hCG). G3 = group 3 (+20 IU/mL hCG) and COCs = Cumulus Oocyte Complexes.

Table 3. Effect of hCG on Maturation Rate (%) of Goat oocytes According to First Polar Body Percentage

Groups	Total Number of Culture Oocytes	No. of Mature Oocytes (MII)	Maturation Rate Mean ±SE	Chi Square Value (P-value)	P-value of F-test
G1	542	102	18.9±0.7[b]	11.39 (0.0034**)	P<0.0001**
G2	506	100	19.84±0.76[b]		
G3	566	150	26.49±0.52[a]		

Data recorded as mean±SE/ a&b = superscripts to be compared statistically within the same column. Values with different letter superscripts are significantly different (P≤0.01). *P value < 0.05 means significant. **P value < 0.01 means highly significant. G1 = group 1 (free hCG). G2 = group 2 (+10 IU/mL hCG). G3 = group 3 (+20 IU/mL hCG) and MII = mature oocytes

Table 4. Effect of hCG on Mitochondrial Florescent Intensity of *In vitro* Matured Goats' Oocytes

Groups	No. of Mature Oocytes (MII)	Mitochondrial Intensity Mean ±SE	P value of F-test
G1	15	79.87±2.45 [b]	P<0.003**
G2	15	89.21±2.13[a]	
G3	15	95.06±4.1[a]	

Data are recorded as meanSE/a&b: superscripts for statistical comparison within the same column. The values with different letter superscripts differ significantly (P 0.01). *P value 0.05 means significant; **P value 0.01 means highly significant. G1 = group 1 (free hCG). G2 = group 2 (+10 IU/mL hCG). G3 = group 3 (+20 IU/mL hCG) and MII = mature oocytes.

(79.87±2.45), as shown in Table 4. In addition, there was no significant difference in the mitochondrial intensity in G3 compared with the G2 group. Additionally, there were also significant differences among all groups in the distribution of active mitochondria in the cytoplasm of *in vitro*-matured goat oocytes (Table 5). A higher proportion (P≤0.01) of oocytes with peripheral mitochondria was observed in the G1 group (73.33%) than in the G2 (26.67%) and the G3 groups (6.67%). Group G2 (60%) had a higher (P≤0.01) percentage of the semi-peripheral distribution of active mitochondria (20%) than G1 (20%) and the G3 groups (73.3%). While the proportion of oocytes with diffused mitochondria increased (P≤0.01) in the G3 group (73.33%) compared with the G1 (6.67%) and the G2 groups (13.33%), as shown in Table 5.

Nuclear Chromatin Evaluation

The nuclear maturation stages of the oocyte progressed with the stage of germinal vesicle

(GV), which was characterized by condensation of the oocytes or slightly diffused chromatin. After that, the oocytes that possessed clumped or strongly condensed chromatin that formed an irregular network of individual bivalents or a metaphase plate but no polar body were classified as a metaphase I (MI) stage. The mature oocytes were characterized by those that reached the MII stage (oocytes with either a polar body or two chromatin masses). In this study, the proportion of MII oocytes in G3 (41.18%) was significantly higher than that in the G2 (23.53%) and G1 groups (11.76%), as presented in Table 6.

In vitro Fertilization Rate

The *in vitro* fertilization results (Table 7) shows, the G3 group had a significantly higher (P≤0.01) rate of zygote formation (18.73±0.94%) than the control and G2 groups (9.46±0.79% and 10.64±0.69%, respectively). The quantity and quality of recovered oocytes from the ovaries of slaughtered animals are essential factors that affect the formation of *in vitro*-produced embryos in goats and other farm animal species. By the slicing approach, an average of 4.91 excellent oocytes (A and B) were recovered per ovary in this study, which represented 15.9% of recovered oocytes per ovary. Oocyte recovery in goats was performed using a variety of procedures; however, the slicing technique reported a greater oocyte recovery rate and quality per ovary than puncture and aspiration methods (AL-Nuaimi *et al.,* 2020). Hormonal stimulation of oocytes during

Table 5. Effect of hCG on Mitochondrial Distribution of *In vitro* Matured Goat's Oocytes

	G1		G2		G3		P value of Chi square test
	N	%	N	%	N	%	
Diffused	1	6.67^{cb}	2	13.33^{b}	11	73.33^{a}	0.000**
Periphery	11	73.33^{a}	4	26.67^{b}	1	6.67^{c}	0.0005**
Semi-periphery	3	20^{b}	9	60^{a}	3	20^{b}	0.02*
Total No.	15		15		15		

Data are recorded as N and %. a, b, c, p value for Chi square test for comparing between the different rows. The values with different letter superscripts differ significantly (P 0.01). *P value 0.05 means significant. **P value 0.01 means highly significant. G1 = group 1 (free hCG). G2 = group 2 (+10 IU/mL hCG). G3 = group 3 (+20 IU/mL hCG). and MII = mature oocytes

Table 6 Effect of hCG on Nuclear Chromatin Evaluation of *In vitro* Matured Goat's Oocytes

	G1		G2		G3		P value of Chi square test
	N	%	N	%	N	%	
GV	5	29.45	3	17.13	1	5.89	0.0162 *
GVBD	7	41.18	6	35.29	3	17.13	0.0128 *
MI	3	17.65	4	23.53	6	35.29	0.1111
MII	2	11.76	4	23.53	7	41.18	0.0026 **
Total No.	17		17		17		

Data are recorded as N and %. a, b, c, = p value for Chi square test for comparing between the different rows and the values with different letter superscripts differ significantly (P 0.01). *P value 0.05 means significant; **P value 0.01 means highly significant. G1 = group 1 (free hCG); G2 = group 2 (+10 IU/mL hCG) and G3 = group 3 (+20 IU/mL hCG. the germinal vesicle (GV) = germinal vesicle breakdown (GVBD). metaphase I (MI). metaphase II (MII) stage.

Table 7. Effect of Adding of hCG in Fertilization Rate (%) of Egyptian Goats' Oocytes

Groups	No. of Culture Oocytes	No. of Fertilized Oocytes	Fertilization Rate Mean ±SE	Chi square value (P value)	P value of F-test
G1	340	32	$9.46±0.79^{b}$	10.70 (0.0047**)	P<0.0001**
G2	186	20	$10.64± 0.69^{b}$		
G3	216	40	$18.73± 0.94^{a}$		

Data recorded as mean±SE/ a&b = superscripts to be compared statistically within the same column. Values with different letter superscripts are significantly different (P≤0.01). **P value < 0.01 means highly significant. G1 = group 1 (free hCG). G2 = group 2 (+10 IU/mL hCG) and G3 = group 3 (+20 IU/mL hCG).

in vitro maturation is of the utmost importance in order to achieve nuclear and cytoplasmic maturation and subsequently, oocytes fertilization (Hatrnaz *et al.*, 2018). Our findings indicated that the group supplemented with a high level of hCG (20 IU/mL) during IVM recorded an increase in maturation and cumulus expansion rates compared to the control and low hCG dose-supplemented groups.

Similar to our results, the addition of hCG at a high level (20 IU/mL) increased cumulus expansion and nuclear maturation in goat oocytes (Kouamo and Kharche, 2014). According to Zombie *et al.* (2018), PMSG and hCG not only have comparable effects to the natural gonadotropin hormones (FSH and LH) but can also induce estrus and ovulation efficiently. Due to its ability to bind to LhCGR, hCG is a good substitute for LH. In this regard, the supplementation of IVM medium with these gonadotropins improved oocyte quality and developmental potential (Bahrami and Cottee, 2022). Moreover, these beneficial actions of these gonadotropins were confirmed in other species, as reported by Dinopoulou *et al.* (2016), who found that the addition of hCG to mouse GV oocytes during IVM accelerated maturation rates. Indeed, the IVM system in goat COCs could be considerably enhanced by adding a hormone combination (20 IU/mL of PMSG + 20 IU/mL of hCG + 1 µg/mL E2) to TCM-199 culture medium.

Oocytes' mitochondrial distribution and metabolic activity (mitochondrial intensity) affect how their cytoplasm and nucleus mature (Reader *et al.*, 2017).

Oocyte quality and embryonic development may be improved by adding hCG and increasing mitochondrial activity. According to our findings, adding hCG helps goat oocytes mature more competently and raises the intensity of the mitochondria when combined with free hCG. The findings suggest that the addition of hCG to the fertilization culture medium may be advantageous for stimulation cycles that contain a large percentage of immature oocytes. *In vitro*-matured good-quality bovine and buffalo oocytes had higher fluorescent mitochondrial intensity than bad-quality oocytes (Ghanem *et al.*, 2021a). Therefore, it seems that increased fluorescent mitochondrial intensity in oocytes matured *in vitro* in a medium supplemented with high hCG enhanced the maturation rate by providing metabolic requirements (Ghanem *et al.*, 2021a; Ghanem *et al.*, 2021b; Torner *et al.,* 2008).

Moreover, the distribution of mitochondria was associated with an enhancement of the nuclear maturation rate of bovine oocytes (Silva *et al.*, 2013). Ghanem *et al.* (2021b) have demonstrated a higher rate of mitochondrial central distribution in buffalo-competent (BCB+) than less competent (BCB-) oocytes. On the other hand, the rate of diffuse mitochondrial distribution was increased in competent (BCB+) compared to less competent (BCB-) porcine COCs after IVM, which was coupled with developmental competence enhancement (Fu *et al.*, 2015). Indeed, peripheral localization is more common in meiotically incompetent oocytes, which showed no sign of cumulus expansion and a clustered mitochondrial distribution pattern (Kirillova, *et al.*, 2021). Therefore, it seems that increased fluorescent mitochondrial intensity in oocytes matured *in vitro* in medium supplemented with high hCG enhanced maturation rate by providing metabolic requirements (Ghanem *et al.*, 2021a; Ghanem *et al.*, 2021b; Torner *et al.*, 2008).

By altering the nutritional environment to boost the energy available for the oocyte to support future development in fertilization and cleavage, LH may also improve the oocyte IVM. Enhanced glycolysis, together with increased mitochondrial glucose oxidations and increased tricarboxylic acid (TCA) cycle activity within cumulus cell-enclosed oocytes, could be used to demonstrate the net metabolic effect of LH exposure during the maturation stage (Mardenli *et al.*, 2021). Overall, supplementing the IVM medium with a high concentration of hCG (20 IU/mL) improved goat oocytes' maturation and fertilization rates by modulating mitochondrial distribution and enhancing their activity.

High levels of hCG in the follicular fluid

have been associated with increased oocyte quality and fertilization potential (Da Broi *et al.*, 2018). Accordingly, our data revealed a high rate of zygote formation in the group supplemented with high hCG (G3) compared to the control and G2 groups. This data is in line with the findings that revealed an increased fertilization rate following IVM in a medium supplemented with hCG in humans (Tantitham *et al.*, 2020). This idea is supported by research that indicates the positive action of gonadotropin supplementation during IVM in improving the developmental capacity of oocytes (Dinopoulou *et al.,* 2016; Bahrami and Cottee, 2022).

CONCLUSION

Based on the findings of this study, it is possible to conclude that maturation of goat oocytes in medium supplemented with 20 IU/mL hCG boosted maturation and fertilization rates by modulating cytoplasmic distribution and promoting utilization of oocyte mitochondria.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publication of this article.

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