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The Impact of Maturation Medium Supplemented with PMSG and HCG Hormones on *In vitro* Maturation of Ovine Oocytes

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ABSTRACT

This investigation aimed to assess the dose-dependent impact of the pregnant mare serum gonadotropin (PMSG) on the *in vitro* maturation of sheep oocytes and the effect of combining the best dose with the human chorionic gonadotropin (hCG), *which is* a cost-effective substitute for FSH and LH hormones. The study was planned as two experiments that included 1134 oocytes to study the impact of varying concentrations (0, 10, 20, and 40 IU/mL) of PMSG addition (1st experiment) as well as the influence of hCG, PMSG, or their combination (2nd experiment) on the ovine's oocyte *in vitro*. The experimental groups of the 2nd experiment were control (G1), 20 IU/mL hCG (G2), 20 IU/mL PMSG (G3), and 20 IU/mL PMSG plus 20 IU/mL hCG (G4). The first experiment demonstrated that the polar body (PB) rate was increased ($P < 0.01$) by all treated groups, being 13.33, 17.76, and 15.70 in G2, G3, and G4 versus 9.87% in G1, respectively. The mitochondrial fluorescent intensity recorded 7.61, 11.92, 12.43, and 12.35, while the lipid fluorescent intensity was 9.88, 29.98, 40.05, and 25.66 for G1, G2, G3, and G4, respectively, being higher ($P < 0.05$) in treatment groups than in the control one. The results of the 2nd experiment showed that the PB extrusion was higher ($P < 0.01$) in G2, G3, and G4 than in G1 (12.60, 14.91, and 14.75 vs. 6.92%, respectively). The mitochondrial fluorescent intensity recorded 8.82, 12.30, 17.58, and 13.36 for G1, G2, G3, and G4, respectively. The lipid fluorescent intensity was 10.16, 6.98, 13.83, and 49.68 for G1, G2, G3, and G4, respectively. In conclusion, the addition of PMSG and hCG to sheep oocyte maturation media improved the oocyte maturation.

Keywords: hCG, IVM, Oocytes, PMSG, Sheep.

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INTRODUCTION

Sheep are an important part of Egyptian agriculture, accounting for around 30 percent of the overall agricultural total revenue. Egypt is promoting the production of sheep to increase human dietary protein intake. Sheep are thus a crucial part of Egypt's food security plan. In the year of 2017, approximately 2.34 million head of sheep provided 72,296 tons of red meat, accounting for around 7.4% of Egyptian output of red meat (Elshazly and Youngs 2019). Recently, in 2021, the number of sheep was reduced to 1.94 million head in Egypt **(FAO, 2023).**

Sheep species are important because of their biological properties, which include short generation intervals, twinning, short growth periods, and moderate requirements for space **(Nawito *et al.*, 2015).** Furthermore, the ability to convert forages and feeds with low nutritional value to high-protein products such as meat and milk **(Elshazly and Youngs 2019).** Egyptian sheep breeds have high fertility rates and a long breeding season, but they have limited prolificacy. **(Gabr *et al.*, 2016; Elshazly and Youngs 2019).** Prolificacy in reproductive variables like follicle development, ovulation rate, and litter size is influenced by factors like age, hormones, genetic mutations, environmental factors, nutrition, and fetal growth. **(Mohanan *et al.*, 2020).** Assisted reproduction technologies (ART) have been developed to produce high-yielding lambs, but in vitro embryo production (IVP) has limitations and requires improvement for healthy lambs. **(Camargo *et al.*, 2006).**

In vitro embryo production (IVP) represents a helpful technique for producing offspring and is composed of numerous laboratory methods, involving in vitro maturation (IVM), fertilization (IVF), and in vitro culture (IVC) **(Lonergan and Fair, 2016; Viana *et al.*, 2018)**. To use IVM technology, immature oocytes

must be recovered from the follicles found on the ovarian cortex and then cultured under optimal conditions to reach maturity **(Wang *et al*., 2014).** Different facets of IVM in mammalian oocytes have been investigated by a number of researchers **(Dunning and Robker, 2018; Yang *et al*., 2021; Jiang et al., 2023).** The maturation of the oocyte is regarded as one of the critical events during IVP since it requires nuclear, biochemical, structural, and cytoskeletal modifications, which are essential for the embryo's early development **(Jiang *et al*., 2023).** In this respect, **del Collado *et al*., (2017)** mentioned that cytoplasmic maturation has been characterized by structural alterations like cortical granule dispersion and reorganization, protein and RNA storing, calcium regulatory mechanism development, and mitochondrial migration to a perinuclear location.

Mature oocytes of good quality are crucial factors in determining blastocyst and fertilization rates. **(Lonergan and Fair 2016; Ferré *et al*., 2020; Widayati and Pangestu 2020).** Gonadotropin binding with receptors and subsequent activation of the corresponding G protein initiate nuclear maturation processes by initiating a sequence of signaling pathway events leading to the phosphorylation of cyclic adenosine monophosphate (cAMP)-dependent protein kinases **(Russell *et al*., 2016),** which works as a secondary messenger associated with the gonadotropin signaling system in the tissue of the ovary. Cyclic AMP has a physiological role in the follicular environment by regulating the meiotic maturation of oocytes **(Botigelli *et al*., 2017)**. Gonadotropins are essential for oocyte maturation **(Kaabi *et al*., 2020; Lee *et al*., 2023)** and gonadotropic hormones are important because they affect ovarian activity, which is required for the development of viable oocytes. IVM of oocytes is commonly carried out in medium enriched via gonadotrophic hormones that include the follicle-stimulating hormone (FSH) as well as the luteinizing hormone (LH), which promote granulosa cell proliferation and initiate meiotic divisions **(Xiao *et al*., 2014; Wei *et al*., 2016).**

Pregnant mare serum gonadotropin (PMSG) has a performance similar to FSH and LH. It possesses a strong attraction for the FSHR and LHR receptors in the ovaries. It was found that PMSG administration might lead to better reproductive function, ovulation enhancement, and increased rates of conception in noncyclic cattle **(Uslu *et al*., 2012)** as well as sheep **(Wei *et al*., 2016)**. Gonadotropins, specifically FSH, can be added to the medium of maturation to boost the mRNA expression of gonadotropic hormone' receptors in cumulus cells, causing them to proliferate and ultimately mature into oocytes. **(Lee *et al*., 2007; Xiao *et al*., 2014).**

Regardless of these advantages, the commonly used swine pituitary FSH is expensive, has a wide range of pharmaceutical purity, comes with several different commercial formulas, and is sometimes tainted by other gonadotropic hormones, which might provide inconsistent or inferior results **(Sha *et al*., 2010)**. Therefore, human chorionic gonadotropins (hCG) and PMSG, two gonadotropic sources that operate similarly to FSH, were successfully employed in the mammalian IVM of oocytes **(Bastos *et al*., 2022).** Moreover, commercially available PMSG is more affordable than FSH and LH. Regarding the current investigation, we aimed to investigate the influence of PMSG concentration and the combination of hCG and PMSG on the *in vitro* matured oocytes in sheep.

MATERIALS AND METHODS

Experimental site

The study was conducted at the Desert Research Center's (DRC) Embryology Manipulation Unit (EMU), Department of Animal and Poultry Physiology, Division of Animal and Poultry Production, Cairo, Egypt.

Ethical endorsement

This study was done in compliance with the Desert Research Center's code of ethics and animal rights standards. Furthermore, our investigation aimed to adhere to the regulations and criteria established in the European Union directive for the protection of experimental animals (2010/63/EU).

Media and chemicals

Chemicals and media elements were purchased from Sigma-Aldrich (St. Louis, Missouri, United States) unless specified otherwise.

Biological material

Ovarian collection: The sheep ovaries (N = 465) were randomly acquired from ewes with unknown reproductive history in a local slaughterhouse (Al-Monib, Giza). Within one to two hours following slaughter, the ovaries were brought to the lab in a thermos flask filled with a warm (35 ºC) normal saline solution (NaCl 0.9%), enhanced with 50 µg/mL gentamicin sulfate, in a thermos flask. Extraneous tissues were separated from the ovaries upon arrival at the lab, and to remove any contaminants, the ovaries were rinsed with 70% ethanol.

Oocyte recovery

The slicing approach was applied to obtain 1,134 oocytes from the ovaries as described by **AbdElkhalek *et al*., (2024).** oocytes were gathered in a disposable, sterilized Petri dish with phosphate-buffered saline. Oocytes were searched for under a stereomicroscope (GX microscope, UK, Range: 8x to 50x) using a fine bore pipette before being put in

another Petri plate containing an oocyte washing medium. Cumulus-oocyte complexes (COCs) were assessed based on morphological appearance according to (El-Sayed *et al*., 2015). Oocytes were divided into two categories. Good quality oocytes consist of grade A with at least four or five layers of cumulus cells and homogenous cytoplasm, and grade B has two to three layers of cumulus cells with homogenous cytoplasm. The second category of bad-quality oocytes included grade C with one layer of cumulus cells, partially denuded oocytes, and denuded oocytes were excluded from the experiment. The oocytes were put in a Petri plate with a washing medium (TCM-199 supplemented with 10% fetal bovine serum (FBS) and 25 mM HEPES) and then drop-washed three times (100 µL each), followed by two washes with a maturation medium according to the experimental design.

Experimental design

Experiment 1

Impact of various dosages of PMSG on sheep oocyte maturation in vitro

Group 1 (control): IVM medium included TCM-199, sodium pyruvate (0.25mM), gentamycin (50µg/mL), estradiol 17-ß (1µg/mL), 20 IU/mL hCG, 10 ng/mL epidermal growth factor (EGF), and 10% FBS (v/v).
Group 2: Control medium supplemented with 10 IU/mL PMSG.
Group 3: The control medium supplemented with 20 IU/mL PMSG.
Group 4: Supplemented with 40 IU/mL of PMSG in the control medium.

Experiment 2:Impact of PMSG and hCG on sheep oocyte maturation *in vitro*

Group 1 (control): The control medium in the experiment 1 without the hCG hormone. Meanwhile, the control medium was supplemented with 20 IU/mL of hCG alone **(G2)**, 20 IU/mL of PMSG alone **(G3)**, and 20 IU/mL PMSG plus 20 IU/mL hCG **(G4)**.

Incubation environment

The oocytes were incubated in 100 µL droplets of a pre-warmed, pre-equilibrated (incubated for at least two hours at 38.5 °C in CO_2 incubator before used) maturation medium, placed on a culture dish with mineral oil on top, and then incubated in a humidified atmosphere of 5% CO_2 in a CO_2 incubator for twenty-four hours at 38.5 °C **(Natarajan *et al*., 2010; Mustafa and Naoman, 2022).**

Evaluating the state of oocyte maturation

After 24 h of IVM, the rate of maturation was assessed based on the cumulus cells expansion and the first polar body extrusion. To examine the first polar body **(Fig. 1.C)**, two minutes of moderate pipetting were applied to remove cumulus cells, and the oocytes were assessed under the inverted microscope (Leitz Fluovert FU Leica Microsystems, Wetzlar, Germany).

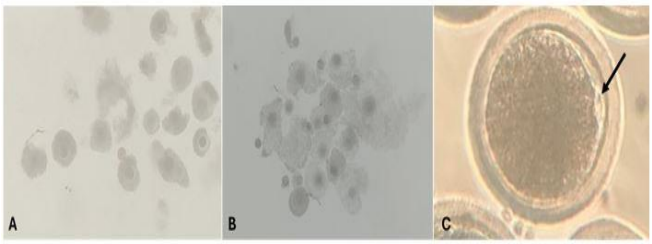

Fig.1. Maturation of oocytes according to cumulus expansion. A: Immature oocytes. B: Matured oocytes with expanded cumulus cells. C: Matured oocyte with a first polar body (black arrow). A and B were observed by stereo microscope (40x), while C was observed using an inverted microscope (400X).

Immunofluorescence staining and evaluation of mitochondrial and lipid intensity

The staining protocol was accomplished in accordance with **AbdElkhalek *et al*., (2024).** Briefly**,** the oocytes were preserved with fixative (4% paraformaldehyde) at 4 °C till the staining time. On the working day, they were washed from the paraformaldehyde with phosphate buffer saline with polyvinylpyrrolidone (PBS-PVP). After washing the oocytes, they were kept for 3 hours in PBS-PVP supplemented with 100 nM of Nile red in the absence of light. Preceded by washing with PBS-PVP **(Ghanem *et al*., 2021)**, the mitochondria of intracellular sheep oocytes were fluorescently stained via 125 nM Mito-Tracker green® (Invirogen-M7514, USA) diluted in dimethyl sulfoxide (DMSO) then incubated for five minutes at room temperature in the absence of light. Followed by the washing of oocytes three times via PVP-PBS.

Then, sheep oocytes were mounted on a clean glass slide with ≈ 3 µL drop of glycerol, under a glass cover. The fluorescent images were acquired via a fluorescent microscope (VE-146YT, Velab, Co. USA) at an emission range of 580 –596 nm. The intensity of mitochondria and lipid content in both experiments were assessed **(Fig. 2 and 3)** to every oocyte separately using the Image J program (National Institutes of Health, USA).

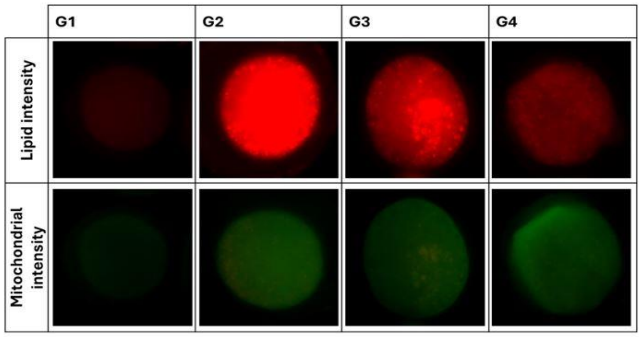

Fig. 2: Representative fluorescence images of sheep oocytes in G1(control), G2(PMSG 10 IU/mL), G3(PMSG 20 IU/mL), and G4 (PMSG 40 IU/mL). Mito-Tracker-Green and Nile red staining visualize the mitochondrial distribution and lipid intensity, respectively.

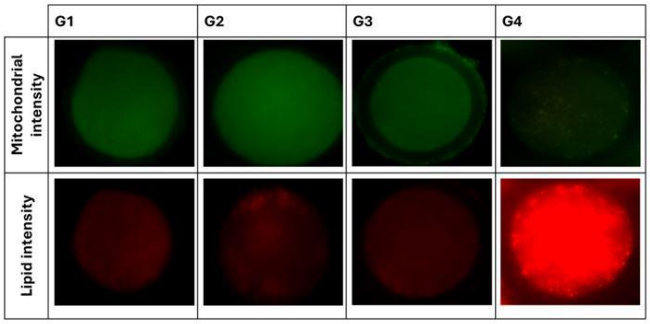

Fig.3: Representative fluorescence images of sheep oocytes in G1 (control group) with no hormonal additives, G2 (20 IU/mL HCG of), G3 (20 IU/mL of PMSG), and G4 (combination of 20 IU/mL PMSG and 20 IU/mL HCG). Mito-Tracker-Green and Nile red staining visualize the mitochondrial distribution and lipid intensity, respectively.

Nuclear maturation

Oocytes that had been denuded were treated for 10 minutes in PBS plus PVP with 10 µg/mL of Hoechst 33342. The nuclear structure was examined using an inverted fluorescence microscope (Nikon, Tokyo, Japan), after rinsing the oocytes with PVP-PBS and mounted on slides. Each oocyte was categorized based on the nuclear configuration stage shown in Figure 4 according to **Satrio *et al*. (2022)**, being germinal vesicle (GV), germinal vesicle breakdown (GVBD), the metaphase-I (MI), and metaphase-II (MII).

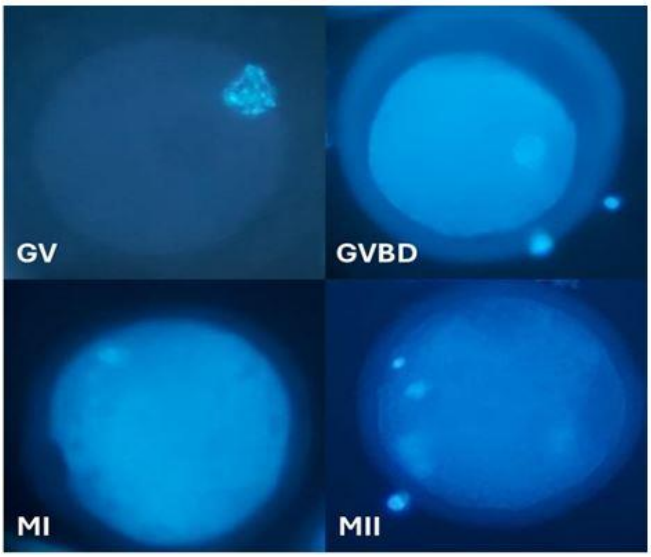

Fig.4: Stages of nuclear maturation of sheep oocytes as observed under fluorescence microscope using Hoechst 33342© stain. (GV: gremial germinal vesicle, GVBD: gremial germinal vesicle breakdown, MI: metaphase-1. MII: metaphase-2. Black arrow points to the first polar body.

Statistical analysis

Data of maturation development were examined using the SAS/STAT® 9.2 software (SAS, 2008). Data of maturation rate were statistically analyzed by one-Chi-square test, while those of mitochondrial and lipid intensity were analyzed by one-way analysis and the statistical model was as follows: $Y_{ij} = \mu + T_i + e_{ij}$ where: Y_{ij} = observation, μ = overall mean, T_i = treatment effect, and e_{ij} = random experimental error. Comparisons among treatment groups were set using Duncan's Multiple Range test at $P<0.05$. Data of stains were expressed as Mean ±SE while maturation data were expressed as percentage (%).

RESULTS

The results of sheep oocyte maturation affected by PMSG concentration are presented in **Table 1.** In this study, the high concentrations of PMSG (20 and 40 IU) in G3 and G4 produced a favorable, substantial ($P < 0.01$) impact on the cumulus expansion rate more than the control and G2. However, there were no substantial variations among the treated groups G2, G3, and G4. Noteworthy to mention is that no noticeable variation was found between G2 and G1 (the control group, **Table 2**). In addition, the polar body extrusion rate was increased ($P < 0.01$) in all groups that received treatment (G2, G3, and G4) in comparison with the untreated group (G1). These results indicated that the addition of 20 IU PMSG/mL of maturation medium showed the highest impact on improving the *in vitro* nuclear maturation of sheep oocytes.

Evaluation of mitochondrial and lipid intensity

The results shown in **Table 2** indicated that the mitochondrial fluorescent intensity of in vitro matured oocytes affected by different PMSG concentrations was higher ($P \leq 0.01$) in G2, G3, and G4 than in G1. In addition, there was no apparent variation in the mitochondrial intensity among the treated groups. On the other hand, the lipid's fluorescence intensity was greater ($P \leq 0.01$) in G3 when compared with G4, G2, and the control group. Moreover, all treated groups were higher ($P \leq 0.01$) than G1. However, no significant differences between the G2 and G4 groups have been found.

Experiment 2: Impact of PMSG and hCG on sheep oocyte maturation *in vitro*

Data from the second experiment showed that the impact of the combination of PMSG and hCG on the cumulus expansion rate in the G2 and G4 groups was greater ($P \leq 0.01$) than in the G1 and G3 groups. However, no variation ($P \leq 0.01$) was noticed between the two sets of groups. Adding to that, the effect of PMSG and hCG combination on the first polar body extrusion was greater ($P \leq 0.01$) in G2, G3, and G4 in comparison to the G1 group. Although, no apparent variations ($P \leq 0.01$) were found between those groups, as demonstrated in **Table 3.**

Table 1: Effect of different concentrations of PMSG on *in vitro* cytoplasmic and nuclear maturation of oocytes in sheep

Group	Total oocytes (N)	Expanded oocytes		Oocytes with first polar body (MII)	
		N	%	n	%
G1 (Control)	52	7	57.24[b]	5	9.87[c]
G2 (10 IU PMSG/mL)	65	11	67.27[ab]	2	13.33[b]
G3 (20 IU PMSG/mL)	52	13	74.34[a]	7	17.76[a]
G4 (40 IU PMSG/mL)	72	32	76.74[a]	7	15.70[ab]
Chi-square value			16.9432		4.3440
P-value			0.0007***		0.0337*

a, b, c: Values within the same column with different letter superscripts are significantly different at P≤0.05.
* Significant differences at P < 0.05. *** Significant differences at P < 0.001.

Table 2: Effect of different concentrations of PMSG on mitochondrial and lipid fluorescent intensity of *in vitro* matured oocytes in sheep

Group	No. of mature oocytes (MII)	Mitochondrial intensity Mean ±SE	Lipid intensity Mean ±SE
G1 (Control)	20	7.61±0.4[b]	9.88±0.83[c]
G2 (10 IU PMSG/mL)	20	11.92±0.82[a]	29.98±1.82[b]
G3 (20 IU PMSG/mL)	20	12.43±0.64[a]	40.05±3.99[a]
G4 (40 IU PMSG/mL)	20	12.35±1.13[a]	25.66±2.58[b]
P-value		0.0001***	0.0001***

a, b, c: Values within the same column with different letter superscripts are significantly different at P≤0.05. *** Significant differences at P < 0.001.

Table 3: Cytoplasmic and nuclear maturation rate of *in vitro* matured oocytes as affected by hCG, PMSG, or their combination supplementation in sheep.

Group	Total oocytes (N)	Expanded oocytes		Oocytes with 1st polar body (MII)	
		n	%	n	%
G1 (control)	130	97	74.62[b]	9	6.92[b]
G2 (20 IU hCG /mL)	127	109	85.83[a]	16	12.60[a]
G3 (20 IU /mL PMSG)	114	89	78.07[b]	17	14.91[a]
G4 (20 IU PMSG+20 IU hCG/mL)	122	105	86.07[a]	18	14.75[a]
Chi-square value		-	8.0742	-	9.868
P-value		-	0.044 *	-	0.0197*

a, b: Values within the same column with different letter superscripts are significantly different at P≤0.05.
* Significant differences at P < 0.05.

Evaluation of mitochondrial and lipid intensity

The results presented in **Table 4** indicated that the fluorescence intensity of mitochondria was greater (P ≤ 0.01) in G3 than in G4, G2, and G1, the control group. Although G2 and G4 were higher (P ≤ 0.01) than the control group, no noticeable variation has been found between those two groups. Additionally, the lipid fluorescent intensity in G4 was substantially greater (P ≤ 0.01) than in all other groups, G3, G2, and the control group. In contrast, the G1, G2, and G3 groups were not significantly different from each other.

Table 4: Effect of hCG, PMSG, or their combination on mitochondrial and lipid fluorescent intensity of *in vitro* matured oocytes in sheep.

Group	No. of mature oocytes (MII)	Mitochondrial intensity Mean ±SE	Lipid intensity Mean ±SE
G1 (control)	20	8.82± 0.45^{c}	10.16±0.62^{b}
G2 (20 IU hCG /mL)	20	12.30± 1.23^{b}	6.98±0.1^{b}
G3 (20 IU PMSG /mL)	20	17.58± 1.28^{a}	13.83±0.67^{b}
G4 (20 IU PMSG+20 IU hCG/mL)	20	13.36± 0.76^{b}	49.68±4.43^{a}
P-value		0.0001**	0.0001**

a, b, c: Values within the same column with different letter superscripts are significantly different at P≤0.05.
** Significant differences at P < 0.01.

DISCUSSION

The current study highlights the importance of supplementing sheep oocytes with gonadotrophic hormones in the IVM medium, as the absence of PMSG hormone reduced cumulus expansion and first polar body extrusion rates**.** The study found that all supplemented groups significantly increased the rate of maturation due to polar body extrusion, regardless of PMSG concentration (1st experiment), and that either PMSG or hCG and the combination of both significantly increased the oocyte IVM rate due to polar body extrusion (2nd experiment) in sheep. The observations were supported by immunofluorescence staining data of mitochondrial activities and lipid content in matured sheep oocytes.

The first experiment's results showed that the highest proportion of oocytes with 1st polar bodies was found in G3, where the PMSG 20 IU/mL concentration was applied. This finding was consistent with **Wei *et al*., (2016)**, who found that sheep oocyte maturation increased with equine chorionic gonadotropin (eCG; also known as pregnant mare serum gonadotropin (PMSG)) incorporation, and apoptosis rates were lower in PMSG-treated groups compared to the control group. Furthermore, our results are in agreement with **Gupta *et al*., (2001),** who claimed that PMSG obtained from commercial sources may be efficiently utilized as a substitute for pure FSH for IVM of buffalo oocytes, which makes it more affordable and cost-saving for IVF investigations. Also, **Kouamo and Kharche (2014)** indicated that PMSG could be utilized as an alternative for gonadotropic hormones for oocyte IVM in sheep.

Similarly, it was found that gonadotropins such as FSH positively affect oocyte maturation, as FSH stimulation improves the cyclic-AMP generation via the COCs that induce the GVBD **(Guler *et al*., 2000)**. This study supported the concept that indicates that gonadotropins produce a boost in the quantity of cyclic AMP in granulosa cells and improve the maturation of oocytes **(Arroyo *et al*., 2020)**. Likewise, the elevation of cAMP levels in granulosa cells can first inhibit the meiotic division resumption; this action is accompanied by a rapid rise in the maturation process speed **(Eppig and Downs, 1988; Sirard and First, 1988)**. A study on the signal transduction system by which FSH influences cumulus expansion, using specific inhibitors of adenylate cyclase (DDA) and PKA (H-89), demonstrated that the stimulatory impact of FSH on the expansion and nuclear maturation in bovine COCs was blocked by both inhibitors **(Roberts *et al*., 2005)**. In addition, the oocyte may directly receive cAMP generated in the cumulus cells, which would cause meiosis to resume. A transient and temporary increase in the amount of oocyte cAMP preceding the commencement of meiosis in rodents. **(Chen *et al*., 2009)** and ovine **(Bilodeau-Goeseels and Magyara, 2012; Richani and Gilchrist, 2022)** oocytes are reported.

Conversely, the oocyte may receive a signal or signals that promote oocyte maturation because of the increased cyclic-AMP content within the cumulus cells. It has been demonstrated that in response to gonadotropin stimulation, rat granulosa cells produce a factor or factors that control the pace of maturation and enhance the fertilization of the oocyte **(Vanderhyden and Armstrong, 1990)**. Furthermore, **Byskov *et al*., (1997)** found that when mouse COC cumulus cells are activated with FSH rather than LH or hCG, they release a meiosis-activating chemical. This thermally stable material flows inside the oocyte and, via a paracrine way, stimulates the continuation of meiosis in both COCs and oocytes without cumulus cells. Also, **Accardo *et al*., (2004)** found that the lowest rate of maturation was in the no-gonadotropin group in sheep. **Ali and Sirard (2005)** showed that FSH increases germinal vesicle breakdown by a process that is mediated indirectly by the cumulus cells. Recently, it was demonstrated that the FSH receptor's mRNA is absent from oocytes produced from small and medium-sized follicles in bovine **(Alam and Miyano, 2020)**.

Furthermore, the culture of rat **(Byskov *et al*., 1997)** cumulus-free oocytes with FSH had no impact on maturation, suggesting that the cumulus cells are the way that FSH influences the meiosis restart. Additionally, the low maturation results found in the untreated group in the current research could be clarified via the findings of **de Frutos *et al*., (2014)**. They established that during the lack of FSH, cumulus cells create an exceptionally thick grid of transzonal projections (TZPs) that culminates at the surface of the oocyte. The presence of these structures is reduced via FSH treatment. In the current work, we reported that the granulosa cell's expansion and polar body rates were reduced in oocytes cultivated without gonadotropins. PMSG and/or hCG have been demonstrated to enhance the growth of granulosa cells in sheep oocytes and additional species of mammals **(Accardo *et al*., 2004; AbdElkhalek *et al*., 2024).**

Mingoti *et al*., (2002) reported that in cumulus cells, PMSG promotes the expression of hCG receptors, permitting the activation of intracellular messengers by hCG. Furthermore, PMSG may attach to both FSH and LH receptors. This could explain the result of **AbdElkhalek *et al*., (2024)**, as they found that the supplementation of gonadotropins, a combination of hormones (20 IU/mL PMSG plus 20 IU/mL hCG), to IVM medium significantly improved the IVM of goat oocytes, which agrees with the findings of the current study. These findings are supported by **Accardo *et al*., (2004)**, who found that a combination of gonadotrophic hormones significantly increased the IVM of sheep oocytes. More specifically, it was reported that adding 20 IU/mL of PMSG to oocyte IVM medium in goats substantially enhanced the IVM rate. This was improved even more via the incorporation of 20 IU hCG/mL into in vitro maturation protocols **(Kouamo and Kharche, 2014).** This may be because employing these gonadotropins for IVM enhances the quality and developing potential of the oocyte by possibly changing metabolic pathways. Moreover, it was reported **(Farag *et al*., 2009)** that enriching TCM-199 via hormone combinations (PMSG + hCG + E2) significantly enhanced the IVM of COCs in contrast to the control (14.75 vs. 3.50, respectively) in sheep.

The significantly higher rate of cumulus expansion in the current study that was found in hCG-supplemented groups (G2 and G4) could be due to the primary mechanism influencing the preovulatory rise in estradiol synthesis being the stimulation of androgen secretion and the release of androgen precursor by theca interna cells. Which is stimulated by hCG during IVM **(Hazarika *et al*., 2019)**. This was demonstrated *in vivo*, via the elevated incidence of hCG irregular spikes, and this might clarify the efficiency of the hCG-containing medium in causing a greater degree of cumulus cell growth via improved IVM of COCs during incubation. Additionally, the significantly increased rates of MII oocytes observed in G3 and G4, in the second experiment, may be because the PMSG plays a mixed role as FSH and LH **(Sha *et al*., 2010)**. **Dinopoulou *et al*., (2016)** demonstrated that when hCG was added to oocytes in the GV stage during IVM, it enhanced the maturation rates in mice. When gonadotropins connect to their receptors, the accompanying G protein is activated, causing the transformation of guanosine triphosphate into guanosine diphosphate (GDP). The GDP binds to the α-subunit of the G protein, prompting adenylate cyclase to produce cyclic-AMP, a key component in regulating oocyte meiosis **(Botigelli *et al*., 2017)**. This finding indicates the relation between gonadotropins and energy production by the mitochondria in the oocyte.

Regarding that, in contrast to the control, the gonadotropin-treated groups demonstrated increased levels of mitochondrial activity. This increase in mitochondrial activities was positively correlated with the maturation rate of sheep oocytes. This may be because once activated, mitochondria guarantee oocytes' functional competence, particularly during cytoplasmic and nuclear maturation according to **Moussa *et al*., 2015)**. Additionally, mature oocytes always exhibit higher levels of mitochondrial activation than immature oocytes **(Tarazona *et al*., 2006)**. Adding PMSG and hCG, as well as improving mitochondrial activity, can increase the quality of the oocyte and the development of the embryo. According to **Ghanem *et al*., (2021)**, combining PMSG with hCG improved goat oocyte maturation and increased mitochondrial intensity.

Lipids are essential for many biological activities, including the composition of cell membranes, storage of energy, cellular signals, metabolism, and epigenetic mechanisms **(de Lima *et al*., 2023)**. The' role of lipids that supports the metabolic activities of oocytes and sperm cells, ovarian follicles, and embryos has been explored **(Marei *et al*., 2010; Sudano *et al*., 2016)**. The lipid content of oocytes was increased in the treated groups in comparison with the control group in both the first and second experiments in the current study. This increase in the lipid content was positively correlated with the maturation rate of sheep oocytes. Although no studies have directly evaluated the significance of lipids as energy sources during buffalo COC maturation, it is widely known that cumulus cells and oocytes store significant quantities of lipids in the shape of lipid droplets **(Mondadori *et al*., 2010)**. According to **Paczkowski *et al*., (2013)**, the content of lipids inside mouse, cattle, and pig COCs determines that they are sensitive to the negative impacts of β-oxidation inhibition during IVM. These findings suggest that

oocytes with higher lipid contents rely more heavily on this metabolic route to provide energy during maturation. Additionally, when both pig and cattle oocytes matured, the triglyceride content decreased, which is in line with the stimulation of lipolytic pathways as stated by **Sturmey and Leese, (2003); Romek *et al*., (2011)**. On the other hand, additional research findings showed that *in vitro*-developed cattle oocytes increased their stock of lipid reserves through the GV to the MII stages of development **(Aardema *et al*., 2011; del Collado *et al*., 2017)**. Thus, additional study is required to identify the processes behind the lipid contribution to sheep oocyte maturation *in vitro*.

CONCLUSIONS

The current investigation demonstrated that adding gonadotropic hormones (hCG and PMSG) to sheep oocyte IVM medium improved the oocytes' rate of maturation. In particular, the addition of 20 IU/mL PMSG and 20 IU/mL hCG to the maturation media substantially improved the maturation rate of ovine oocytes, according to expansion and polar body rates as well as mitochondrial and lipid content results.

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Conflict of interest

The authors declare they have no competing interest.

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