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Effects of Epidermal Growth Factor and Myo-Inositol on Nuclear Configuration and Subsequent Embryonic Development of Sheep Oocytes

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A R T I C L E   I N F O

A B S T R A C T

Growth factors and vitamin-like substances have great positive importance in most biological interactions in the cellular level. The addition of these elements in the culture media will increase the yield of the resulting embryos and improve quality. We examined the effects of epidermal growth factor (EGF) and myo-inositol (MI) on meiotic maturation and yields of blastocyst of Awassi sheep oocyte across two experiments. The oocytes obtained were subjected into three treatments: A (without EGF nor MI), B (10 ng/ml EGF + 20 mmol/l MI) and C (50 ng/ml EGF +40 mmol/l MI). Oocytes were then cultured in Ham's F-10 medium supplemented with 5% (v: v) fetal calf serum and 40 ng/ml follicle - stimulating hormone. In the first experiment, during the 27-h culture period, the oocytes were assessed for germinal vesicle break down, metaphase-I and metaphase-II stages across three-time intervals (9, 21 and 27-h). Results of the experiment showed that EGF and MI enhanced the rates of germinal vesicle break down phase (1.53%; 27-h interval; lowest value), metaphase-I (33.87%; 21-h interval) and metaphase-II (89.23%; 27-h interval). In the second experiment, the oocytes incubated in treatment B achieved the highest rates of cleavage (81.96%), 2-8 cell (62.35%) and blastocyst (45.09%). It is concluded from the present study that incubating sheep oocytes in culture media containing a cocktail of EGF (10 ng/ml) and MI (20 mmol/l) significantly improves the rates of metaphase-II, fertilization and blastocyst rates.

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Introduction

The end of the twentieth century witnessed remarkable developments in many fields related to animal husbandry, where a complete integration of animal breeding, reproduction, nutrition and housing conditions was observed. Since the emergence of assisted reproductive technologies (ART) to the present days, the technology has sought to increase the yield of *in vitro* produced embryos by imitating the basic environment within the female genital tract. Growth factors, which are essentially a subset of the cytokines, are among the most important elements added to the culture media in order to support and stimulate growth, differentiation, survival and proliferation of early embryo cells (Arat., 2016). EGF is a secreted peptide found in many fluids in the body, to name but a few, the submaxillary glands of mice (Savage and Cohen, 1972), human's blood and urine (Aybay et al., 2006), intestinal fluid (Nair et al., 2008) and amniotic fluid (Hofmann and Abramowicz,1990). EGF and its receptors have a wide spectrum in various vital systems and functions within the body, they also play a great supporting role in cell reproduction, division, DNA synthesis, nuclear and cytoplasmic maturation of oocytes, as well as sperm maturation (Yang et al., 2014). Numerous studies have proven the presence of EGF in the follicular fluid of various mammalian ovaries. These studies have also demonstrated the crucial role in the nuclear and cytoplasmic maturation of oocytes through its direct effect on the cumulus cells and their expansion (Gutman et al., 2008; Panigone et al., 2008; Hsieh et al., 2009). MI is a stereoisomer resulted from glucose1-phosphate in a NAD + -catalyzed oxidation/ reduction reaction. In addition to the inclusion of MI in many pathological treatment procedures for humans, MI has a major role in the nuclear maturation of oocytes and subsequent divisions in addition

to the positive role in lipid metabolism and quality improvement in early embryos (Soltani et al., 2012). Within the scope of assistive reproductive biotechnologies (ART) and *in vitro* production of embryos, MI is promising due to its important vital role in cell proliferation and regulation of division. On the other hand, both EGF and MI are important additives but both agents lack a major role in oocyte maturation, as some important elements such as follicle - stimulating hormone (FSH) and fetal calf serum (FCS) must be shared in the culture media. Besides, the vital role of MI in nuclear maturation and support for subsequent embryo division is still not sufficiently studied in most farm animals. Here, the current study targeted the synergistic effect of EGF and MI on nuclear maturation and blastocyst embryo yield by tracing some basic phases of meiosis of sheep oocytes over three-time intervals (9, 21 and 27 hours) and the effect on the subsequent development of preimplantation embryos.

Materials and Methods

Reagents
The chemicals used were from Sigma Chemical Co (St. Louis, USA) unless mentioned otherwise.

Experimental Design
Two basic experiments were conducted:
Experiment-I
The experiment was conducted to study the nuclear maturation progression of oocytes treated with different concentrations of EGF ((cat. #E- 4127) and MI in addition to the control group. The nuclear maturation study included the passage of the oocytes into three main phases: germinal vesicle breaks down (GVBD), metaphase-I (M-I) and metaphase-II (M-II). Groups of oocytes allocated to the different treatments were determined to monitor and follow up the nuclear maturation of the oocyte nuclei across three-time intervals (9,21 and 27- hours).
Experiment-II
In this experiment, all oocytes were destined to evaluate subsequent stages of development, including IVF and cleavage stages down to the blastocyst stage.
The study design falls under the one-factor experimental design (treatment levels) for several traits.

Ovaries and Oocyte Recovery
Ovaries of Awassi breed ewes were obtained from the slaughterhouses in the city of Aleppo and kept in a physiological saline solution at a temperature of 39°C. The collected ovaries were transferred to the biotechnology laboratory at the Faculty of Agriculture – the University of Aleppo within a maximum period of 20 minutes. Cumulus oophorus complexes (COCs) were obtained by slicing method of ≥2 mm follicles of Awassi ewes' ovaries (within the breeding season; of ≥2 years old ewes). Only oocytes completely surrounded by unexpanded cumulus cells (>3 layers) were used.

EGF and MI Levels Determination
For all maturation experiments, the levels of EGF and MI were determined according to two increasing shared concentrations (Table 1):

In Vitro Maturation (IVM)
The resulting COCs specified for the two experiments were washed four times in modified phosphate buffer saline (PBS) supplemented with 40 mg /L pyruvate, gentamycin and 0.5 mg/ml bovine serum albumin (BSA); fraction V. COCs were transferred to 4- well plates (groups of up to 30 oocytes) containing 500 μl of Ham's F-10 medium supplemented with 5% (v:v) FCS and 40 ng/ml FSH. The maturation period lasted for 27-h at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity (according to De Oliveira Bezerra et al. (2019) with some modifications).

Assessment of Nuclear Maturation
Assessment of nuclear maturation was done as described previously (Mardenli, 2020). Briefly, according to the time intervals of incubation for each group of oocytes (experiment I), the oocytes were denuded by repeated pipetting and placed on separate clean dry glass slides. Oocytes were fixed by immersing the slides in a solution of acetic acid and ethanol (1 : 3). Next, the oocytes were washed with 70% ethanol and stained with 1% orcein for 10 minutes. Next, the oocytes were washed with a solution consisted of glycerol, acetic acid and water (1 : 1 : 3) and examined under a phase-contrast microscope at x 400 to monitor meiosis phases upon the specified time intervals (Figure 1).

In Vitro Fertilization (IVF)
As for Experiment II, COCs were washed five times in PBS and three in TALP medium. Next, the COCs were transferred in groups of up to 50 into four -well plates. 250 μl of fertilization medium (TALP) was added to each well contained 10 μg heparin-sodium salt 1200 U/mg. Fresh semen was collected from the testis of proven rams by the electric method (Whitlock et al., 2012). Viable and motile sperms were obtained by centrifugation on a Percoll gradient (2 ml at 45% over 2 ml at 90%) for 45 min at 700 X× g at room temperature. The remaining viable spermatozoa at the bottom were collected, washed in TALP and subjected to centrifugation at 100 X× *g* for 10 min at room temperature. The resulting aliquots of spermatozoa were diluted in TALP medium to give a concentration of 6 X 10^6 spermatozoa/ml. Next, 250 μl of the previous suspension was added to each fertilization well to obtain a concentration of 2 X 10^6 spermatozoa /ml. Afterwords, COCs were incubated for 24 h in 5% CO in humidified air at 39°C (according to De Oliveira Bezerra et al. (2019) with some modifications).

In Vitro Culture (IVC)
To facilitate the cleavage process, the zygotes were stripped of the cumulus cells by performing vortexing for 2 min in 2 ml PBS and washing in PBS + modified synthetic oviduct fluid medium (SOF) three times in each solution. Resulting zygotes were cultured in SOF (1 zygote/μl medium) in presence of BSA under paraffin oil in a humidified atmosphere of 5% CO_2:5% O_2 :90% N2 at 39°C. Cleavage was assessed at 48h of culture. The blastocyst stage was assessed on Days 6—8 of culture (Figure 2) (according to Vajta et al. (1999) with some modifications).
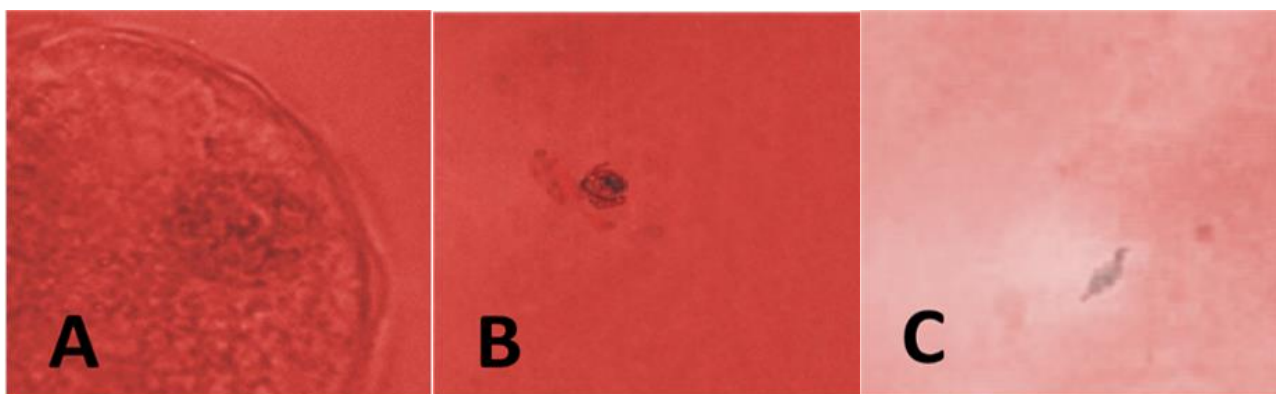
Figure 1. Phases of the nuclear maturation of Awassi sheep oocytes in Experiment I: A: GVBD, B: M-I, C:M-II

Figure 2. Fertilization and cleavage stages of Awassi sheep early embryos in Experiment II: A: zygotes, B: 2-4 cell stage embryos, C: morula and blastocyst.

Statistical Analysis

Chi-square of contingency tables was used to analyze the raw data. Fisher's exact test was used to determine significance across various rates. Analysis was done by applying the SAS Institute Inc. (2017) statistical package.

Results

Experiment -I

Results of the nuclear maturation succession during time intervals 9,21 and 27- h are presented in Tables 2, 3 and 4. After nine hours of incubation, the type of treatment had a significant effect (P=0.03) on the oocytes reaching the M-I, the oocytes of treatment B showed a moderate superiority over the rest of the groups (Table 2). In contrast, there were no real differences between the treatment groups at the GVBD phase.

Up to the 21-hour interval, the oocytes of treatment B showed clear superiority across the three phases of nuclear maturation, the rates reached 3.22%, 33.87% and 19.35%, respectively, followed by the incubated oocytes in treatment C (8.62%, 29.31% and 12.06%, respectively).

In the last time interval of incubation (27 – hour), on the same lines, the oocytes of treatment B clearly outperformed the rest of the oocytes of the rest groups in the previous three stages of nuclear maturation. The rate of M-II oocytes reached 89.23% (P=0.03) with a difference of 37.62% and 14.23% for treatment A and C, respectively. In contrast, high rates of oocytes were observed in both the GVBD (p=0.03) and M-I (p=0.005) in the control group (treatment A), the rates were 11.29% and 27.41%, respectively (Table 4).

Experiment-II

Results of the cleavage, 2-8 and blastocyst stages are presented in Table 5. The oocytes incubated in B treatment continued to outperform by achieving the highest rate of cleavage (81.96%; p=0.0001), 2-8 cell stage (62.35%; p=0.0001) and blastocyst stage (45.09%; p=0.0001) compared to the remaining two groups (A and C treatments). As evidenced previously in Experiment -I, the differences between oocytes treatments B and C were close in all the studied indicators and did not exceed 3% (2-8 cell and blastocyst stages) or 7% (cleavage stage).

Table1. Levels of EGF and MI specified for *in vitro* maturation of Awassi sheep oocytes

Treatment	EGF (ng/ml)	MI (mmol/l)
A	0	0
B	10	20
C	50	40

Table 2. The meiotic progression of *in vitro* matured Awassi sheep oocytes for 9 hours in Ham's F-10 medium across different levels of EGF and MI

Maturation treatment	Incubated oocytes No.	Phase of nuclear maturation					
		GVBD		M-I		M-II	
		No.	%	No.	%	No.	%
A	61	17	27.86	7	11.47[a]	0	0
B	63	8	12.69	19	30.15[c]	0	0
C	65	11	16.92	13	20.00[b]	0	0
P		NS		0.03			

[a-c]Values with different superscripts within the column are significantly different (P< 0.05), GVBD: germinal vesicle breaks down, M-I: metaphase I, M-II: metaphase II, A: without EGF nor MI, B: 10 ng/ml EGF + 20 mmol/l MI, C: 50 ng/ml EGF +40 mmol/l MI, NS: not significant.

Table 3. The meiotic progression of *in vitro* matured Awassi sheep oocytes for 21 hours in Ham's F-10 medium across different levels of EGF and MI

Maturation treatment	Incubated oocytes No.	Phase of nuclear maturation					
		GVBD		M-I		M-II	
		No.	%	No.	%	No.	%
A	55	14	25.45[a]	6	10.90[a]	2	3.33[a]
B	62	2	3.22[b]	21	33.87[b]	12	19.35[b]
C	58	5	8.62[b]	17	29.31[b]	7	12.06[b]
P		0.001		0.01		0.02	

[a-b]Values with different superscripts within the column are significantly different (P< 0.05), GVBD: germinal vesicle breaks down, M-I: metaphase I, M-II: metaphase II, A: without EGF nor MI, B: 10 ng/ml EGF + 20 mmol/l MI, C: 50 ng/ml EGF +40 mmol/l MI

Table 4. The meiotic progression of *in vitro* matured Awassi sheep oocytes for 27 hours in Ham's F-10 medium across different levels of EGF and MI

Maturation treatment	Incubated oocytes No.	Phase of nuclear maturation					
		GVBD		M-I		M-II	
		No.	%	No.	%	No.	%
A	62	7	11.29[a]	17	27.41[a]	32	51.61[a]
B	65	1	1.53[b]	5	7.69[b]	58	89.23[b]
C	60	2	3.33[b]	8	13.33[b]	45	75.00[b]
P		0.03		0.005		0.001	

[a-b]Values with different superscripts within the column are significantly different (P< 0.05), GVBD: germinal vesicle breaks down, M-I: metaphase I, M-II: metaphase II, A: without EGF nor MI, B: 10 ng/ml EGF + 20 mmol/l MI, C: 50 ng/ml EGF +40 mmol/l MI

Table 5. Rates of cleavage, 2-8 and blastocyst stages of *in vitro* matured Awassi sheep oocytes in Ham's F-10 medium across different levels of EGF and MI

Treatment	Incubated oocytes	Cleaved oocytes		Embryonic stage			
				2-8 cell		Blastocyst	
	No.	No.	%	No.	%	No.	%
A	253	159	62.84[a]	109	42.57[a]	71	28.06[a]
B	255	209	81.96[b]	159	62.35[b]	115	45.09[b]
C	256	192	75.00[b]	153	59.76[b]	108	42.18[b]
P		0.0001		0.0001		0.0001	

[a-b]Values with different superscripts within the column are significantly different (P< 0.05), A: without EGF nor MI, B: 10 ng/ml EGF + 20 mmol/l MI, C: 50 ng/ml EGF +40 mmol/l MI.

Discussion

The stage of oocyte maturation includes two closely related types, which are cytoplasmic and nuclear maturation. Both types are associated with a set of complex events, many hormones and growth factors that interact according to a timed timeline to ultimately obtain an oocyte capable of fertilization and subsequent division.

Through the results of the first experiment in our current study, it was clear that the matured oocytes in a mixture of EGF and MI outperformed the control group by achieving high rates during their passage to the three stages of nuclear maturity (GVBD, M-I and M-II; Tables 2,3 and 4). The remarkable increase in the oocyte rates reaching M-II can be explained by the interconnected role between EGF and MI. EGF, mainly, is found in the ovarian follicle; specifically, in the follicular fluid of different species (Das et al., 1992),

while EGF receptors are present on the granulosa layer in preovulatory follicles with high proportions (Feng et al., 1987). EGF and its receptors activate tyrosine kinases and autophosphorylation of the c-terminal- specific tyrosine which in turn receive a variety of signalling molecules harbouring Src homology 2 (SH2) or phosphotyrosine binding (PTB) signals leading to the activation of Ras/Raf/MEK/ERK, JAK/STAT, PI3K/AKT/ mTOR, and P`LCγ/PKC signalling pathways that affect cell proliferation and division (Galvez-Contreras et al., 2013). The important role of MI in nuclear maturation comes through two main mechanisms. In the first mechanism, MI is a major factor in the metabolism of glucose being the second messenger in the act of insulin. MI activate the receptor substrate insulin-1 (IRS-1) and the action of the enzyme phosphatidylinositol-

3-kinase (PI-3K). Thus, phosphatidylinositol 2-phosphate (PIP2) will be converted into phosphatidylinositol-3-phosphate (PIP3) which in turn activates the action of the metabolic pathway protein kinase B (PKB) leading to glycogen synthesis (Huang et al., 1999). In the second mechanism, MI serves as an activator to the pyruvate dehydrogenase kinase isoenzyme 1 (PDK1), which acts on glucotransporter 4 (GLUT4) allowing glucose molecules to pass into the plasma membrane of the cell (Yoshizaki et al., 2004). Although increasing the concentrations of both EGF and MI (treatment C) did not achieve a significant increase in the number of oocytes reaching the M-II or in the yield of embryos reaching the blastocyte stage (Tables 2,3,4 and 5), but the previous results suggest a prolonged effect for both of the studied factors and this may be clarified by the significant increase in previously studied traits. Unfortunately, studies to demonstrate the effect of both EGF and MI on inducing nuclear maturity are almost very rare; however, comparing with our current results, at a concentration of 1 ng/ml of EGF, the rates of GVBD and M-II were 49.6% and 64.5%, respectively (follicles with 3-4 mm of size), as for oocytes derived from follicles with 6-7 mm of size, the rates were 78.8% and 92.2% for both phases, respectively (Procha´ Zka et al., 2000). At 40 -h of incubation, our results in the M-II were higher than what de Ávila Rodrigues and Rodrigues (2004) obtained (40%), meanwhile the GVBD rates were lower (28%). Also, our results were somewhat similar to the results of Khatun et al. (2011) at the 24-hour incubation period, as the rates reached 9.3%, 19.5% and 66.3% for GVBD, M-II and M-II, respectively (oocytes derived from pubertal female goats). In our previous study, at 27-h interval the rates of GVBD ranged between 3.84% and 3.85%, M-I rates ranged between 7.69% and 26.92%, while M-II rates ranged between 56.86% and 84.61% (Mardenli, 2020).

Likewise, the incubated oocytes in the second and third treatment (treatments B and C) continued the true superiority (P= 0.0001; Table 5) in the rates of cleavage, 2-8 cell and blastocyst stages, which is significantly giving a clear explanation of the chronic effect of EGF and MI on the early embryonic stage (Dadi et al., 2007). Not only that, in the literature, most of the studies confirm that the role of EGF is more evident in the stage of fertilization and cell division compared to the oocytes (Vinayak et al., 2018). It has been observed that EGF receptors (EGFR) are located on the cellular membrane of the blastomeres (Wei et al., 2001). Completely in the line, many studies have highlighted the clear effect of MI in supporting early embryo division and increasing zygotes and hatched blastocyst rates (Warner et al., 2003; Colazingari et al., 2014). In a study conducted on bovine oocytes, Zahmatkesh et al. (2018) obtained rates of cleavage and blastocyst of 80.85% and 37.72%, respectively (10 ng/mL EGF; SOF culture media). In another study (Sirisathien and Brackett, 2003), the rates of 2-4 cell and blastocyst stage reached 56.1% and 38%, respectively (50 ng/mL EGF). Iincubating mouse oocytes at a concentration of 30 mmol MI, the fertilization and 2-cell stage rates reached 67.5% and 75.9% respectively (Chiu et al., 2003). Using different concentrations of MI, Soltani et al. (2012) obtained cleavage and blastocyst rates of 66.66% and 36.36% (0.02 g/l), 61.38% and, 37.33% (0.03 g/l) and 62.59% and 46.94% (0.04 g/l), respectively.

In light of the results of the current study, and given the functional characteristics of EGF and MI on the level of ovarian activity and oocyte maturation, it is noted that the effect of each of the studied factors is related to a several other different factors. In addition to the thematic role of follicle size factor (Töpfer et al., 2016), both elements are closely related to the volume and nature of the follicular fluid in the follicle, which is determined based on hormonal balance and hormone concentrations regardless of the ovarian follicular wave dynamics through which the dominant follicle is selected so that the oocyte can complete maturation, fertilization and subsequent divisions (Downes and Macphee, 1990; Saito et al., 2000).

More specifically, the dynamics of EGF action appears to be closely related to specific concentrations of FSH in the follicle, as these concentrations are considered a determining factor for the action (Kyung et al., 2002). In turn, parallel studies proved the important role of luteinizing hormone (LH) in stimulating EGF during ovulation (Panigone et al., 2008). In addition to all the previous considerations, the concept of the developmental potential of the oocytes remains the main factor that gives a clear perception of the extent of the influence of the studied factors. The oocytes used in our experiments derived from follicles of different sizes and ovaries of ewes with a large dispersion in the reproductive cycle. Therefore, the diversity in the diameter of the oocytes contributes to a large degree the heterogeneity of the results obtained (O'Shea et al., 2012; Morohaku et al., 2016).

Conclusion

According to the given results in our study, it can be suggested that enriching culture media with a cocktail of EGF (10 ng/ml) and MI (20 mmol/l) improves the rates of nuclear maturation phases, cleavage and blastocyst yields of Awassi sheep oocytes derived from follicles of > 2 mm of size during the breeding season.

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