Effect of Combination of Follicle Size, FSH and Cysteamine on *In Vitro* Production of Sheep Embryos

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ABSTRAK

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Hubungan partisipatif antara ukuran folikel, *follicle stimulating hormone* (FSH), dan sisteamin (agen antioksidan) berkontribusi dalam produksi embrio yang ditandai dengan jumlahnya yang melimpah dengan kualitas yang baik. Tujuan dari penelitian ini adalah untuk mengevaluasi efektifitas FSH, sisteamin dan ukuran folikel dalam produksi embrio *in vitro* oosit domba Awassi. Ukuran folikel dikategorikan menjadi: folikel kecil (1-2 mm) dan folikel besar (>2 mm). Oosit dimatangkan pada dua kadar FSH dan sisteamin yang ditingkatkan: A (40 ng/ml + 50 μM) dan B (60 ng/ml + 100 μM). Hasil interaksi bilateral menunjukkan perbedaan yang signifikan pada seluruh ukuran folikel (kelompok folikel besar) dan perlakuan pematangan (media B) pada tingkat fertilisasi (nilai tertinggi: 67,51%; p= 0,02), pembelahan (nilai tertinggi: 65,41%; p= 0,01), tahap sel 2-16 (nilai terendah: 2,29%; p= 0,0001), tahap blastokista (nilai tertinggi: 44,82%; p= 0,04), menuju tahap penangkapan morula (nilai terendah: 55,17%; p= 0,04) dan embrio Tipe I (nilai tertinggi: 52,87%; p= 0,03). Demikian juga, oosit matang dari kelompok folikel kecil (medium B) mencapai tingkat tertinggi pada tahap morula (56,60%; p= 0,03). Tidak terdapat perbedaan signifikan pada embrio Tipe II dan Tipe III. Untuk menghasilkan produksi yang tinggi dari embrio berkualitas tinggi, disarankan untuk menambahkan FSH dan sisteamin dengan kadar masing-masing 60 ng/ml dan 100 μM dalam media maturasi oosit *ovine* yang diperoleh dari folikel dengan diameter >2 mm.

Kata Kunci: Sisteamin, Ukuran Folikel, FSH, Produksi Embrio *In Vitro*, Domba

ABSTRACT

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The participatory relationship among the follicle size, follicle stimulating hormone (FSH), and cysteamine (antioxidant agent) contribute to the production of embryos characterized by abundance and good quality. The aim of this study was to evaluate the efficacy of FSH, cysteamine and follicle size on *in vitro* embryo production of Awassi sheep oocytes. Follicles sizes were determined into two groups: small follicles (1-2 mm) and large follicles (> 2 mm). Oocytes were matured across two increasingly shared levels of FSH and cysteamine: A (40 ng/ml + 50 μM) and B (60 ng/ml + 100 μM). Results of the bilateral interaction showed significant differences across the follicle size (large follicles group) and the maturation treatment (B medium) in the rates of fertilization (highest value: 67.51%; p= 0.02), cleavage (highest value: 65.41%; p= 0.01), 2-16 cell stage (lowest value: 2.29%; p= 0.0001), blastocyst stage (highest value: 44.82%; p= 0.04), down to morula stage arrest (lowest value: 55.17%; p= 0.04) and Type I embryos (highest value: 52.87%; p= 0.03). Likewise, matured oocytes of small follicles group (B medium) attained the highest rate of morula stage (56.60%; p= 0.03). No significant differences were observed in Type II and Type III embryos. In order to obtain high yields of good quality embryos, it is advised to add FSH and cysteamine with levels of 60 ng/ml and 100 μM respectively to maturation medium of ovine oocytes obtained from follicles with a diameter > 2 mm.

Key Words: Cysteamine, Follicle Size, FSH, *In Vitro* Embryo Production, Sheep

INTRODUCTION

The deep understanding of the overall processes governing the development of ovarian follicles and oocytes inside the body (*in vivo*) has led to higher *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and cleavage rates in all animal species. Perhaps the emergence of the important trend in the *in vitro* embryo production (IVEP) technology which is the addition of hormones and anti-oxidant agents together in the maturation media was of great interest due to the positive participatory effect. The gonadotropic hormones (FSH, luteinizing hormone (LH) and chorionic gonadotropin (hCG)) are one of the most

important additives in IVM, as the use of these additives during maturation has led to a high rate in the embryos outcome (Kouamo & Kharche 2014; Kalita et al. 2019).

In the ovum pick-up (OPU) technique, FSH plays a key role in increasing the number of follicles and embryo outcome of cattle (Hasler 2014). Some studies indicated the possibility to increase the rate of twining birth rates in lactating cattle following FSH treatment at different stages of the estrus cycle (Situmorang et al. 2010; Situmorang et al. 2012). Beyond that, Aryogi et al. (2013) stated that with the increased levels of FSH, the Ongole cross breed cows with twin birth history could produce more than one of follicle de Graf in an estrus cycle.

Cysteamine is characterized by stability and antioxidativity. Due to the historical popularity and the primary role of cysteamine as an antioxidant agent, its usage has increased recently. However, this usage has also remained limited to specific concentrations, as are hormones. Within the close relationship that governs the follicle size and the size of the oocytes (diameter), it is noted that with the increased size of the follicle and the follicular fluid (FF) the diameters of the oocytes increase (Grabowska et al. 2016), and thus the oocytes acquire the developmental competence that makes them eligible to follow fertilization and subsequent cellular division of the embryos.

In Syria, Awassi sheep are considered a pure breed and of great economic importance because of the distinctive productive characteristics of meat and milk, and there was a need to establish a genetic bank to preserve the genetic resources of this breed. Hence, the aim of this study was to assess the combined effects of follicle size, FSH and cysteamine on the rates of fertilization and cleavage of Awassi sheep oocytes as well to studying the stage and embryo quality.

MATERIALS AND METHODS

Ovaries collection

Ovaries were collected from slaughterhouses located in the city of Aleppo during the reproductive season. Immediately after collection, the ovaries were kept in Dulbecco's Phosphate Buffer Saline (DPBS) and transported to the biotechnology laboratory at the Faculty of Agriculture within 20 minutes (Figure 1; A and B).

Follicle size determination

Follicles diameters were measured by a certain ruler and divided into two main classes: small follicles (SF): 1-2 mm and large follicles (LF): >2 mm.

Oocytes collection

Cumulus oophorus complexes (COCs) were collected in two consecutive ways (aspiration and slicing) as follows: for each group of follicles, the aspiration was done by aspirating the follicular fluid through a sterile 18-g needle attached to a 5 ml syringe containing a sterile saline solution. Next, the slicing method was applied to the same follicles from which the follicular fluid was aspirated by using a surgical blade. The dissected follicles were washed several times with Tissue Culture Medium-199 (TCM-199) supplemented with heparin. Obtained COCs in both ways were received in a Petri dish together (Figure 1; D).

FSH and cysteamine concentrations determination

The concentrations of both the FSH and cysteamine were determined according to two increasingly shared concentrations (Table 1).

Table 1. FSH and cysteamine levels determined in the study

Supplementation medium	FSH (ng/ml)	Cysteamine (μM)
A	40	50
B	60	100

Experimental design

The experiment was designed according to the two-factor design (2 follicle size classes × 2 maturation treatments) for several traits (IVF, cleavage, arrest, embryo stages and embryo quality). A focus was placed on showing the results of bilateral interactions among the factors involved.

IVM and IVF conditions

After adding FSH and cysteamine levels to TCM-199, IVM conditions were done as described elsewhere by de Oliveira Bezerra et al. (2019) with some modifications. In short, every 10-15 oocytes were cultured in TCM-199 supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin sulfate. The selected oocytes were transferred to wells containing the maturation medium under mineral oil and then incubated for 27 hours at 38.5°C with 5% CO_2 and saturated humidity (Figure 1; C). Following incubation, the maturation stage was determined by the expansion of the cumulus cells and the detecting of the second polar body (Figure 1; E and F). IVF was done using

Tyrode's albumin lactate pyruvate (TALP) medium supplemented with 5 mg/ml bovine serum albumin (BSA), 0.2 mm sodium pyruvate, 25 mm sodium bicarbonate, 50 µl/ml penicillamine-hypotaurine-epinephrine (PHE) solution 10 mg/ml heparin and 13 mm of sodium lactate. Fresh semen was collected from proven rams (electrically) and centrifuged in a Percoll discontinuous gradient (1000×g for 10 minutes). The supernatant was discarded, and the pellet containing viable spermatozoa was re-suspended in 1 mm of TALP and centrifuged again at 300×g for 10 minutes. The spermatozoa were then diluted in TALP to achieve a final concentration of 1×10^6 sperm/ml. Every 10-15 oocytes were then transferred to a drop of fertilization medium and co-incubated with spermatozoa for 18–22 hours at 38.5°C, with 5% CO_2 and saturated humidity. The presence of zygote (pronucleus) was investigated under an inverted microscope (Figure 2; A).

In vitro culture (IVC)

Following IVF, the zygotes were washed twice in TALP and once in 50% TALP + 50% modified medium synthetic oviductal fluid (SOF), transferred to SOF supplemented with 50 mg/ml of amikacin, 5 mg/ml of BSA and 2.5% of fetal bovine serum (FBS) (de Oliveira Bezerra et al. 2019) and cultured at 38.5°C, 5% CO_2 and saturated humidity for 7 days. The presence of blastomeres (2-16 cell), morula and blastocyst stages were investigated under an inverted microscope (Figure 2; B, C and D).

Statistical analysis

After arranging the data (Microsoft Excel sheet) using a sophisticated computer, the data were analyzed by applying Pearson Chi-square of contingency tables in SAS Institute Inc. (2017) statistical package. The rates of different traits were presented as a percentage (%) and compared according to the Fisher exact test for each trait.

Embryo grading

Embryo quality was determined as previously described by Wintner et al. (2017) with some modification: Type I (excellent): the cells of the embryo are equal in size while fragmentation entirely is absent (Figure 3; A), Type II (good): the cells of the embryo are equal in size while minor fragmentation only could be seen (Figure 3; B), Type III (poor): the cells of the embryo are of equal or unequal size while fragmentation is moderate to heavy (Figure 3; C).

Reagents

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

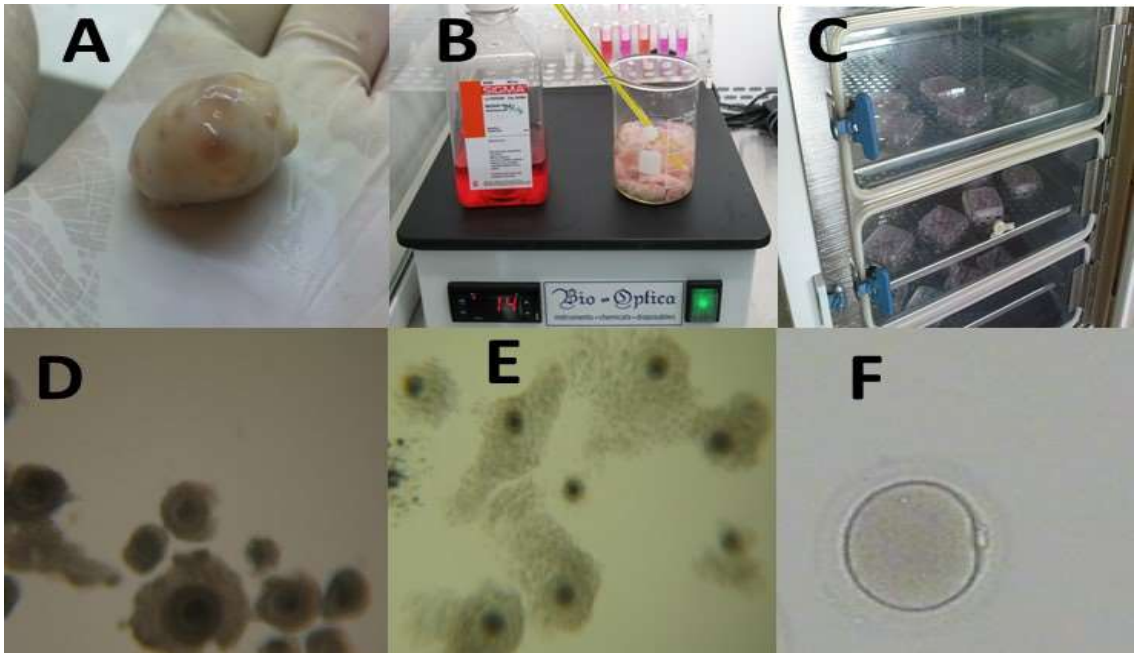

Figure 1. Ovaries collection, oocytes collection and IVM procedures. A: An Awassi sheep ovary. B: Preparing the ovaries for oocytes collection methods. C: Incubating the collected oocytes. D: Awassi sheep oocytes at GV stage. E: Matured oocytes with an expansion of cumulus cells. F: Matured Awassi sheep oocyte with a clear appearance of the second polar body

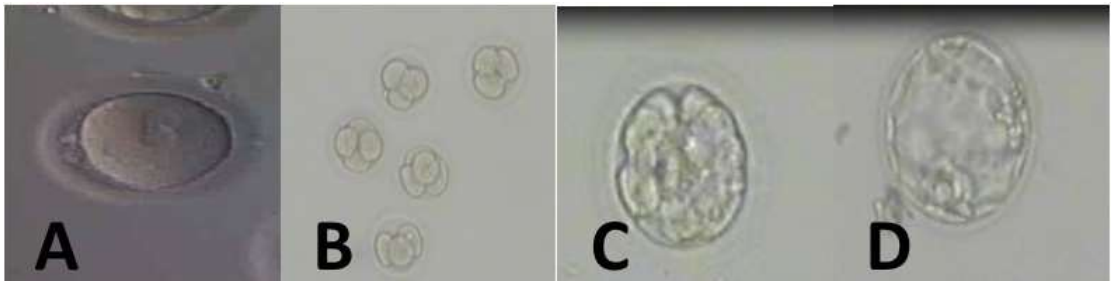

Figure 2. Embryo stages of *in vitro* Awassi sheep produced embryos. A: Zygote. B: Blastomeres (2-4 cell). C: Morula. D: Blastocyst.

Figure 3. Types of embryo quality of *in vitro* Awassi sheep produced embryos. A: Type I. B: Type II. C: Type III.

RESULTS AND DISCUSSION

Results

IVF and cleavage stages

Table 2 shows that matured oocytes in B medium achieved a high response in the IVF (p = 0.002; 67.51%; LF group) and cleavage stages (p = 0.01; 65.41%; LF group). In contrast, the lowest response was observed in oocytes that were matured in A medium (50.81%; SF group, 47.86%; LF group).

Embryo stage and arrest

As indicated in Table 3, the matured oocytes in B medium achieved the highest rates of morula (p = 0.03; 56.60%; SF) and blastocyst stages (p = 0.04; 44.82%; LF group). This was accompanied by achieving the lowest rates at the arrested 2-16 cell stage embryos (p= 0.0001; 2.29%; LF group) and the general rate of arrest (p = 0.04; 55.17%; LF group).

Embryo quality

The quality rates of the embryos ranged between 22.98% and 52.87% and only one significant difference was observed (Type 1), as the matured oocytes in B medium achieved the highest rates (p= 0.03; 52.87%; LF group), while the lowest rates were at embryos whose oocytes matured in A medium (32.14%; LF group) (Table 4).

Discussion

In the current study, the effectiveness of the combined effect of FSH, cysteamine, and follicle size in the studied traits was clearly featured (Tables 3, 4 and 5). The rise in IVF, cleavage and blastocyst rates can be attributed to the vital role resulting from the previous factors. In references, many studies examined the effect of FSH, cysteamine and follicle size separately. The vital roles of the previous factors can be summarized with some of the following points: in the ovary, with regard to FSH, the hormonal regulation plays the basic role in the occurrence of a number of changes that ultimately results in an increase in the size of the follicle, the follicular fluid and the diameter of the oocytes. The previous events would lead the oocytes to reach a stage that enables them to follow cellular divisions and pass the stage of fertilization and division in an optimal way. However, Gougeon (2010) indicated that the optimum size of the follicle in which the oocytes acquire the ideal developmental competence must be at least 2 mm.

The study of Lamb et al. (2011) proved that FSH plays an important role in improving oocyte developmental competence through the mutual relationship between FSH and hCG. In our study, one of the most important factor that plays the key role in determining the previous rates of maturation, fertilization and the ability to follow the cell division of early embryos is that the relationship that governs the follicle size, the diameter of the oocyte, the size of the follicular fluid and the dominant follicle (dynamic follicular wave), which ultimately leads to the retarding

Table 2. Rates of IVF and cleavage of Awassi sheep oocytes under the influence of both follicle size and maturation treatment factors

Source		Incubated oocytes	Fertilized oocytes		Cleaved oocytes	
Maturation treatment	Follicle size	No.	No.	%	No.	%
A	SF	185	94	50.81[a]	46	48.93[a]
A	LF	192	117	60.93[b]	56	47.86[a]
B	SF	201	105	52.23[a]	53	50.47[a]
B	LF	197	133	67.51[c]	87	65.41[b]
p			0.002		0.01	

Values in the same column with different superscript are significantly different at P< 0.05. A = FSH (40 ng/ml) + cysteamine (50 μM), B = FSH (60 ng/ml) + cysteamine (100 μM), SF = small follicles (1-2 mm), LF = large follicles (> 2 mm)

Table 3. Rates of embryonic stages and arrest of Awassi sheep oocytes under the influence of both follicle size and maturation treatment factors

Source		Embryo stage						Arrest (2-16 cell and morula stages)	
Maturation treatment	Follicle size	2-16 cell		Morula		Blastocyst			
		No.	%	No.	%	No.	%	No.	%
A	SF	16	34.78[b]	17	36.95[a]	13	28.26[a]	33	71.73[a]
A	LF	24	42.85[b]	19	33.92[b]	13	23.21[a]	43	76.78[a]
B	SF	6	11.32[c]	30	56.60[a]	17	32.07[a]	36	67.92[a]
B	LF	2	2.29[a]	46	52.87[a]	39	44.82[b]	48	55.17[b]
p		0.0001		0.03		0.04		0.04	

Values in the same column with different superscript are significantly different at P < 0.05. A = FSH (40 ng/ml) + cysteamine (50 μM), B = FSH (60 ng/ml) + cysteamine (100 μM), SF = small follicles (1-2 mm), LF = large follicles (> 2 mm)

Table 4. Rates of embryos types resulted from Awassi sheep oocytes under the influence of both follicle size and maturation treatment factors.

Source		Type I		Type II		Type III	
Maturation treatment	Follicle size	No.	%	No.	%	No.	%
A	SF	16	34.78[a]	15	32.60	15	32.60
A	LF	18	32.14[a]	21	37.50	17	30.35
B	SF	19	35.84[a]	16	30.18	18	33.96
B	LF	46	52.87[b]	21	24.13	20	22.98
p		0.03		NS		NS	

Values in the same column with different superscript are significantly different at P < 0.05. A = FSH (40 ng/ml) + cysteamine (50 μM), B = FSH (60 ng/ml) + cysteamine (100 μM), SF = small follicles (1-2 mm), LF = large follicles (> 2 mm), NS = not significant

inhibitory effect on the development of the follicles at the expense of the dominant follicle (Imron et al. 2016), and thus the negative effect on the developmental competence of oocytes in these non-developing follicles due to the presence of some growth inhibitors in the follicular fluid (FF).

During controlled ovarian stimulation, a close relationship was observed between weight-adjusted recombinant FSH (rFSH) dose and follicular growth, where a decrease in the variability of the follicular sizes and an increase in the rates of mature oocytes were observed (Abbara et al. 2019). On the other hand, the biochemical role of cysteamine can be summarized by neutralizing the harmful effects of reactive oxygen species (ROS) inside the cells, thus decreasing the damage caused by oxygen (O_2) in lipids, protein and nucleic acids during the early embryo development (Kitagawa et al. 2004). Besides, cysteamine stimulates glutathione synthesis (GSH), which in turn improves the developmental competence of oocytes (Gasparrini et al. 2006). In addition, recent studies highlight the important role of cysteamine in the positive effect on different stages of nuclear maturation of oocytes. In a study conducted by Mahmoud et al. (2016) the rates of metaphase II reached 76.2% (with 50% µm cysteamine) and 69.2% (without cysteamine) respectively. Not only that, cysteamine has a prominent role in up-regulating the expression of anti-apoptotic and down-regulating the expression of proapoptotic genes in early embryo stages (Elamaran et al. 2012). As a result, cysteamine has a long-term positive effect on oocyte performance in maturation, fertilization and subsequent development of early embryos (blastocyst stage). Perhaps these vital roles of cysteamine are the main reason for the low rates of arrest presented in our study (Table 3), specifically in embryos resulting from oocytes that have been matured in B medium (LF group). In general, the results of the current study related to the rates of cleavage and blastocyst stage can be compared with the results of some studies (Lojkić et al. 2016; Shabankareh et al. 2015; Muasa 2010; Lunardelli et al. 2016; Merton et al. 2013; Shabankareh & Zandi 2010; Ranjbar et al. 2018) summarized in Table 5 according to the studied factors.

The fertilization rates in the current study came low compared with the rates reached by Izumi et al. (2013) as those rates were 91.2, 90.9 and 85.3% at cysteamine levels of 100, 200 and 500 µM respectively (follicle size = 1 mm). On the other hand, the morula rates in our study were higher than those in the study of Shabankareh & Zandi (2010), as the rate was 50.2%. In the present study, the data of Table 4 indicated a significant difference in embryo quality (Type I; B medium; LF group). In fact, the quality index occupies a large area of importance in IVEP applications. Embryo quality is a basic and critical requirement in embryo transfer (ET) technology. In literature, many studies related to ET have emphasized that the

Table 5. Rates of cleavage and blastocyst stage resulted from maturation with different levels of FSH and cysteamine across different follicle sizes

Follicle size	FSH	Cysteamine	Cleavage	Blastocyst	Source
≤5 mm	75 IU	100 µM	39.5%	8.0%	Lojkić et al. (2016)
>5 mm			58.3%	24.7%	
3–6 mm	0.02 IU/m	-	79.1%	30.8%	Shabankareh et al. (2015)
6–9 mm		-	84.4%	33.6%	
10–20 mm		-	80.5%	38.7%	
1 -3 mm	1ul/ml	-	51.87%	12.07%	Muasa (2010)
> 3 -6 mm		-	55.88%	29.78%	
> 6 mm		-	84.87%	41.25%	
≤ 2 mm	0.5 µg	-	16.6%	40.2%	Lunardelli et al. (2016)
4-8 mm		-	19.0%	50.5%	
Different sizes	4 mg	0.1 mM	59.3%	16.6%	Merton et al. (2013)
2-8 mm	0.5 µg/ml	100 µM	84.5%	35%	Shabankareh & Zandi (2010)
2-8 mm	-	50 µM	48%	20%	Ranjbar et al. (2018)
	-	100 µM	48%	22%	

transferred embryos are of the first type (Type I) in the first importance level, and the Type II in the second importance level. However, the study of Wintner et al. (2017) reported that poor quality embryo does not negatively affect a good quality embryo when transferred together in a double ET. Nevertheless, studies related to embryo quality remained scarce. In contrast, some studies indicated some factors that play a fundamental role in determining the quality of embryos such as the reproductive status of females (Twigg-Flesner et al. 2014), nutritional status (Ashworth et al. 2009; Chundekkad et al. 2020), age (Hammami et al. 2013), reasons related to spermatozoa characteristics used in fertilization (Ervandi et al. 2013; Chapuis et al. 2017), semen quality (Kusumaningrum et al. 2015) and the type of protocol used for super ovulation (Sumantri et al. 2011).

CONCLUSION

Through the outputs of the current study, it was found that maturation of Awassi sheep oocytes originated from follicles with a diameter of more than 2 mm in TCM-199 supplemented with 60 ng/ml of FSH and 100 μM cysteamine resulted in a clear increase in embryos yields as well as in the Type I embryos.

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