EFFECTIVENESS OF OVINE FOLLICULAR FLUID AND EWE AGE ON *IN VITRO* NUCLEAR MATURATION AND EMBRYO EVOLUTION OF AWASSI EWES

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ABSTRACT

Two experiments were conducted to study the efficiency of the ovine follicular fluid ($_O$FF) and the age of ewe on *in vitro* nuclear maturation of Awassi sheep oocytes (Experiment 1) and the subsequent stages of early embryos and their quality (Experiment 2). The $_O$FF has been added to the basic maturation medium (TCM-199) with three different levels (A: 0% (Control group), B:15%, C:25% and D:50%) while the ages of the ewes were determined into two main groups (> 2 and 1-2 year). In the first experiment, across the two age groups, matured oocytes in C solution achieved the highest (p=0.006) rates of metaphase-II (85.70 and 82.35 % respectively). In the second experiment, oocytes belonging to > 2 and 1-2-year ewe age groups (C Solution) achieved the highest (p=0.001) rates of fertilization (23.17 and 30.73% respectively). Similar trend applies to the cleavage stage, the rates reached 35.14 and 41.27% respectively (p=0.01). No significant differences were noticed at the different stages of cleavage (2-4 cell, morula, and blastocyst). The resulting embryos of B (> 2-year ewe age group) and C solution (1-2-year age group) achieved the highest (p=0.04) rates of Type1 embryos (69.56 and 76.92% respectively). A significant difference was noticed in type2 embryos (p=0.02), embryos of the first and second age groups (Solution D) achieved the highest rates (46.15 and 33.33% respectively). It can be concluded that the adding of 25% $_O$FF to the maturation media led to significant improvement in the rates of maturation, cleavage and type1 embryos of sheep oocytes.

Keywords: in vitro embryo production, follicular fluid, ewe age, embryo quality.

تأثير كل من السائل الحويصلي للنعاج وعمرها في النضوج النووي المختبري للبويضات وتطور الأجنة المبكر في الأغنام العواسي

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المستخلص:

أجريت تجربتان لدراسة تأثير كل من السائل الحويصلي النعاج وعمرها (التجربة 1) في النضوج النووي لبويضات الأغنام العواسي مختبريا (التجربة 2) تطور الاجنة المبكر ونوعيتها. تمت إضافة السائل الحويصلي OFFإلى وسط النضج الأساسي (TCM-199) بثلاثة مستويات مختلفة(مجموعة السيطرة A: 0% و B: 15% و C: 25% و D: 50%) بينما تم تقسيم أعمار النعاج إلى مجموعتين رئيسيتين (1-2 سنة واكبر من سنتين). في التجربة الأولى، عبر الفئتين العمريتين، حققت البويضات الناضجة في محلول C أعلى معدلات من النضج النووي Metaphase-II 85.70% و28.35% على التوالي). في التجربة الثانية، حققت البويضات التي تنتمي إلى الفئات العمرية اكبر من سنتين و 1-2 سنوات المحلول C)أعلى(P = 0.001) معدلات الإخصاب 23.17% و 30.73% على التوالي). وينطبق الشيء نفسه على مرحلة الانقسام حيث بلغت المعدلات 35.14% و 41.27% على التوالي. لم تلاحظ فروق معنوية في المراحل المختلفة للانقسام الاجنة (2-4 خلية، مورولا، أريمية. حققت الأجنة الناتجة من نعجة الفئة عمرية أكبر من عامين و فئة عمرية 1-2 سنة والمحلول C أعلى (p = 0.04) معدلات من أجنة النوعA (69.56% و 76.92% على التوالي). لوحظ فرق معنوي (P=0.02) في أجنة النوع B ، حيث حققت أجنة النعاج الأولى والثانية والمحلول D أعلى المعدلات (46.15% و 33.33%على التوالي). يمكن الاستنتاج من نتائج الدراسة الحالية أن إضافة 25% من السائلي الحويصلي إلى وسط النضج أدى إلى تحسن كبير في معدلات النضج والانقسام والنوع الأول لبويضات الأغنام.

الكلمات المفتاحية:انتاج الاجنة مختبريا، السائل الحويصلي، عمر النعاج، نوعية الاجنة

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INTRODUCTION

One of the most important objectives in the field of *in vitro* embryo production (IVEP) is the production of high-quality embryos that are as similar as possible to the conditions of *in vivo* produced embryos (29). Accordingly, attention has been focused on the use of substances and liquids that are present in the natural environment surrounding the oocytes and supporting growth and development (28). The most important of which is follicular fluid (FF) (26). oFF consists of serum and secretions manufactured by ovarian follicle cells that have a key role in supporting the maturation of oocytes due to the richness in many proteins, amino acids, carbohydrates, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and steroids such as androgens, estrogen and progesterone (24, 6) . Also, FF contains many mineral elements, enzymes and salts (1). These substances provide the appropriate medium for oocyte maturation (29, 36). Functionally, FF plays a fundamental role in the cytoplasmic and nuclear maturation of the oocytes and the completion of the developmental competence through its important functions in the fertilization process due to the presence of purine nucleotide (3). During the pre-ovulation stage, FF contains growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF-I) and transforming growth factor α&β(TGF-α, TGF-β) (5). The aforementioned growth factors play an important role in the activity of the ovarian follicles such as cell proliferation, differentiation and the manufacture of steroids (5)**.** the FF has been used in various IVEP studies in cows, ewes and does, but with low concentrations within common media. (2); however, the specific role of FF under low concentrations is still obscure. The embryo quality index is very important to increase pregnancy and implantation rates by selecting good quality embryos. The number and quality of oocytes are influenced significantly by the reproductive status and age (non-adult or adult) (22). Some studies indicated a rise in the effectiveness of fertilization of oocytes derived from ewes more than two years of age (23). Here, In our study, we tried to use high concentrations of $_{O}$FF in the development and maturation of Syrian Awassi ewes oocytes. Also, study the post-embryonic stages and evaluate the quality of the resulting embryos were also investigated.

MATERIAL AND METHODS

The study was carried out at the Reproductive Biotechnology Laboratory; the Department Of Animal Reproductive Physiology; Aleppo University; the Faculty of Agriculture.

Collection and evaluation of ovaries and oocytes

The ovaries were collected from the regional slaughterhouses in Aleppo city, obscure in phosphate buffer saline (PBS); at 39°C and transparted to the laboratory. The time spent from animal slaughter to laboratory did not overtake 1h. Cumulus oocyte complexes (COCs) were removed from follicles of different sizes by slicing process and carried to Petri dishes containing tissue culture media - 199(TCM- 199) with heparin.

Ovine follicular fluid collection and preparation: Ovine follicular fluid was collected from follicles of different sizes by aspiration method using a10 ml syringe with a 19 G needle. The resulting $_{O}$FF was centrifuged at 3000 rpm for 40 minutes at 10^{0} C. Later, the top portion of $_{O}$FF was collected, centrifuged for 15 minutes at 3000 rpm, filtered through a 22µ millipore filter and stored in a deep freeze at -6^{0} C for further use.

Experimental design:

Two experiments were conducted. In the first experiment, the nuclear maturation of the oocytes was studied within a period of 27 hours, the experiment included monitoring of the germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase-I (M-I) and metaphase-II (M-II). In the second experiment, the oocytes were matured in preparation for fertilization and subsequent development down to the blastocyst stage. The process of IVM was carried out across four solutions as described in Table 1. The ages of the ewes were divided into two main groups (> 2 and 1-2 years). The experimental design of the study falls within the two-factor experimental design.

Table 1. Levels of $_{O}$FF and TCM-199 in maturation solutions used in IVM of Awassi sheep oocytes

Maturation solution	TCM-199 (%)	$_{O}$ FF (%)
A(Control)	100	0
B	85	15
C	75	25
D	50	50

***In vitro* maturation (IVM)**

In vitro maturation was done as described previously by Dos Santos-Neto *et al*. (13) with some modifications (used different medium). Substantially, the maturation solution consisted of TCM-199 supplemented with 5% fetal bovine serum, 5 µg/mL FSH, 0.25 mM sodium pyruvate and 100 units/ mL penicillin/streptomycin . The OFF was added to the maturation solution according to the previous three maturation treatments. Oocytes were matured for 27 h at 39°C in 5% CO2 and 95% air. After maturation, cumulus cells were removed from oocytes utilization 1% (wt/vol) hyaluronidase in PBS.

Oocyte nuclear maturation assessment

Denuded oocytes were placed on a glass slide, covered with a coverslip. Oocytes were fixed with aceto-ethanol (acetic acid: ethanol 1:3, V/V) and stained with 1% aceto-orcein. Following drying, the slides were examined under an inverted microscope (700 X) for different nuclear maturation stages (18).

***In vitro* fertilization (IVF)**

Depending on the steps mentioned by De Oliveira Bezerra *et al*. (12) with some modifications (used different medium), to remove the cumulus cells, COCs were mixed for 1 min in 2 ml HEPES- tyrode's albumin lactate pyruvate medium (HEPES-TALP), washed three times in similar medium and twice in TALP medium. The resulting oocytes were carried into four-well plates containing 250 µl of Fertil-TALP. Frozen straws of proven Awassi rams were thawed in a water bath at 38°C for 30 sec. The contents of straws were emptied in a centrifuge tube with 4ml of Hepes-TALP medium. The tube was subjected to centrifugation for 10 minutes at 200 x g. For the swimming-up of spermatozoa, 2 ml of Hepes-TALP medium was appended to 50 µl of the resultıng aliquots and placed in a conical tube. The sperm hang was collected from the upper part of the tube after 1 h and centrifuged for 15 min (400 x g). The resulting sperm pellet was incubated for 45 min at 38.5^{0}C in heparin and Hepes-TALP medium. A haemocytometer was used to count sperm concentration to 3×10^{9} sperms /ml. The resulting sperm suspension was added to the TALP medium to give a final concentration of 1.5×10^{6} spermatozoa/ml. The oocytes were incubated for 17 h in the previous conditions of maturation. The resulting zygotes were elaborated under an inverted microscope to the detective for second polar body formation.

***In vitro* culture (IVC)**

The resulting zygotes were washed three times with TCM -199 and cultured at the previous conditions of incubating. The cleaved oocytes were examined after 24 hours post culturing. further developmental stages (morula and blastocyst; Figure 1) were evaluated during 8 -days of culture (27) with some modifications.

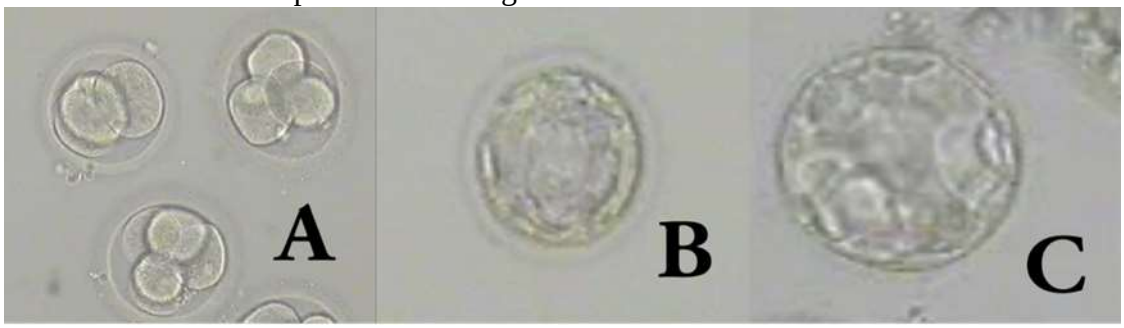

Figure1. Awassi sheep embryos in different stages: A: 2-4 cell, B: morula, C: blastocyst

Embryo grading

According to Wintner et al. (34); Embryos were graded into three main groups as follows (Figure 2):

1. Type 1: cells are of equal size and symmetrical with complete absence of fragmentations.

2. Type 2: cells are of equal size and symmetrical but with few fragmentations.

Type 3: cells are of equal or unequal size with many fragmentations.

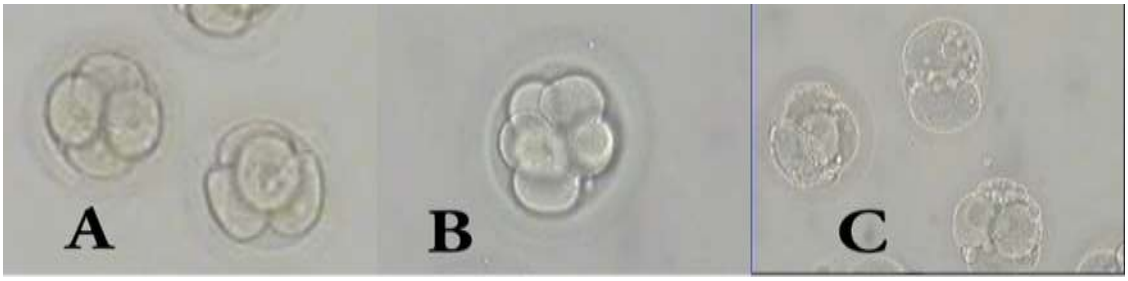

Figure 2. Embryo quality grades of Awassi sheep embryos: A: Type 1, B: Type 2, C: Type 3

Statistical analysis

Across the two experiments, all rates were presented as percentages (%) in tables. Comparison of the rates among different parameters, maturation, fertilization, rate of development and embryo quality was evaluated and based on log-linear analysis of two-way frequency tables of chi-square. Fisher exact test was applied to compare the differences of the rates. Data were analyzed using SAS Institute Inc. (25, 14, 15) statistical package.

RESULTS AND DISCUSSION

In vitro nuclear* maturation*:

Through the progress in the different stages of nuclear maturity, the ewe oocytes, with ages ranged between 1-2 years, showed significant differences in the GBVD phase, where the rates in the fourth group (D) increased to 20.83% ($p \leq 0.05$). Non significant differences were noticed for ewes oocytes (> 2 years of age) through the four maturation solutions (Table 2). In similar table and within the two groups of ewes, a significant ($P \leq 0.006$) difference was observed for oocytes that completed M-II, the rates reached 85.70 and 82.35% in C solution for each age group respectively. The differences in the rates for both groups of age and across the four maturation solutions did not exceed 31.54%.

Table 2. *In vitro* nuclear maturation stages of Awassi sheep oocytes cultured in different maturation solutions supplemented with different levels of of ovine follicular fluid across ewe age groups

Ewe age (year)	Maturation solution	Incubated oocytes (No)	Nuclear maturation phase			
			GV No./%	GBVD No./%	M- I No./%	M- II No./%
>2	A	40	4/10.00	3/7.50^{A}	6/15.00	27/67.50^{aA}
	B	53	4/7.54	2/3.77^{A}	5/9.43	42/79.24^{aA}
	C	49	2/6.12	1/2.04^{A}	4/10.20	42/85.70^{bB}
	D	56	3/5.35	3/5.35^{A}	15/26.78	35/62.50^{aB}
						*P_1 = 0.03
1-2	A	43	4/9.30	6/13.95^{aA}	5/11.62	28/65.11^{aA}
	B	50	6/12.00	5/10.00^{aA}	5/10.00	34/68.00^{aA}
	C	51	1/1.96	3/5.88^{aA}	5/9.80	42/82.35^{aB}
	D	48	5/10.41	10/20.83^{bB}	7/14.58	26/54.16^{aB}
				**P_2=0.05		
				***P_3 =0.02		***P_3 = 0.006

Each subscript letter denotes a subset of the variable whose column proportions do not differ significantly from each other at the assigned probability(p), The lowercase letters are assigned to each ewe age group separately and the upper case letters to the entire column (both ewe age groups),*: P_1:an assigned probability for first ewe age group(>2 years) for each trait separately, **: P_2:an assigned probability for second ewe age group(1-2year) for each trait separately, *:P_3: an assigned probability for whole column(both ewe age groups) for each trait separately**

IVM, IVF and cleavage

Table (3) showed that fertilization rates differed clearly(p=0,000) across the two age groups within the four maturation solutions, where the oocytes that matured in solution C in both age groups achieved the highest rates (32,17 and 30,73 % respectively), the rates of matured oocytes in D group decreased in both age groups (20.46 and 18.50% respectively). Only matured oocytes in the second age group achieved the highest rate of cleavage (C solution; 41.27%; p=0.004). The rates of cleavage stage were very close to each other in the previous groups, the difference did not exceed 25.5%. During the different stages of the early embryos (2-16 cell, morula and blastocyst), although there were differences between the rates; however, those differences were not statistically significant (Table 3).

Table 3. *In vitro* fertilization and cleavage of Awassi sheep oocytes matured in different maturation solutions supplemented with different levels of ovine follicular fluid across ewe age groups.

Ewe age (year)	Maturation solution	Matured oocytes	Fertilized oocytes	Cleaved oocytes	Embryonic stage		
					2-16 cell	Morula	Blastocyst
		No.	No./%	No./%	No./%	No./%	No./%
>2	A	210	63/30.00^{aA}	21/33,33^{A}	3/14,29	5/23,81	13/61,90
	B	242	67/27.68^{aA}	23/34,33^{A}	4/17,39	7/30,43	12/52,17
	C	230	74/32.17^{aA}	26/35,14^{A}	4/15,38	9/34,62	13/50,00
	D	215	44/20.46^{bB}	13/29,55^{A}	5/38,46	4/30,77	4/30,77
			*P_1= 0.03				
1-2	A	180	53/29,44^{aA}	16/30,19^{aA}	2/12,50	4/25,00	10/62,50
	B	195	55/28,21^{aA}	22/40,00^{aA}	3/13,64	8/36,36	11/50,00
	C	205	63/30,73^{aA}	26/41,27^{bA}	4/15,38	9/34,62	13/50,00
	D	200	37/18,50^{bB}	6/16,22^{bB}	1/16,67	2/33,33	3/50,00
			**P_2= 0.023	**P_2= 0.004			
			***P_3= .000	***P_3= 0.01			

Each subscript letter denotes a subset of the variable whose column proportions do not differ significantly from each other at the assigned probability(p), The lowercase letters are assigned to each ewe age group separately and the upper case letters to the entire column (both ewe age groups),*: P_1:an assigned probability for first ewe age group(>2 years) for each trait separately, **: P_2:an assigned probability for second ewe age group(1-2year) for each trait separately, *:P_3: an assigned probability for whole column(both ewe age groups) for each trait separately.**

Embryo quality

The rates of type1 embryos differed clearly (p=0.04) between the two ewe age groups within the various maturation solutions, the embryos in the second age group (C solution) achieved the highest rates (76.92%f). Elsewhere, the rates of the embryos of type2 decreased (p=0.02) and ranged randomly between 0 and 46.15% (Table 4).

Table 4. Rates of Awassi sheep early embryos types in different maturation solutions supplemented with different levels of of ovine follicular fluid across ewe age groups

Ewe age(year)	Maturation solution	Embryos (n)	Embryo quality		
			Type1	Type2	Type3
			No./%	No./%	No./%
>2	A	21	9/42.85^{aA}	9/42.85^{A}	3/14.28
	B	23	16/69.56^{aA}	6/26.08^{A}	1/4.34
	C	26	15/57.69^{aA}	8/30.76^{A}	3/11.53
	D	13	3/23.07^{bB}	6/46.15^{A}	4/30.76
			*P_1=0.04		
1-2	A	16	10/62.50^{A}	3/18.75^{aA}	3/18.75
	B	22	14/63.63^{A}	7/31.81^{bA}	1/4.54
	C	26	20/76.92^{B}	0/0.00^{bB}	6/23.07
	D	6	4/66.66^{A}	2/33.33^{aA}	0/0.00
				**P_2=0.02	
			***P_3=0.04	***P_3=0.02	

Each subscript letter denotes a subset of the variable whose column proportions do not differ significantly from each other at the assigned probability(p), The lowercase letters are assigned to each ewe age group separately and the upper case letters to the entire column (both ewe age groups),*: P_1:an assigned probability for first ewe age group(>2 years) for each trait separately, **: P_2:an assigned probability for second ewe age group(1-2year) for each trait separately, *:P_3: an assigned probability for whole column(both ewe age groups) for each trait separately.**

In the current study, taking into account the factors that contributed to obtaining the current results, it became clear that the addition of OFF to the maturation media by up to 25% has contributed to raising the rates of the different studied traits (Tables 2, 3 and 4). However, using OFF by up to 50% of the entire maturation medium (group D) showed an acceptable performance during the phases of nuclear maturation, despite the high random arresting rates in GV, GBVD and M-I, and a decrease in M-II rates compared to the oocytes in other maturation solutions (Table 1). On the other hand, a significant increase in the rates of oocytes reaching M-II was observed, in which the OFF and TCM-199 media were used in different combination concentration levels (B and C groups) which may reveal a positive participatory effect between the OFF and maturation media. Likewise, the positive participatory effects between maturation media and OFF may form a strong competitive environment against using common media individually in in vitro maturation processes, especially since obtaining OFF is an easy application and with low costs. Simulated by the above, fertilization rates of matured oocytes in participatory maturation solutions increased (Table 3), especially those in which the oFF participated TCM-199 with 25% in both age groups (Group C). Relatedly, the oocytes in the first age group (> 2 years) were characterized by a slight increase in the rates compared to their counterparts in the second group (32.17 Vs. 30.73% respectively). As for cleavage stages, ewes oocytes of the second age group which were matured in a participatory solution containing 15% or 25% $_{O}$FF showed higher cleavage rates than their counterparts in the first age group where the rates were 41.27 and 40.00% respectively (C and B Solutions). At the various stages of cleavage, there were no significant differences across the four maturation solutions within the first group of ewe age, the rates of each stage of cleavage were very close to each other, noting an increase in the rates of embryos reaching the blastocyst stage in group A across the two age groups of ewes (61.90 and 62.50% for groups A respectively). The former differences may be due to the nature of developmental competence of the oocytes which in turn qualifies the oocytes to complete the fertilization process and follow up on the subsequent division processes (7).In general, the results indicated in our experiments can be traced to many factors that relate to the nature and components of the $_{O}$FF and its physiological role within the follicle. where the effect of FF when supplemented to maturation media is not affected by follicle size but that it may be affected by the quality of the follicle from which it is obtained and the factors involved in the increased development of oocytes are not necessarily present in all large follicles but seem to be expressed in selected large follicles (8). In addition, the factors that inhibit the subsequent divisions of the oocyte that are already present in the oFF and that operate according to specific physiological determinants in the ovary, In more detail, within the scenario of follicular wave dynamics whose results in the selection of the dominant follicle, the simulation relationship between the factors inhibiting the growth of the follicle and the oocyte (inhibin A and inhibin B) and the activating factors (activin A, activin AB, activin B and follistatin)will finally lead to a difference in the concentrations of the previous factors in the FF, which in turn leads to affecting the release of oocytes that are essentially arrested in the GV stage (17, 6). In the environment of IVM, the previous factors may have a major role in inhibiting the cytoplasmic and nuclear maturation of the oocyte, allowing the chance for other oocytes to grow. Despite that, it is well recognized that oocytes obtained from large follicles are more developmentally competent than those from smaller follicles (8). We also can explain the results of cleavage in table (3) to the importance of the media after IVF. Tung Huang *et al*. (30) explained that the fertilized oocytes, which were matured in two solutions, the first contained 40% FF and the other contained 100%, which was cluttered after IVF in a culture medium of rabbit oviduct, the cleavage rates increased to 70% and 73% respectively. The dispersion in the significance of the previous results under the influence of ewe age, especially the absence of differences between the different stages of cleavage can be explained that ewes which are intended for

slaughter are in very different ages, especially ewes which excluded out of breeding which can be more than five years old. Theoretically, this may be due to the effect of the physiological phase of ewes and to heterogeneity in the sizes of ovarian follicles and oocytes diameters during these two groups of ages which fall within the reproductive season (breeding season) of Awassi sheep, especially the stage of the reproductive cycle at ewes before slaughter is unknown. In addition, the feeding status of the animals plays an important role in the physiological status of the animal, the change in the animal feed causing rapid changes in the rate of metabolic actions which ultimately affects the developmental competence of the oocyte and quality of resulting embryos (4, 32;10). Data in Table (4) show that the embryos whose oocytes have matured in participatory solutions between the TCM-199 and the $_{O}$FF have given high rates of the Type 1 embryos which in turn may suggest the importance of the involvement of the FF in IVM processes. It is reasonable that some biochemical characteristics and components of the FF surrounding the oocytes may play a major role in determining oocyte quality and the subsequent potential to achieve fertilization and embryo development at optimal involvement (21). In literature, there is still some ambiguity surrounding the mechanism of influence of factors affecting the quality of the embryos. The results of our study (Table 4) suggested the prolonged effect of FF source and steroid hormones (luteinizing hormone (LH)) on quality. The rationale assumes that the acquisition of the developmental potential of the oocytes occurs by the correlation between the increase in the volume of the follicle and the FF and the diameter of the oocyte simultaneously. The increase in the volume of the FF provides the oocytes with all the components that this ability acquires (11). Many studies suggest that the embryo quality is affected by the dominant follicle and dynamic follicular wave (33), the reproductive status of females (31), the vital characteristics of the spermatozoon (16; 9) and semen characteristics (36). It concluded from the current study that adding $_{O}$FF to maturation medium by up to 25% has significantly improved the rates of maturation, fertilization, cleavage and embryo quality of Awassi sheep.

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