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EFFECTS OF FOLLICLE SIZE AND OOCYTES DIAMETER ON DEVELOPMENTAL COMPETENCE AND IN VITRO EMBRYO PRODUCTION OF AWASSI SHEEP EWES OOCYTES

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ABSTRACT

Follicles of different sizes were dissected from ovaries of Awassi ewes and classified according to size into four groups (A: < 1 mm, B: 1- 2 mm <, C: 2 – 3 mm < and D: ≥ 3 mm). The resulting oocytes were classified according to their diameter into four groups (A": < 100 m, B": 100 – 110 m <, C":110 – 120 m < and D": ≥ 120m). Matured oocytes subsequently were subjected to fertilization and culturing was done for six days. Pearson Chi-square was used to analyze the data. For the study the effect of follicular size, significant differences (P < 0.01) were obvserved in the IVM rate among the four groups of A, B, C and D (10.67%, 28.97%, 56.25% and 70.83% respectively), significant difference (P < 0.01) was ovbserved in the IVF rate between the two groups C and D (26.98% and 61.76% respectively), no significant difference was obvserved in the cleavage rate between the two groups C and D (33.33 % and 29.41% respectively). The study of the effect of oocytes diameter results showed significant differences (P < 0.01) in the IVM rate among the four groups A", B", C"and D" (6.16%, 35.59% ,72.30% and 82.02%, respectively), no significant differences were observed in the IVF rates among the three groups B", C" and D" (22.72%, 38.29% and 42.46% respectively), no significant difference was observed in the cleavage rate between the two groups C" and D" (33.33 % and 41.93% respectively).

KEY WORDS: follicular size-oocyte diameter-Awassi ewes-IVM-IVF-cleavage.

INTRODUCTION:

In vitro embryo production at early stages of mammalian oocytes is correlated with ovarian follicular growth and with the increasing in oocyte diameter. This relationship was confirmed in vitro in many species. This occurs with cattle (Pavlok et *al.* 1992; Lonergan *et al.* 1994 ; Hendriksen *et al.* 2000); goats (Crozet *et al.* 1995); sheep (Cognie *et al.* 1998).

Kruip et *al.* (2000) indicated the relationship between follicular size and developmental competence of oocytes. In cows, oocytes derived from follicles 2-3 mm gave moderate rates of cleavage stage (Hendriksen *et al.* 2000).

There is a close positive relationship between follicular size and high IVM, IVF, cleavage rate is attained (Lonergan *et al.* 1994; Otoi *et al.* 1997). Fair *et al.* (1995) found that oocytes of cows are gaining developmental competence by increasing in follicular size, and noted that developmental competence of oocytes in mammals occur when follicular size reaches 3 mm and the corresponding diameter of oocytes 110 µM, as well as 23% of the oocytes stop at the GV stage (germinal vesicle) after 24 hours of maturation. Sato *et al.* (1990) explained the high rates of bovine oocytes that had large diameters, while oocytes were able to pass the GVBD stage (germinal vesicle break down) at diameters 91 µM. As noted, the formation of the first polar body begins at diameters 110 µM. Arlotto *et al.* (1996) found differences in the rates of matured oocytes at diameters 125-134 µM, compared with those had diameters 95-105 µM.

In cows, oocytes derived from follicles > 6 mm reached high rate of blastocyst stage compared with oocytes derived from follicles ≤ 6 mm (TAN and LU., 1990). The vesicular cavity in cattle begins configuring in follicles at diameters 0.12 - 0.16 mm (Mariana and Machado ,1976), while Marion and Gier (1971) referred that vesicular cavity begins configuring in follicles at diameter 0.7 mm. Lussier *et al.* (1987) noted that vesicular cavity develops in a steady level at diameters 0.14 - 0.28 mm. Nuclear maturation can be done in oocytes derived from small follicles while the cytoplasm still immature (Barnes *et al.* 1991). In cows oocytes derived from mature follicles gave high rate of blastocyst stage compared with oocytes derived from small follicles (Pavlok *et al.* 1992), sheep (Cogine *et al.* 1998), and goats (Crozet *et al.* 1995). Luissier *et al.* (1987) showed that oocytes grow inside the follicles slowly for a period may last for 6 months and during that period oocytes gaining ability for nuclear maturation through interaction between oocyte and granular cell layer.

Therefore, the aim of this study was to determine the possible effects of follicular size and oocyte diameter on developmental competence and in vitro embryo production at early stages of Awassi ewes oocytes.

MATERIALS AND METHODS:

The research was conducted in Reproductive Biotechnology laboratories of Agriculture Faculty of Aleppo University, using total number of 418 Awassi ewes oocytes obtained from ovaries were collected from slaughterhouses in Aleppo city and transported to the laboratory in phosphate-buffered saline (PBS) at 35–37 ◦C within 1 h of slaughtering. Cumulus oocyte complexes (COCs) (Fig. 1) were recovered by slicing method from follicles at all diameters at 32–36 ◦C using an specific blade and pooled in 50 ml conical 4-wall plate ,follicles sizes were measured by a ruler while the diameters of oocytes were mea¬sured by using an ocular micrometer (340 magnification) inserted into an inverted-phase microscope, follicles were classified into four groups according to size (*A: <1 mm, B: 1- 2 mm <, C:2 – 3 mm <* and *D: ≥ 3 mm*) and in the same time the oocytes derived from the previous follicles were classified into four groups according to their diameters (*A": < 100 µm, B": 100 – 110 µm <, C":110 – 120 µm <* and *D": ≥ 120µm*).

1. **In vitro maturation (IVM):** COCs were assessed morphologically and only those that had a compact, three or more complete layers of cumulus cells and fully grown oocytes with homogenous cytoplasm were selected for the experiments. All selected COCs were washed three times in HEPES-buffered tissue culture medium TCM199 and once in IVM medium and placed in 4 wall-dishes (10-15 oocytes/wall) of the same medium under sterile silicone oil and matured for 27 h at 38.5°C in an atmosphere of 5% CO_2 in humidified air. 25 Mm HEPES-buffered TCM199 was used, supplemented with 2 mm sodium pyruvate, 1mm l-glutamine, penicillin (75 mg/ml), streptomycin (50 mg/ml), 10% steer serum (Desmedt *et al.* 1994), matured COCs were examined under inverted microscope to investigate for first polar body formation.

2. **In vitro fertilization (IVF) and sperm preparation:** Following maturation, presumptive COCs were denuded of surrounding cumulus cells by vortexing for 1 min in 2 ml HEPES-TALP and subsequently washed three times in HEPES-TALP supplemented with 2% bovine serum albumin (BSA, Fraction V) and twice in IVF-TALP. Oocytes were transferred into four-well plates containing 250 µL of Fertil-TALP. The fertilization medium (Fertil-TALP) was supplemented with a final concentration of 10 µg/ml heparin-sodium salt (185 units/mg heparin), 500 µM epinephrine and 250 µM penicillamine (Martino *et al.* 1994). Frozen-thawed Awassi ram semen was prepared for IVF using previously described methods (Desmedt *et al.*1992) with some modifications. Two frozen semen straws were thawed in a water bath at 38°C for 30 sec and emptied in a centrifuge tube. Four milliliters of Hepes-TALP medium was added to the tube and the tube was centrifuged at 200 x g for 10 min. After discarding the supernatant, an aliquot of sperm pellet was resuspended (1:1) with Hepes-TALP medium. Swim-up in sperm-TALP medium was achieved using 50 µl aliquots of spermatozoa placed at the bottom of a conical tube containing 2 ml of Hepes-TALP medium. After 1 h of placing them at 38.5°C when the actively motile sperm swam up, 0.5 ml of the sperm suspension was collected from the upper part of the tube and centrifuged at 200 x g for 10 min. After discarding the supernatant, the resulting sperm pellet was resuspended with heparin containing (100 µg/ml) Hepes- TALP medium and incubated for 45 min at 38.5°C. The sperm concentration was assessed in a haemocytometer and the sperm pellet was resuspended in TALP to give a final concentration

of 3 x 10^9 sperms /ml. The oocytes were transferred to another dish containing Fert-TALP medium (TALP supplemented with 30 µg penicillamine /ml, 15 mM hypotaurine/ml) under paraffin oil. This was inseminated with prepared sperm in a volume, so as to give a final concentration of 4 × 10^6 sperms/ml. The sperm suspension was added to each fertilization well to obtain a final concentration of 1.5×10^6 spermatozoa/ml. Plates were incubated for 17 h under 5% CO_2 in air with maximum humidity (>95%) at 38.5°C. Fertilized oocytes were rinsed with PBS and examined under inverted microscope to detective for second polar body formation.

3. **In vitro culture (IVC):** Zygotes were rinsed with serum-free TCM199 medium, and cultured in 50 µl droplets (20 oocytes/drop) serum-free TCM199 medium at 38.5°C in an atmosphere of 5% CO_2 in humidified air. Cleavage stages were assessed on days 2-8 of IVC (Martino *et al.*1994).

4. **Statistical analysis**: Pearson Chi-square of contingency table test and exact Fisher test were used to analyze the data among groups for matured oocytes, fertilized oocytes and early stages of embryos, using SAS, 9 Software (SAS. 1996).

Reagents:
All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

RESULTS AND DISCUSSION:

1. **Effect of follicular size:** The results, presented in Table 1, show that most of oocytes in the both groups A and B failed to reach the maturation stage (10.67% and 28.97% respectively) (Table 1). While on the contrary oocytes in the two groups C and D clearly superior (P <0.01) (70.83% and 56.25%, respectively). As evidenced by the contents of the table 1 that oocytes in the two groups A and B failed to reach the fertilization stage, there was difference in IVF rates between the both groups C and D but in a varying degrees (P <0.01) (26.98% and 61.76%, respectively), also there was no difference in cleavage rates between the two groups C and D (29.41% and 33.33%). These results are consisting with what were Desmedt *et al*. 1994 reported that maturation rates of oocytes derived from follicles < 1 mm were very low and did not exceed 10.67%. The current study came to similar conclusions to Stubbs *et al*. (2007); Howell *et al*. (2001) whom indicated that sheep oocytes gaining developmental competence gradually with the continued growth of the follicle. These low rates of maturation in the two groups A and B (Table 1) may be due to the inability of the oocyte to resume meiosis and survival at GV stage, and that deficit in the necessary proteins is needed to follow up the development of manufacturing, therefore the most of the oocytes in A group failed to mature, while oocytes in B group were able to reach maturation rate at 8.97% with the possibility of having the largest diameters compared with the other oocytes in the same group, However that helped to complete the development or as a result of the largest enclosed number of layers of cumulus cells, which led to increased contact between them and the surrounding media (Ledda *et al*. 1997).

It is possible that the lack of cortical granules is distributed along the plasma membrane of the oocytes; As a result it led to the inability of most of the mature oocytes to complete fertilization and division (Patricia *et al*. 2008). Taneja *et al*. (2000) also pointed to existence of an influential factor within cytoplasm in oocytes taken from large follicles may urges evolutionary capacity. The oocytes in the two groups C and D were able to complete maturation (56.25% and 70.83%, respectively), after 27 hours and then they were able to complete the fertilization and the sequence of early embryonic divisions process in acceptable rates successfully, which indicates that the oocytes of these two groups completed the process of growth with completion of the molecules responsible for the maturation of the nucleus, the cytoplasm and the completion of the necessary proteins needed by the oocyte during IVM (Black *et al*. 2008). In addition the cleavage rates in both groups C and D were 29.41% and 33.33%, respectively (Table 1) whereas most of the oocytes had diameters ≥120 µM, allowing the resumption of meiosis, consequently, rates were high compared with other groups. Oocytes derived from follicles ≥ 3 mm can manufacture essential factor of mother (Maternal factor) to support the maturation and fertilization and early embryonic development follow-up (Bogliolo *et al*. 1997). The completion of the development competence of oocytes in Awassi ewes in B, C and D Groups may be due to the gradual acquisition of nuclear maturation, and sequence of proteins manufacturing necessary for the development of the oocytes (Ledda *et al*. 1997).

Table 1: Rates of IVM, IVF and cleavage according to follicular size

Follicular size (mm)		Incubated oocytes	Matured oocytes		Fertilized oocytes		Cleavage (two cells to blastocysts stage)	
		No.	No.	%	No.	%	No.	%
A:	< 1	103	11	10.67a**	0	0	0	0
B:	1- < 2	107	31	28.97b**	0	0	0	0
C:	2 - < 3	112	63	56.25c**	17	26.98[a]**	5	29.41[NS]
D:	≥ 3	96	68	70.83d**	42	61.76[b]**	14	33.33[NS]

NS: insignificant, ****:** (P < 0.01), values with different subscripts (a, b, c and d) differ (P < 0.01) within column

2. **Effect of oocyte diameter:** Our results show (Table 2) that the oocytes in the two groups C" and D" reached the highest rates of IVM (P < 0.01), (72.30% and 82.02%, respectively), compared with the other groups A" and B" (6.16% and 37.28% respectively). As for the results of fertilization it has been observed that oocytes in A" group failed to complete fertilization and division, while no significant differences were observed among the three groups B", C" and D", (22.72%, 38.29% and 42.46% respectively). The study also show insignificant difference in cleavage rates in the two groups C" and D", (33.33% and 41.93 %respectively). These results came close with the results of Fair *et al*. (1995) where IVM rates of ovine oocytes were 21%, 42%, 76%, and 81% respectively. As well as with the results of Luciana *et al*. (2009) where the ovine oocytes were grouped into three classes depending on oocyte diameter (<100 µM, and 100-110 µM < and 110-120 µM), IVM rates were 10.8%, 11.57% and 77.98 % respectively. However, our results were less than what Otio *et al*. (1996) found, where the oocytes diameters increased at a rate of 5 µM when were divided from <110 to larger than 130 µM, IVM rates ranged between 46.9% - 96.4% and rates of fertilization ranged between 45.2% - 78.7%. Pavlok *et al*. (1992) found that the oocytes > 110 µM and oocytes derived from follicles < 3 mm, the nuclear and cytoplasmic maturation are incomplete during fertilization, therefore they are not be able to develop after fertilization. Also oocytes that had diameters < 100 µM are not able to clone and transfer mRNA or the completion the necessary steps after the completion of the transfer of messages that related to proteins manufacturing which they are necessary to the developmental competence and access to the M-II stage (Fair *et al*. 1995)

It was clear in this study the variability of the influence of diameter on oocytes susceptibility during IVM because of the needs to food items which vary according to their stage of development, and this can be found in ovarian follicles while in IVF, the incubated oocytes in specific media could have a negative impact because of the relative lack of some of the components that exist in the vesicular liquid (Suzuki *et al*. 1994)

Table 1: Rates of IVM, IVF and cleavage according to oocyte diameter

Oocyte diameter (µM)		Incubated oocytes	Matured oocytes		Fertilized oocytes		Cleavage (two cells to blastocysts stage)	
		No.	No.	%	No.	%	No.	%
A":	<100	146	9	6.16[a]**	0	0	0	0
B":	100 - <110	118	44	37.28[b]**	10	22.72[NS]	0	0
C":	110 - <120	65	47	72.30[c]	18	38.29[NS]	6	33.33[NS]
D":	≥120	89	73	82.02[d]**	31	42.46[NS]	13	41.93[NS]

NS: insignificant, ****:** (P < 0.01), values with different subscripts (a, b, c and d) differ (P < 0.01) within column

CONCLUSİONS:

It concluded from this study the following:

1. In general there were differences among Awassi ewes oocytes according to their diameter which affected their developmental competence on in vitro embryos production, the oocytes that have diameters > 120 µM gave higher rates of IVM , IVF and cleavage stages compared with those that have diameter less than 120 µM.

2. Awassi ewes follicular size affected significantly in developmental competence of oocyte in IVM, IVF and cleavage stages according to their follicular size, oocytes that have follicular sizes at ≥3 mm gave higher rates of IVM, IVF and cleavage compared with those that have follicular size less than 3 mm in general.

3. Slaughter houses can be good and cheap source for sheep oocytes used in in vitro embryos production.

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