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EFFECTS OF MONTH OF THE YEAR ON IN VITRO EMBRYO PRODUCTİON OF SYRİAN AWASSİ SHEEP

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ABSTRACT

In the present work, we have investigated the influence of months of the year on the ability of Awassi sheep oocytes to produce embryos in vitro. Ovaries were collected during months of the year from local slaughterhouses. All resulting oocytes were subjected to in vitro maturation (IVM), fertilization (IVF) and culture (IVC). Results showed that month of the year greatly affected the IVM rates ($p < 0.0001$) where the highest value was in October and the lowest was in February (80.58 and 30.90% respectively), high significant differences ($p < 0.0001$) were observed in IVF rates among months of the year, oocytes reached the highest IVF rate in September (41.83%) while IVF rate was the lowest in January (17.10%). As for results of cleavage stages, no significant differences were observed among months of the year, where the values ranged between 30 - 42.18%. The month of the year affected significantly the quality of type1 embryos ($p< 0.04$) where the rate rose to 71.4 % (October). Under the conception of breeding and non-breeding season , it concluded that months of the year clearly affected IVM and IVF rates of Awassi sheep oocytes .

KEYWORDS: in vitro embryo production, months of the year, ovine oocytes, embryo quality.

1. INTRODUCTION:

In vitro embryo production (IVEP) techniques play an important role in sheep breeding applications because of the phenomenon of seasonal reproduction. In general, efficient systems of ovine IVEP have two purposes: producing large numbers of good quality blastocysts and producing superiors offspring (Sreenivas et al. 2014). Syrian Awassi sheep are characterized by the seasonal reproduction, where the reproductive season (breeding season) starts in summer and ends in autumn. Activity and duration of estrous are affected by the seasonal variation of environment, nutrition and management (Gwazdauskas. 1985). Lee. (1993) reported that conditions of heat and cold stress reduce the pregnancy rates as noted that these stressors alter the intricate balance of endocrine profiles, leading to lower intensity of estrous behaviour, anestrous, embryonic death and subsequent infertility.

Basically, the reproductive seasonality in the ewe is characterized completely by changes at behavioral, endocrine and ovulatory levels (Rosa and Bryant. 2003). Within the same breed, rams and ewes have seasonal variations in breeding activity, where ewes are more seasonal than rams accompanied by short period of breeding season. Photo-periodical influences can be modulated by factors like temperature, nutrition, social influences, lambing date and lactation period (Scaramuzzi and Martin. 2008 ; Forcada and Abecia. 2006)

Across monthss of the year, The the inhibitory influnce (non stimulatory photoperiods) of the penile gland makes the hypothalamus pituitary axis in sheep quiescent while stimulatory photoperiods allows secretion of the gonadotrophins (Karsch et al. 1989). Rutledge et al. (1999) noted that blastocyst rate was low in mid to late summer in cattle, however, the rate increased from mid to late spring, while there was stability in these rates in winter and fall months. Rocha et al. (1997) reported that seasons had no effects on IVEP of Brahman breed in subtropical environment, as well as bovine breeds (Rivera et al. 2000). Low fertility is associated with the heat stress before mating in cows (Al-Katanani et al. 2002), and in sheep (Dutt. 1963). Reasons of infertility may reflect the malfunction in oocytes developmental competence, Rocha et al. (1998) referred to the possibility of giving the evidence correlated with developmental competence of oocytes during IVF procedures, where they noted that IVM rates were very low in months of summer compared to those in winter. Heat stress lead to decreasing in follicular growth which lead in turn to decreasing in secondary follicles growth (Badinga et al. 1993). Wolfenson et al. (1995) referred that the rising in temperature synchronized with heat stress in cows can directly frustrate the functions of oocytes, in addition , this causes harmful effects to protein manufacturing and essential transcripts necessary for subsequent embryonic development.

We tried in our study to demonstrate circumstantially the effects of months of the year within the conception of breeding and non-breeding season on developmental competence of Awassi sheep oocytes for IVEP.

2. MATERIALS AND METHODS:

2.1. Oocyte collection:

Ovaries were collected from local sluotherhouse in Aleppo city ,and immersed in PBS and transported to the laboratory in an incubator at 39°C . the time elapsed from animal slaughter to laboratory did not exceed 1h. Cumulus oocyte complexes (COCs) were isolated from follicles with different sizes by slicing method and transferred to a petri dishs containing fresh collection medium (TCM-199) with heparin. Supernatant were then evaluated and washed three times in maturation medium(TCM-199).

2.2. IN VITRO MATURATION:

IVM was done as described previously by Desmedt et al. (1994), with some modifications. Briefly, oocytes were matured in vitro in maturation medium (TCM-199 containing 10% fetal bovine serum, 5 µg/mL FSH, 0.25 mM sodium pyruvate, 100 µM cysteamine and 100 units/mL penicillin/streptomycin) for 27 h at 39°C in 5% CO_2 and 95% air followed by cumulus cell removal using 1% (wt/vol) hyaluronidase in PBS.

Maturation rate was then evaluated by observation of the first polar body under the mi-croscope

2.3.Sperm capacitation and in vitro fertilization:

IVF was done as described previously by Desmedt et al. (1992) with some modifications. Briefly, presumptive COCs were denuded of surrounding cumulus cells by vortexing for 1 min in 2 ml HEPES-TALP and 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 (TALP) was supplemented with a final concentration of 10 µg/ml heparin-sodium salt, 500 µM epinephrine and 250 µM penicillamine. Frozen-thawed Awassi ram semen was prepared for IVF using previously described methods by Rocha et al . (1998) with some modifications. Briefly ,two frozen semen straws were thawed in a water bath at 38 °C for 30 sec and emptied in a centrifuge tube with 4ml of Hepes-TALP medium. The tube was centrifuged at 200 x g for 10 min. The resulting aliqut of sperm pellet was resuspended (1:1) with Hepes-TALP medium. Then 2 ml of Hepes-TALP medium was added to 50 µl of aliquots of spermatozoa and placed at the bottom of a conical tube for Swim-up. After 1 h 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. 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 3×10^9 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. Resulting zygotes were rinsed with PBS and examined under inverted microscope to detective for second polar body formation.

2.4. In vitro culture:

The resulting zygotes were cultured in TCM -199 Culture medium at 39ºC, 5% O_2, 5% CO_2 and 90% N_2. Number of cleaved oocytes was recorded after 24 h post culturing. Subsequent developmental stages were evaluated every second day during 8 day culture.

2.5. Embryo grading:
Embryos right morula stage were graded according to their quality into three groups as follows:

1. Type1: Cells are of equal size; no fragmentation seen.
2. Type2: Cells are of equal size; minor fragmentation only.
3. Type3: Cells are of equal or unequal size; fragmentation is moderate to heavy.

2.6. Statistical analysis:
Pearson Chi-square of contingency table and Fisher excat test were used to analyze the data and compare different rates among groups of different months of the year for matured, fertilized, cleaved oocytes and gradede embryos using SAS, 9 software (1996).

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

3. RESULTS:
IVM rates among the months of the year were greatly different ($p < 0.0001$), where the highst value was in October (80.58%) (breeding season) while the lowest was in Februrary (non- breeding season) (30.90 %) (table 1). Also there were high significant differences ($p < 0.0001$) in IVF rates among the months of the year, values ranged between 17.10 % (January) - 41.83 % (September). Differences in cleavage rates among months of the year were slight and did not ecxeed 12.50% between the highst and the lowest value (June and February), however these differences were not significat (table 1).

Table 1: Rates of IVM,IVF and cleavage of Awassi sheep oocytes during months of the year.

Months of the year		Incubated oocytes No.	Matured oocytes		Fertilized oocytes		Cleaved oocytes	
			%	No.	%	No.	No.	%
Breeding season	June	170	128	75.29 ^{a}	40	31.25 ^{a}	17	42.50
	July	166	125	75.30 ^{a}	44	35.2 ^{a}	18	40.90
	August	180	138	76.66 ^{b}	51	36.95 ^{a}	21	41.17
	September	190	153	80.52 ^{b}	64	41.83 ^{b}	27	42.18
	October	170	137	80.58 ^{b}	55	40.14 ^{b}	21	38.18
	November	140	110	78.57 ^{b}	41	37.27 ^{a}	15	36.58
Non-breeding season	December	120	82	68.33 ^{a}	23	28.04 ^{a}	8	34.78
	January	110	76	69.09 ^{a}	13	17.10 ^{b}	4	30.76
	February	110	34	30.90 ^{b}	10	29.41 ^{a}	4	30.00
	March	135	55	40.74 ^{b}	12	21.81 ^{a}	5	41.66
	April	140	92	65.71 ^{a}	23	25 ^{a}	9	39.10
	May	160	115	71.87 ^{a}	25	21.73 ^{b}	10	40.00
P			*		*		NS	

** : p< 0.0001, NS: not significant. Each subscript letter denotes a subset of case categories whose column proportions do not differ significantly from each other at the, 05 level.*

Table 2 indicate that the lowest rates of arrested embryos at 2-16 cell stage were in the months of breeding season were the values ranged between 9.5-13.3 % ($p< 0.03$) While the differences were not significant for the embryos that continued to develop to morula or blastocyst stage across months of the year despite the high rates of embryos that reached blastocyst stage in the non-breeding season months which their values ranged between 20-50%.

Table 2. In vitro development and viability of various stages of Awassi sheep embryos during months of the year

Months of the year		Cleaved oocytes No.	Embryo viability					
			Developed to 2- 16 cell		Developed to morula		Developed to blastocyst	
			No.	%	No.	%	No.	%
Breeding season	June	17	2	11.5 $_{a, b,}$	5	29.4	10	58.8
	July	18	2	11.1 $_{b}$	6	33.3	10	55.6
	August	21	2	9.5 $_{b}$	6	28.6	13	61.9
	September	27	3	11.1 $_{b}$	4	14.8	20	74.1
	October	21	2	9.5 $_{b}$	7	33.3	12	57.1
	November	15	2	13.3 $_{a, b}$	2	13.3	11	73.3
Non-breeding season	December	8	2	25 $_{a, b, c}$	2	25	4	50
	January	4	1	25 $_{a, b, c}$	1	25	2	50
	February	4	1	25 $_{a, b, c}$	1	20	2	50
	March	5	3	16 $_{c}$	1	20	1	20
	April	9	4	44.4 $_{a, c}$	2	22.2	3	33.3
	May	10	5	50 $_{c}$	2	20	3	30
p			**		NS		NS	

*** : p< 0.03, NS: not significant. Each subscript letter denotes a subset of case categories whose column proportions do not differ significantly from each other at the ,05 level*

In the same simulation, the percentages of embryos that were classified within the type1 model increased during the months of the reproductive period ($p< 0.04$) were the values ranged between 55.6-71.4% (table3), for type2 and type3 graded embryos the rates varied randomly without significant differences

Table 3: Rates of types of graded Awassi sheep embryos (from tow cell to morula stage) during months of the year

Months of the year		Cleaved oocytes No.	Embryo quality					
			Type 1		Type 2		Type 3	
			No.	%	No.	%	No.	%
Breeding season	June	17	10	58,8 $_{a, b, c, d, e}$	3	17,6	4	23.5
	July	18	10	55,6 $_{a, b, c, d, e, f}$	3	16,7	5	27.8
	August	21	13	61,9 $_{a, b, c, d, e}$	6	28,6	2	9.5
	September	27	17	63,0 $_{c, d, e}$	3	11,1	7	25.9
	October	21	15	71,4 $_{e}$	3	14,3	3	14.3
	November	15	10	66,7 $_{b, d, e}$	3	20,0	2	13.3
Non-breeding season	December	8	2	25,0 $_{a, b, c, d, f}$	5	62,5	1	12.5
	January	4	1	25,0 $_{a, b, c, d, e, f}$	2	50,0	1	25
	February	4	1	25,0 $_{a, b, c, d, e, f}$	2	50	1	25
	March	5	1	16,7 $_{a, f}$	1	20	3	60
	April	9	2	25,0 $_{a, b, c, d, f}$	3	33,3	4	44.4
	May	10	2	20,0 $_{f}$	3	30,0	5	50
p			***		NS		NS	

****: p< 0.04, NS: not significant. Each subscript letter denotes a subset of case categories whose column proportions do not differ significantly from each other at the ,05 level*

4. DISCUSSION:

As mentioned above, sheep are distinguished by marked seasonal reproduction activity unlike most domestic livestock species. This phenomenon has an important aspect in IVEP technics. On the other hand, it seems reasonable that the possibility of producing embryos of cattle in vitro across months of the year is widely available comparing to small ruminants species like sheep and goat, not only that, but even producing a huge number of good quality embryos.

Unfortunately, the effects of different months of the year on IVEP and embryo quality at animals that characterized by seasonal reproduction had not been studied sufficiently, whereas few studies examined the ability of oocytes to mature in vitro during breeding and non-breeding seasons.

In general, the differences in IVM and IVF rates among the months of the year in our study may be due to the breed of sheep (Goddard and Pratt. 1983) , age (Majerus et al. 1993), the reproductive physiology position (Bavister. 1995) and the variation in follicles sizes and oocytes diameters (Lonergan et al. 1994). Moreover, the unknown stage of reproductive cycle of the ewes during collecting the ovaries in slaughterhouses.

Nutrition also has an important effects on physiologic condition of the animal, in winter , nutrition is limited to what breeders offer to their animals, where these rations are scarce and have low contents of nutrients, in addition to the lack of green forage, on the contrary, in spring, animals basically feed on green forage through extended time intervals. Ocallaghan et al. (2000) noted that the variation in animal nutrition across months of the year causes instant changes in metabolic efficiency especially the balance between insulin and glucose, where the variability in insulin is correlated directly by the variation in IGF-I (Insulin-like Growth Factor) and IGF-II ,this in turn, reflects directly on the content of follicular fluid to this tow factors.

As evidenced in table1, maturation rates was clearly high during the months of both seasons Summer and Autumn that fall under breeding season conception compared to those in other seasons. Although the rarity in studies investigated the effects of months on IVEP, our results disagree with the results of Khairy et al. (2007) whom obtained high IVM rates of Egyptian buffalo oocytes in months of winter and spring (85.5 and 92.5% respectively),also, our results didn't agree with the results of Mor et al. (2017) where the rates of IVM in January , February, March, April, May and June were 79.1,76.5,82.5,81.5,85.1 and 84% respectively.

As for the IVF results in our study, it was clear that IVF rates were slight at months of non-breeding season, consequently , this agree with results of Pugh et al. (1991) whom referred to the possibility of sheep oocytes fertilization and cleavage in months of non-breeding season. Leibfried- Rutledge et al. (1998) reported that high ambient temperature and humidity have harmful effects on oocyte developemental competence.

It is probable that superiority in IVM and IVF rates appeared across months of breeding season in the current study due to the most of oocytes that were collected in the breeding season had follicle sizes ≥ 3 mm and diameters ≥ 110 µm, this agree with results of Desmedt et al. (1994) . In sheep, Rao et al. (2002) found significant differences in oocytes quality derived from ewes at age ≥ 2 year within breeding and none-breeding season.

As for results of cleavage stages in our study,there was a big convergence among the rates of cleavage across months of the year and ranged between 30 - 42.50%, at the same time, the cleaved oocytes showed remarkable superiority in their follow-up of the advanced stages of cellular division in the months of the year of breeding season, where the percentages of oocytes that stopped at 2-16 cell stage were low(table 2), perhaps, this convergence may be due to that most of cleaved oocytes had the same extent of developemental competence which qualified them to continue subsequnt stages of embryounic developement. However ,our results came less than results reached by Davachi et al. (2014) where the cleavage rates ranged between 51.4-71.4% (breeding season) and 41.3- 64.1% (non-breeding season) ,our results also came much lower than the rates of Mor et al. (2017) in January , February, March, April, May and June (63.2,72.4,65.1,66.7,62.1 and 68% respectively). In contrast, embryos at 2-16 cell, morula and blastocyst stage in our study were much higher than the results of Mor et al. (2017) for the same months of the year(January , February, March, April, May and June) where their values ranged between 42 – 47% , 19-21% and 7.2-9 % for 8-16 cell , morula and blastocyst stage respectively.

Our results indicated a significant difference in the type1 embryos during the months of the year and the absence of this difference for type2 and type3 grade(table 3). Mostly, the reproductive season in sheep will give the opportunity to produce oocytes with high capacity which will result in embryos of good quality with the same time should not ignore the other factors that affect the quality of the produced embryos like nutritional factors and heat stress (Sartori et al. 2010)

5. CONCLUSİON:

The object of our study was to evaluate the differences in Awassi sheep oocyte in different months of the year for IVEP, whereas sheep is a seasonal breeder species and their hormonal and systemic changes being affected greatly by different seasons, so, under the conditions of Syrian environment it's preferable to intensify IVEP operations during the summer and autumn months. Beyond that, it would be reasonable to consider a combination of seasonal effects (including months of the year) with other primary factors may affect the success rates of ovine IVEP, also we suggest more studies to investigate the integrity of IVEP techniques assisted with seasonal reproduction of sheep by applying more excessive methods to overcome these problems.

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