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EFFECTS OF SEASON, EWE AGE AND THEIR INTERACTIONS ON *IN VITRO* EMBRYO PRODUCTION OF SYRIAN AWASSI SHEEP

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Abstract

To study the effects of season and ewe age and their interactions on the oocytes competence for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and subsequent development of early embryos in Awassi sheep, Awassi ewes from which oocytes were taken, were classified into three main groups according to their age: A: <1, B: 1-2 and C :>2 year, a total of 1363 oocytes were collected from ovaries by slicing method. Season of the year clearly affected (p=0.00) the IVM and IVF rates (57.98% and 37.91% for summer and autumn, respectively). According to ewe age, a significant differences(p=0.00) were noticed in IVM rates where group B had the highest values comparing with A and C groups (62.67%,Vs 48.92 and 46.39% ,respectively). As for results of interaction between ewe age and seasons of the year, a significant differences (p<0.01) were noticed in IVM rates, the highest value was in autumn (B group) while the lowest was in winter at A group (27.04 % Vs 29.12% ,respectively), a significant differences (p<0.01) were also noticed in IVF rates,the highest value was in spring at group B while the lowest was in winter at group A (46.47% vs 13.33%, respectively), while no significant differences noticed at cleavage phases. It concluded that opertations of *in vitro* embryo production (IVEP) in Awassi sheep could be intensified in summer and autumn across different ages of ewes.

Key words : Oocytes, *in vitro* embryo production, Awassi sheep, season, ewe age.

Introduction

Awassi sheep are seasonally polyoestrous, where the reproductive activity is characterised by the alternation between breeding and anoestrous periods. It is known that the sexual activity in sheep is affected by the breed, nutrition and photoperiod (Delgadillo *et al*., 2004). While Papachristoforou *et al*. (2000) considered that photoperiod is defined as the primary environmental factor controlling seasonal reproduction. Many other factors including breed, nutritional and lactational status, social interactions and the season of parturition, may interact with seasonal reproduction, which is mainly regulated by photoperiod to modulate its effect and thus influence the timing and length of the breeding season (Forcada *et al*., 2006). "Reproductive seasonality in the ewe is characterized by changes at behavioral, endocrine and ovulatory levels, in an absolute fashion, giving rise to an annual alternation between two distinct periods; a breeding season, characterized by the succession at regular intervals (mean of 17 days) of estrous behavior and ovulation, if a pregnancy does not develop, and an anestrous season characterized by the cessation of sexual activity" (Rosa and Bryant, 2003).

Sheep live weights (LWs) and age along with nutrition, weather and season have a huge influence on reproductive performance (Ray and Smith, 1966). Pregnancy rate and multiple births could be icreased by increasing the premating weight and age of ewes (Akta's and Dogan, 2014). AL-Katanani *et al*. (2002) indicated that season of the year clearly affects the rates of IVM and IVF of cows oocytes subjected to IVEP programs

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and operations related to genetic improvement, as observed a significant differences in the rates of produced embryos in estrous periods in autumn. It was explained that the breeding season in sheep is linked to climatic factors primarily (Badinga *et al*., 1993). Crozet *et al*. (2000) explained that season and ovulation of any breed of sheep vary depending on geographic location , environmental conditions and nutritional status, therefore, sexual activity represents quarterly and his term average values as well as deviations from those averages. Moges *et al*. (1997) found that number and quality of oocytes influenced heavily by the reproductive status and age (non-adult or adult). Some studies indicated a rise in the effectiveness of IVF at oocytes derived from ewes mor than tow years of age (Pawshe *et al*., 1994). The oocytes that are derived from the ovaries of slaughtered animals are heterogeneous because they come from ovarian vesicles at different stages of development. Presicce *et al*. (1997) have pointed out that the ovarian follicles in immature animals may stimulated to growth when treated with gonadotrophins, also, it was observed that FSH treatment improves the developmental competence (Ptakg *et al*., 1999). Therefore, the objective of this study is to investigate different maternal age in relation to season of the year and their interactions to produce embryos *in vitro*.

Materials and Methods

Chemicals and reagents

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co. (USA).

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).

In vitro maturation (IVM)

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

Sperm capacitation and *in vitro* fertilization (IVF)

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 ìM epinephrine and 250 ìM 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.

In vitro culture (IVC)

The resulting zygotes were cultured in TCM -199 Culture medium at 39ºC, 5% O_2, 5% CO_2 and 90% N2. Number of cleaved oocytes was recorded after 24 h post culturing. Subsequent developmental stages were evaluated every second day during 8 day culture.

Experimental design and Statistical analysis

The experiment's oocytes were classified according to ewe age into three main groups: A:<1, B: 1-2 and C:>2 year and at the same time the same oocytes were monitored across seasons of the year (winter, spring,

summer and autumn). Data were analyzed based on log-linear analysis of two-way frequency tables of chi sequare using SAS, 9 software.

Results

Effect of season of the year

The contents of table 1 indicate that season of the year significantly affected (p=0.00) mature sheep oocytes rates, the highest values were in summer and autumn (56.87 and 57.98%, respectively) while the lowest were in winter and spring (43.52 and 49.29%, respectively). On the other hand, the highest rate of fertilization was in summer (37.91%; p = 0.00) and the lowest was in winter (16.03%). Despite of the absence of significancy in cleavage stage among seasons of the year, the study have shown the integral failure of the fertilized oocytes to continue division in winter. As for results of cleavage stages, the rates's values for the embryos that stopped at 2-16 cell, morula and blastocyst stages across seasons converged without significant differences.

Effect of ewe age

Age of ewe clearly affected IVM rates (p = 0.00) where B group surpassed on the other groups A and C (62.67% vs 48.92 and 46.39% respectively). No significant difference were noticed for age effects in IVF and cleavage stage. Rates of embryos which stopped at 2-16 cell stage was very low at C group (4.76%) (p<0.04) compared to other groups. It is also noted that the rates of embryos that lasted right blastocyst stage in this group(C) was higher(p<0.04) than in the other two groups (Table 2).

Effect of interactions (season of the year × ewe age)

IVM stage interactions

The data in table 3 shows that the rate of IVM rose to 58.59% (p=0.00) in ewes oocytes in A group within summer. When comparing the age groups with each other over the seasons, the rate of IVM of oocytes in A group was 64.21% in winter (p=0.00) and 72.04% in B group in autumn (p<0.04).

IVF stage interactions

At level of p<0.03 of significancy,the oocytes that were derived from ewes of B group in summer were superior to those of the other two groups A and C in IVF rate (46.47%) (table 4). The same table shows no significant differences were noticed in the three age groups during the seasons in IVF stage.

Cleavage stage interactions

Rates of cleavage stage for all age groups during

Table 1 : Rates of IVM,IVF, cleavage stage and embryos viability according to season of the year in Awassi sheep.

Season of the year	Incubated oocytes (GV)	Matured oocytes		Fertilized oocytes		Cleaved oocytes		Embryo viability					
								Developed to 2-16 cell		Developed to morula		Developed to blastocyst	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Winter	301	131	43.52**a**	21	16.03**b**	0	0.00**b**	0	0.00	0	0.00	0	0.00
Spring	353	174	49.29**b**	46	26.43**a**	15	32.60**a**	6	40.00	5	33.33	4	26.66
Summer	371	211	56.87**b**	80	37.91**b**	29	36.25**a**	4	13.79	12	41.37	13	44.82
Autumn	338	196	57.98**b**	67	34.18**a**	22	32.83**a**	6	27.27	9	40.90	7	31.81
P		*		*		**		*NS*		*NS*		*NS*	

*:p=0.00,**: p< 0.02,NS:insignificant, 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 : Rates of IVM,IVF , cleavage stage and embryos viability according to ewe age in Awassi sheep.

Eww's age (year)	Incubated oocytes (GV)	Matured oocytes		Fertilized oocytes		Cleaved oocytes		Embryo viability					
								Developed to 2-16 cell		Developed to morula		Developed to blastocyst	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A: < 1	466	228	48.92**a**	61	26.75	18	29.50	7	38.88**a**	9	50.00	2	11.11**a**
B :(1-2)	418	262	62.67**b**	87	33.20	27	31.03	8	29.62**b**	8	29.62	11	40.74**b**
C: > 2	479	222	46.39**a**	66	29.72	21	31.81	1	4.76**c**	9	42.85	11	52.38**b**
P		*		*NS*		*NS*		***		*NS*		****	

*:p=0.00, ***:p<0.04, ****: p<0.03, NS:insignificant, each subscript letter denotes a subset of case categories whose column proportions do not differ significantly from each other at the .05 level.

seasons of the year were convergent in values and ranged between 27.77-37.50% (except winter rates) without significancy (table 5).

Discussion

Generally, principles of IVEP in sheep are the same as in cattle, since sheep are considered seasonal breeders, there are some differences of importance for several reasons. The most important of these reasons is the acquisition of embryos with an optimal genetic contents throughout the year.

Our results showed significant differences in the IVM, IVF and cleavage rates among seasons of the year, where these rates rose in both summer and autumn,theoretically, this may be due to the effect of physiological phase of ewes and to heterogeneity in the sizes of ovarian follicles and oocytes diameters during these two seasons 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, feeding of the animals play 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. The absence of significancy in the three stages of the embryos and their viabilty and the complete lack of embryonic ability to follow the division in winter (table 1) can be mainly attributed to the developmental competence of the oocytes and their quality during different seasons (Toy *et al*., 1993). The fact that oocytes at the same level of developmental competence and good quality can be an important reason to reach very close rates for different embryonic stages in our study.

The significant differences in IVM rates and insignificant differences in IVF and cleavage rates according to ewe age among the three groups A,B and C in our study (table 2) may be due to the great similarity in the surrounding environmental conditions that prevailed during animal life, in addition, the oocytes that were used in this experiment were at high degree of development and growth, which means that the oocytes has completed the process of growth and amounted to diameter which enables them to resume meiosis after the completion the synthesis of necessary proteins that they need to follow meiosis upon completion of the necessary requirements needed to mature and attain M -II stage after the emergence of the first polar body as a result of the completion of nuclear maturation and cytoplasmic maturation, in contrast, Wani *et al*. (2000) did not notice significant differences among IVM and IVF rates for goat oocytes, whether those oocytes derived from adult or young goats (72.4 and 64.1% for IVM, respectively

Table 3 : Rates of IVM according to ewe age and season of the year in Awassi sheep.

Season of the year	Ewe age(year)						P
	A:<1		B:(1-2)		C: >2		
	No.	%	No.	%	No.	%	
Winter	30/103	29.12**Ab**	61/95	64.21**Ba**	40/103	38.83**Aa**	*
Spring	56/115	48.69**Aa**	63/117	53.84**Aa**	55/121	45.45**Aa**	NS
Summer	75/128	58.59**Ab**	71/113	62.83**Aa**	65/130	50.00**Aa**	NS
Autumn	67/120	55.83**Aa**	67/93	72.04**Ba**	62/125	49.60**Aa**	***
P	*		NS		NS		

*:p=0.00, ***:p<0.04, NS:insignificant, each subscript letter denotes a subset of case categories whose column(small letters) and line (capital letters) proportions do not differ significantly from each other at the .05 level**.**

Table 4 : Rates of IVF according to ewe age and season of the year in Awassi sheep.

Season of the year	Ewe age(year)						P
	A:<1		B:(1-2)		C: >2		
	No.	%	No.	%	No.	%	
Winter	4/30	13.33	10/61	16.39**a**	7/40	17.50	NS
Spring	16/56	28.57	18/63	28.57**a**	12/55	21.81	NS
Summer	23/75	30.66	33/71	46.47**b**	24/65	36.92	NS
Autumn	18/67	26.86	26/67	38.80**b**	23/62	37.09	NS
P	NS		****		NS		

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

Table 5 : Rates of cleavage stage according to ewe age and season of the year in Awassi sheep.

Season of the year	Ewe age(year)						P
	A:<1		B:(1-2)		C: >2		
	No.	%	No.	%	No.	%	
Winter	0/4	0.00	0/10	0.00	0/7	0.00	NS
Spring	5/16	31.25	6/18	33.33	4/12	33.33	NS
Summer	8/23	34.78	12/33	36.36	9/24	37.50	NS
Autumn	5/18	27.77	9/26	34.61	8/23	34.78	NS
P	NS		NS		NS		

NS: insignificant**.**

and 44.8 and 36.4% for IVF, respectively). One important reason that can not be ruled out is 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 or some lambs that do not exceed the age of five months. However, it was observed that there were significant differences in the stage of 2-16 cell (p<0.04) and blastocyst stage (p<0.03) where the rates of embryos that stopped at the 2-16 cell stage were very low in C group (4.76%), also, the rates of embryos that developed to blastocyst stage were very high in the same group (52.38%) compared with the oocytes in the two other groups A and B.

The interaction relationships of IVM stage in our study showed that less than one year old ewes (group A) were more distinguished (p=0.00) than those of other ages during autumn and summer (breeding season) (table 3), at the same time, the oocytes that were derived from ewes in B group during summer and autumn reached the highest rates of IVF (46.47 and 38.80% respectively; p<0.03; table 4), generally, Awassi sheep reach puberty stage at the age of five months and physical maturity at the age of 8 months, in addition to the previous explanations, it is probable that superiority in IVM and

IVF rates appeared across 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 ≥m, 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.

Despite the full absence of significancy of interactions in all age groups across the different seasons of the year in the rates of cleavage stage, but it was observed that these rates converged strongly so that the difference did not exceed 9.73% (except winter rates; Table 5). This indicates that most of the divided oocytes were at a high level of developmental competence which in turn enabled them to follow up the different stages of embryonic cell division. In cattle, Majerus *et al.* (1999) found that oocyte developmental competence, which seems to be dependent on age of the donor, is lower in prepubertal heifers than in cows, however, prepubertal Holstein heifers between 7-11 months of age have produced oocytes with similar competence to those of cows.

It concluded that slaughterhouses can be used as a source of low cost in IVEP program of sheep. Also, the study showed the possibility of using ewes in all stages of age in IVEP programs and the possibility of intensified action programs during summer and autumn for raising IVEP efficiency of Awassi sheep.

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