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Effect of ovarian morphology on *in vitro* oocyte recovery and quality, and certain follicular fluid steroids hormones concentrations in Ewes

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SUMMARY

The present study was conducted to investigate the effects of ovarian morphology on oocyte quantity and quality, as well as on follicular fluid steroid hormones concentrations. Fifty pairs of ovaries were collected from Barbari ewes and grouped into right, left, CL bearing and non-CL bearing ovaries. The weight, length, width and thickness of the right, left, CL bearing and non-CL bearing ovaries were recorded. The follicles were classified according to their diameter into 3 groups; small (<2mm), medium (2-4mm) and large (>4mm) follicles. Oocytes were classified according to their morphology into 3 grades; COCS (Compact cumulus oocyte complexes), POCS (Partially invested with less than three layers of cumulus cells) and DO (denuded oocyte). The concentrations of progesterone and estradiol 17β in the follicular fluid were estimated. Results indicated that, dimensions of both right and left ovaries were not significantly differed. However, the ovarian dimensions as well as their weights were significantly ($P < 0.05$) affected by the presence of CL, being higher in the CL bearing ovary. The average number of large follicles were significantly ($P < 0.05$) increased in the right ovary when compared to the left one. The recovered COCs number was found to be significantly higher ($P < 0.05$) in the right than left ovaries. A greater number of vesicular follicles and aspirated COCS were found in the non-CL bearing ovary than in the CL bearing ovary. The non CL bearing ovaries provide larger numbers as well as higher quality of COCs when compared to CL bearing ovaries and that the former can be used to collect good quality COCs for in vitro production of sheep embryos. The progesterone concentration of follicular fluid was significantly higher in CL- and non-CL bearing ovaries (27.75 and 12.33 ng/ml; $P < 0.05$,

respectively). Non-CL bearing ovaries had significantly ($P < 0.05$) higher concentration of estradiol 17β than those found in CL bearing ovaries (22.10 vs.8.43 pg/ml, respectively). It can be concluded that non-CL bearing ovaries provide a higher number as well as superior quality of COCs than those obtained from ovaries bearing CL suggesting that the ovaries without CL can be used to collect good quality of COCs in view of in vitro production of sheep embryos (IVP).

Keywords: *Morphology; Ovary; CL; Follicles; Oocytes*

INTRODUCTION

Quantitative aspects of follicle growth have been studied in sheep (Draincourt, 1991; Draincourt et al., 1993; Mohammadpour, 2007) and bovine (Singh and Adams, 2000). In all mammals, follicles begin to grow from a pool of primordial follicles constituted early in life, and grow continuously throughout the life of the female. The comparing weight, length, width and thickness of right and left ovaries in sheep and goat are the same and there are no significant differences (Mohammadpour, 2007). Over the past years, the in vitro embryo-production procedures developed for sheep have been improved significantly, but many factors influencing their efficiency still need to be investigated. In fact, the proportion of viable embryos is still highly variable and this could be due to culture conditions but also to the heterogeneous quality of the oocytes (Bracket and Zuelke, 1993). The optimal rates of embryo production in vitro can be attained by selecting ovaries and follicles, which provide oocytes correlated with their ability to undergo maturation, successful fertilization and subsequent in vitro development (Madison, 1988). Extensive research on in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) of the resulting zygotes has been reported (Cognie et al., 2003) but limited information has been reported on the evaluation of sheep ovaries or methods for the efficient collection and grading of oocyte. The average number of high quality oocytes recovered from goat ovaries without corpora lutea was greater than recovered from the ovaries with corpora lutea, which can be effectively used for IVF (Kumar et al., 2004). The average number of normal follicles was significantly higher in normal breeder than acyclic, cyclic and post partum anestrous Black Bengal does (Salim, 2004). Oocytes recovered from large antral follicles have the ability to develop to the blastocysts stage compared to small and medium size follicles (Crozet et al., 1995). Fulka and Okolski (1981) reported that compact COCs matured in vitro and subsequent

studies further confirmed the observation (Zhang et al., 1989). In addition, it has been shown that a comparable higher maturation rate could be reached within 24 h of culture if the oocyte had a compact cumulus investment. Denuded oocyte with few cumulus cells are usually rejected because of their low capacity of fertilization and or in vitro development (Leibfried-Rutledge et al., 1986). Follicular microenvironment may affect the quality and fertilizability of an oocyte in vivo and in vitro (Testart, 1985). Concentrations of certain follicular fluid compounds upon aspiration could therefore be used as important clinical measures to predicate the developmental ability of an oocyte subjected to in vitro maturation and fertilization (Greve et al., 1989). From that standpoint, the present research has been undertaken to evaluate the morphology of the slaughterhouse ovaries, follicles and determination of some steroids (progesterone and estradiol) with the view of in vitro recovery and quality of sheep oocyte.

MATERIALS AND METHODS

1. Collection of ovaries

One hundred ovaries from Barbari ewes, aged 2-3 years old, weighing 40-45 kg average body weight with unknown reproductive history were obtained from local abattoir during January through March 2010. The ovaries were then kept in a thermos flask containing 0.9% physiological saline supplemented with 100 IU/ml penicillin-G sodium and 100mg/ml streptomycin sulfate at 37 °C and were transported to the laboratory within 2 to 3 hrs .

2. Processing of ovaries

The ovaries were recorded as right and left and with presence or absence of corpora lutea (CL). The ovaries were then washed three times in phosphate buffer saline (Gordon, 1994) supplemented with the above-mentioned antibiotics. After trimming, each ovary was weighed (gm) and its length, width and thickness (cm) was measured using Vernier calliper (Fig.1).The length, width and thickness of each ovary was determined as previously reported (Al-Baggal et al., 1993; Bukar et al., 2006). All antral follicles of each ovary were counted and classified according to their diameters into small (<2mm), medium (2-4mm) and large follicles (≥4mm) as reported by Madison (1988).

3. Oocyte recovery and classification

Follicular oocytes (2-5mm in diameter) were aspirated from the surface of the ovary with an 18-gauge needle fixed on a disposable syringe filled with 1ml of the aspiration solution used Dulbecco □s modified Eagle □s medium(DME, Flow laboratories, UK, Scotland). The media used for aspiration were

enriched with 3% heat inactivated fetal calf serum (FCS, Sigma, USA) and contained the same previously mentioned antibiotics. Oocytes were collected using a stereomicroscope, and were washed twice in the previous media. The average number of oocyte per ovary was recorded and evaluated according to their morphological shape (Eckert and Niemann, 1995) into:

a. COCs (Compact cumulus oocyte complexes) with homogenous evenly granulated cytoplasm possessing at least three layers of cumulus cells (Fig.2/A).

b. POCs (Partially invested with less than three layers of cumulus cells) (Fig.2/B).

c. DO (Denuded oocyte) without cumulus cells (Fig.2/C).

Fig. 1: Morphology of the ovary, A. Non-CLbearing ovaries (* indicates follicles, (*) small follicle, (**) medium follicle, and (***) large follicle). B. CL- bearing ovaries (* indicate corpus luteum).

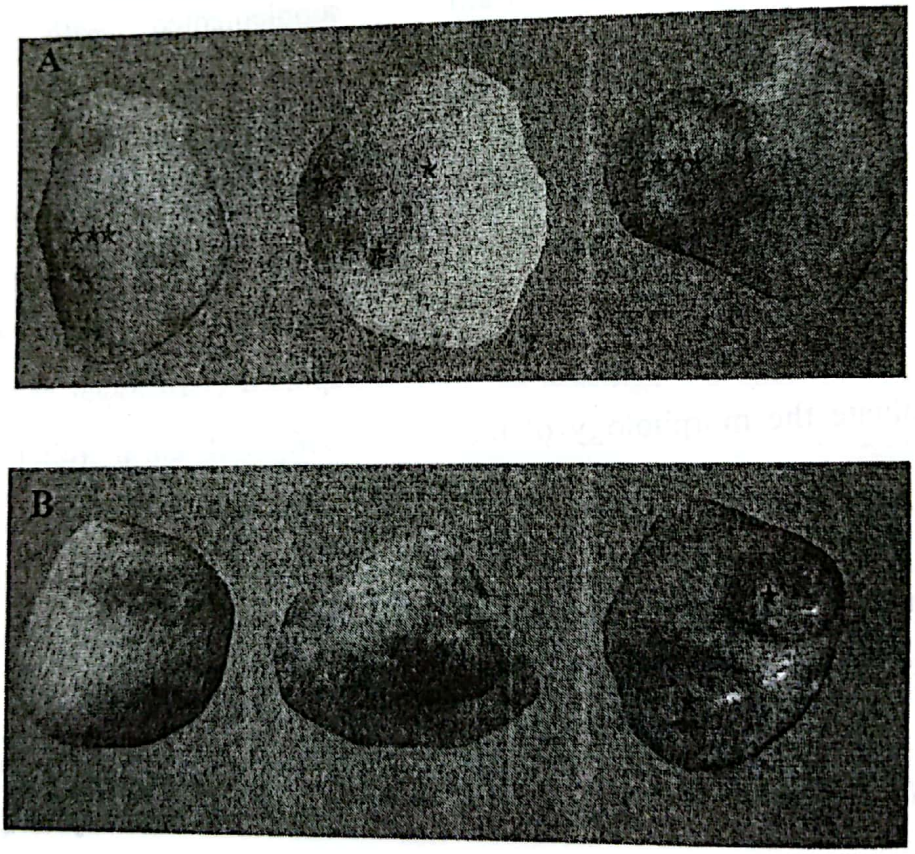
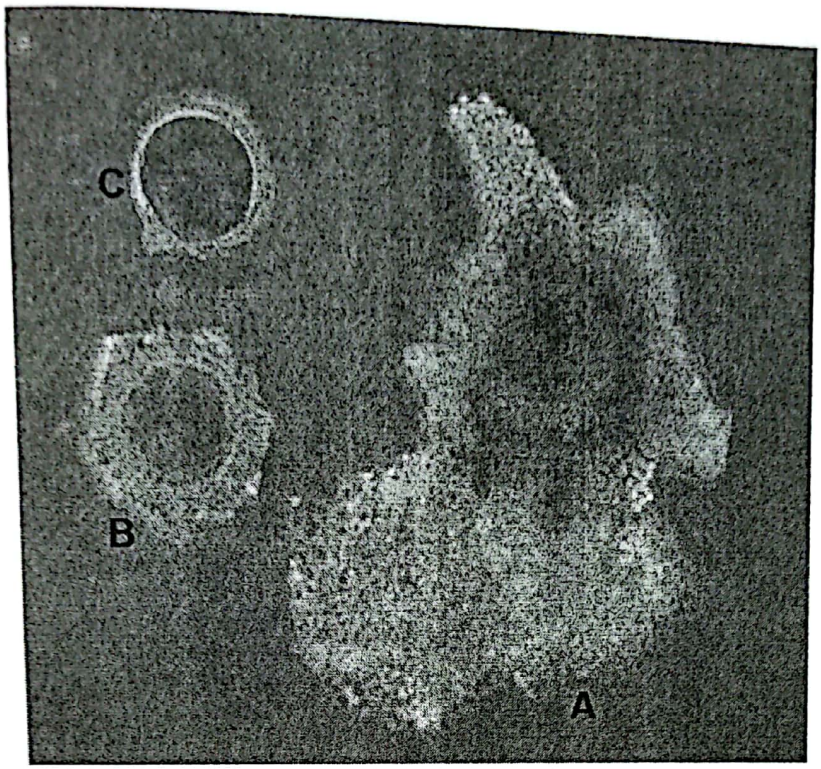


Fig. 2: Different categories of freshly collected immature sheep oocytes (X - 100), A. COCs (Compact cumulus oocyte complexes), B. POCs (Partially invested with less than three layers of cumulus cells), C. DO (denuded oocyte).



4. Hormonal assay

Follicular fluids of small, medium and large follicles of each ovary were aspirated using 1ml insulin syringe. The follicular fluids were pooled and stored frozen at -20°C until assay for progesterone and estradiol 17β hormones according to Dobeli (1980) by using a commercial radioimmunoassay kit (Baxter Merz plus Dade AG, CH 3186 Duedingen). Prior to analysis, the samples of follicular fluid were thawed and centrifuged at 1200 g. for 30 minutes at 4°C to remove the debris and follicular cells. For Progesterone the intra- and inter- assay coefficient of variations were 7.5% and 8%, respectively. For estradiol 17β the intra- and inter- assay coefficient of variations were 10.2% and 11.5%, respectively.

5. Statistical analysis

All values were expressed as means ± SEM. The data were analyzed using general linear model of SAS (1992), while the difference between means was detected by ANOVA and Student "t" Test.

RESULTS

1. Morphometry of ovaries in relation to corpus luteum and follicles

Data presented in Table 1 showed that the mean weight, length, width and thickness of the right ovaries (1.34gm, 1.02, 0.73 and 0.54 cm, respectively) were not significantly differed compared to that of left ovaries (1.27gm, 0.97, 0.71and 0.52 cm, respectively). Out of the 50 examined slaughtered ewes (100 ovaries), 30 ewes (60%) were exhibited CL in their ovaries, 16 ewes had an active right bearing CL and inactive left ovary (53.3%), 14 ewes had an inactive and active left ovary (46.6%), and 20 ewes (40.0%) had inactive ovary (no CL). The ovarian weight was significantly ($P < 0.05$) affected by the presence of CL, where the CL bearing ovaries were heavier than that of non-CL bearing ovaries (1.4 vs.0.77gm). Similar trends were observed in width and thickness (Table 2) being higher in CL bearing ovary (0.82 and 0.84cm, respectively) than that of non-CL bearing ovary (0.66 and 0.60 cm, respectively). However, the length of non-CL bearing ovary was significantly ($P < 0.05$) longer than that in CL bearing ovaries (1.14 vs.0.75 cm, respectively).

Table 1: Comparative dimensions of right and left ovaries (Mean± SEM).

Parameters	Right ovary (n=50)	left ovary (n=50)
Weight(gm)	1.34±0.17[a]	1.27±0.13[a]
Length(cm)	1.02±0.12[a]	0.97±0.13[a]
Width(cm)	0.73±0.07[a]	0.71±0.06[a]
Thickness (cm)	0.54±0.05[a]	0.52±0.06[a]

Within the same row values with same superscripts are not significantly different.

Table 2: Comparative dimensions of CL or no- CL bearing right and left ovary (Mean± SEM).

Parameters	CL- bearing ovary (n=60)	Non CL- bearing ovary (n=40)
Weight(gm)	1.40±0.19[a]	0.77±0.06[b]
Length(cm)	0.75±0.05[a]	1.14±0.09[b]
Width(cm)	0.82±0.07[a]	0.66±0.06[b]
Thickness (cm)	0.84±0.05[a]	0.60±0.06[b]

Within the same row values with different superscripts (a, b) are significantly different at least ($p<0.05$).

The number of follicles visible on the surface in relation to the CL bearing right and left ovaries is listed in Table 3. There were 400 follicles recorded on the surface of the ovaries.

The average numbers of follicles per ovary were 4.0±0.87 and 4.0±0.92 found on the right and left ovaries, respectively (200 follicles in each ovary). The mean numbers of small and medium sized follicles were equivalent without a significant difference between right and left ovary. However, the average number of large follicles were significantly ($P < 0.05$) increased in the right ovary (1.0±0.14) than that in the left ovary (0.6±0.10). Non-CL bearing ovaries had a significantly higher number of total follicles than those of CL- bearing ovaries (5.0 vs. 3.0; $P < 0.05$). A significantly ($P < 0.05$) higher number of medium and large sized follicles in the non-CL bearing ovaries (1.75±0.22; 1.50±0.36, respectively) as compared to those found on CL- bearing ovaries (0.83±0.18; 0.83±0.27 respectively) as shown in Table 4.

Table 3: Follicular development in right and left ovaries (Mean± SEM) of ewes.

Parameters	Number	Right ovary	Left ovary	Number
Small follicle	100	2.0±0.80[a]	2.2±0.88[a]	110
Medium follicle	50	1.0±0.24[a]	1.2±0.22[a]	60
Large follicle	50	1.0±0.14[a]	0.6±0.10[b]	30
Total number of follicles	200	4.0±0.87[a]	4.0±0.92[a]	200

Within the same row values with different superscripts (a, b) are significantly different at least ($p<0.05$).

Table 4: Follicular development in CL and non-CL bearing ovary (Mean±SEM).

Parameters	Number	CL- bearing ovary	Non-CL bearing ovary	Number
Small follicle	100	1.67 ±0.97[a]	1.75±0.68[a]	70
Medium follicle	50	0.83±0.18[a]	1.75±0.22[b]	70
Large follicle	50	0.83±0.27[a]	1.50±0.36[b]	60
Total number of follicles	200	3.34±0.67[a]	5.00±0.97[b]	200

Within the same row values with different superscripts (a, b) are significantly different at least ($p<0.05$).

The mean numbers of aspirated follicles were similar in right ovary (4.0±0.87) and left ovary (4.8±0.92). The collected COCs per follicles number was found to be significantly higher ($P < 0.05$) in right ovaries (2.0±0.13) than that in left one (0.83 ±0.09). However, the mean numbers of POCs and DO were similar between right (1.0±0.27; 0.50±0.01, respectively) and left ovaries (1.0±0.26; 0.25±0.00 oocyte, respectively) in Table 5. A significantly higher numbers of aspirated follicles were found in non-CL bearing ovaries (4.0±0.44 follicles per ovary) than those observed in CL-bearing ovary (2.5±0.51 follicles per ovary). The overall mean number of oocytes per follicles were significantly higher in non-CL bearing ovary than those in CL- bearing one (2.18 vs. 1.67 oocytes, $P < 0.05$; respectively). Furthermore, the average number of COCs per follicles recovered from non-CL bearing ovary was significantly higher ($P < 0.05$) than those obtained from CL-bearing one (1.25±0.09 vs.0.67±0.13, respectively). A similar trend was observed in the overall mean number of oocytes per follicles. Oocytes with few dispersed layers of cumulus (POCs) were recovered from CL bearing ovaries with mean number of 0.67±0.27 oocytes while, a mean number of 0.62±0.26 oocytes obtained from non-CL bearing ovaries without significant difference as shown in Table 6.

Table 5: Effect of site of sheep ovary on the mean number of recovered oocyte and their quality per follicles (Mean± SEM).

Parameters	Number	Right ovary	Left ovary	Number
No. of aspirated follicles per ovary	100	4.0±0.87[a]	4.8±0.92[a]	120
COCs	200	2.0±0.13[a]	0.83±0.09[b]	100
POCs	100	1.0±0.27[a]	1.0±0.26[a]	120
DO	50	0.50±0.01[a]	0.25±0.00[a]	30
Total number of oocytes	350	3.50±0.21[a]	2.08±0.11[b]	250

Within the same row values with different superscripts (a, b) are significantly different at least ($p<0.05$).

Table 6: Effect of presence or absence of CL on the mean number of recovered oocyte and their quality per follicles (Mean± SEM).

Parameters	Number	CL- bearing ovary	Number	Non CL- bearing ovary
Number of aspirated follicles per ovary	150	2.50±0.51[a]	160	4.0±0.44[b]
COCS	100	0.67±0.13[a]	200	1.25 ±0.09[b]
POCS	100	0.67±0.27[a]	100	0.62±0.26[a]
DO	50	0.33±0.01[a]	50	0.31±0.00[a]
Total number of oocytes	250	1.67±0.15[a]	350	2.18±0.27[b]

Within the same row values with different superscripts (a, b) are significantly different at least ($p<0.05$).

3.2. Hormonal assay

Table 7 represented the mean concentrations of progesterone and estradiol 17β of follicular fluid of the CL bearing and the non-CL bearing ovaries. Progesterone concentration of follicular fluid was significantly higher ($P < 0.05$) in CL than those in non-CL bearing ovaries (27.75 vs.12.33 ng/ml respectively). However, Non-CL bearing ovaries had significantly ($P < 0.05$) higher concentration of estradiol 17β than those found in CL bearing ovaries (22.10 vs. 8.43 pg/ml, respectively).

Table 7: Effect of presence or absence of CL on follicular fluid concentrations of Progesterone (ng/ml) and Estradiol17β (pg/ml) of ewes (Mean± SEM).

Type of Ovary	Progesterone	Estradiol
CL bearing ovary	27.75±2.3[a]	8.43±1.4[a]
Non CL bearing ovary	12.33±1.5[b]	22.10±3.3[b]

Within the same column values with different superscripts (a, b) are significantly different at least ($p<0.05$).

DISCUSSION

The mean weight, length and width of ovaries in the present study were nearly similar in both ovaries. Similar findings were recorded by Mohammadpour (2007) in sheep and goat. In agreement with the previous reports of the Casida et al., (1966); Emady, (1976); Scaramuzzi and Downing (1997); Alosta et al., (1998), the presence of CL reported in the study present was more pronounced in the right ovary (53.3%) than that in the left ovary (40%) indicating that the right ovaries are more active than the left ones. The size of ovaries bearing CL in terms of length, width and thickness were significantly ($P<0.05$) higher than those of ovaries without CL. Such variation in ovarian size was expected as the hypertrophy of luteinized granulosa cells, hyperplasty of fibroblasts of the connective tissues and vascularity contribute to an increase in size of the CL (Jablonka-Shariff et al., 1993). The maximum diameter of CL is reached at days 6-9 after ovulation and then regression starts between days 13 and 16 in ewes if maternal recognition does not occur (Jablonka-Shariff et al., 1993). Moreover, Higgins (1986) and EL-wishy (1992) stated that the size and shape of camel ovaries varied with age and their contents of follicles and corpora lutea. The mean number of follicles reported herein was the same in right and left ovaries (4.0±0.87 and 4.0±0.92, respectively). However, the average number of large follicles were significantly ($P<0.05$) increased in right ovary than that in left ovary. This is attributed to that the right ovary is more active than left one (Scaramuzzi and Downing, 1997; Alosta et al., 1998). Moreover, a significantly higher numbers of COCs were recovered from right ovary than that of left one (2.0±0.13; 0.83 ±0.09 respectively).The average number of COCs were found to be lower than 4.00 oocytes per ovary (Wahid et al., 1992) and 2.17 oocytes per ovary (Datta et al., 1993) in sheep .The lower number of COCs per ovary obtained in the present study could be attributed to species variation. Results of the current work indicated that the ovary without corpus luteum had higher follicular activity than that bearing corpus luteum .This phenomenon was also reported in cattle (Bellin et al., 1984; Pierson and Ginther, 1987), camel (Ghoneim, 2001) and buffalo (Amer et al., 2008). The lower number of follicles found in ovaries with CL may attribute to the fact that the CL reduces the growth and increases the atresia of follicles (Hafez, 1993). According to Wani (1995) in sheep, large ovaries yielded significantly higher number of oocytes than small ovaries. The presences of CL on the ovaries, in the present study, explains the role of progesterone on sheep follicular

growth and recovery of oocytes as indicated by higher level of progesterone in follicular fluid of CL bearing ovaries (Table, 7). It was reported that progesterone secreted by luteal cells inhibited estrus and caused a negative feedback to the anterior pituitary gland thus inhibiting GnRH and FSH secretion (Roy et al., 1972; Hafez, 1993). A few number of growing follicles was recorded in CL group ovaries of the present study and it can assumed that the higher numbers of COCs in CL-absent ovaries than those in CL bearing ovaries (1.25±0.09 ;0.80±0.13 oocytes / ovary respectively) might arise from the activity of corpus luteum and the negative effect of progesterone. The presence of CL on the ovaries decreased the number of oocytes per ovary in goats and cows (Agrawal, 1992; Amer et al., 2008), which might be the result of a major portion of the ovary being occupied by the lutein cells (Das et al., 1996; Kumar et al., 1997). Furthermore, Abdoon (2001) showed that total number of oocytes recovered from camel ovaries without CL (9.1/ovary) was higher ($P<0.01$) than those with CL (6.8/ovary). However in bovine, oocyte yield per ovary was higher ($P<0.01$) for ovaries with than without- CL (Varisanga et al., 1998). In contrary, Shamiah (2004) observed similar number of buffalo oocytes on ovaries with or without -CL. In addition, the higher number of COCs recovered from ovaries without- CL than those obtained from CL- bearing ovary explains the role of hormonal balance on sheep folliculogenesis. It can be concluded that the ovaries without CL comprise a higher number as well as superior quality of COCs than those obtained from CL- bearing ovary suggesting that the ovaries without- CL can be used for collecting good quality of COCs in view of in vitro production of sheep embryos (IVP).

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تأثير الشكل المورفولوجى للمبيض معمليا على أنتاج البويضات وجودتها ومستوى بعض الهرمونات الحلقية فى السائل الحويصلى فى النعاج

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أجرى البحث على ٥٠ زوج من مبايض النعاج تم تجميعها من السلخانة و تم تصنفهم الى يمين وشمال ومبيض عليه جسم أصفر وآخر بدون. تم أخذ مقياس المبايض بأنواعها ويشمل الطول والعرض والسمك وكذلك اوزانهم . تم تسجيل وتصنيف الحويصلات المبيضية على كل مبيض على حدة على حسب قطرها الى صغيرة (≤2mm), ومتوسطة (2-4mm) وكبيرة (>4mm). تم تجميع البويضات وتم تصنيفها الى ٣ أنواع COCS وDOوPOCS . وتم قياس هرمون الاستروجين والبروجسترون فى سائل الحويصلات المبيضية. دلت النتائج على أنه لا يوجد فروق معنوية لمقياس المبيض اليمين عن الشمال . ولكن حجم المبايض التى تحمل الجسم الأصفراكبر من المبايض التى لاتحمله. كان متوسط عدد الحويصلات الكبيرة للمبيض اليمين أكبر من الشمال و عدد البويضات COCS المأخوذة من المبيض اليمين أعلى من الشمال وعدد الحويصلات الموجودة على المبايض التى لا تحمل الجسم الاصفر كانت أعلى من التى تحمل جسم اصفروكذلك عدد البويضات المأخوذة من المبايض التى لا تحمل الجسم الاصفر أعلى من المأخوذة من المبايض التى تحمل جسم لأصفر و مستوى هرمون البروجسترون للسائل الحويصلى من المبايض التى تحمل الجسم الاصفر كان أعلى معنويا من المبايض التى لاتحمل الجسم الاصفر(٢٧.٧٥ ضد ١٢.٣٣). بينما هرمون الاستروجين كان أعلى فى المبايض التى ليس بها جسم أصفر (22.10 ضد 8.43)