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IN VITRO MATURATION OF SHEEP OOCYTES AS AFFECTED BY HORMONES, SERUM AND EPIDERMAL GROWTH FACTOR

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ABSTRACT

The present study was designed to evaluate the effects of hormonal additives (FSH 10 IU/ ml, LH 10 IU/ ml and 17 β Estradiol 1 µg/ ml) and serum additives (10% Estrous ewes serum (EES) or 10% Fetal calf serum (FCS)) on oocyte cumulus cells expansion and the percentage of nuclear maturation of sheep oocytes. The effects of adding different concentrations of epidermal growth factor (EGF) (10, 20 and 40 ng/ml) to the tissue culture medium (TCM-199) with hormonal and serum additives on oocyte cumulus cells expansion and percentage of nuclear maturation of sheep oocytes was also examined. The obtained results showed that the highest percentage of oocytes with complete cumulus cells expansion was obtained with adding EES to the medium alone or with hormones additives (83.33% and 74.36%, respectively for class A oocytes). Whereas, TCM-199 supplemented with hormones and EES achieved the highest percentage (51.85 %) of class B oocytes with complete cumulus cells expansion. Significant differences between the treatment groups of class A and B oocytes were found ($P<0.001$). The highest percentage of mature oocytes (MII stage) was achieved from oocytes cultured in TCM-199 + (FSH, LH and 17 β Estradiol) + EES, (53.85 % and 42.59 % for class A and B oocytes, respectively).

EGF supplementation increased the percentage of class A oocytes with complete cumulus cells expansion for the different concentrations, 10, 20, 40 ng/ml (75.68 %, 75.68 % and 73.68 %, respectively). These values reduced considerably for class B oocytes. The differences between concentrations of EGF were not significant for oocytes class A and B. Incubation of oocytes cultured in TCM-199 + 40 ng EGF achieved the highest percentage of oocytes that reached MII (70.27% and 58.46% for class A and B oocytes, respectively).

Furthermore, the presence of hormonal additives and 10% EES in TCM-199 that contain 40 ng/ml EGF during IVM, had the highest percentage of sheep oocytes maturation. It could be deduced that EES alone or with hormonal additives in TCM-199 increased the percentage of oocytes with complete cumulus cells expansion. Similar results achieved by using EGF in *in vitro* maturation medium only for class A oocytes.

Key words: *Sheep, IVM, hormones, serum, EGF.*

INTRODUCTION

Using the *in vitro* technique allows us to obtain a large number of mammalian embryos for research, genetic improvement or commercial purposes **(Accardo et al., 2004)**. The production of a high percentage of normal young from in vitro matured oocytes cultured within intact follicles supported the contention that both control systems are important for full physiological maturation of mammalian oocytes **(Galli and Moor, 1991)**.

Oocyte *in vitro* maturation (IVM) is a reproductive technology that enables mature oocytes to be generated *ex vivo* without the need for ovarian gonadotropin treatment. IVM involves artificial removal of cumulus oocytes complexes (COCs) from the antral follicles and culturing them in essentially standard cell culture condition for 22-24 h until they reach metaphase II **(Robert and Jeremy, 2007)**.

The control of oocytes maturation in vivo includes both, an endocrine component involving the hypophysial-ovarian axis and local autocrine or paracrine regulators, which operate at follicular level **(Wani, 2002)**. Presence of cumulus cells surrounding the oocyte is a limitation factor in oocytes maturation process. Communication between the oocyte and its somatic cells has been shown to be important in oocyte maturation during *in vitro* culture (IVC) and subsequent development of the oocyte **(Shirazi *et al.*, 2007)**. The proportion of oocytes capable of *in vitro* maturation (IVM) is lower when cumulus cells are previously removed **(Zhang *et al.*, 1995)**. In response to the pre ovulatory luteinizing hormone (LH) surge, the oocyte resumes meiosis and the cumulus cells start producing hyaluronic acid (HA) which is deposited into the intercellular spaces and stabilized by accessory proteins. This process is called cumulus expansion **(Zhuo and Kimata, 2001)**.

The addition of exogenous gonadotrophic hormones to the culture medium is reported to increase the number of oocytes reaching metaphase II (MII) and the overall total yield of viable embryos **(Galli and Moor, 1991)**. Also, the addition of gonadotropins was found to be essential for the IVM of oocytes collected from lambs **(Ledda *et al.*, 1997)**.

Various sera are used as media supplements. Employed serum is usually heated at 56°C for 30 min, presumably to inactivate the unfavorable factors. Besides being of nutritive value, serum nurtures the cells surrounding the oocytes rather than the oocytes itself and prevents the oocytes of the Zona hardening when the oocytes are liberated from the follicular environment. Usually sheep serum **(Cognie *et al.*, 1991)**, fetal calf serum **(Wani *et al.*, 2000; Wani, 2002)**, or human serum **(Thompson *et al.*, 1992)** is used to supplement culture media for *in vitro* studies of sheep oocytes. **Birler *et al.* (1999)** cited that the beneficial effects of EES on maturational processes of sheep oocytes mimic that of FCS on bovine oocytes maturation. The contribution of TCM-199 with FSH, LH and 17 β E supplemented with 10 % FCS was the most efficacious medium for *in vitro* maturation and subsequent embryonic development of caprine oocytes **(Pawshe *et al.*, 1996)**.

Growth factors may be a key factor in mediating oocytes nuclear and cytoplasmic maturation. In particular, epidermal growth factor (EGF) and EGF-like growth factors have been proposed to have a positive impact on oocytes maturation *in vivo* and *in vitro*. Inclusion of EGF in maturation medium (concentration range 1 to 100 ng/ml) resulted in an increase of cumulus expansion and maturation in bovine (**Lorenzo *et al.*, 1994; Lonergan *et al.*, 1996**), and in sheep (**Grazul-Bilska *et al.*, 2003**).

This study was designed to evaluate the effects of supplementation of hormones (FSH, LH and 17 β Estradiol) and sera (EES or FCS) and different levels of EGF (10, 20 and 40 ng/ml) on the cumulus cells expansion and nuclear *in vitro* maturation of sheep oocytes.

MATERIALS AND METHODS

1. Oocytes recovery and selection:

Oocytes were recovered from 620 sheep ovaries at various stages of estrous cycle. Ovaries were obtained from local slaughterhouses in Cairo and transported in a 0.9% NaCl aqueous solution containing 100,000 IU/l penicillin and 100 mg/l streptomycin to the Laboratory of Reproduction and Biotechnology, Animal Production Research Institute (APRI), Dokki, Giza. Cumulus-oocyte complexes (COCs) were recovered from 2-6 mm diameter follicles and aspirated using a 21-g needle attached to a 10-ml syringe. Only oocytes with compact cumulus cells (COC) and homogenous cytoplasm were selected using a stereomicroscope then washed three times in a phosphate buffered saline (PBS). The recovered oocytes were divided into two classes, class (A) that oocytes with homogenous cytoplasm and greater than three layers of cumulus cells, while class (B) was oocytes with homogenous cytoplasm and less than or equal three layers of cumulus cells or partially denuded.

2. *In vitro* maturation:

COCs were placed in groups; each group of 5-10 oocytes was kept in a 50 µl droplet of maturation medium (TCM 199) covered with mineral oil (Sigma-Aldrich). Maturation proceeded in the incubator for 23-24 h at 38.5°C in an environment of 5 % CO2 in air with 95% humidity **(Galli and Moor, 1991).**

3. Experimental design:

In vitro maturation (IVM) of sheep oocytes was studied under different conditions as the following experiments:

Exp. 1: The effect of hormones and sera: COCs were matured in the maturation medium (TCM-199) alone or in the presence of the hormonal additives (10 IU FSH/ml (Folligon, Intervet, Holand), 10 IU LH/ml (Pregnyl, Nile Co., ARE) and 1000 ng 17β estradiol /ml (Sigma, Germany)). 10% Estrus ewes serum (EES) or 10 % fetal calf

serum was added to maturation medium. The effects of serum (EES or FCS) in maturation media on sheep oocytes maturation was studied alone and in the presence of hormonal additives (H). In this experiment, oocytes were cultured in TCM-199 alone (as a control), TCM199 + H, TCM199 + H + EES, TCM199 + EES, TCM199 + H + FCS, and TCM199 + FCS for both A and B oocyte classes.

Exp.2: The effects of epidermal growth factor (EGF) on ***in vitro*** **maturation of sheep oocytes**: COCs were matured in the maturation medium (TCM 199) with hormonal treatment and 10% EES. Three levels of EGF (10, 20, 40 ng/ml) were tested to assess the role of EGF in sheep oocytes maturation in vitro.

4. Maturation assessment:

Maturation is assessed either by staining oocytes with 1% aceto-orcine stain for the presence of first polar body or roughly by the degree of cumulus cell mass expansion **(Madison, 1988).**

a. Cumulus cell mass expansion:

Matured oocytes were classified according to **Schellander *et al.* (1990)** by the degree of cumulus cell mass expansion into:

i) Complete expansion: Oocytes with evenly granulated cytoplasm and fully expanded cumulus cells with minimum degenerated cells as seen with light microscope.

ii) Partial expansion: Oocytes had the same appearance except that the innermost 2 to 3 layers of the cumulus cells were not fully expanded.

iii) No expansion: Oocytes with unexpanded cumulus.

b. Fixation and staining of oocytes:

At the end of culture period, the cumulus cells of COCs oocytes were removed by vortexing. The cumulus-free and homogeneous oocytes were then fixed in acetic acid: ethanol (1: 3 v/v) in small culture dishes (35 x 10 mm) for at least 48 h at 4°C. Fixed oocytes were transferred to glass slides; silicon gel was used to maintain a coverslip in contact with the oocytes. The slides were immersed in 1% aceto-orcein stain for 30 min. Then, slides were washed 3 times in increasing concentrations of ethanol to remove the surplus orcein dye as follows: 5 sec in 70% ethanol, then 1 and 3 min in 100% ethanol **(Rao *et al.*, 2002).**

5. Statistical analysis:

Data were analyzed using SAS (1998). Chi square test was used to evaluate the significant differences among treated groups.

RESULTS AND DISCUSSION

3.1. Experiment 1: Effect of hormones and sera:

It is apparent from the results in Figure (1-a) that the percentage of class A oocytes with complete cumulus cells expansion was increased by supplementing the

basic medium (TCM-199) with EES alone or with hormones (FSH, LH and E17β) reached 83.33% and 74.36%, respectively. The percentage of oocytes exposed to the medium with hormonal additives (TCM+H) reached 68.42 % and that enriched with hormones and FCS (TCM+H+FCS), reached 64.86 %. Whereas, oocytes cultured in

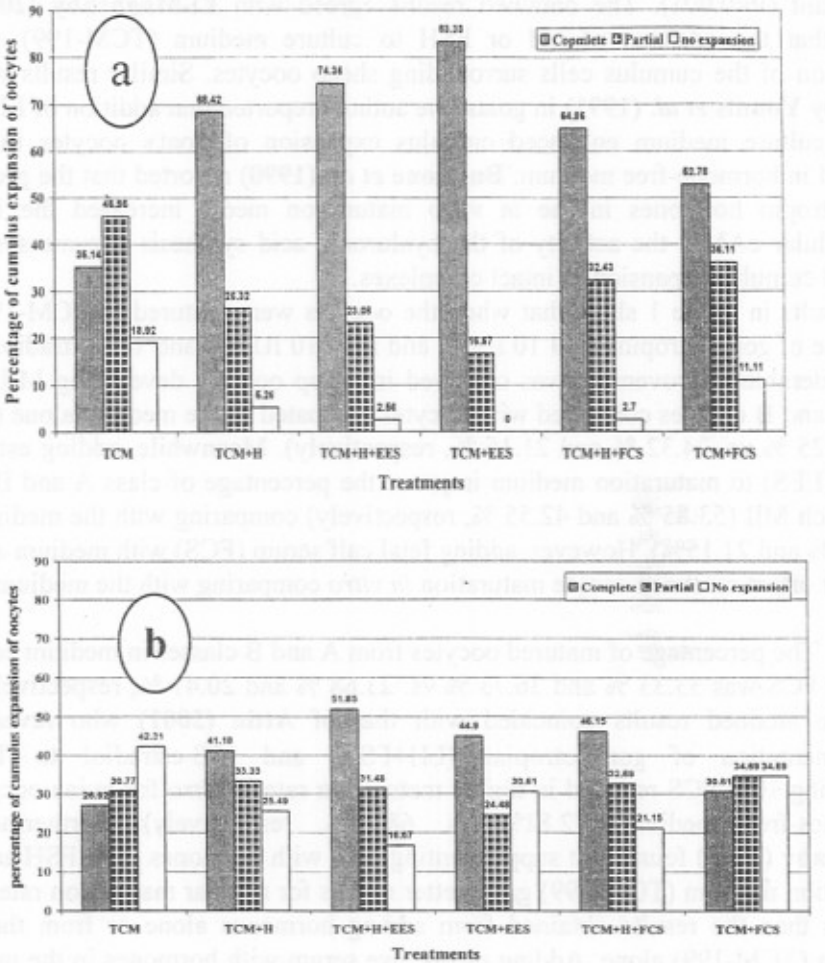

Figure (1): Cumulus expansion status of sheep oocytes cultured *in vitro* in TCM-199 supplemented with hormones and serum. (a) are values of class A oocytes, (b) are values of class B oocytes.

TCM-199 with FCS alone achieved the lowest percentage of cumulus expansion (52.78 %). Differences between treatments on the expansion of cumulus cells of sheep oocytes were highly significant ($P<0.01$). Class B oocytes that cultured in TCM plus hormones and EES achieved the highest percentage (51.85 %) of oocytes in the same class with complete cumulus cells expansion compared with the other treatments (Fig. 1-b). Differences between the effect of treatments on class A and B oocytes were highly significant ($P<0.001$). The obtained results agreed with **El-Maghraby (2004)** who found that the addition of LH or FSH to culture medium (TCM-199) enhanced expansion of the cumulus cells surrounding sheep oocytes. Similar results were also found by **Younis *et al.* (1991)** in goats, the authors reported that addition of LH or FSH to the culture medium enhanced cumulus expansion of goat's oocytes than those cultured in hormone-free medium. **Buccione *et al.* (1990)** reported that the presence of gonadotropin hormones in the *in vitro* maturation media increased the levels of intracellular cAMP, the activity of the hyaluronic acid synthesis enzyme system and induced cumulus expansion in intact complexes.

The results in Table 1 show that when the oocytes were matured in TCM-199 in the presence of gonadotropins (LH 10 IU/ml and FSH 10 IU/ml) and 17β estradiol 1μg/ml, a considerable improvement was observed in sheep oocytes developing MII stage in both A and B oocytes compared with oocytes incubated in the medium alone (42.11 % and 37.25 % vs. 24.32 % and 21.15 %, respectively). Meanwhile, adding estrus ewes serum (EES) to maturation medium improve the percentage of class A and B oocytes that reach MII (53.85 % and 42.55 %, respectively) comparing with the medium alone (24.32 % and 21.15%). However, adding fetal calf serum (FCS) with medium alone had no clear effect on sheep oocyte maturation *in vitro* comparing with the medium alone.

The percentage of matured oocytes from A and B classes in medium containing EES or FCS was 33.33 % and 36.73 % vs. 23.68 % and 20.41 %, respectively (Table 1). The attained results coincided with that of **Attia (2001)** who revealed that supplementation of gonadotropins (LH+FSH) and 17β-estradiol to TCM-199 containing 10% FCS resulted in higher maturation rate *in vitro* for ovine oocytes than hormones-free medium (72.81% vs. 68.21%, respectively). Furthermore, **El-Maghraby (2004)** found that supplementing EES with hormones (LH, FSH and E2) to maturation medium (TCM-199) gave better results for nuclear maturation rate in sheep oocytes than the results obtained from adding hormones alone or from the control medium (TCM-199) alone. Adding estrus ewe serum with hormones in the maturation medium induced higher values of maturation rate compared to the bovine serum albumin (BSA) with hormones (82.6 vs. 78.4). In this context, **Pawshe *et al.* (1996)** reported that estradiol and gonadotropins usually cause synergistic enhancement of nuclear maturation in caprine oocytes.

Table 1. Nuclear maturation stages of sheep oocytes cultured *in vitro* in TCM-199 supplemented with hormones and serum.

Treatments	Oocyte class	Nuclear stages of oocyte maturation				
		GV	GVBD	MI	MII	Deg.
		N %	N %	N %	N %	N %
TCM-199	A	8 21.62	3 8.11	3 8.11	9 24.32	2 5.41
	B	6 11.54	4 7.69	12 23.08	11 21.15	2 3.85
TCM-199 + FSH+LH +E17β	A	5 13.16	3 7.89	3 7.89	16 42.11	0 0.00
	B	4 7.84	6 11.76	7 13.73	19 37.25	2 3.92
TCM-199 +FSH+LH +E17β + EES	A	0 0.00	7 17.95	4 10.26	21 53.85	1 2.56
	B	4 7.41	2 3.70	6 11.11	23 42.59	0 0.00
TCM-199 + EES	A	5 13.89	3 8.33	2 5.56	12 33.33	3 8.33
	B	7 14.29	3 6.12	8 16.33	18 36.73	0 0.00
TCM-199 +FSH+LH +E17β + FCS	A	3 8.11	2 5.41	4 10.81	17 45.95	0 0.00
	B	6 11.54	6 11.54	7 13.46	17 32.69	2 3.85
TCM-199 + FCS	A	5 13.16	5 13.16	10 26.32	9 23.68	0 0.00
	B	5 10.20	3 6.12	8 16.33	10 20.41	1 2.04

Chi-square value =74.71 **P= 0.216**

N: Number of oocytes **GV**: Germinal vesicles

GVBD: Germinal vesicles break down **MI**: First Metaphase

MII: Second metaphase **Deg**: Degenerated

It is clear (Table 1) that the presence of hormones (LH, FSH and $E_{17\beta}$) and serum (EES or FCS) in the maturation medium, obviously improved the rate of oocytes either class A or B developed to MII. The increasing rate was limited with FCS, but

was considerable with EES, 45.95 % and 32.69 % vs. 53.85 % and 42.59 %, for FCS vs. EES groups of A and B oocytes, respectively. Therefore, it could be noticed that class A oocytes were higher in maturation rate than class B oocytes for all studied treatments (Table 1).

3.2. Experiment 2: Effects of epidermal growth factor: It is interesting to show from Fig. 2-a that adding epidermal growth factor (EGF) to the maturation medium (TCM-199) by different concentrations (10, 20, 40 ng/ml) increased the percentage of class A oocytes with complete cumulus cells expansion (75.68 %, 75.68 %and 73.68 %) after 24 h of incubation time. Whereas, adding 10 ng/ml EGF to TCM-199 for class B oocytes achieved the highest percentage of complete expansion compared with 20 and 40 ng/ml (59.38% vs. 51.65 % and 49.23 %, respectively) (Fig. 2-b). However, the differences among EGF concentrations were not significant. These findings are in agreement with **Rieger *et al.* (1998)** who reported that in IVM, exposure of oocytes to EGF+IGF-I resulted in improving expansion of cumulus cells surrounding the oocytes and increased metabolism of pyruvate as well as glutamine within the oocytes.
It is clear (Table 2) that the percentage of class A and B oocytes that reached metaphase II (MII) stage when treated with 40 ng /ml EGF, was obviously higher (70.27 % and 58.46 %, respectively) compared with those oocytes matured in medium containing 0, 10 or 20 ng/ml EGF (50.00 % and 38.33 %, 54.05% and 43.75 %or 54.05 % and 46.88 %, respectively). This positive effect may be mediated through the cumulus cells. Meanwhile, class A oocytes were higher in maturation rate than class B oocytes for all the studied treatments (Table 2). However, Chi-square test revealed that the differences among treated groups were not significant (Table 2). **Harper and Brackett (1993)** demonstrated that oocyte quality as reflected by the proportions of oocytes developing to the morula and blastocyst stages was improved only when oocytes were matured in the presence of EGF in combination with gonadotropins.

It was pointed out that treatment with EGF during IVM improves cAMP production by cumulus oocytes complex (COC) and shortens the time required for germinal vesicle break down (GVBD) **(Downs *et al.*, 1991).** Furthermore, **Park *et al.* (1999)** reported that IVM of bovine oocytes in the presence of EGF stimulated the production of urokinase – plasminogen activators (uPA) by the cumulus cells. It has been shown that uPA plays an important role in cytoplasmic maturation of oocytes. These findings confirm the importance of growth factors in ovarian physiology and their role in the processes of oocyte maturation.

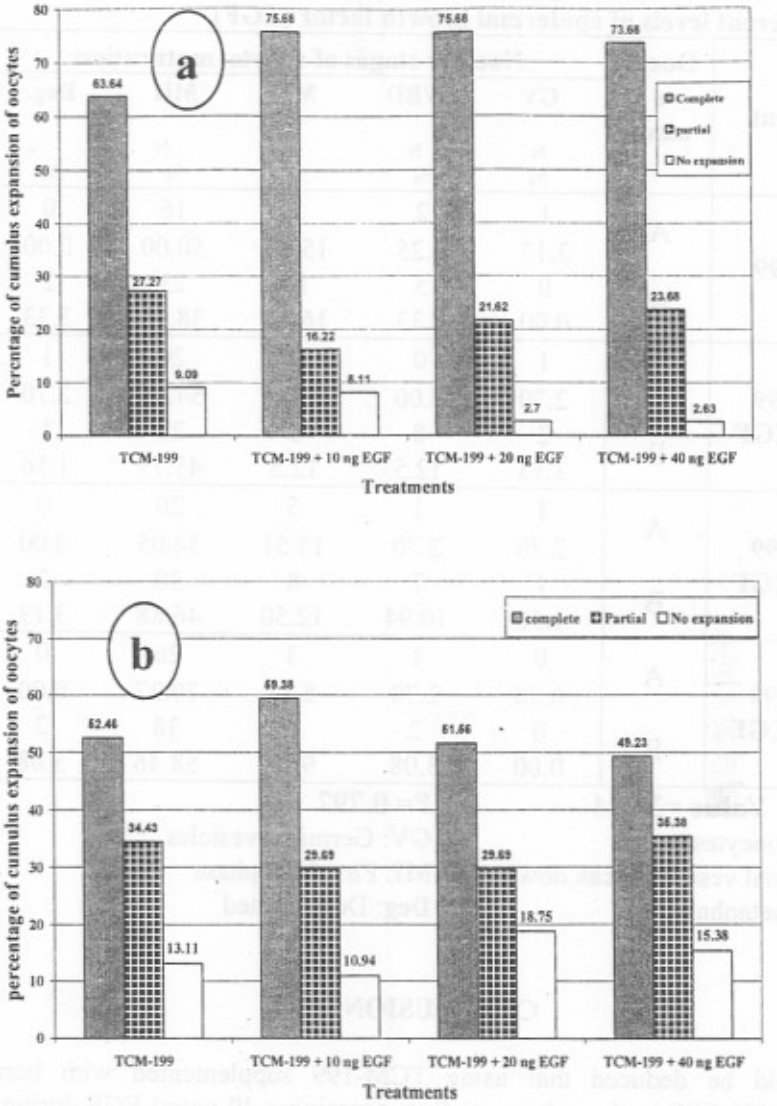

Figure (2): Cumulus expansion status of sheep oocytes cultured *in vitro* in TCM-199 supplemented with Epidermal growth factor (EGF). (a) are values for class A oocytes, (b) are values for class B oocytes .

Table 2: Nuclear maturation stages of sheep oocytes cultured *in vitro* in TCM- 199 containing different levels of epidermal growth factor (EGF).

Treatment	Oocyte asses	Nuclear stages of oocyte maturation				
		GV	GVBD	MI	MII	Deg.
		N %	N %	N %	N %	N %
TCM-199	A	1 3.13	2 6.25	5 15.63	16 50.00	0 0.00
	B	0 0.00	5 8.33	10 16.67	23 38.33	2 3.33
TCM-199 + 10 ng EGF	A	1 2.70	0 0.00	5 13.51	20 54.05	1 2.70
	B	2 3.13	8 12.5	8 12.5	28 43.75	1 1.56
TCM-199 + 20 ng EGF	A	1 2.70	1 2.70	5 13.51	20 54.05	0 0.00
	B	1 1.56	7 10.94	8 12.50	30 46.88	2 3.13
TCM-199 + 40 ng EGF	A	0 0.00	1 2.70	3 8.11	26 70.27	0 0.00
	B	0 0.00	2 3.08	6 9.23	38 58.46	2 3.08

Chi-square : Value =34.24 **P= 0.797**

N: Number of oocytes **GV**: Germinal vesicles

GVBD: Germinal vesicles break down **MI**: First Metaphase

MII: Second metaphase **Deg**: Degenerated

CONCLUSION

It could be deduced that using TCM-199 supplemented with hormonal additives and 10% EES in the culture medium containing 40 ng/ml EGF during IVM, enhanced nuclear maturation and achieved the highest percentage of sheep oocytes maturation. Furthermore, EES alone or with hormonal additives in TCM-199 increased the percentage of oocytes that developed to complete cumulus cells expansion. Similar results were attained by using EGF in *in vitro* maturation medium. After 24 hours of incubation in the maturation medium, class A oocytes had higher developmental capability to reach complete expansion of their cumulus cells than class B oocytes.

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إنضاج بويضات الأغنام معملياً وتأثرها بالهرمونات والسيرم عامل النمو الإبيدرمي

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صمم هذا البحث لتقييم تأثير تدعيم بيئة إنضاج بويضات الأغنام (TCM-199) معمليا بالإضافات الهرمونية بالتركيزات التالية [١٠ وحدات دولية/ مل من الهرمون المنبه لنمو الحويصلات المبيضية (FSH) + ١٠ وحدات دولية /مل من الهرمون المنبه للتبويض (LH) + ١ ميكروجرام/مل من هرمون الاستراديول($E_{17\beta}$)] وتأثير إضافة السيرم بنسبة ١٠% سواء سيرم النعاج الشايعة (EES) أو سيرم أجنة العجول (FCS) على درجة تمدد الخلايا الركامية المحيطة بالبويضة وعلى النسبة المئوية للإنضاج النووي لبويضات الأغنام. حيث تم تحضين البويضات الغير ناضجة في البيئة (TCM-199) فقط و البيئة + الهرمونات والبيئة + الهرمونات + EES والبيئة + EES فقط والبيئة + الهرمونات + FCS والبيئة + FCS فقط). كذلك درس تأثير إضافة تركيزات مختلفة من عامل النمو الإبيدرمي (EGF) (١٠، ٢٠، ٤٠ نانوجرام لكل ملي) مع الإضافات الهرمونية والسيرم في بيئة إنضاج البويضات (TCM-199) على درجة تمدد الخلايا الركامية المحيطة بالبويضة وعلى درجة النضج النووي للبويضات النعاج سواء كانت من الدرجة A أو B.

أوضحت النتائج أن أعلى نسبة تمدد كامل للخلايا الركامية المحيطة بالبويضات كانت باضافة (EES) فقط إلى (TCM-199) أو مع الإضافات الهرمونية (٨٣,٣٣% و ٧٤,٣٦% على التوالي) من البويضات من الدرجة A. في حين حققت البويضات من الدرجة B أعلى نسبة تمدد كامل للخلايا الركامية بأضافة الهرمونات +EES (٥١,٨٥ %) حيث كانت الفروق بين المجاميع المختبرة عالية المعنوية (احتمال أقل من ٠,٠٠١). تحققت أعلى نسبة من نضج البويضات التي وصلت لمرحلة الطور الأستوائي الثاني بأضافة الهرمونات +EES إلى بيئة الإنضاج (TCM-199) حيث بلغت نسبتها ٥٣,٨٥% و٤٢,٥٩ % لكل من البويضات من الدرجة A و B على التوالي.

أدى إضافة عامل النمو الأبيدرمي (EGF) بالتركيزات المختلفة ١٠، ٢٠، ٤٠ نانوجرام/مل إلى زيادة التمدد الكامل للخلايا الركامية حول البويضات من الدرجة A (٧٥,٦٨ %، ٧٥,٦٨%، ٧٣,٦٨ % على التوالي). بينما كان تمدد الخلايا الركامية للبويضات من الدرجة B أقل بأضافة EGF بتركيزاته المختلفة.

حقق تحضين البويضات في البيئة (TCM-199) مع ٤٠ نانوجرام/مل EGF أعلى نسبة مئوية من البويضات التي وصلت للطور الأستوائي الثاني حيث بلغت نسبتها ٧٠,٢٧ % و ٥٨,٤٦ % للبويضات من الدرجة A و B على التوالي. مع ذلك لم يكن هناك فروق معنوية بين التركيزات المختلفة من EGF.

يمكن الأستنتاج بأن اضافة سيرم النعاج الشايعة منفرداً أو مع الأضافات الهرمونية في بيئة TCM-199يرفع النسبة المئوية لتمدد الخلايا الركامية المحيطة بالبويضات. تحققت نتائج مماثلة بإضافة عامل النمو الأبيدرمي EGF في بيئة الإنضاج المعملية وخاصة للبويضات من الدرجة A.