Kafkas Univ Vet Fak Derg
17 (6): 1009-1014, 2011
DOI:10.9775/kvfd.2011.4963

RESEARCH ARTICLE

Effects of Serum, Gonadotropins, Epidermal Growth Factor and Estradiol 17-Beta on Cumulus Expansion and Nuclear Maturation of Bovine Oocytes [1]

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[1] *This study was supported by the University of Ondokuz Mayıs (grant no: PYO.VET.1901.09.002)*
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Makale Kodu (Article Code): KVFD-2011-4963

Summary

The aim of the study was to evaluate the effects of follicle stimulating hormone (FSH), luteinizing hormone (LH), epidermal growth factor (EGF) and estradiol 17-β (E2) in maturation medium on nuclear maturation and cumulus expansion of cumulus-oocyte complexes (COCs) obtained from bovine ovaries. COCs were classified as good and poor quality grades based on cumulus investment. COCs were then subjected to in vitro maturation in TCM-199 in a humidified atmosphere of 5% CO2 in air at 38.5°C for a period of 24 h. The combination of the hormones added to the medium was as follows: T1=10% (v/v) heat inactivated Fetal Bovine Serum (FBS); T2=5 µg/ml bLH, 0.5 µg/ml bFSH and 10 ng/ml EGF; T3=FBS, bLH, bFSH and EGF; T4=1 µg/ml E2; T5=FBS and E2. Supplementation of maturation medium with the FSH, LH, EGF irrespective to supplementation with FBS stimulated expansion of cumulus around the oocytes compared to oocytes in other treatment groups (P<0.05). However there were no significant differences between treatment groups in terms of proportion of oocytes reached to metaphase II (M II) stage determined by nuclear staining. In general cumulus expansion degree increased with the quality of COCs in all treatment groups (P<0.05). In conclusion, hormone and growth factor supplementation (FSH, LH and EGF) stimulated cumulus cell expansion and maturation rate.

Keywords: *Bovine oocyte, IVM, EGF, Estradiol 17-β, Gonadotropins*

Sığır Oositlerinin Kumulus Ekspansiyonu ve Nükleer Maturasyon Üzerine Serum, Gonadotropinler, Epidermal Büyüme Faktörü ve Östradiol 17-Beta'nın Etkileri

Özet

Çalışmanın amacı, sığır ovaryumlarından elde edilen kumulus oosit komplekslerinin (KOK) nükleer olgunlaşma ve kumulus ekspansiyonu üzerine olgunlaşma medyumuna katılan folikül stimüle edici hormon (FSH), luteinleştirici hormon (LH), epidermal büyüme faktörü (EGF) ve östradiol 17-β (E2)'nin etkisini değerlendirmektir. KOK'lar oositlerin kumulus hücreleri ile kuşatılma derecesine göre iyi ve zayıf kaliteli olarak sınıflandırılmıştır. Sonrasında KOK'lar 24 saat süreyle TCM-199 medyumunda 38,5°C ve %5 CO2'li ortamda olgunlaşmaya bırakılmıştır. Medyuma eklenen hormonların kompozisyonu şu şekildedir; T1=10% (v/v) inaktif Fötal Sığır Serumu (FBS); T2=5 µg/ml bLH, 0.5 µg/ml bFSH ve 10 ng/ml EGF; T3=FBS, bLH, bFSH ve EGF; T4=1 µg/ml E2; T5=FBS ve E2. Olgunlaşma medyumuna FBS hariç FSH, LH ve EGF eklenmesinin diğer muamele grupları ile karşılaştırıldığında kumulus ekspansiyonunu artırdığı görülmüştür (P<0.05). Fakat nükleer boyama ile metafaz II (MII) safhasına ulaştığı belirlen oositlerin oranı muamele grupları arasında önemli bir fark göstermemiştir. Genel olarak tüm muamele gruplarında kumulus ekspansiyon derecesi KOK'ların kalitesiyle birlikte artış göstermiştir (P<0.05). Sonuç olarak hormon ve büyüme faktörü ilavesi kumulus ekspansiyonu ve olgunlaşma oranını stimüle etmiştir.

Anahtar sözcükler: *Sığır oositi, IVM, EGF, Östradiol 17-Beta, Gonadotropinler*

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INTRODUCTION

Various approaches have been employed to improve the developmental competence of bovine oocytes *in vitro* [1]. Maturation media have been supplemented with various hormones, growth factors, sera, and follicular fluid in different reports for this aim [2-7]. Improvement of developmental competence of mammalian oocytes has been subjected in many investigations by supplementation of IVM media with gonadotropins, steroid hormones, serum and growth factors. It is well established that gonadotrophins play a major role in triggering the resumption of meiosis in the bovine oocyte and in the expansion of the cumulus oophorus [8]. FSH promotes the mucification of the matrix by stimulation of hyaluronic acid production. FSH has roles in cAMP regulation, protein kinase B (PKB) and mitogen-activated protein kinase (MAPK) signaling within the cumulus cells, and these are believed to assist oocyte development by involving in the processes surrounding both meiotic and cytoplasmic maturation of the oocyte [4,9,10].

Supplementation of the IVM media with a serum source like Fetal Bovine Serum (FBS) or Estrus Cow Serum (ECS) has also been found to be necessary for achieving high maturation rates for bovine oocytes. It is known that, serum contain many components including hormones, trace elements, and growth factors. Foetal bovine serum is routinely used in culture media for oocyte maturation and known to enhance the fertilizability of bovine oocytes. Although, supplementation of serum, gonadotrophins and steroids in the culture media enhances the expansion of cumulus cells [3,10-12]. Expansion of cumulus cells has been reported to depend largely on the culture media used for maturation of the oocytes [13].

In several studies it has been shown that EGF improves oocyte maturation and increases the developmental potential of embryos after fertilization. EGF might act on the cumulus cells surrounding the oocyte and/or on the oocyte itself since mRNA for the EGF receptor is present in the bovine oocyte [4,9]. It was reported by Mingoti et al.[14] the presence of steroids in the follicular fluid before and during maturation suggests that they may play a role in oocyte maturation. In fact, it has been shown that estradiol as well as other steroids is involved in keeping the oocytes in meiotic arrest. Maturation of bovine oocytes in the presence of high concentrations of estradiol had a negative effect on spindle formation and polar body extrusion [13-15]. There are various bovine oocyte maturation systems employed in different laboratories with successful maturation rates and subsequent embryonic development [8]. The main difference between these culture systems is the supplements used in culture media. Some systems require hormonal supplementation while some studies report no beneficial or adverse effects of hormonal supplementation [16,17] in addition to develop serum-free culture systems [18].

This study was designed to evaluate the effects of hormones (FSH, LH and E2) and EGF alone or in combination, in serum-free and serum supplemented TCM-199 medium on cumulus expansion and nuclear maturation *in vitro* of bovine cumulus-oocyte complexes (COCs) with a view to develop a valid maturation systems responsive to the known factors.

MATERIAL and METHODS

All chemicals used in this study were purchased from Sigma-Aldrich, Turkey.

Recovery of Bovine Oocytes

Bovine ovaries at various stages of their oestrous cycle were obtained from a local slaughterhouse and transported to the laboratory in a thermos filled with physiological saline solution (0.9% w/v NaCl) containing 0.1 µl/mL gentamycin sulphate at 34.0±2.0°C within 3 h after slaugther. Ovarian follicles measuring 2-8 mm in diameter were aspirated with an 18-gauge needle attached to a 10 mL dispossible syringe. Aspirated follicular fluid was pooled in conical tubes. The contents the bottom of tubes were searched for oocytes, which were placed in TL Hepes. The follicular materials were then searched under stereomicroscope and COCs were classified into 4 grades based on cumulus investment according to previously described criteria [2]. Briefly as grade A- homogeneous cytoplasm with an intact cumulus cells around oocytes; grade B- homogenous cytoplasm but unevenly surrounding cumulus investment around oocytes; grade C- oocytes without layers of cumulus cells (denuded oocytes); grade D- expanded cumulus investment. Grade A and B oocytes were classified as good quality, and Grade C and D oocytes were classified as poor quality oocytes.

In Vitro Maturation

Selected good and poor quality COCs were washed three times in TL Hepes medium and placed into 500 µl of maturation medium in four-well dishes (Nunc, Roskilde, Denmark), as 25-35 oocytes per well. Each well of maturation medium were covered with 300 µl mineral oil. All incubations were performed at 39°C in a humidified atmosphere of 5% CO_2 in air for a period of 24 h.

Experimental Design

TCM-199 containing Earl salts, L-glutamine and 2.2 mg/ml sodium bicarbonate supplemented with 5.5 µg/ml sodium pyruvate, 1% v/v penicillin-streptomycin (10.000 U/ml penicillin G, 10.000 µg/ml streptomycin) was used as basal medium for maturation. Hormones, serum and EGF were added to the maturation media; there were five different treatments: Treatment 1 (T1) = Basal medium supplemented with 10% (v/v) heat-inactivated FBS; Treatment 2 (T2) = Basal medium supplemented with 5.0 µg/ml bLH, 0.5 µg/ml bFSH, and 10 ng/ml of EGF,;

Treatment 3 (T3)= Basal medium supplemented with 10% (v/v) heat-inactivated FBS, 5.0 µg/ml bLH, 0.5 µg/ml bFSH, and 10 ng/ml of EGF; Treatment 4 (T4) = Basal medium supplemented with 1 µg/ml E2,; Treatment 5 (T5) = Basal medium supplemented with 10% (v/v) heat-inactivated FBS and 1 µg/ml E2.

Assessment of Cumulus Expansion and Meiotic Status of Oocytes

COCs were evaluated and cumulus expansion degree was recorded at the end of the culture period to assess the effect of treatments on cumulus cell expansion (CCE) under a stereomicroscope according to a subjective scoring system from 0 to +3 as follows: Grade 0, no expansion; Grade 1, separation of only the outermost layer of cumulus cells; Grade 2, further expansion involving the outer half of the cumulus oophorus; Grade 3, complete expansion including the corona radiata cells. Oocytes with expanded cumulus considered as matured oocytes.

After maturation for 24 h, the COCs were denuded of cumulus cells by vortexing, and the denuded oocytes were subsequently fixed with acetic acid: ethanol (1:3) for 24 h. The fixed oocytes were mounted on glass slides with 90 % glycerol in dPBS containing 10% (w/v) Hoechst 33342 (pH 7.4) at room temperature for 15 min and stained oocytes were examined under fluorescent microscope. Excitation was induced at 365 nm and the emission is viewed through a 420 nm barrier filter. Nuclear maturation was evaluated without knowing the treatment groups by evaluator and classified as germinal vesicle breakdown (GVBD), metaphase I (M I) (including anaphase 1 and telophase-1), metaphase II (M II), chromosome aberrations and degenerate as described by Lorenzo et al.[4]. The degree of cumulus expansion and M II stage of oocytes were used as endpoint parameters for assessing the effect of treatments on expansion and maturation of bovine oocytes *in vitro*.

Statistical Analysis

To evaluate the differences between experimental groups Chi-square analysis was performed. Because of its independency from the distribution non-parametric tests such as Chi-square are successful in many cases where parametric tests are not [19]. Differences with $P<0.05$ were considered significant.

RESULTS

During the entire study, 526 ovaries were collected and of 505 (96.00%) used for aspiration of oocytes; the remaining 21 (3.99%) ovaries that either devoid of follicles or cystic were discarded. A total of 2115 culturable grade oocytes were recovered from 505 ovaries (4.18 oocytes per ovary). A total of 2043 and 698 oocytes were analyzed for cumulus expansion and nuclear maturation evaluation, respectively.

The maturation rate of bovine oocytes, based on cumulus expansion, matured in five different culture conditions are presented in *Table 1*. Maturation rate was higher in good quality oocytes than poor quality oocytes for all treatment groups ($P<0.05$). Maturation of good quality oocytes in culture media supplemented with oestradiol reduced maturation rate compared to maturation of those in other treatment groups ($P<0.05$). There were no significant differences between other treatment groups for good quality oocytes in terms of maturation rate based on the CCE.

CCE degrees of good and poor quality of bovine oocytes matured in different maturation conditions are presented in *Table 2*. Generally cumulus expansion degree increased with the quality of COCs. Higher proportion of good quality oocytes reached grade 3 cumulus expansion degree compared to poor quality oocytes in all treatment groups ($P<0.05$). Proportion of good quality oocytes reached grade 3 cumulus expansion degree was higher in FSH, LH and EGF supplemented media with or without serum supplementation ($P<0.05$) compared to other treatment groups. Estradiol supplementation in addition to serum had no effect on the proportion of good quality oocytes reached grade 3 degree of cumulus expansion. The proportion of good quality oocytes reached grade 3 degree

Table 1. *The average maturation rate of COCs cultured in different maturation media compositions based on cumulus expansion*

Tablo 1. *Farklı maturasyon medyumlarında kültüre edilen KOK'ların kumulus ekspansiyonuna bağlı maturasyon oranları*

Treatments	Good Quality		Poor Quality	
	(n)	(%)	(n)	(%)
TCM–199+FBS	203	71.4 [a*]	116	48.3 [a]
TCM–199+FSH+LH+EGF	174	87.4 [a]	73	68.4 [a]
TCM–199+FBS+FSH+LH+EGF	509	85.5 [a]	334	57.6 [a]
TCM–199+Estradiol 17-β	145	67.7 [b]	103	41.8 [a]
TCM–199+FBS+Estradiol 17-β	261	74.7 [a]	123	63.4 [a]

** Means in columns with different letters are significantly different at P<0.05. Values within columns with the same letters are insignificantly different (P>0.05)*

TCM = TCM-199 medium; FBS = fetal bovine serum; FSH = follicle stimulating hormone; LH = luteinizing hormone; EGF = epidermal growth factor

Table 2. *Effects of oocyte quality degrees on cumulus expansion grades of COCs cultured in different maturation media compositions*
Tablo 2. *Oosit kalitesinin farklı maturasyon medyumlarında kültüre edilen KOK'ların cumulus ekspansiyon derecesine etkisi*

Treatments	Quality Degree	No. of Oocytes	Cumulus Expansion Grades (%)			
			0	1	2	3
TCM–199+FBS	Good	203	28.6 ^{cd*}	10.3 ^{d}	28.1 ^{abc}	33.0 ^{b}
	Poor	116	51.7 ^{ab}	24.1 ^{ab}	20.7 ^{cd}	3.5 ^{ef}
TCM–199+FSH+LH+EGF	Good	174	12.6 ^{e}	9.2 ^{d}	19.0 ^{cd}	59.2 ^{a}
	Poor	73	31.5 ^{cd}	12.3 ^{cd}	43.8 ^{a}	12.3 ^{cd}
TCM–199+FBS+FSH+LH+EGF	Good	509	14.5 ^{e}	4.7 ^{d}	22.2 ^{bcd}	58.6 ^{a}
	Poor	334	42.5 ^{abc}	9.3 ^{d}	27.0 ^{bc}	21.3 ^{bc}
TCM–199+Estradiol 17-β	Good	145	32.4 ^{cd}	22.1 ^{abc}	35.9 ^{ab}	9.7 ^{de}
	Poor	103	58.3 ^{a}	28.2 ^{a}	12.6 ^{d}	1.0 ^{f}
TCM–199+FBS+Estradiol 17-β	Good	261	25.3 ^{de}	12.6 ^{cd}	31.8 ^{abc}	30.3 ^{b}
	Poor	123	36.6 ^{bcd}	25.2 ^{ab}	26.0 ^{bc}	12.2 ^{cd}

**Means in columns with different letters are significantly different at P<0.05*
TCM = TCM-199 medium; FBS = fetal bovine serum; FSH = follicle stimulating hormone; LH = luteinizing hormone; EGF = epidermal growth factor

Table 3*. Effects of different hormone treatments on nuclear status of in vitro matured bovine oocytes*
Tablo 3. *Farklı hormon muamelelerinin in vitro mature edilmiş sığır oositlerinin nükleer maturasyonu üzerine etkisi*

Treatments	No. of Oocytes	Nuclear Stages (%)				
		DG	CA	GVBD	M I	M II
TCM–199+FBS	191	11.5 ^{b*}	0.5 ^{a}	0.0 ^{a}	26.2 ^{a}	61.8 ^{a}
TCM–199+FSH+LH+EGF	120	34.2 ^{a}	0.8 ^{a}	0.8 ^{a}	22.5 ^{a}	41.7 ^{a}
TCM–199+FBS+FSH+LH+EGF	179	39.1 ^{a}	0.6 ^{a}	2.8 ^{a}	24.0 ^{a}	33.5 ^{a}
TCM–199+Estradiol 17-β	123	27.8 ^{a}	0.7 ^{a}	0.7 ^{a}	18.3 ^{a}	33.9 ^{a}
TCM–199+FBS+Estradiol 17-β	85	24.7 ^{a}	1.2 ^{a}	0.0 ^{a}	17.7 ^{a}	56.5 ^{a}

**Means in columns with different letters are significantly different at P<0.05. Values within columns with the same letters are insignificantly different (P>0.05)*
TCM = TCM-199 medium; FBS = fetal bovine serum; FSH = follicle stimulating hormone; LH = luteinizing hormone; EGF = epidermal growth factor; GVBD = germinal vesicle break down; M I = metaphase I; M II = metaphase II; CA = chromosome aberrations; DG = degenerate

of cumulus expansion was lower in oocytes matured in culture media supplemented with oestradiol alone (P<0.05) compared to oocytes matured in other treatment groups. Oocytes showing no cumulus expansion following 24 h maturation was higher in poorer quality oocytes in all treatment groups compared to good quality oocytes (P<0.05).

Nuclear maturation status of bovine COCs matured in different conditions are presented in *Table 3*. There were no significant differences between treatment groups in terms of proportion of oocytes reached to M II stage determined by nuclear staining, although serum supplementation resulted in numerically higher proportion of oocytes reached M II stage.

DISCUSSION

The results of the present study showed that FSH, LH and EGF supplementation with or without serum supplementation to culture media did not affect CCE in good quality oocytes. However supplementation of culture media with oestradiol alone reduced the maturation rate based on CCE. Interestingly there was no significant difference between treatment groups in terms of oocytes reached M II. Cumulus expansion degree increased with the quality of COCs and hormone supplementation resulted in higher proportion of good quality oocytes reached degree 3 CCE. The effect of hormones studied might be expected because it is known that cumulus cells have receptors for both FSH and LH and also for EGF [3]. Increased cumulus expansion have been attributed to the differential mitogenic effect of FSH on bovine cumulus cells and granulosa cells and the combined action of EGF, FSH and LH on cumulus cells to synthesize pyruvate, thus stimulating the tetraacetic acid cycle leading to an increased availability of ATP for the energy requirement of the oocytes [12]. However the results of the present study showed that the stimulatory effect of FSH when supplemented together with LH and EGF with or without serum on cumulus expansion but no effect was observed on nuclear maturation. In fact, our experimental design does not allow to see individual affects of each hormone

used in the present study. It would be interesting to know whether such effects of FSH, LH and EGF would be evident when they were used alone or in combination. The cumulus expansions of the bovine oocytes obtained in the present study is similar to previous studies of Lorenzo et al.[4], but lowers than Baştan et al.[2] and Wang et al.[13] on cattle oocytes.

It is interesting that stimulatory effect of FSH, LH and EGF was not evident on nuclear maturation status of oocytes in our culture systems presented in this study. Stimulatory effect of these hormones on CCE may not necessarily be reflected on nuclear maturation, if one assumes that these hormones act only at the cumulus cell level but not at on the oocytes directly or indirectly. It may also be possible that culture systems *in vitro* may differ in their response to hormonal effects due to effects of hormonal supplementation to culture media [8,16-18].

With the number of cumulus cell layers surrounding an oocyte, COC grade is one of the most important factors affecting nuclear maturation and cytoplasmic maturation of mammalian oocytes. In the present study, cumulus expansion degree increased with the quality of COCs. Given the pronounced differences in maturation rates for good and poor quality oocytes, results presented in this study indicate extent and integrity of the cumulus cell layer are major determining factors in successful *in vitro* maturation of bovine oocytes as reported also by other investigators [20].

Growth hormone and EGF have been reported to stimulate cytoplasmic maturation of oocytes in cattle [3,4]. Nagar & Purohit [5] found that cumulus expansion of oocytes increased significantly with EGF supplementation in a dose-dependent manner up to 50 ng/mL. In our study, when EGF (10 ng/mL), FSH, LH was used together with or without FBS, the proportion of good quality oocytes showing grade 3 cumulus expansion was higher than in other treatment groups but this effect was not evident in nuclear maturation status of oocytes. Similar results were also reported Warzych et al. [17]. In the study of Lonergan et al. [3] contrary to the present study, addition of EGF to culture media, irrespective of concentration, or 10% FCS to TCM-199 stimulated cumulus expansion as well as significantly increasing the proportion of oocytes attaining M II. Cumulus expansion and maturation rates in the present study were comparable to the in studies utilizing EGF, FSH and LH as supplements during *in vitro* maturation of bovine oocytes [4,11]. This may show that our culture system may support oocyte maturation irrespective of hormonal supplementation because there are culture systems that maturation degree are similar to the level reached in our study [1,16]. When oocytes are removed from antral follicles, they begin spontaneous meiotic maturation, presumably due to release from inhibitory influences of substances in follicular fluid or produced by cumulus cells through gap junctions [13]. Even without any stimulatory agent supplementation oocytes may undergo resumption of meiosis *in vitro*.

The use of serum as a supplement in the *in vitro* maturation media for oocytes seems to be controversial because of the variety of substances that the sera obtained from different sources may contain substances which may have beneficial or harmful effects on oocytes [3,4,8]. Inhibitory effects of estradiol on nuclear maturation of *in vitro* matured bovine oocytes have been observed previously [11]. The results observed in the present study support this view that oesradiol decreases maturation rate of bovine oocytes *in vitro*, at least CCE.

The results presented in this study showed that an *in vitro* bovine oocyte maturation system was developed which is responsive to known stimulatory and/or inhibitory factors of oocyte maturation *in vivo*. Hormone supplementation (FSH, LH and EGF) stimulated CCE and maturation rate. Oocytes selected as good quality had higher rates of maturation in the present maturation system. It is concluded that such a system can be used for to study oocyte maturation *in vitro* and also for further development following fertilization. Further studies are required to determine developmental competence of bovine oocytes matured in this culture system to develop blastocyst stage and embryo quality.

Acknowledgements

We wish to thank Dr. H. Onder for statistical analysis.

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