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Effect of epidermal growth factor on maturation and fertilization *in vitro* of goat follicular oocytes in a serum free or serum supplemented medium

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ABSTRACT

The cumulus expansion, nuclear maturation and fertilization of goat cumulus oocyte complexes (COC) were studied during *in vitro* culture, either in serum supplemented or serum free tissue culture medium (TCM-199) to which different concentrations (10, 20, 50 and 100 ng ml^{-1}) of epidermal growth factor (EGF) were added. There were two experiments with different end points: (1), Cumulus expansion and nuclear maturation after 28 h of culture (8 replicates), and (2), Proportion fertilized 24 h after insemination (8 replicates). Oocytes were cultured in groups of up to five per drop. In Experiment 1, the cumulus expansion of oocytes increased significantly with EGF supplementation in a dose-dependent manner up to 50 ng ml^{-1}. However, there were no differences in serum free or serum supplemented medium. The proportion of oocytes showing +++ cumulus expansion was higher for all the supplements compared to control media. However, the dose dependent effect of EGF supplementation in increasing the proportion of oocytes reaching metaphase II was evident only up to 20 ng ml^{-1}. Supplementation of EGF resulted in a significantly ($P<0.01$) higher proportion of oocytes reaching M II compared to control. The respective proportion of oocytes that reached M II were 55.63%, 64.5%, 52.35%, 49.18% and 34.07% in media supplemented with 10, 20, 50, 100 and 0 (control) ng ml^{-1} of EGF. In experiment 2 EGF supplemented media had a significantly ($P<0.01$) higher proportion of oocytes that were fertilized *in vitro* compared to control. The supplementation of EGF increased the fertilization rates in oocytes in a dose-dependent manner up to 50 ng ml^{-1}. Serum supplementation had no effect on fertilization *in vitro* of goat COC's. It was concluded that EGF at concentrations of 10 to 50 ng ml^{-1} increase the *in vitro* maturation and fertilization of goat COC's. Addition of serum to EGF supplemented media had little benefit in increasing the maturation and fertilization *in vitro* of goat COC's.

Key words: epidermal growth factor, goat, *in vitro* fertilization, oocytes, serum

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Introduction

The technique of *in vitro* maturation (IVM) of follicular oocytes, their *in vitro* fertilization (IVF) with *in vitro* capacitated spermatozoa and the *in vitro* culture of the resulting embryos have been successfully established for both sheep (CROZET et al., 1987) and goat (DE SMEDT et al., 1992; KESKINTEPE et al., 1994) species. However, only about one-third of the eggs resulting from IVM and IVF developed to morula-blastocysts in vivo or *in vitro* (LEIBFRIED RUTLEDGE et al., 1987) which might suggest that hormonal and/or follicular factors are required to improve maturation to obtain normal fertilizability and development rate. Amongst the various factors studied for the complex events that eventually prepare oocytes for fertilization, epidermal growth factor (EGF) is known to enhance oocyte maturation in animal species such as rat (FENG et al., 1989); pigs (REED et al., 1993; WANG and NIWA, 1995); cattle (HARPER and BRACKETT, 1993; LORENZO et al., 1994; NANDI et al., 2003); sheep (GULER et al., 2000), and buffalo (KUMAR and PUROHIT, 2004). The possible mechanisms of EGF action on oocyte maturation are either disruption of oocyte communication with cumulus cells (DECKEL and SHERIZLY, 1985), creation of a positive maturational signal (DOWNS, 1989) and mediation of effect via a tyrosine kinase dependent intracellular mechanism (LORENZO et al., 2001).

Foetal calf serum is routinely used in culture media for oocyte maturation and known to enhance the fertilizability of bovine oocytes (SANBUISSHO and THRELFALL, 1989). However, more recent observations imply that the effect of growth factor enhancement of maturation of oocytes is retarded by serum (SAKAGUCHI et al., 2000).

Although protocols for *in vitro* embryo production in small ruminants vary between various studies the most important component of the procedures are thought to be the capacitation of spermatozoa and that the *in vitro* maturation, fertilization and embryo culture procedures need substantial improvement (COGNIE, 1999). The present study was undertaken to evaluate the effect of epidermal growth factor on *in vitro* maturation and fertilization of goat cumulus oocyte complexes in a serum-free or serum supplemented medium.

Materials and methods

All the reagents and media were purchased from Sigma Chemical Co. (St. Louis, M.O, USA). EGF from mouse sub-maxillary glands (Code E 4127) was used. The experiments were conducted between August and December, 2003. Ovaries were obtained from an abattoir and were transported to the laboratory in PBS (Dulbecco) at 39 °C within 2 h. Oocytes were aspirated from antral follicles (2-8 mm) in diameter using an 18 gauze needle attached to a 10 ml disposable syringe. The COC's were washed 5 times in Hepes buffered washing medium (Tissue culture medium-199; with Earle's salts, L-glutamine and 25mM Hepes, (Product Code M 2520) + 0.25 mM sodium pyruvate + penicillin 100 I.U. ml^{-1} and streptomycin 50 μg ml^{-1}.

Oocytes with a homogenous and evenly granulated cytoplasm and three or more layers of cumulus cells were placed into drops (100 µl) of maturation medium under paraffin oil, and cultured in 35 mM petri-dishes at 39 °C under an atmosphere of 5% CO_2 in air, 95% humidity, for 28 h. The oocytes were matured in bicarbonate buffered TCM-199 with the addition of 0.25 mM sodium pyruvate, penicillin 100 I.U. ml^{-1} and streptomycin 50 µg ml^{-1}. The treatment included different concentrations of EGF with or without addition of 10% foetal bovine serum, (FBS) (Hi-Media, Mumbai, India. Product Code RM 1112). TCM-199 alone or with serum supplementation formed the control. The treatments were i) Control ii) EGF 10 ng ml^{-1} iii) EGF 20 ng ml^{-1} iv) EGF 50 ng ml^{-1}, and v) EGF 100 mg ml^{-1}.

Oocytes were assigned at random to the controls and treatments. The treatments were used in each of the two experiments, each with different end points, which were maturation (1) and fertilization (2). Treatments were represented by more than one drop of ≤ 5 oo cytes within a replicate. The treatments were replicated 8 times in experiments 1 and 2.

Sperm preparation and *in vitro* fertilization (IVF): Frozen thawed buck semen was prepared for IVF by swim up procedure using sperm TALP medium as described previously (PALOMO et al., 1999). Briefly, two frozen straws were thawed and emptied in a centrifuge tube. Four ml of Hepes TALP medium was added to the tube and the tube was centrifuged at 200 × g for 10 minutes. After discarding the supernatant an aliquot of sperm pellet was re-suspended with heparin containing (100 µg ml^{-1}) Hepes-TALP medium and incubated for 45 minutes at 38.5 °C in a CO_2 incubator. The actively motile spermatozoa were allowed to swim up and were used for insemination. Oocytes were inseminated with prepared sperms in Fert-TALP medium supplemented with hypotaurine 1 µg ml^{-1} to give a final concentration of 4×10^6 cells/ml.

In Experiment 1, the COC's cultured using different concentrations of EGF with or without serum supplementation were evaluated for cumulus expansion using procedures described previously for bovine (LORENZO et al., 1994) and bubaline oocytes (KUMAR and PUROHIT, 2004). The culture period was 28 h. Subsequent to subjective evaluation for cumulus expansion, the oocytes were freed of surrounding cumulus cells by repeated pipetting or vortexing and fixed by immersion in acetic methanol (1:3) and stained with 1% orcein in 45% acetic acid. The nuclear morphology was examined at × 200 and classified as germinal vesicle, metaphase I or metaphase II as per a previous study (KUMAR and PUROHIT, 2004). Oocytes from each replicate (n = 8) of each treatment were fixed, stained and evaluated as a group.

Experiment 2. COC's were matured for 28 h in the different treatments. Oocytes were then fertilized as described before. After 24 h of sperm oocyte incubation, the oocytes from replicates (n = 8) within the same treatment were fixed and stained, and evaluated for fertilization at × 400 as a group. Oocytes were considered fertilized when they showed a sperm head in the vitellus or male and female pronuclei.

Statistical Analysis. Cumulus expansion at the end of the culture period was assigned a numerical value corresponding to the degree of expansion achieved, where minimum response (+) equals 1, moderate response (++) equals 2 and maximum response (+++) equals 3. These numerical values were subjected to analysis of variance. The data were analyzed by replication (n = 8) and treatment (n = 4) comparisons between least square means (LSM ± SEM) by 't' test. The arcsine transformed data of the proportion of oocytes reaching M-II stage in Experiment 1 or fertilized in Experiment 2, were compared by ANOVA and Duncan's multiple range test.

Results

A total of 1883 culturable grade oocytes were recovered from 852 ovaries (2.21 per ovary). At the end of experiment 1 (n = 787), the proportion of COC's showing different grades of cumulus expansion and their least square means ± standard errors with different treatment groups are presented in Table 1. Compared to control media supplementation of EGF at all concentrations showed a significantly higher ($P<0.01$) cumulus expansion of +++ grade. The proportion of oocytes showing +++ cumulus expansion increased with EGF supplementation in a dose-dependent manner up to 50 ng ml^{-1} (which also showed the highest +++ cumulus expansion). However, at 100 ng ml^{-1} no further increase was seen. The supplementation of serum had no significant effect on the cumulus expansion of oocytes. The +++ cumulus expansion of serum supplemented and serum-free media was 47.08 and 44.69%, respectively.

Table 1. Effect of different treatments on cumulus expansion of goat follicular oocytes in TCM-199 media

Cumulus expansion									
Treatment	Nº of total oocytes*	Nil %	LSM ± SEM**	+ (%)	LSM ± SEM**	++ (%)	LSM ± SEM**	+++ (%)	LSM ± SEM**
TCM-199	135	60.74%	5.12 ± 0.22^{b}	17.77%	1.91 ± 0.21^{ab}	11.85%	1.22 ± 0.40^{a}	9.62%	1.30 ± 0.87^{a}
TCM+EGF-10 ng ml^{-1}	142	11.97%	2.33 ± 0.33^{a}	16.19%	2.87 ± 0.26^{b}	29.57%	3.54 ± 0.42^{b}	42.25%	4.03 ± 0.71^{b}
TCM+EGF-20 ng ml^{-1}	155	6.45%	1.25 ± 0.32^{a}	7.74%	1.50 ± 0.26^{a}	33.54%	3.49 ± 0.37^{b}	52.25%	5.06 ± 0.69^{bc}
TCM+GF-50 ng ml^{-1}	170	2.35%	1.00 ± 0.45^{a}	7.64%	1.62 ± 0.26^{a}	25.88%	3.74 ± 0.42^{b}	64.11%	6.81 ± 0.69^{c}
TCM+EGF-100 ng ml^{-1}	185	5.94%	1.37 ± 0.32^{a}	10.81%	1.93 ± 0.23^{ab}	29.19%	3.37 ± 0.36^{b}	54.05%	6.7 ± 0.71^{bc}

* Data pooled from 8 independent replicates. (From both serum-free and serum supplemented media).
** Values within column with different superscripts are significantly different ($P<0.01$) ($P>0.05$)

Table 2. Effect of different treatments on nuclear status of *in vitro* matured goat follicular oocytes in TCM-199 media

Nuclear status									
Treatment	N° of total oocytes*	D. G.	LSM ± SEM**	G. V.	LSM ± SEM	M I	LSM ± SEM**	M II	LSM ± SEM***
TCM-199	135	32.53%	1.64 ± 0.25^{a}	22.96%	1.66 ± 0.26	10.37%	1.25 ± 0.22^{a}	34.07%	2.87 ± 0.46^{a}
TCM+ EGF-10 ng ml^{-1}	142	19.01%	1.81 ± 0.24^{ab}	14.78%	2.0 ± 0.28	10.5%	1.54 ± 0.24^{a}	55.63%	4.93 ± 0.46^{b}
TCM+ EGF-20 ng ml^{-1}	155	20%	2.38 ± 0.26^{b}	9.67%	1.7 ± 0.3	5.8%	1.12 ± 0.27^{a}	64.51%	6.25 ± 0.46^{b}
TCM+ GF-50 ng ml^{-1}	170	21.17%	2.50 ± 0.25^{b}	17.05%	2.07 ± 0.24	9.41%	1.6 ± 0.24^{a}	52.35%	5.62 ± 0.46^{b}
TCM+ EGF-100 ng ml^{-1}	185	20%	2.64 ± 0.25^{b}	16.75%	2.21 ± 0.24	14.05%	2.16 ± 0.22^{b}	49.18%	5.68 ± 0.46^{b}

DG = Degenerate, GV = Germinal vesicle, MI = Metaphase I, M II = Metaphase II. * Data pooled from 8 independent replicates. (From both serum-free and serum supplemented media). Values within column with different superscripts are significantly different. ** ($P<0.05$). *** ($P<0.01$

Table: 3 Effect of different treatments on fertilization rate of *in vitro* matured goat follicular oocytes in TCM-199 media

		Nuclear stages					
Treatment	N° of total oocytes*	Arrested	LSM ± SEM**	M II	LSM ± SEM	Fertilized	LSM ± SEM**
TCM-199	234	56.41%	1.78 ± 0.27^{b}	38.03%	5.56 ± 0.87	9.83%	1.43 ± 0.31^{a}
TCM+ EGF-10 ng ml^{-1}	205	30.24%	4.75 ± 0.62^{a}	52.68%	6.75 ± 0.87	17.03%	2.78 ± 0.36^{ab}
TCM+ EGF-20 ng ml^{-1}	215	23.25%	3.75 ± 0.61^{a}	54.88%	7.37 ± 0.87	21.86%	3.47 ± 0.34^{b}
TCM+ GF-50 ng ml^{-1}	244	14.75%	3.18 ± 0.64^{a}	56.96%	8.68 ± 0.87	28.27%	4.87 ± 0.34^{b}
TCM+ EGF-100 ng ml^{-1}	198	30.3%	4.04 ± 0.58^{a}	47.97%	5.93 ± 0.87	21.71%	2.68 ± 0.31^{ab}

* Data pooled from 8 independent replicates. (From both serum-free and serum supplemented media). **Value within column with different superscripts are significantly different ($P<0.01$). M II = Metaphase II

The nuclear status of oocytes achieved after their *in vitro* maturation revealed that a significantly higher ($P<0.01$) proportion of oocytes reached metaphase II in EGF supplemented medium compared to control. A dose-dependent increase in the proportion of oocytes reaching metaphase II was seen up to 20 ng ml^{-1} only. The proportion of oocytes reaching M-II in serum supplemented media was significantly higher (52.42) compared to non-serum supplemented media (50.37%).

Experiment 2. After 24 h of sperm oocyte co-incubation, the proportion of oocytes (n = 1096) that were fertilized were 9.83% (control), 17.03% (EGF- 10 ng ml^{-1}), 21.86% (EGF 20 ng ml^{-1}), 28.27% (EGF 50 ng ml^{-1}) and 21.71% (EGF 100 ng ml^{-1}), respectively (Table 2). EGF supplementation increased the fertilization rates in oocytes in a dose-dependent manner up to 50 ng ml^{-1}. Serum supplements increased the fertilization rates non-significantly (22.28%) compared to non-serum supplemented media (17.35%).

Discussion

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 which may have beneficial or harmful effects. The present study demonstrated that epidermal growth factor at different concentrations when combined with serum supplements have a beneficial effect on the *in vitro* maturation and fertilization of goat follicular oocytes. However, additive effects of serum and growth factor combination were not seen. This reflects that growth factor or sera alone can have beneficial effects on oocyte maturation and fertilization, but the effects of serum cannot be precisely defined because of a wide variety of substances that the commercially available serum contains (LEGUIENNE and HUMBLOT, 1998).

The cumulus expansion of the goat oocytes obtained in the present study is similar to previous studies of YOUNIS et al. (1991) on goat, LORENZO et al. (1994) on bovine, and KUMAR and PUROHIT (2004) on bubaline oocytes. A dose-dependent increase in cumulus expansion of goat oocytes was seen during the present study up to 50 ng ml^{-1}, similar to previous findings of LORENZO et al. (1996) on rabbit oocyte cumulus expansion. Compared to control media EGF stimulated cumulus expansion at all concentrations, similar to previous findings (PARK and LIN, 1993; KEEFER et al., 1994; LORENZO et al., 1994; LORENZO et al., 1996).

The maturation rate obtained in the present study in serum-free or serum supplemented medium were around 50-52%. Previous studies on goat oocytes have shown nuclear maturation rates of 68-97% (YOUNIS et al., 1991; CROZET et al., 1995). However, these studies had utilized hormones as supplements instead of growth factors. Nuclear maturation rates comparable to the present study have been achieved in studies utilizing EGF as supplements during *in vitro* maturation of bovine (LORENZO et al., 1994; KOBAYASHI

et al., 1994) or bubaline (KUMAR and PUROHIT, 2004) oocytes. Retardation of oocyte maturation by serum seen in a previous study (SAKAGUCHI et al., 2000) was not evident in the present study.

The *in vitro* fertilization rates obtained during the present study are similar to those observed by KESKINTEPE et al. (1993) who observed fertilization rates of between 17.0 -25.9% when TCM-199 media was used for *in vitro* fertilization of goat oocytes. However, CROZET et al. (1995) obtained higher fertilization rates in their studies.

A dose-dependent increase in the fertilization rates was evident up to 50 ng ml^{-1}. LORENZO et al. (1996) had obtained similar findings with rabbit oocytes. In conclusion, EGF at concentrations of 10 to 50 ng ml^{-1}, increases the *in vitro* maturation and fertilization of goat oocytes. Addition of serum to EGF supplemented media has little benefit in increasing the *in vitro* maturation and fertilization of goat oocytes.

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SAŽETAK

U radu je proučavana ekspanzija kumulusa, zrenje jezgre i oplodnja kumulusnih oocita u koza u tijeku *in vitro* uzgoja u mediju bez seruma ili u mediju sa serumom (TCM-199) u koje su dodavane različite koncentracije epidermalnog faktora rasta EFR (10, 20, 50 i 100 ng ml^{-1}). Provedena su dva pokusa s ciljem promatranja ekspanzije kumulusa i zriobe jezgre nakon 28 sati uzgoja i omjera oplođenih 24 sata nakon osjemenjivanja. Oocite su uzgajane u skupinama do 5 po kapljici. U prvom pokusu došlo je do ekspanzije kumulusa oocite uslijed dodavanja EFR i to ovisno o dozi do 50 ng ml^{-1}. Nije ustanovljena razlika između medija s dodatkom ili bez dodatka seruma. Omjer oocita koje su pokazale najveću kumulusnu ekspanziju (+++) bio je viši u svih uzgojenih s dodatkom seruma. Učinak EFR na povećanje postotka oocita koje su dosegnule metafazu II bio je vidljiv samo do 20 ng ml^{-1}. Dodavanje EFR dovelo je do većeg broja oocita ($P<0,01$) u M II u usporedbi s kontrolom. Omjer oocita koje su dosegle M II iznosio je 55,63% u mediju s dodatkom 10 ng ml^{-1} EFR, 64,5% s dodatkom 20 ng ml^{-1}, 52,35% s dodatkom 50 ng ml^{-1}, 49,18% s dodatkom 100 ng ml^{-1} te 34,07% bez dodatka EFR. U drugom pokusu ustanovljen je značajno veći udio ($P<0,01$) oplođenih oocita u mediju s dodatkom EFR u odnosu na kontrolu. Dodatak EFR povisio je stopu oplodnje ovisno o dozi do 50 ng ml^{-1}. Obogaćivanje serumom nije imalo učinka na oplodnju *in vitro* kozjih kumulusnih oocita. Dodatak seruma u EFR obogaćeni medij imao je slab učinak na povećavano zrenje i oplodnju kozjih kumulusnih oocita *in vitro*.

Ključne riječi: epidermalni faktor rasta, koza, oplodnja *in vitro,* oocita, serum