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Assessment of nuclear maturation and subsequent *in vitro* embryo development of caprine oocytes with different supplementations in maturation medium

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ABSTRACT

The maturation medium and selection of protein supplements, growth factors, antioxidants and hormones for IVM play an important role in subsequent *in-vitro* fertilization and *in-vitro* embryo development. The objective of the present experiment was to study the effect of exogenous addition of hormones, epidermal growth factor (EGF), insulin and ß-mercaptoethanol into the maturation medium for *in-vitro* maturation of caprine oocytes. A total of 3753 Cumulus oocyte complexes (COCs) were collected from slaughtered goat ovaries, randomly divided and matured into following treatment groups. Group 1 (control): TCM-199 medium containing 10% newborn calf serum (NCS) and 3mg/ml BSA used as base medium, group 2: base medium supplemented with FSH (5µg/ml), LH (5µg/ml) and estradiol-17ß (1µg/ml), group 3: base medium supplemented with insulin (50ng/ml), group 4: base medium supplemented with FSH (5µg/ml), LH (5µg/ml), estradiol-17ß (1µg/ml) and epidermal growth factor (EGF; 10ng/ml), group 5: base medium supplemented with FSH (5µg/ml), LH (5µg/ml), estradiol-17ß (1µg/ml) and ß-mercaptoethanol (50mM/ml) and group 6: COCs were matured in a base medium supplemented with FSH (5µg/ml), LH (5µg/ml), estradiol-17ß (1µg/ml) and insulin (50 ng/ml). After maturation, 1, 540 oocytes from different groups were assessed for the evidence of nuclear maturation. Remaining 2,213 oocytes from different maturation group were allowed for *in vitro* fertilization and subsequent *in vitro* embryo development. The nuclear maturation rates in groups 1 to 6 were 33.6%, 38.0%, 39.7%, 60.0%, 37.4%, and 44%, respectively. The cleavage rate and morula production rate in groups 1 to 6 were 10%, 24%, 27%, 25%, 31%, 28% and 5.12%, 15.6%, 5.55%, 10.41%, 13.3%, 5.49% respectively. Statistical analysis (ANOVA) after arcsin transformation revealed that the maturation rate in group 4 was statistically significant ($P < 0.05$) as compared with other groups. ANOVA also revealed that morula development was significantly higher in group 2, group 4 and group 5 as compared to group 1, group 3 and group 6. The results suggested that the supplementation of EGF along with hormones in maturation medium significantly enhances the *in-vitro* maturation rate of caprine oocytes. These results also indicated that the supplementation of hormones, hormones with ß-mercaptoethanol, hormones with insulin in base medium have significantly higher embryo development competence in comparison with other groups.

Key words: 4', 6-diamidino-2-phenylindole (DAPI), Embryo development, Caprine oocytes, *In-vitro* fertilization, *In-vitro* maturation

Culture media components and culture conditions can affect and even modulate meiotic regulation of mammalian oocytes (Downs *et al.* 1997). Therefore, it is necessary to optimize culture systems taking in account all factors essential for the completion of oocyte maturation *in vitro*. Oocytes resume and complete meiosis spontaneously *in-vitro* in a wide variety of culture media containing serum, hormones and mixture of energy substrates. It is possible that a good quality oocyte may not reach M-II (nuclear maturation) and have no potential to develop to blastocyst stage due to incomplete maturation medium supplement. Thus maturation media is critical in terms of providing correct environment for oocytes to provide nuclear and cytoplasmic maturation. Therefore, the maturation medium, selection of protein supplements, growth factors, antioxidants and hormones play an important role in *in-vitro* fertilization and subsequent *in-vitro* embryo development. The purpose of this study was to examine the role of different supplementations on *in-vitro* maturation medium on *in vitro* nuclear status and *in vitro* embryo development competence of caprine oocytes.

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MATERIALS AND METHODS

Collection and processing of ovaries: Goat ovaries were obtained within 3–4 h of slaughter from the local abattoir and transported to laboratory in a thermos flask containing sterile warm physiological normal saline solution

supplemented with antibiotics. All the ovaries were then subjected to washings (5–6 times) with warm saline fortified with antibiotics.

Recovery of oocytes and in-vitro *maturation:* The oocytes were collected from each ovary into a petriplate containing oocyte collection media (Dulbecco's phosphate-buffered saline with 3 mg/ml BSA) by follicle puncture method using 18-G needle. Each petri dish containing oocytes was evaluated under a stereo zoom microscope and grade A, B, C oocytes were chosen for *in-vitro* maturation by the method described by Kharche *et al*. (2008). Selected oocytes were washed 4 to 5 times in oocyte holding medium (TCM-199 with Hepes modification, EGS 10%, sodium pyruvate 0.25 mM, gentamycin 50 µg/ml, glutamine 100 µg/ml, BSA 3 mg/ml). Washed oocytes were randomly divided into following treatment groups.

Group	Maturation treatment
1.	TCM-199 medium containing 10% new born calf serum (NCS) and 3mg/ml BSA used as a base medium for control
2.	Base medium supplemented with FSH (5µg/ml), LH (5µg/ml) and estradiol-17ß (1µg/ml)
3.	Base medium supplemented with insulin (50ng/ml)
4.	Base medium supplemented with FSH (5µg/ml), LH (5µg/ml), estradiol-17ß (1µg/ml) and epidermal growth factor (EGF, 10ng/ml)
5.	Base medium supplemented with FSH (5µg/ml), LH (5µg/ml), estradiol-17ß (1µg/ml) and ß-mercaptoethanol (50mM)
6.	Base medium supplemented with FSH (5µg/ml), LH (5µg/ml), estradiol-17ß (1µg/ml) and insulin (50 ng/ml)

These COCs were matured at 38.5°C in 5% CO2 in air for 27 h.

Evaluation of in vitro *maturation:* After the maturation, oocytes were separated from cumulus cells by treatment with 0.1% hyaluronidase and by passing through a fine pipette. They are then fixed in 2.5% glutaraldehyde, stained with 4', 6-diamidino-2-phenylindole (DAPI) and observed under fluorescent microscope for evidence of nuclear maturation.

In vitro *fertilization:* Remaining 2,213 oocytes were subjected for *in vitro* embryo production to assess cleavage and *in vitro* embryo development competence of oocytes matured in different groups. After 27 h of *in-vitro* maturation oocytes were denuded by hyaluronidase enzyme and by passing repeatedly through fine pipette. The oocytes were washed 4–5 times in fertilization medium (TALP solution containing 10% estrous goat serum, 8 mg/ml fatty acid free BSA and 10 µg/ml heparin) and placed in 50 µl drop of fertilization medium in culture petridish. An amount of 50 µl neat semen was diluted with 5 ml of sperm TALP containing 10% EGS and 10 µg/ml heparin and washed 2–3 times by centrifugation at 1200 rpm for 5 min. The supernatant was discarded in each washing. Finally 50 µl pellet of sperm was diluted with 1 ml of fertilization media. Fertilization drops containing oocytes were inseminated with 25 to 50 µl of finally diluted semen so as to obtain a sperm concentration of 2–4 millions sperm/ml. The sperm oocytes were co-incubated for 18 h in a CO_2 incubator maintained at 38.5° C, 5% CO_2 and high humidity.

In-vitro *embryo development:* Following 18 h of sperm oocytes co-incubation, the oocytes were washed in embryo development medium (KSOM medium) containing 10% NCS and 3 mg/ml BSA and then cultured in 50 µl drop of embryo development medium. The zygotes were cultured under mineral oil in 5% CO_2 in air with high humidity at 38.5°C.

Evaluation of in-vitro *embryo development:* After every 48 h, oocytes were observed for embryo development competence and half of the culture medium was replenished with fresh medium upto 10 days. Embryos were morphologically evaluated under inverted phase contrast microscope.

Statistical analysis: Analysis of the data was carried using Chi-square test (χ^2). Cleavage rates between the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 95% level of confidence (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

In this study, we compared the effect of hormones (FSH, LH and estradiol), growth factors (EGF and insulin), and antioxidant (ß-mercaptoethanol) in TCM-199 medium and assessed nuclear maturation status, cleavage rate and *in vitro* embryo development competence.

Statistical analysis (ANOVA) after arcsin transformation revealed that the maturation rate in group 4 was statistically significant ($P < 0.05$) as compared to those in groups 1, 2, 3, 5 and 6. ANOVA revealed that cleavage rate was significantly higher in group 2 to group 6 as compared with control (group 1). No significant difference was found in cleavage rate between different supplementation groups except control i.e. group 2-group 6. Although the development of embryos upto morula was significantly higher in group 2, 4 and 5 as compared to group 1, 3 and 6.

In this study, significantly higher cleavage rate and embryo development rate was found in hormonal supplemented group (group 2) as compared with control group (group 1). Hormonal stimulation of oocytes during the course of *in-vitro* maturation is of paramount importance in achieving nuclear and cytoplasmic maturation which is essential for the preparation of oocyte for fertilization (Stubbing *et al.* 1988). Researchers are using FSH and LH to determine the developmental potential of *in-vitro* produced embryos as gonadotropins not only supports oocytes maturation but also induce major alteration in the protein profile of oocytes (Schroter and Meinecke 1995). The inclusion of gonadotropins in IVM medium is reported to enhance oocytes

Table 1. Meiotic competence of *in-vitro* matured oocytes in different supplementation medium

Groups	Total no. of ovaries	Total no.of oocytes for maturation	Total no. of M_2 stage oocytes	% maturation rate
Group1 (control)	100	220	74	33.6 [a]
Group 2 (hormones)	175	300	114	38.0[a, b]
Group 3 (insulin)	135	235	93	39.57[a, b, c]
Group 4 (EGF+ Hormones)	140	250	150	60[d]
Group 5 (ß-mercaptoethanol+ hormones)	173	310	116	37.4[a, b, c, e]
Group 6 (insulin+iormones)	110	225	99	44[a, b, c, e, f]

[a.b, c, d, e, f] The values bearing different superscript with in a column differed significantly (P<0.05).

Table 2. Effect of different supplementation in maturation medium on cleavage rate and embryo development of goat oocytes

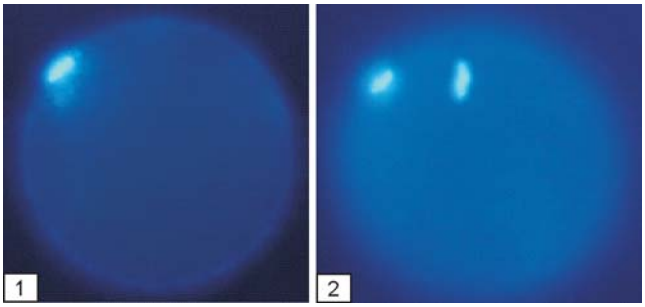
Groups	Total no. of oocytes used in fertilization	Total no. of cleaved oocytes	% cleavage rate	Morula formation rate (n)	% Morula formation
Group 1 (Control)	390	39	10[a]	2	5.12 [a]
Group 2 (hormones)	400	96	24[b]	15	15.6 [b]
Group 3 (insulin)	330	90	27[b, c]	5	5.55 [ac]
Group 4 (EGF + hormones)	410	144	25[b, c, d]	15	10.41 [bd]
Group 5 (ß- mercaptoethanol+ hormones)	358	112	31[b, c, d, e]	15	13.3 [bd]
Group 6 (insulin+hormones)	325	91	28[b, c, d, e, f]	5	5.49 [acf]

[a.b, c, d, e, f] The values bearing different superscript with in a column differed significantly (P<0.05).

quality and developmental potential by possible alterations in metabolic process (Kharche *et al.* 2011). Estradiol may be involved in ooplasmic maturation by stimulating DNA polymerase ß and enhancing the synthesis of presumed male nucleus growth factors (Rahman *et al.* 2008). Pawshe *et al.* (1996) observed maturation rate (90%) in goat oocytes when all the hormones were added together to the maturation medium (FSH 0.5 µg/ml + LH 5 µg/ml + estradiol 0.5 µg/ml) than the other treatments in which hormones (FSH, LH, estradiol) alone used in maturation medium. We also found inclusion of hormones in the base medium affect *in-vitro* maturation rate as compared with control group. However the addition of hormones is still controversial, as we found higher maturation rate (39.57%) in insulin supplemented group (group 3) as compared with hormone supplemented group (group 2, 38.0%). However, the combination of hormones and insulin (group 6) shows numerically higher (44%) nuclear maturation rate as compared to individual hormones and insulin supplemented group (group 2 and group 3). Some workers also found no significant difference in the number of oocytes reaching metaphase-II or oocytes forming blastocyst in the absence of exogenous hormones (Wahid *et al.* 1992, O-Brian *et al.* 1994).

This result also shows supplementation of antioxidant (ß-mercaptoethanol) in IVM media group 5 has higher nuclear maturation rate and significantly higher embryo development competence than control medium which might be due to intracellular GSH level in IVM oocytes. With bovine oocytes, it was demonstrated that addition of ß-mercaptoethanol to the maturation medium causes an increase in intracellular GSH synthesis (De Matos and Furnus 2000). Sutovsky and Shatten (1997) also suggested that the depletion of GSH during bovine oocyte maturation blocks the decondensation of the male pronucleus without dis-assembling of the sperm tail connecting piece and pronuclear apposition during fertilization. Abeydeera *et al.* (1998) also shows that ß-Mercaptoethanol in IVM media has beneficial effects on subsequent development of IVM-IVF embryos.

In this study we found statistically significant (P <0.05) nuclear maturation rate in EGF supplemented group (group 4, 60%) as compared with other groups used in this study. We also found significantly higher embryo development competence upto morula stage in group 2, 4 and 5 as compared with group 1, 3 and 6. The results suggest that the supplementation of EGF in maturation medium significantly enhances the *in-vitro* maturation rate and embryo development competence of caprine oocytes.The mechanism whereby growth factors regulate or modulate resumption of meiosis in oocytes may be mediated via the granulosa and/or cumulus cells (Dekel *et al.* 1985, Brucker *et al.* 1991). Goat cumulus cells express EGF receptors (Gall *et al.* 2004) and EGF triggers signaling through the MAPK pathway during IVM in goat COCs (Gall *et al.* 2005).The intrinsic tyrosine kinase of EGF receptor (EGF-R) is activated by binding of EGF, resulting in EGF-R autophosphorylation and subsequent tyrosine phosphorylation of numerous substrates



Figs 1–2. **1.** M II oocyte with polar body. **2.** M II oocyte with two chromatin spot

within the cell (Carpenter and Cohen 1990).

Epidermal growth factor (EGF) improves not only nuclear maturation but also cytoplasmic maturation of cumulus enclosed caprine oocytes *in-vitro* (Cognie *et al.* 2004), cleavage rate (Rieger *et al*. 1998) as well as embryo development (Sirisathien *et al.* 2003). In the bovine, EGF alone present during IVM increased the proportion of oocytes reaching the blastocyst stage following IVF and IVC (Lonergan *et al.* 1996).

Gaud *et al*. (1998) revealed higher number of oocytes cultured in the absence of cumulus cells completed nuclear maturation in medium supplemented with EGF in comparison with their sibling oocytes cultured without EGF. This might be because of EGF modulates differentiation and plays an important role in ovarian folliculogenesis. In sheep, EGF-R was recently detected in ovarian tissues and EGF has a physiological role in the regulation of oocyte maturation via the tyrosine kinase pathway (Lorenzo *et al.* 2001). During the growth phase, EGF acts as a mitogenic factor inducing granulosa cell proliferation, whereas in antral follicles, it regulates granulosa cell differentiation and maturation of oocytes (Goritz *et al.* 1996).

The results indicated that the EGF in combination with hormonal supplementation regulates the significantly higher oocyte maturation rate as compared with other supplementations used in the study. These results also indicate that the supplementation of hormones, hormones with EGF or hormones with ß -mercaptoethanol in control medium also have significantly higher embryo development competence in comparison with control, insulin, insulin plus hormone supplemented groups. Thus the presence of hormones, hormones with EGF and hormones with ß-mercaptoethanol in maturation medium could also be beneficial for subsequent embryo development.

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