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Dose dependent effect of pregnant mare serum gonadotropin and human chorionic gonadotropin on *in vitro* maturation of goat oocytes

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ABSTRACT

This study was aimed to evaluate the dose dependent effect of pregnant mare serum gonadotropin and human chorionic gonadotropin on *in vitro* maturation goat oocytes. The study was planned in 2 experiments. In the first experiment effect of pregnant mare serum gonadotropin supplementation on nuclear maturation of goat oocytes using 5 different concentrations of PMSG, was studied. The treatment was divided into group 1 (control medium containing TCM-199, sodium pyruvate (0.25mM), gentamycin (50µg/ml), L-glutamine (100µg/ml), estradiol 17-ß (1µg/ml), BSA (3mg/ml), supplemented with 10% FBS); group 2 (control medium + 10 IU/ml PMSG); group 3 (control medium + 20 IU/ml PMSG); group 4 (control medium + 30 IU/ml PMSG); group 5 (control medium + 40 IU/ml PMSG); and group 6 (control medium + 50 IU/ml PMSG). The oocytes of these treatment groups were matured in maturation media for 27 h in humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator. The maturation rates achieved in groups 1–6 were 20.0, 48.51, 74.63, 74.15, 75.0 and 76.33%, respectively. In experiment 2, the effect of hCG supplementation on nuclear maturation of goat oocytes using 3 different concentrations of PMSG, was studied. Second experiment was divided into group 1 (control medium + 20 IU/ml PMSG +10IU/ml hCG), group 2 (control medium + 20 IU/ml PMSG +20IU/ml hCG) and group 3 (control medium + 20 IU/ml PMSG +30IU/ml hCG). The maturation rates achieved in groups 1–3 were 77.0, 85.19 and 84.22%, respectively. It can be concluded that supplementation of 20 IU/mL PMSG and 20IU hCG in maturation media significantly increased the maturation rate.

Key words: Goats, hCG, *In vitro* maturation, Oocytes, PMSG

Small ruminants are good models for developing technologies like *in vitro* embryo production, transgenesis and cloning (Freitas *et al*. 2007). Selection of protein supplements and hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) for IVM medium is an important event in the subsequent *in vitro* fertilization (IVF) and embryonic development (Pawshe *et al*. 1996). 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). The beneficial effect of gonadotropins in the IVM medium proved to be more pronounced for the oocytes of pre-pubertal females (Ledda *et al*. 1997). Researchers are using FSH ranged from 0.1 µg/ml (Cognie *et al*. 2003) to 10 µg/ml (Jimenez-Macedo *et al*. 2007), LH 3 µg/ml (Ongeri *et al*. 2001) to 100 µg/ml (Keskintepe *et al*. 1994) and estradiol up to 1µg/ml (Jimenez-Macedo *et al.* 2007**,** Kharche *et al*. 2008, Yadav *et al*. 2010) to determine the developmental potential of *in vitro* produced embryos as gonadotropins support oocytes maturation and also induce major alteration in the protein profile of oocytes (Schroter and Meinecke 1995). Caprine culture media supplemented with gonadotropins and estradiol-17ß improve maturation rates (Keskintepe *et al*. 1994, Pawshe *et al*. 1996, Mogas *et al.* 1997a, b, Kharche *et al*. 2008, Yadav *et al*. 2010, Kharche *et al*. 2011). Moreover, FBS increased cleavage rate over goat serum in an LH, FSH and E2 hormonal milieu (54%; $P<0.05$, Seydou *et al*. 1999).

Commercial PMSG is less expensive than FSH and LH. However, to our knowledge, dose dependent effects of PMSG and PMSG+hCG supplementation on *in vitro* maturation of goats oocytes have not been previously investigated in detail. Thus, in the present study attempts were made to study the effect of different concentrations of PMSG and the effect of different concentrations of hCG in media supplemented with PMSG on *in vitro* maturation of goats oocytes.

MATERIALS AND METHODS

Collection of ovaries: Goat ovaries (355) were obtained within 4 h of slaughter from a local abattoir and were transported to the laboratory in a thermos flask containing sterile warm (35–37°C) physiological normal saline solution

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(NSS) supplemented with antibiotics (100 IU/ml penicillin G and 100µg/ml streptomycin sulphate). All ovaries were cleared off the attached tissue and mesovarium (trimming). The trimmed ovaries were subject to washings (5–6 times) with warm saline fortified with antibiotics and transferred into laminar flow. All subsequent experimental procedures were conducted in laminar flow.

Recovery of oocytes: Oocytes (1,216) were recovered from ovaries by slicing (Yadav *et al.* 2007) with a sterile surgical blade separately in a sterile disposable Petri-dish containing oocyte collection medium (Dulbecco's phosphate-buffered saline (D-5773) with 3 mg/ml BSA). The oocytes were searched in a Petri-dish under stereozoom microscope. Oocytes were searched, picked up with the help of a fine bore pipette and subsequently placed in another Petri-dish containing oocyte holding medium. The collected oocytes were finally graded as excellent (A), good (B), fair (C) and poor (D) quality under the inverted phase contrast (Kharche *et al.* 2008). Only grade A and B quality oocytes were chosen. The before said graded cumulus oocyte complexes (COCs) were selected, picked up and placed in a sterile disposable vile containing oocyte holding medium (TCM-199 containing L-glutamine (100 µg/ml), sodium pyruvate (0.25 mmol) and gentamycin (50 µg/ml)). The selected oocytes were picked up from vile and subjected to serial drop washing. Each oocyte was washed 14 times (100 ml each) through oocyte holding medium (OHM).

Experiment 1: Dose dependant effect of pregnant mare serum gonadotropin (PMSG) on in vitro maturated goat oocytes

After washing with oocyte holding medium, selected cumulus oocyte complexes (n=856) were divided into 6 groups. Oocytes were then washed 3–4 times with different maturation media depending on the group selected.

Group 1 (control medium): TCM-199 medium containing sodium pyruvate (0.25mM), gentamycin (50µg/ml), L-glutamine (100µg/ml), estradiol 17-ß (1µg/ml), BSA (3mg/ml), supplemented with 10% fetal bovine serum (FBS).

Group 2: Control medium + 10 IU/ml PMSG.

Group 3: Control medium + 20 IU/ml PMSG.

Group 4: Control medium + 30 IU/ml PMSG.

Group 5: Control medium + 40 IU/ml PMSG.

Group 6: Control medium + 50 IU/ml PMSG.

Oocytes of each group were matured in 100 µl droplets of maturation media (MM) covered with sterile mineral oil in a polystyrene culture dish (35mm × 10mm), previously incubated for 2h in humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator for 27 h.

After 27 h of *in-vitro* maturation, cumulus cells were removed by treatment with 0.1% hyaluronidase enzyme (H-4272) followed by gentle pippeting for 1 min. Denuded oocytes after exposing in hypotonic solution of KCl for 5 min were ûxed on a rectangular cover glass slide (22 × 30 mm) with 2.5% glutaraldehyde for 5 min at room temperature followed by staining with 0.1µg/ml 4,6-diamidoino-2-phenylindole (DAPI).

The different stages of oocyte maturation were evaluated as per the method of Kharche *et al*. (2006).

Experiment 2: Dose dependant effect of human chorionic gonadotropin (hCG) on in vitro *matured goat oocytes*

Following the results of experiment 1, an attempt was made to know if the addition of human chorionic gonadotropin at different concentration in maturation media with 20IU/ml PMSG and 1µg/ml estradiol (based on previous results from experiment 1) may increase the maturation rate. Oocytes were recovered from ovaries by slicing technique and selected cumulus oocyte complexes (n=360) were transferred to a 100 µl droplets of maturation media (MM) covered with sterile mineral oil in a polystyrene culture dish (35mm × 10mm), previously incubated for 2h in humidified atmosphere of 5% CO_2 at 38.5°C in a CO_2 incubator for 27 h. Maturation media consisted of:

Group 1: control medium + 20 IU/ml PMSG +10IU/ml hCG

Group 2: control medium + 20 IU/ml PMSG +20IU/ml hCG

Group 3: control medium + 20 IU/ml PMSG +30IU/ml hCG

After 27 h of *in-vitro* maturation, cumulus cells were removed by treatment with 0.1% hyaluronidase enzyme (H-4272) followed by gentle pippeting for 1 min. Denuded oocytes after exposing in hypotonic solution of KCl for 5 min. were fixed on a rectangular cover glass slide (22×30mm) with 2.5% glutaraldehyde for 5 min at room temperature followed by staining with 0.1µg/ml 4,6-diamidoino-2-phenylindole (DAPI).

Statistical analysis: The maturation stage of oocytes was calculated as a percentage. Maturation rates between the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

Collection, quality and quantity of oocytes harvested from goat slaughterhouse ovaries are important for *in vitro* embryo production. In this study, on an average 3.40±0.06 oocytes (A and B) recovered per ovary using slicing technique. Several techniques were used for oocytes recovery in goats and sheep, and they reported better recovery and quality of oocytes per ovary than puncture and aspiration technique in sheep and goats (Martino *et al*. 1994, Wani *et al*. 2000, Wang *et al*. 2007, Yadav *et al*. 2007) and cattle (Yoo *et al*. 1993). 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 oocytes for fertilization. Our results demonstrated that maturation rate increases with addition of 20IU/mL PMSG (Table 1; Fig. A, B, C, D, E and F). Furthermore, supplementation with higher concentrations of PMSG (from 30IU/mL to 50IU/mL) did not increase the progression to metaphase II. Feng *et al.* (2002) studied the effect of serum and

Table 1. Nuclear status of goat oocytes matured *in vitro* with different concentrations of PMSG

Groups	Treatment	Total COCs	GV(%)	GVBD (%)	MI(%)	MII(%)
Group 1	(Control)	100	21 (21)	27 (27)	32 (32)	20 (20.0)[a]
Group 2	(Control+10IU/ml PMSG)	101	1(1)	12 (11.88)	39 (38.61)	49(48.51)[b]
Group 3	(Control+20IU/ml PMSG)	205	1 (0.49)	28 (13.66)	23 (11.22)	153 (74.63)[c]
Group 4	(Control+30IU/ml PMSG)	147	2 (1.36)	14 (9.52)	22 (14.97)	109 (74.15) [c, d]
Group 5	(Control+40IU/ml PMSG)	164	0 (0.00)	16 (9.76)	25 (15.24)	123(75.00)[c,d,e]
Group 6	(Control+50IU/ml PMSG)	131	0 (0.00)	11(8.4)	20(15.27)	100(76.33)[c,d,e,f]

Values in a column with different superscripts are significantly different (P < 0.05).

gonadotropins on cumulus expansion and nuclear maturation of goat oocytes and reported that at 24h of culture the nuclear maturation rates of oocytes cultured in TCM-199 containing PMSG was higher than that of oocytes in TCM-199 without PMSG but the difference in nuclear maturation rate between 2 groups become significant (Pß 0.05) when examined at 27 h. Typically, most caprine IVM culture media are supplemented with gonadotropins (FSH and LH) and estradiol-17ß which improved maturation rates (Keskintepe *et al*. 1994). FSH generally upholds the developmental competence during folliculogenesis. It maintains follicular growth and is vital for upregulation of the LH receptor during the final stages of follicular development (Sirard *et al*. 2007). With the basic media (TCM-199), Mogas *et al*. (1997a,b) reported the addition of FSH, LH and estradiol-17ß as well as estrus goat serum (EGS) to increase the maturation rate to 72.4 and 64.1% in adult and prepubertal goats, respectively. Similarly, Pawshe *et al*. (1996) also reported a maturation rate of 62.6% following the supplementation of hormones (FSH and LH) and EGS. Chauhan *et al*. (1996) observed that addition of foetal bovine serum and hormones to TCM-199 resulted in higher percentage of cumulus expansion (77.9%) and nuclear maturation (77.7%). Although FSH and LH are necessary for spontaneous oocyte maturation, it is generally believed that only these hormones can improve oocyte cytoplasmic maturation by significantly altering a range of cumulus cell activities. With the replacement of FSH and LH by PMSG, the maturation rate obtained from 20IU/mL to 50IU/mL is in agreement with Chauhan *et al*. (1996) and Mogas *et al.* (1997a,b). Gupta *et al*. (2001) suggested that commercially available PMSG at 40 IU/mL can effectively be used in place of pure follicle-stimulating hormone for *in vitro* maturation of buffalo oocytes, making it cost effective for IVF studies. Similar to Gupta *et al*. (2001), our results showed that PMSG (20IU/mL) can be used as replacement of FSH and LH for *in vitro* maturation goat oocytes (Table 1).

The second experiment was aimed to know the addition of human chorionic gonadotropin to maturation media with 20IU/mL PMSG can increase the maturation rate. Although a high maturation rate of 85.19% (Table 2) was obtained with 20IU/mL hCG , but it did not differ significantly with supplementation of 10IU/mL and 30IU/mL concentration of hCG. In Egyptian sheep, Farag *et al.* (2009) demonstrated that the supplementation of hormone combinations (PMSG+hCG+E2) plus FBS to culture media (TCM-199) improved maturation rate (41.25%) of sheep COCs as compared to the control media (3.5%). Serum provides nutrients to an oocyte and apart from this, tends to nurture the cells surrounding the oocyte, rather than the oocyte itself. It also decreases the possibility of the zona pellucida (ZP) hardening when an oocyte is liberated from its follicular environment. The addition of serum to the culture medium during *in vitro* maturation of oocytes is partly responsible for the induction of maturation. Combination of FBS and BSA are most commonly used protein source for *in vitro* culture system goat oocytes. Therefore the supplementation of hormone combination (20IU/mL PMSG+ 20IU/mL hCG+1µg/mL E2) with 10% FBS to culture media TCM-199 could significantly improve the IVM of goat oocytes especially COCs. Inclusion of these gonadotropins for IVM enhances oocyte quality and developmental potential by possibly altering metabolic processes (Brackett and Zuelke 1993). The results indicated that supplementation of 20 IU/mL PMSG in maturation media significantly (P<0.05) increased maturation rate which was further enhanced by addition of 20IU hCG and may be used in *in vitro* maturation protocols.

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Table 2. Nuclear status of goat oocytes matured *in vitro* with different concentration of hCG.

Groups	Treatment	Total COCs	GVBD (%)	MI(%)	MII(%)
Group 1	Control+20IU/ml PMSG+10IU/ml hCG	113	10 (8.84)	16 (14.16)	87 (77)[a]
Group 2	Control+20IU/mlPMSG +20IU/mL hCG	135	3 (2.22)	17 (12.59)	115 (85.19)[a]
Group 3	Control+20IU/mlPMSG +30IU/ml hCG)	102	7 (6.86)	9 (8.82)	86 (84.32)[a]

Values in a column with different superscripts are significantly different (P < 0.05).

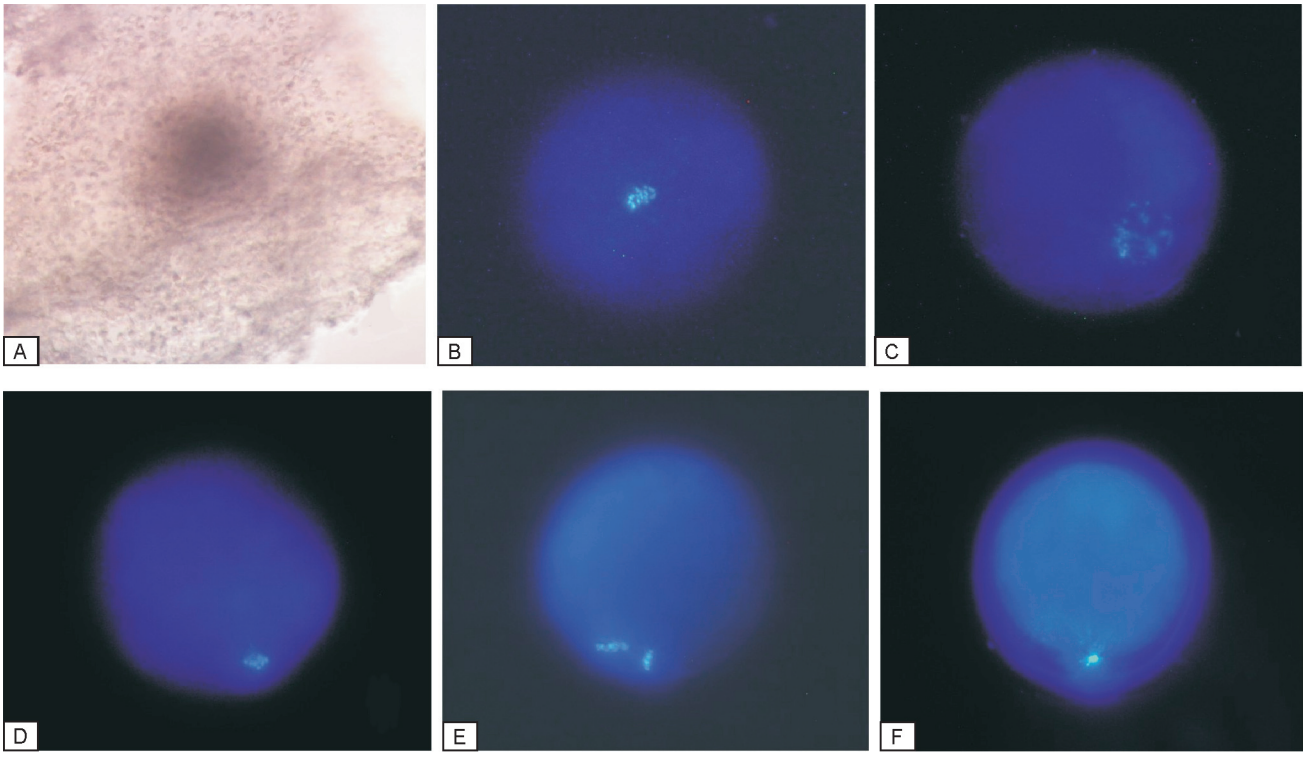

Fig. 1. A. Mature oocytes; B. germinal vesicle; C. germinal vesicle breakdown; D. metaphase I; E. mataphase II with 2 chromatin spots; F. metaphase II with polar body.

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