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In vitro production of meiotically competent oocytes from goat preantral follicles

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Abstract
An experiment was conducted to establish *in vitro* maturation protocol for production of MII stage oocytes from preantral follicles. Goat preantral follicles (PFs) (diameter ranging from 150-400µ) were cultured in culture medium to support growth and development *in vitro* for a period of eight days. After culture period of 8 days, oocytes were retrieved from cultured preantral follicles (T_1-T_5), were kept for *in vitro* maturation for a period of 27hrs in different IVM media (IVM-I, IVM-II, IVM-III and IVM-IV) and antral oocytes were kept for IVM as control-II in different IVM media. After 27 hours of *in vitro* maturation period, oocytes were denuded and evaluated for nuclear status by staining with propidium iodide. Among *in vitro* maturation protocols of our study the proportion of oocytes exhibiting M-II stage were maximum in IVM-III. And among treatments T_3 (T_4+FSH+GH+EGF) had a highest percentage of M-II stage in all IVM media and these results were statistically different ($P \leq 0.05$) with oocytes of other treatments. From the results of the present study, it can be concluded that, *in vitro* development of goat PFs could be significantly improved through the addition of complement inactivated caprine follicular fluid to the *in vitro* maturation media.

Keywords: Goat, pre antral follicles, hormones, IVM media

Introduction
In mammals, the preantral follicles serve as store-house of majority of oocytes. The ovulatory follicle develops from primordial follicle by a process of folliculogenesis. Through this process, a healthy oocyte is usually selected for maturation. Recent advancements in the area of reproductive biotechnology, particularly the animal biotechnology is reaching to the level where it can be successfully utilized for augmenting the livestock productivity. Few current reproductive technologies used for increasing animal productivity are super ovulation, *in vitro* embryo production, nuclear transfer, stem cell culture, transgenic animal production etc. Since these technologies depend on availability of large number of fertilizable oocytes, due to which need arises to produce large number of meiotically competent oocytes from *in vitro* culture of preantral follicles. Several *in vitro* studies suggest that the preantral follicles are able to generate competent oocytes that are able to undergo subsequent embryo development *in vitro* (Wu *et al*. 2001a; Gupta *et al*. 2008; Arunakumari *et al*. 2010; Magalhães *et al*. 2011b) [24, 8, 11, 4, 6]. Moreover, the follicular development is regulated by various endocrine and paracrine factors (Araújo *et al*. 2011) [6].

In several mammalian species different IVM culture systems have been established to study oocyte development, and have applied to assisted reproduction in humans and livestock animals. Mouse is the only species in which *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes from PFs resulted in the birth of live offspring (Liu *et al*. 2001) [14]. However among farm animals, success has been achieved up to embryo development using oocytes from cultured PFs of adult ovaries in pig (Wu *et al*. 2001a and b) [24, 23], buffalo (Gupta *et al*. 2008) [8, 11], sheep (Arunakumari *et al*. 2010) [4] and goat (Magalhaes *et al*. 2011a) [17] but none of these studies progressed to develop blastocyst except pig. Addition of GH (50ng/ml) resulted in growth of oocytes form goat preantral follicles that were acceptable for IVM and IVF Magalhaes *et al*. (2011b) [16]. Addition of VEGF-A_{165} to the culture medium improved the development of goat PFs cultured *in vitro*, allowing the production of mature oocytes (Araujo *et al*. 2011) [1, 3]. In view of the above, the present investigation was undertaken to study the effect of different maturation media for *in vitro* maturation of oocytes obtained from cultured PFs to get maximum number of meiotically competent oocytes which is an important mean for successful *in vitro* fertilization.

Materials and Methods

Unless otherwise stated, all culture media, hormones, growth factors, PBS and chemicals were purchased from Sigma (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All media were incubated at 39ºC under a humidified atmosphere of 5% CO_2 in air for 2 h prior to use. Phosphate buffered saline (PBS), collection medium for PFs [HEPES buffered tissue culture medium 199 supplemented with 0.5% bovine serum albumin (BSA), 50 µg/mL gentamicin sulphate, 0.23 mM of sodium pyruvate, 2 mM l-Glutamine and 25 IU/mL heparin], handling medium (collection medium without heparin), stock solutions of EGF and FSH were all prepared as described by Tamilmani *et al*. (2005) [22]. The preparations of thyroxin, GH and IGF-I solutions were made as per Arunakumari *et al*. (2007, 2010) [5, 4]. Bicarbonate buffered tissue culture medium 199 supplemented with 50 µg/mL gentamicin sulphate was used as control medium.

TCM 199B, supplemented with 0.5 μgml^{-1} FSH, 100 μgml^{-1} LH, 1 μgml^{-1} estradiol 17µ, 50 $\mu gml^{-}1$, gentamicin sulphate, 10 μgml^{-1} BSA and 20% (v/v) heat-treated oestrus goat serum was used for the *in vitro* maturation (IVM) of oocytes collected from the cultured PFs (Rao *et al*. 2002) [18]. This was stored at 4ºC for up to 1 week and equilibrated for 1 h at 39 ºC in 5% CO_2 in air, prior to use.

In vitro maturation media

Control media was supplemented with 10 µg/ml FSH, 10 µg/ml LH, 1 µg/ml Estradiol 17β, 50 µg/ml gentamicin sulphate, 10µg/ml of Bovine serum albumin (FAF) and 10% fetal bovine serum (IVM-I). In addition to these components 50 ng/ml EGF was added to IVM-II. Whereas, IVM-III was prepared by adding 200 µl of complement inactivated caprine follicular fluid to the 800 µl of control media and to the IVM-IV 200 µl of complement inactivated oestrus goat serum was added to the 800 µl of control media. All the prepared media were stored at 4 ºC for up to 1 week in 5 ml disposable syringe with 0.22 µm filter attached. Filtered medium were equilibrated for 1 h at 39 ^{0}C in 5% CO_2 in air under humidified atmosphere prior to use.

Preparation of propidium iodide solution

Stock solution of propidium iodide (PI) was prepared by reconstituting 5 mg of lyophilized desiccate of propidium iodide (P 81845, Sigma, USA) in 5 ml of filtered PBS to yield concentration of 1 mg/ml (1 µg/µl). 10 µl of this solution was diluted with 990 µl of filtered PBS to yield a final concentration of 10 µg/ml PI solution. This solution was stored in 1 ml of plastic aliquots at 4ºC in darkness till used.

Collection of ovaries, isolation, selection and culture of preantral follicles

Collection of ovaries, isolation of PFs, classification and selection of PFs for cultures were performed, as described by Tamilmani *et al*. (2005) [22], Arunakumari *et al*. (2010) [4] and Amin *et al*. (2013) [2]. Briefly each ovary was cut into two halves along its longitudinal axis. The medulla is scooped out from each half. Then, each half of the ovarian cortex was dissected into thin slices using a 26 gauge needle and sterile surgical blade. Under a stereo zoom microscope (Nikon, Japan), these cortical slices were subjected to micro dissection in collection medium for isolation of the PFs, in the size range of 150-400 µm. The diameter of PFs was measured using Scopetek software, China. Care was taken to leave a small amount of stromal tissue attached to the basement membrane of the follicles. PFs having a centrally placed spherical oocyte with an intact basement membrane were chosen for culture. The selected PFs were washed thrice in handling and culture medium and placed individually in 20 µL droplet of culture medium in 35 mm tissue culture dishes. The micro droplets were overlaid with autoclaved pre-equilibrated mineral oil and cultured for 8 days in 5% CO_2 incubator at 39ºC. Half of the culture medium was replaced with fresh culture medium every 48 h.

In vitro maturation of oocytes from in vitro cultured follicles

The *in vitro* cultured follicles were carefully opened (if the oocytes were not extruded itself) using two 26G needles attached to 1ml syringe barrels, to release the oocyte inside after the end of 8 days culture period. The collected oocytes were washed three times in holding medium followed by three washings in the *in vitro* maturation medium before being placed individually in ~20 µl droplets of IVM medium in 35-mm tissue culture dishes. The droplets were overlaid with autoclaved, pre-equilibrated lightweight mineral oil. These culture dishes were incubated at 39ºC in 5% CO_2 for 24 h in a CO_2 incubator. This procedure regularly supports meiotic maturation of more than 80% of oocytes collected from the antral follicles in sheep (Rao *et al*. 2002) [18].

Staining of *in vitro* matured oocytes

At the end of the IVM period of 27 hours, the oocytes were denuded of cumulus cells either by repeated pipetting through a fine-bore glass pipette or by placing COCs in 200 µl of hyaluronidase solution for 1 minute and the cumulus cells were denuded off the oocytes by repeated pipetting through a fine bore glass pipette. The denuded oocytes were washed twice in filtered PBS to remove any hyaluronidase solution adhering to the oocytes. Denuded oocytes were placed separately 200 µl PI (Propidium Iodide) stain solution for 15 minutes in darkness in normal atmosphere. After the end of 15 minutes staining period, oocytes were washed twice in filtered PBS to remove excess PI stain particles adhering to the oocytes.

Morphological evaluation of cultured preantral follicles and nuclear status of oocytes

Morphological evaluation of PFs was done as described by Aruna kumari *et al*. (2007) [5], Amin *et al*. (2013) [2]. However, the stained oocytes were examined through an inverted microscope at magnification of 400x with fluorescent illumination equipped with an excitation filter 510-530 nm, emition filter 590 nm and dichromatic mirror 570 nm and the nuclear maturation of oocytes was evaluated by observing the GV (Germinal Vesicle, GVBD (Germinal Vesicle Breakdown), M-II (Metaphase-II), UC (Unclassified) stages of oocyte captured by using a digital camera coupled to microscope.

Statistical Analysis

Two way ANOVA procedure, followed by Duncan's multiple range test in SPSS 17 was performed. Percentage data (proportions) was subjected to arcsine transformation prior to comparisons.

Results

In the present study a total of eleven hundred (1125) preantral follicles were kept for *in vitro* culture, out of these a total number of 931 oocytes were retrieved from preantral follicles of different treatments after 8 days of culture period, while remaining were damaged during culture and retrieval time. Oocytes, having at least two layers of cumulus cells and evenly granular cytoplasm, retrieved from preantral follicular culture after a period of eight days were kept for *in vitro* maturation in incubator at 38.5ºC, 5% CO_2 in different IVM media for 27 hours (Table 1-5). The oocytes collected by aspiration method from antral follicles of ovaries were kept for *in vitro* maturation as control-II. In order to evaluate the nuclear status of oocytes, surrounding cumulus cells were denuded off and stained with propidium iodide (PI). The nuclear status was determined by marking the changes like exhibition of metaphase II stage, Germinal vesicle breakdown (GVBD), shedding of polar body after putting oocytes in different IVM media. From the present results (Table 1-5) it can be confirmed that treatment T_3 showed higher rate of nuclear maturation as compared to other treatments T_1, T_2, T_4, T_5 and T_6. Oocytes from T_3 in the IVM medium-I did not differ significantly with T_1, T_2, T_4, T_5 and T_6 with respect to parameters like GV and GVBD. However, the maturation rate in the control medium was significantly lower ($P \leq 0.05$) than other treatment groups (Table 1). In IVM-II majority (45.10±3.72) of oocytes from T_3 showed M-II stage and was significantly different than other treatment groups (Table 2). However, GVBD do not show any significant difference between treatment groups. A Table 3 show the maturation rate of oocytes in IVM-III in which GVBD is higher in oocytes of treatment group T_4 but does not show statistical difference among other treatments. However, oocytes from treatment T_3 showed highest percentage of M-II stage oocytes (60.05±1.85) and is significantly higher than other treatments groups ($P \leq 0.05$). In IVM-IV the M-II oocytes were highest in treatment T_3 (49.80±2.32) and were statistically higher than other treatments groups (Table 4). The present study was further proceeded to perform the comparative study on meiotically competent oocyte (M-II stage) production, after *in vitro* maturation in different IVM media for oocytes obtained from PFs cultured for a period of eight days in different treatments. Oocytes retrieved from treatment T_1 (T_4+FSH) had reached maximum percentage to M-II stage (40.15±1.69) in IVM-III media which had significant difference with IVM-I (19.07±2.17), IVM-II (30.27±1.83) and IVM-IV (34.85±1.28) (Table 5). Oocytes retrieved from follicles cultured in treatment T_2 (T_4+FSH+GH) had exhibited highest percentage of meiotically competent (M-II stage) oocytes in IVM-III (44.85±1.92). However, these were significantly different with IVM-I (26.12±2.81), IVM-II (31.83±1.79) and IVM-IV (39.66±1.60). Oocytes obtained from treatment T_3 (T_4+FSH+GH+EGF**)** were exhibited maximum percentage of M-II stage (60.05±1.85) matured in IVM-III only and these oocytes had a significant difference with IVM-I (38.06±2.93), IVM-II (45.10±3.72), IVM-IV (49.80±2.32).IVM-III media exhibited maximum percentage of M-II stage (52.33 ±2.6) for treatment T_4 (T_4+FSH+IGF+EGF) obtained oocytes and these were statistically significant with oocytes matured in IVM-I (29.61 ±4.11), IVM-II (36.65.±2.50), and IVM-IV (42.81±2.51) media. However, none of the oocyte reached to M-II stage for PFs cultured in control media (T_5) in four protocols of IVM media. Antral oocytes kept for *in vitro* maturation in different IVM media had significantly reached maximum from IVM-III (70.40±2.45) media only and these oocytes had a significant difference with oocytes matured by other IVM media **(**IVM-I (45.74±1.70), IVM-II (53.88±2.00) and IVM-IV (63.54±2.08)**.** overall results of the study indicated that, *in vitro* development of goat PFs could be significantly improved through the addition of complement inactivated caprine follicular fluid to the *in vitro* maturation media.

Table 1: Nuclear status of oocytes obtained from cultured PFs after 27hrs *in vitro* maturation in IVM-I

Treatments	Combinations (Replicates/ No. of oocytes)	GV (%)	GVBD (%)	M-II (%)	UN Classified (%)
T_1	T4 +FSH (6/48)	21.38±2.21^{a}	34.44± 3.8^{b}	19.07±2.17^{b}	25.09±3.71^{c}
T_2	T4+FSH+EGF (6/41)	17.07±1.44^{a}	31.68±4.21^{b}	26.12±2.81^{bc}	20.88 ± 4.56^{bc}
T_3	T4+FSH+GH+EGF (6/46)	26.97±2.24^{a}	26.03±2.77^{b}	38.06±2.93^{d}	10.73±2.33^{ab}
T_4	T4+FSH+IGF+EGF (6/46)	24.24±2.96^{a}	34.81±3.84^{b}	29.61 ±4.11^{c}	11.28±3.80^{ab}
T_5 (Control-I)	Control-I (6/40)	55.64±2.81^{b}	0.00^{a}	0.00^{a}	44.35±2.81^{d}
T_6 (Control-II)	Control-II (6/75)	17.17±1.97^{a}	32.62±3.19^{b}	45.74±1.70^{e}	4.44±2.04^{a}

Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Table 2: Nuclear status of oocytes obtained from cultured PFs after 27hrs *in vitro* maturation in IVM-II

Treatments	Combinations (Replicates/ No. of oocytes)	GV (%)	GVBD (%)	M-II (%)	UN Classified (%)
T_1	T4 +FSH(6/53)	21.87 ±2.89^{b}	34.82±4.77^{b}	30.27±1.83^{b}	13.01±3.26^{a}
T_2	T4+FSH+EGF (6/40)	21.83±2.94^{b}	31.12±4.55^{b}	31.83±1.79^{b}	15.15 ± 5.70^{a}
T_3	T4+FSH+GH+EGF (6/43)	23.00 ±1.34^{b}	24.62±3.44^{b}	45.10±3.72^{c}	7.20±3.47^{a}
T_4	T4+FSH+IGF+EGF (6/43)	26.63 ±1.76^{b}	24.78±5.12^{b}	36.65. ±2.50^{b}	11.90±5.00^{a}
T_5 (Control-I)	Control-I (6/40)	51.21 ±3.01^{c}	0.00^{a}	0.00^{a}	48.88±3.07^{b}
T_6 (Control-II)	Control-II (6/75)	10.76±1.60^{a}	28.96±1.99^{b}	53.88±2.00^{d}	5.23±2.51^{a}

Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Table 3: Nuclear status of oocytes obtained from cultured PFs after 27hrs *in vitro* maturation in IVM-III

Treatments	Combinations (Replicates/ no. Of oocytes)	GV (%)	GVBD (%)	M-ii (%)	Un classified (%)
T_1	T4 +FSH(6/50)	26.39±4.14^{c}	26.16±4.61^{bc}	40.15±1.69^{b}	7.26±3.47^{a}
T_2	T4+FSH+EGF (6/51)	19.36±2.01^{bc}	27.75±2.73^{c}	44.85±1.92^{b}	7.98 ± 2.59^{a}
T_3	T4+FSH+GH+EGF (6/56)	9.20 ±2.04^{a}	23.11±3.64^{bc}	60.05±1.85^{d}	7.50±3.37^{a}
T_4	T4+FSH+IGF+EGF (6/50)	11.73±2.67^{ab}	28.2±2.58^{c}	52.33 ±2.6^{c}	7.68±2.45^{a}
T_5 (Control-I)	Control-I (6/40)	62.66 ±4.10^{d}	0.00^{a}	0.00^{a}	37.30±4.10^{b}
T_6 (Control-II)	Control-II (6/79)	8.67±2.44^{a}	17.77±1.18^{b}	70.40±2.45^{e}	3.05±1.94^{a}

Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Table 4: Nuclear status of oocytes obtained from cultured PFs after 27hrs *in vitro* maturation in IVM-IV

Treatments	Combinations (Replicates/ No. of oocytes)	GV (%)	GVBD (%)	M-II (%)	UN Classified (%)
T_1	T4 +FSH(6/52)	20.92 ± 3.23^{b}	34.44 ± 3.59^{c}	34.85 ± 1.28^{b}	9.76 ± 3.79^{ab}
T_2	T4+FSH+EGF (6/50)	17.59 ± 1.53^{ab}	31.50 ± 3.08^{bc}	39.66 ± 1.60^{bc}	17.83 ± 2.00^{bc}
T_3	T4+FSH+GH+EGF (6/54)	18.76 ± 2.2^{ab}	25.06 ± 2.83^{bc}	49.80 ± 2.32^{d}	6.28 ± 2.95^{a}
T_4	T4+FSH+IGF+EGF (6/49)	14.11 ± 4.84^{ab}	22.21 ± 5.65^{b}	42.81 ± 2.51^{c}	20.83 ± 5.27^{c}
T_5 (Control-I)	Control-I (6/40)	64.15 ± 4.04^{c}	0.00^{a}	0.00^{a}	36.01 ± 4.1^{d}
T_6 (Control-II)	Control-II (6/79)	10.01 ± 1.17^{a}	25.31 ± 2.09^{bc}	63.54 ± 2.08^{e}	1.11 ± 1.11^{a}

Values with different superscripts within a column are significantly different ($P\leq0.05$).

Table 5: Effect of different combinations of hormonal and growth factors and IVM protocols on MII stage oocytes production

	T1	T2	T3	T4	T5	T6
	T4 +FSH	T4+FSH +EGF	T4+FSH +EGF +GH	T4+FSH +EGF +IGF	Control-I	Control-II
IVM-I	$19.07\pm2.17^{(a)(q)}$	$26.12\pm2.81^{(a)(q,r)}$	$38.06\pm2.93^{(a)(s)}$	$29.61\pm4.11^{(a))(r)}$	$0^{(p)}$	$45.74\pm1.70^{(a)(t)}$
IVM-II	$30.27\pm1.83^{(b)(q)}$	$31.83\pm1.79^{(a)(q)}$	$45.10\pm3.72^{(a,b)(r)}$	$36.65\pm2.50^{(a,b)(q)}$	$0^{(p)}$	$53.88\pm2.00^{(b)(s)}$
IVM-III	$40.15\pm1.69^{(c)(q)}$	$44.85\pm1.92^{(b)(q)}$	$60.05\pm1.85^{(c)(s)}$	$52.33\pm2.6^{(c)(r)}$	$0^{(p)}$	$70.40\pm2.45^{(d)(t)}$
IVM-IV	$34.85\pm1.28^{(b)(q)}$	$39.66\pm1.60^{(b)(q,r)}$	$49.80\pm2.32^{(b)(s)}$	$42.81\pm2.51^{(b)(r)}$	$0^{(p)}$	$63.54\pm2.08^{(c)(t)}$

Values with different superscripts (a, b, c, d) within a column are significantly different with respect to IVM media, and different superscripts (q, r, s, t) within a row are significantly different with respect to Treatment (PFs culture media) ($P\leq0.05$)

Discussion

The present study demonstrated the effect of various growth factors and hormones like IGF, EGF, T_4, FSH and GH in different combinations on *in vitro* development of goat preantral follicles. The present study advocated that the highest proportion of PFs exhibiting growth, increase in diameter, antrum formation and *in vitro* ovulation were in treatment groups cultured in combination of hormones and growth factors T_4+FSH+GH+EGF (T_3). These results are comparable with the findings observed by Zhou and Zhang (2005) who reported that, in presence of FSH, IGF-I in combination with EGF produced a higher survival rate of adult caprine preantral follicles than any other growth factor did individually. Findings of present study are in accordance with findings of Magalhaes *et al*. (2011b) [16] in high rate of follicular survival and antrum formation when media supplemented with Growth hormone (GH). Furthermore, the influence of the thyroxin on PFs appears to depend on the physiological status of the PFs at the time of collection (Gupta *et al*. 2007) [9, 10]. Hence the present study proved that the *in vitro* development of goat PFs could be significantly improved through addition of T_4, FSH, GH, EGF, and IGF-I in different combinations in the culture medium. Moreover, according to Gupta *et al*. (2007) [9, 11] age of the animal and season of the year at the time of isolation will also influence the *in vitro* development of preantral follicles.

However, immature oocytes *in vivo* remain arrested in the first meiotic prophase until maturation is induced by the interaction of steroids, gonadotropins and other follicular constituents. Full oocyte maturation involves not only acquisition of meiotic competency, but also cytoplasmic maturation. Use of different culture and maturation media *in vitro* with different supplements might improve the understanding of the minimal requirements for oocyte growth and maturation, expected to provide a reasonable yield of meiotically competent oocytes. Earlier studies indicated that different *in vitro* maturation media were able to produce meiotically competent oocytes from goat PFs (Saraiva *et al*. 2010; Magalhaes *et al*. 2011b) [16]. The influence of different hormones, growth factors, follicular fluid and estrus goat serum on maturation of oocytes retrieved from preantral follicle culture *in vitro* on comparative way was meager. Hence the present study was planned to carry out the role of different hormones, follicular fluid and estrus goat serum on maturation of oocytes derived from PFs cultured for a period of eight days. Oocytes retrieved from PFs were grown in different culture media. In IVM- I oocytes obtained from T_3 treatment (T_4+FSH+GH+EGF) had maximum percentage of M-II stage oocytes (38.6%) in contrast to the findings of Arunakumari *et al*. (2010) [4] in sheep, the difference might be due to variation in culture media or species difference. However, these findings were in accordance with the Magalhaes *et al*. (2011b) [16] on production of matured oocytes by supplementation of Growth hormone at a concentration of 10 ng/ml and 50 ng/ml. The present findings were in accordance with the results of Saraiva *et al*. (2012) [20] who studied the effect of both FSH and LH on oocyte maturation. Furthermore, oocytes retrieved from treatment T_3 of cultured preantral follicle had maximum percentage of M-II stage (45%) in IVM-II media. Recent study in goat preantral follicles revealed that the addition of LH to a culture medium containing FSH and epidermal growth factor (EGF) influences the development of the oocytes (Silva *et al*. 2011) [3-6-21]. However, a very small number of these oocytes became competent to resume meiosis. Similar results were obtained in the present study also. Meiotic resumption of oocytes from the present study was similar to the observations made by Saraiva *et al*. (2012) [20] with the media supplemented with LH at concentration 50 ng/ml. However, difference was noticed with LH supplemented at 100 ng/ml. It was reported by earlier studies that the effect of LH on the *in vitro* culture of preantral follicles depends on the follicular category, the concentration used and the timing of addition of LH to the culture medium (Tamilmani *et al*. 2005; Silva *et al*. 2011) [22, 3, 6, 21]. IVM-III consisting of Follicular fluid (FF), is a serum transudate modified by follicular metabolic activities, contains specific constituents such as steroids and glycoproteins synthesized by the cells of the follicle wall. Oocytes retrieved from treatment T_3 were kept for *in vitro* maturation in this media, which resulted in maximum percentage of M-II stage (60%) oocytes. The present results support the study by Duarte *et al*. (2011) [1, 3, 6] who also reported a high percentage of follicular growth, meiosis resumption and early antrum formation with the addition of bFF (bovine Follicular Fluid) at the beginning of culture. However, oocytes retrieved from treatment T_3 resulted in maximum percentage of M-II stage (49%) in IVM-IV, consisting of oestrus goat serum. This is the first report on

IVM of cultured preantral follicular oocytes in oestrus goat serum. Lu and Gordon (1987) [15] used estrus cow serum (OCS) as the main protein source in bovine IVM studies and found that estrus cow serum (OCS) had a significant and marked effect, compared with foetal calf serum (FCS). The present results were in accordance with Lu and Gordon (1987) [15]. Younis *et al*. (2005) [25] suggested that proestrous cow serum may be more effective than estrus cow serum (OCS). Karami *et al*. (2010) [12] studied on antral oocytes of sheep and reported that the supplementation with human menopausal serum, estrus sheep serum and estrus goat serum supported better rates of *in vitro* maturation, *in vitro* fertilization and embryo development. The results of present study on antral oocytes (control-II) were similar with those reported by Kharche *et al*. (2006) [13] on *in vitro* maturation; who also reported the successful embryo production from goat antral oocytes by using 20% estrus goat serum in IVM media. The comparative study of *in vitro* maturation of oocytes obtained from preantral follicle culture with different IVM media revealed that IVM-III had shown best results among different IVM media employed in the present study and oocytes retrieved from treatment T_3 preantral follicles had best results among all treatments. Oocytes from antral follicles were kept for IVM as control-II, which had shown maximum percentage of M-II stage oocytes with IVM-III media only.

Conclusion

From the results of the present study, it can be concluded that, in *vitro* development of goat PFs could be significantly improved through the addition of complement inactivated caprine follicular fluid to the *in vitro* maturation media. In vitro maturation of oocytes from cultured preantral follicles could be improved by using various *in vitro* maturation protocols. However, further investigation is needed for optimization of *in vitro* culture systems that include not only *in vitro* maturation, but also *in vitro* fertilization and culture (IVFC) of embryos.

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