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# Effect of co-culture of sheep preantral follicles with ovarian somatic cells (cumulus cells and granulosa cells)

**Deepa Pathipati, CH Srinivasa Prasad, AVN Siva Kumar, B Punya Kumari, RV Suresh Kumar, B Rambabu Naik and VH Rao**

**Abstract**
The study was conducted to evaluate the effects of co-culture of preantral follicles with cumulus cells or granulosa cells for six days. Preantral follicles (PFs') were isolated from the ovarian cortical slices using micro dissection method under stereo zoom microscope. The cumulus cells were obtained by repeated pipetting of cumulus oocyte complexes from medium and large sized follicles on surface of the ovary and granulosa cells were aspirated from the oocytectomized preantral follicles. Isolated PFs' were cultured in TCM 199B which serves as control group or in standard culture medium (TCM 199B supplemented with FSH (2.5 µg/mL), IGF-I(10 ng/mL), GH(1 mIU/mL) and $T_4$(1 µg/mL). PFs' were co-cultured with cumulus or granulosa cells in TCM199B and/ standard medium (T3-T6) for six days. Our results indicated that co-culture of PFs' with Cumulus cells in TCM 199B (T3) yielded better growth and development of preantral follicles *in vitro* when compared to others.

**Keywords:** Sheep, preantral follicles, cumulus cells, granulosa cells

## 1. Introduction
The normal physiology of ovary is tuned to support growth of only limited number of ovarian follicles from preantral to antral and to ovulation while rest of them undergoes atresia. This limits the efficient utilization of female reproductive germplasm. Therefore to bridge the gap, preantral follicles are isolated, cultured *in vitro* and *in vitro* fertilization of the oocytes from cultured PFs' resulted in successful production of embryos in the Mouse (Eppig and Schroeder *et al.,* 1989) [9], Pig (Wu *et al.,* 2001), Buffalo (Gupta *et al.,* 2008) [14], Sheep (Arunakumari *et al.,* 2007) [2] and Goat (Magalhaes *et al.,* 2011) [19]. However, production of live offspring from the *in vitro* fertilized ova in the cultured PFs' was reported only in the mouse (Eppig and Schroeder *et al.,* 1989, Xu *et al.,* 2006) [9, 28]. Despite the fair knowledge on the folliculogenesis and events associated with it, the growth of Preantral follicles *in vitro* culture conditions is poor than *in vivo*. This indicates the need to improve the culture systems appropriately that stimulates the development of PFs' *in vitro*. Cumulus Cells (CCs) originate from granulosa cells (GCs) which differentiate during follicular antrum formation into mural GCs, that line the wall of the follicle with primarily a steroidogenic role whereas cumulus cells encircle and maintain bi directional communication with the oocyte to regulate its growth and developmental competence (Gilchrist *et al.,* 2008) [12]. CCs express many of the same genes as GCs but their transcriptome also displays some unique features, such as expression of Slc38a3 (which codes for SLC38A3, a sodium-coupled neutral amino acid transporter; Eppig *et al.,* 2005) [8] and Amh (Anti-Mu¨llerian hormone; Salmon *et al.,* 2004) [23] at higher levels than GCs. Published reports have indicated that the Co-culturing of preantral follicles with different ovarian cells like cumulus cells, granulosa cells, mesenchymal cells, skin fibroblasts etc., had considerable influence on the *in vitro* preantral follicle development (Eppig and schroeder., 1989; Itoh and Hoshi., 2000; Wu *et al.,* 2002; Ramesh *et al.,* 2007; Li *et al.,* 2011) [9, 27, 21]. However, the effect of Co-culture of preantral follicles with cumulus or granulosa cells *in vitro* in Sheep has not been studied before. Therefore, the present study was undertaken to evaluate the effects of co-culturing of preantral follicles with cumulus or granulosa cells in TCM 199 B and/standard culture media.

## 2. Materials and Methods
A total of ~210 ovaries from sheep were collected irrespective of age, body condition, stage of oestrous cycle and season from local slaughter house and were utilised in this study.

The ovaries were removed within 30 minutes of slaughter and washed in phosphate buffered saline (PBS) supplemented with 50 µg/ml Gentamicin sulphate to remove blood and extraneous material. The washed ovaries were transported at $37^0$C in a thermos flask in the same media to the laboratory within 30 minutes. The extra-ovarian tissues were trimmed off and the ovaries were washed with PBS to remove blood clots and superficial bacterial contamination. Then they were immersed in 70 percent alcohol for 3-5s and rinsed twice in PBS supplemented with 50µg/ml Gentamicin sulphate.

**2.1 Isolation of preantral follicles**

Ovaries were cut into two halves and the medulla was scooped out. After removal of medulla, the ovaries were placed in 35mm plastic culture dishes (153066, Nalge Nunc, Denmark) containing handling medium for follicles (HEPES buffered Tissue culture medium 199 supplemented with 0.23 mM of sodium pyruvate, 2 Mm L-glutamine and 50µg/ml Gentamicin sulphate). The ovarian cortex was cut into thin slices using a sterile surgical blade. These cortical slices were placed in the follicular handling medium in a 35mm culture dish. Intact preantral follicles in the size range of 250-400 µm were mechanically isolated by micro dissection under a stereo zoom microscope (SMZ 2T, Nikon corporation, Japan). PFs' in the size range of 250 – 400 µm having visible centrally placed oocytes with no signs of atresia and with intact basement membrane and no antral cavity were considered as good quality follicles and were used for culture.

**2.2 Isolation of cumulus cells and granulosa cells**

The medium to large sized follicles on the surface of the ovary were aspirated for the collection of cumulus oocyte complexes which were further subjected to Repeated pipetting to release cumulus cells from oocytes. After the isolation of large PFs', oocytes were removed from them by using 22 G needles and the granulosa cells were collected from the broken PFs'. The cumulus cells and granulosa cells were washed thrice in the culture medium before using them for the experiments.

**2.3 Culture of Preantral follicles**

Preantral follicles were washed thrice in the culture medium and cultured in 80µl droplets of culture medium in 35 mm plastic culture dishes (cat. No. 153066, Nalge Nunc, Denmark) as explained below and the entire experiments were repeated 30 times.

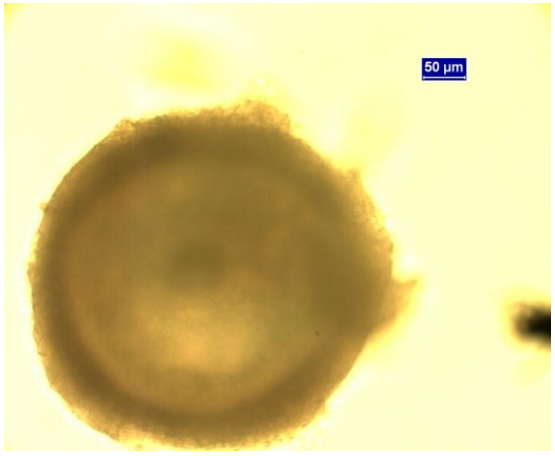
**2.3.1 Experimental Design**

In each Experimental group, three to four preantral follicles (PFs') were randomly allocated and were cultured for six days in different culture conditions as mentioned below.

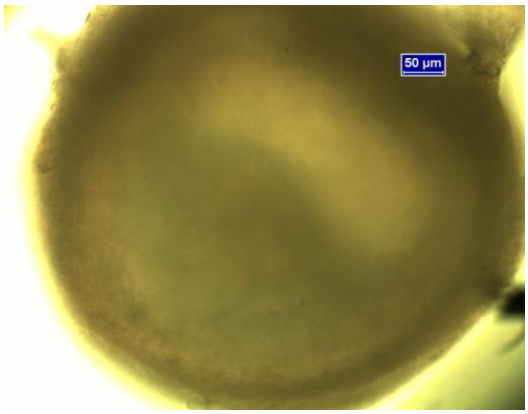
**Experiment 1:** Preantral follicles without Co-culture in TCM 199B (CONTROL - I)
**Experiment 2:** Preantral follicles without Co-culture in Standard medium
**Experiment 3:** Co-culture of PFs' with Cumulus cells in TCM 199B
**Experiment 4:** Co-culture of PFs' with Cumulus cells in Standard medium
**Experiment 5:** Co-culture of PFs' with Granulosa cells in TCM 199B
**Experiment 6:** Co-culture of PFs' with Granulosa cells in Standard medium.

The culture droplets were overlaid with sterilized lightweight mineral oil (M 8410, Sigma Aldrich, USA) which was pre equilibrated with the medium overnight at 39ºC and 5% $CO_2$ in air. The culture media for all the experiments were preincubated for 1h at 39ºC under humidified atmosphere in 5% $CO_2$ in air. These culture dishes were incubated for six days at 39ºC under humidified atmosphere in 5% $CO_2$ in air. On every alternate day half of the medium was removed and replaced with equal volume of fresh medium. The Preantral follicles were morphologically evaluated every 48h during the culture period using an ocular micro meter attached to inverted microscope (Leica, DMIRB, Germany; 20 X Magnification) for increase in the follicular diameter and antrum formation (Fig. 1 A-D).

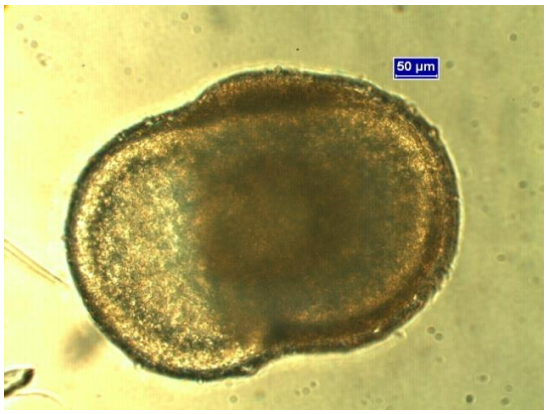

A: Freshly isolated preantral follicle (20 X)




B: 2-day cultured follicle (20 X)




C: 4-day cultured follicle (20 X)




D: 6-day cultured follicle (20 X)

**Fig 1:** Preantral follicles during different days of six day culture period

### 2.4 *In vitro* maturation (IVM) of oocytes obtained from cultured PFs'

After six days of culture, the cumulus oocyte complexes were isolated by opening the cultured PFs' mechanically under stereo zoom microscope which were further subjected to *in vitro* maturation for another 24h (Fig 2). The selected COCs were washed three times in the IVM medium (TCM199B supplemented with 10µg/ml FSH, 10 µg/ml Luteinizing hormone, 1 µg/ml estradiol-17β, 50 µg/ml Gentamicin sulphate, 10µg/ml bovine serum albumin (BSA) (A8412, Sigma, USA) and 10% (v/v) oestrous sheep serum). After being washed the COCs were placed individually in 20µl droplets of the same medium in 35mm plastic culture dishes, covered with pre equilibrated lightweight mineral oil, and incubated for 24h as described above.

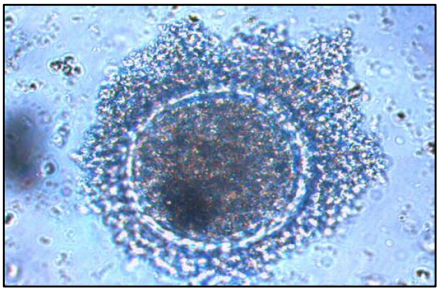


**Fig 2:** Cumulus oocyte complex after IVM

After IVM, the oocytes were denuded off cumulus cells by repeated pipetting through a fine bore glass pipette. Subsequently the oocytes were washed in Hoechst 33342 fluorescent stain solution (B 2261) and incubated in a 50µl droplet of the same solution for 15 minutes at 39ºC (Rao *et al.*, 2002) [22] which were examined under fluorescent light on an inverted microscope (Leica, Germany; excitation: 352-455nm and emission 460-490nm) for M II stage (Fig. 3).

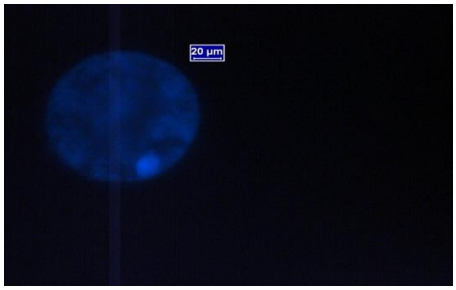



**Fig 3:** Metaphase II stage of oocyte from cultured preantral follicles

### 2.5 Statistical analysis

The significant differences between the follicular diameter, and meiotic maturation of oocytes to MII were analysed by one way ANOVA and Duncan's multiple range test. The mean values were considered significant when the P values were less than 0.05. SPSS 20 software was employed for all the above analysis.

## 3. Results

### 3.1 Morphological evaluation of Preantral follicles

In the present study a total of nine hundred and ten (910) preantral follicles were cultured in *in vitro* conditions in six different treatments (T1-T6). Among all the treatments preantral follicles co-cultured with cumulus cells in TCM 199 B (T3) was found to be the best combination, as proportions of preantral follicles exhibiting growth in terms of follicular diameter, antrum formation and meiotic maturation of oocytes to MII are significantly higher in T3 than in other treatments (Table 1). It is noteworthy that among the different treatments, the descending order of proportion of PFs' exhibiting growth, increase in diameter and antrum formation is in the order of T3>T4>T5>T6>T2>T1.

From Table 1 it was observed that growth and development of preantral follicles was significantly higher in the co-cultured experiments (T3-T6) than the groups without Co-culture (T1 and T2). However, among the co-cultured groups, co-culturing of PFs' with cumulus cells was found to yield significantly highest proportion of follicles to grow with respect to all parameters studied (T3 and T4). This observation is remarkably evident on day six of the culture period. T3 and T4 (T3>T4) showed significantly increased proportion of PFs' showing antrum formation than remaining treatments. Our findings in this study revealed that, culturing of PFs' with Cumulus cells in TCM 199B supported higher proportion of follicles to grow, increase their diameter, antrum formation as well as maturation of the oocytes in COCs isolated at the end of culture and subjected to *in vitro* maturation (IVM) for 24h.

### 3.2 Assessment of nuclear status of oocytes

The nuclear status was identified by observing the Metaphase-II stage which is considered as mature stage. It is observed that co-culturing of preantral follicles with cumulus cells in TCM 199B (T3; 16%) yielded significantly proportion of oocytes reaching MII (Table 1). However, all the other stages of oocytes obtained after IVM *viz*., Germinal vesicle (GV), Germinal vesicle break down (GVBD) and Metaphase-I (M-I) were considered as immature oocytes (Fig. 3).

**Table 1:** Effect of c- culturing of preantral follicles in cumulus cells or granulosa cells in different combinations on *in vitro* development of sheep preantral follicles.

| Experiment | Proportion of PFs' exhibiting growth (Mean ± SE) | Average increase in diameter (µM) (Mean ± SE) | Proportion of PFs' exhibiting antrum formation (Mean ± SE) | Proportion (%) of PFs' matured to MII* (Mean ± SE) |
|---|---|---|---|---|
| T1 (4/160) | 56.40±5.10$^{a}$ | 11.60±2.10$^{a}$ | 37.20±3.10$^{a}$ | 7±2.48$^{a}$ |
| T2 (4/180) | 73.96±7.70$^{b}$ | 21.74±2.90$^{a}$ | 52.21±8.30$^{b}$ | 11±1.85$^{a}$ |
| T3 (4/140) | 98.54±2.69$^{c}$ | 123.71±6.18$^{b}$ | 96.52±1.22$^{c}$ | 16±1.75$^{b}$ |
| T4 (4/140) | 94.89±3.02$^{c}$ | 91.35±4.97$^{c}$ | 73.97±7.70$^{d}$ | 13±1.47$^{a}$ |
| T5 (3/130) | 85.81±4.40$^{bc}$ | 54.32±9.40$^{d}$ | 68.30±5.10$^{d}$ | 14±0.69$^{a}$ |
| T6 (4/160) | 80.21±6.50$^{c}$ | 47.90±6.20$^{d}$ | 53.50±7.90$^{b}$ | 12±0.56$^{a}$ |

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

* Meiotic maturation of oocytes in COCs isolated from six day cultured follicles in different treatments and subjected to IVM for additional 24h

## 4. Discussion

In the present study the effect of Co-culture of preantral follicles with cumulus cells and granulosa cells is systematically investigated for the first time in sheep. This approach may be useful in improving the culture conditions of sheep preantral follicles *in vitro*. In our study, in all the treatments increase in the follicular diameter was observed by the end of the culture period when compared to day zero. However, co-culture of PFs' with cumulus cells in TCM 199 B (T3) showed significantly higher diameter, growth rate and meiotic maturation of oocytes than in all other treatments. Co-culturing of preantral follicles with somatic cells (cumulus cells and granulosa cells) resulted in positive growth of the PFs' in terms of follicular diameter, antrum formation and meiotic competence of the oocytes to metaphase-II. Glucose metabolism plays a significant role in every aspect of oocyte maturation including synthesis of nucleic acid and purine, substrates for matrices production, mucification, cellular homeostasis, cellular signalling and nuclear maturation (Sutton *et al.*, 2003) [24] and any alterations in this mechanism may influence oocyte competence (Sutton-McDowall *et al.*, 2010) [25]. But the oocyte itself has a relatively poor capacity to utilize glucose (Biggers *et al.*, 1967; Sutton-McDowall *et al.*, 2010) [25]. Therefore, these somatic cells supply intermediate metabolites of glucose like pyruvate which might support the development and maturation of the oocyte. Cumulus Cells are known to metabolize the bulk of glucose consumed by the COC, supplying metabolic intermediates like pyruvate, mainly via glycolysis, to the oocyte. Cumulus Cells support the development and maturation of the oocyte during the antral phase, and in turn, the oocyte responds to and regulates its surroundings in tandem with CCs to ensure conditions are conducive for its growth (Gilchrist *et al.*, 2006) [13]. Positive growth of the PFs' might be due to the paracrine factors secreted by the somatic cells during *in vitro* conditions. Also it was reported earlier that cumulus cells play an important role in coordinated follicular development (Fauser *et al.*, 2011) [10]. During *in vitro* conditions, presence of cumulus or granulosa cells might secrete factors that are essential for the growth and development of the preantral follicles. These results are parallel with the findings of Wu *et al.*, 2002 [27] and Ramesh *et al.*, 2007 [21] wherein they also reported the increased growth rate of preantral follicles when co-cultured with cumulus cells or granulosa cells. Foong *et al.*, 2006 [11] reported that cumulus cells from the pre ovulatory follicles contain higher extent of LH-receptors whose activation by supplemented LH in IVM medium results in triggering of varied intra cellular signalling pathways of ovulation. This can be correlated to the higher MII % in the present study in the oocytes from PFs' cultured along with cumulus cells.

Even though Co-culturing of preantral follicles with both somatic cells i.e., cumulus cells and granulosa cells resulted in positive growth of the PFs, significantly higher growth was observed in PFs' co-cultured with cumulus cells as compared to granulosa cells. This could be attributed to the differential expression of Slc38a3 and Amh. The expression of Slc38a3 which codes a sodium-coupled neutral amino acid transporter is found to be limited to Cumulus Cells with little or no expression in granulosa cells (Eppig *et al.*, 2005) [8] which appears to enhance amino acid transport to support oocyte development. Similarly, Amh expression is restricted to Cumulus Cells during antrum formation. Yihui Zhang *et al* (2014) [30] reported that the addition of 100 ng/ml of rh-AMH to IVM medium improved oocyte quality and they suggested that Anti-Mu¨llerian hormone improves oocytes quality by up-regulating GDF9 and BMP15 mRNA expressions during IVM. Their results also indicated that AMH mRNA expression and protein were localized only in cumulus cells. In our study, better growth observed in PFs' co-cultured with cumulus cells compared to granulosa cells could be attributed to the expression of Amh, which improves oocytes quality by up-regulating GDF9 and BMP15 mRNA expressions. Higher Proportion (%) of PFs' maturation to MII in PFs' co-cultured with cumulus cells can also be attributed to a diffusible heat stable meiosis activating substance produced by cumulus cells only (Byskov *et al.*, 1997) [5].

Earlier reports also indicated the effects of interaction between oocyte secreted factors and somatic cells on cellular proliferation and differentiation (Brankin *et al.*, 2003) [4]. In porcine species it was reported that cumulus expansion enabling factor (CEEF) produced by the oocytes, a key factor in cumulus expansion. Such production of associated paracrine factors was essential for the development of PFs' *in vitro*. In our study co-culture of PFs' with cumulus cells in TCM 199B supported better development of PFs' than in with standard medium. Although standard medium was proved to be better for development of PFs' *in vitro* (Arunakumari *et al.*, 2010) [1], the effects of co-culture were more pronounced in the PFs' cultured in TCM 199B than in standard medium particularly with respect to co-culture with cumulus cells. It could be due to attenuating effect of Anti-Mu¨llerian hormone secreted by cumulus cells on follicle stimulating hormone (FSH) in standard medium (Dewailly *et al*., 2016; Durlinger *et al.*, 2016) [6, 7].

Therefore it is concluded that culturing of PFs' in TCM 199B in association with cumulus cells had supported better development of PFs' *in vitro*. However, the paracrine factors secreted by cumulus or granulosa cells and their expression pattern during *in vitro* co culture needs further investigation to elucidate the effects in more detailed manner.

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