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Influence of TCM 199B, α-MEM, Waymouth MB 752/1 culture media, VEGF, Estradiol-17β, GDF-9 and FGF on in vitro development of preantral follicles in sheep.

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ABSTRACT

A total of 2792 preantral follicles (PFs') isolated from 750 ovaries of sheep were cultured in four different experiments. The efficacy of three commercially available culture media viz., TCM 199B, α-MEM and Waymouth MB 752/1 on the growth of sheep PFs' was tested in experiment I. Among the three media TCM 199B supported better development of PFs' in culture. The remaining experiments established the best concentrations of vascular endothelial growth factor (VEGF), Estradiol-17β (E2), GDF-9, Fibroblast growth factor (FGF) and their best combinations for the in-vitro development of PFs'. Inclusion of VEGF at 10 ng/mL, Estradiol-17β at 5 ng/mL, GDF-9 at 10 ng/mL or FGF at 10 ng/mL individually in a standard medium (SM) (containing FSH, IGF-I, GH and T4) supported better nuclear maturation of the oocytes to MII stage. Different combinations of VEGF, Estradiol-17β, GDF-9 and FGF supplemented in the SM promoted similar overall follicular growth. However, (a) SM + VEGF(10 ng/mL) + E2(5 ng/mL) supported higher increase in the diameter, (b) SM without any supplements induced antrum formation in greater proportion of follicles, and (c) SM + VEGF(10 ng/mL) + GDF 9(10 ng/mL) or SM + E2 (5 ng/mL) + FGF(10 ng/mL) supported high proportion of oocytes to reach MII stage. To conclude, TCM 199B appeared to be a better medium for development of sheep PFs'. VEGF, Estradiol-17 β, GDF-9 and FGF have beneficial influence on the development of sheep PFs' when supplemented in TCM 199B

Abbreviations
Preantral follicles (PFs')
Vascular endothelial growth factor (VEGF)
Estradiol-17β (E2)
Growth differentiation factor-9 (GDF-9)
Fibroblast growth factor (FGF)
Bicarbonate-buffered tissue culture medium 199 (TCM199B)
α-minimum essential medium (α-MEM)
In vitro Maturation (IVM) medium
Estrus sheep serum (ESS)
HEPES-buffered tissue culture medium 199 (TCM 199H)
Cumulus oocyte complexes (COCs)
Standard medium (SM)

1. Introduction

Oocytes in primordial and preantral follicles (PFs') in the ovaries represent a huge resource of female germplasm. Therefore, efforts are continuing to harvest, mature and fertilize the oocytes in PFs' of laboratory and domestic animals. While live offspring from cultured PFs' could be produced only in the murine species (Hasegawa et al., 2006, Mochida, Ogasawara & Koyama, 2006), in domestic animals, in vitro embryo production from the oocytes in cultured PFs' was reported in the pig (Wu et al., 2001, Emery & Carrell, 2001), buffalo (Gupta et al., 2008, Ramesh, Manjunatha, Nandi & Ravindra, 2008), sheep (Arunakumari et al., 2010) and goat (Magalhaes et al., 2011). However, the frequency of maturation of oocytes in the cultured PFs' in different species has been relatively low (Kamalamma et al., 2016; Silva et al., 2014). Earlier efforts in the laboratory to improve the development of sheep PFs' in vitro included the studies on the influence of several

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growth factors, hormones and their combinations (Arunakumari et al., 2010, 2007; Hemamalini et al., 2003; Kamalamma et al., 2016; Rajarajan et al., 2006; Tamilmani et al., 2005). However, the influence of different commercially available culture media, vascular endothelial growth factor (VEGF), Estradiol- 17β (E2) growth differentiation factor-9 (GDF-9) and fibroblast growth factor (FGF) on in vitro development of PFs' in sheep were not investigated earlier. Accordingly, the present study analyzed the ability of different (i) commonly used culture media - α-MEM, Bicarbonate-buffered tissue culture medium 199 (TCM199B) and Waymouth MB 752/1 and (ii) concentrations of VEGF, Estradiol-17β, GDF-9 and FGF to support in vitro development of PFs' in sheep. In addition, the effects of inclusion of these growth factors individually and in different combinations in a medium that routinely supports good development of sheep PFs' in the laboratory (Arunakumari et al., 2010, 2007;Chakravarthi et al., 2015, 2016a, 2016b; Kumar et al., 2019; Lakshminarayana et al., 2014, Praveen Chakravarthi, Brahmaiah & Rao, 2014; Srividya et al., 2017) were also examined.

2. Materials and methods

This study was undertaken as per the guidelines of the Institutional Research and Animal Ethics Committees of the College of Veterinary Science, Tirupati. Unless otherwise mentioned, the culture media, hormones, growth factors and all the other chemicals were procured from Sigma Chemical Co. (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). However, fetal calf serum (FCS) was supplied by PAN Biotech, Germany and Waymouth medium by GIBCO life technologies. All the hormones and growth factors used were cell culture tested and endotoxin free. Barring the media supplemented with different growth factors and hormones, all the other solutions were filtered through a 0.22 μm sterilizing filter (Sartorius, Germany) prior to use. All the procedures employed in the study were previously reported from the laboratory (Arunakumari et al., 2010, 2007; Chakravarthi et al., 2015, 2016a, 2016b; Hemamalini et al., 2003; Kamalamma et al., 2016; Kona et al., 2016; Kumar et al., 2019; Lakshminarayana et al., 2014; Rajarajan et al., 2006; Srividya et al., 2017;; Tamilmani et al., 2005;). However, a sufficiently brief explanation of all the methods employed in this study is presented.

2.1. Culture media

Bicarbonate-buffered tissue culture medium 199 (TCM199B) (M4530), α-minimum essential medium (α-MEM) (M8042), and Waymouth MB 752/1 (11220–035) were all routinely supplemented with gentamicin sulfate (10 mg/mL) filtered through 0.22 μm filter and stored at 4 °C till used.

2.2. Preparation of stock solutions of different growth factors and hormones

Stock solution of VEGF (V4512) was prepared by reconstituting 5 μg in 50 μL of cell culture grade water (W3500) to get a concentration of 0.1 μg/μL. Ten microliters of the above solution was reconstituted to a final volume of 1 mL to yield a concentration of 1 ng/μL.

Stock solution of Estradiol-17β was prepared by dissolving 0.1 mg of powder (E4389, water soluble, Bio availability was 50 mg/g of powder) in 5 mL of cell culture grade water to get a concentration of 1 ng/μL.

Stock solution of GDF-9 was prepared by reconstituting 20 μg of GDF-9 (SRP4872) in 200 μL of cell culture grade water to get a concentration of 0.1 μg/mL. Ten microliters of the above solution was reconstituted to a final volume of 1 mL to yield a stock concentration of 1 ng/μL.

Stock solution of FGF was prepared by reconstituting 10 μg of lyophilized desiccate of FGF (F3133) in 100 μL of TCM 199H containing 0.1% BSA (A8412) to yield a concentration of 0.1 μg/μL. Ten microliters of this solution was diluted with 990 μL of TCM 199H to yield a stock concentration of 1 ng/1 μL.

Stock solutions of all the above growth factors/hormones were stored separately in 100 μL aliquots at -20 °C until used

Supplementation of with one or more of the hormones/growth factors was achieved by appropriately mixing suitable volumes of the culture medium and stock solutions. For example 1998, 1980 and 1800 μL of TCM 199B was added with 2, 20 and 200 μL of VEGF stock solution (1 ng/ μL) to achieve a concentration of 1, 10 and 100 ng/mL of VEGF.

2.3. Preparation of the standard medium

Bicarbonate buffered tissue culture medium 199 (TCM 199B) was supplemented with 50 μg/mL gentamicin sulfate, 1 μg/mL L-thyroxine (T_4), 2.0 μg/mL follicle stimulating hormone (FSH), 10 ng/mL insulin like growth factor-I (IGF-I) and 1MIU/mL of growth hormone (GH). Unlike in our previous studies (Arunakumari et al., 2010, 2007; Chakravarthi et al., 2015, 2016a, 2016b; Hemamalini et al., 2003; Kamalamma et al., 2016; Kona et al., 2016; Kumar et al., 2019; Lakshminarayana et al., 2014; Rajarajan et al., 2006; Srividya et al., 2017; Tamilmani et al., 2005), FSH (F8174) and LH (L5269) used in the present study were of ovine rather than porcine and equine origin respectively. This medium referred to as the standard medium in the present study, routinely supports good development of sheep PFs' in vitro (Arunakumari et al., 2010, 2007; Chakravarthi et al., 2015, 2016a, 2016b; Hemamalini et al., 2003; Kamalamma et al., 2016; Kona et al., 2016; Kumar et al., 2019; Lakshminarayana et al., 2014; Rajarajan et al., 2006; Srividya et al., 2017; Tamilmani et al., 2005).

2.4. Preparation of in vitro maturation (IVM) medium

TCM 199B was supplemented with 10 μg/mL Follicle Stimulating Hormone, 10 μg/mL Luteinizing hormone, 1 μg/mL Estradiol-17, 50 μg/mL gentamicin sulfate and 10% (v/v) estrus sheep serum (ESS). This was stored at 4 °C for up to 1 week. The medium was equilibrated for 1 h at 39 °C in 5% CO_2 in air under humidified atmosphere prior to use.

2.5. Collection and processing of ovaries

Ovaries were collected at a slaughter house immediately and transported to the laboratory within 1 h in plastic sachets containing PBS placed in a thermos flask containing warm water (37 °C). On reaching the laboratory, the ovaries were placed in 35 mm plastic culture dishes (153066, Nalge Nunc, Denmark) and were trimmed off adherent tissues and ligaments. They are washed twice in handling medium (HEPES-buffered tissue culture medium 199 (TCM 199H) supplemented with 0.23 mM of sodium pyruvate, 2 mM L-glutamine and 50 μg/mL gentamicin sulfate). All the subsequent procedures were carried out in a laminar air flow (Nuaire, Class II type A B3, USA).

2.6. Isolation and selection of preantral follicles

The ovaries were cut into two halves and the medulla was removed. The ovarian cortices were cut into thin slices using a sterile surgical blade. These cortical slices were placed in the handling medium and intact preantral follicles (100–400 μm) in the size range of 250–400 μm were mechanically isolated by micro dissection under a stereo zoom microscope (SMZ 2T, Nikon corporation, Japan) using two 26 gage needles fitted to 1 mL syringe barrels. Care was taken to leave small amount of stromal tissue to avoid damage to the basement membrane. Preantral follicles having centrally placed, spherical oocytes with no signs of atresia and with intact basement membrane were considered good for culture (Fig 1a).

2.7. Culture and in vitro evaluation of development of preantral follicles

Differently supplemented culture media based on the experimental

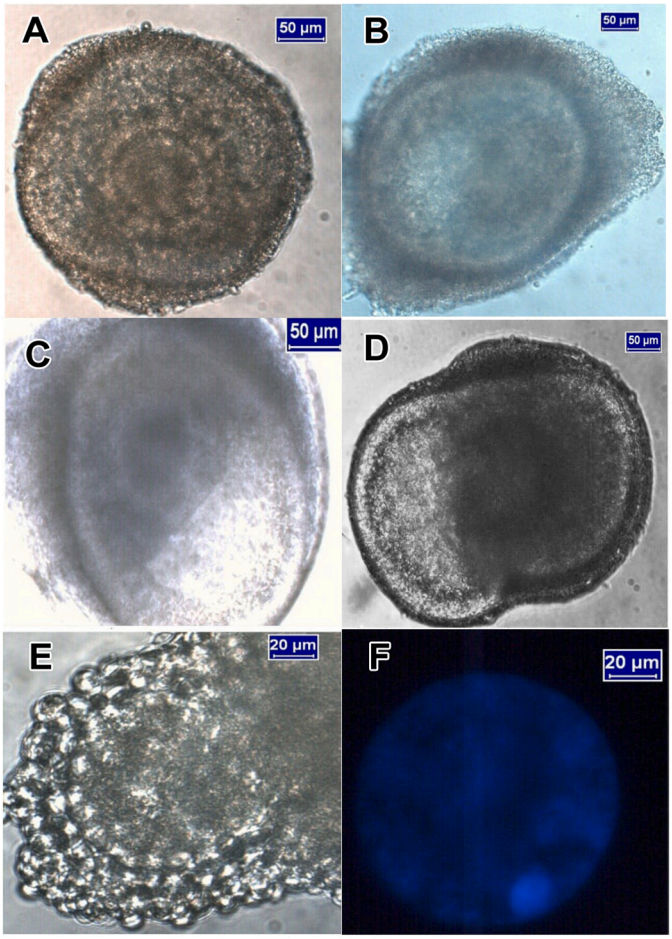

Fig. 1. In vitro development of preantral follicles in sheep (A) Typical preantral follicle selected for culture (B) Preantral follicle cultured for 2 days. Note the early development of antrum (C) Preantral follicle cultured for 4 days. Note the large antrum pushing the COC to one side within the follicle (D) Preantral follicles cultured for 6 days. (E) COC collected from 6 day cultured Preantral follicles. (F) Oocyte from COC of 6 day cultured follicle matured in vitro for 24 h to MII stage as observed by Hoechst Staining.

protocol was pre-incubated for 1 h at 39 °C under humidified atmosphere in 5% CO_2 in air. The selected follicles were washed thrice in the culture medium and subsequently placed individually in 20 µL droplets of the culture medium in 35 mm plastic tissue culture dishes. To avoid evaporation of the medium, the micro-droplets were overlaid with autoclaved lightweight mineral oil (M 8410) pre-equilibrated with the medium overnight at 39 °C in 5% CO_2 in air. These culture dishes were incubated at 39 °C in 5% CO_2 under humidified atmosphere in air for up to 6 days. Day on which the PFs' were placed in culture was designated day zero and subsequent days as 1, 2, 3 and so on. Half of the medium was replaced by an equal volume of fresh medium every 48 h. This culture procedure supported good development of sheep PFs' in earlier studies (Chakravarthi et al., 2015, 2016a, 2016b; Kamalamma et al., 2016; Kona et al., 2016; Kumar et al., 2019; Lakshminarayana et al., 2014; Srividya et al., 2017). Each follicle was morphologically evaluated every 24 h during culture period using an inverted microscope (Leica, DMIRB, Germany) for the proportions of follicles exhibiting growth, increase in the diameter and antrum formation (Fig. 1b, c, d). At the end of the culture, the follicles were carefully opened using two 26 gage needles attached to 1 mL syringe barrels, to release the cumulus oocyte complexes (COCs).

2.8. In vitro maturation of oocytes from in vitro cultured follicles

The cumulus oocyte complexes (COCs) (Fig. 1e) were washed thrice in the in vitro maturation medium (2.4). The COCs were placed individually in 20 µL droplets of IVM medium in 35 mm tissue culture dishes. The droplets were overlaid with autoclaved and pre-equilibrated lightweight mineral oil and incubated at 39 °C in 5% CO_2 under humidified atmosphere for 24 h. At the end of IVM period 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 and incubated in a 50 µL droplet of the same solution for 15 min at 39 °C (Rao et al., 2002). Later the oocytes were examined for MII stage under fluorescent light (Leica BG 38 filter cube; excitation 352–455 nm and emission 460–490 nm) on a Leica DMIRB inverted microscope. (Fig. 1f)

2.9. Experiments

Four different experiments were conducted in this study:

1 Three commercially available media viz., α-MEM, TCM 199B and Waymouth Medium MB 752/1 were tested for their ability to support in vitro growth of PFs' in sheep.
2 The medium that fared better in experiment 1 was supplemented with VEGF, Estradiol-17β, GDF-9 or FGF individually. Each of these growth factors was supplemented at three different concentrations based on a preliminary investigation (data not shown) as there are no prior publications on the use of these growth factors in the culture of PFs'.
3 Concentrations of growth factors that supported similar development of preantral follicles in experiment 2 were supplemented in the standard medium (SM).
4 Standard medium was supplemented with various combinations of the best individual concentrations of the growth factors arrived at in experiments 2 and 3 and used for the culture of PFs' as above in an attempt to improve the development of the PFs'.

2.10. Statistical analysis

Comparisons of proportion of follicles exhibiting growth, increase in mean follicle diameter, proportions of follicles exhibiting antrum formation and maturation of the oocytes to MII stage were undertaken by analysis of variance separately in different experiments using SPSS 15.0. This was followed by Duncan's multiple range test (DMRT) to test the significance of difference among different treatment groups for the various parameters studied.

3. Results

TCM 199B induced significantly higher proportion of PFs' to exhibit growth, increase in diameter and antrum formation. However, none of

Table 1
Influence of TCM 199B, α-MEM and Waymouth MB 752/1 media on in vitro development of sheep preantral follicles

Media (replicates / no. follicles: 8/64)	% PFs' exhibiting growth	Increase in diameter (µm)	% PFs' exhibiting antrum formation	% Oocytes in cultured PFs' matured to MII
TCM 199B	73.9 ± 7.7^{a}	21.7 ± 2.9^{a}	52.2 ± 8.3^{a}	00.0
α –MEM	47.1 ± 7.3^{b}	10.2 ± 2.0^{b}	36.5 ± 6.4^{ab}	00.0
Waymouth MB752/1	57.8 ± 10.7^{ab}	13.1 ± 1.8^{b}	23.5 ± 6.9^{b}	00.0

Values are expressed as Mean ± SE.
Figures with different superscripts within a column are significantly different ($P \leq 0.05$).

the media supported oocytes in COCs isolated from cultured PFs' to mature to MII stage subsequently (Table 1). As TCM 199B appeared to support better development of PFs' than the other media it was used in subsequent experiments.

The effects of different concentrations of the growth factors/hormones on in vitro development of the PFs' are shown in Table 2. VEGF at 1 and 10, Estradiol-17β at 5 and 25, GDF-9 at 10 and 50 and FGF at 10 and 50 ng/mL supported similar (>20%) development of the sheep PFs' in vitro (Table 3).

Standard medium supplemented with either VEGF(10 ng/mL) + GDF-9(10 ng/mL) (T2, Table 4) or E2(5 ng/mL) + FGF(10 ng/mL) (T5, Table 4) afforded significantly higher proportion of the oocytes to mature to MII stage.

4. Discussion

This is the first study that compared three different commercially available media viz., TCM 199B, α-MEM and Waymouth MB 752/1 for their ability to support in vitro development of PFs' in sheep. Further individual and combined influences of VEGF, Estradiol-17β, GDF-9 and FGF on in vitro development of sheep PFs' were also investigated for the first time.

Present observation that TCM 199B allowed better development of the PFs' is in agreement with reports in sheep, goat (Andrade et al., 2014; Arunakumari et al., 2010, 2007; Chakravarthi et al., 2015, 2016a, 2016b; Kamalamma et al., 2016; Kona et al., 2016; Kumar et al., 2019; Lakshminarayana et al., 2014; Magalhaes et al., 2011; Silva et al., 2014; Srividya et al., 2017) and in bovines (Castro et al., 2014; Rossetto et al., 2013) although Waymouth medium was not tested in those studies. Another study in bovines claimed that TCM 199 could be replaced by α-MEM only if fresh medium was continuously added (Araújo et al., 2015). In a recent study (Jimenez et al., 2016), it was reported that α-MEM supported better development of the ovarian cortical slices in the bovine. However, relative to TCM 199 and Waymouth media, α-MEM reportedly supported better growth of PFs' in the mouse (Kim

Table 2
Effect of different concentrations of VEGF, Estradiol-17β, GDF-9 and FGF on in vitro development of sheep preantral follicles.

Growth factor/ hormone ng/ml (replicates/no. follicles)	% PFs' exhibiting growth	Increase in diameter (μm)	% PFs' exhibiting antrum formation	% Oocytes in cultured PFs' matured to MII
VEGF				
0 (8/48)	56.4 ± 5.1	11.6 ± 2.1	37.2 ± 3.1	2.0 ± 2.1^{c}
1 (8/72)	56.3 ± 4.8	13.4 ± 2.1	19.6 ± 9.4	13.0 ± 3.5^{ab}
10 (8/72)	60.5 ± 6.2	16.5 ± 2.9	28.5 ± 6.4	20.0 ± 9.6^{a}
100 (8/72)	44.4 ± 5.3	9.6 ± 1.9	24.2 ± 9.0	10.0 ± 2.4^{b}
Estradiol-17 β				
0 (8/48)	56.4 ± 5.1 ^{a}	11.6 ± 2.1 ^{b}	37.2 ± 3.1	2.0 ± 2.1 ^{c}
5 (8/56)	58.6 ± 4.4 ^{a}	8.2 ± 1.8 ^{ab}	48.6 ± 8.5	17.0 ± 2.3 ^{a}
25 (8/56)	53.3 ± 10.0 ^{a}	8.3 ± 1.9 ^{ab}	31.0 ± 9.7	15.0 ± 3.8 ^{ab}
50 (8/56)	32.5 ± 7.6 ^{b}	3.9 ± 1.3 ^{a}	25.2 ± 9.0	10.0 ± 2.4 ^{b}
GDF-9				
0 (8/48)	56.4 ± 5.1	11.6 ± 2.1 ^{ab}	37.2 ± 3.1	2.0 ± 2.1^{b}
10 (8/64)	63.8 ± 10.1	15.4 ± 2.4 ^{a}	42.3 ± 9.4	20 ± 6.7^{a}
50 (8/64)	57.8 ± 8.9	13.9 ± 2.3 ^{ab}	35.9 ± 10.7	14 ± 7.3 ^{a}
100 (8/64)	40.6 ± 5.6	7.8 ± 1.7 ^{b}	22.5 ± 7.7	05.0 ± 3.9^{b}
FGF				
0 (8/48)	56.4 ± 5.1 ^{a}	11.6 ± 2.1	37.2 ± 3.1 ^{a}	2.0 ± 2.1^{b}
10 (8/64)	63.2 ± 9.3 ^{a}	16.2 ± 2.9	40.1 ± 9.3 ^{a}	23.7 ± 6.7 ^{a}
50 (8/64)	63.5 ± 6.9 ^{a}	13.9 ± 2.4	39.3 ± 6.4 ^{a}	17.4 ± 3.0 ^{a}
100 (8/64)	38.9 ± 7.5^{b}	10.5 ± 2.3	22.4 ± 5.4^{b}	15.0 ± 4.2 ^{a}

Values are expressed as Mean ± SE.
Figures with different superscripts within a column are significantly different ($P \leq 0.05$).

Table 3
Influence of supplementation of the standard medium (SM) with VEGF, Estradiol 17β, GDF-9 or FGF on in vitro development of sheep preantral follicles.

Growth factor concentration (replicates/no. follicles)	% PFs' exhibiting growth	Increase in diameter (μm)	% PFs' exhibiting antrum formation	% Oocytes in cultured PFs' matured to MII
VEGF				
SM (7/49)	65.3 ± 5.1	27.3 ± 4.4	27.7 ± 6.4	10.8 ± 4.4 ^{a}
SM +1 ng/ml (7/70)	79.6 ± 6.5	17.8 ± 4.7	26.2 ± 4.5	13.2 ± 2.9 ^{a}
SM +10 ng/ml (7/70)	75.2 ± 4.3	24.8 ± 4.3	24.2 ± 6.1	28.8 ± 5.7 ^{b}
Estradiol-17 β				
SM (5/50)	65.3 ± 5.1	27.3 ± 4.4	27.7 ± 6.4	10.8 ± 4.4 ^{a}
SM + 5 ng/ml (5/50)	62.0 ± 10.3	20.5 ± 5.5	24.5 ± 5.6	38.8 ± 9.6 ^{a}
SM + 25 ng/ml (5/50)	69.6 ± 9.1	26.1 ± 6.0	16.4 ± 5.4	14.6 ± 8.4 ^{b}
GDF-9				
SM (7/49)	65.3 ± 5.1	27.3 ± 4.4	27.7 ± 6.4	10.8 ± 4.4 ^{b}
SM + 10 ng/ml (7/56)	63.5 ± 9.1	16.5 ± 2.5	18.5 ± 5.9	32.4 ± 3.6 ^{a}
SM + 50 ng/ml (7/56)	57.1 ± 12.4	20.8 ± 4.7	28.4 ± 3.7	13.3 ± 5.2 ^{b}
FGF				
SM (6/48)	65.3 ± 5.1	27.3 ± 4.4	27.7 ± 6.4	10.8 ± 4.4 ^{b}
SM + 10 ng/ml (6/60)	65.8 ± 10.1	22.5 ± 6.0	11.7 ± 4.5	37.6 ± 7.6 ^{a}
SM + 50 ng/ml (6/60)	58.6 ± 12.8	21.2 ± 7.1	19.1 ± 7.3	14.1 ± 4.6 ^{b}

Values are expressed as Mean ± SE.
Figures with different superscripts within a column are significantly different ($P \leq 0.05$).

Table 4
Influence of supplementing the standard medium with various combinations of growth factors / hormones on in vitro development of sheep preantral follicles.

Growth factors & hormone combinations (ng/ml) (replicates / no. follicles:9/81)	% PFs' exhibiting growth	Increase in diameter (μm)	% PFs' exhibiting antrum formation	% Oocytes in cultured PFs' matured to MII
T1. VEGF:10 + E 2:5	80.2 ± 8.7	46.6 ± 10.0 ^{c}	23.4 ± 3.4 ^{a}	30.1 ± 4.1 ^{de}
T2. VEGF:10 + GDF 9:10	69.6 ± 5.5	27.4 ± 6.7^{abc}	39.9 ± 6.1 ^{ab}	36.1 ± 3.6 ^{e}
T3. VEGF:10 + FGF 10	62.3 ± 8.7	30.1 ± 7.8^{abc}	28.9 ± 7.5 ^{a}	24.4 ± 2.2 ^{cd}
T4. E 2.5 + GDF 9:10	76.1 ± 8.5	34.2 ± 5.4^{abc}	26.5 ± 5.3 ^{a}	15.2 ± 3.2 ^{a}
T5. E 2:5 + FGF:10	75.4 ± 8.3	23.3 ± 7.2 ^{ab}	53.5 ± 7.9 ^{b}	35.5 ± 4.9 ^{e}
T6. GDF 9:10 + FGF:10	80.9 ± 6.5	20.2 ± 4.2^{ab}	43.8 ± 8.8^{ab}	15.6 ± 2.7^{a}
T7. VEGF:10 + E 2:5 + GDF 9:10	84.2 ± 5.2	40.1 ± 6.8^{bc}	36.3 ± 5.2 ^{ab}	13.4 ± 0.8 ^{ab}
T8. VEGF:10 + E 2:5 + FGF:10	85.8 ± 4.4	23.5 ± 5.8 ^{ab}	43.2 ± 9.8 ^{ab}	18.3 ± 2.2^{abc}
T9. VEGF:10 + GDF 9:10 + FGF:10	63.3 ± 4.4	16.7 ± 3.4^{a}	26.0 ± 5.2 ^{a}	24.3 ± 4.0^{bcd}
T10. E 2:5 + GDF 9:10 + FGF:10	82.7 ± 6.3	32.6 ± 5.5^{abc}	21.8 ± 5.0 ^{a}	16.6 ± 4.8^{abc}
T11. VEGF:10 + E 2:5 + GDF 9:10 + FGF:10	85.2 ± 5.9	30.1 ± 7.0^{abc}	39.3 ± 11.0 ^{ab}	32.14 ± 2.7 ^{de}
T12: SM (Control)	73.1 ± 6.6	37.9 ± 6.2^{abc}	54.3 ± 9.4^{b}	23.6 ± 3.0 ^{bcd}

E2 = Estradiol −17β.
Values are expressed as Mean ± SE.
Figures with different superscripts within a column are significantly different ($P \leq 0.05$).

et al., 2008, Seong & Lee, 2008). In another study TCM 199B and α-MEM fared similarly in supporting the growth of murine PFs' (Heidari et al., 2012, Malekshah, Azami & Mirhoseini, 2012). It is enticing, from these studies, to suggest that TCM 199 may be better suited for the culture of PFs' in farm animals and α-MEM in lab animals. It is also possible that the higher growth rate of PFs' in TCM 199B in the present and earlier studies in the farm animals was due to the exclusive presence of retinol tocopherol and calciferol specifically required in the farm animals (Moellers & Riese, 1988; Schweigert & Zucker, 1988). However, such specific requirements need be empirically confirmed. It was speculated that the adenosine sulfate present in TCM 199 protects the PFs' from oxidative stress during culture (Rossetto et al., 2013). However, if this was the case, TCM 199 should have supported better development of PFs across various species studied. Laboratory specific factors such as the use of ovarian cortical slices (Andrade et al., 2014; Castro et al., 2014; Jimenez et al., 2016), culture in groups (Andrade et al., 2014; Castro et al., 2014; Jimenez et al., 2016), individual PFs', (Araújo et al., 2015; Heidari et al., 2012; Kim et al., 2008; Rossetto et al., 2013), length of culture (Andrade et al., 2014; Araújo et al., 2015; Castro et al., 2014; Heidari et al., 2012; Jimenez et al., 2016; Kim et al., 2008; Rossetto et al., 2013), composition and frequency of change/addition of the culture medium (Andrade et al., 2014; Araújo et al., 2015; Castro et al., 2014; Heidari et al., 2012; Jimenez et al., 2016; Kim et al., 2008; Rossetto et al., 2013) initial size and quality of the PFs' (Andrade et al., 2014; Araújo et al., 2015; Castro et al., 2014; Heidari et al., 2012; Jimenez et al., 2016; Kim et al., 2008; Rossetto et al., 2013) might also be responsible for the observed differences in the ability of different media to support in vitro development of PFs' in different species.

The influence of supplementation of TCM 199B with 1 or 10 ng/mL of VEGF on the growth of PFs' was similar to that reported in the bovines (Yang & Fortune, 2007) and goats (Bruno et al., 2009). However, better nuclear maturation with 100 ng/mL of VEGF reported earlier in goats (Araújo et al., 2011) was not observed in the present study. This difference might be due to the differences in species, basic culture medium (TCM 199B in the present study versus Waymouth MB 752/1 or α-MEM in earlier studies) and/or the use of cortical slices in earlier reports. Interestingly supplementation of the SM with VEGF (10 ng/mL) afforded better development of preantral follicles in the present study. As the mean increase in size of PFs' was better in the standard medium (Arunakumari et al., 2010) and since innate expression of VEGF increases as the follicle growth progresses (Chowdhury et al., 2010, Scaramuzzi, Wheeler-Jones & Khalid, 2010; Fisher et al., 2013) lower dose of VEGF was apparently adequate to support better development of the PFs' in this study.

It was presumed that the supplementation of the culture medium with Estradiol-17β might be needed for the in vitro development of the PFs' based on earlier observation in the laboratory that the in vitro environment suppressed the expression of P450 aromatase gene needed for steroidogenesis (Lakshminarayana et al., 2014). This presumption was borne out by better nuclear maturation of the oocytes in the PFs' cultured in TCM 199B supplemented with either 5 or 25 ng/mL of Estradiol-17β. Interestingly SM was needed to be supplemented with only a lower dose of Estradiol-17β (5 ng/mL) to improve the nuclear maturation of the oocytes since it contained GH and FSH known to stimulate steroidogenesis in growing ovarian follicles in mammals (Roy & Greenwald, 1989; Serafim et al., 2015). Indeed, higher dose of Estradiol-17β in the present study decreased the nuclear maturation alluding to the possible toxicity.

The fact that the two lower doses of GDF-9 (10 and 50 ng/mL) appeared to perform equally and that a higher concentration (100 ng/mL) was no better than the control indicates a biphasic influence of GDF-9 on in vitro development of sheep PFs'. Thus continuous presence of GDF-9 at higher concentrations in the culture medium may not be desirable. The fact that nuclear maturation of the oocytes was favorably influenced by GDF-9 (10 ng/mL) without a significant influence on other growth parameters indicates the subtle and remote nature of the influence of this growth factor on PFs'. Adverse influence of the higher dose of GDF-9 on the nuclear maturation of the oocytes in cultured PFs' could be attributed to increased and stage specific expression of GDF-9 stimulated by FSH and thyroxine (Kobayashi et al., 2009; Kona et al., 2016) present in the standard medium.

Since FGF was known earlier to favorably influence DNA synthesis, antrum formation and nuclear maturation of the oocytes in cultured follicles in the hamster and buffalo (Gupta & Nandi, 2010; Gupta et al., 2001; Roy & Greenwald, 1991), present results provide an important confirmation in yet another mammalian species. Just as for GDF-9, minimum dose of FGF interacted better with other growth factors in the standard medium. Since there is a significant drop in the nuclear maturation of oocytes with 50 ng/mL of FGF and as maximum frequency of nuclear maturation was obtained with minimum dose, there may be scope for further improvement in the nuclear maturation between 10 and 50 ng/mL that needs to be explored in future studies.

In the earlier study in the laboratory (Arunakumari et al., 2010), the concentration of several of the growth factors/hormones were tested for their efficacy to support good development of PFs' in vitro while designing the medium referred to as the standard medium in the present study. However, supplementation of the standard medium with additional growth factors/hormones did not improve the in vitro development of PFs' in sheep. While several different combinations of the growth factors and hormones supported similar improvements in different growth parameters including the nuclear maturation of the oocytes, no single treatment proved to be better than the rest. This signposts several caveats in the present study: (i) If the so called standard medium was already optimized to support the best in vitro development of preantral follicles in sheep, naturally no further improvement could be expected. Since some combinations did perform better although statistically not significant SM is not yet optimized to support the best development of the PFs'. Further. Studies involving larger number of follicles and replicates might clarify the situation; (ii) The two concentrations of each of the growth factors that supported good development of the PFs' when supplemented in TCM 199B were subsequently tested by inclusion in the standard medium. This was done considering the possible interactions among them and also because several hormones and growth factors are known to act through yet other growth factors and/or hormones. Indeed, this strategy was able to differentiate between the two concentrations of the individual growth factors. However, inclusion of different combinations of growth factors in SM did not lead to improvements in the development of PFs'. Obviously, the doses of growth factors supplemented alone in TCM 199B or SM, when used in combination with the others are not suitable for the supplementation of the standard medium. It may be necessary to test much lower doses than tested in the present study. (iii) Finally, it was observed in the laboratory that several growth factor genes were expressed in a stage-specific manner in in vitro grown ovarian follicles (Chakravarthi et al., 2015, 2016a, 2016b; Kona et al., 2016; Lakshminarayana et al., 2014; Srividya et al., 2017) in sheep necessitating the supplementation with different (and possibly different levels of) growth factors/hormones at different stages of the development rather than making them available throughout the culture period. Such a strategy would exponentially increase the complexity of the experiments as the culture components, concentrations and times of exposure increase.

5. Conclusion

It is concluded that

(1) TCM 199B appeared to be a better medium for the culture of sheep PFs',
(2) VEGF, Estradiol-17β, GDF-9 and FGF all have beneficial influence on the development of sheep PFs' cultured in TCM 199B

CRediT authorship contribution statement

S.S.R. Kona: Conceptualization. **A.V.N. Siva Kumar:** Conceptualization. **B. Punyakumari:** Conceptualization. **R.V. Suresh Kumar:** Conceptualization. **V.H. Rao:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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