



Quantification of Estradiol 17 β (Er β) Receptor Gene in Cultured and *in vivo* Grown Ovarian Follicles of Sheep

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10.18805/IJAR.B-5473

ABSTRACT

Background: The study aimed at quantifying the estradiol 17 β receptor gene in *in vivo* developed and cultured ovarian follicles of sheep in different culture media by RT-q PCR. Since, estradiol 17 β is a key steroid hormone for antrum formation, preovulatory follicle maturation, expression of genes involved in ovarian differentiation and follicular rupture during ovulation of mammals and the local action of any hormone on the target cell or tissue depends on the expression of its receptor.

Methods: Estradiol 17 β receptor mRNA expression in sheep oocytes and cumulus cells was studied using quantitative RT-PCR for: (i) *In vivo* grown preantral, early antral, antral, large antral follicles and cumulus-oocyte complexes (COCs) obtained from large antral follicles subjected to 24 h of *in vitro* maturation and (ii) Accordingly, the cumulus cells and oocytes were collected from different development stages of preantral follicles cultured *in vitro* in TCM199B (Group I), TCM199B + E₂ (5 ng/mL) (Group II) and Standard medium + E₂ (5 ng/mL) (Group III) for 3 min, two, four or six days and subsequently matured *in vitro* for 24 h.

Result: Estradiol 17 β receptor was observed at all stages of ovarian follicles in both cumulus cells and oocytes. The upregulation of the ER β gene was noted as the follicle grew in both the media supplemented with estradiol 17 β . Irrespective of *in vitro* groups, cumulus cells from COCs of the large antral follicles matured *in vitro* for 24 h exhibited the highest gene expression of the estradiol 17 β receptor. Among the three groups under study, the follicular cells of PFs' cultured in group III showed the highest expression of the ER β gene at any point of their development. The supplementation of estradiol 17 β in low doses stimulated synergism with growth factors and hormones to express its receptors in cultured preantral follicles of sheep.

Key words: Estradiol 17 β receptor, Estradiol 17 β , Expression, *in vitro*, Preantral follicle.

INTRODUCTION

Estradiol 17 β is a key steroid hormone for antrum formation (Tasaki *et al.*, 2013), preovulatory follicle maturation (Drummond and Fuller, 2012), expression of genes involved in ovarian differentiation (Emmen *et al.*, 2005) and follicular rupture during ovulation of mammals. Estradiol 17 β is essential to support the growth and maturation of bovine primary follicles *in vitro* (Sun and Li, 2013).

Estradiol-17 β is synthesized by aromatase, present in the granulosa cells and its production depends on gonadotrophin secretion (Nelson and Bulun, 2001). In our earlier studies, it was noticed that expression of the aromatase 450 enzyme gene was suppressed during *in vitro* culture of sheep preantral follicles (PFs') (Lakshminarayana *et al.*, 2014). To circumvent this subsequently, the effect of supplementation of Estradiol-17 β (5 or 25 ng/mL) was studied during *in vitro* culture and found that a lower dose of estradiol-17 β (5 ng/mL) improved the nuclear maturation of the oocytes along with standard medium since it contained GH and FSH known to stimulate steroidogenesis in growing ovarian follicles in mammals and higher doses reduced the nuclear maturation (Kona *et al.*, 2021). Liu *et al.* (2017) showed that the estradiol (E2)-estrogen receptors (ERs) system in follicular granulosa cells has a dominant role in controlling oocyte meiotic resumption in mammals.

The ability of estradiol-17 β to exert various autocrine and paracrine actions is most likely dependent on the

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How to cite this article: Supriya, B., Kumar, A.V.N.S., Jamuna, K.V., Deepa, P. and Ramayya, P.J. (2025). Quantification of Estradiol 17 β (Er β) Receptor Gene in Cultured and *in vivo* Grown Ovarian Follicles of Sheep. Indian Journal of Animal Research. 1-6. doi: 10.18805/IJAR.B-5473.

Submitted: 14-08-2024 **Accepted:** 11-11-2024 **Online:** 10-01-2025

expression of its receptor subtype in specific ovarian cells. Estrogen receptor beta belongs to the nuclear receptor superfamily (Mangelsdorf *et al.*, 1995). Cardenas *et al.* (2001) identified the reading frame of ovine ER β consisting of 1581 nucleotides and its predicted translation produces a protein with 527 amino acids. Further quantification of the estrogen receptor gene will help to understand the role

of estradiol at different developmental stages of *in vitro* cultured preantral follicles and develop a suitable medium for PFs' culture. Therefore, the present study was undertaken to compare the quantitative expression of the estrogen receptor gene in the sheep oocytes and granulosa cells obtained from the *in vivo* grown and cultured ovarian follicles from preantral to Graafian follicle stages.

MATERIALS AND METHODS

The work was conducted in the Embryo Biotechnology Laboratory, Sri Venkateswara Veterinary University, Tirupati from 2020 to 22.

Collection of ovaries and isolation of different stages of ovarian follicles

Ovaries recovered after sheep slaughter were transported to the laboratory within 1h after slaughter in sterile, warm (37°C) phosphate-buffered saline. A total of 1017 intact preantral follicles (PFs') isolated from the collected ovaries in the size range of 250-400 μ m (Standardized in our lab) were used in the study. The PFs' having visible centrally placed oocytes without any signs of atresia and with intact basement membrane and no antral cavity were considered good for the culture. *In vivo* developed follicular stages including intact preantral (PFs'), early antral, antral and large antral follicles were mechanically isolated by microdissection method from ovarian cortex under a stereo-zoom microscope (SMZ 2T, Nikon Corporation, Japan) and also cumulus-oocyte complexes (COCs) were aspirated from Graafian follicles (Kona *et al.*, 2016). The PFs' were cultured for up to six days in different groups (three groups mentioned in 2.2) with subsequent *in vitro* maturation of the cumulus oocytes complex (COC) for an additional 24 h. The number of follicles obtained from each developmental stage of the follicle from *in vivo* and the three groups of cultured follicles are 30-50.

Culture media

TCM 199B without any supplementation (Group-I), TCM 199B supplemented with 5 ng/mL of estradiol-17 β (Sigma; Cat No. E4389, USA) (Group-II) and Standard medium (SM) (TCM 199B supplemented with 1 μ g/mL T4, 2.5 μ g/mL FSH, 10 ng/mL IGF-1, 1 mIU/mL of GH) supplemented with 5 ng/mL of estradiol-17 β (Group-III).

Selection and culture of the preantral follicles

Preantral follicles were cultured in bicarbonate buffered tissue culture medium 199 (TCM 199B) supplemented with 50 mg/mL Gentamicin sulphate and the Culture medium was pre-incubated for 1 h at 39°C under a humidified atmosphere of 5% CO₂ in the air. The selected follicles were washed thrice in the culture medium and placed individually in 20 μ L droplets of the culture medium in 35 mm plastic culture dishes (cat. No. 153066, Nalge Nunc, Denmark). To avoid evaporation of the medium, the microdroplets were overlaid with autoclaved lightweight mineral oil (Sigma M 8410). These culture dishes were incubated at 39°C under a humidified atmosphere in 5% CO₂ in the air for up to six days. Half the medium was replaced by an equal volume of fresh medium every 48 h. The cumulus cells and oocytes from preantral follicles (PFs') cultured for 3 min (Pre antral follicle), 2 days (early antral follicle), 4 days (Antral follicle), or 6 days (Large antral follicle) (Fig 1) in three differently supplemented media (mentioned in 2.2) were used for this study.

After six days of culture, the cumulus-oocyte complexes (COCs) were isolated mechanically with a 24G needle under a stereo-zoom microscope. These isolated COCs were subjected to further *in vitro* maturation for 24 h. The COCs were washed thrice in the IVM medium (TCM199B supplemented with 10 μ g/mL FSH, 10 μ g/mL Luteinizing hormone, 1 μ g/mL estradiol-17 β , 50 μ g/mL, gentamicin sulphate, 10 μ g/mL bovine serum albumin (BSA) (A8412,

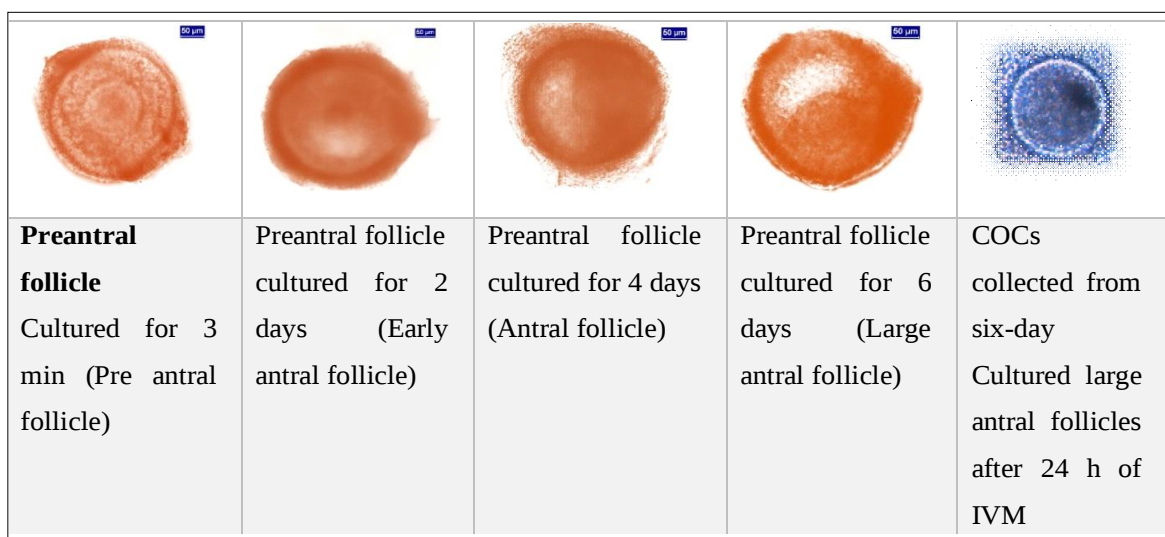


Fig 1: Different development stages of the *in vitro* cultured preantral follicles of sheep.

Sigma, USA) and 10 % (v/v) estrous sheep serum). After washing, the COCs were placed in microdroplet culture for 24 h. After IVM, the COCs were subjected to repeat pipetting through a fine-bore glass pipette during which the oocytes were denuded of cumulus cells. At the end of the culture, various cells of the follicles were used for RNA isolation.

Quantitative expression of estradiol 17 β receptor (ER β) gene

Isolation and quantification of total RNA from cumulus cells and oocytes in each group

Isolation of RNA was carried out by pooling cumulus cells and oocytes from 30-50 follicles at each development stage of follicles from *in vivo* and the three groups of cultured follicles (Groups I–III) using patented Medox-Easy spin column Total RNA Mini prep Kits (Pragna *et al.*, 2021) according to the manufacturer's instructions (Medox Biotech India Pvt. Ltd., Chennai, India). The concentration and purity of RNA were determined using Nanodrop lite (Thermo scientific S.No.1354). RNA samples having purity (Absorbance at 260/280) in the range of 1.8-2.1 only were used in the expression studies. The RNA sample was stored at -70°C till analyzed.

Reverse transcription (RT) and real-time PCR

High-capacity reverse transcription kit (Applied Biosystems, USA) was used for the reverse transcription. RT reaction was carried out for 10 min at 25°C, for 120 min at 37°C and for 5 min at 85°C in a thermocycler (Eppendorf Master Cycler Gradient) according to the manufacturer's instructions. In a comparison of twelve commonly used reference genes RPLPO, HPRT1 and 18SrRNA were the three most stably expressed genes in the sheep ovarian (unpublished observations in the laboratory). Therefore, the geometric mean of these three genes (Mamo *et al.*, 2007) was used as the normalizer in analyzing the expression of the estradiol 17 β receptor.

Real-time RT-qPCR was performed on the Applied Biosystems 7500 machine. Each 25 μ L reaction mix contained 12.5 μ L of Taq Man Universal PCR Master Mix (2x), 1.25 μ L of 20X gene expression assay mixture and 25 ng of cDNA sample in 11.25 μ L nuclease-free water. Thermal cycling conditions were Erase UNG (Uracil N-glycosylase) activation 2 min at 50°C, Ampli Taq Gold DNA polymerase activation 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Extreme Ct (threshold cycle number) values and 'no detection' in some of the samples were discarded before calculating RQ (relative quantification-RQ) values resulting in an unequal number of observations in different groups. For the calculation of the expression levels (RQ values) of different target genes, first the Ct values of target and reference genes were converted to quantity inputs using the formula 2 minimum Ct - sample Ct. Expression of the target genes was the ratio of target quantity input to that of the geometric mean of quantity inputs of reference genes (Table 1).

Experimental design

Quantitative expression of the estradiol 17 β receptor gene was studied at different development stages of the *in vivo* growth and corresponding stages of the three groups of cultured ovarian follicles (Table 2). The entire experiment was repeated four times. Triplicate samples of complementary DNA (cDNA) from each replicate of the experiment (4 $\times$ 3 =12 cDNA samples for each *in vivo* and *in vitro* stage) were subjected to a reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Statistical analysis

Stage of development and source (*in vivo* or *in vitro*) were the independent variables and the expression of the genes was the dependent variable. Log RQ values were analysed by two-way ANOVA (General Linear Model) with an unequal number of observations followed by Tukey–HSD multiple

Table 1: Primers details of target and reference genes.

Gene name	Symbol	Primers and probe sequences	Accession number
Estradiol 17 β receptor	ER17 β	F. Primer: CTGGTGCAGCGGAGAAG R. Primer: GCGAGGCCGAAGGA Probe: 6FAMACGTGTCCGCCTACATAMARA	AF110817
Reference (House keeping) genes			
Large ribosomal protein	RPLPO	F. primer: GCTCTGGAGAACTGTTGCC R. primer: CCAGCAGCATGTCCCTGAT Probe: 6FAM-AGGTCCTCCTTGGTGAACACGAAGC-BBQ	NM_001012682
Hypoxanthine-guanine phosphoribosyl transferase	HPRT1	F. primer: GCTCGAGATGTGATGAAGGAGAT R. primer: TCCAACAGGTCGCAAGAA Probe: 6FAM-AGCCCCCTTGAGCACACAGABBQ	AF176419
18S ribosomal RNA	18SrRNA	F. primer: AACAAATACAGGACTCTTTCGAGGC R. primer: CAGACTTGCCCTCCAATGGA Probe: 6FAM-CCACTTTAAATCCTTCCGCGAGGAT-BBQ R. primer: CCAGCAGCATGTCCCTGAT Probe: 6FAM-AGGTCCTCCTTGGTGAACACGAAGC-BBQ	AM711875.1

comparison tests (SPSS version 20, IBM Corp). P values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Quantitative expression of estradiol 17 β receptor gene in the cumulus cells at different development stages of *in vivo* grown ovarian follicles

Estradiol 17 β receptor gene expression in the cumulus cells isolated from the *in vivo* grown ovarian follicles increased insignificantly from the preantral to early antral follicle stage, with a significant increase and highest from early antral follicles to large antral follicles followed by an insignificant increase in the cumulus cells from COCs of the large antral follicles matured *in vitro* for 24 h (Table 2).

Expression of estradiol 17 β receptor gene in the cumulus cells at different development stages of *in vitro* cultured preantral follicles in different media

Irrespective of *in vitro* groups, cumulus cells from COCs of the large antral follicles matured *in vitro* for 24h exhibited the highest expression of the estradiol 17 β receptor gene (Table 2). Whereas, in the cumulus cells from group-I cultured PFs', the pattern of estradiol 17 β receptor gene expression was different from that of *in vivo* as the insignificant decrease in expression from preantral follicles (PFs' exposed for 3 min) to early antral follicular stage (2-days cultured PFs'). Then there was a gradual increase in the

expression towards the antral stage (4 -d cultured PFs') and large antral stage (6-days cultured PFs') and the steep increase observed in cumulus cells from COCs of the large antral follicles matured *in vitro* for 24 h. Estradiol 17 β receptor gene expression in group II (TCM 199B + E₂), increased insignificantly from the preantral to the early antral follicle and after that, increased significantly in the antral follicle. The antral and large antral follicles showed almost the same estradiol 17 β receptor gene expression. There was a steep increase in the estradiol 17 β receptor gene expression from large antral follicles to the cumulus cells from COCs of the large antral follicles matured *in vitro* for 24 h (Table 2). In group III, significantly increased gene expression at the early antral (2-days cultured PFs'), large antral follicles stage (6-days cultured PFs') and in cumulus cells from COCs of the large antral follicles matured *in vitro* for 24 h (Table 2).

Expression of estradiol 17 β receptor gene in the oocytes at different development stages of *in vivo* grown ovarian follicles

The oocytes from COCs of the large antral follicles matured *in vitro* for 24 h showed the highest expression of the estradiol 17 β receptor among all the *in vivo* stages of follicles. There was an insignificant decrease in the expression of the estradiol 17 β receptor gene from pre-antral to the early antral follicle, later there was a significant increase in antral followed by large antral stages. Among

Table 2: Expression of estradiol-17 β gene in cumulus cells and oocytes of *in vivo* and *in vitro* grown follicles in TCM 199B, TCM 199B + E₂ and Standard medium + E₂.

Source	Estradiol-17 β gene expression (log ₁₀ Relative quantification)				
	Cumulus cells				
	<i>In vivo</i>		<i>In vitro</i>		
		PFs' cultured in medium	TCM 199B	TCM 199B + E ₂	Standard medium + E ₂
Preantral follicles	17.41 $\pm$ 1.09 ^{Ab}	Exposed for 3 min	11.32 $\pm$ 0.92 ^{Ab}	20.42 $\pm$ 0.92 ^{Ba}	26.78 $\pm$ 0.82 ^{Ba}
Early antral follicles	23.65 $\pm$ 2.14 ^{Ba}	Exposed for two days	9.48 $\pm$ 0.54 ^{Aa}	21.82 $\pm$ 0.84 ^{Ba}	34.78 $\pm$ 1.06 ^{Cb}
Antral follicles	38.36 $\pm$ 2.16 ^{Cb}	Exposed for four days	16.74 $\pm$ 0.68 ^{Abc}	34.65 $\pm$ 1.24 ^{Bb}	41.76 $\pm$ 1.24 ^{Bb}
Large antral follicles	107.48 $\pm$ 2.67 ^{Cc}	Exposed for six days	21.56 $\pm$ 0.72 ^{Ac}	36.79 $\pm$ 1.35 ^{Bb}	119.32 $\pm$ 3.08 ^{Cc}
Cumulus cells from COCs after IVM	198.64 $\pm$ 2.84 ^{Cc}	COCs from 6 days cultured PFs' matured <i>in vitro</i> for 24 h	78.64 $\pm$ 1.06 ^{Ac}	134.74 $\pm$ 4.68 ^{Bc}	312.78 $\pm$ 4.61 ^{Cd}
	Oocytes				
	<i>In vivo</i>		<i>In vitro</i>		
		PFs' cultured in medium	TCM 199B	TCM 199B + E ₂	Standard medium + E ₂
Preantral follicles	21.05 $\pm$ 0.64 ^{ABab}	Exposed for 3 min	16.81 $\pm$ 0.63 ^{Aa}	35.41 $\pm$ 1.08 ^{Ba}	31.53 $\pm$ 1.18 ^{Ba}
Early antral follicles	17.84 $\pm$ 0.32 ^{Aa}	Exposed for two days	30.81 $\pm$ 1.09 ^{Ab}	40.82 $\pm$ 1.12 ^{Bb}	51.47 $\pm$ 1.09 ^{Cb}
Antral follicles	30.06 $\pm$ 1.52 ^{Abc}	Exposed for four days	55.81 $\pm$ 1.54 ^{Bc}	39.54 $\pm$ 1.09 ^{Ab}	60.54 $\pm$ 1.45 ^{Bb}
Large antral follicles	96.54 $\pm$ 2.06 ^{Bd}	Exposed for six days	59.45 $\pm$ 1.89 ^{Ac}	183.72 $\pm$ 4.32 ^{Cc}	117.43 $\pm$ 1.89 ^{Bc}
Oocytes from COCs after IVM	172.81 $\pm$ 2.81 ^{Be}	COCs from 6 days cultured PFs' matured <i>in vitro</i> for 24 h	74.89 $\pm$ 1.69 ^{Ac}	267.89 $\pm$ 6.51 ^{Bd}	284.52 $\pm$ 2.09 ^{Cd}

Values with the same superscripts between corresponding *in vivo* and *in vitro* stages grown in TCM 199B, TCM 199B + E₂ and Standard medium + E₂ media are not significantly different (P $\leq$ 0.05).

the three groups under study, the oocytes of PFs' cultured in group III exhibited the highest expression of the ER α gene at any point of their development (Table 2).

Expression of estradiol 17 β receptor gene in the oocytes at different development stages of *in vitro* cultured preantral follicles in different media

Irrespective of *in vitro* groups, oocytes from COCs of the large antral follicles subsequently matured *in vitro* for 24h showed the highest gene expression of the estradiol 17 β receptor (Table 2). Estradiol 17 β receptor gene in the oocytes of group I, significantly increased in the levels at the early antral follicles (2-days cultured PFs') and antral follicle (4-days cultured PFs') stages with a subsequent insignificant increase toward the large antral stage (Table 2).

In group II (TCM 199B + E₂), increased estradiol 17 β receptor gene expression in the oocytes of pre-antral follicles (PFs' exposed for 3 min) to early antral follicles (2-days cultured PFs'), followed by a significant increase from antral follicles to large antral follicles. However, the estradiol 17 β receptor gene expression in the oocytes in COCs from large antral follicles matured *in vitro* for 24 h was significantly higher (Table 2). In group III (Standard medium + E₂), the estradiol 17 β receptor gene expression significantly increased at the early antral (2-days cultured PFs'), large antral follicles stage (6-days cultured PFs') and there was a steep increase in oocytes from COCs of the large antral follicles matured *in vitro* for 24h. Among the three groups under study ER β expression of oocytes of PF's grown in group III is higher in early antral, antral and COC'S extracted from 6-day cultured follicles cultured *in vitro* is highest. The oocytes of the PF's exposed for 6 d (Large antral follicles) showed the highest expression of the estradiol 17 β receptor in Group II (Table 2).

This is the first study on the quantitative patterns of expression of ER β in PFs' during different *in vivo* and *in vitro* developmental stages *i.e.*, from preantral to Graafian follicles. Earlier, estradiol 17 β receptor mRNA expression was detected in the ovaries (*in vivo* stages) by several techniques in different species, *i.e.*, by RT-PCR (Enmark *et al.*, 1997; Cardenas *et al.*, 2001), RNase protection assay (Enmark *et al.*, 1997), *In situ* hybridization (Enmark *et al.*, 1997; Juengel *et al.*, 2006).

In the present study, expression of ER β mRNA was observed both in granulosa and oocytes of all *in vivo* stages of follicles, in line with Juengel *et al.*, (2006) in small ovarian follicles of sheep. The expression of ER β receptor mRNA in both granulosa cells and oocytes was highest in COCs extracted from the large antral follicles and subsequently matured *in vitro* for 24 h. In the granulosa cells and oocytes, the expression of ER β receptor mRNA was increased from preantral to large antral follicles. However, the oocytes of the 2-day cultured follicle (in TCM199B medium) showed a slight non-significant decrease in the ER β expression. This might be because the RQ values in the present study were the average of triplicate RNA samples obtained from morphologically similar but perhaps physiologically

different follicles or it may not have a role during this transition period.

This is also the first report on the estradiol 17 β receptor gene expression during different development stages of sheep ovarian follicles grown from preantral follicles (PFs') cultured *in vitro* in three different culture media. No reports are available to compare and contrast the present observations. Hence, it is an effort/attempt to identify the expression patterns of estradiol 17 β receptor during PFs' development *in vitro*. In all the three *in vitro* groups (Group I-II and III Group), cumulus cells from COCs of the large antral follicles matured *in vitro* for 24h exhibited the highest gene expression of the estradiol 17 β receptor in comparison to the other developmental stages of their group. This may be due to the presence of estradiol in the maturation medium. In Group II and Group III (which were supplemented with E₂), the expression of estradiol 17 β receptor expression significantly increased from the antral to the large antral stage. Earlier reports found that the expression of the aromatase 450 enzyme gene was downregulated by the *in vitro* culture system in sheep (Lakshminarayana *et al.*, 2014). In their studies, they did not add estradiol in the culture medium and study the effect of estradiol on PFs' development unlike our studies. In the present study, significantly higher expression of estradiol 17 β receptor was noted in antral and large antral follicular stages of PFs' grown in group III. This expression of receptors in early antral, antral and large antral follicles shows the role of estrogen in initiation and improving antrum formation. This supports and extends the results of Tasaki *et al.* (2013) in pigs. Ciesiolka *et al.* (2016) recognized E₂ as a potential estrogen receptor agonist since esrrb3 (estrogen-related receptor beta3) expression is upregulated in both acute and prolonged E₂ treatment of porcine CGs during *in vitro* real-time cell proliferation. In agreement with the present results, supplementation of E₂ improved antrum formation in bovine OGCs and exogenous E₂ enhanced the expression of genes those were reportedly highly expressed in large healthy follicles (Hayashi *et al.*, 2010). In the present study, supplementation of estradiol 17 β (5 ng/mL) along with other hormones or growth factors in the culture media in group III could have stimulated its receptor gene expression better than *in vivo* or other media used for the culture of preantral follicles. This fact is supported by the observation that a decrease in aromatase expression and therefore less aromatization to estrogens leading to anovulation due to suppressed levels of FSH was reported by Zarei *et al.* (2021). Detection of estrogen receptor protein and its gene in *in vivo* cultured and *in vitro* grown PFs' showed that it has a local role in modulating the growth and maturation of PFs through classical receptor-mediated pathways. The expression of receptors in granulosa cells may be associated with their maturation and differentiation (Tomanek *et al.*, 1997). It was also reported that ER- β mRNA in rat granulosa cells is regulated hormonally by gonadotrophins, where low levels of gonadotrophins appear to slightly decrease ER- β mRNA

levels but high doses reduced the expression (Fitzpatrick *et al.*, 1999). One of the reasons for better expression of ER 17 β protein and gene could be due to synergistic effects among E2, IGF, GH, FSH and Thyroxine in the culture medium.

CONCLUSION

It is concluded that estradiol 17 β supplemented in low concentration (5 ng/mL) stimulated its receptor expression in granulosa cells and oocytes from the preantral stage to the large antral stage. Further, this stimulatory effect of estradiol 17 β on its receptor showed synergism in the preantral follicles cultured in a medium supplemented with estradiol 17 β with other growth factors and hormones.

ACKNOWLEDGEMENT

This work was supported by a research grant from the Science and Engineering Research Board (DST No: EMR/2017/000851) to A.V.N. Siva Kumar.

Conflicts of interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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