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Differential expression of angiopoietin-1 in ovarian follicles of cross bred malabari goats by immuno-histochemical method

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Abstract
Angiopoietin-1 (ANPT-1) is an angiogenic growth factor, and a ligand for the receptor-"Ties" (a family of receptor tyrosine kinases). Present study aimed to determine the differential protein expression of Angiopoietin-1 in ovarian follicles of cross bred Malabari goats by immuno-histochemical method. Protein expression of Angiopoietin-1 gene could be detected in the ovarian follicular cells *viz.*, granulosa cell, theca interna cells, endothelial cells of blood vessels, follicular fluid and germinal epithelium. Also it was observed that percentage of positivity (number of positive cell/ total number of cells in the follicle) for Angiopoietin-1 was higher in initial stage of follicular development when compared with later stages of follicular development, hence it was concluded that the protein expression of Angiopoietin-1 decreases with increase in follicular size. The staining intensity and the number of positive staining cells were more in the granulosa cell layer than in the theca interna cells of antral follicle. Overall scoring of staining intensity was also more in granulosa cell layer compared to the theca interna cells.

Keywords: Angiopoietin-1, Ovarian follicles, Malabari Goat and Immunohistochemical

Introduction
Goat is known as "poor man's cow" and 90% of goats are present in the developing countries (Iqbal *et al*., 2008) [1]. Two native goat breeds in Kerala are Malabari and Attappady black. Malabari goats are medium sized animals, commonly white in colour along with admixtures and black (Acharya, 1982; Verna *et al.*, 2009) [2, 3]. Goat ovaries are almond-shaped, paired, and are developed as complex endocrine organs (Gillman, 1948) [4]. Ovary consisting of ovarian follicles, corpus luteum, interstitial glands and stroma (Zhou *et al.*, 2008) [5]. Main functional units in the ovary are follicles and corpus luteum. Two parts of the ovary are cortex, predominate tissue and medulla. Ovarian follicular pool in the cortex consists of different categories of follicles *viz.* primordial, primary, secondary and pre-antral follicles which grow under the influence of various hormones and growth factors. Primordial follicles are the ones which are in the resting stage of folliculogenesis. The primary follicles symbolizes the initial stage of follicular development with one layer of granulosa cells and oocyte (Bezerra *et al.*, 1998; Gougeon, 2003) [6, 7]. Secondary follicles has two or more layers of granulosa cells and small number of theca cells (Araujo *et al*., 2014) [8]. Tertiary follicle is the fully matured whole antral follicular stage of ovarian folliculogenesis, consisting of matured oocyte surrounded by zona pellucida, fluid filled antrum, six to nine layers of granulosa cells, a basal lamina, a theca interna and a theca externa. Tunica albuginea is the connective tissue layer of ovary surrounded by epithelial layer called as germinal epithelium (Bezerra *et al.*, 1998; Erickson *et al.*, 2008) [6, 9]. Follicular granulosa cells are avascular, while the theca cells are vascular endocrine cells lying outside the granulosa cell layer in the outermost layer of a follicle. From the secondary follicle stage, specialized inter-follicular stroma layers develop into theca interna cells and theca externa cells, outside the granulosa cells (O'Shea *et al.*, 1978) [10]. Zona pellucida is a thin extracellular matrix seen between oocytes and surrounding granulosa cells. Production of steroid hormone is the key function of ovarian theca cells and granulosa cells. (Hummitzsch *et al.*, 2013) [11]. Angiogenesis is the generation of new blood vasculature arising from the pre-existing ones, which occurs by breakage in basal membrane, migration of endothelial cells, proliferation of the endothelial cells and development of capillary lumen (Folkman and Klagsbrun, 1987) [12]. And it is augmented by members of local ovarian angiogenic factors like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), angiopoietins, insulin like growth factor-1 etc.

Angiopoietins are soluble secreted glycoproteins, which exist in multimeric forms, with a molecular weight of approximately 70kDa and are decisive in the growth, development and stability of capillaries (Drenkhahn *et al*., 2004) [13]. The angiopoietin family includes Angiopoietin-1 and Angiopoietin-2, as well as receptors, tyrosine kinase with immunoglobulin-like and EGF-like domains 1(Tie1) and 2 (Tie2) (Shimizu *et al.*, 2007, Hayashi *et al*., 2004) [14, 15]. The first ligand member of the angiopoietin family to be discovered, Angiopoietin-1, was identified by its ability to bind Tie2 extracellular domain. Angiopoietin-1 and 2 shares approximately 60% amino acid identity (Shimizu *et al.*, 2007) [14]. Angiopoietin-1 which displays a body wide expression, has a strong impact on angiogenesis (Stouffer *et al*., 2001) [16]. Present work could be looked upon as a basic research attempt to learn the protein expression patterns of a crucial local ovarian angiogenic factor, viz., angiopoietin-1 in ovarian follicles of cross bred Malabari goats, at different stages of follicular growth, by making use of immunohistochemistry techniques.

2.3 Protein Expression

2.3.1 Immunohistochemistry

Ovaries were cut into pieces and were fixed in 10% neutral buffer formalin (NBF) for two days. The tissues were dehydrated in a series of graded alcohols for one hour interval, cleared by treatment with xylene for 40 minutes and embedded into paraffin blocks. Thin sections of 5µ paraffin embedded tissues were taken on to silane coated slides. The sections were heat fixed on to the slides and were immersed in two changes of xylene for five minutes each. The deparaffinized tissues were rehydrated with distilled water after immersing in a series of graded alcohol for five minutes each. Heat induced antigen retrieval was done in Tris EDTA buffer. The sections were washed thrice with TBST (Tris-*buffered* saline and Tween 20) buffer and blocked the endogenous peroxidase activity by adding enough drops of hydrogen peroxide block (Abcam) on to the slides to cover the sections and incubated at room temperature. At the end of incubation, the slides were washed well with TBST buffer. Added protein blocking agent (Abcam) to minimise non-specific binding of antibodies to highly charged sites on the tissue sections. After protein block incubation, the section was treated with pre diluted antibody (Rabbit Anti-ANG-1 Polyclonal antibody) at a concentration of 1:200 and incubated at 4 °C overnight. Then washed the sections four times with TBST buffer. Incubated the slides with secondary antibody (Biotinylated goat anti polyvalent) for 10 minutes at room temperature and again washed the sections with TBST buffer for four times, which was again followed by incubation of slides with streptavidin-peroxidase for 10 minutes at room temperature after which the slides were washed four times with TBST buffer. The colour was developed after incubation with substrate DAB (Abcam) for 2-3 minutes and the slides were washed again four times with TBST buffer. The slides were then counterstained with Mayer's hematoxylin for 1-2 minutes. Omission of primary antibodies was used for negative control. The slides were examined under a microscope, and pictures captured employing Leica DM2000 LED trinocular microscope.

2.3.2 Angiopoietin-1 expression-Scoring method for staining intensity

The intensity of the immunostaining with all the antibodies was evaluated by dividing the staining reaction in four groups: (Vakkala *et al.*, 1999) [17]

1 = Weak staining intensity.
2 = Moderate staining intensity.
3 = Strong staining intensity.
4 = Very strong intensity.

The quantity of the immunostaining was evaluated as follows

0 = No positive Immunostaining.
1 = < 25% of cells showing positivity.
2 = 25–50% of cells showing positivity.
3 = 50–75% of cells showing positivity.
4 = > 75% of cells showing positivity.

A combined score for the immunostaining, based on both qualitative and quantitative immunostaining, was composed by adding both the qualitative and quantitative score, which was then divided into three groups:

+ = no or weak immunostaining; score 0-2.
++ = moderate immunostaining; score 3-5.
+++ = strong immunostaining; score 6-8.

Results

3.2 Immunolocalization of Angiopoietin-1

Immunohistochemistry of ovarian section of tropical goats revealed the protein expression of Angiopoietin -1 in all developmental stages of ovarian follicles including primordial, primary, small and large antral stages. Angiopoetin-1 expression was detected in the granulosa cell, theca interna cells, follicular fluid, endothelial cells of capillaries and germinal epithelium of goat ovaries. Microscopic examination of stained ovarian section revealed brown areas of Angiopoietin-1 expression (Fig. 1, Fig. 3 and Fig. 5). The negative controls, without primary antibodies showed only a weak background staining (Fig. 2, Fig. 4, and Fig. 6).

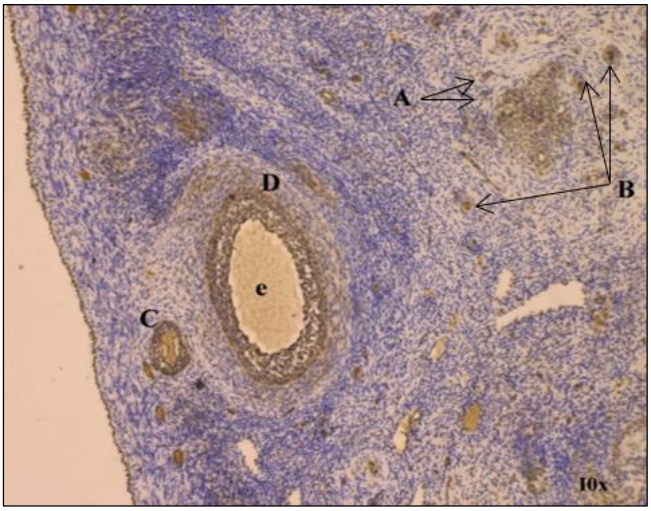

Fig 1: Angiopoietin-1 expression during follicular development: (A)-primordial follicles (B)-primary follicles, (C)-small antral follicle, (D)-large antral follicle (e)-follicular fluid in antral follicle

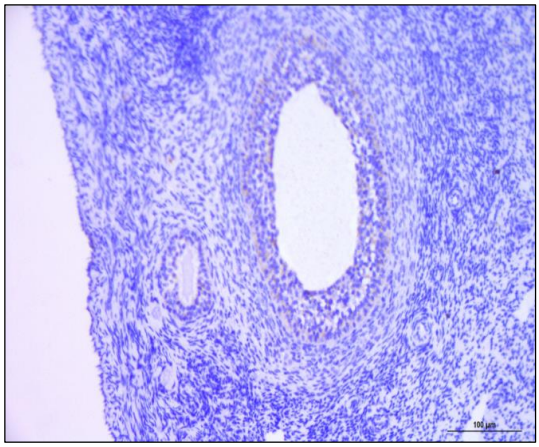

Fig 2: Negative control

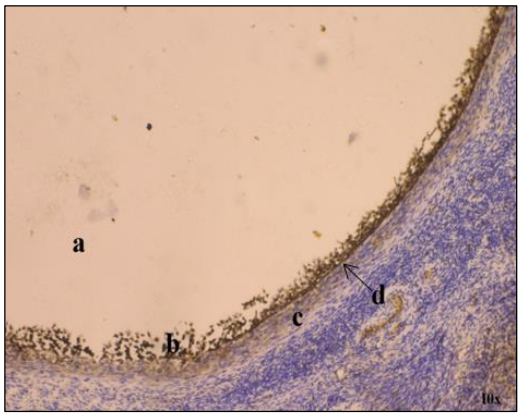

Fig 3: Antral follicle: (a)-antrum, (b)-mural granulosa cells, (c)-theca interna cell (d)-basal lamina

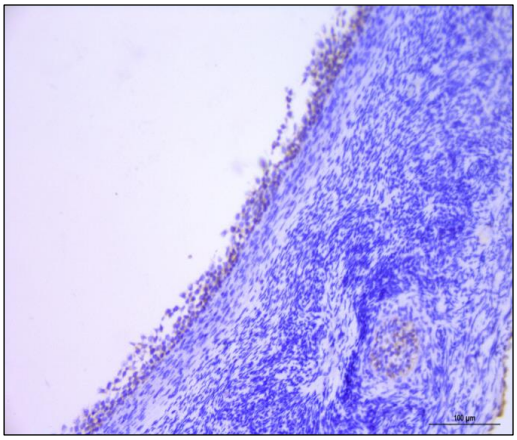

Fig 4: Negative control

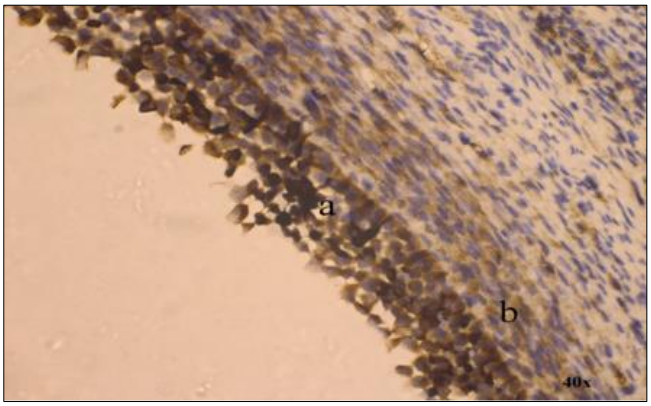

Fig 5: Angiopoietin-1 expression in (a)-mural granulosa (b)-theca interna cells of antral follicle

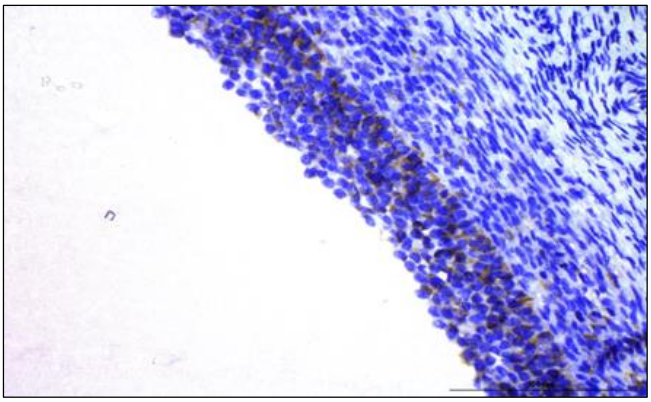

Fig 6: Negative control cells

3.3 Angiopoietin-1 Expression–Combined Scoring Method Based on Percent Positivity and Staining Intensity

Intensity of expression of Angiopoietin 1 was detected using the combined scoring method which determines the quantitative per cent of positivity (number of positive cell/ total number of cells in the follicle) and qualitative (intensity of immunostaining) methods.

3.3.1 Quantitative Method-Percentage of Positivity of Angiopoietin-1 Expression

Percentage of positivity was higher in initial stages of follicular development and decreased with later stages of follicular development (Table. 3.1). By comparing the Angiopoietin -1 expression in granulosa cells (Table 3.2) and theca interna cells (Table 3.3) of antral follicles, we observed that the expression of Angiopoietin -1 is higher in granulosa cells than theca interna cells.

Table 3.1: Percentage of positivity of Angiopoietin-1 expression during follicular development (GC = granulosa cells, TI = theca interna cells)

Follicles	Number of cell in follicle	Number of cells showing positivity	Percentage of positivity
Primordial follicle (*contains only GC*)	7	7	100%
Primary follicle (*contains only GC*)	18	17	94%
Small antral follicle (*contains GC&TI cells*)	212	195	92%
Large antral follicle (*contains GC&TI cells*)	420	350	83.33%

Table 3.2: Percentage of positivity of Angiopoietin-1 expression in granulosa cells of antral follicle

Field No:	Number of cells in granulosa Layer (at x400 magnification)	Number of cells in granulosa layer showing positive (at x400 magnification)	Percentage of positivity
1.	384	334	87%
2.	246	218	88.61%
3.	180	160	88.88%
4.	260	234	90%

Table 3.3: Percentage of positivity of Angiopoietin-1expression in theca interna (TI) cells of antral follicle

Field No:	Number of cells in granulosa Layer (at x400 magnification)	Number of cells in granulosa layer showing positive (at x400 magnification)	Percentage of positivity
1.	298	200	67.11%
2.	324	270	67.90%
3.	340	180	52.94%
4.	280	210	75%

3.3.2 Qualitative method-Immunostaining intensity of granulosa and theca interna

Staining intensities of theca interna and granulosa cells of antral follicle are listed in (Table 4.4 and 4.5). On comparison, immunostaining intensity was more in the granulosa cell than the theca interna cells.

Table 3.4: Combined score for Immunostaining of granulosa cells

Field No:	Intensity of the immunostainings	Percentage positivity of the immunostainings	Combined score	Scoring
1.	4	4	8	+++ (strong staining)
2.	4	4	8	+++ (strong staining)
3.	4	4	8	+++ (strong staining)
4.	4	4	8	+++ (strong staining)
Average	4	4	8	+++ (strong staining)

+ = no or weak immunostainings, ++ = moderate immunostainings, +++ = strong immunostaining

Table 3.5: Combined score of immunostainings of theca interna cells

Field No:	Intensity of the immunostainings	Percentage positivity of the immunostainings	Combined score	Scoring
1.	2	3	5	+++ (moderate staining)
2.	2	3	5	+++ (moderate staining)
3.	2	3	5	+++ (moderate staining)
4.	2	3	5	+++ (moderate staining)
Average	2	3	5	+++ (moderate staining)

+ = no or weak immunostainings, ++ = moderate immunostainings, +++ = strong Immunostaining

Thus it was concluded that the Angiopoietin-1 protein expression in ovarian follicular cells decreased with increase in follicle size and development. Moreover, based on the combined scores for immunostaining (staining intensity and the number of positive staining cells), the Angiopoietin-1expression was detected more in the granulosa cell layer compared to theca interna cells of antral follicle in tropical goats.

5. Discussion

5.1 Expression of Angiopoietins in Ovarian Follicle

The present work was aimed to find out the protein expression of Angiopoietin-1 in the ovarian follicles of cross bred Malabari goats during various stages of follicular growth, development and maturation. It was evident by the immunohistochemical staining of caprine ovarian sections, that angiopoetin-1 is expressed in the granulosa cell, theca interna cells and germinal epithelium of ovaries of cross bred Malabari goats.

Goede *et al*., (1998) [18] reported that Angiopoietin -1 protein is expressed in ovarian follicular cells throughout the ovarian cycle in cow. Theca interna and granulosa cells of ovarian follicles in cow were found to express Angiopoietin-1 mRNA (Hayashi *et al*., 2003) [19] and the mRNA expression was also detected in bovine ovarian follicles secreting increased levels of estradiol (Hayashi *et al*., 2004) [15]. Muller *et al*., (2009) [20] found out that in mares, entire population of granulosa and a large proportion of theca interna cells of primordial (no the cal layer) follicles and tertiary follicles expressed angiopoietin-1 protein. While Maisonpierre *et al*., (1997) [21] found out that the expression of Angiopoietin-1 mRNA is restricted to the theca interna of the preovulatory follicle of rat, Abramovich *et al*., (2009) [22] observed that expression of angiopoetin-1 protein could not be found in granulosa cells from immature hormone-treated as well as non-treated rats during stage of any follicular development. Angiopoietin-1 protein was expressed in follicular fluid of women (Nishigaki *et al*., 2011) [23] and in primates, limited Angiopoietin-1 protein production was detected in slow-growing and fast-growing follicles after antrum formation (Fisher *et al*., 2013) [24].

Angiopoietin-1 mRNA expression in follicular cells *viz.* granulosa, and theca interna indicates its indispensable role, as part of angiopoietin family, in follicular growth, development and maturation (Hayashi *et al*., 2003 and 2004, Mishra *et al.*, 2016) [19, 15, 25].

5.2 Expression of Angiopoietin 1 in Endothelial Cells of Capillaries and Ovarian Germinal Epithelium

In the present study we observed angiopoietin-1 protein expression in endothelial cells of capillaries and germinal epithelium (outer covering of ovary) in goat ovarian follicle. Similar findings of local growth factors being expressed in extra follicular cells of ovaries were reported.

It was reported in an earlier study that VEGF mRNA expressed in cells surrounding to growing ovarian vasculature and endothelial cells of both inactive and proliferating blood vessels of rat (Shweiki *et al*., 1993) [26]. In women VEGF expression was detected in benign ovarian neoplasms epithelial lining, employing immunohistochemistry method (Bamberger and Perrett, 2002) [27].

Gospodarowicz *et al*., (1989) [28] derived basic FGF from ovarian germinal epithelial cells. Cultured bovine ovarian epithelial cells respond to FGF with an increased rate of proliferation. Campos and Romero (2007) [29] reported complete absence or low concentration of Nerve growth factor and its receptor trkA in normal ovarian surface epithelium of women.

5.3 Differential Expression of Angiopoietin in Different Stages of Ovarian Follicular Development

In this study, Angiopoietin-1expression in ovarian follicular cells of cross bred Malabari goats was found to show a decrease, consequent to a rise in follicle size and the expression of Angiopoietin-1 was detected more in the granulosa cell layer compared to theca interna cells of antral follicle in cross bred Malabari goats. In consensus with our findings, the expression of Angiopoietin-1mRNA was seen to be decreased in granulosa cells of medium and large follicles compared to small follicles in gilts (Shimizu *et al*., 2003) [30]. In common marmoset monkeys the highest levels of Angiopoietin-1gene expression was noticed in granulosa cells of initial follicular development, while a decrease was noted in late secondary follicles (Wulff *et al*., 2001) [31]. A decrease in Angiopoietin-1 protein concentrations in follicular fluid was observed in women, in line with rise in volume of follicular fluid, and hence it was concluded that the follicular size, and the change in Angiopoietin-1 levels might be closely interwoven with growth and development of follicular and associated angiogenesis occurring in the preovulatory period (Nishigaki *et al*., 2011) [23].

Hayashi *et al*., (2004) [15] noted that the expression of Angiopoietin-1 mRNA did not exhibit any significant variation across various phases of follicular development in cattle. Angiopoietin-1 is crucial for the final stages of follicular development and (Hayashi *et al*., 2003) [19] observed that in bovine antral follicles, mRNA expression for Angiopoietin-1 fluctuated during various stages of follicular development and atresia. In humans a uniform expression of Angiopoietin-1protein was noticed in the cal layer during follicular development (Fraser, 2006) [32]. In an earlier work it was observed that Angiopoietin-1protein expression in the rat remained unaltered with rise in follicular diameter (Maisonpierre *et al*., 1997) [21].

However, contradictory findings of increase in Angiopoietin-1 expression with follicular size and development were reported by some workers. Chowdhury *et al.*, (2010) [33] detected an increase in Angiopoietin-1 mRNA expression in ovine ovarian antral follicles as the diameter of follicles increased. Angiopoietin tie system was found to be consistently expressed in greater levels in theca cells compared with granulosa cells, and the protein expression in theca cells increased in parallel with follicular development in rats (Abramovich *et al.*, 2009) [1]. In buffalo the gene as well as protein expression of Angiopoietin-1 was found to be the greatest in pre-ovulatory follicles as compared to small follicles (Mishra *et al.*, 2016) [25]. Saju *et al*., [34] reported that in goats, though insignificant, the expression of Angiopoietin-1 mRNA was found to be decreasing from small to large follicles.

Our finding of Angiopoietin-1 expression in follicular cells however, gets decreased with follicular growth, might be due to the reason that it is the interrelationship between Angiopoietin-1, with angiopoietin-2, their receptors, and VEGF, which is going to be decisive in developing the ovarian follicle towards maturity, rather than Angiopoietin-1 acting in isolation. Angiopoietin-1, together with Angiopoietin-2, their receptors, and VEGF are involved in the angiogenesis of the growing ovarian follicle in farm animals, and the resulting exuberant capillary growth in turn brings about supply of nutrients, oxygen, hormones, growth factors preferentially to developing follicles (Hayashi *et al*., 2003 and 2004, Mishra *et al*., 2016) [19, 15, 25].

7. References

1. Iqbal A, Khan BB, Tariq M, Mirza MA. Goat a potential diary animal: present and future prospects. Pakistan journal of agricultural sciences. 2008; 45:227-230.
2. Acharya RM. Sheep and goat breeds of India. Food and agriculture organization of the United Nations, 1982.
3. Verna NK, Dixit SP, Dangi PS, Aggarwal RAK, Kumar Z, Joshi BK. Malabari goats: characterization, management, performance and genetic variability. Ind. J Anim. Sci. 2009; 79:813-818.
4. Gillman J. Contributions to Embryology. Carnegie Institution of Washington; Washington DC. The development of the gonads in man, with a consideration of role of fetal endocrines and histogenesis of ovarian tumors, 1948, 83-131.
5. Zhou L, Li R, Wang R, Huang HX, Zhong K. Local injury to the endometrium in controlled ovarian hyperstimulation cycles improves implantation rates. Fertil steril. 2008; 89:1166-1177.
6. Bezerra MB, Rondina D, Lima AKF, Oliveira LC, Cecehi R, Lucci CM *et al*. Quantitative and qualitative aspects of prenatal folliculogenesis in caprine species. Ciencia Anim. 1998; 8:47-56.
7. Gougeon A. The early stages of follicular growth. Biology and pathology of the oocyte, 2003, 29-43.
8. Araujo VR, Gastal MO, Figueiredo JR, Gastal EL. *In vitro* culture of bovine preantral follicles: a review. Reprod. Biol. Endocrinol. 2014; 12:78.
9. Erickson GH, Mangoffin DA, Dyer CA, Hofeditz C. The ovarian androgen producing cell: a review of structure/ function relationships. Endocrine Reviews. 1985; 6:371-399.
10. O'shea JD, Hay MF, Cran DG. Ultrastructural changesin the theca interna during follicular atresia in sheep. J Reprod Fert. 1978; 54:183-187.
11. Hummitzsch K, Irving-Rodgers HF, Hatzirodos N, Bonner W, Sabatier L, Reinhardt DP *et al*. A new model of development of the mammalian ovary and follicles. PloS one. 2013; 8:1-6.
12. Folkman J, Klagsbrun M. Angiogenic factors. Science. 1987; 235:442-447.
13. Drenkhahn M, Gescher DM, Wolber EM, Meyhoefer-Malik A, Malik E. Expression of angiopoietin 1 and 2 in ectopic endometrium on the chicken chorioallantoic membrane. Fertil. Steril. 2004; 81:869-875.
14. Shimizu T, Berisha B, Schams D, Miyamoto A. Expression of angiopoietin (ANPT)-1, ANPT-2 and their receptors in dominant follicles during periovulatory period in GnRH-treated cow. Reprod Domest Anim. 2007; 42:221-224.
15. Hayashi KG, Berisha B, Matsui M, Schams D, Miyamoto A. Expression of mRNA for the angiopoietin-Tie system in granulosa cells during follicular development in cows. J Reprod. Dev. 2004; 50:477-480.
16. Stouffer RL, Martı´nez-Chequer JC, Molskness TA, Xu F, Hazzard TM. Regulation and action of angiogenic factors in the primate ovary. Arch Med Res. 2001; 32:567-575.
17. Vakkala M, Paakko P, Soini Y. Expression of caspases 3, 6 and 8 is increased in parallel with apoptosis and histological aggressiveness of the breast lesion. Br J Cancer. 1999; 81:592-599.
18. Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab. Invest. 1998; 78:1385-

1394.
19. Hayashi KG, Thomas J Acosta, Berisha B, Matsui M, Schams D, Masayuki O *et al*. Involvement of Angiopoietin-Tie System in Bovine Follicular Development and Atresia: Messenger RNA Expression in Theca Interna and Effect on Steroid Secretion. Biol. Reprod. 2003; 69:2078-2084.
20. Muller K, Ellenberger C, Schoon HA. Histomorphological and immunohistochemical study of angiogenesis and angiogenic factors in the ovary of the mare. Res Vet Sci. 2009; 87:421-431.
21. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C *et al*. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. Science. 1997; 277:55-60.
22. Abramovich D, Rodriguez CA, Hernandez F, Tesone M, Parborell F. Spatiotemporal analysis of the protein expression of angiogenic factors and their related receptors during folliculogenesis in rats with and without hormonal treatment. Reproduction. 2009; 137:309-320.
23. Nishigaki A, Okada H, Tsuzuki T, Cho H, Yasuda K, Kanzaki H. Angiopoietin 1 and angiopoietin 2 in follicular fluid of women undergoing a long protocol. Fertil Steril. 2011; 96:1378-83.
24. Fisher TE, Molskness TA, Villeda A, Zelinskil MB, Stouffer RL, Xu J. Vascular endothelial growth factor and angiopoietin production by primate follicles during culture is a function of growth rate, gonadotrophin exposure and oxygenmilieu. Hum Reprod. 2013; 28:3263-3270.
25. Mishra SR, Thakur N, Somal A, Parmar MS, Yadav VP, Bharati JB *et al*. Expression and localization of angiopoietin family in buffalo ovarian follicles during different stages of development and modulatory role of angiopoietins on steroidogenesis and survival of cultured buffalo granulosa cells. Theriogenology. 2016; 86:1818-1833.
26. Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin. Invest. 1993; 91:2235-2243.
27. Bamberger ES, Perrett CW. Angiogenesis in epithelian ovarian cancer. J Clin. Pathol: Mol. Pathol. 2002; 55:348-359.
28. Gospodarowicz D, Plouet J, Fujii DK. Ovarian germinal epithelial cells respond to basic fibroblast growth factor and express its gene: implications for early folliculogenesis. Endocrinology. 1989; 125:1266-1276.
29. Campose X, Romero C. Nerve growth factor and its high-affinity receptor trkA participate in the control of vascular endothelial growth factor expression in epithelial ovarian cancer. Gynecol Oncol. 2007; 104:168-175.
30. Shimizu T, Iijima K, Sasada H, Sato E. Messenger ribonucleic acid expressions of hepatocyte growth factor, angiopoietins and their receptors during follicular development in gilts. J Reprod Dev. 2003; 49:203-211.
31. Wulff C, Wiegand SJ, Saunders PTK, Scobie GA, Fraser HM. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (Vascular Endothelial Growth Factor TrapA40). Endocrinology. 2001; 142:3244-3254.
32. Fraser HM. Regulation of the ovarian follicular vasculature. Reprod Biol Endocrinol. 2006; 4:18.
33. Chowdhury MWH, Scaramuzzi RJ, Wheeler-Jones CPD, Khalid M. The expression of angiogenic growth factors and their receptors in ovarian follicles throughout the estrous cycle in the ewe. Theriogenology. 2010; 73:856-872.
34. Saju SS, Plakkot B, Babitha V. Expression of angiopoietin-1 (ANPT-1) gene in different categories of ovarian antral follicles in tropical goats, 2020.
35. Saju SS, Plakkot B, Vazhoor B. A review article on angiopoietins: A crucial ovarian local growth factor, 2018.