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Expression of *Angiopoietin 2* in ovarian follicles of crossbred Malabari goat#

M.S. Adithian[1*], V. Babitha[2], R. Uma[3], I. S. Sajitha[4], and E. S. Navya[5]
Department of Veterinary Biochemistry,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur, 680651
Kerala Veterinary and Animal Sciences University, Kerala, India

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Abstract

Angiopoietin-2 (Ang-2) is a local ovarian growth factor belonging to the angiopoietin/Tie (tyrosine kinase with Ig and EGF homology domains) signalling pathway, one of the main pathways involved in ovarian follicular angiogenesis. The main objective of this study is to assess the differential protein expression of Ang-2 in the developmental phases of ovarian follicles of cross-bred Malabari goats employing an immunohistochemical technique. Ang-2 protein expression was found in all developmental phases of ovarian follicles, including primordial, primary, small, and large antral ovarian follicles. Ang-2 was found in the granulosa cells, theca interna cells, follicular fluid (granulosa cells in the FF), endothelial cells of capillaries and germinal epithelium of the goat ovaries. The Quantitative Method-Percentage Positivity of Ang-2 expression revealed that it was higher in the initial stages of ovarian follicular development and decreased with later stages of follicular development in cross-bred Malabari goats. Moreover, the protein expression of Ang2 was found to be higher in granulosa cells (GC) than in theca interna (TI) cells of antral follicles as evident from the higher staining intensity in GC cells compared to TI cells. It is therefore concluded that the Ang-2 protein expression in ovarian follicular cells decreased with an increase in follicle size and development and based on the combined scores for immunostaining (staining intensity and the number of positive staining cells), the Ang-2 was found to be expressed more in the granulosa cell layer compared to the theca interna cells of the antral follicle in crossbred Malabari goats.

Keywords: *Angiopoietin-2 (Ang-2), goat, ovarian follicles, immunohistochemistry, granulosa cells, theca interna, protein expression*

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1. *MSc. scholar*
2. *Associate Professor, Department of Veterinary Physiology*
3. *Associate Professor, Department of Veterinary Biochemistry*
4. *Assistant Professor, Department of Veterinary Pathology*
5. *MVSc. Scholar*

**Corresponding author: adithianms0007@gmail.com Ph: 9747371653*

Goats, a member of the family Bovidae, are one of the earliest species of animals domesticated by man. There are more than 210 different goat breeds across the globe, and they come in a wide assortment of colours. According to the Livestock Survey 2019, the total goat population in Kerala was 13-14 lakhs, of which the major part belongs to Malabari and Attappady breeds. Malabari goats are found in Kerala's districts of Calicut, Kannur, Wayanad, and Malappuram. Goats are raised mainly for milk and meat (Rani *et al*., 2010), and this species of small ruminant is often considered a 'future animal' intended to cater to the increasing demand for food for the expanding population of the country (CIRG, 2013).

As in any other species, both genetic as well as environmental elements play crucial roles in the reproductive physiology of caprine. Goats have round, oval, or almond-shaped ovaries and when the ovary is split into two sections, the outer cortex and inner medulla become visible (Annie *et al*., 2021). The cortex contains follicles and oocytes in various phases of development and degeneration, and the medulla includes blood vessels and connective tissue. The ovarian follicular pool in the cortex is made up of numerous types of follicles, including primordial, primary, secondary, and antral follicles, all of which develop in a pre-programmed fashion as dictated by the genetic make-up, various hormones, locally derived growth factors, nutritional status etc, to name a few. Follicular cells of endocrinological significance include granulosa cells and theca interna. Flattened granulosa cells initially form a single layer surrounding the oocytes in the primordial follicle. As the follicle develops additional granulosa cell layers are added, and theca layer also develops outside the granulosa layer with a basement membrane separating them. Thecae encircling the follicle are separated into two parts: an inner vascularized layer called the theca interna, and an outer fibrous connective tissue called the theca externa.

Angiogenesis is the vascular development process that occurs in tandem with folliculogenesis ensuring a preferential supply of oxygen, nutrients, hormones and growth factors to the developing follicles in a follicular wave. Several local ovarian growth factors, synthesized and secreted from follicular cells viz., vascular endothelial growth factor (VEGF), angiopoietins, insulin-like growth factor, basic fibroblast growth factor etc., are all instrumental in regulating neoangiogenesis in ovarian follicles (Müller *et al.,* 2009). Angiopoietins (*Ang*) are a four-membered growth factor family that interacts with the tyrosine kinase Tie2 endothelial receptor to bring about their biological functions. It is worth mentioning that the role of angiopoietins 1 and 2 on ovarian follicular angiogenesis is found to be influenced by the ratio between the two factors as well as the levels of VEGF (Fraser*.,* 2006). By means of their angiogenic activity, Ang- Tie system is observed to be actively involved in the regulation of ovarian follicular dynamics encompassing all the growth and developmental stages of folliculogenesis till ovulation in various species as rats (Maisonpierre *et al*., 1997), cow (Hayashi *et al.,* 2003, Shimizu *et al.,* 2007), ewes (Chowdhury *et al.,* 2010, Christensen *et al.,* 2014), buffalo (Mishra *et al*., 2016a), monkey (Hazzard *et al.,* 1999), human (Nishigaki *et al.* 2011), gilts (Shimizu et *al.,* 2003) etc. Though the expression studies on various local ovarian angiogenic factors are plenty in most species of domestic animals, there is a noticeable paucity of research attempts in this direction in caprine. Hence the present study has looked into the protein expression pattern of *Ang-2* in ovarian follicles of crossbred Malabari goats at various stages of development, employing the technique of immunohistochemistry as well as evaluation of the same using scoring method based on staining intensity.

Materials and methods

Immunohistochemistry

Ovaries of crossbred Malabari goats were collected from local slaughterhouses in a thermo flask containing neutral buffer formalin (NBF). 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 a one-hour interval, cleared by treatment with xylene for 40 minutes and embedded into paraffin blocks. Thin sections

(5µm) of paraffin-embedded tissues were taken onto silane-coated slides. The use of tissue adhesive (silane) in the slides was important since without an adhesive, tissues tend to get detached from the slides during the antigen retrieval process. The sections were heat fixed onto the slides by placing them in an oven at 37° C for 10-15 minutes.

Slides were immersed in two changes of xylene for ten minutes each for removing paraffin wax. The deparaffinised tissues were rehydrated with distilled water after immersing in 100%, 90%, 80%, 70% and 60% alcohol for three minutes each.

Antigen retrieval

The slides were taken out and blotted well using tissue paper. Heat-induced antigen retrieval was done in sodium citrate buffer (pH 6) at a temperature of 90°- 95° C for 20 minutes. The sections were washed thrice with Tris-*buffered* saline (TBS) buffer and the tissue area was encircled with a hydrophobic pen. During the application of high temperature much care was taken that the sections did not detach from the slide and also that it did not get dried up.

Endogenous peroxidase activity was blocked by adding enough drops of hydrogen peroxide block (Abcam) onto the slides to cover the sections and incubated for 20 minutes at room temperature. At the end of incubation, the slides were washed well with TBS buffer. A protein blocking agent provided in the kit (protein block; Abcam) was added to the sections for 20 minutes at room temp to minimise non-specific binding of antibodies to highly charged sites on the tissue sections.

Primary antibody incubation

The reagent was drained off and the slide gently blotted around the section. Pre-diluted antibody (Rabbit ANGPT2 Polyclonal antibody) at a concentration of 1:100 was applied over the sections and incubated at 4°C overnight. The sections were rinsed well four times with TBS buffer after incubation. A drop of enhancer was added to the sections and incubated for 30 minutes at room temperature to increase signals and again rinsed with TBS buffer.

The slides were incubated with the secondary antibody (Super Sensitive Label in PBS with carrier protein and preservative) for 30 minutes at room temperature. Then the sections were washed with TBS buffer four times.

The colour was developed after incubation with substrate DAB (Abcam) for 20-30 minutes and the slides were washed again with TBS buffer, and then counterstained with Mayer's haematoxylin for 5 minutes. The addition of the primary antibody step was omitted in the negative control. The slides were examined, and pictures were captured using Leica DM2000 LED trinocular microscope.

Angiopoietin- 2 expression - scoring method for staining intensity

The intensity of the immunostainings with the antibodies was evaluated by allocating the staining reaction into four groups (Vakkala *et al*. 1999):

1 = weak cytoplasmic staining intensity
2 = moderate cytoplasmic staining intensity
3 = strong cytoplasmic staining intensity
4 = very strong cytoplasmic staining intensity.

The quantity of the immunostaining was evaluated as follows:

0 = No positive immunostaining

1 = < 25% of cells showing cytoplasmic positivity

2 = 25–50% of cells showing cytoplasmic positivity

3 = 50–75% of cells showing cytoplasmic positivity

4 = > 75% of cells showing cytoplasmic positivity.

A combined score for the immunostaining, based on both qualitative and quantitative immunostaining, was composed by adding both the qualitative and quantitative scores, 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 and discussion

Immunolocalisation of Ang-2

Microscopic examination of the stained ovarian section revealed brown areas of *Ang-2* expression (Fig.1, Fig.3, Fig.5, Fig.7 and Fig. 9). The negative controls, without primary antibodies, showed only a weak background staining (Fig.2, Fig.4, Fig.6 and Fig.8).

Immunohistochemistry results showed that *Ang-2* protein was expressed in all developmental stages of ovarian follicles, *viz.*, primordial, primary, small, and large antral ovarian follicles. *Ang-2* expression was detected in the granulosa cell, theca interna cells, follicular fluid (granulosa cells in the FF), endothelial cells of capillaries and germinal epithelium of goat ovaries. *Ang-2*

Maisonpierre *et al.* (1997) could detect significant numbers of *Ang-2* transcripts inside the GC layer of rats., while Hayashi *et al.* (2003) observed that both TI and GCs displayed mRNA *Ang-2* signals in bovine ovarian follicles. Müller *et al*. (2009) investigated ovaries of mares immunohistochemically and observed that *Ang-2* was expressed in every GC and several TI of primordial follicles, tertiary follicles, and postovulatory follicle walls. The authors noticed that the most intense staining for *Ang-2* was in the tertiary follicles and the postovulatory follicle wall. Abramovich *et al.* (2009) detected *Ang-2* protein by immunochemistry and western blot analysis, and they found that *Ang-2* was weakly expressed in GCs at all follicular stages in rats, while in TI, *Ang-2* expression increased significantly from preantral follicle to early antral follicle and preovulatory follicle. Even though the expression pattern in GCs was contradictory, other results were in consensus to our findings. Mishra *et al*. (2016a) detected the expression of *Ang-2* mRNA in GC and TI and the expression was found to be highest in F2 follicles and lowest in F1 and F4 follicles of buffalo, wherein F1, F2, F3 and F4 indicates follicle classification from smallest to largest size.

In situ hybridization demonstrated punctate *Ang-2* protein expression at the invasive front of vascular sprouts, where bovine endothelial cells showed clear *Ang-2* protein expression. This highlighted the prospect that cultivated endothelium cells may generate *Ang-2* and so could serve as a model for studying *Ang-2* expression regulation *in vitro* (Mandriota and Pepper, 1998). Ang-2 was observed to be abundantly expressed in endothelial cells of large and medium size blood arteries in human ovarian cancer tissues, as per IHC results (Zhang *et al.,* 2003). Mishra *et al.* (2016b) found that angiopoietin family members including *Ang-2* were expressed in endothelial cells of buffalo ovarian follicles. The authors opined that *Ang-2* might be involved in promoting follicular angiogenesis by destabilizing blood vessels and causing endothelial cell migration, proliferation and differentiation.

Ang-2 expression in caprine ovarian follicular cells–a combined scoring method based on per cent positivity and staining intensity

The intensity of expression of *Ang-2* was detected using the combined scoring method which determines the quantitative / per cent positivity (number of positive cells / total number of cells in the follicle) and qualitative (intensity of immunostaining) methods.

Quantitative Method-Percentage of positivity of Ang-2 expression

The percentage of positivity was higher in the initial stages of follicular development and decreased with later stages (Table 1). By comparing the *Ang-2* expression in GCs (Table 2) and TI cells (Table 3) of antral follicles, we observed that the expression of *Ang-2* is higher in GCs than in TI cells.

Vakkala *et al*. (1999) used the combined scoring method on the protein expression of caspases 3, 6, and 8 by IHC with specific antibodies. The intensity of the immunostaining was evaluated by dividing the staining reaction into four groups: weak, moderate, strong, and very strong cytoplasmic staining intensity, and the quantity of the immunostaining was evaluated as the total percentage of cells showing positivity. The immunostaining was then given a composite score based on both qualitative

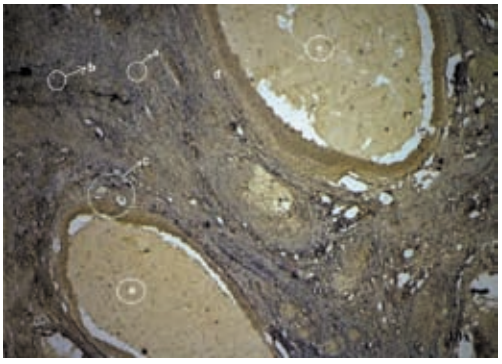

Fig.1 *Ang-2* expression during follicular development: (a) primordial follicles (b) primary follicles, (c) small antral follicle, (d) large antral follicle (e) follicular fluid in antral follicle

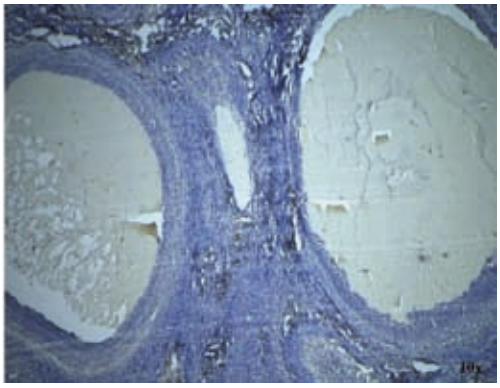

Fig.2 Negative control of Fig.1

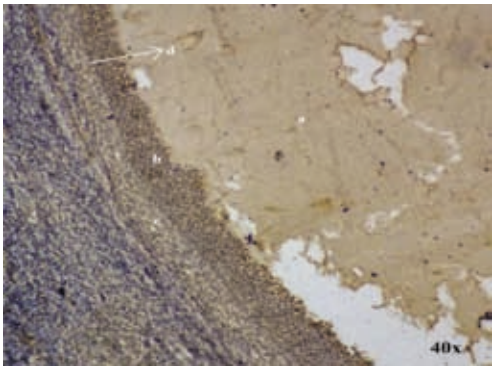

Fig.3 Antral follicle: (a) antrum, (b) mural granulosa cells, (c) theca interna cells (d) basal lamina

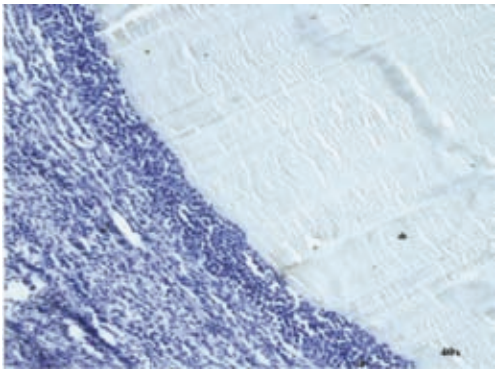

Fig.4 Negative control of Fig.3

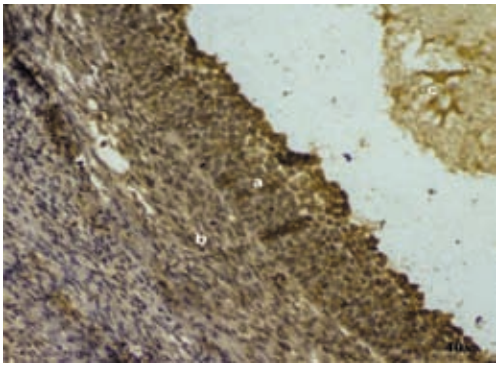

Fig.5 *Ang-2* expression Magnified version (a) mural granulosa cells, (b) theca interna cells of antral follicle (c) follicular fluid

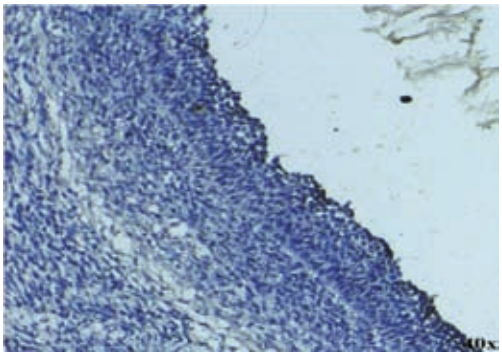

Fig.6 Negative control of Fig.5

and quantitative immunostaining, which was calculated by combining the qualitative and quantitative scores together. Recently, Nishitha et al. (2020) had employed the same type of combined scoring method in their work to estimate the protein expression of *Ang-1* in the ovarian follicles of crossbred Malabari goats. The degree of staining in TI and GCs of the antral follicle demonstrated that the GC cells were more intense than the TI cells. Also, the authors pointed out that the percentage of positivity was greater in the early phases of follicular development and reduced as follicular development progressed, very similar to our finding of *Ang-2* expression in caprine ovarian follicles.

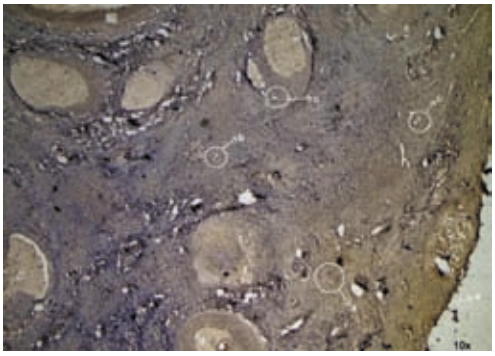

Fig.7 *Ang-2* expression during follicular development: (a) primordial follicles (b) primary follicles, (c) small antral follicle, (d) large antral follicle, (e) germinal epithelium

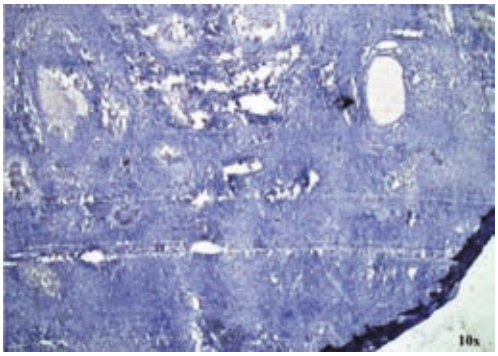

Fig.8 Negative control of Fig.7

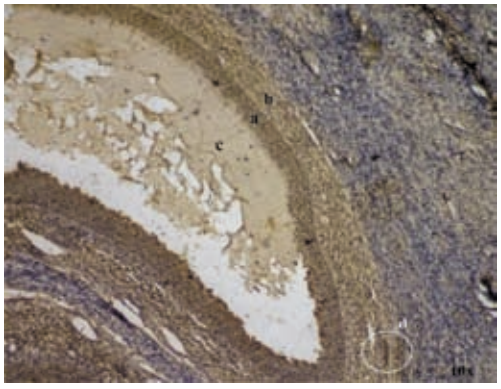

Fig.9 (a) mural granulosa cells, (b) theca interna cells, (c) follicular fluid (d) Capillary endothelial cells

Qualitative method- Immunostaining intensity of granulosa and theca interna

Staining intensities of granulosa cells and theca interna of the antral follicle are listed in (Table 4 and Table 5). On comparison, the intensity was more in the GCs than in the TI cells. Though GCs do not possess vascular supply, they rely on vessels within the surrounding stroma.

Thus, it was found that the *Ang-2* protein expression in ovarian follicular cells decreased with an increase in follicle size and development. Moreover, based on the combined scores for immunostaining (staining intensity and the number of positive staining cells), the *Ang-2* expression was detected more in the granulosa cell layer compared to theca interna cells of the antral follicle in crossbred Malabari goats.

In consensus with our findings, Hayashi *et al*. (2003) found that both the TI and GCs of mature bovine follicles contained signals encoding for mRNA *Ang-2* wherein, RT-PCR analysis was used to look *Ang-2* expression and follicular development. Similar to the current findings, they had reported, *Ang-2* mRNA expression was lower in mature follicles (with E2 > 180 ng/ml FF) during follicular development in TI than in the other developing follicles. As the follicles became larger, the expression of *Ang-2* mRNA in granulosa cells was reduced. The mRNA level in the thecal tissues was unaffected by the different follicle sizes. Shimizu *et al*. (2003) in eCG-treated gilts using RT-PCR found that the expression patterns of angiopoietin mRNA were altered during the follicular development phase. As the follicles became larger, the expression of *Ang-2* mRNA in granulosa cells was reduced. In small follicles, the mRNA levels of *Ang-2*

Table 1. Percentage of positivity of *Ang-2* expressions during follicular development

Follicles	Number of cells in the follicle	Number of cells showing positivity	Percentage of positivity
Primordial follicle (*contains only GC*)	5	5	100%
Primary follicle (*contains only GC)*	23	21	91.3%
Small antral follicle (*contains GC & TI cells)*	176	155	88.06%
Large antral follicle *(contains GC & TI cells)*	576	457	79.34%

Table 2. Percentage of positivity of *Ang-2* expression in granulosa cells of antral follicle

Field No.	Number of cells in granulosa layer (at 40x magnification)	Number of cells in granulosa layer showing positive (at 40x magnification)	Percentage of positivity
1.	464	376	81.03%
2.	295	262	88.81%
3.	545	478	87.7%
4.	278	236	84.89%

Table 3. Percentage of positivity of *Ang-2* expression in theca interna cells of antral follicle

Field No.	Number of cells in thecal Layer (at 40x magnification)	Number of cells in thecal layer showing positive (at 40x magnification)	Percentage of positivity
1.	304	223	73.35%
2.	288	173	60.06%
3.	383	256	72.93%
4.	273	154	56.41%

Table 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.	3	4	7	+++ (Strong staining)
3.	4	4	8	+++ (Strong staining)
4.	3	4	7	+++ (Strong staining)
Average	3.5	4	7.5	+++ (Strong staining)

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

Table 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.	1	3	4	++ (Moderate staining)
Average	1.75	3	4.75	++ (Moderate staining)

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

increased substantially in the eCG-treated group than in the control group. However, no difference in the expression of *Ang-2* mRNA in granulosa cells of medium and large follicles was reported and the mRNA level in the thecal tissues was unaffected by the different follicle sizes. The authors concluded that it could be the conflicting actions of *Ang-1* and *Ang-2*, and their relative ratio (*Ang-2/Ang-1*) that was instrumental in guiding the follicular development rather than individual proteins separately. Hayashi *et al*. (2004) detected a higher *Ang-2* mRNA expression in small follicles with E2<5ng/ml, which got significantly reduced in larger follicles with E2>5ng/ml in bovine ovaries. Shimizu *et al*. (2007) found that the changes in *Ang-2* mRNA expression in the follicles of GnRH-treated cows were evident at different developmental stages. Incidentally in tropical goats of Kerala, a strikingly similar pattern, of decrease in gene expression of another member in the angiopoietin family viz., angiopoietin 1, with an increase in ovarian follicle growth and development, was reported (Saju *et al*., 2020)

In contradiction to our findings, Chowdhury *et al.* (2010) explained that, in ewes, larger follicles displayed higher levels of *Ang-2* expression in theca cells, but small and medium

follicles did not vary in expression at any point of the oestrous cycle, and *Ang-2* expression in GCs was highest in larger follicles and least in small and medium follicles. The authors found that at all follicular phases, theca cells expressed more *Ang-2* than granulosa cells. Christensen *et al.* (2014) assessed the protein expression of *Ang-2* in ewes and observed it was considerably higher in the TI than in the GC or TE of smaller follicles ($P < 0.05$).

The contradictory observations reported by various studies, demands for in-depth and exhaustive research attempts regarding the spatiotemporal expression pattern of various angiogenic factors including *Ang-2*, along with the interactions between the various local ovarian factors to figure out the mechanism of actions followed by these local factors alone and/or in combination in bringing about angiogenesis in growing ovarian follicles in a wave, in a well-balanced and favourable manner.

Conclusion

Based on the findings it could be concluded that the change in *Ang-2* levels might be closely interwoven with angiogenesis occurring in concert with various stages of follicular development, either in isolation and/or on interaction with other related angiogenic factors in caprine. We observed that *Ang-2* expression in follicular cells was steadily getting decreased with follicular growth and development, and the *Ang-2* protein expression was detected more in the granulosa cell layer compared to theca interna cells of caprine antral ovarian follicles. The research outcome in turn indicates the temporal and spatial pattern of expression of this crucial local angiogenic factor ovarian follicle during follicular development in the Malabari breed of goats. More *in vivo* and *in vitro* studies in goats are necessary in this regard, to gain an exhaustive insight into the individual and combined roles of such various local ovarian angiogenic factors and how they regulate the overall ovarian follicular dynamics, by looking into their protein and gene expressions. In fact, much of the intricacies of ovarian follicular physiology remain still unknown and only a comprehensive understanding of such minute details would help the scientific community to come up with suitable strategies to bring about an overall improvement in the production and reproduction capabilities of farm animals in the long run.

Conflict of interest

The authors declare that they have no conflict of interest

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