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Expression of angiopoietin-1(ANPT-1) gene in different categories of ovarian antral follicles in tropical goats

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Abstract
The present study was undertaken to find out the pattern of mRNA expression of Angiopoietin-1(ANPT-1) gene in different categories of ovarian antral follicles in tropical goats of Kerala. The ovaries collected from 12 goats at proestrous stage were subjected to study. Whole follicles were isolated from the ovaries and categorized them as small (1-3 mm) and large (>3 mm). From our study it became evident that, though insignificant, the mRNA expression of ANPT-1 was found to be decreasing from small to large ovarian follicles. It is concluded that the reducing trend of expression of ANPT-1, on interaction with other local ovarian angiogenic factors would be having a crucial role in the angiogenesis, development, maturation and ovulation of follicle in tropical goats of Kerala.

Keywords: ANPT-1, mRNA expression, ovarian follicles, tropical goats, angiogenesis

1. Introduction
Pre-ovulatory follicle formation is the first milestone towards a successful reproduction, hence an exhaustive understanding of follicle development is critical to improve and control the vital reproductive functions. Even though the pertinent role played by the classical hormones, gonadotropins, in regulation of folliculogenesis has been widely accepted, the variable fates of different follicles in same ovary raises the possibility of existence of other Intraovarian modulatory systems (Franchimont and Channing., 1981) [1]. This lead to the identification of the existence and importance of local ovarian growth factors which were found to be as indispensable as that of gonadotropins in the development, maturation, and ovulation of follicles.

Among the numerous local ovarian growth factors, Angiopoietin (ANPT)-Tie system (ANPT-1, 2 and Tie-1, 2 receptors) has a decisive role in ovarian follicular development and attainment of dominance in later stages by increasing vascularisation, which in turn causes increased blood flow to individual follicles, and plays a crucial role in the selective maturation of pre-ovulatory follicles (Zeleznik *et al*., 1981) [2]. These findings emphasise that the rate of vascular development, and therefore the action of local ovarian angiogenic factors including ANPT system is decisive in the ovarian follicular growth, development, maturation and ovulation.

The first ligand member of the angiopoietin family to be discovered was ANPT-1, and it was identified by its ability to bind Tie2 extracellular domain. Angiopoietin-1 acts as an agonist which phosphorylates the Tie-2 receptors and thus promotes vascular stabilisation by congregating the perivascular cells to maintain vascular integrity (Maisonpierre *et al.,* 1997) [3].

An increase in the ANPT-2: ANPT-1 ratio was considered as the primary requisite for new blood vessel formation which is closely interwoven with the destabilization of blood vessels. The ratio of ANPT-1 and ANPT-2 along with vascular endothelial growth factor (VEGF) determined vascular stabilization and regression (Thurston *et al*., 2000 [4], Schams and Berisha. 2004) [5].

Destabilise blood vessels awaits any of these two fates, i.e when VEGF is high, active angiogenesis occurred and resulted in the formation of a new blood vessel network (high ANPT-2: ANPT-1 ratio, high VEGF), whereas a lack of VEGF support resulted in a regression of blood vessels (high ANPT-2: ANPT-1 ratio, low VEGF). A low ANPT-2: ANPT1 ratio with low VEGF resulted in a stabilization of blood vessels (Hayashi *et al*., 2003) [6].

Changes in mRNA expression of ANPT-1, ANPT-2, and Tie-2 in theca interna, associated with follicular development and atresia and the ANPT-Tie system had a pivotal role in the regulation of ovarian follicular dynamics that comprised of development, maturation, and

ovulation of follicles in rat (Maisonpierre *et al.,* 1997) [3], cow (Goede *et al.,* 1998) [7], monkey (Hazzard *et al.,* 1999) [8], human (Yancopoulos *et al.,* 2000) [9], pig (Shimizu *et al.,* 2003) [10], ewe (Chowdhury *et al.,* 2010) [11], and buffalo (Mishra *et al*., 2016) [12].

No studies had been reported yet about the expression of Angiopoietins in goats, hence the present study was envisaged as a preliminary step to disclose the mRNA and protein expression of ANPT-1 in different stages of follicular development in tropical goats of Kerala.

2. Materials and Methods

2.1 Follicle collection and classification

Adult female goats were selected from the culling list of University goat farm, Kerala Veterinary and Animal Sciences University, Mannuthy. The criteria for selection of animals were, age (within a range of 2 to 5 years) and a history of at least two Kiddings. Twelve non pregnant goats were selected for the study and synchronized oestrous of these twelve goats. Oestrous in selected animals was confirmed by the presence of external signs like thick white coloured vaginal discharge, vaginal edema, hyperemia and behavioral signs like bleating, tail wagging, mounting on animals etc. The day in which animal came in heat was considered as day 0, by about 15th day (approximately follicle size reached optimum), the animals were slaughtered in the Meat Technology Unit, KVASU, Mannuthy. The ovaries were collected in chilled Phosphate Buffered Saline and transported in ice to the laboratory without delay. Whole follicles were isolated from ovary, after measuring the ovarian follicular diameter using Vernier Caliper, classified them into small (1-3mm) and large (>3mm). Immediately after isolation the follicles were transferred to labelled eppendorf containing RNA later solution (Qiagen, Life technologies, USA). Follicles with different diameters were stored separately at -80^{0}C.

2.2 Gene expression

2.2.1 RNA isolation and cDNA synthesis

Isolation of RNA from the follicles was performed using RNAeasy fibrous tissue kit (Qiagen, Life technologies, USA) by following the manufacture's protocol with slight modification. The yield and purity of isolated RNA samples were measured by NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, USA). The quality and integrity of the RNA were assessed electrophoretically (0.8% agarose gel) and confirmed the presence of 28s and 18s RNA. The total RNA was reverse transcribed into cDNA using Verso cDNA synthesis kit (Thermo Scientific, USA) as per manufacturer's protocol using random hexamer primers. Protocol for cDNA synthesis included initial incubation at 25 °C for five min followed by 42 °C for 30 minutes for cDNA synthesis and 95 °C for two minutes for inactivation.

2.2.2 Quantitative RT-PCR

The qRT-PCR of target gene (ANPT-1) and housekeeping genes (β-actin) were done in duplicate using SYBR green chemistry (Maxima SYBR green qPCR master mix, Thermo scientific, USA). A total reaction volume of 12.5 µL [1µl cDNA, 6.25 µL master mix, 0.5 µL of forward and reverse primer each (10 pM/µl), 4.25 µL of nuclease free water] was subjected to qRT-PCR. Exon spanning primers were designed using online NCBI primer design software (Primer3, http://bioinfo.ut.ee/primer3/) and specificity of the primer was checked and confirmed by using the BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and quality of the primer was assessed by using the software Primerstat.

Table 1: Target genes, primer sequences and product size for qRT-PCR

Gene Name	Primer sequence (5"- 3")	Product Size (bp)	Gene Accession No
ANPT-1	FP: - TCATGCTAACAGGAGGCTGG	135	XM_005689163.3
	RP: AGGAGTAACTGGGCCCTTTG		
β-actin	FP: -AGATCAAGATCATCGCGCCC	108	NM_001314342.1
	RP: -ACTCCTGCTTGCTGATCCAC		

The following qRT-PCR protocol was followed - single cycle, initial denaturation (enzyme activation) at 95 ^{0}C for 3 minutes, 40 cycles of denaturation: - 94 ^{0}C for 30 seconds, annealing: - 61 ^{0}C for ANPT-1 and 60 ^{0}C for β-actin for 30 seconds followed by the programme for melt curve analysis: denaturation at 95 ^{0}C for 15 seconds followed by annealing at 55 ^{0}C for 15 seconds, and 95 ^{0}C for 15 seconds. The thermal cycler (Eco-Illumina Real-time Thermal cycler, USA) was pre-programmed for temperature and cycling conditions specified. The cycle threshold (Ct) values were used for the relative expression study. For relative quantification by comparative method, the values were expressed relative to control sample called calibrator, with β-*actin* used as internal control. The Ct of target gene and Ct of control gene were determined for each sample and calibrated (ΔΔCt method) (Livak and Schmittgen, 2001) [13].

2.2.3 Statistical analysis: Results were expressed as means (± SE). The statistical significance of difference were analyzed by Independent t-test using the software Statistical Product and Service Solutions (SPSS), version 24.0 and the differences were considered statistically significant at 5% level (P<0.05) (Snedocor and Cochran, 1994) [14].

3. Results

In tropical goats, the mRNA for gene ANPT-1 could be detected in both small and large follicles. When we compared the expression of ANPT-1 in small and large follicles of goats it was found that the expression of the gene was decreasing from small to large follicles and the decrease was 0.80 fold in large follicles when compared to the small follicles (control group). However there was no significant difference between the expression of ANPT-1 gene in small and large follicles.

Table 2: Comparison of *ANPT-1* gene expression within goats between follicles of different sizes (ns-non significant)

Tissue	Mean C_T ± S.E		ΔC_T±S.E	$\Delta\Delta C_T$±S.E	Fold change from control ($2^{-\Delta\Delta CT}$)	p-Value
	ANPT-1	B-Actin				
Small	24.26±0.26	17.35±0.68	6.89±0.72	0.00±0.72	1	0.72
Large	23.59±0.16	16.38±0.57	7.21±0.59	0.31±0.27	0.80^{ns}	

4. Discussion

According to our study the presence of mRNA for ANPT-1 gene could be detected in both small and large follicles of tropical goats in Kerala.

In consensus with our findings, the expression of ANPT-1 mRNA was seen to be decreased in granulosa cells of medium and large follicles compared to small follicles in gilts (Shimizu *et al*., 2003) [10]. In common marmoset monkeys the highest levels of ANPT-1 gene expression was noticed in granulosa cells of initial follicular development, while a decrease was noted in late secondary follicles (Wulff *et al*., 2001) [15]. A decrease in ANPT-1 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 ANPT-1 levels might be closely interwoven with growth and development of follicular and associated angiogenesis occurring in the preovulatory period (Nishigaki *et al*., 2011) [16].

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Goede *et al*., (1998) [7] reported that ANPT -1 is expressed in ovarian follicular cells throughout the ovarian cycle in cow. Hayashi *et al.* (2003) [6] detected the signals encoding mRNA of ANPT-1 in the theca internal and granulosa cells of bovine follicles and found that the mRNA expression of ANPT-1 did not vary in follicles with different oestradiol concentrations of follicular fluid. Hayashi *et al*. (2004) [17] failed to detect ANPT-1 mRNA in follicles with 5 to 10 mm diameter in bovines and concluded that the ANPT-1 expression did not change among different follicular developmental stages. Shimizu *et al.* (2007) [18] reported that in cows treated with GnRH hormone the expression of ANPT-1 decreased during the LH surge stage. Muller *et al*., (2009) [19] found out that in mares, entire population of granulosa and a large proportion of theca internal cells of primordial (no thecal layer) follicles and tertiary follicles showed ANPT-1expression. While Maisonpierre *et al*.,(1997) [3] found out that the expression of ANPT-1mRNA is restricted to the theca internal of the preovulatory follicle of rat, Abramovich *et al.*,(2009) [20] observed that angiopoetin-1 expression could not be found in granulosa cells from immature hormone-treated as well as non-treated rats during stage of any follicular development. ANPT-1 expression was detected in follicular fluid of women (Nishigaki *et al*., 2011) [16] and in primates, limited ANPT-1 production was detected in slow-growing and fast-growing follicles after antrum formation (Fisher *et al*., 2013) [21].

However in ovines, Chowdhury *et al.* (2010) [11] observed the maximum expression of ANPT-1 in large follicle just before the LH surge, in comparison to small and medium follicles. The authors could detect ANPT-1 expression in all follicles at all stages of oestrous cycle, though the expression did not vary significantly between small and medium follicles. Mishra *et al*. (2016) [12] reported that in buffaloes the mRNA expression for ANPT-1 was greater in follicles with a diameter greater than 14mm and the difference was significant when compared with small follicles.

6. Conclusion

Failure to detect a significant difference in expression of ANPT-1 with follicular growth in caprine species could be due to the fact that follicular growth, development and dominance depends not solely on ANPT-1 expression, but more on the ratio of ANPT-2: ANPT-1, together with whether vascular endothelial growth factor (VEGF), yet another crucial local ovarian angiogenic factor, is expressed or not, and to what extent (Mishra *et al*., 2016) [12]. The pattern and extent of angiogenesis and consequent follicular development is dependent on such cross talks between local ovarian factors, which in turn lead to profuse capillary growth, through which a rich supply of nutrients, oxygen, hormones, and growth factors are channelised to developing follicles (Hayashi *et al*., 2003) [6].

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