The Pharma Innovation Journal 2019; 8(9): 188-190

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(9): 188-190
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www.thepharmajournal.com
Received: 22-07-2019
Accepted: 24-08-2019

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Expression of CYP19 gene in different categories of ovarian antral follicles in goats

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Abstract
The present work was undertaken to study the expression pattern of CYP19 in ovarian antral follicles of tropical goats. CYP19 is a gene which codes for the enzyme aromatase. Oestradiol is synthesized from androgen in granulosa cells of the ovarian follicles by the action of aromatase. Our results showed that in caprines at proestral stage there was no significant difference between the expression of CYP19 in different categories of antral follicles (small follicles: - 1-3mm and large follicles: - < 3 mm diameter). An increase in the expression of CYP19 gene was observed in large follicles when compared to small follicles, the reason for the increased expression might be the formation of more granulosa cells towards later stage of follicular development.

Keywords: CYP19, aromatase, oestradiol, caprines, gene expression

1. Introduction

The aromatase gene (CYP19) belongs to cytochrome P450 enzyme family. The enzyme complex called aromatase catalyzes the synthesis of estrogens from androgens (Conley and Hinshelwood 2001) [1]. Granulosa cells of the ovarian follicle aromatize C19-steroids produced by cells of the theca interna to estrogens in response to FSH. Binding of FSH to receptors on granulosa cells increases intracellular cAMP, which, in turn, results in an enhanced *CYP19* expression in both humans and rats (Hickey *et al*., 1988) [2]. The classical two-cells-two-hormones model details about the significant role of theca and granulosa cells (follicular cells) and gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) in steroidogenesis and secretion in the ovary. By the
action of 17β hydroxysteroid dehydrogenase, androstenedione synthesized from progestogens is converted to testosterone in theca cells under the LH stimulus and the androgen is transported passively to the granulosa cells where the substrate formed is converted to estrogen by the action of aromatase under the stimulus of FSH (Miller *et al*., 1994) [3]. Stocco, (2008) [4] reported that in most mammals CYP19 gene contained a large regulatory region encoded by the enzyme aromatase, which is essential for the synthesis of estrogen. Largest follicle in mammals had significantly a greater aromatase activity than that of smaller follicles on all days of oestrous cycle (Rhodes *et al*., 2001) [5]. Steroidogenesis includes a cascade of events in which the androgens viz androstenedione, testosterone, and dihydrotestosterone are synthesized from cholesterol which are produced from the ovary in sequential manners along with other sex steroids, where each steroid serves as a substrate for the subsequent one (Palermo, 2007) [6]. Therefore, the coordinated and cell-specific expression of the *aromatase* (*Cyp19*) gene in the ovary plays a key role in the normal progression of the oestrous cycle. Researches are plenty in exploring the follicular dynamics of most farm animals while goats remain as a neglected species. Hence we designed our work with the aim 1) to find out the presesnce of CYP19 in different catogeries of follicles 2) to compare the expression of CYP19 in two different categories of follilces.

2. Materials and methods

2.1 Follicle collection and classification

Twelve non pregnant goats from the culling list of University Goat Farm, Mannuthy was selected for our study. The age of animals were within a range of two to five years. Oestrous in animals was confirmed by detecting the physiological and behavioural signs, also confirmed the open cervix in animals using the vaginal speculum method. After fifteen days from the day of oestrous ultra sound scanning was performed to assess about the follicular development

and regression of corpus luteum (CL). When the CL regressed, just before the exhibition of next oestrous cycle the animals were slaughtered in the Meat Technology Unit, KVASU, Mannuthy. The ovaries were collected in chilled Phosphate Buffered Saline and kept in ice. The samples were immediately transported to the working laboratory and isolated the follicles. Categorised the follicles as small (1-3mm) and large (>3mm) based on the follicular diameter. Stored the isolated follicles in RNA later until further processing (Qiagen, Life technologies, USA).

2.2 RNA isolation and cDNA synthesis

Isolated the RNA from the follicles 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 was 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.

2.3 Quantitative RT-PCR

The resulting cDNA was used as template for qRT-PCR. The qRT-PCR of target gene (CYP19) and housekeeping genes (β-actin) were performed 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
CYP19	FP: - CCCCTTGGATGAAAGTGCCA	193	XM_01396704 6.2
	RP: AGCTTCTCTGCTGTTGAAATCC		
β-actin	FP: -AGATCAAGATCATCGCGCCC	108	NM_00131434 2.1
	RP: -ACTCCTGCTTGCTGATCCAC		

The following qRT-PCR protocol was followed - single cycle, initial denaturation (enzyme activation) at 95 ^{0}C for 3 minutes for one cycle, 40 cycles of denaturation: - 95 ^{0}C for 30 seconds, annealing: - 61 ^{0}C for CYP19 and 600C for β-actin at 45 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. *β-actin* was used as internal control. The Cq of target gene and Cq of control gene were determined for each sample and calibrated (ΔΔCq method) (Livak and Schmittgen, 2001) [7].

$$\Delta Cq = Cq(\text{target gene}) - Cq(\text{reference gene}) \quad (1)$$

$$\Delta\Delta Cq = \Delta Cq(\text{test sample}) - \Delta Cq(\text{reference sample}) \quad (2)$$

$$RQ = 2^{-\Delta\Delta Cq} \quad (3)$$

2.4 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 ($P < 0.05$) (Snedecor and Cochran, 1994) [8].

3. Results

In caprines, the mRNA for gene *CYP19* could be detected in both small and large follicles category. When we compared the expression of *CYP19* in small and large follicles of does it was found that the expression of the gene was increasing from small to large follicles and the increase was 2. 49 fold in large follicles when compared to the small follicles (control group). However there was no significant difference between the expression of CYP19 in small and large follicles.

Table 2: Comparison of *CYP19* gene expression within goats between follicles of different sizes

Tissue	Mean CT ± S.E		ΔCT± S.E	ΔΔCT±S.E	Fold change from control (2-ΔΔCT)	p- Value
	CYP19	B- Actin				
Small	21.81±0.64	16.97 ±0.73	4.84±0.97	0.00±0.97	1	0.16
Large	20.30±0.96	16.77 ±0.54	3.53± 1.10	-1.31±0.64	2.49ns	

4. Discussion

In our study the expression of *CYP19* in large follicles was found to be increased by 2.49 folds from control group (small follicle) in goats. The increase, though insignificant, might be due to increase in granulosa cell layers occurring in tandem with follicular development and maturation.

According to Rhodes *et al.* (2001) [5], largest follicle had significantly a greater aromatase activity than that of smaller follicles in ewes, on all days. The granulosa cells of preovulatory follicles in ovary expressed maximum aromatase than that of the small follicles (Hickey *et al.*, 1988) [9]. Yuvan (2008) [10] reported that in ovines aromatase mRNA was expressed in all antral follicles irrespective of the size, and the expression significantly increased during the follicular growth. Huet *et al.* (1997) [11] reported that in ovines aromatase expression was detected only in follicles with a diameter greater than 3.5mm. Badinga *et al.* (1992) [12] who reported that the granulosa cells of dominant follicles had

significantly greater aromatase or *CYP19* activity than that of cells from subordinate follicle.

From our study it was concluded that the mRNA for *CYP19* gene was present in all antral follicles devoid of the difference in diameter and it was also noticed that though not significant the expression was increased from small to large follicles. Hence it is concluded that the increasing expression of CYP19 in large follicle might play role in increased aromatization of androgen to oestradiol than that in small follicle and thereby supports the enhances the follicular development.

5. Conclusion

From the present study it is concluded that the expression of CYP19 gene which codes for the enzyme aromatase is evident both small and large follicles of goats irrespective of the size. The expression was found to be increasing from the small to large follicle though the increase was not significant

6. Acknowledgement

The authors would like to thank Kerala Veterinary and Animal Sciences University, Pookode for funding our research work and we would also like to thank the Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Mannuthy for providing necessary facilities to conduct the work.

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