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Expression profile of insulin-like growth factor-1 (IGF-1) and insulin-like growth factor receptor-1 (IGFR-1) gene and the localisation of IGF-1 protein in granulosa cells of malabari goats

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Abstract
The study was conducted to investigate the expression profile of *IGF-1* and *IGFR-1* in granulosa cells and its relation with size of goat ovarian follicles, to detect the IGF-1 protein localization in granulosa cells and also to assess the gonadal steroid status in the follicular fluid. The ovaries of healthy cycling Malabari goats were collected and preserved. The follicular fluid from selected follicles of two groups; group I (medium-3-5mm) and group II (large->5mm) was separately aspirated. Follicular fluid (FF) pooled from seven follicles of same size were considered as one sample and six such samples of each group was used for the study. RT-PCR analysis demonstrated a significant difference ($p \leq 0.05$) in the expression levels of *IGF-1* and *IGFR-1* between granulosa cells from group-I and group-II follicles. The follicular fluid progesterone and oestrogen concentrations were significantly ($p \leq 0.05$) higher in the larger follicles. The immunohistochemistry of ovarian sections showed a positive immunoreaction for IGF-1 protein in granulosa cells of antral follicles. In the present study an increase in the expression of *IGF-1* and *IGFR-1* along with the increase in follicular fluid oestrogen concentration with the progression of follicular size indicate the role of IGF-1 and oestrogen in follicular development in caprines.

Keywords: *IGF-1, IGFR-1,* granulosa cells, malabari goats

1. Introduction
Goat also called as poor man's cow in India is the major contributor of rural income in the country and also component of world biodiversity. Hence understanding the goat follicular dynamics may help in better exploitation of the beneficial aspects of goat reproduction. Follicular development in goats occurs in wave like pattern, unlike cattle follicular dominance is less apparent. In general, four predominant follicular wave with 5 to 10 follicles having ≥ 3mm diameter are present in goat ovaries during the estrus cycle and ovulation occurs from follicles of 6-9 mm diameter (Ginther and Kot, 1994; Rubianes and Menchaca, 2003; Medan *et al*., 2005 Lehloenya *et al.*, 2008) [4, 21, 14, 10]. The development of small to large antral follicles occurred mainly under the influence of gonadotropins (Medrano *et al*., 2012) [15] while locally produced hormones and growth factors played a facilitator role in follicular growth (Monget and Bondy, 2000) [15]. Insulin-like growth factor is one such growth factor. The involvement of insulin-like growth factor system in follicular development was proposed by presence of insulin-like growth factor-1 (*IGF-1)* and insulin-like growth factor receptor-1 (*IGFR-1)* in ovary, oviduct and uterus (Giudice, 1992) [5]. The coexpression of IGFR-1 with gonadotropin releasing hormone in rat brain indicated a potential anatomic locus where IGF-1 regulate reproductive development. Moreover the presence of IGFR-1 in ovine pars tuberalis and median eminence of rat pituitary indicated that the influence of IGFs on gonadal axis start at the central level (Williams *et al*., 1995) [28]. These insulin-like growth factors are reported to be produced by granulosa cells (GC), theca cells (TC) and luteal cells in different animals thus forming an intraovarian autocrine and paracrine system (Spicer and Echternkamp, 1995) [25] affecting the reproduction efficiencies. *In vitro* studies in rat, and porcine granulosa cells demonstrated that IGF-1 enhanced follicle stimulating hormone (FSH) and stimulated steroidogenesis in both follicular cells and luteal cells (Adashi *et al*., 1985; Giudice, 1992) [1, 5]. This occurs due to the increased aromatase activity and lutenizing hormone (LH) receptor synthesis in GCs by synergistic actions of IGF-1 and FSH as demonstrated by culture studies in rat granulosa cells Adashi *et al*. (1985) [1].

Wood and Strauss (2002) [29] reviewed that in pigs and rodents, IGF-1 is the major IGF ligand produced by GCs whereas IGF-2 is the major one produced by GCs and TCs in humans. But its expression in ruminants remains controversial with *in vitro* (Martins *et al.*, 2010) [11] and *in vivo* (Yu *et al.*, 2003) [30] studies in caprine follicles suggesting the presence of it and *in vivo* studies in ovine follicles demonstrating low expression of mRNA encoding IGF-1 (Hastie and Haresign, 2006). Mikawa *et al*. (1995) [12, 16] reported that *IGF1* gene was expressed in goat ovary and its expression increased during development of goat from young to adulthood. Magalhaes *et al*. (2013) [12] have demonstrated the involvement of IGF-1 gene in secondary and antral follicle development in goats using microarray analysis but information regarding its expression in granulosa cells of antral follicle of goats is scanty. Hence the present study was conducted in Malabari goats, to know the *in vivo* differential expression profile of IGF-1 and IGFR-1 genes in granulosa cells in different size of antral follicles, localization of IGF-1 protein in GCs and also to assess the gonadal steroid status in the follicular fluid.

2. Materials and Methods

2.1 Animals and sample collection

Ovaries (n=30) from fifteen adult healthy Malabari goats were collected at Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuthy, in acoordance with law of university ethical committee. Immediately after collection the ovaries were stripped of surrounding adipose tissue and rinsed in chilled normal saline. Ovaries were transported to the laboratory within 90 minutes after slaughter in thermo flask containing chilled phosphate buffered saline solution. Twenty four ovaries were used to evaluate the mRNA expression and hormone analysis and the rest were used for immunohisto chemical localization of IGF-1 protein.

2.2 Granulosa cell isolation and follicular fluid separation

Only follicles which appeared healthy (well vascularised and having transparent follicular wall and fluid) and with diameter more than 3 mm were used for the study. The follicular fluid from selected healthy follicles of two groups; Group I (medium-3-5mm) and Group II (>5mm) was separately aspirated. Follicular fluid pooled from seven follicles were considered as one sample and six such samples were used for the study. The oocyte present in the follicular fluid was removed under the stereozoom microscope and the granulosa cells suspended in the follicular fluid were harvested by centrifugation of follicular fluid at 500×*g* for 10 min[26] (Supriya, 2016). The supernatant follicular fluid was further centrifuged at 12,000×g for 20 min, to remove cellular debris, and stored at -20°C until used for hormone quantification.

2.3 RNA isolation and cDNA synthesis

The total RNA was isolated manually from these pelleted GCs using TRIzol™ reagent (ambion by Life technologies, USA) according to the instructions of manufacturer with slight modification. The samples were then treated with DNase I to remove the genomic DNA contamination. The purity and concentration of RNA was assessed spectrophotometrically using Nanodrop (Fig 1). The integrity of RNA was analysed on 0.8% agarose gel (Fig 2). One microgram of RNA was reverse transcribed into cDNA using Thermo Scientific Verso cDNA synthesis (USA) kit in a reaction volume of 20 µL and stored in -80 untill use.

2.4 Standardisation of PCR

Gene specific primers for IGF-1, IGFR-1 and β-actin (housekeeping gene) were designed using online NCBI primer design software (Primer3, http://bioinfo.ut.ee/primer3/) and specificity of the primer was checked using BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) to amplify the target genes. PCR reactions were carried out in 25µL of the mixture. The details of primer used and PCR conditions are given in Table 1 & 2 respectively. The sizes of the amplified product run on two per cent agarose gel were in agreement with the length of the amplicons specified in the primer designing tool and the product size of IGF-1, IGFR-1 and β-actin were found to be 111bp, 115bp and 54bp respectively (Fig 3). The efficiency checking of PCR reactions were determined by using standard curve drawn with three serial dilutions of cDNA that is 1, 1:10 and 1:100. Annealing temperature determined for three genes was referred for real time PCR reactions.

2.5 Real-time PCR analysis

Quantification of mRNA for IGF-1, IGFR-1 and β-actin in isolated GCs were performed using Maxima SYBR green/ROXqPCR Master Mix (2X) (Thermofisher Scientific, USA). The RT-PCR reaction was carried out in a 10µL reaction volume using 1µL of template cDNA in 5µL of SYBR Green, 3.8µL of nuclease free water and 0.4µM of each primer. The PCR reactions were carried out in triplicates (technical triplicates). In addition, one non template control (NTC) for each gene, transcription minus (RT minus) control for each sample and negative control (with only nuclease free water) were also included in the reaction. Tubes were centrifuged briefly to force the reaction mixture to the bottom of the tubes and to remove any possible bubbles and the proper tube setup was planned. Three steps thermal cycling protocol was followed for the Real-time PCR. The details of the conditions found optimum for the amplification of *IGF-1*, *IGFR-1* and *β- actin* transcript cDNA giving product of desired size repeatedly in Real time PCR is described in table 3. It also included the dissociation step to draw the melt curve in order to check the Real-time PCR reaction specificity.

Pfaffl, (2001) [20] method of real-time PCR analysis was followed to study the relative expression of *IGF-1* and *IGFR-1* transcript in granulosa cells of group-I and group-II follicles of goat. The Group-I was used as control for obtaining relative mRNA expression. Beta-actin was used as house keeping or reference gene.

2.6 Hormonal assay

The oestrogen and progesterone concentration in the samples were analyzed using the commercial ELISA kits (Pathozyme ® Oestradiol, Omega Diagnostics Ltd, Pathozyme ® progesterone, Omega Diagnostics Ltd, Thermofisher) as per manufacturers instructions. The absorbance was read at 450nm on the ELISA plate reader (Thermofisher, Finland)

2.7 Immunohistochemistry

Localization of IGF protein was done on serial 5µm sections cut from the ovaries (n=12) of six different adult goats. The immunohistochemistry was carried out in cut sections using chemicals procured from biogenex, USA as per manufacturer's instructions with slight modifications. These sections were mounted on poly-L-lysine coated slides dried overnight at 37 °C, deparaffinised in xylene and rehydrated in a graded ethanol series. Then the epitopes were activated by

microwaving the sections for 20 min at 300W in 0.01M citrate buffer (pH6). The sections were rinsed with distilled water. The endogenous peroxidase was blocked by incubating the sections in 3 per cent hydrogen peroxide for 10 min. The sections were then rinsed with PBS buffer before being incubated for 10 min with powerblock to minimize non specific binding. The sections were then incubated overnight in moist chamber with polyclonal anti IGF-1 diluted 1:200 (Abcam, UK). After this incubation sections were washed 3 times with PBS and incubated for 45 min with secondary antibody. Next the sections were rinsed with PBS and incubated for 30 min with HRP Conjugate. The sections after washing 3 times with PBS were stained with diaminobenzidine. The sections after rinsing with PBS was couterstained for 3min in Mayers haematoxylin. Finally sections were rinsed with tapwater for 30sec and mounted in DPX mount for histological examination. Sections were visualised in Leica DM 2000 LED microscope.

2.8 Statistical analysis

Statistical significance between the two groups was calculated by student t- test for gene expression study and hormonal assay using the software Statistical Product and Service Solutions (SPSS), version 21.

3. Results and Discussion

The present study analysed the differential expression of mRNAs encoding *IGF-1* and *IGFR-1* in granulosa cells of group I (medium-3-5mm) and group II (large->5mm) follicles, localization of IGF-1 protein in antral follicle and gonadal steroid status in the follicular fluid of two groups of follicles. There was significant difference ($p \leq 0.05$) in the expression levels of *IGF-1* and *IGFR-1* between two groups of follicles (Table no: 4). In our study it was observed that IGF-1 mRNA expression increased with increase in follicular size. Similar to our results Yu *et al*. (2003) [30] demonstrated higher *IGF-1* levels in the follicular fluid of large follicles (>5mm) compared to medium (3-5mm) follicles in cycling does of Lubei White breed. *In vivo* studies in beef cattle revealed an increase in concentration of IGF-1 and oestrogen in the follicular fluid of large follicles (>8mm) compared to medium (5-8mm) follicles (Echternkamp *et al*., 1994) [3]. The present immunohistochemical study detected the strong immunoreaction for IGF-1 in granulosa cells of antral follicle (Fig 4) which were in agreement with Martins *et al.* (2010) [11] who demonstrated strong immunoreaction for IGF-1 in GCs of caprine antral follicles.

In the present study increase in *IGFR-1* expression was noticed with increase in the size of follicles which was in agreement with Spicer *et al.* (1994) [24] who reported that granulosa cells from large (>8mm) bovine follicles have more IGF-1 receptors than small (3-5mm) follicles. Consistent with the present study Singh *et al.* (2015) [22] also noticed an increase in the *IGFR-1* expression with increase in the follicular size in water buffalo and thus, it seems that granulosa cells acquire a greater number of IGF-1 receptors during the process of differentiation. The increase in the expression of IGFR-1 mRNA in the caprine GCs may indicate its steroidogenic capacity as Zhou *et al*. (1991) [30] suggested that higher levels of IGFR-1 mRNA were observed in corpus luteum of rats than in growing follicles indicating its steroidogenic capacity than proliferative ability. Moreover an increase in the steroidogenesis was observed in the present study to support this observation.

In the present study the follicular fluid estradiol-17β level was significantly ($p \leq 0.05$) higher in the group II follicles than the group I follicles (Table 5) which was in accordance with the reports of Mohanan *et al.* (2017) [17] who observed significantly higher concentration of oestrogen in the follicular fluid from large (3-6mm) follicle compared to small (1-3mm) follicles in Malabari goats. The result of this study was almost in agreement with Henderson *et al.* (1982) [8] in bovine, Kalmath and Ravindra, (2007) [9] in buffalo, and Tungal *et al.* (2014) [27] in goat follicular fluid. The analysis of data revealed that mean follicular fluid progesterone level was significantly ($p \leq 0.05$) higher in group II follicles than the group I follicles (Table 5). Our result was in agreement with Henderson *et al.* (1982) [8] that in bovine progesterone concentrations increased with follicle size. However, Berisha *et al.* (2000) [2] reported that healthy bovine follicles of all sizes had relatively constant progesterone level. In the present study we found that the higher concentration of both progesterone and estradiol-17β in group II follicles than the group I follicles. It might be due to the increased steroidogenic activity of follicular cells with increase in size of follicle. Similarly, increase in progesterone concentration in group II follicles might be due to the production of progesterone in the synthetic pathway of follicular production of oestrogen.

The IGFs were the local mediators of gonadotropins in the ovary hence synergistically with gonadotropins it increased the proliferation and steroidogenesis in follicular cells. The IGF-1 acting through IGFR-1 promote the proliferation of granulosa cells, may be by stimulating the cell cycle events and increasing the production of intracellular proteins like c-jun and c-myc (Mazerbourg *et al*., 2003; Spicer and Aad, 2007) [13, 23]. The IGF system synergistically with gonadotropins is said to increase the generation of cAMP, the gonadotropin receptor second messenger. The increased cAMP activated protein kinase-A, in turn, can directly influence the expression and/or activity of components of steroidogenic machinery like the aromatase and the steriodogenic acute regulatory (StAR) protein (Wood and Strauss, 2002) [29]. Moreover, the IGF-1 is as effective or more as FSH in stimulating oestrogen synthesis (Giudice, 2001) [6].

Our study indicate protein localisation of IGF-1 in GCs and greater expression of IGF-1 and IGFR-1 gene in GCs with the progression of follicular size in goat. This may be indicative of autocrine action of IGF-1 in the granulosa cells of caprines as evidenced from the mRNA expression of *IGF-1* and *IGFR-1* in antral follicles and they may stimulate proliferation and steroidogenesis of granulosa cells as an increase in expression of IGF-1 and IGFR-1 was also found to be associated with an increased concentration of oestrogen in the follicular fluid.

4. Figures

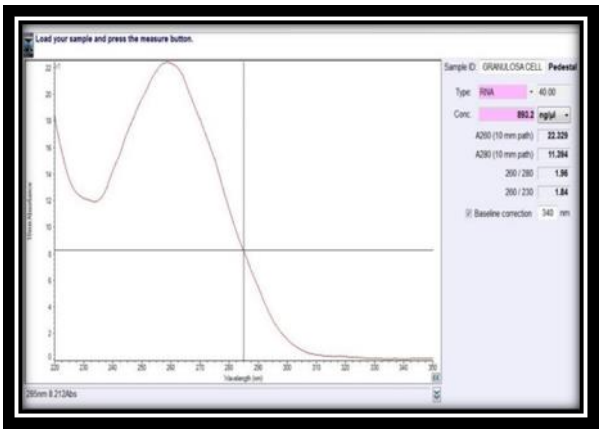

Fig 1: Nano Drop spectrophotometer measurement of the concentration and optical density of RNA

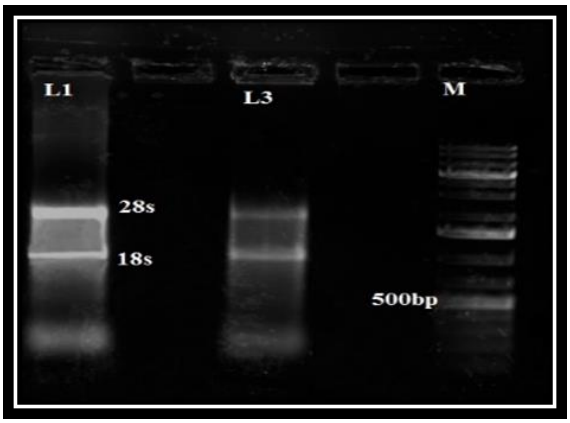

Fig 2: Agarose gel electrophoresis of RNA isolated from granulosa cells

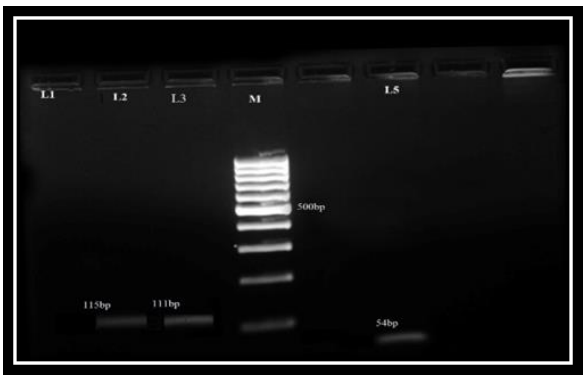

Fig 3: Agarose gel electrophoresis of PCR products IGFR-1:115bp, IGF-1:111bp and *β actin:* 54bp (100bp ladder)

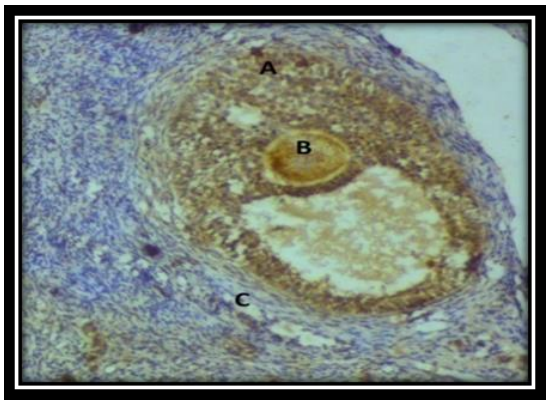

Fig 4: IGF-1 ligand immunoreactivity in different structures of goat anral follicles Intense immunoreactions for IGFI in GCs, oocyte and comparatively less in theca cells (Original magnification X400) A- Granulosa cells B-Oocyte C-Theca cells

5. Tables

Table 1: Primer details

Gene	Primers	Sequence (5'-3')	Product size	Accession no
IGF-1	Forward	5'CATCCTCCTCGCATCTCTTC3'	111bp	D11378
	Reverse	5'ACTGGAGAGCATCCACCAAC3'		
IGFR-1	Forward	5' TGCGCTTCTGTTGATAGTGG3'	115bp	XM_018065947.1
	Reverse	5'AAACGTGGGTCTCTGATTGG3'		
β-actin	Forward	5'AGCTCGCCATGGATGATGA3'	54bp	DQ661647
	Reverse	5' TGCCGGAGCCGTTGT 3'		

Table 2: PCR conditions for gene amplification

Steps		Temperature	Time
Initial denaturation		**95 ^{0}C**	**3min**
35 cycles of	Denaturation	95 ^{0}C	15 sec
	Annealing *IGF-1* *IGFR-1*and *β-actin*	60 ^{0}C 59 ^{0}C	30 sec
	Extension	72 ^{0}C	30sec

Final extension: 72 ^{0}C for 30 sec

Table 3: Real-time PCR programme for amplification of *IGF1*, *IGFR1*and *β- actin* Genes

SI. No.	Step	Temperature	Time
1	Initial denaturation cycles	95 ºC	3 min
2	40 cycles of		
	Denaturation	95 ºC	15 sec
	Annealing *IGF-1* *IGFR-1* and *β-actin*	60 ºC 59 ºC	30 sec
	Extension	72 ºC	30 sec
3	Dissociation step	95 ºC	15 sec
		55 ºC	30sec
		95 ºC	15 sec

Table 4: The relative expression ratio of *IGF1* and *IGFR1*

Gene	*ΔCT-target*	*ΔCT-reference*	Fold change	p-value
IGF1	3.31±0.63	2.14±0.26	1.56*	0.002
IGFR1	4.50±1.23	2.14±0.26	3.19*	0.028

* - significant at 0.05 level

Table 5: Mean (±SE) Progesterone and Oestrogen concentration in the follicular fluid

Hormone	Average follicular fluid hormone level (ng/ml)	
	Group-1	Group-2
Progesterone	10.56 ± 2.3	20.53 ± 3.82 *
Oestrogen	12.08 ± 2.78	22.08 ± 1.97 *

* - significant at 0.05 level

6. Conclusion

The results of our study concludes the presence of IGF-1 and IGFR-1 in Malabari goat antral follicle and that IGF-1 acting via IGFR-1 in an autocrine manner in the granulosa cells may stimulate the proliferation and steroidogenesis in GCs. The expression and protein quantification of all the components of IGF family in antral follicles has to be analysed to identify the exact role of IGF family in folliculogenesis. Findings in the present study will be helpful in understanding the growth factors which regulate ovarian follicle development in caprines which in turn will be helpful in getting better reproductive efficiency.

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