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Research Article

Isolation of Theca Cells from Goat Ovarian Follicles and Expression of *CYP19* Gene in the Isolated Cells

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Abstract

Ovarian Theca Cells (TCs) isolation is quite difficult as they are attached to connective tissue and usually isolated TCs have contamination with Granulosa cells (GCs). Contamination of TCs with GCs can be detected by the marker genes which are expressed only in GCs. *Cytochrome P450 aromatase* (*CYP 19*) is a marker for GCs which is responsible for the formation of enzyme *aromatase* for the synthesis of oestradiol from androgen. This study was undertaken to isolate TCs from caprine ovarian follicles by two different methods and to assess the efficacy of these methods to isolate theca cells with minimum contamination by GCs. In the study, follicles measuring three to six-millimetre diameter were used for isolation of TCs. Theca cells were isolated by two different methods - Trypsin and Collagenase methods. In the first method [1], trypsin with hyaluronidase was used and in the second method [2] different concentrations and combinations of collagenase and hyaluronidase enzymes were used. The isolated TCs were visualised under the inverted microscope and samples from both the methods were subjected to real-time qPCR for the presence of GCs specific marker *Cytochrome P450 aromatase* (*CYP 19*). *Cytochrome P450 17 α-hydroxylase* (*CYP 17*), marker for TC, gene expression was also studied to estimate the yield of TC by two methods. From this study, it was confirmed that collagenase was the better method for TCs isolation.

Keywords: *Caprine Ovarian Follicles; Theca Cells; CYP 17; CYP 19; qRT-PCR*

Introduction

An ovarian follicle is multi-compartmental and it is responsible for the formation and maturation of female gametes and steroidogenesis. The vital components of ovarian follicles (Graafian follicles) consist of an oocyte surrounded by GCs and TCs [3]. Folliculogenesis generally refers to the growth and differentiation of follicles from the primordial population. Follicular development starts by transformation of primordial germ cells into oocytes and their enclosed structures called follicles. Follicles are classified into type 1 (Primordial), type 2 (Primary), type 3 and 4 (Preantral), and type 5 (early antral) [4-6]. During secondary follicle formation, specialised stroma layers

of TCs accumulate as a layer around GC-oocyte structure. In the tertiary follicle formation, TCs are transformed into Theca Interna Cells (TICs) and Theca externa cells (TECs). The TECs are loosely organised non-steroidogenic cells, contains abundant collagen. The TICs are three to five layers thick and highly vascularised steroidogenic cells, responsible for the production of androgen and thereby synthesis of oestrogen by GCs. After rupture of the ovarian follicle, the TICs differentiated into theca lutein cells or small luteal cells of the corpus luteum. The adjacent layer of GCs with basement membrane consists of mural GCs which are steroidogenically more active than cumulus cells in the production of oestradiol. The GCs express *CYP 19* gene, which is responsible for the formation of the enzyme *aromatase,* used for oestradiol biosynthesis.

It is necessary to isolate the pure population of somatic cells, both GCs and TCs from follicles which enable us to study their functions and regulation of follicle development and ovarian steroidogenesis by culture methods. Methods are available for isolation of GCs. However, only a few methods currently exist for the isolation of a pure population of TCs from ovarian follicles. TCs isolation is quite difficult as they are attached to connective tissue and usually isolated TCs have contamination with GCs. Contamination of TCs with GCs can be detected by the marker genes which are expressed only in GCs [7].

Therefore, the present study was undertaken to compare the efficacy two methods for isolation of TCs from goat ovarian antral follicles and to assess the isolated cells by analysing expression of marker genes.

Materials and Methods

Theca cell isolation methods from goat ovarian follicles

Caprine ovaries were collected from slaughterhouses in and around the College of Veterinary and Animal Sciences campus, Mannuthy. Isolation of TCs was done by two different methods [1,2] and was compared for the purity of isolation by quantification of GCs marker, *CYP 19*. In trypsin method, as suggested by Stoklosowa., *et al*. [1] the collected ovaries were washed thoroughly in fresh Phosphate Buffer Saline (PBS). Ovaries were washed with Dulbecco's PBS (DPBS) without calcium and magnesium solution in the collagenase method [2]. Adipose tissues surrounding the ovaries were removed and the diameter of ovarian follicles was measured with vernier callipers and recorded.

Isolation of TCs - Trypsin method [1]

Three to six mm diameter of follicles (Figure 1) were cut open and spread in a Petri dish in such a way that the interior GCs layer was turned upwards. GCs were removed from the ovarian follicles using a platinum loop under the dissection microscope. To remove the detached GCs, PBS in Petri dish was changed frequently. Using a sharp scalpel blade, follicles were excised and placed in a Petri dish with PBS. GCs attached to the basement membrane was further removed by incubating with Hyaluronidase (Sigma Chemical Co., St Louis, MO) enzyme in 10 IU/mL of PBS for two minutes. The procedure was repeated three times to ensure complete removal of GCs.

Figure 1: *3 - 6 mm follicles for theca cell isolation.*

Theca layers thus separated were vigorously stirred in Medium 199 (Sigma-Aldrich, Cat. No. M2520) with 10 per cent foetal bovine serum (FBS, Whittaker Bioproduct, Walkersville, MD) to stop the activity of Hyaluronidase enzyme. Then, the tissue was transferred with forceps to a clean and sterile Petri dish containing fresh Medium 199 and was energetically pipetted with a capillary pipette. The medium was changed several times until no GCs clusters were visible when checked under the microscope. The isolated theca tissue was washed, cleaned and minced with scissors and exposed to routine gentle trypsinisation with 0.25 per cent trypsin (Sigma-Aldrich, Cat. No. T4049) enzyme for 10 minutes at 37^0C for three times. TCs were dispersed and washed with medium 199 with 10 per cent FBS. The resulting cell suspension contained viable cells many of which were ovate or polygonal shape under an inverted microscope (Figure 2).

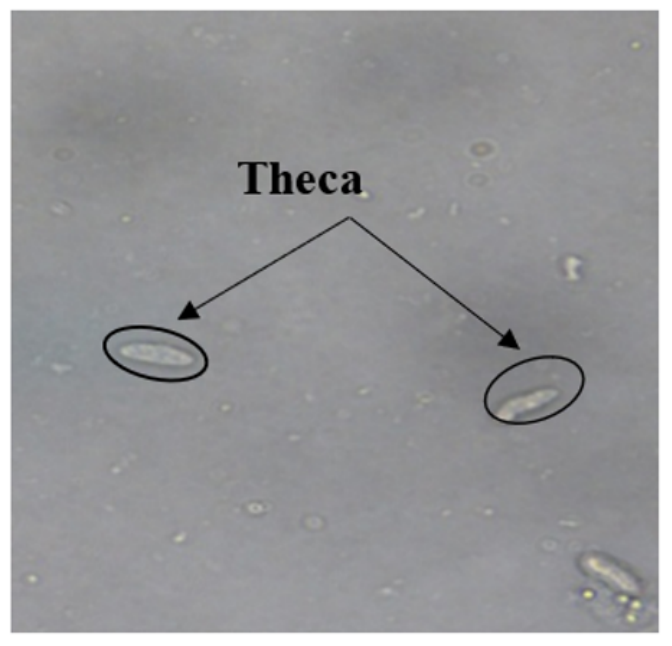

Figure 2: *Microscopical image of theca cells under stereo zoom inverted microscope.*

Isolation of TCs - Collagenase method [2]

Whole follicle (3 - 6 mm diameter) was cut in half with a pair of the fine dissection scissors. From inside the follicles, GCs were removed by gently scraping with a platinum loop and scalpel blades under a dissecting microscope. The remaining GCs were enzymatically detached by Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Cat. No.R0883) containing 0.024 per cent collagenase (Sigma-Aldrich, Cat. No. C9891) and 0.001 per cent hyaluronidase, by incubation for 10 minutes at 37^0C in shaking water-bath. Then, tissue was filtered through one mm square steel mesh basket and washed out with a vigorous stream of a large quantity of CMS-PBS to eliminate the detached GCs from thecal sheets. The theca tissues were enzymatically digested in a shaking water bath for one hour at 37^0C in RPMI 1640 containing 0.12 per cent collagenase and 0.005 per cent hyaluronidase. The digested tissue was then filtered through a 100 µm nylon mesh to collect detached TCs. The isolated cells were washed thrice with fresh RPMI 1640 medium and visualised under an inverted microscope (Figure 2).

Expression studies of *CYP 17* in theca Cells and *CYP 19* in granulosa cells

The isolated cell suspension containing cells, their purity and the efficiency of isolation methods were checked by quantifying the expression of *CYP 17* and *CYP 19* genes. In the present study, this technique was used to find relative expression of *CYP 17* as marker gene of TCs and *CYP 19* genes in GCs using β-*actin* as a reference gene. The relative quantification of gene expression was carried out with cDNA as a template for amplification using specific gene by Illumina Eco® qRT-PCR system using SYBR green chemistry.

Complementary DNA (cDNA) synthesis for expression studies

RNA was isolated from the pure TCs samples of two different methods by RNeasy mini kit (Qiagen, Life technologies, USA). The quality and integrity of isolated RNA were checked by NanoDrop (Thermo Scientific, NanoDrop™2000 Spectrophotometer, USA) (Figure 3) and agarose gel electrophoresis. Complementary DNA was synthesised from isolated total RNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Cat# K1622). For cDNA synthesis, 500 ng of RNA from each sample of both methods was reverse transcribed.

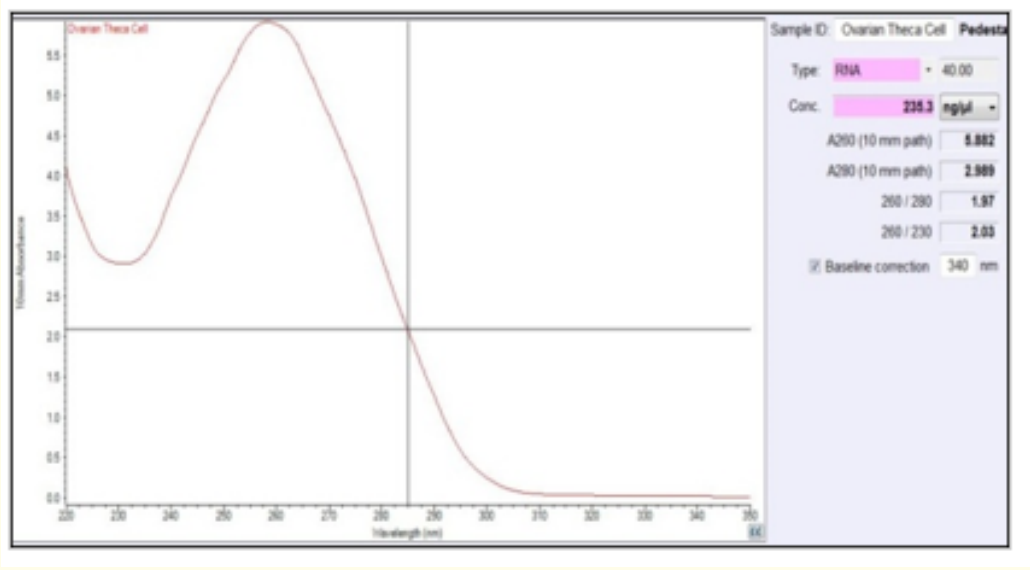

Figure 3: *Measuring the concentration and assessing purity of RNA using NanoDrop spectrophotometer.*

Quantification of *CYP17* and *CYP19* in isolated goat ovarian theca cells

The relative quantification of gene expression was carried out using Illumina Eco® qRT-PCR system using SYBR green chemistry. Relative expression of *CYP 17* as TCs specific, *CYP 19* as GCs specific gene was carried out using β-actin as the reference gene. Exon-spanning primers were designed for *CYP 17* (NM_001314145.1) and *CYP 19* (XM_013967046.2) using online primer design software Primer 3 (v.0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 1). The primer specificity was checked using BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Accession Number	Primer	Primer Sequence (5^1-3^1)	Product Size (bp)
NM_00131 4145.1	*CYP 17*	GCAAGGAATTCTCTGGGCGT	186
		CGTTGGCTTCCTGATTAATGATCT	
XM_01396 7046.2	*CYP 19*	CGACCTTCCCCTGAGATCAA	183
		GCTGGGACCTGGTATTGAGG	
NM_00131 4342.1	β- actin	AGATCAAGATCATCGCGCCC	108
		ACTCCTGCTTGCTGCTGATCCA	

Table 1: *Primers for caprine CYP 17, CYP 19 and β-actin genes.*

Quantitative real-time PCR

Six biological replicates were taken from two isolation methods under study and in each sample; two technical replicates were taken for conducting real-time expression studies. Separate reaction plates were kept for each gene of interest as the annealing temperature varied between genes of interest under study. In addition to the sample, specific control reactions were also kept for Non Template Control (NTC) - reaction assay with all necessary components except template, RT minus control (RTC) - reaction assay with all necessary components containing DNase treated RNA, Negative control, where only nuclease-free water was in place of template in master mix for real-time PCR reaction.

After the end of 40 cycles, melt curve analysis was performed to check the specificity of the amplification and also to discriminate from non- specific amplifications or primer dimers formed. The fluorescence signal was captured at every 0.3^0C rise in temperature during the final denaturation stage.

Statistical analysis

The cycle threshold (CT) values obtained at the end of the reaction generated by the machine were recorded and further analysis was done based on the $2^{-\Delta\Delta CT}$ method [8].

Results

In this study, the ovarian follicles were measured and classified as large, medium and small follicles respectively by vernier callipers and follicles with three to six mm diameter were isolated for further processing. The study was aimed to compare two different methods (Trypsin and Collagenase) to isolate ovarian TCs from caprine ovarian follicles with less contamination by GCs. Samples from both methods were subjected to qRT-PCR for a specific marker *CYP 19* to detect the presence of GCs. *CYP17* was quantified to assess the efficacy of the methods in isolation of TCs.

Theca cells identification

When observed under an inverted microscope, the viable cells in isolated TCs suspension were ovate or polygonal in shape (Figure 2).

Isolation of total RNA from ovarian theca cells

The concentration of total RNA isolated from collected TCs was checked using NanoDrop 2000C spectrophotometer, RNA samples having optical density ratio (OD) 260/280 above ~2 were used for this study. Average value of OD 260/280 and 260/230 ratio for isolated ovarian TCs were 2.06 ± 0.09 and 2.05 ± 0.18, respectively.

Quantitative real-time PCR of isolated theca cells

Quantitative real-time PCR was carried out with cDNA as a template for amplification using gene-specific primers. Primers used for real-time PCR were standardised using conventional PCR for its annealing temperature. Specificity of primers was checked by visualisation of amplified products in two per cent agarose gel under gel documentation system. The size of the amplified product of *CYP 17* was 186 bp (Figure 4), *CYP 19* was 183 bp (Figure 5) and *β- actin* was 108 bp (Figure 6). *B-Actin* was used as an internal control or reference gene for studying the expression pattern of *CYP17* and *CYP19*. A single peak was observed on melt curve analysis for each gene which confirmed the absence of any non-specific product or primer dimer.

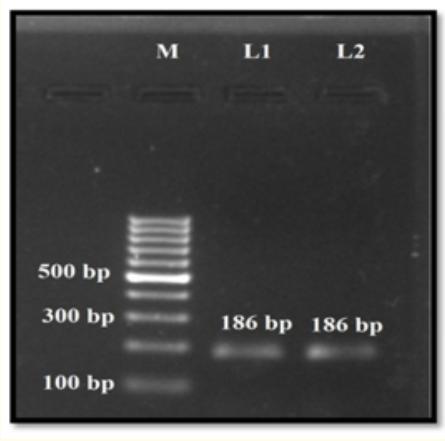

Figure 4: *Agarose gel electrophoresis of PCR products of CYP 17 by trypsin (L1) and collagenase (L2) along with 100 bp DNA ladder (M).*

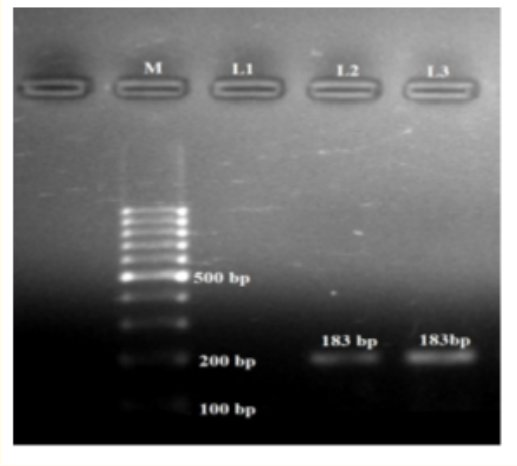

Figure 5: *Agarose gel electrophoresis of PCR products of CYP 19 by trypsin (L1) and collagenase (L2) along with 100 bp DNA ladder (M).*

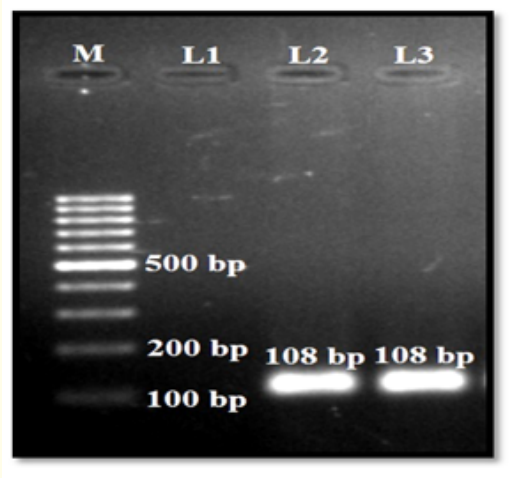

Figure 6: *Agarose gel electrophoresis of PCR products of β-actin by trypsin (L1) and collagenase (L2) along with 100 bp DNA ladder (M).*

Relative quantification of CYP17 and CYP19 gene expression between trypsin and collagenase methods

The expression of target genes (*CYP 17 and CYP19*) and reference gene (*β-actin*) was compared to calculate Δ^{CT} (Table 3). In the present study, *the CYP 17* gene expression was found to be lesser (0.18 folds)in trypsin method when compared with the collagenase method. *CYP 19* gene was found to be higher (5.38 folds) in TCs isolated by trypsin method in comparison with the collagenase method.

The result (Table 2 and 3) obtained by qRT-PCR, confirmed that the collagenase method is better than trypsin method for TCs isolation.

Method	Mean CT ± S.E (n = 6)		ΔCT ± S.E	ΔΔCT ± S.E	Fold change from control ($2^{-\Delta\Delta CT}$)	P-Value
	CYP 17	*β actin*				
Trypsin	24.65 ± 0.62	18.41 ± 0.14	6.23 ± 0.64	2.46 ± 0.64	*0.18	0.016
Collagenase	21.12 ± 0.11	17.36 ± 0.52	3.76 ± 0.53	0.00 ± 0.53	1	

Table 2: *Comparison of CYP 17 gene expression between methods (Trypsin and collagenase).*
**Significant at P value ≤0.05.*

Method	Mean CT ± S.E (n = 6)		ΔCT ± S.E	ΔΔCT ± S.E	Fold change from control ($2^{-\Delta\Delta CT}$)	P-Value
	CYP 19	*β actin*				
Trypsin	26.03 ± 0.65	18.44 ± 0.16	7.59 ± 0.67	-2.41 ± 0.67	*5.38	0.028
Collagenase	27.37 ± 0.11	17.36 ± 0.52	10.0 ± 0.53	0.00 ± 0.53	1	

Table 3: *Comparison of CYP 19 gene expression between methods (Trypsin and Collagenase).*
**Significant at P value ≤ 0.05.*

Discussion and Conclusion

Theca cells and granulosa cells are the fundamental components of ovarian follicles essential for follicle development including ovulation and provide precursor material for oestrogen synthesis and are considered to be essential cell models for basic research. However, no method currently exists for simultaneously isolating TCs without contamination of GCs from ovarian follicles.

This study intended to assess the efficacy of Trypsin and Collagenase methods [1,2] to isolate ovarian TCs from caprine ovaries with less contamination by GCs.

Caprine ovaries (n = 6) were collected in solution of PBS for trypsin method and other (n = 6) in DPBS for collagenase method, the ovarian follicles were measured and classified as ≥ 5 mm, ≥ 4 mm and ≥ 2 mm of large, medium and small follicles respectively by vernier callipers in accordance with the method was adopted by Sangha., *et al* [9]. The follicles with three to six mm diameter were isolated for further processing.

Stoklosowa., *et al.* [1] had purified and dispersed theca cells by a combination of mechanical and enzymatic procedures. This method was adopted for the current study of TCs isolation. In the study, the attached GCs to basement membrane in ovarian follicles were removed enzymatically by incubation with hyaluronidase in PBS. Hyaluronidase is a polysaccharide with specific action against hyaluronic acid and chondroitin sulfate. It is used for the dissociation of tissues, usually in combination with other proteases.

Kataoka., *et al.* [2] isolated TCs from the porcine ovary with less than three per cent GCs contamination by a method in which they utilised different concentrations and a combination of collagenase and hyaluronidase enzyme. Also, this method was chosen for the second study of isolation of caprine ovarian TCs. In this study, the tissue layers were incubated in a mild concentration of hyaluronidase (0.005 per cent) and collagenase (0.024 per cent) with RPMI 1640. Collagenase can attack and degrade the triple-helical native collagen fibrils commonly found in connective tissue. Here, the hyaluronidase enzyme was used in combination with collagenase enzyme for dissociation of connective tissues. The remaining thecal sheets were minced and treated with very mild concentrations of RPMI 1640 with collagenase (0.012 per cent) and hyaluronidase (0.001 per cent). The TCs were observed under an inverted microscope for their oval or polygonal shapes.

Similarly, the TCs were isolated with collagenase by a mechanical and enzymatic method from Immature Sprague-Dawley intact female rats [10,11]. Also, followed by, Liu., *et al*. [12] isolated TCs from mouse pre-antral follicles by collagenase I enzymes, to study steroidogenesis.

Structure and shape of ovarian theca cells

The isolated theca interna cells appeared as epithelioid in shape, which is typical of steroidogenic cells [13]. An epithelial-like appearance and androgenic capacity of theca cells were maintained when co-cultured with granulosa cells; however their shape became fibroblastic and produced less androgen when cultured alone [14]. This suggested that the presence of granulosa cell factors were essential for theca cells to retain their function and morphology. [15]. Theca cells were of fibroblast-like long fusiform or an anomalistic triangular shape, while the GCs were polygonal or cuboidal, when observed by an inverted microscope [16].

Expression studies of marker genes

To compare the efficacy of these two methods quantification of *CYP17* and *CYP 19* was done by qRT-PCR. *CYP 17* is required for the synthesis of androgens in TCs and *CYP 19* responsible for the conversion of androgenic precursors into oestrogens by the GCs. Both *CYP*

17 and *CYP 19* are located in the endoplasmic reticulum of theca and GCs, respectively. *CYP 17* gene product is a dual functioning enzyme since it catalyses 17α-hydroxylation of steroid substrates and cleavage antioxidation of their side-chains. *CYP 19* synthesise oestrogen by aromatisation of the A ring of the androgenic steroid substrates.

The purity of TCs was verified by the TC-specific marker gene, *CYP17* by real-time PCR in mouse preantral follicle [12].

As the dominant follicle grows, the GCs acquire the potential to produce large amounts of estradiol. The FSH-mediated induction of P450 from (*CYP19* gene) expression in the GCs is causal to the acquisition of the oestrogen potential of the follicle [17]. Skinner., *et al*. [18] revealed that expression of aromatase (CYP 19) changes among small, medium and large follicles and theca cells have an insignificant expression in small and medium follicles of bovine was confirmed by real-time PCR. Hatzirodos., *et al.* [7] studied the transcriptome profiling of GCs from small to large antral bovine ovarian follicles and they identified *CYP 19* as a marker for GCs which could be assessed to check the purity of TCs. Hatzirodos., *et al.* [7] confirmed that *CYP 19* was ovarian GC gene marker, which could be used to study the degree of cross-contamination during the TCs isolation.

Conclusion

The isolated TCs from two different methods were visualized under the inverted microscope and samples from both the methods were subjected to real time qPCR for the presence of GC specific marker Cytochrome P450 aromatase (CYP 19). Cytochrome P450 17 α-hydroxylase (CYP 17), marker for TC, gene expression was also studied to estimate the yield of TC by two methods. Gene expression confirmed that collagenase method was the better for isolation of theca cells from ovarian follicles without much contamination with granulosa cells.

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