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Isolation and culture of chicken growing follicles in 2- and 3-dimensional models

Dan Zhao [a], Imdad Hussain Leghari [a, b], Jian Li [a], Yuling Mi [a], Caiqiao Zhang [a, *]

[a] Department of Veterinary Medicine, College of Animal Sciences, Zhejiang University, Hangzhou, 310058, PR China
[b] Department of Poultry Husbandry, Sindh Agriculture University Tandojam Hyderabad, Pakistan

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ABSTRACT

The isolation and culture of ovarian follicles is essential for the studies of follicular development and function. In contrast to the relative ease of culture for mammalian follicles, developing *in vitro* cultures of high viability for the much larger avian follicles has always proven to be more challenging. In this study, the growing follicles from domestic hens (*Gallus domesticus*) were isolated using enzymatic and mechanical methods and then investigated for the optimized conditions for culture. Assessments of viability and hormonal responsiveness were also considered. A larger percentage of healthy follicles was achieved by mechanical separation than enzymatic dissociation (83% vs. 55% by collagenase I or 63% by trypsin), despite a lower recovery yield for the former (126 vs. 275 by collagenase I or 261 by trypsin) from each ovary. All of the mechanically isolated follicles (800 μm) survived when cultured in the 3-dimensional (3D) system for 7 days whereas only 93% of the follicles survived in the 2-dimensional (2D) group. Follicles cultured in the 3D system also had a higher cell proliferation rates but lower apoptotic rates as assessed by BrdU incorporation and TUNEL assays. Ultrastructural examination showed that the granulosa cells in the 3D group were organized tightly with adjacent layers in contrast to the loose attachment in the 2D system group. After treatment with follicle-stimulating hormone in the 3D culture for 3 days, the mechanically isolated follicles (800 μm) displayed elevated mRNA expression of steroidogenic enzymes, cytokines and cell cycle-regulating proteins. The 3D culture model established in this study thus provides a useful tool for *in vitro* culture using growing follicles in a large diameter to study the mechanisms of growing follicle development in the avian species.

1. Introduction

In avian species, the ovary contains thousands of follicles and provides a unique model for the study of the strict hierarchy of avian follicular development. The avian ovarian follicle is composed of an oocyte surrounded by layers of granulosa cells that are in contact with the oocyte plasma membrane and the theca cell layer surrounding the granulosa cells. Folliculogenesis is a complex process that involves a series of sequential steps in which a growing follicle either develops to the ovulation stage or undergoes atresia [1,2]. Over the past several years, considerable attentions have been paid to the study of preovulatory follicles [3], primordial germ cells (PGCs) [4] and follicular cells [5] in the poutry species, but far less attention has been given to the developmental mechanisms of early growing follicles. A number of studies have suggested aspects of the molecular mechanism that governs the folliculogenesis (from growing follicle to prehierarchical follicles) [6–9], but the complete mechanism responsible for the development of growing follicle in the avian species remains elusive. Ease of culture of individual isolated follicles would greatly facilitate and provide a technical platform for investigations into the regulation of follicular development.

Several reports have shown that follicles at different stages can be obtained from the mammalian ovaries using respective isolation methods. Recently, follicular isolation techniques were comprised by mechanical [10–12] or enzymatic [13] methods have been applied to various species such as bovine [11], mouse [12], goat [14] and human [15]. The main objective of each method is to recover follicles of sufficient number that they are capable of being preserved and cultured. The isolation of viable mammalian primordial and primary follicles has greatly facilitated the studies relating to

* Corresponding author. College of Animal Science, Zhejiang University, No. 866 Yuhangtang Road, Hangzhou 310058, PR China.
E-mail address: cqzhang@zju.edu.cn (C. Zhang).

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the mechanism underlining follicle activation, antrum formation and other aspects of further development. However, such works fell far behind for avian species due to the particular structure and size of avian follicles making them much more difficult to culture [16,17].

Since in avian follicles, the oocyte is relative large and surrounded by a loose vitelline membrane, which is followed by the granulosa and theca layers, with a final tightly compact connective layer [18], the development of a specifically modified protocol for the isolation and culture of avian follicles is required. Here we develop an effective isolation method for the collection of a sufficient number of growing follicles of hens that have integral structures and high viability for *in vitro* culture. The establishment of such a method provides a platform for investigations on the regulation of the development of the growing avian follicle and of any nutritional or pathological aspects of developmental influence.

2. Materials and methods

2.1. Animals

Hyline white hens (*Gallus domesticus*) were raised in a local commercial farm. All pullets were raised in accordance with the conventional standards of the commercial farms. All procedures related to the animals were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals of Zhejiang University* (ZJU20170660). The experimental protocols were approved by the Committee on the *Ethics of Animal Experiments of Zhejiang University*.

2.2. Isolation of follicles

Ovaries were obtained from 15-week-old Hyline hens under sterile conditions and extra tissues were removed using fine tweezers and scalpels. Ovaries were washed in DMEM (Hyclone, Utah, USA) supplemented with 200 IU/mL penicillin and 200 μg/mL streptomycin. The ovaries for enzymatic isolation were chopped on a plastic surface using a sharp sterile razor blade into small pieces (~2 mm^3). These pieces were incubated in 1.2 mg/mL collagenase I (Sigma Aldrich) solution or 0.15% trypsin-EDTA (Sigma Aldrich) for 15 min at 37 °C. After every 5 min the ovarian tissues were gently shaken to facilitate proteolytic digestion and liberate more follicles. Enzymatic digestion was halted by the washing of the ovarian pieces with fresh DMEM and by centrifugation for 7 min at 800 rpm. The mechanical isolation method was carried out according to a modification of the method of Tagler et al. [19]. Briefly, individual follicles (100–1600 μm in diameter) were mechanically isolated using insulin gauge needles in dissection medium (Leibovitz's L-15 medium) supplemented with 5% fetal bovine serum (HyClone, Tauranga, New Zealand), 100 IU/mL penicillin and 100 μg/mL streptomycin. The isolated follicles were picked up with the aid of a mouth-operated micropipette under a stereomicroscope and the number and diameter of isolated follicles was recorded.

2.3. Follicular live and dead assays

Neutral red, a water-soluble and non-toxic dye, was proposed as a non-invasive tool used to conduct live and dead assays based on the ability of living cells to incorporate it into their lysosomes [20,21]. Here neutral red was adopted to assess the live and dead ratio of the isolated follicles and cultured follicles owing to its non-delecterious effects on living tissue and cells, and its high safety index compared with other strains. The follicles were stained with the neutral red staining solution at a dose rate of 20 μg/mL (C0125, Beyotime, China) at room temperature for 7 min and then washed twice in PBS and observed under a stereoscope (Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan). On the basis of external appearance, the follicles were categorized into the viable and dead where the live follicles were stained red, and dead follicles remained unstained, although it could be observed that some dispersive red particles were still occurring in the oocyte.

2.4. Culture of follicles

Since a high viability of growing follicles was achieved by the mechanical separation method, these isolated growing follicles (800 μm) were cultured in encapsulated form in alginate hydrogels (Sigma Aldrich) for 3-dimensional (3D) culture. Concurrently, follicles were placed on the plate bottom for 2-dimensional (2D) culture in a 96-well culture plate (Corning Costar #3596) for 7 days. The follicles for 3D culture were transferred into 30 μl solutions of 0.25% alginate and immersed into a 50 mM $CaCl_2$ and 140 mM NaCl solutions 1–2 min for crosslinking. The beads were then placed into separate wells of a 96-well flat-bottom tissue culture plate. The follicles were cultured in 200 μl/well of DMEM high glucose replenished with 1 × ITS, 5% fetal calf serum (FCS, Hyclone, Utah, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin and incubated at 38.5 °C and 5% CO_2. Half of the media (100 μl) was replaced with fresh media every day. Follicular size and shape were taken as important parameters to judge follicular viability and growth conditions. Images of the cultured follicles were collected and their diameter was measured every day using an inverted Nikon Eclipse microscope (TE2000-U). An average of two perpendicular diameter measurements was used for each follicle at each time point. Follicles were determined as live and dead with neutral red staining at the end of each day.

2.5. Collection of follicles for morphological observation

The follicles for 3D culture were dissolved in 3 IU/mL alginate lyase (A1603, Sigma Aldrich) for 15 min at 37 °C and the follicles of the 2D culture were slightly pipetted. The collected follicles were then fixed in 4% formaldehyde for 24 h, dehydrated, waxed in paraffin. The follicles were totally sectioned at a 5 μm thickness and stained with hematoxylin and eosin.

2.6. RNA extraction and real time PCR analysis

Total RNA was extracted from the cultured follicles using Trizol reagent (15596018, Invitrogen Co., Carlsbad, CA, USA). The total RNA (2 μg) was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific) according to the manufacture's instruction and further amplified by PCR. The sequences of the primers are listed in Table 1. Real time PCR was carried out on a BIO-RAD RealTime system (BIO-RAD, C1000 Touch Thermal Cycler) with a reaction volume of 20 μl consisting of 2 μl cDNA, 0.4 μM of each gene-specific primers, and 10 μl ChamQ SYBR qPCR Master Mix (Vazyme. Nanjing, China). Individual samples were analyzed in triplicate and experiments were performed twice. All samples were normalized against β-actin using the comparative cycle threshold (Ct) method ($2^{-[\Delta][\Delta]Ct}$).

2.7. BrdU incorporation

After 24 h, 72 h or 120 h culture, 25 μg/mL BrdU (B5002, Sigma Aldrich) was added into ovarian culture medium and the incubation was continued for additional 24 h for incorporation. The follicles for BrdU assay were fixed in 4% paraformaldehyde and the paraffin ovarian sections were deparaffinized and hydrated