i. Running title: Bovine primordial follicles isolation

Method of isolation and in vitro culture of primordial follicles in bovine animal model

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ii. Summary/Abstract

The mammalian ovary is a substantial source of oocytes arranged into follicles at various stages of folliculogenesis, from the primordial to the ovulatory ones. Primordial follicles constitute the most abundant source of gametes inside the mammalian ovary at any given time.
The isolation of a high number of primordial follicles, together with the development of protocols for in vitro follicle growth, would provide a powerful tool to fully exploit the female reproductive potential and boost the rescue and restoration of fertility in assisted reproduction technologies in human medicine, animal breeding, and preservation of threatened species. However, the most significant limitation is the lack of efficient methods for isolating a healthy and homogeneous population of viable primordial follicles suitable for in vitro culture. Here we provide a fast and

high-yield strategy for the mechanical isolation of primordial follicles from limited portions of the ovarian cortex in the bovine animal model.

iii. Key Words

Primordial follicle, preantral follicle, culture, mechanical isolation, viability, folliculogenesis, in vitro growth, oocyte, ovary

1. Introduction

The mammalian ovary contains a fixed number of non-growing primordial follicles established before birth representing the ovarian reserve that declines with age and culminates at the end of the reproductive lifespan [1]. The number of follicles in the ovaries of mammals is remarkably variable at birth, ranging, for example, from 350.000 to 1.100.000 in humans [2,3] and approximately 14.000 to 250.000 in cattle [4,5].

Folliculogenesis begins during fetal life and proceeds until the end of reproductive capacity. It starts with the recruitment of primordial follicles followed by the cyclic recruitment that brings to the development of the preovulatory follicle containing an oocyte, which is ovulated and is able to be fertilized and become an embryo [6]. Of the initial pool recruited to grow, only a few follicles reach the preovulatory stage, and less than 1% escape the process of atresia at various stages of development, particularly during the preantral to early antral transition, which is the most susceptible to this process [6,7]. In women, of the original primordial follicle stockpile at birth, only approximately 400 will fully mature into secondary oocytes, being ovulated and ready to be fertilized during a woman's reproductive lifespan [8,9] while the vast majority are destined to undergo atresia [10-12].

Primordial follicles represent the largest population of the ovarian reserve in mammals at any given time, thus constituting the most relevant repository of the female reproductive potential in mammals [13].

Efficient culture systems for the primordial follicles may enhance fertility preservation opportunities in women, expand genetically important livestock breeds and support conservation programs for endangered species [14]. In the bovine model, the current assisted reproductive technologies can rely only on a limited number of follicles, namely the population of fully-grown

oocytes isolated from the medium-large antral follicles (Table 1) [15-17] and with relative success from oocytes isolated from early antral follicles [18].

Recruiting preantral follicles (from primordial to secondary follicle stage), and particularly primordial follicles, for in vitro growth would enormously broaden the availability of gametes for a massive exploitation of the reproductive potential of a female individual. The development of in vitro follicle growth system would also deepen our knowledge of the processes that initiate mammalian folliculogenesis, allowing investigation of folliculogenesis and oogenesis in a tightly controlled environment.

In the last two decades, several attempts to develop follicle culture systems for preantral follicles *in situ*, i.e., in the intact ovary or fragments of the ovarian cortex, have been made in several species [19]. Although advances in current systems have enhanced oocyte growth and maturation to some extent, further optimization is required to improve oocyte competence with genetic integrity for proper embryonic development. Most of the attempts were ineffective, with little success regarding follicle development, and limited to mice [20] as proof of principle, while in large mammals, the techniques are still considered experimental [21,22,19].

The ability to rescue a high number of primordial follicles and the development of in vitro follicle 3D growth protocols, as recently suggested in mice [23], may provide a powerful means of fully exploiting the female reproductive potential. However, the most significant limitation in all the species considered so far is the lack of efficient methods for isolating a homogeneous population of primordial follicles.

The currently available techniques for isolating primordial follicles - or preantral follicles, in general - have been optimized in several labs to develop the most efficient method (mechanical, enzymatic, or a combination of both [24-26]) to increase follicle yield. In large mammals, despite considerable progress in the methodology for the isolation of preantral follicles, regardless of the isolation method, most of the studies have reported the recovery of primary and secondary

follicles [27-32], very often from fetal ovaries [24,33-35], occasionally in adult individuals [24,36], and frequently processing a high number of ovaries [37,26,38]. Surprisingly, the yield rate is only occasionally declared, but when available, the number of isolated primordial follicles is lower than 5, while primary and secondary follicles vary from 2 to about 40.

Here we provide a fast and effective strategy to maximize the isolation of primordial follicles in the bovine model from limited portions of the ovarian cortex. Specifically, starting from a 0.5-1 mm thick ovarian cortex slice of 2 cm^2 in size, the present methodology allows for recovering 166.5 ± 40.8 (N=10) primordial follicles of 34.5 ± 3.8 in diameter (n=176). After collection and 1 hour of culture, 88% of the primordial follicles were viable. Furthermore, the entire mechanical isolation procedure lasts 30-40 min from the time of isolation of the 2 cm^2 fragment of the ovarian cortex.

Finally, we propose the bovine animal model because bovine and human reproductive biology share numerous characteristics [39]. For example, cows and women have similar folliculogenesis length [3,40-42], are monovular, cycle continuously while not pregnant, have a gestation period of approximately 9 months, and their ovaries are similar in size (approximately 3 cm × 2 cm × 1.5 cm), morphology [43], and architecture [44,45], 2009 #3;Roberts, 2022 #10048;Sirard, 2017 #6755}.

To conclude, considering the limited success of the *in situ* culture system in cattle and humans [46,47], isolating a high number of primordial follicles to be used in suitable in vitro culture systems (2D and 3D) is extremely encouraging. The development of in vitro primordial follicles growth systems in the bovine model can provide a tool for deepening our knowledge of mammalian folliculogenesis, overcoming logistical and ethical limits in using human ovarian

samples, and studying tailored approaches, minimizing the invasiveness of the interventions to preserve female fertility.

2. Materials

Disposable sterile plasticware is from NUNC IVF Line, SARSTEDT Green line (for suspension cells), and Sterilin™ by ThermoScientific. Final filtration of all stock solutions and the preparation of working solutions are performed using sterile techniques under a biohazard laminar flow cabinet or a horizontal laminar flow hood to keep sterility. All glassware is exclusively dedicated to gamete and embryo culture and is high-pressure steam-sterilized by autoclaving at 121°C for 20 min. After use, glassware is washed and rinsed with running tap water for 30 minutes, rinsed three times with 18.2 mΩ water, then dried thoroughly and covered with aluminum foil until sterilization. All the procedure's dedicated steel instruments (forceps, spatula, scalpel handle) are high-pressure steam-sterilized by autoclaving at 121°C for 20 minutes.

All the procedures are conducted at room temperature (26°C) unless otherwise specified.

2.1 Manipulation solution and media

1. Collection and washing solution: Prepare 0.9% saline solution by adding 9 gr of NaCl in 1 L of sterile ultrapure 18.2 mΩ water. Supplement the saline solution with penicillin 100 U/mL and streptomycin 0.1 mg/mL.
2. Isolation Medium: Leibovitz Medium supplemented with 0.3% Bovine Serum Albumin, 0.164 mM Penicillin and 0.048 mM Streptomycin (*see* **Note 1, 2**).
3. For homogenization procedure, prepare aliquots of 15 ml isolation medium into 50 ml Falcon tubes.

2.2 Culture Medium

1. Culture Medium: αMEM supplemented with 0.1% Bovine Serum Albumin fatty acid-free, 1mg/ml r-hInsulin, 0.55 mg/ml hTransferrin, 0.5 µg/ml Sodium Selenite, 10^{-4} IU/ml r-hFSH, 0.164 mM Penicillin, and 0.048mM Streptomycin (*see* **Note 3**).
2. Prepare a 4-well plate filled with 500 µl of Culture Medium and equilibrate at 38.5°C and 5% CO_2 in air, maximum humidity, for at least 4 hours before use.

2.3 Dual-fluorescence Viability Assay

1. Manipulation Buffer: Polyvinyl Alcohol dissolved in Phosphate Buffer Saline to a final concentration of 0.1%.
2. Dual staining solution: Fluorescein Diacetate (FDA) and Propidium Iodide (PI) diluted to a final concentration of 1 µg/mL each in the previously prepared Manipulation Buffer.

2.4 Equipment

1. Scalpel handle with a surgical blade no. 22.
2. Single-edge carbon steel razor blades 1.5” with aluminum back.
3. High-density polyethylene cutting board.
4. IKA ULTRA-TURRAX® T25 Digital Advanced Homogenizer.
5. IKA Plastic Disperser Tool S25D-14G-KS (Stator diameter: 14mm, Rotor Diameter: 9.5mm).
6. Cell strainer of 300, 100, 70, 40, and 30 µm mesh size.
7. Mouth pipette with pulled glass capillary (inner diameter about 100 µm).
8. Culture petri dish 35 and 60 mm, for suspension cell culture.

3. Methods

3.1 Isolation and Culture of Primordial Follicles

The passages described below are illustrated in **Figure 1**.

1. Collect bovine ovaries from Holstein Friesian cattle subjected to routine veterinary inspection and following the specific health requirements. Transport to the laboratory on ice within 1 hour in a 50 ml tube with sterile collection saline solution.
2. Under a horizontal laminar flow hood, place one ovary on a sterile cutting board. Using surgical blade no. 22 mounted on a scalpel handle, cut a 0.5-1 mm thick ovarian cortex slice of 2 cm^2 in size (*see* **Note 4**). Chop the cortical slices into tiny fragments with 1.5" single-edge razor blades and carefully mince them on the sterile cutting board.
3. Wash the minced ovarian cortex by transferring the fragments with a spatula into a sterile 60 mm Petri dish containing 3 ml of isolation medium **(***see* **Note 5)**.
4. Remove the isolation medium using a pipette and transfer the washed minced cortical pieces to a 50 ml falcon tube containing 15 ml isolation medium **(***see* **Note 6)**.
5. Place the 50 ml Falcon tube containing the minced cortical pieces dispersed in 15 ml of isolation medium under the IKA ULTRA-TURRAX® T25 Homogenizer with the Disperser Tool S25D-14G-KS.
6. Homogenize the minced fragments in the 50 ml Falcon tube at 3000 rpm for 6 minutes **(***see* **Note 7).**
7. Filter the homogenate through a 300 µm strainer placed at the top of an open empty 50 ml Falcon tube. Wash the strainer by pipetting 1 ml of isolation medium 5 times.
8. Pour the filtrate through a 100 µm strainer placed atop an open empty Falcon tube. Wash the strainer by pipetting 1 ml of isolation medium 5 times.
9. Pour the filtrate through a 70 µm strainer placed atop a falcon tube. Wash the strainer by pipetting 1 ml of isolation medium 5 times.

10. Pour the filtrate through a 40 µm strainer placed atop a Falcon tube. Wash the strainer by pipetting 1ml of isolation medium 5 times (*see* **Note 8).**
11. Pour the filtrate through a 30 µm strainer placed atop a falcon tube. Wash the strainer by pipetting 1 ml of isolation medium 5 times.
12. The 30 µm mesh traps the bovine primordial follicles. Flip the 30 µm strainer upside down and stably hover over a 60 mm Petri dish. Wash the strainers by pipetting 1ml of isolation medium 5 times. **(***see* **Note 9)**.
13. Under the stereomicroscope, select the primordial follicles from the resultant filtrate with a mouth pipette and transfer them into a 35 mm Petri dish with 2 ml of manipulation medium **(***see* **Note 5, 8, 9, 10).**
14. Collect groups of 20 primordial follicles and place them in a 4-well plate containing 500 µl of the previously prepared Culture Medium at 38.5°C and 5% CO_2 in air, maximum humidity.

3.2 Viability Assessment

1. Assess primordial follicle viability after 1 hour of incubation in the culture medium **(***see* **Note 11)**.
2. In a 35 mm Petri dish, make a 50 µl drop of the previously prepared manipulation buffer and dual staining solution.
3. Collect individual groups of primordial follicles cultured from the 4-well plate in maximum 5 µl of media and wash them in the droplet of manipulation buffer.
4. Transfer the primordial follicles in the dual stain droplet and observe them under a fluorescence microscope at appropriate wavelengths (PI: λ 510nm, FDA: λ 580nm).
5. Count as live follicles those showing green fluorescence in all cells (intact) or <10% of dead (red) cells [48] (**Figure 2**).

4. Notes

1. Ensure that the isolation medium is at room temperature (26°C) prior to use.
2. L-15 should be with phenol red, GlutaMAX™, sodium pyruvate, and galactose, and without glucose, HEPES and sodium bicarbonate.
3. From previous reports in bovine and human species, it is recommended the use of Medium αMEM with specific nucleosides, nucleoside triphosphate, and ribonucleosides to preserve the morphology, morphometry, and ultrastructure of pre-antral follicles and ensure their survival and growth [49-52].
4. As described by Van Wezel and Rodgers [53], bovine ovary have a major distinct polarity from the surface to the medulla, in histological sections, and is composed of at least five identifiable zones. The zones containing primordial follicles are substantially avascular and localized in the thickness between 0.5 and 1 mm of the depth of the ovarian cortex [53].
5. Ensure that the Petri dishes (60 and 35 mm) used during all the procedures are suspension cell culture dishes, not those for adherent cells. It will prevent the adhesion of follicles on the bottom of the plate, thereby avoiding damage due to applying mechanical force during the collection with glass or plastic tips.
6. Steps 2-4 should be performed within 5 minutes.
7. Position the falcon tube under the homogenizer such that the level of isolation medium in the tube is above the minimum (indicated as ‘MIN’) line on the IKA Plastic Disperser tool. Stabilize the Falcon tube by providing support to the walls and tip of the tube, allowing it to remain upright independently during the homogenization. This procedure will avoid foam formation during the homogenization process. Foam could entrap follicles that may subsequently be lost during filtration.
8. The serial filtrations with decreasing mesh size allow for isolating a homogeneous population of primordial follicles with minimum debris. Primordial follicles can also be

found entrapped in the 40 µm strainer due to the prolate shape of bovine primordial follicles [53], which have three dimensions measured as length (45.4 ± 2.4 µm), breadth (26.8 1.5 µm), and depth (30.4± 1.4 µm (mean ± SEM). They can be recovered by washing the 40 µm strainer as described for the 30 µm one in step 12.

9. To optimize yield and viability, steps 7-13 should be completed within 25-30 mins, as also recently reported in mice [23] and bovine [38].
10. Use a pulled glass capillary with an inner diameter of approximately 100 µm to collect a clean population of PMF.
11. Before assessing the viability of the primordial follicles at the time of collection, incubate the follicles for at least 1 hour to allow the cells to recover [23].

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Figure captions

Figure 1. Schematic representation of the workflow described in section 3 (Methods). Created with BioRender.com (December 28, 2022)

Figure 2. Representative images of primordial follicles subjected to dual-fluorescence viability assay using Fluorescein Diacetate (FDA, green, live cells) and propidium iodide (PI, red, dead cells). Scale bar = 100 μm.

Table 1. Extent of follicle reserve and follicle categories in 1-8 years old bovine ovaries (data were based on the estimation from [54,42,40,5,55,56].

Follicle category	Primordial	Primary	Secondary	Early antral	Mid-large antral
Number/ovary (Heifers)	84,000	21,000	5,000	120	25
Number/ovary (Cows)	64,000	23,000	1,800	120	25
Incidence of Atresia	<2%	<5%	8%	30%	60%

Figure 1

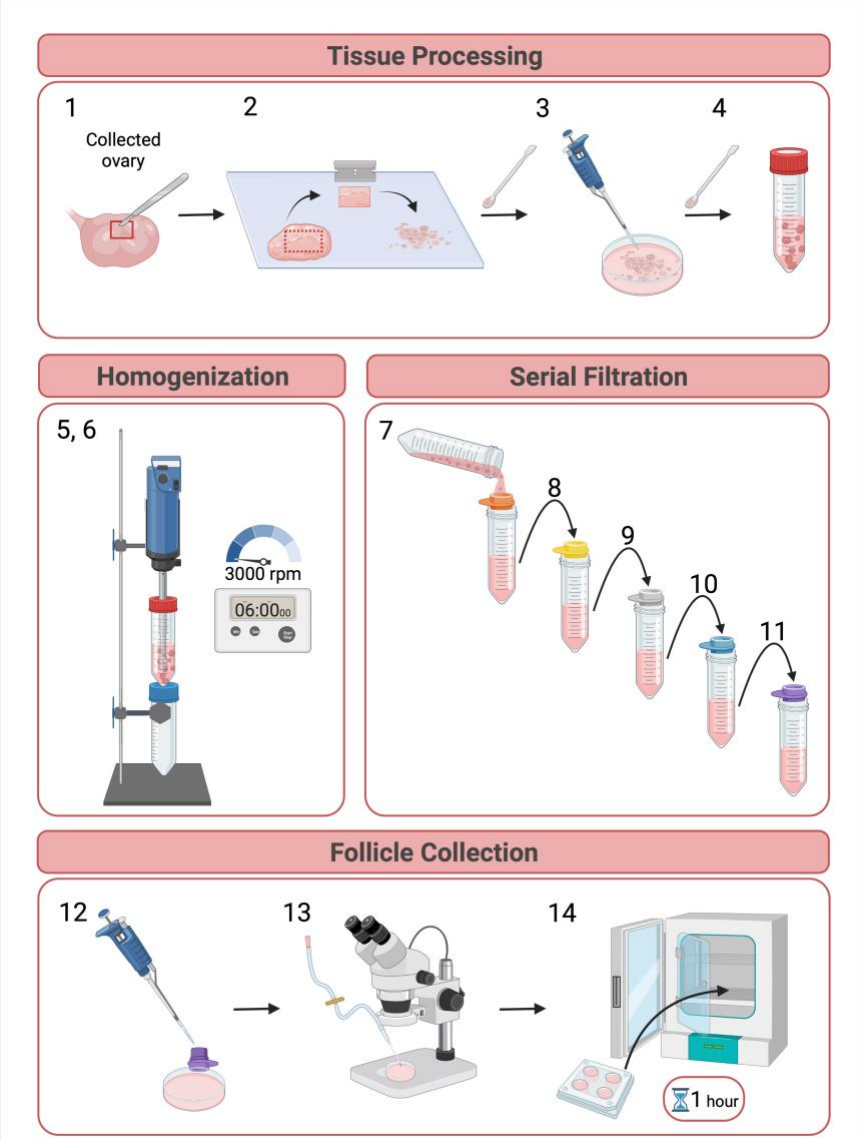
Tissue Processing
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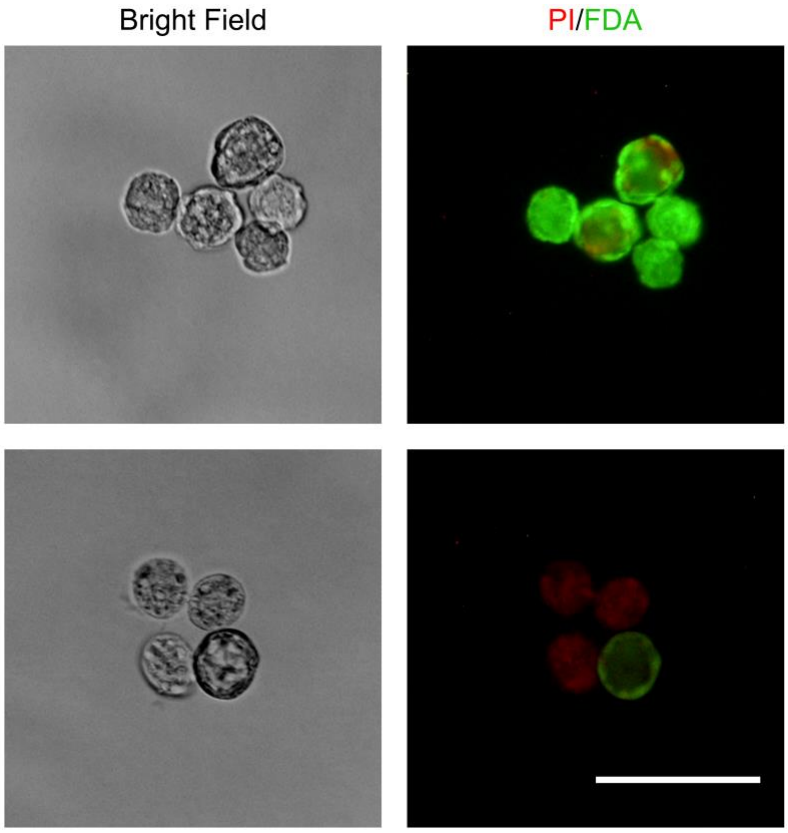
Figure 2



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