Recreating the Follicular Environment: A Customized Approach for In Vitro Culture of Bovine Oocytes Based on the Origin and Differentiation State

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i. Running title: Strategies for bovine oocytes in vitro culture

Recreating the follicular environment: a customized approach for in vitro culture of bovine oocytes based on the origin and differentiation state

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ii. Summary/Abstract

The mammalian ovary is a large source of oocytes organized into follicles at various stages of folliculogenesis. However, only a limited number of them can be used for in vitro embryo production (IVEP) while most have yet to complete growth and development to attain full meiotic and embryonic developmental competence. While the in vitro growth of primordial follicles in the ovarian cortex has the potential to produce mature oocytes, it is still at an experimental stage. The population of early antral follicles (EAFs), instead, may represent a reserve of oocytes close to completing the growth phase, which might be more easily exploited in vitro and could increase the number of female gametes dedicated to IVEP.

Here we present in vitro culture strategies that have been developed utilizing physiological parameters to support the specific needs of oocytes at distinct stages of differentiation, in order to expand the source of female gametes for IVEP by maximizing the attainment of fertilizable oocytes. Furthermore, these culture systems provide powerful tools to dissect the molecular processes that direct the final differentiation of the mammalian oocyte.

iii. Key Words

Oocyte, cumulus cells, gap-junction, chromatin, germinal vesicle; intercellular communication; meiotic arrest, prematuration, oogenesis, in vitro oocyte growth

1. Introduction

IVEP technologies developed to allow the transfer of embryos obtained through in vitro maturation (IVM) of immature oocytes, followed by in vitro fertilization (IVF) and in vitro culture (IVC) of early embryos. However, the efficiency is still limited, and the small supply of mature, fertilizable oocytes is one of the main limitations to the success rate of assisted reproduction, in cattle as well as in other mammals.

The mammalian ovary is a potential large source of oocytes that are enclosed into follicles at various stages of development, from the primordial to the ovulatory ones. Nonetheless, only a limited number of such oocytes can be readily submitted to routine IVEP protocols and generate viable embryos. These oocytes are the ones that have reached the so-called fully-grown stage and are enclosed in middle and large antral follicles (MAFs and LAFs, respectively). Yet, not all of them reach the blastocyst stage of embryonic development when subjected to standard IVEP. Furthermore, most of the oocytes in an ovary have yet to reach the fully-grown stage and ultimately acquire full meiotic and embryonic developmental competence [1]. Specifically, these oocytes span from the ones enclosed in primordial follicles to the ones enclosed in EAFs, that are still in the growing phase.

While in vitro growth of primordial follicles enclosing the resting oocyte pool in the ovarian cortex has the potential to produce mature oocytes [2], this technique is still experimental, and an efficient exploitation of this reserve seems far from being applicable on a large scale. By contrast, the population of oocytes enclosed in EAFs may represent a more readily exploitable source that would increase the number of female gametes that can successfully undergo IVEP [3] (**Table 1**). Finally, optimization of culture systems for fully-grown oocytes isolated from MAFs may increase the overall efficiency.

Oocytes enclosed in EAFs and MAFs represent an extremely heterogeneous cell population, with very distinct morphological and functional characteristics [4,5]. In bovine, nearly all of the oocytes isolated from EAFs (0.5-2 mm) are still growing, the chromatin appears mostly uncondensed and dispersed throughout the nucleoplasm, in the so-called

GV0 configuration [6]. At this stage, oocytes are still transcriptionally active and are functionally fully coupled through gap junctions (GJs) to the surrounding cumulus cells [6-8]. On the other hand, oocytes from MAFs (2-8 mm), which are the most commonly used for IVEP, are characterized by various degrees of progressive chromatin compaction, named GV1, GV2 and GV3 configurations. The 3 classes are equally represented within the populations of oocytes collected from MAFs [6,9]. The transition from GV0 to the higher classes of chromatin compaction, up to GV3, is accompanied by progressive transcriptional silencing [7], changes in the epigenetic signatures such as global DNA methylation [10] and histone modifications [11,12] and changes in cytoplasmic organelle redistribution and nuclear architecture [7]. More importantly, these changes are also accompanied by a gradual acquisition of meiotic and developmental competence [6,7,3,13,14].

In the present work, we describe cultural strategies customized to better fulfill the physiological needs of oocytes at the distinct stages of differentiation. The described procedures induced an increased chromatin compaction, that was accompanied by a progressive and significant increase in developmental capacity (**Figure 1**). Notably, that the transition to higher degrees of chromatin compaction is associated with an increase in developmental competence has recently been observed also in vivo [15,16]. Specifically, oocytes that were 'synchronized' at the GV2 stage using a mild FSH stimulation before ovum pick up (OPU), reached significantly higher blastocyst rates when submitted to IVM/IVF/IVC [15].

Precisely, in this method article we will describe:

- the long in vitro culture of oocytes (L-IVCO), targeted to growing oocytes isolated from EAFs [17];
- the prematuration (pre-IVM), mostly beneficial to the GV1-enriched population of oocytes isolated from MAFs [9];
- the IVM, mostly beneficial to the GV2 and GV3-enriched population of oocytes isolated from MAFs [9];

The rationale adopted in designing and optimizing the above-listed treatments stems from the idea of recreating the follicular environment in which a developmentally competent oocyte grows and differentiates until the first meiotic division is completed.

Each of the proposed protocols has been optimized considering the following main physiological characteristics and experimental evidences.

Once the oocytes have acquired the molecular machinery necessary to resume meiosis, the follicular environment acts to keep the cell cycle arrested by supporting the intra-oocyte content of cyclic nucleotides [18]. In vivo, the protracted arrest ensures that the oocyte undergoes the final steps of differentiation that confer developmental competence, by avoiding untimely meiotic resumption. This condition was recreated in vitro by supplementing the L-IVCO and pre-IVM media with cilostamide [3,14,19-21], an inhibitor of phosphodiesterase-3 (PDE3) which is specific to the oocyte in bovine as in most mammals [22]. As an alternative to pharmacological treatment, also the use of natriuretic peptide precursor C (NPPC) has been shown to be effective [14,23-28]. NPPC is the natural activator of the guanylyl cyclase-coupled natriuretic peptide receptor type-2 (NPR2) [29] that induces the production of 3'-5' cylic guanosine monophosphate (cGMP), which is then transferred via GJs to the oocyte [30] where it inhibits PDE3A, thus maintaining the meiotic arrest [31,32].

The hormonal milieu, including estradiol (E_2), testosterone (T), progesterone (P_4), and follicle stimulating hormone (FSH), was studied to mimic as much as possible the follicular conditions in EAFs [33-36]. E_2 and T, which also acts as an estrogen precursor, primarily support oocyte and follicle growth [37,21,38], by promoting granulosa cells intercellular communication and meiotic competence acquisition [39]. P_4 has instead a role in inhibiting the apoptosis in granulosa cells [40]. Since a specific hormonal composition was also found in the follicular fluid at the time of selection for dominance [41-44,28], the pre-IVM medium can be similarly supplemented with E_2, T, and P_4 [28].

Studies conducted in several mammalian species show that supplementation of the IVM medium with FSH improves oocyte quality [45-50], prompting the widespread use of FSH as hormonal supplementation in IVM protocols. Even though there is not consensus on the exact signaling mode, FSH at high dosage seems to exert its effects through the activation of the epidermal growth factor (EGF) network [51,45,52,53,50], which is physiologically triggered in vivo by the surge in luteinizing hormone (LH) [54]. However, FSH levels similar to the ones used in IVM proved to be harmful for the culture of growing oocytes, that underwent early closure of the GJ-mediated communications with the cumulus cells, precocious meiotic resumption and insufficient growth [3]. The preservation of cumulus oocyte communication functionality during in vitro growth and prematuration protocols represent a key point and specifically the intracellular cAMP concentration evoked by FSH stimulation plays a crucial role [55-60,3,61-63,20,64,13,19,65]. By conducting dose/response curves, it was established that one thousand times less FSH was instead beneficial during the growing phase, sustained the functionality of the GJs, and promoted transcriptional activity and oocyte growth and differentiation [3].

During pre-IVM, FSH concentrations similar to the ones used in L-IVCO also improved oocyte quality by sustaining GJ coupling with the cumulus cells [13,14,9,28].

Other aspects that are instead peculiar to the L-IVCO aimed at promoting a 3D structure and supporting the transcriptional activity. Specifically, the first was achieved by increasing the viscosity of the medium to mimic the physiological viscosity of the follicular fluid, and by culturing the cumulus oocyte complexes (COCs) on a collagen coated surface (**Figure 2**) [17,66].

Finally the inclusion of zinc sulphate is supported by recent results showing the role of this trace element in promoting the differentiation and transcriptional activity of bovine growing oocytes [67].

The procedures described herein represent multiple culture strategies that have been developed to support the specific oocyte needs in relation to the developmental step, with the aim of expanding the source of oocytes usable for IVEP. Nonetheless, these protocols

will provide useful tools to dissect the cellular and molecular process that control the final oocyte development.

2. Materials

All media are prepared with embryo tested ultra-pure water. Disposable, sterile plasticware is from BD Falcon by Corning, NUNC IVF Line, and Sterilin™ by Thermo Scientific, where duly specified. Final filtration of all stock solutions, as well as preparation of working solutions, is performed using sterile techniques under a biohazard laminar flow cabinet or laminar flow hood to keep sterility. All glassware is exclusively used for embryo culture media preparation and is high pressure steam-sterilized by autoclaving at 121°C for twenty minutes. After use, glassware is immediately washed and rinsed with running tap water for 30 minutes, rinsed three times with 18.2 mΩ water, then dried completely and covered with aluminum foil until sterilization.

All the procedures are conducted at 26-28 °C unless otherwise specified.

2.1 Collection media

1. Prepare HEPES-based manipulation medium (HM199) from Medium 199 with Earle′s salts supplemented with 0.68 mM L-glutamine, 25 mM HEPES, 0.4% Bovine Serum Albumin (BSA) Fraction V, 0.164 mM penicillin, 0.048 mM streptomycin and 1790 u/L heparin. HM199 are aliquoted in 20 mL in Sterilin tube (Thermo Scientific) and stored at 4°C for 6 months.

2. Prepare meiotic arrest holding medium (HM199-Cilo) with HM199 supplemented with 5 μM of the selective PDE3 inhibitor cilostamide [14] (see **Note 1)**.

3. Warm sealed aliquots of HM199 and HM199-Cilo at 38.5°C in a dry thermostatic chamber before use.

2.2 Long in vitro oocyte culture (L-IVCO) medium

1. Prepare the basic oocyte culture medium (bM199) from Medium 199 with Earle′s salts supplemented with 25 mM sodium bicarbonate, 21.3 µg/ml phenol red, 75 µg/mL kanamycin, and 4% polyvinylpyrrolidone (PVP; 360k molecular weight) (see **Note 2**).

2. Five hours before culture (see **Note 3**), prepare the L-IVCO medium by supplementing the above bM199 with 2 mM GlutaMAX^{TM}, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 0.15 µg/mL zinc sulphate, 10^{-4} IU/mL recombinant human FSH (r-hFSH), 10 ng/mL E_2, 50 ng/mL T, 50 ng/mL P_4 and 5 µM Cilostamide [17].

3. Fill a BioCoat™ Collagen I 96-well plate (Corning) with 200 µl of L-IVCO medium and equilibrate for at least 4 hours before use for oocyte culture at 38.5°C and 5% CO_2 in air, maximum humidity. For all additives' storage, see **Note 4**.

2.3 Pre-IVM medium

1. Prepare the oocyte pre-IVM medium from Medium 199 with Earle′s salts supplemented with 25 mM sodium bicarbonate, 2 mM GlutaMAX^{TM}, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, 10^{-4} IU/mL r-hFSH and 10 µM Cilostamide [14,9] (see **Note 5**). Equilibrate in the incubator at 38.5°C and 5% CO_2 in air, maximum humidity (see **Note 6**).

2. Fill each well of a NUNC IVF four-well plate (Thermo Scientific) with 500 µl of pre-IVM medium and equilibrate for at least 4 hours before use at 38.5°C and 5% CO_2 in air, maximum humidity.

2.4 IVM medium

1. Prepare IVM medium from Medium 199 with Earle′s salts supplemented with 25 mM sodium bicarbonate, 2 mM GlutaMAX^{TM}, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/ml of kanamycin, 0.1 IU/ml of r-hFSH.

2. Fill each well of a NUNC IVF four-well plate with 500 µl of IVM medium and equilibrate for at least 4 hours before use for oocyte culture at 38.5 °C and 5% CO2 in air, maximum humidity.

3. Methods

The recommended operative procedure is to first proceed with the isolation of COCs from MAFs (2-8 mm) by aspiration through a needle connected to a vacuum pump (3.1). These COCs will be divided in subgroups and either subjected directly to IVM (3.2) or to pre-IVM (3.3) followed by IVM as detailed below. COCs from EAFs (0.5-2 mm) are isolated by individual follicle dissection from slices of ovarian cortex (3.4) taken from the ovaries that already underwent MAFs aspiration. These oocytes are submitted to L-IVCO (3.5) and, at the end of L-IVCO, can be transferred to IVM.

All the procedures are conducted at 36-38°C, unless otherwise indicated.

3.1 Isolation of COCs from MAFs

1. Collect bovine ovaries from 4-8 years old Holstein dairy cows subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications, at the abattoir. Transport to the laboratory, within 3 h, in sterile saline maintained at 28° C.

2. Retrieve COCs from 2-8 mm follicles with an 18-gauge needle mounted on an aspiration pump (COOK-IVF, Australia) with a vacuum pressure of 28 mm/Hg (see **Note 7**). Once the content of the MAFs has been aspirated, keep the ovaries in warm saline for procedures described in 3.4.

3. Examine the morphology of the COCs under a stereomicroscope and select the complexes medium brown in color, with five or more complete compact layers of cumulus cells [68,69]. These COCs will be further divided in 3 subclasses, according to previously described morphological features [70,71,9]: Class 1, with homogeneous ooplasm and compact cumulus cells; Class 2, with minor granulation of the ooplasm and compact

cumulus cells; Class 3, with highly granulated ooplasm and slight expansion of cumulus cell layers (**Figure 3**). As previously demonstrated, Class1 is GV1-enriched and Class 2/3 are GV2/3-enriched [9].

3.2 IVM

Group the Class 2 and Class 3 COCs, wash twice in HM199, then culture in groups of 30-34 in 500 µl of the IVM medium for 24 h, in four-well dishes at 38.5°C under 5% CO_2 in humidified air (see **Note 8**).

3.3 Pre-IVM

Wash twice the Class 1 COCs in HM199, then culture in groups of approximately 30 COCs in 500 µl of the pre-IVM medium for 6 h, in four-well dishes (NUNC) at 38.5°C under 5% CO_2 in humidified air. After pre-IVM culture, wash COCs of Class 1 twice in HM199, then transfer them in 500 µl of IVM medium for 24 h, in four-well dishes (NUNC) at 38.5°C under 5% CO_2 in humidified air (see **Note 8**).

3.4 Isolation of COCs from EAFs

1. Prepare ovarian cortex slices as previously described [17]. Under a horizontal laminar flow hood, with warm plate and a stereomicroscope equipped with a heating stage, place one ovary at the time on a sterile Teflon cutting board. Using a surgical blade n. 22 mounted on a scalpel handle, cut slices 1.5-2 mm thick of ovarian cortex and parallel to the major axis of the organ. Maintain the slices of ovarian cortex in a sterile glass Petri dish covered with HM199 on a warm plate at 38.5 °C.

2. Place the ovarian cortex slice in a 60 mm glass Petri dish with 2-3 mL of HM199. Under the stereomicroscope select the follicles between 0.5-2 mm by using a micrometer-equipped eyepiece. Remove the ovarian stroma surrounding the follicle to expose it on the edge using a surgical blade n. 22 mounted on a scalpel handle.

Carefully, holding the slice with tweezers, make a slit on the follicle wall using a 26G needle mounted on a syringe to release the follicular content, including the COC, follicular fluid and clumps of cells.

3. Under the stereomicroscope select COCs medium brown in color, with five or more complete compact layers of cumulus cells, intact zona pellucida and homogeneous cytoplasm. The diameter of the oocyte, excluding the zona pellucida, must be within a range of 100 - 110 µm (see **Note 9**). Place the isolated COCs in HM199-Cilo.
Limit each round of COCs isolation to 30 min, then incubate the selected COCs in a 35 mm Petri dish with 2 ml of L-IVCO at 38.5°C under 5% CO2 in humidified air, until the collection procedure is completed and the COCs can be transferred individually into a well of the L-IVCO 96-well plate.

3.5 Long in vitro oocyte culture

1. Place each selected COC singularly in the center of a well of the 96-well plate containing the previously prepared L-IVCO medium (see 2.2) and incubate the plate for 5 days at 38.5°C and 5% CO_2 in air, maximum humidity.

2. On day 2 and day 4 renew half of the medium by removing 100 µL of medium and replacing with 100 µl of freshly prepared and incubator-equilibrated L-IVCO medium. Perform the medium renewal gently under the stereomicroscope to avoid detaching of the COCs from the bottom of the well.

3. On day 5, select all COCs with compact cumulus cell investment, with no sign of cumulus expansion and cell degeneration while discard COCs showing abundant loss of cumulus cells extending for more than 50% of the oocyte surface, and signs of cell degeneration and cell debris, as previously described in [17].

4. Wash the selected in vitro grown COCs twice in HM199 then transfer them in 500 µl of IVM medium for 24 h, in four-well dishes (NUNC) at 38.5°C under 5% CO_2 in humidified air (see **Note 8**).

4. Notes

Note 1: Meiotic arrest during the collection procedure can also be maintained by using the non-selective PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) at the final concentration 0.5 mM [13].

Note 2: To prepare the 4% PVP basic L-IVCO, first prepare an 8% PVP solution by dissolving 40 gr of PVP in 500 ml of embryo tested cell culture water in a sterile bottle. Seal the cap tightly and keep in a water bath at 100 °C for 40 minutes. Leave to cool. Prepare basic L-IVCO 2X by dissolving the Medium 199 with Earle′s salts powder in 500 ml of embryo tested cell culture water and supplement with 25 mM sodium bicarbonate, 21.3 µg/ml of phenol red, 75 µg/mL of kanamycin. Adjust the pH to 7.4 and filter-sterilize. Mix basic L-IVCO 2X and 8% PVP solution 1:1 to obtain basic L-IVCO medium.

Note 3: All $NaHCO_3$-buffered media must be equilibrated in the incubator for a minimum of 4 h before use. This step allows the gas exchange with the correct CO_2 pressure to obtain a pH of approximately 7.4.

Note 4: Additives such as sodium pyruvate and cysteamine are prepared as stock solutions 100X and stored at -20°C for up to three months. The BSA stock solution is 50X and is stored at +4°C for up to three months. The r-hFSH is prepared as 100 IU/ml stock in PBS containing 0.1% of BSA and stored at -20°C up to three months. Steroids are prepared as stock solutions 1000X in absolute Ethanol and stored at -20°C for up to one year.

Note 5: For pre-IVM, depending on the experimental design, cilostamide can be replaced with 100 nM natriuretic peptide precursor C (NPPC) [14,28,72].

Note 6: In order to further reproduce the follicle environment during the phase of selection for dominance, pre-IVM medium can additionally be supplemented with 500 ng/mL E_2, 50 ng/mL P_4, 50 ng/mL T [28,16].

Note 7: During follicle aspiration, aspirate aliquots of about 0.5 ml of HM199 through the needle into the tube circuit every 2 ml of aspirated liquid to prevent formation of clots. Avoid excessive dilution of follicular fluid with HM199 to effectively maintain oocytes in meiotic arrest [73]. The whole procedure (follicle aspiration and COCs selection) must be performed in approximately 60 min to avoid meiotic resumption of Class 1 oocytes. Alternatively, use the HM199-Cilo as holding medium to pool all Class 1 COCs during selection.

Note 8: After IVM, oocytes can be submitted to IVF and presumptive zygotes cultured for 7 days as previously described [3,14,9,28].

Note 9: Measure the diameter of the oocytes using a micrometer-equipped eyepiece or other appropriate tools such as a microscope-mounted camera and software that allows measurements. The diameter of the oocyte excluding the zona pellucida must be within a range of 100 - 110 µm. Discard COCs with larger or smaller oocytes and with irregular and not-rounded shaped oocyte.

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Figure Captions

Figure 1. Multiple approach: a customized culture system for each stage specific need.

Figure 2. Schematic representation of the COCs growing in the BioCoat™ Collagen I 96-well plate during L-IVCO. To promote the three-dimensional organization of cells, the COCs are cultured in L-IVCO medium where viscosity is increased by adding 4% PVP to mimic the physiological viscosity of the follicular fluid and by culturing the COCs on a collagen coated surface that promote the formation of a 3D-like culture [17,66].The stem of granulosa cells adheres to the coated bottom while the cumulus oophorous protrudes into the medium in the center of the well.

Figure 3. Representative pictures of COCs isolated from MAFs. After collection, COCs are separated according to morphological criteria. Class 1: homogeneous ooplasm and absence of expansion of outer layer of cumulus cells; Class 2: minor granulation of the

ooplasm and/or beginning of expansion of outer layer of cumulus cells; Class 3: highly granulated ooplasm and few cumulus cells layers showing expansion.

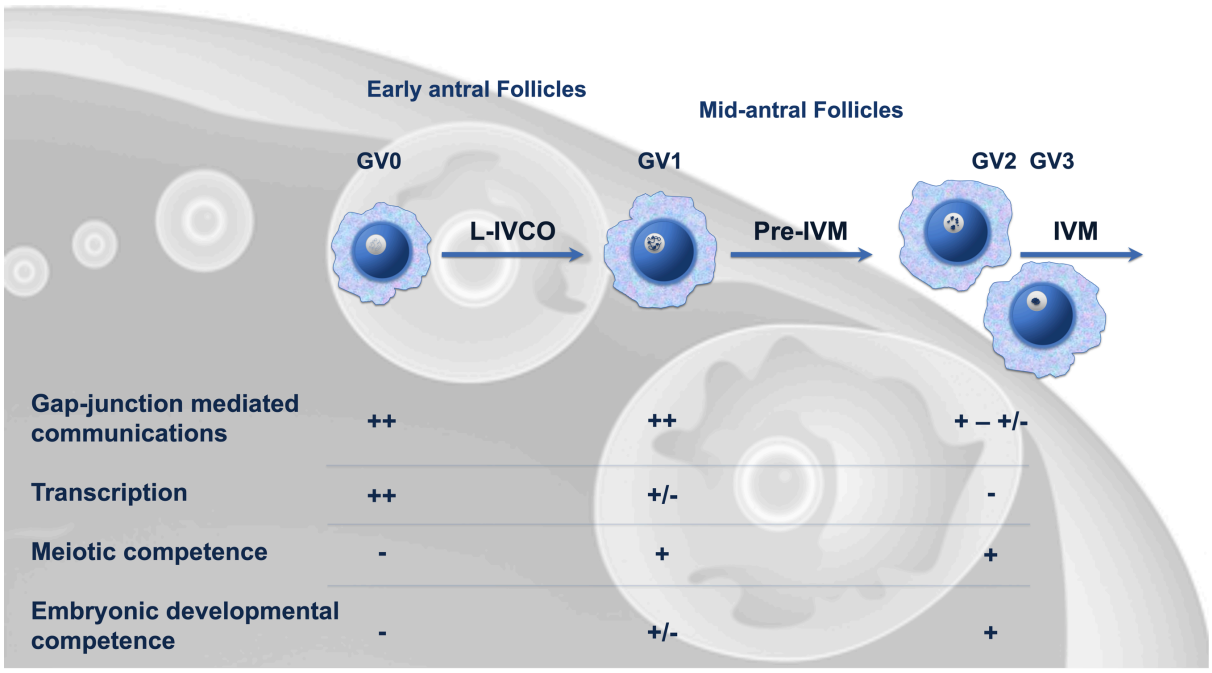
5

Table Captions

Table 1. Extent of follicle reserve and follicle categories in 4-8 years old bovine ovaries (data were extrapolated from [74-77,44]). The bottom part of the table are the proposed optimal exploitation strategies.

Follicle category	Primordial (<0.1 mm)	Primary and Secondary >0.1 to <0.5	Early antral (≥0.5 to <2 mm)	Antral (2 to 8 mm)
Number/ovary	65,000	25,000	120	25
Atresia	<5%	5%	30%	60%
Chromatin	-	-	GV0	GV1, GV2, GV3
Optimal exploitation strategies				
Ex-vivo (following culling) ovaries	Freezing/culture [2]	Freezing/culture [2]	L-IVCO [17,3]	Pre-IVM (GV1) and direct IVM (GV2 and GV3) [70,9]
In vivo	-	-	-	Synchronization followed by OPU and direct IVM [15]

Figure 1



5 Figure 2

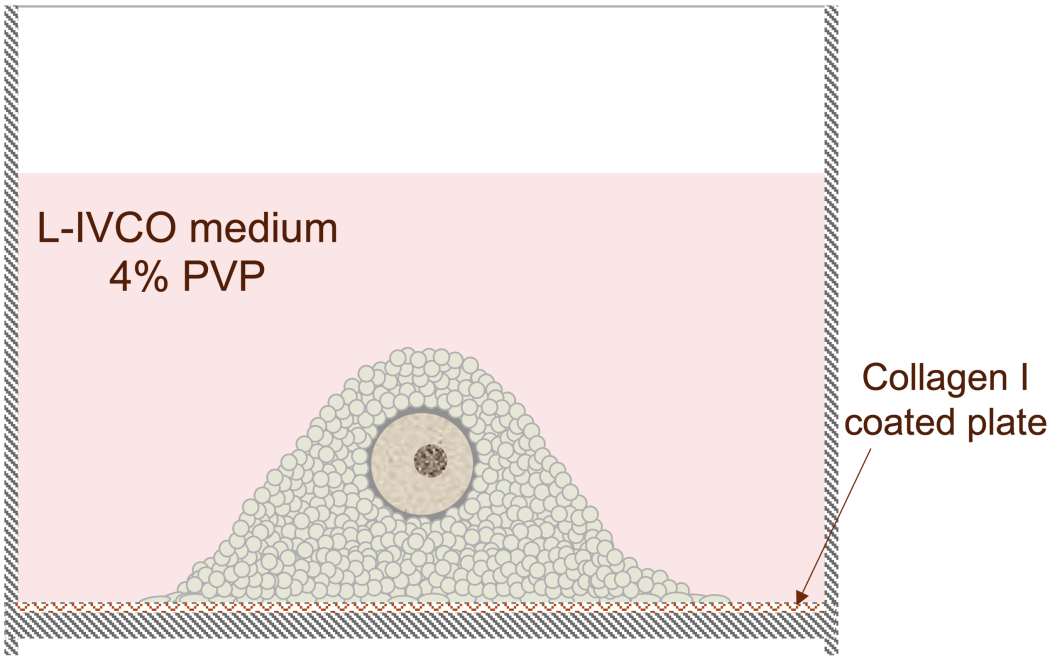


Figure 3