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REVIEW

Human oocyte capacitation culture: Essential step toward hormone-free assisted reproductive technology

Johan Smitz[1] | Flor Sánchez[2] | Sergio Romero[3] | Heidi Van Ranst[1] | Ellen Anckaert[1] | Robert B. Gilchrist[4] | Tuong M. Ho[5,6] | Lan N. Vuong[7] | Yoshiharu Morimoto[8]

[1]Follicle Biology Laboratory, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium

[2]Centro de Estudios e Investigaciones en Biología y Medicina Reproductiva, Lima, Peru

[3]Centro de Fertilidad y Reproducción Asistida, Lima, Peru

[4]Fertility & Research Centre, Discipline of Women's Health, School of Clinical Medicine, University of New South Wales Sydney, Sydney, New South Wales, Australia

[5]IVFMD, My Duc Hospital, Ho Chi Minh City, Viet Nam

[6]HOPE Research Center, Ho Chi Minh City, Viet Nam

[7]Department of Obstetrics and Gynecology, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Viet Nam

[8]HORAC Grand Front Osaka Clinic, Osaka, Japan

Correspondence
Johan Smitz, Follicle Biology Laboratory, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, Brussels, Belgium.
Email: johan.smitz@gmail.com

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Abstract

Background: In vitro oocyte maturation (IVM) is not a novel concept; however, its wide-scale practice has been limited because of the lower clinical outcomes compared to conventional assisted reproductive technologies.

Methods: This comprehensive review addresses the significant advances made in oocyte in vitro maturation with the biphasic capacitation (CAPA)-IVM strategy applied to small ovarian antral follicles in humans over the last 10 years. CAPA-IVM consists of a prematuration phase wherein immature oocytes are temporarily meiotically arrested to gain competence before undergoing meiotic resumption.

Main findings: The integration of knowledge from basic research in animal models into clinical practice has led to a reevaluation of IVM for policystic ovary syndrome (PCOS) and onco-fertility patients. The introduction of meticulously conceived growth factors, hormonal supplements, and culture conditions led to an integrated biphasic CAPA-IVM system that promotes oocyte competence. A series of prospective randomized controlled studies validated the reproducible improvements in clinical outcomes and the safety of CAPA-IVM. So far, nearly 1000 babies have been born using this approach.

Conclusion: The use of CAPA-IVM in clinical studies has set the tone for major progress in the field and is achieving a safer, less expensive, and less emotionally loaded IVF experience, currently validated for PCOS patients.

KEYWORDS
biphasic in vitro maturation, CAPA-IVM, cumulus–oocyte complexes, in vitro maturation, IVM

1 | INTRODUCTION: ANIMAL REPRODUCTIVE SCIENCE INSPIRED IMPROVEMENT OF HUMAN IVM

The purpose of this review article is to summarize the improvements made in the field of in vitro maturation (IVM) of small ovarian follicles in humans over the last 10 years and to present a comprehensive list of these advances to clinicians and embryologists eager to apply them in their clinics. This review does not intend to discuss IVM as an add-on method to mature MI or germinal vesicle (GV) oocytes, which are side products from fully hormone-stimulated IVF cycles (i.e., rescue-IVM).

From a practical point of view, IVM is widely applied in livestock breeding programs. In the context of ART in bovine, ovine, and porcine, the female gamete at the start of the culture is most often an immature oocyte (GV stage) that gets isolated from the follicle either by ultrasound-guided needle aspiration or by isolation out of ovaries obtained at the slaughterhouse. In the large majority of cases, no stimulation of follicle growth with hormones is practiced, but care is taken to select the GV oocytes from follicles of a restricted diameter group (4–6 mm diameter) to allow for a uniform maturation time.[1–4] Application of a stringent selectivity in follicle diameters for IVM is feasible because the animals are generally young and have a good ovarian reserve.

In order to broaden the possibility to mature oocytes from smaller follicular diameters (predominant sizes), the concept of prematuration ("capacitation") was first introduced in bovine IVM practice.[5] Experiments in bovine, ovine, and porcine have been a very generous source of ideas and techniques that would otherwise never have led to the applications of an efficient IVM system for humans. Although the work in livestock breeding has been inspirational and a valuable reference for the efficacy and safety of IVM, its application had never been broadly tested in clinical human ART before the year 2000. Reasons for this are thatthe available culture media for IVM were sparse and expensive, the entire lab process for IVM was poorly described, the clinical pretreatment of patients was fairly arbitrary between centers, and precise clinical indications for IVM (besides policystic ovary syndrome (PCOS)) were lacking.

The changes in practice to make IVM successful do not require major financial investments. Clinicians should consider a "training" period to learn the COC pick-up from follicles between 2 and 9 mm in diameter. Embryologists should learn how to recognize and select out of curetted intrafollicular cellular material the small unexpanded COCs. In the hands of experienced IVF experts, these two changes to their current practice should not take more than 20–30 cases to obtain the required key performance indicators (KPI).

In the following pages, we provide the rationale for providing cumulus-enclosed immature oocytes with an extra culture step under an in vitro environment that is compatible with a healthy stepwise maturational process.

For the patient eligible for IVM, the Capacitation (CAPA)-IVM technology reduces treatment burden, time, and costs. Compared to current IVF, the reduced medicalization might attract infertile couples into infertility treatment mode at an earlier age, which in itself would already increase their chances of success.

2 | OVARIAN FOLLICULAR SIZES TO TARGET FOR OBTAINING CUMULUS OOCYTE COMPLEXES SUITABLE FOR CAPA-IVM

Follicular sizes targeted for IVM differ substantially from those used in COS cycles: CAPA-IVM targets small antral follicles of 2–9 mm in diameter.

In the human ovary, initiation of antral cavity formation occurs under the influence of FSH in follicles with a diameter of 250 μm.[6] At these very early antral stages, until follicles reach ~2 mm in diameter, only a low proportion of them show atresia: ~15%–30%.[6] Follicles grow to 2–5 mm in diameter (also known as the selectable stage) under baseline gonadotropin levels. From a diameter of 2 mm onward, follicles become more dependent on FSH, and the proportion of atretic follicles tends to increase unless increasing FSH levels rescue them from atresia.[7] Follicular growth beyond 2–5 mm depends on the intercycle rise of FSH. Follicles that fail to react to threshold gonadotropin are highly susceptible to atresia. Indeed, the mean percentages of healthy follicles from 2 to 5 mm and 6 to 10 mm were reported to be ~42% and ~23%, respectively, demonstrating that in 6–10 mm follicles, atresia can be as high as >70%.[7]

In most IVM studies, mild gonadotropin priming strategies (either with hCG, FSH, or hCG + FSH) were used to stimulate follicular growth.[8–10] The COCs were usually retrieved from 6 to 12 mm follicles,[11,12] suggesting that most of these could already be undergoing atretic changes.

Despite the fundamental role of FSH, the benefit of using FSH priming in IVM remains controversial.[8,13–16] Currently, most clinical programs use a few[2–5] days of FSH stimulation before collecting oocytes for IVM. The rationale of FSH pretreatment in IVM cycles is based on the high expression of FSH receptors in 2–6 mm human follicles and the beneficial effects of FSH on follicular health.[17,18] In normo-ovulatory women, FSH priming reduced, by half, the incidence of apoptosis in granulosa cells of immature follicles compared to unstimulated normal ovaries (~29% vs. ~46%).[15] Until today, there is no consensus on either the dose or duration of FSH priming to be applied in IVM cycles. A short course of FSH treatment usually involves 150 IU FSH daily for 2–3 days, initiating from day 2 or 3 of the menstrual cycle or after a progestin withdrawal bleed.[19–21] Despite previous evidence of the need for FSH with monophasic ("standard") IVM culture, results from a recent RCT demonstrated that using capacitation in vitro maturation (CAPA-IVM), pretreatment of oocytes in PCOS patients with either FSH and/or hCG is superfluous.[22]

On a similar note, cases for fertility preservation where ovarian tissue IVM is used because there is no time for stimulation treatment before chemotherapy, the application of CAPA-IVM yielded more blastocysts for cryopreservation than monophasic IVM.[23]

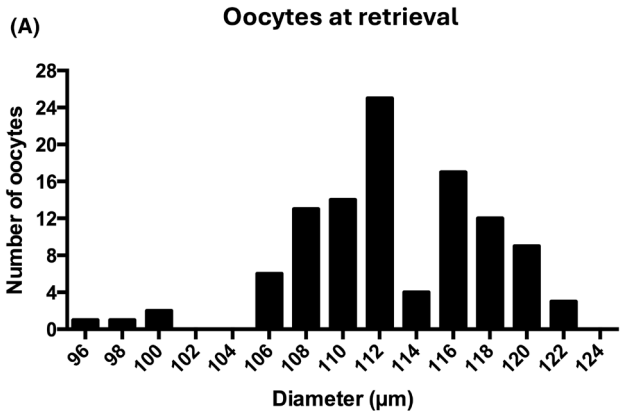
Notably, in IVM cycles where PCOS women receive 2–3 days of FSH priming (but no hCG priming), the majority (≥80%) of COC are retrieved from follicles <6 mm in diameter,[21,24–26] a range where a higher percentage of follicles do not show signs of atresia.[7] A smaller proportion of COC (~20%) are from follicles larger than 6 mm with a higher risk of atresia. Follicular size and oocyte diameter are important determinants of IVM outcomes.[27–32] Likewise, the capability of oocytes to resume and complete meiosis in vitro is dependent on oocyte size.[30–32]

Nevertheless, oocyte and follicle growth do not occur in a synchronous manner (except during the early stages until follicle diameter is ~124 µm and the oocyte is on average, 80 µm). Final oocyte size is reached at a faster pace, apparently during the small antral stages.[32,33]

In ovaries obtained from patients requesting fertility preservation before cancer treatment, Pors[32] demonstrated that human small antral follicles ranging from 0.5 to 3 mm are found in a high number in the medulla of cryopreserved ovaries. In these antral follicles, oocytes displayed an average diameter of 115 µm following IVM, but those that reached the MII showed larger diameters, on average 123 µm.[32] This report confirms previous findings on the relationship between oocyte size and maturation potential.[30,31,34] Later data confirm that fully grown oocytes, in size, can be present in a high proportion in a population of yet underdeveloped antral follicles.[33] Besides oocyte size, other important aspects need to be considered when maturing oocytes in vitro. In PCOS patients receiving mild FSH priming for IVM, ~80% of cumulus-enclosed oocytes derived from follicles <10 mm had diameters ≥110 µm (on average 115 µm) at retrieval[34] (Figure 1). Yet, at this oocyte size, only 53% of these cumulus-enclosed oocytes displayed condensed chromatin and were transcriptionally quiescent (Figure 2), and only 20% showed perinuclear distribution of mitochondria, all being patterns associated with meiotic and cytoplasmic maturity (Figure 3). The other fraction revealed mostly features associated with immaturity at cellular and molecular levels (Figure 4).

When the effectiveness of the different IVM strategies is considered, a higher maturation rate was observed for COCs from follicles >6 mm compared to <6 mm[21,25,26] (Table 1). However, when applying a biphasic system (e.g., CAPA-IVM), compared to conventional monophasic or Standard IVM, significantly more top-quality embryos were available for transfer from <6 mm follicles than from follicles >6 mm.[25] For the first time in human ART, a live birth was achieved successfully from such small (<6 mm) antral follicles.[21]

Hence, it could be inferred that the more suitable target follicle population to focus on for IVM is the class of <6 mm. The most suitable follicle class to culture is the more uniform cohort of (1) healthy follicles, (2) with COCs having strong interconnected (unexpanded) cumulus cells, and (3) a healthy, nearly fully grown oocyte capable of sustaining maturation, embryo development, and live birth.[21] In conclusion, selection of a proper IVM system is crucial for the successful clinical applicability of IVM programs in the clinic. Our data show that oocytes found in small follicles (i.e., <6 mm) profit most from a sophisticated IVM system that supports the completion of essential processes to acquire full oocyte developmental potential before they are exposed to a maturation trigger (see Section "Cellular markers of nuclear and developmental competence of CAPA-cultured oocytes").



(B)

<105µm	≥105 and <110µm	≥110µm
4	19	84
3,7%	17,8%	78,5%

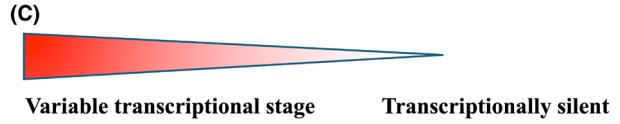


FIGURE 1 Frequency distribution of oocyte diameters from human small antral follicles at the time of oocyte retrieval. Oocyte size was calculated as the average after measuring maximum and minimum diameters, without including the zona pellucida (A). Number and proportions of oocytes analyzed at retrieval in three different size categories (B). The majority of oocytes (78.5%) displayed diameters >110 µm (mean diameter in this group: 115 µm). A representation of the transcriptional activity in the three oocyte diameter categories (C).

3 | PREDOMINANT FOLLICLE PHYSIOLOGY

3.1 | Gonadotropin receptors in small follicles from PCO and normal ovaries: Does the patient need any gonadotropin before CAPA-IVM?

LHCGR and *FSHR* are expressed in cumulus cells from both immature and mature follicles and are regulated differently during oocyte maturation in vivo and in vitro.[17,35–37] In granulosa cells, *LHCGR* levels are upregulated in connection with ovarian stimulation and during in vitro maturation, whereas levels of FSHR are downregulated. These changes in expression levels are related to exogenous FSH administration, either in vivo or in vitro.[17,35,37] However, compared to mural granulosa cells, *LHCGR* expression is very low in cumulus, while *FSHR* is expressed at higher levels, including in CCs of

	Oocyte diameter category	Chromatin configuration: Dispersed (%)	Intermediate (%)	Condensed (%)
small	< 105 µm	3 (75)	0	1 (25)
medium	≥ 105 < 110 µm	3 (27.3)	3 (27.3)	5 (45.5)
large	≥ 110 µm	20 (31.3)	10 (15.6)	34 (53.1)
	Total (N=79)	**26 (32.9)**	**13 (16.5)**	**40 (50.6)**

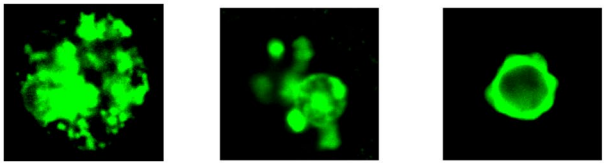


FIGURE 2 The three pictures show the chromatin status in relation to oocyte diameter in human germinal vesicle-stage oocytes from small antral follicles immediately analyzed after retrieval in IVM cycles. Oocyte classification is according to the condensation status of the chromatin. Nucleolus-like bodies (NLBs) are "dispersed" (left), "intermediate" (middle), or "condensed" (right) chromatin. The single chromatin ring around an NLB is also known as the perinucleolar rim. The table shows the distribution of the chromatin status in three different categories of oocyte diameter as measured at the time of retrieval.

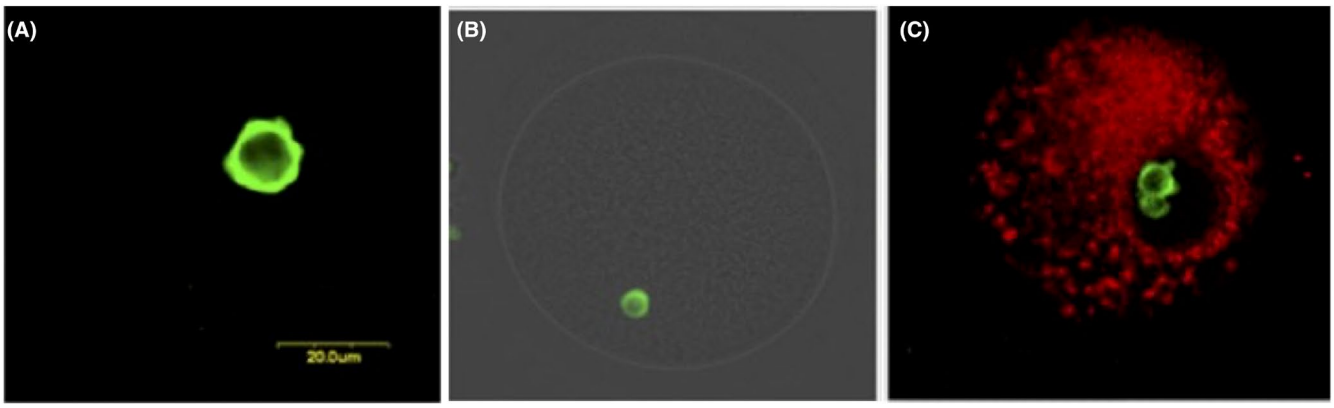


FIGURE 3 Examples of oocytes displaying characteristics associated with meiotic and cytoplasmic competence. (A–C) In all figures, a condensed chromatin ring surrounding the oocyte nucleolus can be visualized while (B) transcriptional activity has ceased, (C) mitochondria have been translocated toward the nucleus, perinuclear distribution. Original pics from Sánchez et al. (2015).

follicles <6 mm retrieved for IVM[35] and in CCs from follicles <3 mm from ovarian tissue.[37] Rationally, gene expression findings on cumulus cells do not suggest the in vitro use of an LHCGR ligand to induce maturation for IVM cycles.

Interestingly, in women with PCOS, Owens[36] reported an aberrant expression profile of crucial regulatory hormone receptors that are possibly involved in follicle arrest. Granulosa cells of small antral follicles ranging from 2.4 to12.4 mm were retrieved from unstimulated PCO, and their expression profiles were compared to those of normal ovaries in a fertility preservation program. Granulosa cells from PCO ovaries showed an altered profile, with a reduced expression of *CYP11A1*, *FSHR* and AR; and an increased expression of *STAR* and *CYP19*. Furthermore, although overall, no difference was found in *LHCGR* expression, a subpopulation (20%) of PCO ovaries displayed abnormally high levels of *LHCGR* transcripts. Based on this, the authors suggest that the aberrant expression of both gonadotropin receptors is among the crucial factors involved in the premature arrest of antral follicle growth observed in PCOS women.[36]

The studies performed over the last decade using ovarian tissue from fertility preservation programs, have provided valuable insights into the gene and protein expression of gonadotropin receptors, steroid receptors, and oocyte growth factors in the follicular contents of small antral follicles <6 mm.[17,18,36,37]

In ovarian tissue from women with normal endocrinology (non-PCOS), a marked difference between *FSHR* and *LHCGR* expression levels has been found in human granulosa cells from small antral follicles in unstimulated ovaries. Although *FSHR* gradually decreases as the follicle increases in size and maturity, *FSHR* was highest in granulosa cells from small antral follicles (3–6 mm), compared to *LHCGR*.[17]

On the other hand, in cumulus cells from <6 mm follicles, no changes in expression levels of both gonadotropin receptors were

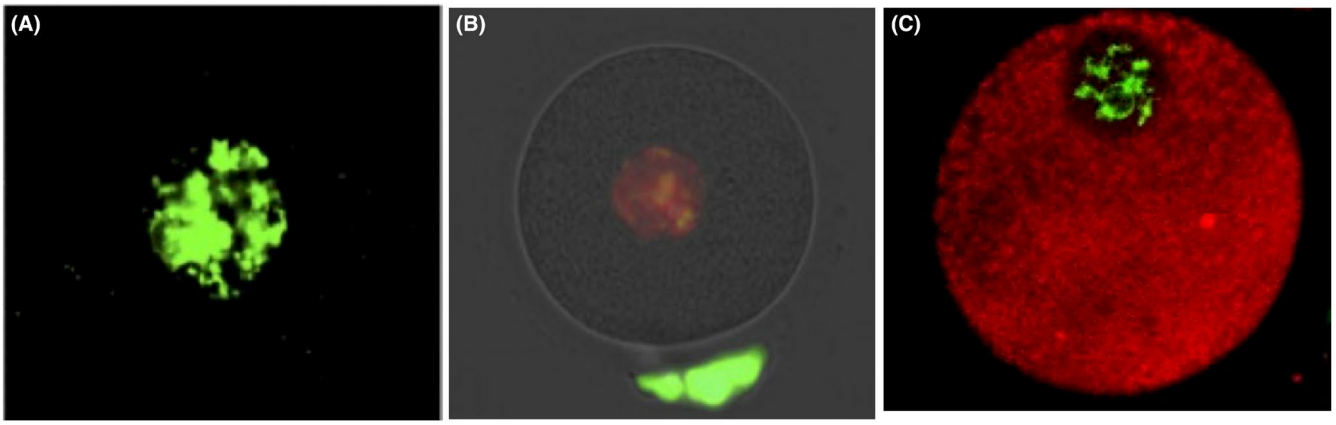


FIGURE 4 Examples of oocytes displaying characteristics associated with meiotic and cytoplasmic incompetence. (A) A disperse chromatin pattern throughout the oocyte nucleus. (B) An oocyte displaying strong transcriptional activity while chromatin is dispersed. (C) Oocyte with a homogenous mitochondria distribution pattern throughout the cytoplasm, while chromatin is in the process of condensation. Original pics from Sánchez et al. (2015).

TABLE 1 In vitro maturation and embryology outcomes in 40 patients.

Oocyte developmental outcomes	CAPA-IVM <6 mm, *N* = 20	STD IVM <6 mm, *N* = 20	*p*-Value	CAPA-IVM ≥6 mm, *N* = 14	STD-IVM ≥6 mm, *N* = 15	*p*-Value
Number of COCs	236	178	–	69	60	–
Maturation (MII) rate, % (*n*)	61 (144)	44.4 (79)	0.055	65.2 (45)	58.3 (35)	0.517
Fertilization rate per ICSI, % (*n*)	86.1 (124)	84.8 (67)	0.912	84.4 (38)	62.9 (22)	0.118
EQ1 + EQ2 rate per fertilized, % (*n*)	43.5 (54)	29.9 (20)	0.138	44.7 (17)	31.8 (7)	0.573
EQ1 rate per fertilized, % (*n*)	16.9 (21)	9 (6)	0.258	15.8 (6)	13.6 (3)	0.258

Note: Outcomes of COC are analyzed in relation to their follicular origin, being follicles <6 mm, and ≥6 mm. The performance is shown for CAPA-IVM and Standard IVM in accordance with follicle diameter of origin. For COC from follicles <6 mm, capacitation culture produced more MII oocytes than Standard IVM ($p = 0.055$), which provided more embryos of good morphological quality. Values are presented as percentages and total number of COC included between brackets. Adapted from Sánchez et al. (2019).

Abbreviations: CAPA-IVM, capacitation in vitro maturation; COC, cumulus–oocyte complex; EQ1, embryo quality 1 (Top quality); EQ2, embryo quality 2; MII, Metaphase II.

observed between PCOS and normovulatory women, before or after undergoing IVM culture.[35]

LHCGR gene is expressed in granulosa cells from follicles with diameters of 5–6 mm and until ovulation[17] and its expression is ~10-fold lower in small antral follicles (~6–9 mm) compared to preovulatory follicles (~15 mm).[17,36] As reported by Jeppesen,[17] *LHCGR* becomes measurable only around a diameter of 5–6 mm since granulosa cell samples showing no *LHCGR* expression were mostly derived from follicles of diameters <5–6 mm.

Based on previous findings, the widely used practice of injecting an hCG trigger in IVM cycles[9,11] where usually small antral follicles (up to 12 mm diameter) are targeted seems not to be backed up by scientific rationale. Triggering with 5000 or 10 000 IU hCG could induce inappropriate signaling within the follicle,[38] with undesired consequences for the COCs, such as asynchrony in oocyte maturation stage (as evidenced by the typical retrieval of a heterogeneous population of COCs containing a mixture of immature and mature oocytes).[39,40] Furthermore, the induction of the expansion process in cumulus cells leads to interruption of essential communication between oocytes and cumulus cells, which is incompatible with the acquisition of developmental competence in oocytes. Finally, clinical studies that compared hCG-triggered cycles with untriggered cycles did not find a difference in pregnancy outcomes, demonstrating there is no clinical benefit in using hCG-triggering in IVM cycles.[41]

4 | OPTIMAL TIME AND TREATMENT SCHEME TO COLLECT CUMULUS–OOCYTE COMPLEXES (COCs) WITH HIGH DEVELOPMENTAL POTENTIAL

The CAPA-IVM strategy brings a radical paradigm shift in infertility treatment in many ways. It moves the infertility specialist away from the principle that "quality" oocytes for ART can only be obtained from large preovulatory follicles. So far, gonadotrophins have been instrumental in overcoming the strong natural selection process among human follicles in the ovary. However, instead of targeting follicles of >15–17 mm, a diameter above which follicles generally deliver an MII oocyte after an HCG trigger, with CAPA-IVM the

aim is to collect COCs from the follicles between 2 and 9 mm, before dominance is established in the ovary. Follicles between 2 and 6 mm, at the end of their gonadotropin-independent growth phase, are present at all times in the ovary.[42–44] In the absence of gonadotrophin treatment, most follicles >6 mm are, at any moment of the menstrual cycle, in variable stages of early atresia.

In the particular case of PCOS, the follicles are arrested at the stage of 4–6 mm and are resistant to FSH stimulation for further growth. The COCs from such small follicles, when aspirated for CAPA-IVM on day 5 after a withdrawal bleeding, even without any prior exposure to FSH or hCG, nevertheless contain oocytes with high developmental capacity.[21,45] As an infertility treatment for PCOS women, the use of CAPA-IVM without any gonadotrophin administration resulted in a 38% live birth rate after the first blastocyst transfer.[22]

With a consistent average live birth rate of 35% over several RCT studies (see Table 3), CAPA-IVM could be considered a superior treatment for PCOS compared to protracted ovulation induction treatments which, with or without intra-uterine insemination, generally produce live birth results that rarely exceed 20%.

Until today, the clinical studies in PCOS patients using CAPA-IVM have timed the oocyte retrieval after: (1) withdrawal bleeding (obtained after 10–14 days of contraceptive pill) followed by (2) 2 or 3 days of gonadotrophin stimulation (150 IU/day r-FSH). The oocyte retrieval was scheduled 42 h after the last FSH injection. In case no gonadotrophins were preferred, the oocyte retrieval was performed on day 5 after the last day of taking the OC pill.[22]

Currently, a simplified treatment scheme (Figure 5), without prior oral contraceptive use is proposed for PCOS patients. For patients with a regular cycle, the random retrieval of small follicles could also be proposed. Alternatively, for more convenient scheduling of the OPU in a busy clinic, the use of OC or progesterone might be implemented.

For the patients with normo-ovulatory cycles, there is not yet much experience with the CAPA-IVM technique. In principle, oocyte retrieval could be performed from follicles from 2 to 6 mm at any time of the cycle, as this class is always present.[42] Perhaps the peri-ovulatory period would be a period to avoid doing OPU in these patients. However, in case that the initial aim was to also collect the oocyte from the dominant follicle, like in Teramoto's study,[39] it is foreseeable that a fraction of the smaller follicles (those <6 mm) would be usable for CAPA-IVM, while the COCs from follicles between 7 and 10 mm are on the path to atresia. Teramoto et al.[39] have demonstrated that in combination with a dominant follicle, oocytes from small follicles are capable of developing into blastocysts and giving live births.

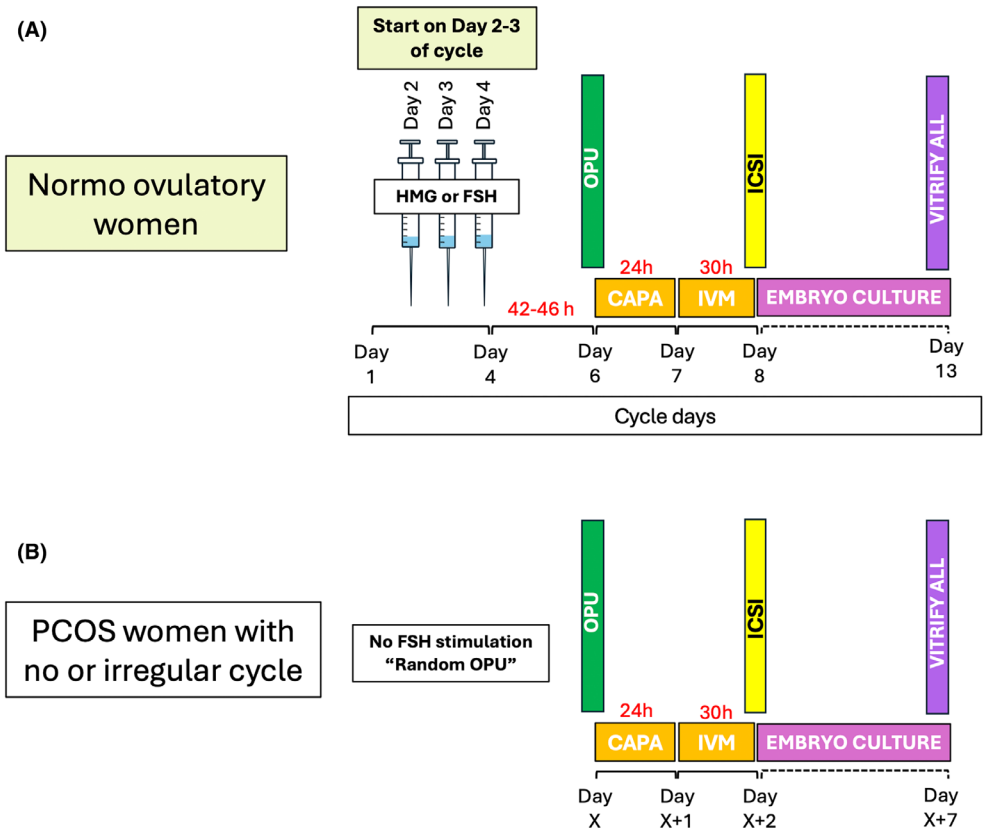


FIGURE 5 Representative scheme showing the clinical protocol for CAPA-IVM cycles and the key steps following oocyte retrieval. (A) In women with normal cycle (≤35 days), no oral contraceptive is needed. CAPA-IVM treatment starts on Day 2 or 3 of cycle. (B) In PCOS women with no cycle or irregular cycles, OPU for CAPA-IVM can be performed randomly on any day. Similarly, in oncological patients, gonadotropin injection can be avoided.

5 | TECHNICAL ASPECTS OF INTACT COC RETRIEVAL FROM SMALL FOLLICLES

The size of the unstimulated ovary to puncture in IVM is considerably smaller and is thus more mobile in the pelvis. This, in combination with the smaller follicles to puncture, requires particular attention from the clinician. Particularly for CAPA-IVM, it is more the amount of intact COCs (i.e., well surrounded by several layers of cumulus cells) than overall oocyte number that determines success (see Figure 8). The retrieval technique used so far for most patients in CAPA-IVM is with a double lumen needle (17G external and 19G internal from Kitazato (Japan)). Suction pressure is set at 100–120 mm Hg. The inner needle is spun between the index finger and thumb to perform a curettage of every little follicle accessed under ultrasound guidance. During the procedure of oocyte retrieval, the use of insufficient aspiration pressure in combination with a thin needle can result in obstruction of the needle lumen by blood clots or fine tissue fragments. Needles can be rinsed with flushing media to circumvent the formation of blood clots, but often the addition of heparin is applied. Some clinics make use of pseudo double lumen needles for follicle aspiration in IVM cycles.[46] The development of such closed-circuit needle flushing systems initially aimed to increase the number of oocytes retrieved in IVM cycles. Although similar retrieval rates were obtained after using single lumen or pseudo double lumen needles, the use of these latter needles showed some advantages, mainly in the handling of the COCs in the embryology lab. They mainly avoid blood clots in the aspirated follicular fluid, which results in an IVM retrieval more like IVF for the embryologists.[46] In experienced hands, the use of a (co-axial) double or single lumen needle in patients with ≥5 ng/mL can yield an average of 20 COCs suitable for culture.[22] In CAPA-IVM cycles, hCG trigger before follicle aspiration is never used.[21,22] This makes the retrieval of COC from small follicles quite different from large follicles, where hCG expands the innermost cumulus layers by mucification. The absence of any hCG-triggering in CAPA-IVM keeps the COC firmly lodged in the follicular wall. Hence, it is logical that a follicle curettage by the spinning movement of the needle followed by aspiration would collect COCs more easily in a small space (small follicle of <6 mm) than COCs from larger follicles (>6 mm follicles). The COC recovery rates in small follicles <6 mm is 80%, in contrast to ~20% in the 6–10 mm diameter follicles.[25] Hence, it is a majority of COCs from <6 mm follicles (the healthier cohort) that will be cultured in a CAPA-IVM system.

In general, oocyte retrieval in IVM cycles is usually more challenging compared to standard IVF collections. Physicians need training, and there is a learning curve to perform the aspiration of such small antral follicles.

6 | CAPA-IVM CULTURE METHODOLOGY

The steps involved in the biphasic CAPA-IVM procedure until an in vitro competent MII oocyte is obtained are summarized in Figure 6.

6.1 | General laboratory aspects considered for CAPA-IVM

Different from regular IVF cycles with a full FSH and hCG treatment course, where an embryologist is mostly used to handling and searching for fully expanded COCs following OPU; in standard IVM and CAPA-IVM cycles, embryologists are challenged with fully compacted GV-stage COCs. The processing of bloody follicular aspirates and the identification of immature compacted COCs retrieved from small antral follicles require a period of training. Nonetheless, this is

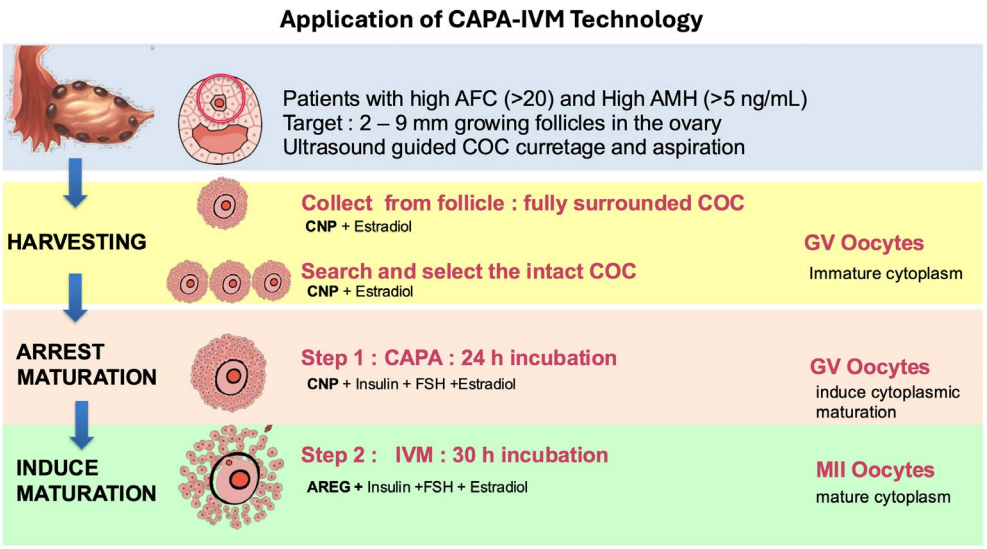


FIGURE 6 Steps of CAPA-IVM procedure in patients with high antral follicle count (AFC) and the effects that each step produces within the COC. AMH, antimüllerian hormone; AREG, Amphiregulin; CAPA-IVM, capacitation in vitro maturation; CNP, C-type natriuretic peptide; COC, cumulus–oocyte complex; GV, germinal vesicle; IVM, in vitro maturation.

not particularly demanding, and the learning curve is substantially less than, for example, an embryologist learning ICSI or embryo biopsy procedures.

The main disposable device used for this purpose is a 70-µm pore cell strainer, composed of a nylon mesh, through which follicular aspirates collected in tubes are passed. Following a continuous rinsing with a buffered medium, follicular aspirate is sieved, and COCs are separated from other smaller components such as red blood cells and cellular debris.

Cumulus oocyte complexes and some clumps of granulosa cells retained in the cell strainer are then washed and transferred to a new dish for COC identification under a stereomicroscope. Because the type of follicular aspirates received in the lab requires a more laborious process prior to the isolation of COCs, it is important to initially allocate double searching time to collect all the COCs for further culture.

A crucial aspect when applying the biphasic CAPA-IVM protocol is that during the aspiration procedure, collection, and isolation, appropriate methods need to be used that are nondisruptive to cumulus–oocyte connections. COCs should keep their "compact" integrity, which is needed to maintain oocytes under meiotic arrest. Both cell types are equally critical and rely on each other, as proper communication between cumulus cells and the oocyte will maintain oocytes under meiotic arrest via the C-type natriuretic peptide (CNP)/NPR2 system. Consequently, oocytes cultured in the biphasic CAPA-IVM system will acquire cytoplasmic and nuclear maturity during the CAPA pre-IVM culture period and will resume meiosis in a more homogenous manner following the maturation trigger.[25]

6.2 | Culture supplements of the CAPA-IVM system

The CAPA-IVM system involves different specific media for COC collection/searching, prematuration, and oocyte maturation procedures.

With the aim of mirroring, as much as possible, the in vivo microenvironment, the main two physiologically relevant components included in the CAPA-IVM system are CNP, present in the pre-IVM capacitation (CAPA) medium and amphiregulin, present in the second-step IVM medium. Basal factors present in both prematuration and maturation medium are FSH, insulin, and estradiol (E2) as originally described.[24,47] In order to evaluate the potential of CNP as a physiological nuclear arrestor, our laboratory chose the most stringent mouse model: juvenile unstimulated animals. The surprising results showed an increased yield of good-quality embryos from COCs from juvenile mice by using CAPA-IVM.[47]

Almost all previous work in animal models from others had modeled IVM in the mouse by using eCG-stimulated mice, which are known to increase the proportion of surrounded nucleolus (SN) oocytes.[48] Hence, this might mask the importance of other paracrine factors that are needed in the human model when the purpose is to minimize the daily injections of gonadotrophins in infertility treatment. Translation of the CAPA-IVM methodology developed in the murine model was subsequently implemented into the human clinical embryology laboratory using donated oocytes.[24] Similar favorable results of applying the culture supplements of CAPA-IVM to human COCs were evidenced in minimally stimulated PCOS volunteers.[24] The judicious selection of supplements to add to the basal IVM media extrapolated from the CAPA system in humans relies on various publications in animal models and in human studies.

6.2.1 | Capacitation medium composition

CNP, the natural meiosis-inhibiting substance produced in the granulosa compartment of the follicle, was elected to be added to the prematuration (CAPA) medium. In vivo, high levels of cAMP within the oocyte inhibit the activation of maturation-promoting factor, resulting in oocyte meiotic arrest. CNP signals through its receptor, NPR2, a guanylyl cyclase natriuretic peptide receptor 2, present both in mural granulosa and cumulus cells. CNP regulates cGMP levels in granulosa cells.[49] Cyclic GMP diffuses into the oocyte via gap junctions and inhibits phosphodiesterase (PDE) 3.[50,51] PDE3 is a cGMP-dependent cAMP phosphodiesterase. The inhibition of PDE3 prevents oocyte meiotic resumption.[50] Hence, CNP is the natural oocyte meiotic inhibitor in vivo and can readily be used to arrest oocyte maturation in vitro.

Making use of CNP in in vitro prematuration protocols in different animal models and in human oocytes was shown not only to efficiently maintain oocytes under meiotic arrest but also to preserve transzonal projections and keep gap-junctional communication between the oocyte and CCs[3,24,47,52,53]; therefore, favoring developmental competence acquisition.

The effectiveness of the CNP/NPR2 system needs a minimal amount of estradiol (E2) to maintain the expression of the NPR2 receptor on granulosa and cumulus cells, as demonstrated by Zhang et al. in the mouse model.[49,54]

Also, the presence of FSH at critical doses during the different steps of in vitro culture is essential. FSH promotes an efficient intercellular coupling between the oocyte and their surrounding cumulus cells.[5,47] High FSH doses would induce a premature retraction of TZPs,[55] a low dose of FSH is used in the CAPA pre-IVM phase. Furthermore, there is a molecular rationale for the use of a higher dose of FSH in the maturation media as well. A study revealed that FSH, through interaction with the EGF network and activation of the PI3K/AKT signaling pathway, regulates the translation of maternal mRNAs in the oocyte, leading to improved oocyte developmental potential.[56] FSH may accelerate oocyte development by its activity to promote cumulus cell proliferation and by stimulation of specific genes (KITL) that promote oocyte growth.[48]

6.2.2 | Maturation medium composition

The addition of amphiregulin (AREG), an epidermal growth factor (EGF)-like peptide in the maturation medium, in combination with FSH, was selected to stimulate the EGF signaling network to induce

oocyte maturation in vitro. Amphiregulin is the most abundant EGF-like factor found in the follicular fluids of primates and humans, and its concentration is positively correlated with human oocyte developmental competence.[57,58] In mouse, primate, and human models, EGF-like peptides such as epiregulin and/or amphiregulin are the most suitable additives to IVM medium compared to FSH or EGF[47,59,60] and were shown to improve oocyte developmental competence.[57–61] In human COCs retrieved from <6 mm follicles undergoing CAPA-IVM culture, the presence of AREG in the maturation medium significantly increased the oocyte maturation rate compared to using standard maturation medium containing FSH, LH, and growth hormones. Using the AREG trigger upregulated genes responsible for progesterone synthesis and redox metabolism.[26]

The combination of the components and their variable fine-tuned concentrations in biphasic IVM sustained cumulus-oocyte dynamic communication and led to in vitro meiotic and developmental competence acquisition of human oocytes from the 2–6 mm follicle class, which would otherwise have poor developmental outcomes.[21,24,25]

Further optimization of the CAPA culture medium with the potential addition of components such as cumulin, midkine, melatonin, or antioxidants may continue increasing the efficacy of the CAPA-IVM system, as shown already in animal models, but needs to be researched in humans.[62–68] Recent work from Cava-Cami[69] on human COCs from hyperresponsive patients demonstrated subcellular effects in cumulus and oocyte after supplementing the COC with pro-cumulin.

6.3 | Significance of a prolonged prematuration incubation in biphasic IVM culture systems

Currently, compared to conventional IVF, the standard IVM protocol requires one additional day of oocyte culture in the laboratory, whereas the biphasic IVM protocols, which include a prematuration step, require two additional days of oocyte culture.

The rationale behind the development of biphasic IVM approaches is that despite being almost fully grown, oocytes retrieved from underdeveloped (mostly 2–10 mm) antral follicles are still transcriptionally active and acquiring/rearranging their cellular machinery (cytoplasmic maturation) needed to support the first stages of embryo development.[31,32,34] These cytoplasmic processes are normally taking place in vivo during the several days of a follicular phase before natural ovulation. In vitro, prior to being exposed to a meiotic stimulus, there is a requirement to induce these cytoplasmic and nuclear changes by providing appropriate culture conditions. Pre-IVM aims to provide an environment where oocytes are intentionally kept meiotically arrested and where oocyte–cumulus crosstalk (via TZP and GJC) is uninterrupted so they can continue to develop in vitro.

Several protocols and time periods using prematuration have been reported, where oocytes have acquired partly or fully nuclear and cytoplasmic maturation in vitro (discussed further in the next section). When using cAMP modulators in prematuration culture systems, culture times as minimal as 1 h (initial models where cAMP modulators were present during collection of COCs), and up to ~24 h, have been studied (Reviewed in Gilchrist et al., 2024).[40] Recent studies in bovine and sheep models showed that a prolonged culture (known as "long in vitro oocyte culture") from up to 5 days was able to sustain the development of COCs derived from early antral follicles.[4,70] In the mouse model, oocytes have been successfully cultured in prematuration medium, particularly employing CNP as a meiotic inhibitor (CNP-mediated pre-IVM), for periods from 2 h to 48 h (reviewed in Gilchrist et al., 2024[40]). A temporally dependent improvement in oocyte developmental competence has been reported in the mouse and bovine model after using biphasic IVM in the presence of cAMP[60,71] or CNP.[52] Furthermore, an inverse relationship between the length of prematuration and the developmental status (follicle size and FSH-priming dose or duration) of COCs at the moment of retrieval has been hypothesized.[52,72] This hypothesis is mainly supported by findings in animal models but also from experience in humans using biphasic IVM.[24] In mouse, a prolonged CNP-mediated pre-IVM culture of 48 h successfully allowed oocytes originating from the smallest follicles—deriving from unstimulated mice—to acquire nuclear and cytoplasmic maturation, resulting in higher rates of blastocyst development compared to directly applying standard IVM.[47] A more recent study from Zhao et al.,[52] in mildly (23 h post-PMSG) stimulated mice, CNP-mediated pre-IVM culture of 24 h enhanced oocyte developmental competence (in terms of blastocyst and hatching rates), more efficiently than shorter pre-IVM intervals. Interestingly, in mice that received full (46–48 h post-PMSG) stimulation, an improvement in blastocyst development and pregnancy rates was already evident following a 2 h, rather than a 24 h, CNP-mediated pre-IVM culture period compared to control conditions (without pre-IVM).[53]

Prematuration culture systems for human oocytes have also succeeded in maintaining oocyte meiotic arrest and TZPs and/or GJC, but initially for not longer than 6 h.[73,74] In this initial work with human oocytes by Nogueira et al.,[73,75] pharmacological compounds (small-molecule competitive inhibitors of PDE3) had strong biological activity but did not succeed in keeping the oocyte and cumulus tightly connected. As a result, the embryos obtained after this kind of induced arrest were not of better morphological appearance. In contrast, using CNP in pre-IVM, human oocytes cultured for 24 h gained developmental potential and were able to maintain cumulus–oocyte TZPs[24] for up to 46 h.

In human oocytes retrieved from small antral follicles from PCOS patients stimulated for 2–3 days with hMG, 24 h and 46 h of CNP-mediated pre-IVM culture (CAPA-IVM system) efficiently prevented spontaneous meiotic resumption and retrained cumulus–oocyte TZP. Nonetheless, no gain in maturation rates, oocyte quality, and blastocyst yields was evidenced when the capacitation period was extended for COC from the same follicle class (2–9 mm), from 24 h to 46 h.[24] Recent reports from Garcia Barros et al.[4] and Ebrahimi et al.[70] on COCs derived from unstimulated animals (bovine, ovine) support the hypothesis that developmental competence can be successfully promoted in vitro in oocytes from very small antral follicles (0.5–2 mm) after using a more elaborate extended cAMP-mediated

pre-IVM protocol of 5 days. This work has implications for the future development of human pre-IVM culture systems.

6.4 | In vitro connectivity between cumulus cells and oocytes during CAPA-IVM determines embryo quality after IVF/ICSI

During follicle development, oocytes depend on differentiated cumulus cells, which are highly specialized somatic cells that provide them with nutrients and regulatory signals needed to promote oocyte growth and the acquisition of nuclear and cytoplasmic competence (reviewed in[76]). However, oocytes greatly influence granulosa cell functions such as proliferation, differentiation, and regulating the metabolic activity of cumulus cells (amino acid uptake, glycolysis, and cholesterol biosynthesis), which indeed is recognized as an important mutual cooperativity within the COC.[77–79] Based on this, it is essential to emphasize that, as an initial condition for CAPA-IVM to properly support the acquisition of oocyte nuclear and cytoplasmic maturation, the aspiration procedure should be nondisruptive for cumulus–oocyte connections. As mentioned earlier, CNP is a key component of the capacitation culture medium. The generation of cGMP by cumulus cells via the CNP/NPR2 system and its diffusion into the oocyte via gap junctions is crucial to preventing oocyte meiotic resumption.[49,54] Currently, the biphasic CNP-mediated prematuration step, especially the CAPA-IVM system, has been shown to improve oocyte quality and subsequent embryology (Table 2) and clinical outcomes (Table 3) (reviewed in[40,80]). A crucial step for these improvements was made possible by successfully maintaining COC integrity and supporting oocyte–cumulus cell communication as revealed at the cellular level, by maintaining TZP and GJC in mouse, bovine, and human (reviewed in[40]). Figure 7 shows the preservation of TZP integrity throughout CAPA culture in human COCs. Furthermore, evidence of oocyte–cumulus interactions affecting cumulus cell function has been shown by Zhao et al.[52] and Gong et al.,[81] reporting an increase in cumulus cell proliferation and expansion, and changes in cumulus cell metabolism in a mouse model.

Inevitably, although at a low incidence, there is a fraction of COCs that are only partially surrounded by cumulus cells at retrieval (Figure 8). An initial study using CAPA showed that both after 24 h and 46 h pre-IVM CAPA culture, fully enclosed oocytes (surrounded by cumulus cells) displayed 100% meiotic arrest, whereas in oocytes (a minority) that were only partially surrounded by cumulus layers, meiotic arrest at 24 h and 46 h was maintained less efficiently: at ~83% and 75%, respectively.[24] Furthermore, after undergoing the IVM step, partially cumulus-connected oocytes end up being most often totally disconnected.[25,26] Rationally, the poorly surrounded oocytes may have insufficient cGMP to firmly maintain meiotic arrest throughout the pre-IVM period and are prone to being released from the cumulus–oocyte complex during the IVM step.[25,81] A recent publication showed that in spite of this, maturation rates of both types

TABLE 2 In vitro maturation and embryology outcomes in CAPA-IVM and standard IVM (RCT): CAPA-IVM produced significantly higher maturation rates and yielded more frozen embryos per patient than Standard IVM.

Oocyte developmental outcomes	CAPA-IVM ($N=40$)	Standard IVM ($N=40$)	Between-group difference (95% CI)[a]	p Value[b]
Number of COCs	17.5 [11.0, 23.0]	16.5 [9.8, 21.0]	1 (−3, 7)	0.39
% Maturation (MII)	63.6 [55.0, 75.0]	49.0 [35.9, 62.1]	14.6 (5.5, 24)	<0.001
% Pronuclear stage per ICSI	84.0 [72.9, 100.0]	84.50 [72.0, 100.0]	−0.5 (−10.7, 11.9)	0.80
% Grade 1 or 2 embryos per pronuclear stage	37.5 [23.8, 50.0]	35.40 [16.4, 50.0]	2.1 (−11.5, 14.6)	0.60
% Grade 1 or 2 embryos per metaphase II	30.0 [13.9, 43.3]	26.80 [14.3, 40.7]	3.2 (−8.6, 16.2)	0.70
% Grade 1 or 2 embryos per COC	18.9 [8.5, 26.9]	12.7 [7.3, 20.4]	6.2 (−1.5, 12.4)	0.11
No embryo, *n* (%)	1 (25)	1 (25)	–	–
Frozen embryos remaining after first ET, *n*	2.5 ± 2.5	1.3 ± 1.9		0.02

Note: Values are median [interquartile range], number of patients (%), mean ± standard deviation, or difference (95% confidence interval). Original table from Vuong et al. (2020).

Abbreviations: CAPA, capacitation culture; CI, confidence interval; COC, cumulus–oocyte complex; EQ1, day 3 embryo quality grade 1; EQ2, day 3 embryo quality grade 2; ICSI, intracytoplasmic sperm injection; IQR, interquartile range; IVM, in vitro maturation.

[a]Bootstrapping and resampling 1000 times.

[b]Wilcoxon rank sum test, *p*-value.

TABLE 3 RCTs from CAPA-IVM cycles and their principal clinical outcomes to date.

Study	Patient type	AMH mean (ng/mL)	Stimulation (mean IU FSH/patient)	Cycles (*n*)	COC (*n*)	MII (%)	GQED3/COC (%)	Embryos transferred (mean)	LBR (%)
Sanchez et al. (2017)	PCOS	12.9	680	15	117	70	43	NA	Safety study
Sanchez et al. (2019)	PCOS	10.4	450	20	305	62	24	NA	Safety study
Vuong et al. (2020b)	PCOS		379	40	700	64	19	2	47
Vuong et al. (2020a)	PCOS	8.5	373	268	3806	64	21	1.9	35
Vuong et al. (2021)	PCOS	–	300	40	732	67	23	2	60
Akin et al. (2021)	PCOS	10.5	300	30	555	67	20	1.9	47
Vuong et al. (2024)	PCOS	9.36	300	60	798	65	NA	1.2	31.7
Vuong et al. (2024)	PCOS	10.7	0	60	786	62	NA	1.1	38.3
Kirillova et al. (2021)	Gynecological malignancies		0	10	105	56	NA	NA	NA
Total in studies treated with CAPA-IVM				543	7904	62%–70%			31%–60%

Abbreviations: AMH, antimüllerian hormone; CAPA-IVM, capacitation IVM; COC, cumulus–oocyte complex; GQED3, good quality embryo on Day 3; LBR, live birth rate; MII, metaphase II; PCOS, polycystic ovary syndrome.

of COC morphologies were similar. However, embryo formation from oocytes showing a pattern of release from the COC was significantly reduced after the IVM step.[25,82]

Interestingly, in small antral follicles <3 mm, deriving from ovarian medulla in fertility preservation programs, a high and positive correlation between oocyte maturation rates and the size of cumulus cell mass has been found. Unsurprisingly, oocytes surrounded by a large mass of cumulus cells were more prone to mature compared to oocytes enclosed in small cumulus complexes or naked oocytes.[65] Likewise, the COC size was significantly and positively associated with the MII rate and oocyte diameter.[32]

Overall, former studies highlight the crucial role of the somatic cell compartment for human oocyte maturation strategies. A rescue strategy to compensate for the diminished number of somatic cells in partially surrounded, almost naked, or small-sized COCs at retrieval is promising,[83] demonstrating that in the mouse model, partially denuded COCs can reconnect and reestablish a fully enclosed COC structure, similar in morphology and functionality to control intact COCs during CAPA pre-IVM culture. Considerably, more partially denuded COCs were restored after 4 days compared to 2 days in CAPA culture. The reconnected COCs likely reestablish a functional TZP connection, as no significant differences in meiotic and developmental competence were shown between reconnected and control intact COCs. Later findings were recently confirmed by experiments from Morahaku's team.[84] Recent data on gene expression in human oocytes and cumulus cells from PCOS patients revealed that the addition of pro-cumulin to CAPA-IVM culture medium helps in the rescue of partially denuded COCs.[69] Despite the potential possibilities of rescuing COC function in vitro, it is of fundamental importance to adjust the COC retrieval procedure by matching the suction strength of the pump to the needle diameter and to advise a gentle follicle curettage.

7 | CELLULAR MARKERS OF NUCLEAR AND DEVELOPMENTAL COMPETENCE OF CAPA-CULTURED OOCYTES

Most monophasic IVM systems have focused on increasing the number of oocytes reaching maturation (MII stage); however, by removing COCs from the ovarian follicle and placing them in maturation medium, a disconnection between the somatic compartment (cumulus cells) and the oocyte occurs, interrupting the ongoing metabolic, molecular, and cellular signaling systems, leaving the oocyte's cytoplasm in an underdeveloped state. On the contrary, biphasic IVM systems (i.e., CAPA-IVM) have deliberately dealt with a more complex issue: the synchronization of the oocyte's nuclear and developmental competence prior to triggering meiotic resumption.

Being aware that an oocyte's meiotic competence is set before developmental competence, a prematuration phase (in which meiosis is prevented) was thought to provide the appropriate conditions for the oocytes to reach a developmental status that will ultimately

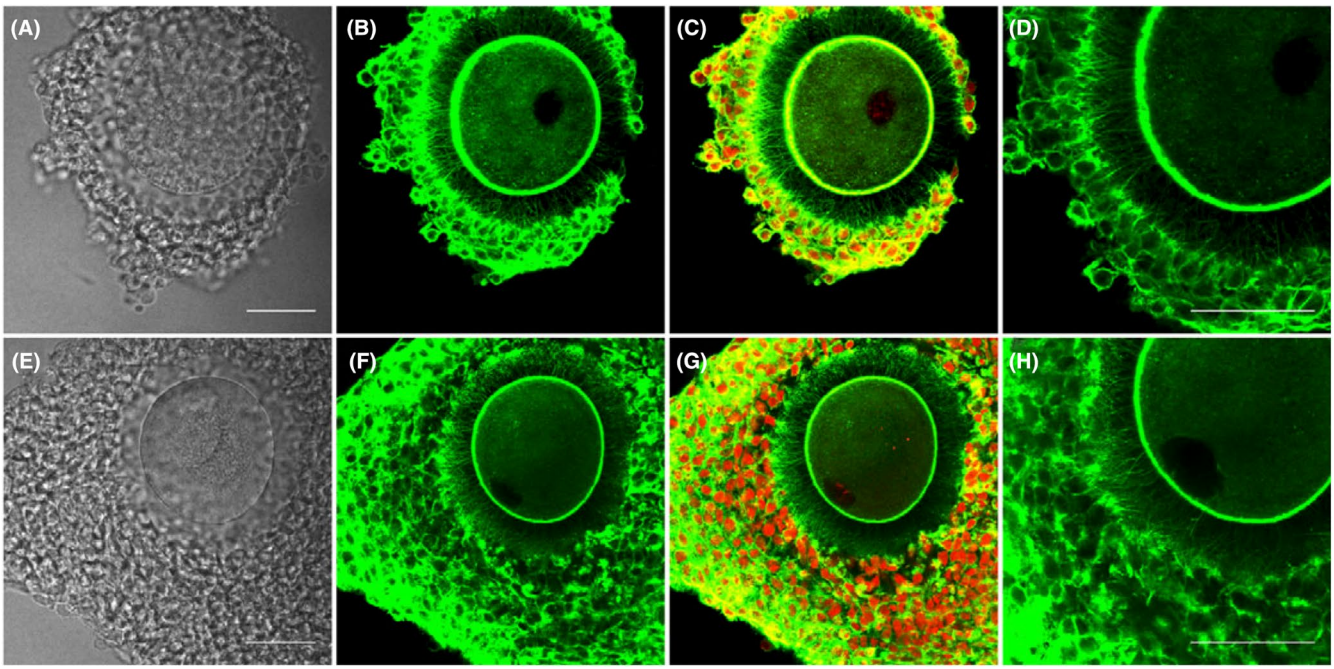


FIGURE 7 Preservation of cumulus-oocyte TZP after extended CAPA culture time. Immunofluorescent labeling of human COCs indicates maintenance of TZPs (green) integrity after 24 h (A–D, $n = 8$) and 46 h CAPA culture (E–H, $n = 10$). F-actin staining (green) shows vertically oriented TZPs on the tangential plane at the oocyte–cumulus cell interface (B, F). Cumulus cell nuclei and oocyte GV (ethidium homodimer-2, red) are shown in the merge (C, G). Original magnification, ×40, zoom 1.5. Scale bar: 50 µm. Magnified TZPs details are depicted in panels (D) and (H). Original magnification ×40, zoom 2.5. Scale bar: 50 µm. GV, germinal vesicle. Original figure from Sánchez et al. (2017). CAPA, capacitation; COC, cumulus–oocyte complex; TZP, transzonal projections.

produce good quality embryos. Acknowledging this is of relevance, since the analysis of oocytes subjected to a standard IVM protocol indicated that they are of an "unripe nature" or are less developed[34] (see Figures 3 and 4).

The CAPA-IVM system was conceived on the premise that oocytes derived from small antral follicles are in the process of acquiring the competencies to undergo maturation, fertilization, embryo development, and offspring production. One such process is the synthesis and storage of transcripts that are needed in the early stages of embryo development, prior to embryonic genome activation. During oogenesis, oocytes are initially transcriptionally active; however, transcription decreases and then eventually ceases when the oocytes approach the preovulatory stage. Transcriptional activity is related to chromatin organization in the oocyte nucleus. A more disperse/noncondensed chromatin is accessible to the transcription factors, while a more compact/condensed chromatin reflects cessation of transcriptional activity. Germinal vesicle (GV) stage oocytes that undergo IVM have been shown to have different degrees of chromatin condensation, both before and after IVM.[34]

In mice and rats, a condensed chromatin status together with the eccentric position of the germinal vesicle has been positively correlated with an oocyte's developmental competence.[85] It is unknown whether this phenomenon is replicated in humans; however, due to the combined toxicity of the dye and UV light exposure, such a study cannot be performed with human eggs.

At the molecular level, among other factors, Oct-4 has been suggested as a key regulator in the acquisition of an oocyte's developmental competence. For instance, it has been shown that Oct-4 expression (and Oct-4-dependent regulated genes) is dysregulated in MII oocytes derived from oocytes with noncondensed chromatin (in comparison to MII derived from condensed chromatin oocytes).[86]

Similarly, being aware that meiosis is an energy-demanding process, the pattern of mitochondria distribution changes prior to (and during) meiotic reinitiation.[34,87] It is not unexpected that altered mitochondria load, distribution, and quality will impact oocyte competence.[81]

Under CAPA-IVM, the pre-IVM step facilitates the transition from a dispersed to condensed oocyte chromatin configuration.[24,47] This means that while maintaining meiotic arrest, the capacitation culture allows for a time window in which more oocytes achieve the status of transcriptional silencing, instead of transitioning from uncondensed chromatin to chromosomes, which abruptly stops RNA transcription and accumulation (like in monophasic IVM) (Table 4).

The capacitation culture also preserves TZPs and gap-junctional communication between the oocyte and cumulus cells[24,47,52,81] (Figure 7). Such communication has been reported to favor the proliferation of cumulus cells, energy metabolism, and the appropriate expression of genes related to cumulus expansion and extracellular matrix formation.[52] Furthermore, goat oocytes cultured in CAPA regulate, in a dynamic way, the lipid content during culture and showed increased expression of *ATGL* (adipose triglyceride lipase) and *PLIN2* (perilipin 2) genes, involved in lipolysis and lipogenesis, respectively, which might be likely

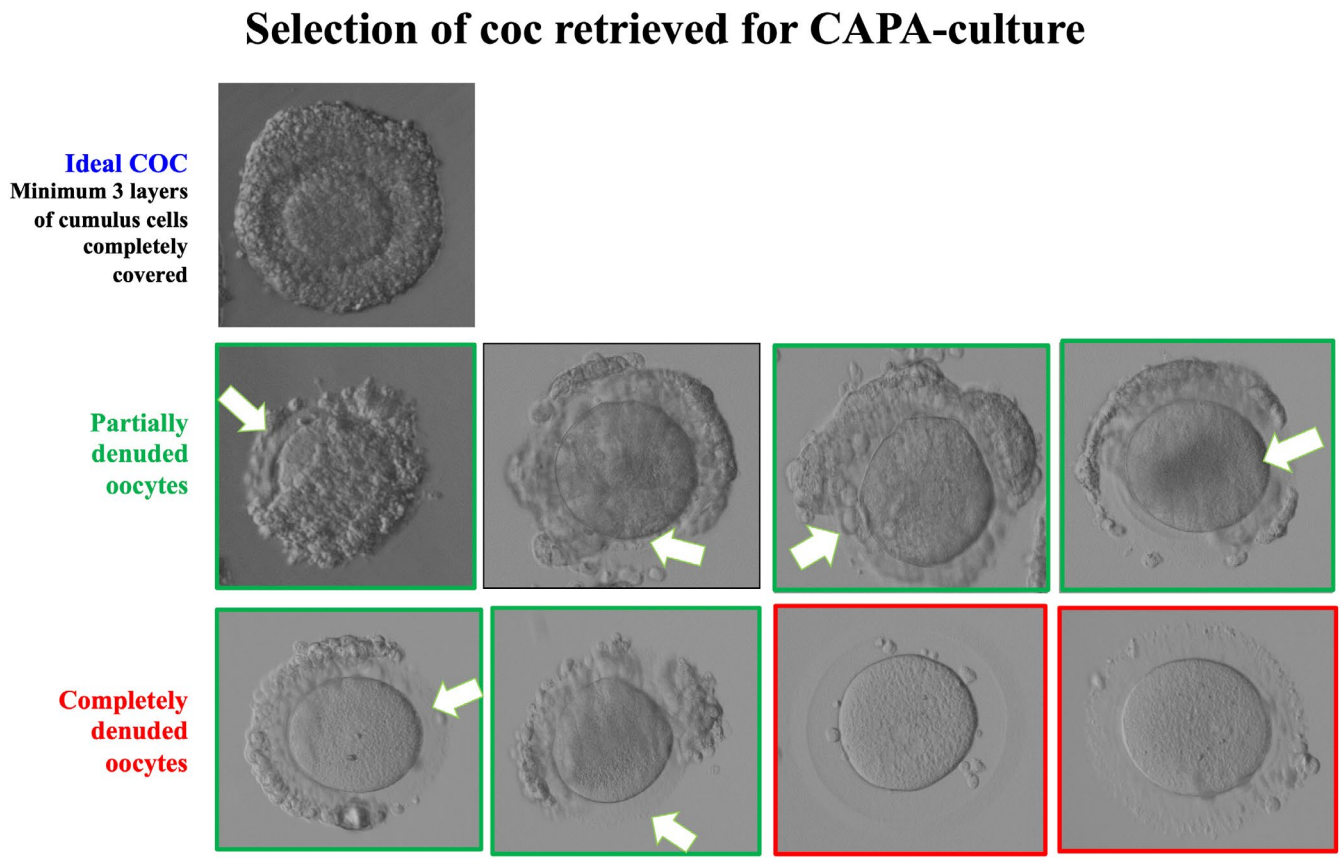


FIGURE 8 Morphological characteristics of COCs retrieved from IVM cycles prior to be subjected to CAPA-IVM culture. The first row shows the ideal morphology of COCs to be put in culture. Integrity is understood as cumulus cells firmly attached and completely surrounding the oocyte. In the second row, oocytes are partially denuded from their cumulus; at the white arrows, the cumulus cells are lacking. Denudation can be at variable degrees. In the third row, the two left oocytes are almost naked, and the two at the right fully naked. CAPA-IVM, capacitation in vitro maturation; COC, cumulus–oocyte complex.

related to the maintenance of lipid stocks necessary to support embryo development.[88]

In the second phase of CAPA-IVM (the IVM phase), the effects of the first phase are notable. The gap junctions preserved throughout the capacitation culture are maintained for some hours even after triggering meiosis in vitro.[52] CAPA-IVM promoted better cumulus expansion[52,81] and promoted antioxidant gene expression.[81] Furthermore, it boosted cytoplasmic quality by reducing reactive oxygen species (ROS) levels, improving mitochondrial function, preserving spindle integrity, and maintaining a normal CG distribution.[81] Oocytes exposed to capacitation culture are more synchronized to reinitiate meiosis. They resume meiosis faster, and the maturation rate is enhanced. More importantly, the improvements observed with the biphasic IVM system are finally translated into better oocyte and embryo quality.[21,24,25,47,52,88]

8 | CLINICAL INDICATIONS FOR CAPA-IVM

Although IVM is being increasingly investigated as an infertility treatment option in women with PCOS who have an indication for ART,[10,20,45,89,90] it is not currently part of first-line strategies to manage infertility.

Live birth rates in PCOS women treated by ovulation induction with letrozole are consistently higher than rates in those treated with clomiphene citrate,[91,92] leading to recommendations for this as first-line ovulation induction therapy in women with PCOS.[93] The conclusion of a multinational RCT on ovulation induction was that FSH may be a more appropriate first-line treatment for infertile women with PCOS.[94]

Data suggest that both IVM and OI-IUI are safe treatment options for infertile couples where the female partner has PCOS and there is no or only a minor male factor in the couples' etiology of infertility. Cumulative live birth rates from CAPA-IVM trials were greater (up to 44%) compared with published data on OI-IUI using letrozole or clomiphene citrate in a comparative population (retrospective data on file of MyDuc Center). Prospective studies should provide support to confirm their comparative effectiveness for earlier use of CAPA-IVM or OI-IUI in patients with PCOS, to evaluate the costs and other impacts of first-line use of CAPA or OI-IUI in this patient population.[95,96]

The current pioneering experience at the MyDuc clinic in Vietnam over the last 6 years with CAPA-IVM shows that patients

TABLE 4 Studies reporting on morphological and functional oocyte quality parameters in different species comparing CAPA-IVM with standard IVM.

Features and processes needed for and involved in gaining cytoplasmic maturity	Detailed description	Studies using CAPA and CAPA-IVM
During pre-IVM (Capa phase)	*Compared to STD IVM*	
Chromatin remodeling	Promotes chromatin transition to condensed stage	Mouse,[47] Human[24]
Preservation of oocyte-cumulus communication	Maintenance of TZP and Gap junctions	Mouse,[47,52,81] Human[24]
Oocyte growth	Increase in oocyte diameter	Mouse,[47] Human[25]
Proliferation of CC	Increase in CC DNA content	Mouse[52]
Energy metabolism	Increase in CC ATP and ADP content	Mouse[52]
Regulation of lipid content within the oocyte	Decreased fluorescent intensity of Nile red. Upregulation of *ATGL* and *PLIN2*	Goat[88]
Post-IVM		
Cumulus expansion	Increased mRNA expression of *Has2* and *Tnfaip6* at 6 h of IVM	Mouse[52,81]
ROS levels in mature oocytes and expression of antioxidant genes	Lower ROS levels and Increased expression of *Sod2* and *Sirt1* and *Egfr*	Mouse[81]
Mitochondrial distribution and MMP	Lower % of oocytes with an abnormal mitochondrial distribution (similar to control OS group)	Mouse[81]
Spindle/chromosome structure and CG distribution in mature oocytes	Lower % of oocytes with an abnormal spindle and chromosome structures	Mouse[81]
Intracellular and mitochondrial Ca^{2+} levels	Maintenance of intracellular Ca_2 in the oocyte	Mouse[81]
Enhanced maturation potential	Higher proportion of oocytes resuming meiosis and reaching the MII stage	Mouse,[47,52,81] Human,[24,25] Goat[88]
Embryo development and quality	Increased embryo quality and/or rate of embryo development	Mouse,[47,52] Human,[24,25] Goat[88]

Abbreviations: *ATGL*, adipose triglyceride lipase; CAPA-IVM, capacitation IVM; CC, cumulus cells; *Egfr*, EGF receptor; IVM, In vitro oocyte maturation; MMP, mitochondrial membrane potential; *PLIN2*, perilipin 2; Pre-IVM, Prematuration in vitro maturation; ROS, reactive oxygen species; *Sirt1*, Sirtuin 1; *Sod2*, superoxide dismutase 2; TZP, transzonal projections.

with PCOS presenting for infertility treatment are provided with information about the first-line therapy treatment options available. For their first-line therapy, some couples choose to have ovulation induction combined with IUI, whereas others choose IVM.

IVM is an alternative assisted reproductive technology that has been consistently shown to eliminate the risk of OHSS.[8,97,98] The suitability of IVM as an alternative to IVF in patients with PCOS has been demonstrated in a consistent number of studies.[10,20,45,89,90]

The use of IVM for patients with resistant ovaries (due to a defect in the FSH receptor), who after multiple IVF attempts fail to become pregnant, has been published.[99–101] The CAPA-IVM system implements factors (targeting the EGF receptor) in the media, which overcomes in vitro this rare defect that these patients experience.

In the oncofertility clinic, where COCs are isolated during the dissection of cortex from medulla, the CAPA-IVM system has been shown to be capable of generating blastocysts from COCs dissected from ovarian tissue in patients with cancers of the reproductive tract.[23] A recent report from Kashutina et al.[102] stressed that the CAPA-IVM media contributed to a significant decrease in large polar body formation compared to standard (monophasic) IVM media.

9 | SAFETY ASPECTS

There has been a concern about the safety of embryos produced after IVM of human oocytes and the long-term safety of children conceived after IVM. This reasonable concern mainly relates to the possible increased risk of chromosomal abnormalities and the potential epigenetic impact (i.e., increased risks of epigenetic alterations and imprinting defects) of in vitro manipulation of human oocytes during prolonged in vitro culture.

9.1 | Human embryo genetic and epigenetic analysis

So far, studies of IVM oocytes and blastocysts produced after IVM are reassuring. Kuhtz et al.[103] could not detect major epigenetic alterations in oocytes cultured after conventional IVM. IVM did not affect the quality of embryos nor the aneuploidy rate of embryos.[104]

Analysis of day 3 embryos produced after IBMX-mediated pre-IVM followed by a short biphasic IVM approach was not different in the rate and type of chromosomal abnormality compared to those produced from in vivo matured oocytes.[105]

In relation to the CAPA-IVM system, an initial analysis of 20 blastocysts obtained from 10 patients, generated in a pre-IVM system using CNP, did not display increased genetic aberrations, as shown by NGS sequencing for aneuploidy screening.[24]

Further collected safety data concentrated on epigenetic risks. Epigenetic effects are best studied in blastocysts if the safety of an ART is to be evaluated. Using the CAPA with CNP and IVM protocol described in Sanchez et al.,[24] blastocysts of CAPA-IVM and blastocysts of standard controlled ovarian stimulation (COS) cycles in PCOS patients were compared for DNA methylation patterns and RNA expression analysis. The results indicate that CAPA with CNP and IVM did not alter DNA methylation levels at germline differentially methylated regions (gDMR). Neither did CAPA-IVM alter expression levels of imprinted genes OR EMI. Until today, there is no evidence that CAPA-IVM using CNP as a meiotic inhibitor followed by IVM using AREG poses increased risks to methylation maintenance through preimplantation development (compared to blastocysts from standard COS).[106]

9.2 | Follow-up of children

Outcome of pregnancy and the 2-year follow-up of children born from CAPA-IVM are available upon request.[107]

Pre-IVM has principally been applied in Vietnam to women with PCOS or a high AFC, who are at increased risk of exaggerated ovarian response, OHSS, ovarian torsion, and related risks of very high concentrations of steroid hormones after ovarian hyperstimulation. So far, no cases of OHSS have been recorded in any of the CAPA-IVM trials. No significant differences have been noted between COS and CAPA-IVM with respect to the occurrence of pregnancy complications, obstetric and perinatal complications, preterm delivery, birth weight, and neonatal complications.[45]

To assess the development of children, the authors prospectively used, in a first study, the screening tools ASQ-3 and Red Flags questionnaire at three time points (ages 6, 12, and 24 months). There were no significant differences in ASQ-3 scores between the CAPA-IVM and IVF groups. Children from both groups showed normal growth with respect to body weight over the first 24 months.[107] In a second prospective cohort study in Vietnam, children born after CAPA-IVM were propensity score-matched with those born after natural conception and followed up to a maximum of 24 months.[108] The mean age of children at the end of follow-up was 15 months. The proportions of babies with any abnormal ASQ-3 score or with a developmental red flag were not statistically different between children from the CAPA-IVM group and babies conceived naturally.[108] The latter study further supports the lack of evidence of any negative effect of CAPA-IVM on childhood physical and mental development.

10 | CONCLUSIONS

The worldwide data in humans produced with the CAPA-IVM system, which were initially developed from animal experiments and from human oocyte donors at the Follicle Biology Laboratory from the Free University Brussels (VUB), Belgium, demonstrate that the system is transferable to other embryology labs. The clinical applications and clinical validation studies with CAPA-IVM in human ART have been developed mainly through long-standing rigorous collaborations between VUB (Belgium) and MyDuc Hospital in Ho Chi Minh City (Vietnam), the Horac Group in Osaka (Japan), and the University of New South Wales Sydney (Australia). As of today, more than 1000 healthy children have been born using CAPA-IVM.

The CAPA-IVM system has been validated clinically in ovulatory and anovulatory infertile PCOS patients, high responder patients (AMH >5 ng/mL) and in patients with gonadotrophin receptor mutations. In onco-fertility patients, CAPA was used to mature the COC dissected from medullary ovarian tissue (ex vivo IVM or OTO-IVM) (Kulakov Institute, Moscow).

The technology of prolonged culturing of the immature oocyte in connection to cumulus also opens a new field: application of oocyte therapeutics. The potential to mature the oocyte without prior burdensome hormonal pretreatments unwraps new exciting possibilities for fertility preservation in disease and for age banking.

Finally, the potential to obtain COCs without having to stimulate the ovary could reduce the threshold to treatment, the burden for the patient, and the cost of treatment to the society.

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CONFLICT OF INTEREST STATEMENT

JS, FS, and SR are co-inventors on a granted patent on a method for in vitro oocyte maturation, CAPA-IVM (WO2016094970). JS reports having shares in Lavima Fertility Inc., a Spinoff company of the Vrije Universiteit Brussel (VUB), where he has a role as a Corporate Scientific Officer (CSO). JS has received consulting fees from Lavima Fertility Inc. FS and SR report consulting fees from Lavima Fertility Inc., received via their Institution. SR reported to receive a research grant from Lavima Fertility Inc. received by his Institution. HVR and EA have no conflicts of interest to declare. RBG reports grants and fellowships from the National Health and Medical Research Council (NHMRC); a research gift from Open Philanthropy; and consulting fees from City Fertility CHA Global, received by his Institution. RBG has received speaker fees from Ferring, Cook Medical, and Gedeon Richter, and conference travel fees for an educational event from CooperSurgical. TMH has reported receiving grants from Lavima Fertility Inc., and he is a member of IVM SIG, ASRM. LNV has

received speaker fees and conference travel fees from Merck, Merck Sharpe & Dohme, and Ferring. MY is an Editorial Board member of Reproductive Medicine and Biology and a co-author of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

ORCID

Flor Sánchez https://orcid.org/0000-0003-1762-6184
Yoshiharu Morimoto https://orcid.org/0000-0003-2834-2726

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