Laboratory and embryological aspects of hCG-primed in vitro maturation cycles for patients with polycystic ovaries

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BACKGROUND: In this review, recent advances in the laboratory as well as embryological aspects of hCG priming in vitro maturation (IVM) cycles are described.

METHODS: This report is based on publications from literature searches and the authors’ experience.

RESULTS: In IVM cycles, priming with hCG permits the recovery of a certain number of oocytes with an expanding/dispersed cumulus pattern which facilitates its identification within follicular fluid as compared with non-primed IVM cycles. The immature oocytes with dispersed cumulus cells (CC) at collection have high IVM rates and embryo development potentials. Moreover, a few in vivo matured oocytes can be obtained, and these have produced good quality embryos. hCG can be given to patients when a dominant follicle reaches 10–12 mm to avoid negative effects on the sibling immature oocytes. ICSI should be performed at least 1 h after the first polar body extrusion. Embryo transfer time depends on quantity and quality of the embryos produced after IVM. Compared with slow freezing, vitrification is a more efficient method for freezing the embryos produced from IVM.

CONCLUSIONS: The data from the meta-analyses suggests that the effect on clinical outcome of gonadotrophin priming of IVM still needs to be studied. In order to improve the IVM programs, it is essential to define not only the clinical aspects but also the laboratory and embryological aspects.

Key words: hCG priming / immature oocyte / in vitro maturation / laboratory aspects / polycystic ovary syndrome
Introduction

In vitro fertilization (IVF) is known worldwide as a successful technique for the treatment of infertility. In order to obtain more oocytes and embryos in attempt to increase chances for pregnancy to occur, ovarian hyperstimulation is carried out by administration of exogenous gonadotrophins (Tan et al., 1992).

However, ovarian stimulation prior to the collection of mature oocytes carries the risk of ovarian hyperstimulation syndrome (OHSS) and none of the strategies used to predict and prevent this potentially life-threatening complication have been proven to eliminate the risk (Buckett et al., 2005). Therefore, the most reliable way to prevent OHSS is to avoid stimulating the ovaries by hormonal therapies (Buckett et al., 2005).

In vitro maturation (IVM) of oocytes was first suggested by Pincus and Enzmann (1935) and later by Edwards (1969). The first birth after IVM of immature oocytes occurred from oocytes collected during gynecological surgeries for oocyte donation (Chia et al., 1991), whereas IVM using a patient’s own oocytes was first reported as a treatment for women with polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS) in 1991 (Trounson et al., 1994). Many improvements in both clinical and laboratory aspects have been achieved in the last 18 years. According to our database, more than 1000 IVM babies have been reported worldwide until 2008 (R.C. Chian, personal communication).

Infertile women who have PCO form the main category of patients to whom IVM has been applied for the clinical purpose of avoiding OHSS caused by exogenous gonadotrophin stimulation (Table I, modified from Jurema and Nogueira, 2006). The second group is regularly cycling women who have normal ovaries with few antral follicles (Table II, modified from Jurema and Nogueira, 2006). Recently, the indications are widening to include various diagnoses of infertility, including IVM for over responders (Lim et al., 2002) and poor responders (Liu et al., 2003), for PGD (Ao et al., 2006), for oocyte donation (Holzer et al., 2007) and for fertility preservation (Rao et al., 2004; Holzer and Tan, 2005).

Since the efficiency of IVM techniques is suboptimal compared with that of controlled ovarian hyperstimulation (COH) cycles, some studies have tried to improve the quality and quantity of oocytes by in vivo stimulation with gonadotrophins, either with FSH in the early follicular or late luteal phases or with hCG before immature oocyte collection (Wynn et al., 1998; Chian et al., 2000; Sulkari et al., 2000).

Regarding FSH priming, it seems that there may be a beneficial effect on oocyte maturation in patients with regular cycles (Wynn et al., 1999). However, pregnancy rates per started cycle seem to be similar when data from comparative studies of FSH-primed versus non-primed cycles are pooled together (Fig. 1A: Mikkelsen et al., 1999; Fadini et al., 2009a). In the case of PCOS patients, Mikkelsen and Lindenberg (2001) reported a beneficial effect of FSH priming on the clinical outcome, whereas others found no significant differences (Trounson et al., 1998; Son et al., 2006).

Regarding hCG priming, Lim et al. (2009) recently reported high pregnancy and implantation rates (40.4 and 17.8%) after hCG priming in patients with regular cycles. However, Fadini et al. (2009a) reported that hCG priming alone has no beneficial effect on the clinical outcome (Table II). For PCOS patients, Chian et al. (2000) reported a higher maturation rate following hCG priming (10 000 IU) compared with non-priming. On the other hand, other studies did not demonstrate any significant difference (Chung et al., 2000; Soderstrom-Anttila et al., 2005). Nevertheless, there is a trend towards higher pregnancy rates with hCG priming in women with PCO even though the difference has not reached a statistical significance in a meta-analysis (Fig. 1B; Chian et al., 2000; Son et al., 2006).

Lin et al. (2003) found that no additional or beneficial effect of FSH stimulation in hCG-primed IVM cycles was observed in PCOS women. However, Fadini et al. (2009a) recently reported, after randomized prospective studies, a significantly higher clinical outcome of combined FSH-hCG priming in patients with regular cycles compared with FSH priming or hCG priming alone. Clearly, further research is still needed to confirm the benefits of priming with gonadotrophins in IVM cycles.

In fact, various clinical outcomes in hCG-primed IVM cycles for PCO or PCOS patients have been reported (Table I). In Table I, the pregnancy and implantation rates are described and both rates varied significantly among the authors (ex: 17–52% pregnancy and 6–27% implantation). Thus, priming with hCG in unstimulated ovaries would need to be optimized for utilization in human IVM programs.

It is important to find innovative ways to improve laboratory procedures as well as clinical aspects to make the use of immature oocytes more efficient. The clinical aspects of managing a patient for IVM cycles have been described previously in several papers (Tan et al., 2002; Papanikolaou et al., 2005; Rao and Tan, 2005; Jurema and Nogueira, 2006; Demirtas et al., 2008; Reinblatt and Buckett, 2008). However, there is a paucity of published data regarding the laboratory procedures on IVM of human oocytes.

In this review, we have summarized the laboratory and embryological aspects in hCG-primed IVM cycles for PCO or PCOS patients and as well as their related clinical significance, rather than focusing only on the clinical outcome of hCG priming. In addition, we compared these cycles to non- or FSH-primed IVM cycles.

Methods

This review is based on the electronic literature searches via PubMed for articles that were published in the English language. Combinations of the following keywords were used to identify relevant articles: ‘FSH priming’, ‘hCG priming’, ‘immature oocyte’, ‘IVM’ and ‘polycystic ovary syndrome’. The literature search was also supplemented with key publications that were known by the authors.

Oocyte identification

The follicular aspirate is collected in collection tubes containing 0.9% saline with 2 U heparin to prevent the formation of blood clots during retrieval. Multiple needle punctures are needed because lower aspiration pressure is used and bloody aspirates may block the thin needle lumen (Rao and Tan, 2005).

There are two ways to look for and to identify cumulus-oocyte complexes (COC) from follicular aspirates. First, the follicular aspirates are poured directly into a Petri dish and examined for COC under a microscope (Cha et al., 2000; Chian et al., 2000). The oocytes aspirated from IVM cycles have a small amount of cells compared with...
<table>
<thead>
<tr>
<th>Authors (year)</th>
<th>No. of cycles</th>
<th>No. of embryo transfer cycles</th>
<th>Gonadotrophin priming</th>
<th>Mean No. of oocytes retrieved</th>
<th>Culture time (h)</th>
<th>In vitro maturation rate (%)</th>
<th>Implantation rate (%)</th>
<th>Clinical pregnancy rate/embryo transfer (%)</th>
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<td>74.8</td>
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<td>70.7</td>
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<td>106</td>
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<td>34</td>
<td>Metformin + FSH + HCG</td>
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<td>48</td>
<td>76.7</td>
<td>15.3</td>
<td>38.2</td>
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<tr>
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<td>171</td>
<td>HCG</td>
<td>18.9</td>
<td>48</td>
<td>65.3</td>
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<td>67.1</td>
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<td>48</td>
<td>79.7</td>
<td>6.4 (ongoing)</td>
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</table>
those from COH cycles (Figs 2 and 3) and most likely will sink to the bottom of the tube. It is, therefore, better that the bottom portion of the tube holds a small volume of follicular aspirate to make the identification of oocytes easier. Especially in hCG- and combined FSH-hCG IVM cycles, the cumulus cells (CC) expansion (dispersal) of the oocytes caused by a high dosage of hCG facilitates detachment and expulsion of the COC mass from the follicle during the aspiration and thus makes oocyte collection easier. Furthermore, oocytes having a dispersed CC are easy to identify under a stereomicroscope similar to that in the standard IVF technique. In the combined FSH-hCG IVM cycles, there is an increased possibility of retrieving more oocytes with dispersed CC having similar aspects to those of conventional IVF.

The other method of identifying COCs is to use a cell strainer device made of a nylon mesh of 70-μm pores (Mikkelsen et al., 2000; Son et al., 2002a). The follicular aspirates in collection tubes are filtered with the cell strainer. After filtering, the collected aspirates can be washed with medium containing human serum albumin (HSA) and HEPES buffer to remove red blood cells and small cells. They are then transferred to a Petri dish to search for COC under a microscope. This method is mainly performed in non- or FSH-primed IVM cycles, since immature oocytes are the same color as the granulosa cells and are difficult to identify under the stereomicroscope (Fig. 3A).

Some IVF centers, such as our own, are utilizing both methods even in hCG-primed IVM cycles (Son et al., 2008a, b, c). The oocytes with a small amount of CC from follicular aspirates are harder to identify than oocytes with a dispersed CC directly under a stereomicroscope (Fig. 3B). Therefore, after identifying oocytes with dispersed CC under stereomicroscope, the remaining aspirates are filtered through a cell strainer to facilitate identification of those oocytes with a small amount of CC.

### Oocyte characteristics and their clinical significance

**Cumulus oocyte morphological patterns in relation to gonadotrophin priming**

COC morphological differences exist between oocytes collected from non-, FSH (hMG)-primed or from hCG-primed cycles (Son et al., 2006; Fig. 3). COC retrieved from non- and FSH(hMG)-primed ovaries have similar morphological patterns (Fig. 3A). Basically, collected oocytes from IVM cycles can be morphologically classified as having dispersed, compacted or sparse CC at the time of oocyte retrieval (Fig. 4). The oocytes with dispersed CC morphological pattern can be encountered only in IVM cycles where hCG has been administered (Figs 3B and 4A). The rate of collected oocytes with dispersed CC has been found to be 42.6%, 36 h after hCG priming (Son et al., 2006).

In the non-primed IVM cycles, the immature oocytes with multilayer CC have shown higher embryo developmental potential than oocytes with sparse CC even though there has been no difference in IVM and fertilization (Cobo et al., 1999; Russell, 1998).

In hCG-primed IVM cycles, Chian et al. (2000) reported a higher and faster IVM of oocytes than in the non-primed group in PCOS...
patients. Son et al. (2006) also confirmed that the IVM rate after Day 1 (24–30 h) of culture was significantly higher in Germinal Vesicle (GV)-stage oocytes obtained from hCG-primed IVM cycles (51.4%) than from non- (45%) or hMG-primed cycles (40%), although the total IVM rate (48–52 h) was similar (73 versus 74.8 versus 70.7%). In 2009, Fadini et al. (2009a) obtained similar results in IVM cycles for patients with a regular cycle.

In order to understand why oocyte IVM occurs faster in hCG-primed IVM cycles than in non-primed cycles, we analyzed the in vitro oocyte maturation from GV-stage oocytes based on COC morphology in hCG-primed IVM cycles in PCO patients. We found that oocytes with dispersed CC showed faster and higher IVM rates than oocytes with compacted CC or sparse CC (Fig. 5; Yang et al., 2005). Similar results were obtained by Nogueira et al. (2006) in immature oocytes obtained in COH cycles. Moreover, those oocytes that reach maturation on Day 1 can produce better quality embryos and more blastocysts than those reaching maturation on Day 2 in hCG-primed IVM cycles (Son et al., 2005a, 2008a). Furthermore, oocytes matured later (Day 2: 48–52 h) showed lower fertilization and cleavage rates as well as higher chromosomal abnormalities compared with fast-maturing oocytes (Son et al., 2005a, 2008a; Benkhalfa et al., 2009). Smitz et al. (2007) also showed that in FSH-primed IVM cycles oocytes matured in vitro for 30 h had lower aneuploidy rates than oocytes matured for 36 h compared with in vivo matured oocytes. Oocytes reaching the M-II stage most rapidly seemed the most competent to develop into blastocysts in un-primed IVM cycles (Barnes et al., 1995). Therefore, better pregnancy rates can be obtained in the IVM cycles that have transferred embryos derived from faster in vitro matured oocytes than from late ones in PCO patients (Son et al., 2008b).

In addition, it seems that a higher blastocyst rate (40%) results from immature oocytes with dispersed CC compared with oocytes of compacted (23.3%) or sparse CC (23.1%) pattern (Yang et al., 2005). Therefore, the fact that oocytes matured faster in vitro and exhibited better embryonic developmental potential in hCG-primed IVM cycles...
compared with those of non- or hMG-primed IVM cycles could be due to in vivo stimulation of the oocytes by a high dose of hCG (10,000 IU) leading to the development of oocytes with a dispersed CC at the time of retrieval.

In vivo matured oocytes in hCG-primed IVM cycles

Within those oocytes with a dispersed COC morphology at the time of collection, few of those have already resumed meiosis and/or extruded polar body (PB) (Son et al., 2002a, 2006, 2008a, b, c; Lim et al., 2007). We have reported that the number of good quality blastocysts produced from in vivo matured oocytes is significantly higher than that of in vitro matured oocytes in PCO patients (Son et al., 2005a). In addition, embryos developed from in vivo matured oocytes show better embryonic developmental quality at cleavage-stage than those of in vitro matured oocytes, resulting in higher clinical pregnancy rates in the cycles with in vivo matured oocytes compared with the cycles without in vivo matured oocytes in women who had regular cycles (Lim et al., 2007) or PCO (Son et al., 2008a). Interestingly, Fadini et al. (2009a) showed an additive effect of FSH on the number of in vivo matured oocytes retrieved in combined FSH-hCG IVM cycles in women with regular cycles.

The in vivo matured oocytes can sometimes be collected from small follicles measuring <10 mm at the time of retrieval and often more than one in vivo matured ovum can be retrieved in hCG-primed IVM cycles of PCO patients (Son et al., 2008c). We have found that the matured oocytes retrieved from small follicles (<10 mm) generated embryos of similar developmental potential to oocytes derived from larger follicles (≥10 mm), resulting in better pregnancy rates (Table III; Son et al., 2007a). In the last 3 years (2006–2008), we achieved clinical pregnancy and implantation rates of 49.1 and 18.2%, respectively, for PCO patients, who received more than one embryo derived from in vivo matured oocytes (Son et al., 2009a).

We reported (Son et al., 2006) that more than 90% of the patients had in vivo matured oocytes when the largest follicle size was ≥12 mm at retrieval. Up to now, from our experience, the lowest diameter of follicle aspirated that contained an M-II stage (i.e. the first PB extruded) oocyte was 6 mm and the highest number of M-II stage oocytes collected with only hCG priming in an IVM cycle was 18. However, further research is necessary to confirm the functional role of hCG priming on small follicles.

The morphology of M-II stage oocytes retrieved from small antral follicles in hCG-primed IVM cycles is different from that seen in mature oocytes retrieved from fully grown follicles in standard IVF (Fig. 6).

To evaluate oocyte maturity in hCG-primed or combined FSH-hCG IVM cycles, two techniques can be used; the sliding method or the

**Table III** Comparison of fertilization, cleavage and embryo development in in vivo matured oocytes collected from different follicular size of sibling follicles in hCG-primed IVM cycles of PCO patients (n = 28; Son et al., 2007a).

<table>
<thead>
<tr>
<th>Variables</th>
<th>&lt;10 mm</th>
<th>≥10 mm</th>
<th>P-value</th>
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<tr>
<td>No. of M-II oocytes retrieved</td>
<td>57</td>
<td>34</td>
<td>0.001</td>
</tr>
<tr>
<td>No. of zygotes (%)</td>
<td>44 (76.4)</td>
<td>28 (82.4)</td>
<td>NS</td>
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<td>No. of cleaved embryos (%)</td>
<td>43 (97.7)</td>
<td>27 (96.4)</td>
<td>NS</td>
</tr>
<tr>
<td>No. of good quality embryos on Day 3* (%)**</td>
<td>24 (54.5)</td>
<td>16 (57.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Six to 8 cells contained <20% anucleate fragments and exhibited no apparent morphological abnormalities.**The number of good quality embryos divided by number of zygotes. NS, non-significant.
The spreading method (Son et al., 2006; Fadini et al., 2009a). In the sliding method, the COC is allowed to slide slowly from one side to the other at the bottom of the Petri dish whereas being observed under the microscope. In the spreading method, follicular aspirates in the Petri dish are first removed with the remaining small amounts of fluid and then the identified COC are put into the Petri dish to allow them to spread so as to observe the oocyte cytoplasm under the dissecting microscope. If no GV is observed in the oocyte cytoplasm, CC are removed to observe maturity 1 h after collection. Generally, the oocytes with a more expanded corona radiata are collected from follicles of larger size (Fig. 7; Son et al., 2008a). However, although the oocytes may be retrieved from similar sized follicles and show similar COC morphology (dispersed CC), the nuclear maturity of oocytes can be different (Fig. 7a versus b, c versus d, e versus f). Also, different corona radiate appearances can be observed in the oocytes collected from different sizes of follicles, but the maturity of the oocytes can still be at the M-II stage (Fig. 7a versus c versus e, b versus d versus f). Therefore, it is important to clearly identify the in vivo matured oocytes in hCG-primed IVM cycles. If the embryologist overlooks M-II oocytes retrieved, the oocytes will be aged until removal of CC (24 h later). The developmental competence of such aged oocytes might therefore be reduced by prolonged arrest (Miao et al., 2009).

**Influence of hCG timing and dosage on oocyte developmental quality**

On the basis of these observations, the dispersed CC pattern at the time of aspiration in hCG-primed IVM cycles can be predictive of fast, high maturation rates, of the likely presence of some in vivo matured oocytes and of subsequent embryonic developmental competence. Therefore, it is very important to find ways to increase the number of oocytes with dispersed CC at the time of retrieval in hCG-primed IVM cycles.

Two methods can be considered to increase the number of oocytes with dispersed CC in hCG-primed IVM programs: a time-dependent and a dose-dependent response on hCG priming. The effect of a dose-effective response 36 h after hCG priming was studied on embryological aspects in IVM cycles of PCO patients (Gulekli et al., 2004). In that study, the authors concluded that there is no dose-dependent effect of hCG on in vitro oocyte maturation between 10 000 and 20 000 IU. However, the difference in CC appearance and number of in vivo matured oocytes was not mentioned. Further studies are therefore warranted.

Regarding the time-dependent effect of hCG in PCO patients, a higher number of oocytes with dispersed CC appeared in the 38 h group (64.3%) compared with that in the 35 h group (37.5%; Son et al., 2008b). Accordingly, we found that there was a higher number of oocytes matured in vivo and matured in vitro on Day 1 in the 38 h group compared with the 35 h group. In both groups (35 and 38 h post-hCG priming), the clinical pregnancy rate in cycles...
where embryos were derived from in vivo or/and Day 1 in vitro matured oocytes was higher than that where embryos were derived from oocytes matured later. Most of the cycles in the 38 h group (97.7%, 43/44) had transferred embryos derived from oocytes matured in vivo and/or in vitro on Day 1 compared with cycles in the 35 h group (68.4%, 52/76). Therefore, the trend of better pregnancy outcome in the 38 h group is attributed to an increase in the quantity of embryos produced from oocytes matured in vivo and matured faster in vitro (Son et al., 2008b).

Selection of the optimal day for oocyte retrieval

For the optimal timing of the IVM collection, the most common view shared by investigators is based on the presence of a dominant follicle (DF).

In the non-primed IVM cycles for women who had a regular cycle, some investigators reported that the immature oocytes retrieved from a cohort of small follicles could produce viable embryos in the cycles where the DF size is 12 mm (Mikkelsen et al., 2000), < 13 mm (Fadini et al., 2009b) or < 14 mm (Russell, 1998). However, other investigators believe it is detrimental and proposes canceling the cycle if the DF is over 10 mm (Cobo et al., 1999; Ge et al., 2008; Table IV). Cobo et al. (1999) found that once the selection of the leading follicle (>10 mm) has occurred, the blastocyst developmental potential of the remaining oocytes is impaired although the authors did not transfer those embryos. On the contrary, it has been reported that the rates of oocyte maturation, fertilization and early embryonic development of the sibling oocytes are not affected by the presence of a leading follicle (Mikkelsen et al., 2000; Fadini et al., 2009b; Table IV). Mikkelsen et al. (2000) showed similar clinical pregnancy rate between cycles with a DF of <12 and ≥12 mm. However, Fadini et al. (2009b) reported a lower pregnancy rate in the cycles with DF of >13 mm than other groups (Table IV). So, it is difficult to conclude from the literature whether or not the DF is affecting clinical outcome in non-primed IVM cycles.

In the hCG-primed IVM cycles, some investigators suggest that sibling immature oocytes exposed to any size of DF could contribute to the overall pregnancy success, although it was not properly evaluated whether IVM embryos could implant or not (Paulson et al., 1994; Thornton et al., 1998; Chian et al., 2004; Lim et al., 2007). In our study, although there was no difference in the embryological aspects of oocytes collected, lower clinical pregnancy and implantation rates were achieved in the group where oocytes were collected when the DF size was >14 mm compared with the group where the DF size was ≤14 mm (Table IV; Son et al., 2008c). In the group where the DF was >14 mm at collection, only one pregnancy was observed when only IVM embryos were transferred (5.9%, 1/17). Although our results show that sibling immature oocytes collected from IVM cycles with >14 mm of DF can be normally fertilized and used to increase the number of embryos available for transfer, the increase in number of embryos derived from immature oocytes cannot be efficiently translated into pregnancies.

In the Group where the DF was 10–14 mm at the time of collection, however, the clinical pregnancy rate was comparable between the cycles with transferred embryos generated from at least one in

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<tr>
<th>References</th>
<th>DF at collection (mm)</th>
<th>No. HCG priming</th>
<th>No. IVM M-II (%)</th>
<th>2 PN (%)</th>
<th>Cleavage (%)</th>
<th>Blastocyst (%)</th>
<th>CP (%)</th>
<th>IR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobo et al. (1999)</td>
<td>&lt;10</td>
<td>Normal</td>
<td>68</td>
<td>53.2</td>
<td>86.7</td>
<td>35.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mikkelsen et al. (2000)</td>
<td>&lt;10</td>
<td>None</td>
<td>44</td>
<td>37.2</td>
<td>70.4</td>
<td>26.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lim et al. (2007)</td>
<td>&lt;10</td>
<td>None</td>
<td>248</td>
<td>37.2</td>
<td>70.4</td>
<td>26.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Son et al. (2008a)</td>
<td>&lt;10</td>
<td>None</td>
<td>328</td>
<td>33.7</td>
<td>63.7</td>
<td>30.9</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mikkelsen et al. (2009b)</td>
<td>≥14</td>
<td>HCG</td>
<td>114</td>
<td>30.1</td>
<td>54.1</td>
<td>23.4</td>
<td>13.6</td>
<td>13.6</td>
</tr>
<tr>
<td>Son et al. (2008c)</td>
<td>≥15</td>
<td>PCO</td>
<td>67</td>
<td>33.3</td>
<td>60.3</td>
<td>25.2</td>
<td>13.6</td>
<td>13.6</td>
</tr>
</tbody>
</table>

DF: dominant follicle; CP, clinical pregnancy; IR, implantation rate; NA, non-available.
vivo matured oocyte (41.7%, 15/36) and with only IVM embryos (38.7%, 12/31). These data suggest that in order to increase the successful pregnancy rate of immature oocytes in hCG-primed IVM cycles, the best time for oocyte collection is when a DF reaches a diameter of 10–14 mm. If indeed oocyte collection is performed after hCG priming, it is important to know when the best time for hCG priming is. For this purpose, follicle growth from hCG priming to oocyte retrieval was evaluated. We found that the optimal time for hCG priming (10,000 IU) is when the largest follicle reaches 10–12 mm (Son et al., 2008c).

For anovulatory PCOS patients, where the follicles would not grow to 10 mm, oocyte collection can take place when the largest follicle size is ≤10 mm since the pregnancy and the implantation rates observed were reasonable (27.0 and 13.6%, respectively; Son et al., 2008c).

**IVM of immature oocytes**

**Culture medium for IVM and supplements**

Very few reports based on human data are available on the composition of culture media for human oocyte maturation. This is because very few human GV oocytes have been available to make meaningful comparisons. Therefore, the composition of most media used for human IVM is based on experiences with other mammalian species. Complex culture media, such as tissue culture medium 199 (TCM 199), Ham’s-F10, P-1 and Chang’s medium have been most widely used in research or clinical application of oocyte IVM (Cha and Chian, 1998; Trounson et al., 1997). There have been some reports comparing IVM rates between different culture media. Söderström-Anttila et al. (2005) reported that the maturation rate of immature oocytes was 58.9% (945/1604) for TCM 199-based medium and 56% (122/218) for Medi-Cult IVM medium for 28 h culture. Filali et al. (2008) found similar results for IVM, fertilization, subsequent embryo development and pregnancy rates, between TCM 199-based and Medi-Cult IVM groups. However, de Araujo et al. (2009) reported significant differences between TCM 199 and human tubal fluid regarding maturation rate (82 versus 56.9%), fertilization rate (70 versus 39.4%) and embryo quality (81.3 versus 41.7%). Therefore, although new commercialized culture media for human IVM are currently available, they may still be suboptimal to support IVM of human immature oocytes. Further research remains to be done in order to define optimal culture medium for human immature oocytes.

Culture medium for human IVM is usually supplemented with serum (Cha et al., 1991; Trounson et al., 1994; Son et al., 1996; Park et al., 1997). The most commonly used protein sources in human IVM are fetal cord serum, fetal bovine serum and human follicular fluid. Owing to potential sources of infectious agents, it is advisable not to supplement with serum sources from other patients or from animals, and therefore the patient’s own serum has been used. Some IVF clinics have used HSA or synthetic serum substitute as protein supplementation in IVM. However, significantly higher rates of maturation (63 versus 41%), pregnancy (21 versus 0%) and implantation (30 versus 0%) have been obtained from oocytes matured in culture medium with serum supplementation compared with oocytes matured in medium supplemented with HSA, indicating that factors other than albumin in maternal serum may play an important role in maturation and subsequent developmental capacity of human oocytes (Mikkelsen et al., 2001). Serum can contain several factors such as epidermal growth factor (EGF), inhibins and activins, which are thought to be important for nuclear and cytoplasmic maturation of immature oocytes. We reported the presence of EGF receptors in the oocyte, including GV-stage oocytes (Son et al. 1997: Fig. 8). Therefore, the EGF in serum supplemented-IVM medium could help immature oocytes with a small amount of CC to achieve oocyte maturation. Actually, Goud et al. (1998) reported that EGF supplementation in the IVM medium improved the nuclear maturity in cumulus-denuded human GV-stage oocytes and increased the fertilization rate in cumulus-intact immature oocytes.

Currently, most IVM protocols also supplement oocyte culture medium with FSH and/or LH based on their physiological role in oocyte maturation in vivo (Richards, 1980). However, their effects on oocyte maturation and embryonic development are still controversial. FSH is important for the development of pre-ovulatory follicles in vivo (Abir et al., 1997) and for induction of LH receptors, and is normally added to the culture medium. Studies in humans provide support for the responsiveness of human oocytes to gonadotrophins during IVM. Improvements in human oocyte maturation and embryo cleavage in the presence of FSH and LH have been reported (Durinzi et al., 1997; Anderiesz et al., 2000). Durinzi et al. (1997) showed that the addition of FSH to oocyte maturation media tends to increase oocyte maturation of the immature oocytes retrieved from ovariectomy samples. Anderiesz et al. (2000) reported that

![Figure 8 Expressions of epidermal growth factor receptor (EGFR) in different nuclear maturation stages of human oocytes.](https://academic.oup.com/humupd/article-abstract/16/6/675/739935/fig8)
human embryonic developmental competence was improved by maturing oocytes in the presence of a 1:10 ratio of rFSH:rLH after 24 h culture with FSH than with FSH alone. HCG and LH are equally effective in promoting oocyte maturation in vitro (Hreinsson et al., 2003). However, a recent report suggests that hCG in the culture medium had no effect on IVM and embryonic development (Ge et al., 2008), but that fetal bovine serum supplemented as a basic protein source may possibly contain certain amounts of LH/hCG to support enough IVM. Therefore, further studies are needed to clarify the role of gonadotrophins in culture medium. In addition, there is still a need to consider the optimal concentration of gonadotrophins to improve the developmental capacity of the oocytes. These gonadotrophins may not play the same role in oocyte maturation in vitro since mural cells play a role in mediating LH/hCG signals involved in in vivo maturation, similar, for instance, to those up-regulating EGF-like growth factors, amphiregulin, epiregulin and β-cellulin, in vivo (Park et al., 2004).

**Culture time for assessing IVM of immature oocytes**

As we mentioned before, it is important to identify in vivo matured oocytes at the time of collection in the hCG-primed IVM cycles. It is not difficult to identify the oocytes with and without GV in the cytoplasm using the spreading method. However, identification of M-II stage oocytes with extruded the first PB from the Germinal Vesicle Breakdown (GVBD)-stage oocytes is actually hard before removing CC because of the orientation of the first PB on the Petri dish. In our experience, 6% of collected oocytes are at GVBD-stage at the time of removing CC in the hCG-primed IVM cycles. About 30% of those GVBD oocytes can mature on the same day and if any of them become M-II stage, the oocytes would be inseminated. On Day 1, oocyte maturity would be evaluated again early in the morning and ICSI would be performed immediately if they are any M-II oocytes. By the end of Day 1, the presence of zygotes would be evaluated and separated from the other oocytes that show no sign of fertilization but had been injected at the same time. For those oocytes where zygotes were not observed, signs of fertilization would be checked again the next morning. The oocyte maturity of GV-stage oocytes at the time of collection in hCG-primed IVM cycles does not need to be assessed again on retrieval day.

Significant asynchrony of IVM after culture has been observed and a number of M-II stage oocytes can be obtained after 24 h of maturation from GV-stage oocytes collected in both non- or gonadotrophin-primed IVM cycles (Söderström-Anttila et al., 2005; Son et al., 2006; Ge et al., 2008). Most of the IVM studies have shown 40–60% rates of IVM after Day 1 culture (24–30 h) in IVM cycles (Son et al., 2006; Ge et al., 2008). In some early studies, however, oocytes were inseminated 48 or 56 h after maturation in non-primed IVM cycles without assessing the oocyte maturity after culture for Day 1 (24–30 h) (Trounson et al., 1994; Barnes et al., 1995; Son et al., 1996; Park et al., 1997; Cha et al., 2000). If considering that oocytes were matured after 24 h of culture, they may have been arrested at the M-II stage for 24–30 h before insemination. This places them well past the optimal fertilization time. Therefore, oocyte maturity should be assessed after Day 1 culture in the entire IVM program.

**Insemination of mature oocytes produced from IVM cycles**

Historically, ICSI has been used to increase the chances of fertilization, whether or not a male factor has been detected, as a result of the theoretical concern of zona pellucida hardening during the IVM process (Nagy et al., 1996). A study comparing ICSI versus IVF for the insemination of IVM oocytes reported that ICSI resulted in a higher rate of fertilization (84.1%) than IVF (56.3%) (Hwang et al., 2000). However, the developmental potential of the fertilized oocyte was similar irrespective of the insemination method. A more recent study (Söderström-Anttila et al., 2005) reported a higher fertilization rate (69.3%) with ICSI compared with IVF (37.7%), but a higher pregnancy and implantation rate with IVF (23.8 versus 24.2%) compared with ICSI (17.1 versus 14.8%) even though ongoing pregnancies were similar. Therefore, it is not clear whether ICSI is definitively beneficial or absolutely necessary to effectively inseminate IVM oocytes in the absence of impaired sperm parameters. Further studies are still needed to determine the optimal technique for insemination of IVM oocytes. However, in the majority of IVM studies, ICSI has been the preferred method of insemination to reduce the risk of any unexpected failed fertilization. Furthermore, it is more difficult to identify extrusion of the first PB with intact CC after culture. If the CC are removed to evaluate the oocyte maturity, it would lead to low fertilization after IVF. This is because the CC excrete sperm inducing factors such as progesterone (Oren-Benayou et al., 2008), which has been reported to stimulate, or be involved in, a number of sperm functions, including capacitation, hyperactivation, acrosome reaction, binding to the oocyte’s zona pellucida and penetration into the oocyte (Calogero et al., 2000; Yamano et al., 2004).

**Optimal ICSI timing**

One of the characteristics in the human IVM cycles is that asynchronous maturation of the immature oocytes is observed following in vitro culture compared with COH cycles. We have reported that about 18% or more are able to mature on the same day from GVBD-stage at denudation after 24 h culture on Day 1 (Son et al., 2005a; Hyn et al., 2007). Since the number of embryos fertilized is an important factor to increase the chance of successful pregnancy in an IVM program as occurs for COH (Child et al., 2001; Hyn et al., 2007), it is important to determine optimal ICSI timing to induce successful fertilization and embryo development when GBVD stage oocytes become M-II on the same day, especially for patients who have few M-II oocytes after culture. An experiment on immature oocytes obtained from COH cycles indicated that the IVM oocytes needed at least 3 h before insemination to obtain reasonable fertilization rates (61%) in comparison to the rates recorded for the oocytes matured in vivo (77%) (Balakier et al., 2004). Meanwhile, it has been shown that IVM oocytes generated from COH cycles are sensitive to post-maturation aging, and delayed sperm injection resulted in a high incidence of one pronucleus, pronucleus size asynchrony and cleavage failure (Goud et al., 1999). Thus, defining the optimal interval between the first PB extrusion and ICSI is crucial to increase fertilization and embryo development rates especially in human IVM programs. Recently, an optimal ICSI time after the first PB extrusion of oocytes that have been matured in vitro in hCG-primed IVM cycles were
proposed by Hyun et al. (2007). The authors reported that the fertilization rate of the GVBD-stage oocytes injected within 1 h after the first PB extrusion was low (15.8%; 6/38) ($P < 0.01$ versus all other times). In contrast, the fertilization rate was 80, 92.3, 82.1 and 85% for oocytes injected 1–2, 2–4, 4–6 and 6–8 h after the first PB extrusion, respectively. Interestingly, all the oocytes injected within 1 h after the first PB extrusion were in Telophase-I stage (Fig. 9). They concluded that human oocytes matured in vitro needed at least 1 h after the first PB extrusion to complete nuclear maturation and the use of a live spindle imaging system would help decide the timing of ICSI for oocytes matured in vitro. It is important to note that completion of nuclear maturation after the first PB extrusion will depend on the source of immature oocytes (from COH cycles or IVM cycles) as well as on each IVM culture system.

### Determination of embryo transfer time

Most IVF centers are doing Day 2 or Day 3 embryo transfers in IVM cycles in order to avoid the possibility of blastocyst development failure since the quality and quantity of embryos obtained from IVM are not sufficient. As implantation rates are relatively low after IVM, more embryos in an IVM program are transferred to obtain acceptable pregnancy rates and sometimes may cause multiple pregnancies (Demirtas et al., 2008). However, multiple pregnancies rates after IVM are comparable with those of conventional IVF (Demirtas et al., 2008). There is no doubt that blastocyst transfer is a way to obtain a high implantation rate whereas reducing multiple pregnancies and synchronizing between the embryo and the endometrium (Yoon et al., 2001b). Some case reports have shown that in IVM cycles embryo transfer at the blastocyst-stage can achieve implantation (Barnes et al., 1995; Son et al., 2002a, b). In a study performed by our group (Son et al., 2007b), IVM patients with more than seven zygotes and three or more good quality embryos on Day 3 after oocyte collection underwent blastocyst transfer. We obtained 41.6% blastocyst formation and high clinical pregnancy and implantation rates (51.9%, 26.8%) after transferring one or three blastocysts depending on quality without any triplet pregnancies. Therefore, we proposed that determination of the stage at which embryos should be transferred based on the number and quality of embryos on Day 3 after oocyte collection may help obtain an acceptable pregnancy rate whereas minimizing blastocyst development failure and triplet multiple gestations.

### Cryopreservation

There are two possible reasons to freeze IVM embryos. The first is to store the remaining good quality embryos after embryo transfer. The second is due to inadequate endometrial thickness at the time of fresh embryo transfer (Son et al., 2009b; Son and Tan, 2009). Although cryopreservation is now a routine procedure in human IVF programs, there have only been some case reports in which embryos generated from IVM programs were frozen at zygotes (Thorton et al., 1998; Chian et al., 2001; Kyono et al., 2002) and at cleavage-stage (Godin et al., 2003; Chen et al., 2007) using the slow-cooling method. Generally, in vitro produced oocytes or embryos are more sensitive to freezing than the in vivo derived counterparts (Leibo, 1998). Suikkari et al. (2000) found that the cryosurvival rate of in vitro matured cleaved embryos was very poor, compared with that of embryos generated from in vivo matured oocytes by using the slow-cooling method. We also reported poor results using the slow-cooling method to freeze cleavage-stage embryos produced from IVM cycles (Son et al., 2009b; Son and Tan, 2009). Thus, clinically satisfactory results using the slow-cooling method with embryos generated from IVM oocytes have not been obtained up to now.

Vitrification would be a very attractive alternative to the conventional slow freezing method with the advantage of lack of ice crystal formation and ease of operation (Son and Tan, 2009). Several successful pregnancies have been reported after cryopreservation using vitrification of M-II stage oocytes (Chian et al., 2009a), zygote- (Hashimoto et al., 2007) or blastocyst-stage embryos produced from IVM cycles (Son et al., 2002c, 2005b). For the oocytes, we have reported significantly lower survival, fertilization rate and embryo quality using M-II stage oocytes produced from hCG-primed IVM cycles compared with those of conventional COH cycles using the vitrification method (Chian et al., 2009b). However, the differences in the implantation rate per embryo (19.1 versus 9.6%), clinical pregnancy rate per cycle started (44.7 versus 20.0%), and live birth rate per cycle started (39.5 versus 20.0%) were not statistically significant.

Using vitrification on cleavage-stage embryos produced from IVM cycles, we observed a high survival rate (85.5%), a 25.0% clinical pregnancy rate per embryo transfer and 15.4% implantation (Son et al., 2009b). In addition, Lee et al. (2007) described the clinical outcome of cycles where blastocysts produced from hCG-primed IVM cycles were vitrified and thawed, and reported 92% survival, 43.8% clinical pregnancy and 23.6% implantation rates. More recently, Zech et al. (2009) reported 80, 53 and 35% of survival, ongoing pregnancy and implantation rates, respectively, after the transferring of vitrified/thawed blastocysts produced from combined FSH-hCG IVM cycles, although the number of cycles was small.

These results suggested that oocytes and embryos produced from IVM cycles can be safely cryopreserved through vitrification, although more studies are necessary to improve the vitrification method for oocytes produced from IVM cycles.
Summary and conclusion

Conventional IVF stimulation requires high doses of costly recombinant hormones, gonadotrophin-releasing hormone agonists and antagonists. The complexities of their use as well as cost of ovarian stimulation are being challenged by alternative simple approaches such as controlled natural cycle IVF and minimal stimulation IVF. These options allow the mild approaches to be clinically effective and cost-effective with the advantages of better tolerability for patients and less time needed to complete an IVF cycle and are now practiced in an increasing numbers of IVF clinics. In addition, IVM of immature oocytes retrieved from unstimulated ovaries has also been a reproduc tive technology of increasing interest. Despite the remarkable progress of the clinical management of these cycles, it is important to improve the quality of the laboratory procedures in IVM cycles.

Laboratory manipulation of oocytes collected from IVM cycles is more time consuming and technically different than the traditional IVF cycles. Therefore, before starting an IVM program, the embryologists should obtain adequate training and experience by a supervisor skilled in this technique (Jurema and Nogueira, 2006).

On the basis of the information gathered in this review, we can summarize some very important embryological aspects of IVM as follows: (i) There are two methods for oocyte identification, the direct and the filter method. (ii) Oocytes with dispersed CC appearance can be obtained at the time of oocyte retrieval only in hCG-primed IVM cycles. (iii) The immature oocytes with dispersed CC have a faster and higher IVM and embryo developmental potential than those with compacted or sparse CC. (iv) A few in vivo matured oocytes with dispersed CC at the time of collection can be obtained even in IVM cycles, and these have produced better quality embryos than those derived from in vitro matured oocytes. The in vivo matured oocytes retrieved from the cohort of small follicles can produce the same quality of embryos as those of large follicles, resulting in higher pregnancy rates in the cycles in which more than one embryo produced from in vivo matured oocytes are transferred. (v) Extending the period of hCG priming before oocyte retrieval promotes oocyte maturation in vivo and increases the IVM rate of immature oocytes, probably improving subsequent pregnancy outcomes in IVM cycles. (vi) hCG could be given to patients when a DF reaches 10-12 mm to ensure the presence of in vivo matured oocytes on the day of collection and to avoid detrimental effects on the sibling immature oocytes. (vii) The insemination for mature oocytes produced from IVM cycles probably requires ICSI rather than IVF. ICSI should be performed at least 1 h after the first PB extrusion and the use of a live spindle imaging system could help decide the time of ICSI performance. (viii) The embryo transfer day will depend on the quantity and quality of embryos produced after IVM just as in conventional IVF. (ix) Vitrification is more efficient than slow freezing for freezing embryos produced by IVM.

IVM holds great promises as another type of assisted reproductive technologies, and when fully developed, it may be the procedure of choice not only for infertile patients but also for obtaining oocytes for donation or fertility preservation. Actually, the indications for IVM treatment have tremendously expanded in recent years. Success rates, however, are lower in human IVM cycles and so far, this has restricted its widespread clinical implementation. Therefore, further research is needed to improve IVM technology in order to achieve success rates comparable with gonadotrophin stimulated cycles. In conclusion, in order to improve the efficiency of the IVM programs, emphasis should be placed on the laboratory and embryological aspects as well as the clinical aspects.

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