Reproductive BioMedicine Online (2012) 24, 47–53

www.sciencedirect.com
www.rbmonline.com

ARTICLE

# Combination of IVF and IVM in naturally cycling women

Mikael Tang-Pedersen [a,*], Lars Grabow Westergaard [b,1], Karin Erb [b], Anne Lis Mikkelsen [c]

[a] *University of Southern Denmark, Winsloewvej 21, 5000 Odense C, Denmark;* [b] *Fertility Clinic, Odense University Hospital, Sdr. Boulevard 29, 5000 Odense C, Denmark;* [c] *Fertility Clinic, Holbaek Hospital and University of Copenhagen, Denmark*
[*] Corresponding author. *E-mail address:* mikael.tp@gmail.com (M Tang-Pedersen). [1] Deceased author.

Mikael Tang-Pedersen graduated as MB BS in 2002 from the University of Southern Denmark, Odense in Denmark and worked initially in the department of gynaecology and obstetrics at Esbjerg Hospital, Esbjerg. He joined the University of Southern Denmark to study for his PhD in the fertility clinic at Odense University Hospital in the field of in-vitro maturation of oocytes in natural-cycle IVF and for polycystic ovary syndrome patients, which was awarded in September 2011. Mikael is currently employed at the department of gynaecology and obstetrics at Odense University hospital. His main areas of interest are reproductive physiology and endocrinology.

**Abstract** This study investigated the combination of an unstimulated IVF cycle with in-vitro maturation (IVM) of additional immature cumulus–oocyte–complexes (COC) from the same cycle collected at the same time as the spontaneous preovulatory follicle. This could potentially improve rates of embryo transfer and pregnancy/live births compared with conventional unstimulated IVF treatment and at the same time eliminate the risk of ovarian hyperstimulation syndrome. This prospective trial included 77 women with regular menstrual cycles. Age at inclusion was between 20 and 37 years. Results showed a retrieval rate of mature oocytes of 50/80 (62.5%) per cycle started and immature COC were collected in 74/80 (92.5%) cycles. The embryo transfer rate was 28/80 (35.0%) with mature oocytes and increased in total to 43/80 (53.8%) with IVM oocytes. Corresponding birth rates per transfer were 3/28 (10.7%) and 4/43 (9.3%). Birth rates per aspiration were 3/76 (3.9%) and 4/76 (5.3%). It is concluded that the protocol described here shows proof of concept, but the impact of the IVM procedure only reached a significant level regarding embryo transfer, not with live births. The reason for this is yet unclear, but asynchrony between endometrial factors and IVM oocytes together with unknown competence of IVM embryos is suspected.



**KEYWORDS:** IVF, IVM, natural cycle, oocyte

## Introduction

The era of IVF started with unstimulated, spontaneous cycles and the birth of Louise Brown in 1978 (Steptoe and Edwards, 1978). However, over time the spontaneous cycles have been replaced by stimulation protocols due to higher live-birth rates per cycle. The cost of this approach is a number of side effects from gonadotrophins and in some cases ovarian hyperstimulation syndrome (OHSS) (Heijnen et al., 2007; Macklon et al., 2006; Nargund and Frydman, 2007).

IVF in natural cycles offers a simpler, faster and less invasive form of IVF than the more conventional stimulated

1472-6483/$ - see front matter 
doi:10.1016/j.rbmo.2011.10.005

cycles (Edwards, 2007). However, in natural-cycle IVF treatment, a number of other problems arise, including spontaneous ovulation or an increased risk of no oocytes being retrieved during oocyte retrieval and no embryos available for transfer. This means that the cancellation rate is high, between 20% and 30% (Pelinck et al., 2002; Vlaisavljevic, 2007).

In-vitro maturation (IVM) is a treatment where final oocyte maturation is achieved in the laboratory. The basis of IVM is the maturing *in vitro* of oocytes from the germinal vesicle (GV) stage of development to the metaphase II (MII) stage. Oocytes are retrieved transvaginally under ultrasound guidance from antral follicles of 2–10 mm diameter and are matured *in vitro*. A high proportion of the in-vitro matured oocytes are able to resume meiosis and reach the MII stage. Their ability to be fertilized after 28–36 h of IVM is also high and a similar proportion is able to undergo early cleavage-stage development comparable to conventional IVF and intracytoplasmic sperm injection (ICSI) embryos (Child et al., 2001; Jurema and Nogueira, 2006). Recent data taken together suggest that natural-cycle IVF combined with IVM might be a practical treatment for women with various causes of infertility (Fadini et al., 2009a; Son et al., 2008).

Chian et al. (2004) described in a case report three pregnancies and two live births resulting from IVF of mature oocytes from dominant follicles in natural cycles combined with IVM of immature oocytes retrieved from small follicles. Retrospective analyses of IVF treatments comparing natural-cycle IVF combined with IVM, IVM alone and ovarian stimulation with gonadotrophins did not detect any difference in implantation rate in these three groups (Lim et al., 2009). However, in these studies, embryos from in-vivo and in-vitro matured oocytes were pooled, and it is unknown whether the pregnancies were obtained from in-vivo or in-vitro matured oocytes.

Natural-cycle IVF combined with immature oocyte retrieval is in accordance with public expectations for a more patient-friendly IVF procedure – single-embryo transfer – avoiding multiple pregnancies and OHSS (Edwards, 2007; Hreinsson and Fridström, 2004).

The study centre therefore initiated a prospective study to evaluate the pregnancy rate per cycle by combining natural-cycle IVF with IVM in regular-cycling women. In cases where an embryo was obtained from in-vivo matured oocytes, this embryo was transferred and when available the surplus embryos from in-vitro matured oocytes were cryopreserved. In cases where no embryos were obtained from in-vivo matured oocytes, 2 pronuclei (2PN) gametes or embryos from in-vitro matured oocytes were transferred when available. Pregnancy rates per aspiration and per transfer were calculated, taking into account cumulative pregnancy rates by combining transfer of fresh embryos and cryopreserved embryos.

## Materials and methods

### Patients

This prospective observational study included 77 regular-cycling women referred for IVF/ICSI at the fertility clinic at Odense University Hospital (FertilitetsKlinikken OUH), between 1 April 2006 and 1 January 2009. A total of 80 aspirations were performed. The participating women had to be aged between 18 and 37 years, to have ovulatory cycles with a mean duration of 26–35 days and a body mass index (BMI) of 18–29 kg/m$^2$.

Excluded were all patients where infertility was caused by endocrine abnormalities, such as hyperprolactinaemia, and patients with expected low ovarian reserve evaluated on day 3 by an antral follicle count of less than six follicles of 2–5 mm in diameter and/or an increased concentration of FSH >15 IU/l. Excluded also were patients who had previously failed to conceive with conventional IVF after three attempts, and patients with possible poor quality of the oocytes, i.e. patients with a low (<20%) cleavage rate after conventional IVF and women with polycystic ovarian syndrome. If serum human chorionic gonadotrophin (HCG) control on the day of oocyte aspiration turned out to be <2 IU/l, the patient was excluded due to incorrect or missing injection of recombinant HCG (rHCG) 34 h before oocyte retrieval.

### Monitoring during the follicular phase

A transvaginal ultrasound was performed on cycle day 3. Cycle day 1 was defined as the first day of menstrual bleeding. In the case of an ovarian cyst (follicle >10 mm) on day 3, the cycle was cancelled. The second ultrasound was performed on days 8–10, and from then onwards an ultrasound was performed daily or with an interval of 2 or 3 days, depending on the follicle size. Ovum retrieval was scheduled when the dominant follicle reached at least 16 mm in diameter and the endometrium had a thickness of at least 8 mm. At this point, an HCG injection of 6500 IU (Ovitrelle; Merck-Serono, Geneva, Switzerland) was applied 34 h before oocyte retrieval.

### Ultrasound measurements

Follicular diameters were measured by the same observer during transvaginal ultrasound scanning using a 6.5–8.0 MHz transvaginal transducer (Sonoline GS 50; Siemens). The follicular diameter was calculated as the mean of the longest follicular axis and the axis perpendicular to it. The endometrial thickness was measured in a sagittal plane between hyperechoic inner borders of the endometrium. Any fluid in the cavity was not included in the endometrial thickness value. From day 7, it was observed whether an endometrial triple-layer structure was present.

### Priming with oestradiol

Of 80 cycles, 36 were primed with oestradiol tablets (Femanest; Schering-Plough) 2 mg three times daily from previous cycle day 25 or at least 3 days before expected first cycle day, according to earlier work by de Ziegler et al. (1999). The duration was between 4 and 10 days until the first upcoming Thursday in a week. Usually it was stopped on the first Thursday unless the patient had a special interest in postponing the oocyte aspiration. This was due to a desire to implement the procedure in the daily clinical procedures

and to programme the oocyte retrieval within a few days in the middle of a working week. The oestradiol priming was performed during the last half of the study period. No significant difference was observed in the primed versus non-primed groups regarding the number of retrieved oocytes and rates of maturation and fertilization (data not shown).

## Aspiration of mature and immature oocytes

Aspiration of the leading follicle was performed transvaginally with a Cook double-lumen 16G needle with a suction pressure of 200–250 mmHg and with flushing of the follicle. These follicles ranged in size from 16–20 mm. The follicular fluids from these dominant preovulatory follicles were intended to be stored and frozen for later analysis.

Aspiration of the small follicles of size 2–10 mm was also performed transvaginally and immediately after aspiration of the dominant follicle. A single-lumen Wallace 17G needle was used with a suction pressure of 80–100 mmHg and without flushing of the follicle. The aspirates were transferred in tubes containing preheated flushing medium (cat. no. 10760125; MediCult, Denmark). Follicular aspirates were filtered (Falcon 1060, 70 μm mesh size; Falcon, USA) to remove erythrocytes and small cellular debris. Oocytes with signs of atresia (dark or shrunken, irregular cytoplasm) or mechanical damage were discarded.

## IVF and IVM

The mature oocyte from the dominant follicle was washed in flush medium (MediCult) and incubated for 3 h in culture medium (IVF-medium; MediCult) at 37°C and 6% $CO_2$.

Healthy appearing immature oocytes from the small follicles were matured for 2 h in maturation medium and subsequently moved to fresh IVM medium (cat. no. 82214010; MediCult) for the next 28–30 h. Patient serum (10%) was added to the medium along with 0.075 IU recombinant FSH (Gonal-F; Merck-Serono) and 0.1 IU HCG. Oocytes were evaluated on the day of aspiration and examined daily for evidence of germinal vesicle breakdown, polar body extrusion and atretic changes and determined to be: (i) prophase I (PI) if the germinal vesicle was present; (ii) metaphase I (MI) if the germinal vesicle had disappeared and the first polar body was still absent; or (iii) MII if the first polar body was present.

## Sperm preparation and fertilization

Sperm preparation and insemination as well as ICSI were performed as common routines in the laboratory. Fresh human ejaculates were prepared by liquefaction at 37°C followed by discontinuous density-gradient centrifugation (80% + 40%) for 20 min at 300 *g* at room temperature. The bottom fraction was aspirated and washed twice at 300 *g* for 10 min, followed by concentration and motility assessment.

IVF or ICSI was alternatively used in the fertilization of both in-vivo matured oocytes and in-vitro matured oocytes according to sperm quality criteria applied in conventional treatment at the study clinic. Insemination was performed in case the sperm quality was satisfactory (>2 million spermatozoa/ml) with 150,000 spermatozoa per well or else ICSI was performed. Only ICSI oocytes were denuded. IVF oocytes were evaluated with intact cumulus before insemination.

Before the ICSI procedure, the cumulus–corona cells were removed through a short exposure to bicarbonate-buffered medium containing hyaluronidase (Hyadase, MediCult) followed by gentle aspiration in and out of a denudation pipette (SweMed, Sweden). The oocytes were placed individually in 5-μl droplets of HEPES-buffered SpermPrep medium (MediCult) and spermatozoa were placed in a central 4-μl droplet of polyvinylpyrrolidone solution (MediCult) in a Petri dish (Falcon) and covered with liquified paraffin (MediCult).

After insemination, the oocytes were cultured in Petri dishes in 25 μl droplets of cleavage medium ISM1 (MediCult) according to the clinic's standard procedures.

Fertilization was defined as the presence of 2PN 16–19 h post IVF/ICSI.

## Embryo culture, transfer and freezing

Fertilized oocytes were cultured in ISM1 media until day 2 and then transferred from UTM media (MediCult).

Embryo transfer was scheduled on day 2 or 3 after aspiration. Primarily the embryo from the in-vivo matured oocyte was transferred on day 2 and if a surplus of fertilized or cleaved embryos from in-vitro matured oocytes were present, they would be cryopreserved. If no embryo from in-vivo matured oocytes was available, a maximum of two embryos/fertilized oocytes from in-vitro matured oocytes were transferred on day 3.

Before transfer, 2PN oocytes and embryos were scored on a scale from 0–3: 0 being pronuclear oocytes at 2PN stage; 1 being embryos with <10% fragmentation and equal blastomer size; 2 being embryos with <10% fragmentation and unequal blastomers; and 3 being embryos with >10% fragmentation and unequal blastomer size.

A Cook 5000 soft-tip catheter was used for embryo transfer in most cases. A few required a tight-difficult-transfer catheter for successful embryo transfer.

A slow-freezing protocol with propandiol was applied for pronuclear oocytes and embryos (Fabbri et al., 1998). Thawing was initiated 1 day before planned embryo transfer.

## Endometrial priming and pregnancy ultrasound

For luteal support, two HCG injections of 6500 IU were applied on the day of embryo transfer and again 4 days later, intending to stimulate progesterone production from mural granulosa cells in the mature follicle.

A blood sample was taken 13–15 days after embryo transfer and analysed with solid-phase, sandwich, fluoroimmunometric methodology for quantification of βHCG (Auto-DELFIA 1235; Perkin Elmer). The test was considered positive when >2 mIU/ml.

Clinical pregnancy was defined as at least one intrauterine gestational sac with fetal heart beat 5–6 weeks after embryo transfer. The number of intrauterine sacs was recorded.

The study was approved by the local ethical committee of southern Denmark (Den Videnskabsetiske komité, Region SydDanmark; project S-VF-20050164). All couples who participated in the study gave their oral and written consent. The study was carried out in accordance with the Declaration of Helsinki.

## Statistical analysis

Statistical analysis was performed using the *t*-test, Wilcoxon rank sum and Fisher's Exact tests as appropriate. A *P*-value $<0.05$ was considered statistically significant. All levels of significance from Fisher's Exact test (two-tailed) were calculated with GraphPad QuikCalc online software. Wilcoxon rank-sum and *t*-tests were calculated with STATA version 10.1 (STATA Corporation, Texas, USA).

## Results

**Table 1** shows the basic characteristics of the women who participated in the study. No patients were excluded due to insufficient follicle growth or insufficient endometrial development. Diameter of the dominant follicle and endometrial thickness on last monitoring before the administration of HCG are shown in **Table 2**.

The outcomes of cycles using in-vivo matured oocytes are presented in **Table 3**. In 16/80 cycles (20%), spontaneous ovulation had taken place before aspiration. In 64 cycles, aspiration from a dominant follicle was possible. Fifty MII oocytes and no immature oocytes were obtained from the dominant follicle.

The outcomes of in-vitro matured oocytes are presented in **Table 4**. All immature oocytes were obtained from non-dominant follicles. Calculated from antral follicle count on the first monitoring day, the recovery rate from non-dominant follicles was overall approximately 30%. No difference in the rates of maturation and fertilization of immature oocytes was observed when cycles with in-vivo matured oocytes were compared with cycles without in-vivo matured oocytes. Cycles without in-vivo matured oocytes were due to either spontaneous ovulation or no oocyte obtained from the dominant follicle (data not shown). In four out of 16 cycles with ovulation, embryo transfer with in-vitro matured oocytes were performed. More top-quality embryos were obtained from in-vivo matured oocytes compared with in-vitro matured oocytes (**Table 5**). All clinical pregnancies resulted in live birth (**Tables 3** and **4**). The rate of biochemical pregnancies did not differ between embryos obtained after in-vivo maturation and embryos obtained after in-vitro maturation.

In four cycles, neither mature nor immature oocytes were obtained. In two of these cycles, no HCG in serum on the day of aspiration could be detected. Furthermore, in two cycles with only immature oocytes, no HCG in serum on the day of aspiration could be detected. Hence, a possible deviation from the protocol with HCG injection 34 h prior to aspiration of oocytes was observed in a total in four cycles. The combination of IVF and IVM increased the number of cycles with transfer of fresh embryos from 28/80 (35%) to 43/80 (53.8%). The birth rate per transfer by combination of IVF with IVM was 4/43 (9.3%). Birth rate per aspiration by combination of IVF with IVM was 4/80 (5.0%). The

**Table 1** Basic characteristics of the study group.

| *Characteristic* | *Study population (n = 77)* |
|---|---|
| Age (years) | 31.5 ± 0.39 |
| AFC | 11.7 ± 0.38 |
| BMI (kg/m$^2$) | 24.3 ± 0.38 |
| FSH (IU/l) | 6.83 ± 0.20 |
| Tubal factor | 19 (24.7) |
| Male factor | 42 (54.5) |
| Unexplained | 16 (20.8) |
| Primary infertility | 56 (72.7) |
| Secondary infertility | 21 (27.3) |

Values are mean ± SEM or *n* (%).
AFC = antral follicle count; BMI = body mass index.

**Table 2** Ultrasound measurements on last monitoring before human chorionic gonadotrophin (HCG) administration.

| *Measurement* | *HCG day −1 (n = 17)* | *HCG day (n = 47)* |
|---|---|---|
| Diameter of dominant follicle (mm) | 15.7 ± 0.242 | 16.1 ± 0.128 |
| Endometrial thickness (mm) | 8.25 ± 0.509 | 8.93 ± 0.197 |

Values are mean ± SE.

**Table 3** Cycle outcome for in-vivo matured oocytes.

| *Outcome* | *Study population* |
|---|---|
| Cycles | |
| Patients with started cycles | 77 |
| Started cycles | 80 |
| Spontaneous ovulation | 16 |
| Aspiration from dominant follicle | 64 |
| Aspiration of in-vivo matured oocytes | 50 |
| Oocytes retrieved | 50 |
| 2PN fertilization (rate per oocyte) | 32 (64.0) |
| Embryos available for transfer (rate per 2PN) | 28 (87.5) |
| Embryo quality (rate per embryos available) | |
| Grade 1 | 9 (32.1) |
| Grade 2 | 15 (53.6) |
| Grade 3 | 4 (14.3) |
| Transfer cycles (*n* = 28) | |
| Biochemical pregnancies (per cycle started) | 8 (10.0) |
| Biochemical pregnancies (per transfer) | 8 (28.6) |
| Clinical pregnancies (per transfer) | 3 (10.7) |
| Live birth (per started cycle) | 3 (3.8) |
| Live birth (per transfer) | 3 (10.7) |

Values are *n* or *n* (%).

**Table 4** Cycle outcome for in-vitro matured oocytes.

| *Outcome* | *Study population* |
|---|---|
| Cycles | |
| Patients with started cycles | 77 |
| Started cycles | 80 |
| Aspiration of immature oocytes | 74 |
| Immature oocytes retrieved | 253 |
| Maturation | 84 (33.2) |
| Fertilization | 49 (58.3) |
| 2PN oocytes/embryos available for transfer and cryopreservation | 31 |
| 2PN oocytes/embryos used for fresh transfer | 18 |
| Quality of embryos used for fresh transfer | |
| Grade 0 (2PN) | 7 |
| Grade 1 | 1 |
| Grade 2 | 8 |
| Grade 3 | 2 |
| Cycles with transfer of fresh embryos | 15 |
| Biochemical pregnancies (per transfer) | 3 (20.0) |
| Clinical pregnancy (per transfer) | 1 (6.7) |
| Live birth (per transfer) | 1 (6.7) |
| Cryopreserved embryos | 13 |
| Cycles with cryopreservation of embryos due to transfer of embryos obtained after in-vivo maturation | 8 |
| Embryos survived after thawing | 2 |
| Cycles with transfer | 1 |
| Pregnancies after freezing and thawing | 0 |

Values are *n* or *n* (%).

**Table 5** Quality of transferred embryos assessed by morphology.

| *Embryo* | *Embryo grade* | | | | *Total* |
|---|---|---|---|---|---|
| | *0 (2PN)* | *1* | *2* | *3* | |
| Transferred | | | | | |
| Total | 7 | 10 | 23 | 6 | 46 |
| In-vitro matured | 7 | 1 | 8 | 2 | 18 |
| In-vivo matured | 0 | 9 | 15 | 4 | 28 |
| Resulting in positive HCG test (>2 IU/l) | | | | | |
| In-vitro matured | 1 | 0 | 2 | 0 | 3 |
| In-vivo matured | 0 | 5 | 2 | 1 | 8 |
| Resulting in live births | | | | | |
| In-vitro matured | 1 | 0 | 0 | 0 | 1 |
| In-vivo matured | 0 | 3 | 0 | 0 | 3 |

Values are *n*.
HCG = human chorionic gonadotrophin; 2PN = 2 pronuclei.

supplementation of cryopreserved–thawed 2PN oocytes and embryos did not impact on these figures. Thirteen in-vitro matured embryos were cryopreserved. After thawing, two embryos survived and were transferred in one cycle. This patient had not obtained pregnancy after transfer of an embryo obtained after an in-vivo matured oocyte.

## Discussion

In the present study, a retrieval rate of in-vivo matured oocytes of 62.5% was found and embryos for transfer were obtained in 35.0% cycles with a clinical pregnancy rate of 6.25% per aspiration and 9.3% per transfer. The proportion of male factor diagnosis was 54.5% in all cycles and, of the 50 retrieved in-vivo matured oocytes, 22 had ICSI performed. This might be a confounding factor regarding fertilization rate since the extra handling of gametes and lower capacity of spermatozoa impair overall results.

Spontaneous ovulation occurred in 20% of cycles. The delivery rate was 3.8% per aspiration and 10.7% per transfer for cycles using in-vivo matured oocytes. These figures are comparable with other studies (Pelinck et al., 2002). The results of 1800 IVF natural cycles in a systematic review by Pelinck et al. (2002) gave an embryo transfer rate of 45.5%, a pregnancy rate per cycle of 7.2% and a pregnancy rate per transfer of 15.8% (Pelinck et al., 2002). No delivery rate was reported. A retrospective analysis of 1024 oocyte retrievals in natural cycles showed a delivery rate per oocyte retrieval of 6.9% (Vlaisavljevic, 2007).

One of the major problems in natural-cycle IVF is an increased risk of no oocyte being retrieved during collection and no embryo available for transfer; therefore, combining natural-cycle IVF with IVM has been suggested. In this study, such an approach increased the number of available oocytes per aspiration and increased the number of embryos available for transfer. However, this study only obtained the birth of one child after transfer of 18 fresh zygotes/embryos, resulting from in-vitro matured oocytes, in 15 cycles. Cryopreservation followed by thawing did not enhance the outcome in terms of pregnancies and live-birth rates. This may be due to impaired developmental capacity of in-vitro matured oocytes or asynchrony between the in-vitro matured embryos and the development of the endometrium.

The developmental capacity of immature oocytes may depend on the size of the follicle together with the concentrations of intrafollicular hormones and growth factors. Since the volume of each aspirated antral follicle was far less than a normal preovulatory follicle, the aspiration did not allow us to separate and count for each single aspirated follicle regarding size and whether or not it was a preselectable (2–5 mm), selectable (6–9 mm) or selected (>10 mm) follicle and if the cumulus–oocyte–complex (COC) was retrieved from it. Rigorous counting and measuring would prolong the procedure beyond an acceptable level. Even in general anaesthesia, the safety of the aspiration procedure would be questionable if puncture of the ovary was repeatedly done for each follicle. Lately double-needle systems specially designed for IVM have been made commercially available and it can be speculated if such a set-up for aspirating COCs could have contributed to a more accurate registration of aspirated follicles.

It has been questioned whether the developmental competence of the immature oocyte is adversely affected by the dominant follicle (Chian et al., 2004; Fadini et al., 2009a; Thornton et al., 1998). While some studies have

recommended that immature oocyte collection in natural cycles should be performed when the dominant follicle was between 10 and 14 mm due to early atresia of non-dominant follicles (Mikkelsen et al., 2000; Soderstrom-Anttila et al., 2005; Son et al., 2008), other investigators have raised some doubts with regard to the significance of this and Thornton et al. (1998) obtained pregnancies from human oocytes retrieved in unstimulated cycles during mid-cycle aspiration of immature oocytes. In the present study, aspiration was scheduled when the leading follicle was at least 16 mm. This may have been detrimental to the developmental potential of some fraction of the immature oocytes obtained.

In order to improve the quality of the immature oocytes, a potential beneficial effect of priming with HCG and/or FSH before has been examined. Chian et al. (2000) demonstrated in a prospective randomized study on 17 patients with polycystic ovary syndrome that HCG hastened the maturation rate. No effect was demonstrated on pregnancy. In a prospective randomized study including 400 regular cycling women, HCG priming, FSH priming and combined FSH/HCG priming was compared with non-primed cycles (Fadini et al., 2009b). HCG priming increased the maturation rate, but showed no significant effect on clinical outcome. Interestingly, they found, that also the number of in-vivo matured oocytes increased. Not only did they obtain in-vivo matured oocytes from mature follicles, but also from smaller follicles less than 14 mm in diameter. This observation supported earlier observations from Chian et al. (2000) where HCG priming has been recommended as a routine before IVM. The developmental capacity of the different oocytes cannot be evaluated from these studies, as embryos from in-vivo matured and in-vitro matured embryos were pooled at transfer. In the present protocol design, the effect of HCG priming on IVM oocytes cannot be evaluated directly. The main reason for application of HCG in the present study is the planned aspiration of an in-vivo matured oocyte; hence, the timing of the second meiotic division and final nuclear maturation must be predictable (Lenton, 2007). This may have had unanticipated effects regarding the endometrium with asynchrony between the endometrium and the embryo obtained after in-vitro maturation of the oocyte. Transfer was performed on day 4 or 5 after HCG and the embryos were developed corresponding to day 2. The time span from day 4 versus day 5 after HCG induction might be critical. Supportive of this view is the fact that the only live birth from an IVM embryo in this study was transferred at 2PN stage on day 2 after aspiration (due to logistical factors).

There is conflicting evidence whether follicular aspiration in a spontaneous cycle leads to a defective luteal phase and, therefore, it is not clear whether luteal-phase support is necessary for IVF in the natural cycle or not. In IVM, commonly used protocols for endometrial preparation consist of 17β-oestradiol 6 mg/day from the day of oocyte retrieval. Two days after oocyte retrieval, the luteal phase was supported by intravaginal progesterone 400–600 mg daily. These protocols are based on experiences from donor–recipient studies and frozen–thawed embryo-transfer programmes (Escribá et al., 2006; Gelbaya et al., 2008). Prospective studies comparing different protocols for optimal endometrial preparation in IVM protocols are lacking.

In the present study, HCG was chosen due to simpler and fewer administrations, with presumed equally beneficial effect on implantation and pregnancy rate as exogenous hormones. A positive test for HCG was defined as >2 IU/l. In four cycles were measured HCG values between 3 and 8 IU/l. Since the luteal support consisted of two injections of 6500 IU rHCG on the day of embryo transfer and 4 days later, these measurements may be due to a prolonged half-life for some patients. According to the manufacturer, the half-life of rHCG is approximately 30 h in plasma and its bioavailability is 40%. This does leave a possibility of false-positive HCG test after 10 days as the pregnancy test was performed 14 days after embryo transfer. On the other hand, these cases, or some of them, may represent a terminated implantation of the pre-embryo. The very low concentrations of HCG corresponded to a lower morphological embryo score.

In the present study, no detectable HCG was measured on the day of aspiration in four cycles. In these cycles, no oocyte could be aspirated from the dominant follicle. It can be speculated why this was found. All patients received individual instructions by experienced nurses on HCG injections as well as written information was handed out. One hypothesis is that these women had a wish for a treatment without exogenous hormones at all, leading to a missing HCG injection. The fraction of patients with no serum HCG on day of oocyte aspiration is unknown in IVF treatments generally. As far as is known, there are no larger studies where serum concentrations of HCG were measured on day of aspiration.

In conclusion, despite higher embryo transfer rates the overall improvement in live-birth rates in this study is not substantial enough to recommend aspiration of both mature and immature oocytes as a routine application. This conclusion is also due to longer procedure time and repeated punctures for oocyte retrieval compared with aspiration of a single mature follicle, which can be repeated for a number of consecutive cycles, as described in a study by Nargund et al. (2001). The present results showed proof of concept regarding the possibility of a live birth from an IVM-derived embryo with preovulatory mid-cyclic COC aspiration after HCG induction of an in-vivo matured follicle as well. The implantation rate of IVM embryos appeared lower than expected. Possible explanations for this might be asynchrony between endometrial factors and the embryo as well as lower competence regarding embryogenesis due to some degree of insufficiency of the cytoplasmic maturation process in IVM oocytes.

## References

Chian, R.C., Buckett, W.M., Jalil, A.K.A., Son, W.Y., Sylvestre, C., Rao, D., Tan, S.L., 2004. Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential approach in infertility treatment. Fertil. Steril. 82, 1675–1678.

Chian, R.C., Buckett, W.M., Tulandi, T., Tan, S.L., 2000. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. Hum. Reprod. 15, 165–170.

Child, T.J., bdul-Jalil, A.K., Gulekli, B., Lin Tan, S., 2001. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. Fertil. Steril. 76, 936–942.

de Ziegler, D., Brioschi, P.A., Benchaa, C., Campana, A., Ditesheim, P.J., Fanchin, R., Bulletti, C., 1999. Programming ovulation in the menstrual cycle by a simple innovative approach: back to the future of assisted reproduction. Fertil. Steril. 72, 77–82.

Edwards, R.G., 2007. IVF, IVM, natural cycle IVF, minimal stimulation IVF – time for a rethink. Reprod. Biomed. Online 15, 106–119.

Escribá, M.J., Bellver, J., Bosch, E., Sánchez, M., Pellicer, A., Remohí, J., 2006. Delaying the initiation of progesterone supplementation until the day of fertilization does not compromise cycle outcome in patients receiving donated oocytes: a randomized study. Fertil. Steril. 86, 92–97.

Fabbri, R., Porcu, E., Marsella, T., Primavera, M.R., Seracchioli, R., Ciotti, P.M., Magrini, O., Venturoli, S., Flamigni, C., 1998. Oocyte cryopreservation. Hum. Reprod. 13, 98–108.

Fadini, R., Dal Canto, M.B., Renzini, M.M., Brambillasca, F., Comi, R., Fumagalli, D., Lain, M., De Ponti, E., 2009a. Predictive factors in in-vitro maturation in unstimulated women with normal ovaries. Reprod. Biomed. Online 18, 251–261.

Fadini, R., Dal Canto, M.B., Renzini, M.M., Brambillasca, F., Comi, R., Fumagalli, D., Lain, M., Merola, M., Milani, R., De Ponti, E., 2009b. Effect of different gonadotrophin priming on IVM of oocytes from women with normal ovaries: a prospective randomized study. Reprod. Biomed. Online 19, 343–351.

Gelbaya, T.A., Kyrgiou, M., Tsoumpou, I., Nardo, L.G., 2008. The use of estradiol for luteal phase support in in vitro fertilization/intracytoplasmic sperm injection cycles: a systematic review and meta-analysis. Fertil. Steril. 90, 2116–2125.

Heijnen, E.M., Eijkemans, M.J., De Klerk, C., Polinder, S., Beckers, N.G., Klinkert, E.R., Broekmans, F.J., Passchier, J., Te Velde, E.R., Macklon, N.S., Fauser, B.C., 2007. A mild treatment strategy for in-vitro fertilisation: a randomised non-inferiority trial. The Lancet 369, 743–749.

Hreinsson, J., Fridström, M., 2004. In vitro oocyte maturation for safer treatment of infertility. The risk of ovarian overstimulation syndrome is minimized. Läkartidningen 101, 3665–3668.

Jurema, M.W., Nogueira, D., 2006. In vitro maturation of human oocytes for assisted reproduction. Fertil. Steril. 86, 1277–1291.

Lenton, E.A., 2007. Natural cycle IVF with and without terminal HCG: learning from failed cycles. Reprod. Biomed. Online 15, 149–155.

Lim, J.H., Yang, S.H., Xu, Y., Yoon, S.H., Chian, R.C., 2009. Selection of patients for natural cycle in vitro fertilization combined with in vitro maturation of immature oocytes. Fertil. Steril. 91, 1050–1055.

Macklon, N.S., Stouffer, R.L., Giudice, L.C., Fauser, B.C.J.M., 2006. The science behind 25 years of ovarian stimulation for in vitro fertilization. Endocr. Rev. 27, 170–207.

Mikkelsen, A.L., Smith, S., Lindenberg, S., 2000. Possible factors affecting the development of oocytes in in-vitro-maturation. Hum. Reprod., 11–17, 15-12-2000. Ref. Type: Generic.

Nargund, G., Frydman, R., 2007. Towards a more physiological approach to IVF. Reprod. Biomed. Online 14, 550–552.

Nargund, G., Waterstone, J., Bland, J.M., Philips, Z., Parsons, J., Campbell, S., 2001. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. Hum. Reprod. 16, 259–262.

Pelinck, M.J., Hoek, A., Simons, A.H.M., Heineman, M.J., 2002. Efficacy of natural cycle IVF: a review of the literature. Hum. Reprod. Update 8, 129–139.

Soderstrom-Anttila, V., Makinen, S., Tuuri, T., Suikkari, A.M., 2005. Favourable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. Hum. Reprod. 20, 1534–1540.

Son, W.Y., Chung, J.T., Herrero, B., Dean, N., Demirtas, E., Holzer, H., Elizur, S., Chian, R.C., Tan, S.L., 2008. Selection of the optimal day for oocyte retrieval based on the diameter of the dominant follicle in hCG-primed in vitro maturation cycles. Hum. Reprod. 23, 2680–2685.

Steptoe, P.C., Edwards, R.G., 1978. Birth after reimplantation of a human embryo. Lancet 2, 366, 12-8-1978. Ref. Type: Generic.

Thornton, M.H., Francis, M.M., Paulson, R.J., 1998. Immature oocyte retrieval: lessons from unstimulated IVF cycles. Fertil. Steril. 70, 647–650.

Vlaisavljevic, V., 2007. Embryo transfer and luteal support in natural cycles. Reprod. Biomed. Online 14, 686–692.

*Declaration: The authors report no financial or commercial conflicts of interest.*

Received 23 March 2011; refereed 6 October 2011; accepted 6 October 2011.