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Contribution of in-vitro maturation in ovarian stimulation cycles of poor-responder patients

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Abstract This cohort study evaluated whether rescue spontaneous maturation (RSM) could be a valid method to increase the number of embryos available for transfer and whether transfers with RSM-derived embryos would contribute to clinical outcomes of poor-responder patients in ovarian stimulation cycles. The study included 440 patients undergoing intracytoplasmic sperm injection cycles in which fewer than five metaphase II (MII) oocytes and at least one immature oocyte were retrieved after follicle aspiration. Patients were allocated into two groups based on the injected oocytes' nuclear maturation status: MII group ($n = 330$), in which only embryos derived from MII oocytes were transferred, and RSM group ($n = 110$), in which at least one embryo derived from an RSM oocyte was transferred. No differences between the MII and RSM groups were observed for pregnancy (16.7% versus 16.5%) or miscarriage (25.5% versus 29.4%) rates, respectively. The RSM group had a higher number of transferred embryos (1.87 ± 1.24 versus 2.35 ± 1.22 ; $P < 0.001$), a lower embryo transfer cancellation rate (14.5% versus 6.36%; $P = 0.025$) and lower implantation rate ($15.4 \pm 31.5\%$ versus $10.5 \pm 22.3\%$; not significant). These findings suggest that RSM did not contribute to the outcomes in poor-responder cycles. 

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Introduction

The first successful childbirth resulting from IVF was produced with a natural cycle of IVF following in-vitro maturation (IVM) (Steptoe and Edwards, 1978). The success rate of assisted reproduction technology, however, is strictly dependent on the number of oocytes and top-quality embryos obtained. Due to such limitation, the use of ovarian stimulation was developed, replacing the natural cycles of IVF (Johnston et al., 1981; Lopata et al., 1978).

During ovarian stimulation, women are usually treated with an agonist or antagonist of gonadotrophin-releasing hormone (GnRH) to block the action of the pituitary. In addition, ovarian stimulation using gonadotrophins is performed in order to induce the development and final maturation of multiple follicles (Eldar-Geva et al., 1999). After stimulation, due to a lack of perfect maturation synchronicity, oocytes of different developmental stages are retrieved (Cha and Chian, 1998; Trounson et al., 2001). In such cases, some immature oocytes, retrieved at either prophase I (PI) or metaphase I (MI) stages, have the potential to undergo nuclear maturation and further development (Magli et al., 2006). Thus, in-vitro nuclear maturation may be an alternative to increase the number of embryos obtained from ovarian stimulation cycles, especially in cases of poor-responder patients (Strassburger et al., 2004).

A dynamic assessment of the ovarian reserve could be associated with the ovarian response to stimulation with gonadotrophins during IVF treatment (Nikolaou et al., 2002). Previous studies suggest that, among patients undergoing IVF treatment, the prevalence of poor ovarian response is from 9% to 24% (Kumbak et al., 2009), which is defined based on the number of oocytes retrieved after ovarian stimulation.

Oocyte maturation remains a poorly understood process, generally defined as the period from the initiation of germinal vesicle breakdown to the completion of the nuclear changes leading to the extrusion of the first polar body (Lin and Hwang, 2006). The completion of the nuclear changes to produce a metaphase II (MII) oocyte does not, however, identify developmental competence and does not reflect the oocyte's molecular and structural maturity (Trounson et al., 2001).

Previous studies reported successful fertilization, embryo development and pregnancy using immature human oocytes (Chian et al., 2004; Jaroudi et al., 1999; Lim et al., 2007; Papanikolaou et al., 2005). However, the performance of these oocytes is poor, resulting in reduced developmental potential (De Vos et al., 1999; Jaroudi et al., 1999).

Attempts have been made to promote maturation of immature human oocytes retrieved from stimulated cycles and, even though successful fertilization, embryo development and pregnancy have been reported (Chian et al., 2002; Kim et al., 2000; Liu et al., 2003), the literature on the developmental potential of these oocytes in ovarian stimulation cycles is still scarce.

The goal for this work was to investigate whether rescue spontaneous maturation (RSM) of immature oocytes retrieved from ovarian stimulation cycles of poor-responder patients, may improve the intracytoplasmic sperm injection (ICSI) outcomes. The primary endpoints of this trial were pregnancy and implantation rates. Secondary endpoints

were the number of embryos available for transfer, cycle cancellation and miscarriage rates.

Materials and methods

Experimental design

This cohort study included 440 poor-responder patients undergoing ICSI cycles, in which less than five MII oocytes and at least one immature oocyte (MI or PI oocyte) were retrieved after follicle aspiration. Written informed consent was obtained, in which patients agreed to share the outcomes of their cycles for research purposes. The study was approved by the local institutional review board.

Cycles were split into two groups based on the nuclear maturation status of the injected oocytes. Cycles in which only embryos derived from MII oocytes were injected (MII group, $n = 330$), were age matched with cycles in which at least one immature oocyte maintained in culture for spontaneous maturation was injected (rescue spontaneous maturation, RSM group; $n = 110$).

The rates of in-vitro spontaneous nuclear maturation, fertilization and high-quality embryos were evaluated. In addition, the rates of embryo transfer cancellation, pregnancy, implantation and miscarriage when RSM oocytes were injected/transferred were also investigated.

The implantation rate was defined as the total number of gestational sacs divided by the total number of embryos transferred. Clinical pregnancy was defined as the presence of a gestational sac with fetal heart beat visualized by ultrasound 4–6 weeks after embryo transfer; miscarriage was defined as the spontaneous loss of a pregnancy before 24 weeks' gestation.

All cycles from RSM group had at least one embryo derived from RSM transferred.

Ovarian stimulation

Ovarian stimulation was achieved by long pituitary down-regulation using a gonadotrophin-releasing hormone agonist (GnRHa, Lupron Kit; Abbott S.A Societ e Fran aise des Laboratoires, Paris, France) followed by ovarian stimulation with recombinant FSH (Gonal-F; Serono, Geneva, Switzerland). Follicular dynamic was followed by ultrasound, starting on day 4 of gonadotrophin administration. When adequate follicular growth and serum oestradiol concentrations were observed, recombinant human chorionic gonadotrophin (HCG, Ovidrel; Serono) was administered to trigger final follicular maturation. Oocytes were collected 35 h after HCG administration by transvaginal ultrasound ovum retrieval.

Preparation of oocytes

After retrieval, oocytes were incubated in culture medium (G-MOPS-V1; Vitrolife, Kungsbacka, Sweden) covered with mineral oil (Ovoil; Vitrolife) at 37°C and 6% CO₂ for 5 h. Cumulus cells were removed with a 30 s exposure to HEPES-buffered medium containing 80 IU/ml hyaluronidase (Irvine Scientific, Santa Ana, USA), after which coronal cells were manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA).

The denuded oocytes were then assessed for nuclear status. Oocytes that were observed to have released the first polar body were considered mature and used for ICSI; immature oocytes were cultured *in vitro*.

Rescue spontaneous maturation

Immature oocytes were classified as PI- or MI-stage based on whether or not a germinal vesicle was visible. Oocytes with no visible germinal vesicle were considered as MI-stage cells. Immature oocytes were incubated in culture medium (G-1-V1; Vitrolife), at 37°C and 6% CO₂. After 24 h in culture, oocytes that had undergone nuclear maturation and reached the MII stage underwent ICSI, if suitable.

ICSI

For ICSI, oocytes were placed individually in 4 µl droplets of buffered medium (G-Mops-V1). Sperm was placed in a central 4 µl droplet of polyvinylpyrrolidone solution (PVP; Irvine ScientificUSA) in a 50 × 40 mm glass culture dish (WillCo dish; Willco Wells, Vineland, New Jersey, USA) covered with warm mineral oil (Ovoil). Sperm injection was carried out on the heated stage (37°C) of an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan) 40 h after HCG trigger for MII-stage retrieved oocytes and 64 h after HCG trigger for immature oocytes that had undergone nuclear maturation.

Assessment of fertilization, embryo quality and embryo transfer

Fertilization was assessed 18 h after ICSI and normal fertilization was declared when two clearly distinct pronuclei were present. Embryo quality was evaluated under an inverted microscope (Eclipse TE 300). The following parameters were recorded: (i) the number of blastomeres; (ii) the fragmentation percentage; (iii) variation in blastomere symmetry; (iv) the presence of multinucleation; and (v) defects in the zona pellucida and the cytoplasm.

Embryo transfer was performed on day 2 or 3 of development. High-quality (grade A) embryos were defined as those having all of the following characteristics: either 4–6 cells

on day 2 or 8–10 cells on day 3 of development, less than 15% fragmentation, symmetric blastomeres, absence of multinucleation, colourless cytoplasm with moderate granulation with no inclusions, absence of perivitelline space granularity and absence of zona pellucida dysmorphism. Embryos lacking any of the above characteristics were considered as low quality.

For each couple, one to four embryos were transferred, depending on the embryo quality and the female's age. Embryo transfer was cancelled if there are no embryos available, when patients in each group had complete fertilization failure or the embryos failed to divide. In the RSM group, priority for transfer was given for embryos derived from MII oocytes.

Statistical analysis

Results are expressed as mean ± standard deviation (SD) for numeric variables and percentages for categorical variables. Mean values were compared by Student's *t*-test, and proportions were compared by the chi-squared or Fisher's exact test, where appropriate.

To study the influence of the injection of RSM oocytes on the ICSI clinical outcomes, logistic regression models were conducted. For continuous variables such as the number of transferred embryos and implantation, linear regression analyses were performed and the results were expressed as regression coefficients (RC) and *P*-values. For dichotomic variables such as embryo transfer cancellation, pregnancy and miscarriage, binary logistic regression analyses were used and the results were expressed as odds ratios (OR), 95% confidence intervals (CI) and *P*-values.

Results were considered to be significant at the 5% critical level (*P* < 0.05). Data analysis was carried out using the statistical analysis program Minitab version 14.

Results

General characteristics

Experimental groups were similar for female age, number of aspirated follicles, number of MII retrieved oocytes, percentage of immature oocytes and total dose of FSH

Table 1 General characteristics of the study groups in which only embryos derived from metaphase II (MII) oocytes were transferred or at least one embryo derived from a rescue spontaneous maturation (RSM) oocyte was transferred.

	Oocyte stage at retrieval	
	MI (n = 330)	RSM (n = 110)
Female age (years)	37.69 ± 2.86	36.46 ± 4.05
Aspirated follicles	7.03 ± 4.89	6.81 ± 2.97
MI retrieved oocytes	2.48 ± 1.18	2.49 ± 0.98
Immature retrieved oocytes	28.0	32.0
Total dose of FSH administered	2547.70 ± 628.90	2583.70 ± 765.60
Transferred embryos	1.87 ± 1.24 ^a	2.35 ± 1.22

Values are mean ± SD or %.

^aMI group was significantly lower than RSM group (*P* < 0.001).

Table 2 Number of retrieved oocytes, in-vitro matured oocytes, fertilized oocytes and high-quality embryos in relation to the oocyte nuclear maturation status.

	Nuclear maturation status			Total	P-value
	MII (n = 1345)	MI (n = 227)	PI (n = 263)		
In-vitro matured oocytes	—	172 (75.8) ^a	99 (37.6) ^b	271	<0.001
Injected oocytes	1318 (98.0) ^a	140 (81.4) ^b	72 (72.7) ^b	1530	<0.001
Fertilized oocytes	987 (74.9) ^a	91 (65.0) ^b	25 (34.7) ^b	1103	<0.001
High-quality embryos	572 (58.0) ^a	24 (26.4) ^b	6 (24.0) ^b	602	<0.001

Values are number (%).

Values within rows with different superscript letters differ significantly ($P < 0.001$).

MII = metaphase II; MI = metaphase I; PI = Prophase I.

administered for ovarian stimulation, although the number of transferred embryos was higher in the RSM group (Table 1).

Nuclear maturation status and spontaneous in-vitro maturation

The overall number of retrieved oocytes was 1835. From these oocytes, 1345 (73.3%) were in MII stage, 227 (12.4%) were in MI stage, and 263 (14.3%) were in PI stage. Metaphase II stage was achieved *in vitro* in 271 (55.3%) oocytes. A significantly higher percentage of oocytes derived from the MI stage achieved the MII stage *in vitro* when compared with those derived from the PI stage (75.8% versus 37.6%, $P < 0.001$; Table 2).

Fertilization and embryo quality

ICSI was performed in 1530 oocytes: 1318 derived from in-vitro matured oocytes, 140 derived from MI-stage oocytes and 72 derived from PI-stage oocytes (RSM oocytes) (Table 2).

Fertilization rates were significantly higher for in-vitro matured oocytes when compared with RSM oocytes (74.9% versus 54.7, respectively; $P < 0.001$) and a significantly higher percentage of high-quality embryos was derived from in-vitro matured than RSM oocytes (58.0% versus 24.0%, respectively; $P < 0.001$).

Embryo transfer and clinical outcomes

Most embryos transfers was performed on day 3 for both groups (89.3% and 91.8% for MII and RSM groups, respectively). No differences were observed in pregnancy (16.7%

versus 16.5% for MII and RSM groups, respectively) or miscarriage rates (25.5% versus 29.4% for MII and RSM groups, respectively) when RSM and MII groups were compared.

On the other hand, the number of transferred embryos was higher (1.87 ± 1.24 versus 2.35 ± 1.22 for MII and RSM groups, respectively; $P < 0.001$) and the embryo transfer cancellation (14.5% (48/330) and versus 6.36% (7/110) for MII and RSM groups, respectively; $P = 0.025$) was lower in the RSM group. Moreover, a non-significant trend for lower implantation rate in the RSM group was noted (15.4% versus 10.5% for MII and RSM groups, respectively). These most likely did not reach statistical significance because of the small number of cycles evaluated in this trial.

The cycles were also split according to the percentage of embryos derived from RSM oocytes transferred, and no significant difference in the pregnancy and implantation rates was noted (Table 3).

In 17 cycles, only embryos derived from RSM oocytes were available for transfer and two pregnancies were achieved. The implantation rate was 4.7%, the mean number of transferred embryos was 1.3 and the high-quality embryo rate was 22.7%. In this group, 10 patients had only immature oocytes retrieved and in seven patients, the retrieved MII oocytes failed to fertilize. In patients from whom only immature oocytes were retrieved, the mean age was 36.5 ± 2.5 years, the total dose of FSH administered was 2500 IU and ovarian factor was the cause of infertility of all patients.

Discussion

In stimulated cycles, pharmacological doses of gonadotrophins create a supraphysiological hormonal environment

Table 3 Pregnancy and implantation rates of patients in which the percentage of embryos derived from rescue spontaneously matured (RSM) oocytes transferred was 0%, 25%, 50%, 75% and 100%.

	Percentage of RSM oocytes transferred				
	0 (n = 282)	25 (n = 36)	50 (n = 30)	75 (n = 20)	100 (n = 17)
Pregnancy rate	16.7 (47/282)	19.4 (7/36)	16.7 (5/30)	15.0 (3/20)	11.8 (2/17)
Implantation rate	15.4	11.1	8.8	11.2	4.7

Values are expressed as % (number/total). percentage.

No statistically significant differences were found.

that induces the growth of a cohort of follicles, which, under natural conditions, would become atretic and regress (Fortune, 2001). De Vos et al. (1999) showed that ovarian stimulation leads to the retrieval of oocytes at various stages of meiotic maturity and that some of these oocytes may complete maturation spontaneously *in vitro* (Chian et al., 2002).

This study evaluated the usefulness of RSM of oocytes retrieved from ovarian stimulation cycles. It found that, while RSM potentially increases the number of embryos available for transfer and therefore decreases embryo transfer cancellation, it yields oocytes showing lower rates of fertilization and production of high-quality embryos than MII-retrieved oocytes. In addition, no contribution from the use of RSM oocytes was observed for pregnancy and implantation.

Previous studies demonstrated that implantation rates of in-vitro matured oocytes are significantly lower when compared with the rates found for in-vivo matured oocytes, suggesting that the maturation process may be affected by the in-vitro culture conditions (De Vos et al., 1999). Additionally, it is well recognized that complete oocyte maturation depends not only on nuclear maturity but also on the quality and maturity of the ooplasm and the plasma membrane system (Bao et al., 2000; Ji et al., 1997).

Although nuclear maturation can be easily accomplished *in vitro*, a concomitant maturation of the cytoplasm does not seem to occur properly, as indicated by the absence of specific proteins in the cytoplasm of in-vitro matured oocytes (Anderiesz et al., 2000). In fact, according to Van Blerkom (1996), a higher incidence of meiotic errors may be a consequence of defective cytoplasmic maturation or loss of synchrony between nuclear and cytoplasmic maturation.

In the present experiment, oocytes were retrieved from ovarian stimulation cycles. It would not be surprising to find a suboptimal developmental capacity in oocytes that failed to mature *in vivo* despite the exposure to supraphysiological concentrations of exogenous gonadotrophins. Therefore, even though RSM was able to decrease the chance of embryo transfer cancellation, the implantation rate was lower when at least one embryo derived from RSM oocyte was transferred compared with the group in which only embryos derived from MII oocytes were transferred.

Moreover, when exclusively RSM embryos were transferred, the implantation rate was extremely low. This special group of patients in which only RSM embryos were available for transfer is of much interest. Although only 17 cycles are present in this group, this study tried to characterize a profile for these cycles and it noted that the mean age and the total dose of FSH were similar when compared with the MII group. The high-quality embryo rate was much lower than the MII group and slightly lower than the RSM group.

The onset of preovulatory maturation is marked by a dramatic rise in gonadotrophin release from the pituitary, especially LH (Webb et al., 2004). Trounson et al. (2001) suggested that follicles containing immature oocytes after the administration of large doses of HCG must lack sufficient blood supply to receive the ovulatory stimulus or have insufficient LH receptors to induce oocyte maturation *in vivo*, as substantiated by the frequent non-expansion of the corre-

sponding cumuli (Magli et al., 2006). Furthermore, when recovered after ovarian stimulation, immature oocytes may have a delayed maturation process; the reduced developmental competence compared with mature MII oocytes may be related to the inability of the follicles to respond to HCG administration synchronously with other larger follicles.

Despite positive results presented in previous studies (Mikkelsen et al., 1999; Soderstrom-Anttila et al., 2005; Yoon et al., 2001), IVM has not yet become a mainstream assisted reproduction technique. This is primarily because of the lower pregnancy rate as compared with conventional IVF/ICSI. In fact, in order to achieve pregnancy, more attempts are necessary using IVM to compare with IVF (Suikkari and Soderstrom-Anttila, 2007). In addition, there are still concerns regarding the safety of this procedure (Chian et al., 2004). Mikkelsen (2005), reported one case of cleft palate in 47 children born after IVM. Furthermore, Cha et al., (2005) demonstrated, that IVM does not adversely affect the miscarriage rate, gestational age and birthweight of delivered infants or the rate of pregnancy complications; however, a major malformation rate following IVM of 5.3% was observed. Another report showed good obstetric and perinatal outcomes of IVM pregnancies (Soderstrom-Anttila et al., 2006).

These results show that oocytes derived from the PI stage present lower maturation and developmental competence as compared with those derived from MI-stage oocytes. Considering that nuclear maturation consists of germinal vesicle breakdown, resumption of meiosis and first polar body extrusion, it is assumed that the exposure to the in-vitro environment during a more complex phase of development may have important consequences for the potential of human oocytes. Furthermore, as suggested by Chian et al. (1997), the initiation of the maturation process *in vivo* may be important to the acquisition of full developmental competence by the oocyte.

Despite the difference in the number of patients in the studied groups, every patient in the RSM group was age matched with three patients in the MII group. Moreover, the groups were homogeneous regarding the number of aspirated follicles, number of MII retrieved oocytes, percentage of immature retrieved oocytes and total dose of FSH administered. This could diminish a possible bias of the study.

In summary, these results raise a question about the contribution of RSM in poor-responder ovarian stimulation cycles. Although previous studies demonstrated that RSM may increase the number of embryos available for transfer and transfers of exclusively RSM oocyte-derived embryos may result in few pregnancies, the pregnancy and implantation rates observed in this trial suggested that RSM did not contribute to the ICSI outcomes in poor-responder cycles.

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