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The outcome of ICSI of immature MI oocytes and rescued *in vitro* matured MII oocytes

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BACKGROUND: The use of immature oocytes is limited to cases where these are the only available oocytes, and they are usually only microinjected with sperm after having undergone maturation *in vitro*. This study compares the outcome of injection of sperm into metaphase I oocytes immediately after their denudation (MI) performed 2 h after their retrieval, with the outcome of injection of sperm into rescued *in vitro* matured metaphase II (IVM MII) oocytes after their short incubation in routine laboratory conditions. METHODS: ICSI was performed on MI oocytes, rescued IVM MII oocytes and on MI oocytes that were incubated but failed to extrude their first polar body (arrested IVM MI). Fertilization and cleavage rates were compared with those achieved in mature metaphase II oocytes (MII). RESULTS: ICSI of MI oocytes showed impaired performance compared with ICSI of rescued IVM MII oocytes and MII oocytes, in terms of oocyte degeneration rate (11 versus 6 versus 4%; $P < 0.0001$), fertilization rate (28 versus 44 versus 68%; $P < 0.0001$) and multipronucleated fertilization (10 versus 4 versus 4%; $P < 0.01$). The cleavage rate was lower in rescued IVM MII oocytes compared with MII oocytes (86 versus 95%; $P < 0.01$). Arrested IVM MI oocytes showed similar results to those of MI oocytes but had a lower cleavage rate (72 versus 96%; $P < 0.01$). CONCLUSIONS: The injection of rescued IVM MII oocytes is preferred to the injection of MI oocytes.

Key words: fertilization/ICSI/*in vitro* maturation/MI oocytes

Introduction

Under controlled ovarian stimulation, some of the collected oocytes are immature (Smith *et al.*, 2000). These oocytes are usually discarded due to the possibility of abnormal embryonic development, or an increased rate of abortion. However, in cases of poor responders and in patients with an unsynchronized cohort of follicles, where the presence of immature oocytes is frequent after stimulation (Smitz and Cortvrindt, 1999), the use of immature oocytes for IVF is important in order to increase the number of embryos obtained in each cycle. Based on the assumption that oocyte maturity is a prerequisite for obtaining normal fertilization, attempts have been made in mammals and in human to mature germinal vesicle (GV) and metaphase I (MI) oocytes *in vitro* (Lanzendorf *et al.*, 1990; Janssenswillen *et al.*, 1995; Meng and Wolf, 1997; Huang *et al.*, 1999). Despite the use of varying culture techniques and oocytes from different treatment procedures, *in vitro* matured (IVM) oocytes yield lower fertilization rates, frequent cleavage blocks and overall retarded cleavage rate compared with MII oocytes (Lanzendorf *et al.*, 1990; Meng and Wolf, 1997; De Vos *et al.*, 1999; Huang *et al.*, 1999; Chen *et al.*, 2000; Bonu *et al.*, 2001). In addition, normal pregnancies and live births derived from IVM oocytes are mainly limited to case reports (Nagy *et al.*, 1996; Edirisinghe *et al.*, 1997; Liu *et al.*, 2003). It seems that culture systems adequately support nuclear maturation in immature human oocytes but fail to produce oocytes with cytoplasmic competency, which can ensure the formation of bipolar spindles with aligned chromosomes that will enable subsequent events such as fertilization (Mikkelsen *et al.*, 2000; Cekleniak *et al.*, 2001; Combelles *et al.*, 2002).

The aim of the present study was to clarify whether the injection of sperm to rescued matured MII (IVM MII) oocytes (oocytes that underwent nuclear maturation) is preferential to the injection of MI oocytes immediately after denudation, and thus to asses the advantage of the extrusion of the first polar body (PB; nuclear maturation) in terms of ICSI outcome.

Materials and methods

The study had approval from the ethical committee of Assaf Harofeh Medical Center. The study group comprised 237 women undergoing 436 ICSI cycles between July 1999 and July 2000, in which a long protocol was used for controlled ovarian stimulation. Female patients were not pre-selected, but were included only if at least one harvested MI oocyte per cycle was present. The 237 patients had an average age of 31.1 ± 5.3 years. In 178 cycles (101 women), only one MI oocyte was found. These 178 cycles were randomly divided, according to the patient, into two groups: one in which the MI oocyte was immediately injected, and the other one where the MI oocyte was incubated for *in vitro* maturation. The average age of the patients in both groups was

Table I. Fertilization rate of MI, arrested IVM MI, rescued IVM MII and MII injected oocytes

	MI ocytes	Arrested IVM MI oocytes	Rescued IVM MII oocytes	MII oocytes	*P*-value
Injected oocytes (*n*)	249	141	116	4668	
Degeneration (%)	27 (11)[1]	15 (11)[2]	7 (6)	187 (4)[1,2]	<0.001[a]
2PN (%)	69 (28)[3,4]	47 (33)[5]	51 (44)[3,6]	3174 (68)[4,5,6]	<0.01[a]
Embryos resulting from 2PN 1-cell zygotes (%)	66 (96)[7]	34 (72)[7,8]	44 (86)[9]	3047 (95)[8,9]	<0.01[a]
1PN (%)	12 (5)	8 (5)	6 (5)	233 (5)	NS[b]
3PN (%)	26 (10)[10]	9 (6)	5 (4)	186 (4)[10]	<0.0001[b]
No. of blastomeres (mean ± SD)	3.3 ± 1.0	3.1±1.0	3.1 ± 1.1	3.4 ± 1.0	NS[c]
Morphology score (mean ± SD)	1.5 ± 0.7	1.5 ± 0.5	2.0 ± 1.0	1.9 ± 0.7	NS[c]

[a]χ^2 test; [b]Fisher's exact test; [c]one-way ANOVA.
Comparisons were made between numbers denoted with the same superscript.

similar (32.2 ± 5.5 versus 31.9 ± 5.04 years). The indications for assisted reproductive technology (ART) were distributed similarly. In 199 of the cycles, at least two MI oocytes were identified, and divided into two groups (sibling oocytes). The women had normal gonadotrophin levels with normal response to controlled ovarian stimulation. HCG was administered when the leading follicle was at least 20 mm in diameter. The mean estradiol (E_2) level on the day of HCG administration was 2414.2 ± 1318.7 pg/ml, and the mean progesterone was 1.6 ± 1.9 ng/ml.

Oocyte preparation

Oocyte retrieval was performed by vaginal ultrasound-guided puncture. At 2 h after retrieval, the cumulus–corona cells were removed after exposure to hyaluronidase (type IV-S, Sigma) 20 IU/ml in human tubal fluid medium (HTF; Irvine Scientific, Santa Anna, CA) for no more than 1 min. Oocyte evaluation was performed using an inverted microscope (Diaophot 300; Nikon, Japan) with an enlargement of ×400. GV oocytes were discarded. MI oocytes were represented by the absence of a PB and no discernible GV nucleus. MI oocytes were separated from the MII oocytes and randomly divided into two groups (sibling oocytes). In the first group, MI oocytes were injected immediately after denudation (MI oocytes). In the second group, MI oocytes were incubated *in vitro* in 25 µl droplets of medium (G1.2. Vitrolife, Molndalsven, Goeteborg, Sweden) without any supplementation of serum or hormones, under light weight paraffin oil (Sigma, Israel, 8042-47) for 1.5–4 h before injection. The incubated MI oocytes were evaluated for maturation after 1.5, 2.5 and 4 h. After their incubation period, the oocytes were re-evaluated and divided into those which extruded their PB (rescued IVM MII oocytes) and those that failed to do so (arrested IVM MI). Both rescued IVM MII oocytes and arrested IVM MI oocytes were injected. The MII oocytes that were retrieved in the same treatment cycles served as the control group (MII oocytes).

ICSI procedure

The procedure was carried out according to the methodology described by Van Steirteghem *et al.* (1993). Culture of the injected oocytes took place in 25 µl medium droplets ($G_{1.2.}$Vitrolife) under oil. Oocytes were observed for fertilization 16–18 h after micromanipulation. The criterion for normal fertilization was the presence of two extruded PBs and two pronuclei. Cleavage was assessed 24 h later and the embryos were classified according to their morphological appearance. Embryos were classified as grade I, symmetrical blastomeres with no anucleated fragments; grade II, asymmetrical blastomeres without or with <20% of the volume filled with anucleated fragments; grade III, with 20–50% of the volume covered with fragments; and grade IV, extreme asymmetrical blastomeres and >50% of the volume filled with anucleated fragments. Embryos which originated from MI oocytes were transferred into the uterus only in cases where there were not enough embryos from MII oocytes. Generally, transfers included retrieved MII oocytes, except for four cases (see Results) where none were available.

Statistical analysis.

Statistical evaluation was performed using multivariant analyses and then χ^2 and Fisher's exact tests, where appropriate. A *P*-value of <0.05 was considered statistically significant.

Results

Of the 5843 oocytes retrieved, 349 (6%) were either degenerated before or due to denudation. Of the healthy oocytes, 4668 (80%) were at the MII stage and underwent ICSI, 506 (8.7%) oocytes were MI oocytes and 320 (5.5%) were GV oocytes; the latter were discarded. Of the immature MI oocytes, 249 were injected immediately after denudation and 257 MI oocytes were cultured. From the last group, 116 (45.1%) matured *in vitro* (rescued IVM MII). The time interval from oocyte denudation to completion of *in vitro* maturation of MI oocytes was as follows: 15 (13%) of 116 oocytes underwent maturation by 1.5 h of incubation, another 48 (41%) completed their incubation by 2.5 h and the remaining 53 (46%) matured after 4 h. Mean incubation time was 2.1 ± 1.1 h. There were also 141 oocytes (55%) which failed to extrude their first PB (arrested IVM MI oocytes) after the maximum incubation period of 4 h. All of these oocytes underwent ICSI.

Table I compares ICSI outcome of immediately injected MI oocytes with arrested IVM MI, rescued IVM MII oocytes and MII oocytes. The degeneration rate was significantly higher in MI oocytes compared with rescued IVM MII oocytes and MII oocytes (11 versus 6 versus 4%), $P < 0.0001$. The fertilization rate of 2PN 1-cell zygotes from MI oocytes was significantly lower than that found in rescued IVM MII oocytes and in MII oocytes (28 versus 44 versus 68%), $P < 0.0001$. The incidence of multinucleated zygotes (3PN) was similar between rescued IVM MII and MII oocytes but higher in non-incubated MI oocytes (4 versus 10%), $P < 0.01$. Multipronucleated zygotes of the last three groups had two PBs at an incidence of 75, 77 and 78%, respectively. Cleavage rates were similar in both MI and MII oocytes, but significantly lower among the rescued IVM MII (95 versus 86%), $P < 0.01$.The number and quality of the blastomeres were similar in all the three groups. There were

141 arrested IVM MI oocytes injected after incubation. When comparing ICSI outcome between arrested IVM MI and MI oocytes, similar degeneration, fertilization and 1PN rates could be found. The cleavage rate of 2PN embryos was significantly higher among the MI oocytes with no incubation period compared with arrested IVM MI oocytes (96 versus 72%; $P < 0.001$). The number of blastomeres as well as embryo morphology was similar.

In 504 (94%) of the cycles, 1462 embryos were transferred. Out of these, in 104 (20.6%) of the cycles, mixed embryos from both mature and at least one MI originating embryo were transferred. In 49 (47%) of the 104 transfers, the embryos resulted from MI oocytes that were injected immediately after denudation, in 37 (35.5%) they were from rescued IVM MI oocytes, and in 18 (17.3%) of the cycles the embryos were from arrested IVM MI oocytes. In four cycles, only embryos originating from MI oocytes were available and were transferred as follows: case 1, two embryos resulting from two rescued IVM MII oocytes and one from an arrested IVM MI oocyte; and case 2, two embryos resulting from MI oocytes; neither woman conceived. Case 3 was a single embryo originating from a rescued IVM MII oocyte. The woman conceived but the pregnancy terminated with a missed abortion. In case 4, a single MI oocyte was retrieved and after 4 h of incubation failed to mature. This arrested IVM MI oocyte was injected, fertilized and underwent cleavage. The embryo was transferred, implanted and developed to a full-term pregnancy. A healthy neonate was born with a normal karyotype.

Discussion

The clinical use of MI oocytes for ICSI is questionable, even when *in vitro* maturation is achieved in these immature oocytes (Smitz and Cortvrindt, 1999). There seems to be no agreement regarding the optimal incubation period for maturation. In the present study, the outcome of ICSI in MI oocytes that were injected with sperm immediately after denudation was compared with that of rescued IVM MII oocytes, arrested IVM MI oocytes and sibling mature MII oocytes. All these procedures were done within the framework of daily laboratory practice.

Forty-six percent of the MI oocytes that were left for the maximum 4 h incubation in culture medium matured spontaneously. This percentage was higher than those reported in other groups: 24% after a 4 h incubation (Junca *et al.*, 1995) and 27 (De Vos *et al.*, 1999) or 16% after 9 h (Chen *et al.*, 2000). In a kinetic experiment to characterize the timing of meiotic progression of GV oocytes, Combelles *et al.* (2002) showed that 89% of GV oocytes resume meiosis by 6 h in culture. After 18 h, 45 and 50% of the oocytes were in MI and in telophase, respectively, meaning that altogether 95% of the oocytes reached what we consider the MI stage after 18 h. Of these MI oocytes, 67% reached the MII stage after an additional 6 h of incubation. The rational for an incubation period of 4 h was based upon the time needed for oocyte maturation, convenience of working hours in the laboratory, and a need to reduce the risk of an ageing effect. We found that an incubation of 1.5 h led to 13% maturation, and 2.5 h yielded another 41% matured oocytes. This means that the majority of MI oocytes (54%) mature *in vitro* already after 2.5 h of incubation.

Longer incubation periods of MI oocytes, such as 24 and 36 h, were reported to yield higher maturation rates of 64 and 88%, respectively (Chen *et al.*, 2000), or 73 and 84% after 24 and 48 h, respectively, in P1 culture medium (Cekleniak *et al.*, 2001). However, embryos originating from aged oocytes had increased incidence of abnormal cytoskeletal organization and chromosomal imbalance (Racowsky and Kaufman, 1992; Plachot *et al.*, 1998). The variation in the maturation rate might also be related to the composition of the culture medium, which changes the pyruvate glucose uptake and lactate production by the IVM oocytes (Chian and Tan, 2002; Roberts *et al.*, 2002)

The injection of MI oocytes immediately after denudation, and the injection of arrested IVM MI oocytes showed a high degeneration rate that can be explained by increased fragility of the oolemma following ICSI. The extrusion of the first PB may indicate the changes in the oolemma or the cytoskeleton leading to the improved elasticity of the membranes, which is important for successful ICSI (Bourgain *et al.*, 1998).

The fertilization rate of MI oocytes that were injected immediately after denudation and of arrested IVM MI oocytes was lower than the fertilization rate of their sibling rescued IVM MII oocytes and less than half compared with that found among sibling MII oocytes. The improved rate of fertilization in the rescued IVM MII oocytes could be partially due to the fact that MI oocytes which are unable to undergo maturation as a result of complete absence of spindle formation (Windt *et al.*, 2001; Levran *et al.*, 2002) or failure of the oocyte to enter M phase (Hartshone *et al.*, 1999) were not included in this group.

A review of the current literature did not reveal any publication concerning ICSI outcome in MI oocytes that were injected without extrusion of their PB. On the other hand, reports exist concerning outcome of ICSI performed only in MI oocytes that matured *in vitro* after incubation period of 8 h, showing a 24–34% decline in fertilization rate compared with fertilization rates found among MII oocytes (Bonada *et al.*, 1996; Ben-Shlomo *et al.*, 1998). In a large series of 1210 MI oocytes that matured *in vitro*, De Vos *et al.* (1999) showed 52.7% of fertilizations compared with 70.8% in 8803 sibling MII oocytes after 4 h of incubation. These results concur with our results of 44% fertilization in rescued IVM MII oocytes versus 68% in the MII oocytes. Huang *et al.* (1999) showed significantly higher fertilization rates in MII oocytes after ICSI compared with IVM oocytes (81 versus 62%). However, his study group included GV as well as MI oocytes. The reduced fertilization rate of rescued IVM MII oocytes can be explained by cytoplasmic immaturity of these oocytes in contrast to their nuclear maturation (Goud *et al.*, 1998; Russel, 1999).

In the present study, a high rate of multipronucleated oocytes was found in MI oocytes that were injected immediately after denudation. In 78% of them, a second PB could be recognized. This result demonstrates that injection of oocytes in their early stages of maturation causes DNA disorganization.

The cleavage rate was significantly lower among the 2PN 1-cell zygotes that derived from both rescued IVM MII and arrested IVM MI oocytes, namely oocytes that were incubated

in vitro, in comparison with MI oocytes injected immediately after denudation or MII oocytes. This difference was not shown in other studies (De Vos *et al.*, 1999; Chen *et al.*, 2000); however, in the literature, it was shown that the main damage in long *in vitro* maturation was in the cleavage rate as well as in the chromosomal constitution (Chen *et al.*, 2000; Nogueira *et al.*, 2000).

Embryo quality and mean number of blastomeres in our study were similar among the groups whether the embryos resulted from MI oocytes that were immediately injected, MI oocytes that were incubated or mature MII oocytes. De Vos *et al.* (1999) reported slight differences regarding the number of good quality embryos resulting from rescued IVM MII oocytes. In contrast, Chen *et al.* (2000) found that MI oocytes incubated for long periods (24–36 h) led to the development of poorer quality embryos and less blastocysts compared with the outcome from MII oocytes. This difference could be explained by the different incubation times (Plachot *et al.*, 1988; Racowsky and Kaufman, 1992).

We conclude that the injection of rescued IVM MII oocytes is preferred to the injection of MI oocytes since rescued IVM MII oocytes lead to an increased fertilization rate (44 versus 28%) and a higher rate of embryo formation. The use of rescued IVM MII oocytes seems feasible; incubation time can be as short as up to 4 h and suitable for the laboratory routine timetable. The new method of spindle visualization by PolScope may contribute to discriminate rescued IVM MII oocytes with chromosome misalignment (Wang and Keefe, 2002), which might contribute to minimizing the use of oocytes with abnormal chromosome distribution.

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