Article

Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection

Dr Björn Heindryckx is a post-doctoral Research Scientist in the Department for Reproductive Medicine, Ghent University Hospital, Belgium. He obtained his PhD in Medical Sciences in 2006 at the Ghent University Hospital researching the optimisation of nuclear transfer and haploidization in mice and the oocyte activation mechanism. His major areas of interest include the study of parthenogenetic activation of human oocytes; failed ICSI; the optimisation of somatic cell nuclear transfer in humans for therapeutic stem cell purposes; and human embryonic stem cell derivation and differentiation into gametes.

Dr Björn Heindryckx

Björn Heindryckx[1], Stefanie De Gheselle, Jan Gerris, Marc Dhont, Petra De Sutter
Department for Reproductive Medicine, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium
[1]Correspondence: Tel: +32 9 332 4748; Fax: +32 9 332 4972; e-mail: Bjorn.Heindryckx@UGent.be

Abstract

Failed fertilization after intracytoplasmic sperm injection (ICSI) can occur due to an oocyte activation defect. In these cases assisted oocyte activation (AOA) may help but efficiency is still unknown. Prior to AOA, the mouse oocyte activation test (MOAT) can be carried out by injecting human spermatozoa into mouse oocytes to evaluate their activating capacity. According to the MOAT activation percentage achieved, patients were classified into three groups: 0–20% (16 patients); 20–85% (seven patients); 85–100% (seven patients). For AOA, $CaCl_2$ was injected together with spermatozoa followed by a double Ca^{2+} ionophore treatment. The fertilization rates before application of AOA in 50 cycles were 6%, 22% and 14% in, respectively, groups 1, 2 and 3 without any pregnancy. Fertilization and pregnancy rates after AOA in 61 cycles were significantly increased to 75% and 34% for group 1, 73% and 43% for group 2, and 75% and 17% for group 3 ($P < 0.0001$ and $P < 0.004$, respectively). Application of AOA results in normal fertilization and pregnancy rates in patients whose spermatozoa show deficient activation. When MOAT reveals no activation deficiency in spermatozoa, AOA still allows for high fertilization and acceptable pregnancy rates. The obstetric and neonatal outcomes after AOA were normal as no malformations were observed.

Keywords: *failed fertilization, ICSI, ionophore, oocyte activation*

Introduction

Intracytoplasmic sperm injection (ICSI) is currently the most efficient variant of assisted fertilization for treatment of predominantly male infertility and for patients who experienced previous fertilization failure using conventional IVF. Despite the long-term experience that many assisted reproduction laboratories have with this technology, fertilization failure after ICSI still occurs in some patients and may recur in subsequent cycles (Mahutte and Arici, 2003). The term 'failed fertilization' typically refers to failure of all the available mature metaphase II (MII) oocytes to fertilize. This can occur by chance when very few MII oocytes are available for ICSI, or can be due to the lack of motile spermatozoa on the day of oocyte retrieval. Still, the principal cause of failed fertilization has been attributed to an oocyte activation deficiency (Sousa and Tesarik, 1994), also occurring in cycles with a normal number of retrieved MII oocytes. Failure of oocyte activation is observed in approximately 40% of unfertilized oocytes exposed to ICSI (Rawe *et al*., 2000), while others have reported that activation failure was present in >70% of the unfertilized oocytes (Sousa and Tesarik, 1994; Flaherty *et al*., 1995, 1998; Yanagida, 2004).

Oocyte activation relies both on sperm-related and oocyte-related oocyte-activating factors (Tesarik *et al*., 2002) that transduce the sperm-derived signal to the oocyte's cell-cycle controlling systems via specific mechanisms. The first cellular event observed in all activated mammalian oocytes is an increase in the intracellular Ca^{2+} concentration. This initial trigger of Ca^{2+} increase starts within a few minutes of spermatozoa–egg fusion and takes the

form of a wave originating at the point of sperm entry (Tesarik and Mendoza, 1999). During ICSI, this trigger function is taken over by an artificial calcium influx from the surrounding medium produced by the micromanipulation procedure itself (Tesarik *et al.*, 2000). The first Ca^{2+} trigger is followed by a series of shorter high-frequency Ca^{2+} transients, the so-called Ca^{2+} oscillations that continue for several hours after fertilization or ICSI (Tesarik and Mendoza, 1999). Many research and concomitant theories have been described in the last decade about the oocyte activation mechanism and the origin of these Ca^{2+} oscillations (Swann and Ozil, 1994). From literature, there is now general support for the sperm factor hypothesis identified as phospholipase C zeta (PLCζ) (Saunders *et al.*, 2002), a protein factor released by the spermatozo0n into the egg cytosol. The exact role of the oocyte in the activation mechanism warrants further research.

For patients that have experienced failed or low fertilization rates after ICSI, it is of major importance to have a diagnostic test to assess the underlying cause of failure. The crucial question is how to distinguish the activation deficiency due to a lack or a depressed activity of a sperm factor from the impaired ability of the oocyte to respond to this factor in order to be activated. A heterologous ICSI model to evaluate the activation capacity of human spermatozoa by injection into mouse oocytes has been proposed (Rybouchkin *et al.*, 1996; Heindryckx *et al.*, 2005). After this diagnostic mouse oocyte activation test (MOAT), couples with an activation deficiency might benefit from a combined treatment of ICSI and artificially induced activation. Artificial activating agents such as ethanol, Ca^{2+} ionophores and electrical pulses cause a single and prolonged rise in Ca^{2+} in animal oocytes (Swann and Ozil, 1994). However, artificially produced Ca^{2+} rises do not always mimic precisely the sperm-induced Ca^{2+} oscillations, but might offer a solution for activation failure after ICSI.

A series of 17 patients undergoing assisted oocyte activation (AOA) has been previously reported (Heindryckx *et al.*, 2005). Chemical assisted oocyte activation treatment using calcium ionophore has been reported in six case reports (Heindryckx *et al.*, 2005; Ahmady and Michael, 2007), the use of electrical oocyte activation and Sr^{2+} ions has been proven successful in two case reports (Yanagida *et al.*, 1999, 2006), while others reported a mechanically modified ICSI technique to overcome activation failure (Tesarik *et al.*, 2002; Ebner *et al.*, 2004). Due to the limited number of patients described in the former studies, the efficiency of AOA in terms of pregnancy rates is still unknown. Since MOAT was not always performed in previous studies, little information has been gained about the efficiency of AOA, and in particular in correlation to MOAT.

In the present study, patients have been classified according to the MOAT results and, as far as is known, this is the first large-scale report describing the efficiency of fertilization and pregnancy rates after application of AOA for a series of couples who experienced failed or low fertilization after ICSI. The obstetric and neonatal outcome of established pregnancies after AOA is for the first time described.

Materials and methods

Institutional Review Board approval was obtained for performing MOAT and AOA in the present study for patients who showed failed or low fertilization in previous ICSI cycles or who had well-known sperm-borne activation deficiencies such as globozoospermia. The MOAT and AOA protocol were previously described in detail (Heindryckx *et al.*, 2005).

Mouse oocyte activation test

Metaphase II (MII) oocytes were collected 13–14 h after human chorionic gonadotrophin administration from three to four superovulated B6D2F1 mice. Potassium simplex optimized medium (KSOM) and KSOM-HEPES containing 0.2 mmol/l glucose and supplemented with 0.4% bovine serum albumin (BSA, MP Biomedicals, Asse-Relegem, Belgium) were used as oocyte culture medium and manipulation medium, respectively. Frozen–thawed spermatozoa from patients were centrifuged twice at 350 *g* for 10 min. Four experimental groups were set up in each MOAT: (i) injection with patient spermatozoa; (ii) injection with donated spermatozoa with proven fertilization capacity (positive control); (iii) sham injection (negative control); and (iv) non-manipulated oocytes to exclude spontaneous parthenogenetic activation (medium control). Conventional injection of motile human spermatozoa was performed at 15–17°C in KSOM-HEPES supplemented with 20% fetal bovine serum (FBS, cat. no. 101108–157; Gibco BRL, USA) by gentle aspiration of the mouse oocyte cytoplasm. It appears that reduction of the temperature during injection and the addition of a higher concentration of FBS might increase the survival rate of mouse oocytes following the conventional method of ICSI as was already demonstrated for ICSI in mouse by Kimura and Yanagimachi (1995) and Suzuki and Yanagimachi (1997) and by the study group (Heindryckx *et al.*, 2001, 2006; Rybouchkin *et al.*, 2002).

After injection, the oocytes of the four different groups were put in KSOM and, the next day, oocyte activation was examined and defined by the percentage of 2-cell formations in the surviving injected MII oocytes (MOAT activation percentage). The MOAT was considered reliable when 2-cell formation was observed in at least 90% of the positive controls, with less than 10% 2-cell formations in both the negative and medium control.

Assisted oocyte activation

The interval between oocyte retrieval and ICSI with AOA was at least 6 h in order to have fully mature MII oocytes regardless of the MOAT activation percentage. Oocytes were kept at 37°C in a 6% CO_2 air atmosphere in Cook Cleavage medium (Cook Ireland Ltd, Limerick, Ireland). For ICSI with AOA, spermatozoa resuspended in HEPES-buffered Oocyte Wash (Cook Ireland Ltd) and an equal volume of 8% polyvinylpyrrolidone (PVP ICSI-100, VitroLife Sweden AB, Kungsbacka, Sweden) was immobilized by pressing the tail to the bottom of the dish and was drawn up into an injection pipette and the sperm head was kept at the very tip of the pipette. Then the pipette was moved to a drop of 0.1 mol/l $CaCl_2$ and an amount of $CaCl_2$ was aspirated into the injection pipette, which corresponded to the diameter of the oocyte. Oocytes were conventionally injected with the spermatozoa and $CaCl_2$ and kept in Cook Cleavage medium for 30 min. Injected oocytes were exposed for 10 min in the incubator to 10 µmol/l Ca^{2+} ionophore (Ionomycin, cat. no. 159611; MP Biomedicals) dissolved in Cook Cleavage medium and subsequently washed intensively and put in Cook Cleavage for 30 min in the incubator. Finally, ionophore treatment was

repeated during 10 min and after intensive washing, oocytes were placed in Cook Cleavage medium for culture. Oocytes were checked for signs of normal fertilization at around 16 h after ICSI and embryo transfer was carried out on day 2 or 3. Fertilization rate was defined by the number of oocytes showing two pronuclei (PN) and two polar bodies (PB) as a proportion of the total number of injected MII oocytes, and pregnancy rates were defined by the number of clinical pregnancies (gestational sac with fetal heartbeat at 6–7 weeks) as a proportion by the number of fresh cycles.

Recent studies have suggested that the maturity and quality of human MII oocytes can be determined to some extent using the Oosight system (Oosight Imaging System, CRi inc, Woburn, Mass, USA) that allows non-invasive visualisation of the oocyte spindle-chromosome complex (Cohen *et al*., 2004, De Santis *et al*., 2005; Rienzi *et al*., 2005). This spindle imaging was tested in one patient who had a history of high rates of abnormal PN formation (three or more PN in previous cycles).

Statistics

All data were analysed by contingency table analysis followed by Fisher's exact test for independence. The level of significance was set at $P \leq 0.05$.

Results

According to the MOAT activation percentage, 30 patients were classified into three categories. In group 1 (16 patients) the MOAT activation percentage was between 0% and 20%, pointing to a sperm deficiency. Nine of these patients had total globozoospermia, while one patient was diagnosed with partial globozoospermia, with both the normal morphology and the globozoospermic spermatozoa showing an activation deficiency after heterologous injection. In the remainder of this group, oligoasthenoteratozoospermia (OAT) was observed at both a moderate degree (two patients, from whom normal morphology spermatozoa were selected for MOAT) and at an extreme degree (four patients). In group 2 (seven patients) the MOAT activation percentage was between 20% and 85% pointing to either a sperm or an unknown deficiency, and in group 3 (seven patients) the a MOAT activation percentage was between 85% and 100%, suggesting an oocyte deficiency.

The fertilization and pregnancy rates before and after application of AOA for 30 female patients classified according to the MOAT activation percentage are presented in **Table 1**. The mean ± SD age of these 30 patients was 33.5 ± 3.838, 33.86 ± 4.706 and 34.29 ± 4.152 years in the three groups respectively. Ten patients from group 1 had 19 previous ICSI cycle attempts that resulted in 6% fertilization and no pregnancies. Fertilization and pregnancy rates after application of AOA in group 1 were 75% and 34% respectively. The seven patients in group 2 with a moderate OAT had a fertilization history of 22% in previous ICSI cycles (18 cycles, no pregnancies) and had shown low or nearly failed fertilization in at least two ICSI attempts before application of MOAT and AOA. The MOAT revealed a reduced activation capacity of the patient's spermatozoa compared with the positive controls in each MOAT. After application of AOA, fertilization and pregnancy rates were raised to 73% and 43% respectively. Seven patients in group 3 with a moderate OAT had a failed or low fertilization history after ICSI of 14% (13 cycles, no pregnancies); two patients had undergone one ICSI cycle, four had undergone two ICSI cycles and one had undergone three ICSI cycles before application of MOAT and AOA. The MOAT resulted in normal oocyte activation capacity by the patient's spermatozoa. After AOA, fertilization and pregnancy rates were raised to 75% and 17% respectively.

Overall, 61 cycles with AOA were performed. The fertilization rate before application of AOA was 14% in 50 cycles without any established pregnancy. Following AOA treatment in these 30 patients, fertilization rates were significantly increased to 75% and the pregnancy rate was increased to 33%. No increase in three or higher PN formation and equal embryonic developmental potential was observed after the application of AOA in all three groups compared with the normal ICSI population (data not shown).

Fertilization and pregnancy rates before and after application of AOA in a subdivision of group 1 according to the different sperm morphology parameters are presented in **Table 2**. For nine total globozoospermic patients, characterized by round-headed spermatozoa lacking an acrosome, three patients had undergone normal ICSI cycles without AOA. This resulted in total fertilization failure in one patient (0/8 oocytes) in this study clinic and, in the other two patients in another clinic, 2/24 fertilized oocytes (8%) after three cycles and 3/17 fertilized (18%) after one cycle. No pregnancies were established in these cycles. After application of AOA for the group of nine total globozoospermic patients, 76% of the injected oocytes were normally fertilized and a pregnancy was established in 42% (8/19) of cycles. The patient with partial globozoospermia, characterized by the presence of both normal and globozoospermic cells had a history of nearly failed fertilization (3%, three cycles) after ICSI, but after application of AOA, the fertilization rate was increased to 76% and a pregnancy was achieved after two cycles (50%). Two patients with a moderate OAT had a history of nearly failed fertilization after normal ICSI (1/27, 4%, two cycles, no pregnancy; 0/24, 0%, three cycles, no embryo transfer, respectively) while MOAT revealed 0% activation capacity and a decreased (19%) activation capacity respectively in these two patients although normal morphology spermatozoa was used for injection in MOAT. After application of AOA, the fertilization rate was normalized (70%) and both patients became pregnant after three cycles (2/3, 67%). Four patients with extreme OAT sperm characteristics had a history of nearly failed fertilization after normal ICSI (2/14, 14%, two cycles; 2/22, 9%, two cycles; 0/8, 0%, one cycle; 0/10, 0%, one cycle). The MOAT revealed complete or nearly complete failure of oocyte activation by these patients' spermatozoa (0%, 0%, 9% and 20% MOAT, respectively). Fertilization and pregnancy rates after AOA application in these four patients were 76% and 9% (1/11), respectively.

In all, 17 out of 20 clinical pregnancies went to term resulting in 13 singleton infants and four sets of twins. No major or minor congenital malformations were observed. The obstetric and neonatal outcomes of these pregnancies are presented in **Table 3**. One pregnancy was ectopic, another one ended in a miscarriage at 7 weeks and one is still ongoing.

One patient has not been described in the above mentioned results. This patient (30 years) had a history of three ICSI

Table 1. Fertilization and pregnancy rates before and after application of assisted oocyte activation (AOA) in a group of patients with failed or low fertilization after normal intracytoplasmic sperm injection classified according to the mouse oocyte activation test (MOAT).

MOAT group[a]	*Before AOA* *Fertilization rate*[b]	*Pregnancy rate*[c]	*After AOA* *Fertilization rate*[b]	*Pregnancy rate*[c]
1 (n = 16)	6 (0–18)[d] (11/186)	0[e] (0/19)	75 (50–92)[d] (307/407)	34[e] (12/35)
2 (n = 7)	22 (8–42)[d] (36/164)	0[e] (0/18)	73 (67–100)[d] (81/111)	43[e] (6/14)
3 (n = 7)	14 (0–50)[d] (19/138)	0 (0/13)	75 (67–100)[d] (78/104)	17 (2/12)
Total (n = 30)	14 (0–50)[d] (66/488)	0[e] (0/50)	75[d] (466/622)	33[e] (20/61)

[a]According to the MOAT activation percentage achieved, patients were classified into three groups: group 1 = 0–20%; group 2 = 20–85%; and group 3 = 85–100%.
[b]Values are percentage (range) (no. of normally fertilized oocytes/no. of injected metaphase II oocytes).
[c]Values are percentage (no. of clinical pregnancies/no. of fresh cycles).
[d]$P < 0.0001$ within the same row, Fisher's exact test.
[e]$P < 0.004$ (at least) within the same row, Fisher's exact test.

Table 2. Fertilization rate before and after application of assisted oocyte activation and pregnancy rate in group 1 (MOAT activation 0–20%), classified according to the sperm-borne activation deficiency indication.

Group 1 sperm deficiency	*Fertilization rate (%)* *Before AOA*	*After AOA*	*Pregnancy rate (%)*
Total globozoospermia (n = 9)	10 (5/49, 5 cycles); no pregnancy	76 (179/235, 19 cycles)[a]	42 (8/19)
Partial globozoospermia (n = 1)	3 (1/32, 3 cycles); no pregnancy	76 (31/41, 2 cycles)	50 (1/2)
Moderate OAT (n = 2)	2 (1/51, 5 cycles); no pregnancy	70 (32/46, 3 cycles)	67 (2/3)
Extreme OAT (n = 4)	7 (4/54, 6 cycles); no pregnancy	76 (65/85, 11 cycles)	9 (1/11)

AOA = assisted oocyte activation; MOAT = mouse oocyte activation test; OAT = oligoasthenoteratozoospermia.
[a]12 cycles were incorporated from previously reported results (Heindryckx *et al.*, 2005).

Table 3. Obstetric and neonatal outcomes in pregnancies after assisted oocyte activation following intracytoplasmic sperm injection.

Outcome	*Singleton (n = 13)*	*Twin (n = 4)*	*Total babies (n = 21)*
Obstetric outcome			
Preterm delivery <37 weeks	1 (7.7)	0 (0)	1 (4.8)
Vaginal delivery	10 (76.9)	5 (62.5)	15 (71.4)
Caesarean section	3 (23.1)	3 (37.5)	6 (28.6)
Neonatal outcome			
Mean birth weight (g) ± SD (range)	3458 ± 439 (2720–4140)	2822 ± 462 (2310–3385)	3246 ± 532 (2310–4140)
Birth weight <2500 g	0 (0)	2 (25.0)	2 (9.5)
Apgar score <7 at 5 min	0 (0.0)	0 (0.0)	0 (0.0)
Treatment in neonatal intensive care	0 (0.0)	1 (12.5)	1 (4.8)
Perinatal mortality	0 (0.0)	0 (0.0)	0 (0.0)
Gender			
Male	4 (30.8)	5 (62.5)	9 (42.9)
Female	9 (69.2)	3 (37.5)	12 (57.1)

Values are number (percentage) unless otherwise stated.

cycles in the study centre with a low normal fertilization rate (32% 2PN) and a high rate of abnormally fertilized embryos (50% ≥3PN). Of the 13 normally fertilized oocytes in these three cycle attempts, four zygotes showed 2PN arrest, seven embryos showed multinucleated blastomeres while only two embryos showed no multinucleation. Embryo transfer in these three cycles did not result in a pregnancy. The MOAT was performed to exclude a sperm factor deficiency in these cycles, but confirmed a normal activation capacity of the patient's spermatozoa with normal 2PN formation in the mouse injected oocytes. It was decided to perform AOA on half of the oocytes during the next ICSI cycle attempt and to evaluate the oocytes before ICSI using the Oosight system that allows non-invasive visualisation of the oocyte spindle–chromosome complex. Spindle visualisation revealed only one MII oocyte of the 14 retrieved MII oocytes showing a visible spindle located near the first PB. AOA was applied to half of the oocytes. In the group without AOA, only one oocyte was normally fertilized (the one oocyte with a visible spindle) while the rest was all ≥3PN abnormally fertilized. Four of the seven oocytes subjected to AOA showed normal 2PN fertilization (57%) and gave rise to embryos without multinucleation. Embryo transfer was carried out with one embryo originating from the group without AOA (from the one oocyte with visible spindle) and one embryo from the AOA group and subsequently a clinical pregnancy was established which is still ongoing (8.5 months).

Discussion

These findings demonstrate that assisted oocyte activation is highly efficient for couples suffering from low or failed fertilization rates in previous ICSI cycles and concomitant failure to establish a pregnancy. Fertilization rates after AOA application were restored to a normal level (75% average fertilization rate) and successful pregnancies were established in all groups of patients (33% average pregnancy rate). All pregnancies that went to term after AOA application gave rise to healthy infants with no major or minor malformations. Finally, it has been shown that the use of spindle evaluation and AOA might be helpful for patients whose oocytes have an inferior quality or an activation disorder.

There are no large-scale studies on assisted oocyte activation during ICSI describing restoration of fertilization rates and outcome of resulting pregnancies. Tesarik *et al.* (2002) reported a high efficiency using the modified ICSI method after heterologous ICSI, by which five of six patients became pregnant after one or two cycles. However, the number of patients was limited in that study and since then, only one paper confirmed these results although using a slightly different method of modified ICSI (Ebner *et al.*, 2004). The latter reported on 14 patients who experienced failed fertilization in previous cycles (0% fertilization, 17 cycles) and obtained a fertilization rate of 54% and a clinical pregnancy rate of 33% after 15 cycles with modified ICSI. Unfortunately, no heterologous ICSI was performed in the latter study, so no classification of the patients could be made regarding sperm- or oocyte-borne activation deficiency. Attempts have been made to reproduce both modified ICSI methods but the success rate was significantly lower compared with the standard method of AOA using Ca^{2+} injection and ionophore treatment (authors' unpublished data). Another recent study described the use of ionophore activation in selected cases with different types of sperm abnormalities with previously failed or low fertilization after ICSI (Moaz *et al.*, 2006). These authors found that assisted ionophore activation could increase the fertilization rate in patients with amorphous and tapered head spermatozoa. Since the study was done on sibling oocytes, with and without activation, and mixed transfers were applied, pregnancies were not reported. Because no diagnostic heterologous test was performed and considering the fact that the oocytes without assisted oocyte activation showed a fertilization rate of 36–39%, it cannot be concluded from that study whether abnormally shaped spermatozoa are associated with a deficient activation capacity.

The AOA outcomes in 17 patients have been previously reported, but the clinical pregnancy rates were not reported due to the limited number of cycles (Heindryckx *et al.*, 2005). Overall, efficiency of AOA in terms of fertilization is similar in all three MOAT groups and fertilization rates were significantly increased compared with previous ICSI cycles without AOA. Pregnancy rates tended to be higher in groups 1 and 2 compared with group 3 where MOAT indicated an oocyte-borne oocyte-activation deficiency. If the subgroups of group 1 are examined in more detail, it becomes clear that pregnancy rates were very high in the globozoospermic patients, the partial globozoospermic patient and the patients with moderate OAT (42–67%). Only the group of patients with extreme OAT showed a lower pregnancy rate after AOA (9%). It was interesting that even the normal-looking spermatozoa from the partial globozoospermic patient had no activation capacity as shown in MOAT, a finding that, as far as is known, has never been previously reported so far. In the intermediate group 2 with MOAT activation percentages ranging between 20% and 85% both oocyte and sperm deficiency could be involved. It has to be taken into account, however, that the results of MOAT cannot be strictly extrapolated to human oocytes. In this context, Yanagida *et al.* (1999) reported that the MOAT activation rate in one patient was almost doubled when two instead of one spermatozoa was injected, indicating that in some patients the oocyte activating defect of spermatozoa can also have a quantitative aspect. Quantification of PLCζ might reveal some subtle differences in spermatozoa from patients with a sperm-borne activation deficiency. In support of this theory, Cox *et al.* (2002) have shown that human PLCζ exhibits greater potency compared with mouse and monkey PLCζ. They demonstrated that the minimal amount of human PLCζ cRNA required to cause Ca^{2+} oscillations in mouse oocytes was nearly a hundred times less than mouse PLCζ (Swann *et al.*, 2006). Conversely, mouse PLCζ is less effective in generating Ca^{2+} oscillations in human oocytes. Additionally Yu *et al.* (2008) recently reported that development of embryos to the blastocyst stage and the inherent quality depends on a specific range of human PLCζ expression. Because fertilization and pregnancy rates were highest in groups 1 and 2, AOA is particularly efficient for sperm-borne activation deficiencies and suggests that this is also the main problem in patients belonging to the intermediate group (20–85% MOAT activation capacity). Nevertheless, the fertilization rate in group 3 was significantly increased after AOA application, which may question the necessity of performing MOAT for all patients. In this respect, it was emphasized to the patients that MOAT is primarily a diagnostic tool in order to explain to patients the underlying reasons for their low or failed fertilization after normal ICSI and to confirm a sperm-borne activation deficiency. Patients have to be aware that these activation deficiencies might be transferred to their progeny, especially the sperm-borne deficiencies distinguished by MOAT, if a genetic disorder is responsible for the activation deficiency. Currently investigations are underway as to whether abnormalities are present in the PLCζ gene or in

the expression of it within the AOA-patient population classified according to the MOAT result. As for the MOAT classification that is currently in place, the population group needs to be extended in order to confirm whether AOA is more efficient in some MOAT categories in terms of pregnancy rates, whether an oocyte-borne activation deficiency is really responsible for the failed or low fertilization after normal ICSI in group 3 or whether subdivisions in our MOAT classification system exist in terms of patients that respond better to AOA or not and in terms of pregnancy rates because fertilization rates are equally significantly increased in all three MOAT groups. At the same time, it needs to be stressed that Ca^{2+} ionophore treatment should be applied cautiously and for well-defined indications because the clinical experience is thus far too limited.

Several reports have confirmed that although Ca^{2+} oscillations fulfil a critical role in the oocyte activation mechanism, they can also affect long-term developmental events (Ducibella *et al.*, 2002). In mouse and rabbit oocytes, the number, frequency as well as the amplitude and duration of Ca^{2+} rises not only affect the activation efficiency (Vitullo and Ozil, 1992; Ducibella *et al.*, 2006), but also influence the embryo and blastocyst quality (Bos-Mikich *et al.*, 1997; Ozil *et al.*, 2006; Rogers *et al.*, 2006; Yu *et al.*, 2008) and also have long-term effects on both gene expression and development to term (Ozil *et al.*, 2006; Rogers *et al.*, 2006). The results of the present study indicate that the artificially induced Ca^{2+} rises are sufficient to obtain good-quality embryos and establish pregnancies at a similar rate as other assisted reproduction patients, which is contradictory to the above-mentioned studies showing the necessity of normal Ca^{2+} signalling for successful activation and subsequent pre- and post-implantation development. It remains unclear what the activation mechanism is after AOA in this series of patients, where only three artificially induced Ca^{2+} rises take place. A single but large increase in internal Ca^{2+} concentration activates oocytes and promotes parthenogenetic preimplantation development in several mammalian species (Rougier and Werb, 2001; Ozil *et al.*, 2005). Parthenogenetic activation and development can also be achieved in human oocytes where mostly a combination of a single induced Ca^{2+} transient by ionophore is used followed by a protein synthesis inhibitor. Still, development of human parthenogenetic embryos is compromised, and alternative methods like the injection of human PLCζ might promote better blastocyst formation in the future (Rogers *et al.*, 2004). However recent reports show that preimplantation development until the blastocyst stage of human parthenogenetic embryos was efficient when in-vivo-matured donated oocytes were used (Paffoni *et al.*, 2007; Revazova *et al.*, 2007; de Fried *et al.*, 2008).

It is important to emphasize the time interval of >6 h between oocyte retrieval and start of ICSI with AOA. Young mouse oocytes are not easily activated by single artificially induced Ca^{2+} rises (Vincent *et al.*, 1992) and aged oocytes are more susceptible for activation (Fissore *et al.*, 2002). Others have suggested that oocytes need to be cytoplasmically fully mature before ICSI (Kovacic and Vlaisavljevic, 2000) and that the Ca^{2+} releasing mechanism is developed and modified during oocyte maturation (Carroll *et al.*, 1996). Of interest in this respect is the fact that in-vitro-matured oocytes showed a single large spike after artificial oocyte induction with thimerosal which induces Ca^{2+} oscillations very similar to those seen during fertilization, however, subsequent Ca^{2+} oscillations were not observed, indicating that these oocytes failed to develop fully competent Ca^{2+} signalling mechanisms during culture *in vitro* (Herbert *et al.*, 1997). Due to the fact that oocytes obtain an optimal sensitivity of Ca^{2+} release just before the optimal time for fertilization, a minimum of 6 h after collection is recommended before performing ICSI with AOA.

Another new finding in the present study was that spindle visualisation might predict the outcome of normal fertilization in patients who possess inferior quality oocytes with a subsequent abnormal fertilization pattern and that AOA can increase the number of zygotes showing a normal fertilization pattern. These findings will have to be confirmed by additional studies on more patients. Spindle presence and characteristics and zona inner layer characteristics have been reported to correlate with developmental potential to the blastocyst stage (Wang *et al.*, 2001; Rama Raju *et al.*, 2007) and pregnancy rates (Shen *et al.*, 2005; Madaschi *et al.*, 2007). Still, patients who lack spindles in (almost) all the available oocytes on the day of oocyte retrieval have not yet been reported.

In conclusion, it has been shown that AOA can restore fertilization and pregnancy rates in patients with failed or low fertilization after ICSI. This study did not reveal any toxic effect of the ionophore treatment on the developmental capacity of embryos. Moreover, the obstetric and neonatal outcome was normal and no major or minor malformations were observed. Nevertheless, Ca^{2+} ionophore treatment should be applied cautiously and for well-defined indications because the clinical experience is too limited to exclude cytotoxic, teratogenic and mutagenic effects on embryos and offspring. A preceding diagnostic test, such as MOAT, in order to confirm whether a sperm-borne activation deficiency is present in these patients is useful in this respect.

Acknowledgements

The authors would like to thank Ms L Vanhoutte and Ms S Lierman for assistance in MOAT. P De Sutter is holder of a fundamental clinical research mandate by the Flemish Fund of Scientific Research (FWO-Vlaanderen).

References

Ahmady A, Michael E 2007 Successful pregnancy and delivery following intracytoplasmic injection of frozen-thawed nonviable testicular sperm and oocyte activation with calcium ionophore. *Journal of Andrology* **28**, 13–14.

Bos-Mikich A, Whittingham D, Jones KT 1997 Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Developmental Biology* **182**, 172–179.

Carroll J, Jones KT, Whittingham DG 1996 Ca^{2+} release and the development of Ca^{2+} release mechanisms during oocyte maturation: a prelude to fertilisation. *Reviews of Reproduction* **1**, 137–143.

Cohen Y, Malcov M, Schwartz T *et al.* 2004 Spindle imaging: a new marker for optimal timing of ICSI? *Human Reproduction* **19**, 649–654.

Cox LJ, Larman MG, Saunders CM *et al.* 2002 Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. *Reproduction* **124**, 611–623.

de Fried EP, Ross P, Zang G *et al.* 2008 Human parthenogenetic blastocysts derived from noninseminated cryopreserved human oocytes. *Fertility and Sterility* **89**, 943–947.

De Santis L, Cino I, Rabellotti E *et al.* 2005 Polar body morphology and spindle imaging as predictors of oocyte quality. *Reproductive BioMedicine Online* **11**, 36–42.

Ducibella T, Schultz RM, Ozil JP 2006 Role of calcium signals in early

development. *Seminars in Cell and Developmental Biology* **17**, 324–332.
Ducibella T, Huneau D, Angelichio E *et al*. 2002 Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Developmental Biology* **250**, 280–291.
Ebner T, Moser M, Sommergruber M *et al*. 2004 Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. *Human Reproduction* **19**, 1837–1841.
Fissore RA, Kurokawa M, Knott J *et al*. 2002 Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction* **124**, 745–754.
Flaherty SP, Payne D, Matthews CD 1998 Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. *Human Reproduction* **13** (Suppl. 1), 155–164.
Flaherty S, Payne D, Swann N *et al*. 1995 Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Human Reproduction* **10**, 2623–2629.
Heindryckx B, Van der Elst J, Dhont M 2006 Culture medium preferences of pre-implantation cloned mouse embryos. *Methods in Molecular Biology* **348**, 59–78.
Heindryckx B, Van der Elst J, De Sutter P *et al*. 2005 Treatment option for sperm or oocyte related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. *Human Reproduction* **20**, 2237–2241.
Heindryckx B, Rybouchkin A, Van der Elst J *et al*. 2001 Effect of culture media on in vitro development of cloned mouse embryos. *Cloning* **3**, 41–50.
Herbert M, Gillespie JI, Murdoch AP 1997 Development of calcium signalling mechanisms during maturation of human oocytes. *Molecular Human Reproduction* **3**, 965–973.
Kimura Y, Yanagimachi R 1995 Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction* **52**, 709–720.
Kovacic B, Vlaisavljevic V 2000 Configuration of maternal and paternal chromatin and pertaining microtubules in human oocytes failing to fertilize after intracytoplasmic sperm injection. *Molecular Reproduction and Development* **55**, 197–204.
Madaschi C, Carvalho de Souza Bonetti T, Paes de Almeida Ferreira Braga D *et al*. 2008 Spindle imaging: a marker for embryo development and implantation. *Fertility and Sterility* **90**, 194-198.
Mahutte NG, Arici A 2003 Failed fertilisation: is it predictable? *Current Opinion in Obstetrics and Gynecology* **15**, 211–218.
Moaz MN, Khattab S, Foutouh IA *et al*. 2006 Chemical activation of oocytes in different types of sperm abnormalities in cases of low or failed fertilization after ICSI: a prospective pilot study. *Reproductive BioMedicine Online* **13**, 791–794.
Ozil JP, Banrezes B, Tóth S *et al*. 2006 Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Developmental Biology* **300**, 534–544.
Ozil JP, Markoulaki S, Toth S *et al*. 2005 Egg activation events are regulated by the duration of a sustained $[Ca^{2+}]_{cyt}$ signal in the mouse. *Developmental Biology* **282**, 39–54.
Paffoni A, Brevini T, Somigliana E *et al*. 2007 In vitro development of human oocytes after parthenogenetic activation or intracytoplasmic sperm injection. *Fertility and Sterility* **87**, 77–82.
Rama Raju GA, Prakash GJ, Krishna KM *et al*. 2007 Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: a preliminary study using PolScope imaging. *Reproductive BioMedicine Online* **14**, 166–174.
Rawe VY, Olmedo SB, Nodar FN *et al*. 2000 Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Molecular Human Reproduction* **6**, 510–516.
Revazova ES, Turovets NA, Kochetkova OD *et al*. 2007 Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells* **9**, 432–449.
Rienzi L, Ubaldi F, Iacobelli M *et al*. 2005 Meiotic spindle visualization in living human oocytes. *Reproductive BioMedicine Online* **10**, 192–198.
Rogers NT, Halet G, Piao Y *et al*. 2006 The absence of a Ca^{2+} signal during mouse egg activation can affect parthenogenetic preimplantation development, gene expression patterns, and blastocyst quality. *Reproduction* **132**, 45–57.
Rogers NT, Hobson E, Pickering S *et al*. 2004 PLCζ causes Ca^{2+} oscillations and parthenogenetic activation of human oocytes. *Reproduction* **128**, 697–702.
Rougier N, Werb Z 2001 Minireview: parthenogenesis in mammals. *Molecular Reproduction and Development* **59**, 468–474.
Rybouchkin A, Heindryckx B, Van der Elst J *et al*. 2002 Developmental potential of cloned mouse embryos reconstructed by a conventional technique of nuclear injection. *Reproduction* **124**, 197–207.
Rybouchkin A, Dozortsev D, Pelinck MJ *et al*. 1996 Analysis of the oocyte activation capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. *Human Reproduction* **11**, 2170–2175.
Saunders CM, Larman MG, Parrington J *et al*. 2002 PLCζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* **129**, 3533–3544.
Shen Y, Stalf T, Mehnert C *et al*. 2005 High magnitude of light retardance by the zona pellucida is associated with conception cycle. *Human Reproduction* **20**, 1596–1606.
Sousa M, Tesarik J 1994 Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. *Human Reproduction* **9**, 2374–2380.
Suzuki K, Yanagimachi R 1997 Beneficial effect of medium with high concentration serum for direct sperm injection into mouse oocytes using a conventional pipette. *Zygote* **5**, 111–116.
Swann K, Ozil JP 1994 Dynamics of the calcium signal that triggers mammalian egg activation. *International Review of Cytology* **152**, 183–222.
Swann K, Saunders CM, Rogers NT *et al*. 2006 PLCzeta(zeta): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. *Seminars in Cell and Developmental Biology* **17**, 264–273.
Tesarik J, Mendoza C 1999 In vitro fertilization by intracytoplasmic sperm injection. *Bioessays* **21**, 791–801.
Tesarik J, Rienzi L, Ubaldi F *et al*. 2002 Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne oocyte activation failures. *Fertility and Sterility* **78**, 619–624.
Tesarik J, Mendoza C, Greco E 2000 The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. *Fertility and Sterility* **74**, 1245–1247.
Vincent C, Cheek TR, Johnson MH 1992 Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. *Journal of Cell Science* **130**, 389–396.
Vitullo AD, Ozil JP 1992 Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. *Developmental Biology* **151**, 128–136.
Wang WH, Meng L, Hackett RJ *et al*. 2001 Developmental ability of human oocytes with or without birefringent spindles imaged by PolScope before insemination. *Human Reproduction* **16**, 1464–1468.
Yanagida K 2004 Complete fertilization failure in ICSI. *Human Cell* **17**, 187–193.
Yanagida K, Morozumi K, Katayose H *et al*. 2006 Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilisation. *Reproductive BioMedicine Online* **13**, 801–806.
Yanagida K, Katayose H, Yazawa H *et al*. 1999 Successful fertilization and pregnancy following ICSI and electrical oocyte activation. *Human Reproduction* **14**, 1307–1311.
Yu Y, Saunders CM, Lai FA, Swann K 2008 Preimplantation development of mouse oocytes activated by different levels of human phospholipase C zeta. *Human Reproduction* **23**, 365-373.

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

Received 23 January 2008; refereed 26 February 2008; accepted 20 June 2008.