Human Reproduction, Vol.27, No.7 pp. 1977–1984, 2012
Advanced Access publication on May 2, 2012 doi:10.1093/humrep/des097

human reproduction

ORIGINAL ARTICLE *Embryology*

Assisted oocyte activation is not beneficial for all patients with a suspected oocyte-related activation deficiency

F. Vanden Meerschaut*, D. Nikiforaki, S. De Gheselle, V. Dullaerts, E. Van den Abbeel, J. Gerris, B. Heindryckx, and P. De Sutter

Department for Reproductive Medicine, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

*Correspondence address. De Pintelaan 185, B-9000 Ghent, Belgium. Tel: +32(0)9-332-02-89; E-mail: frauke.vandenmeerschaut@ugent.be

Submitted on October 4, 2011; resubmitted on December 30, 2011; accepted on February 8, 2012

BACKGROUND: Despite the success of ICSI, total fertilization failure (TFF) still occurs in 1–3% of all ICSI cycles. ICSI followed by assisted oocyte activation (ICSI-AOA) can restore fertilization, most efficiently in cases of sperm-related fertilization deficiency. The indication for ICSI-AOA is less obvious when the capacity of the sperm to activate oocytes is considered normal, as proved by a heterologous ICSI model, such as the mouse oocyte activation test (MOAT). In this study, we verified whether ICSI-AOA is beneficial for patients in whom an oocyte-related activation deficiency is suspected.

METHODS: A prospective study was conducted including patients presenting with a history of TFF or low fertilization (LF) following conventional ICSI in our centre (*in-house* cases, $n = 2$) or elsewhere (*out-house* cases, $n = 12$). In all cases a sperm deficiency was refuted by the MOAT. In a next treatment cycle, ICSI-AOA was performed on half of the sibling metaphase II oocytes and conventional ICSI on the rest ('split ICSI-AOA cycle'). The main outcome parameters were fertilization, pregnancy and live birth rates.

RESULTS: Overall, ICSI-AOA was able to improve fertilization rates in couples with a suspected oocyte-related fertilization problem, with a mean fertilization rate of 74.2% following ICSI-AOA compared with 43.5% following conventional ICSI ($P < 0.001$). Cumulative pregnancy rate and live birth rate per cycle were 35.7 and 14.3%, respectively. Considering the *out-house* patients only, fertilization rates with ICSI-AOA were higher in couples with previous TFF than with conventional ICSI ($P < 0.001$). Interestingly, for *out-house* patients who had experienced low, but not zero, fertilization elsewhere, ICSI-AOA could not enhance the fertilization rate. For the two *in-house* patients, both suffering from previous LF following conventional ICSI, the ICSI-AOA procedure enhanced the mean fertilization rate (25 versus 75%, respectively).

CONCLUSIONS: For patients with a suspected oocyte-related activation deficiency, as diagnosed by a heterologuous ICSI model, the indication for ICSI-AOA still remains debatable. Our data show that ICSI-AOA is very efficient in patients with a suspected oocyte-related activation deficiency and previous TFF after conventional ICSI. In contrast, when there was a history of LF in another centre, one should be careful and test the efficiency of ICSI-AOA on half of the sibling oocytes, because ICSI-AOA is not always beneficial for patients with previous LF and a suspected oocyte-related activation deficiency. For these patients, a split ICSI-AOA cycle using sibling oocytes can help to distinguish between a molecular oocyte-related activation deficiency and a previous technical or other biological failure. Moreover, this split ICSI-AOA strategy enables us to set the appropriate strategy for future treatment cycles. Further research with larger groups of patients is now required.

Key words: failed fertilization / oocyte activation deficiency / assisted oocyte activation / ionophore / mouse oocyte activation test

Introduction

ICSI is used in two-thirds of the artificial reproduction technology (ART) cycles in European fertility clinics (Mouzon *et al.*, 2010). ICSI was developed in the early 1990s and millions of couples suffering from severe male infertility or previously failed IVF conceived by this technique. On average, ICSI leads to fertilization rates of approximately 70–80% (Palermo *et al.*, 2009). Unfortunately, total fertilization

© The Author 2012. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please email: journals.permissions@oup.com

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

failure (TFF) still occurs in ~1–3% of all ICSI cycles and can recur in subsequent cycles (Flaherty et al., 1995; Esfandiari et al., 2005). Failure of oocyte activation is considered to be the main cause of fertilization failure following conventional ICSI (Liu et al., 1995; Flaherty et al., 1998; Rawe et al., 2000). Less common causes include failed sperm head decondensation, premature sperm chromatin condensation, spindle defects or sperm aster defects and incorrect sperm injection (Swain and Pool, 2008). Furthermore, fertilization failure can be caused by technical problems, a limited availability of mature or morphologically normal oocytes, a lack of motile spermatozoa or severe forms of teratozoospermia, such as globozoospermia (Yanagida et al., 2004; Dam et al., 2007).

When a mammalian oocyte is activated, the first observed cytoplasmic event is an increase in intracellular calcium concentration. The initial calcium increase (trigger) starts within a few minutes of sperm–oocyte fusion in IVF, whereas following ICSI this trigger is provoked immediately during the injection of the sperm by the artificial calcium influx from the surrounding injection medium (Tesarik et al., 2000). This trigger is followed by a typical pattern of calcium oscillations which are crucial for normal fertilization and further embryo development (Ducibella et al., 2002; Parrington et al, 2007; Kashir et al., 2010). Substantial evidence suggests that these calcium oscillations are the result of the release of the sperm phospholipase C zeta (PLCζ) into the oocyte cytosol (Saunders et al., 2002; Kashir et al., 2010). During final oocyte maturation, the ability to generate calcium oscillations is developed within the oocyte. This means that successful fertilization also depends on the inherent quality and cytoplasmic maturity of the oocyte (Ajduk et al., 2008). Hence, both the sperm and the oocyte play a role in the oocyte activation mechanism, fertilization and further embryo development: the sperm by supplying the protein PLCζ and the oocyte by its responsiveness to PLCζ and downstream molecular pathways (Tesarik and Mendoza, 1999).

In our centre, a diagnostic test was developed to distinguish sperm- from oocyte-related causes of fertilization failure (Rybouchkin et al., 1996). The mouse oocyte activation test (MOAT) is offered to patients with TFF or low fertilization (LF) following conventional ICSI. Test sperm is injected by heterologous ICSI into mouse oocytes, followed by assessment of the percentage of oocyte activation. The MOAT enabled the classification of patients into three groups (Heindryckx et al., 2008). Patients with an oocyte activation percentage of <20%, being the upper limit of the negative control, are classified as *MOAT Group 1* patients. In these cases, a sperm-related activation deficiency is extremely likely. In contrast, when 85% or more mouse oocytes are activated, being the lower limit of the positive control, patients are classified as *MOAT Group 3* patients. In this group, a sperm deficiency can be excluded. Less distinct are *MOAT Group 2* patients, in which 21–84% of the mouse oocytes are activated, pointing to either a sperm or an unknown deficiency.

Recently, there has been growing interest in the use of ICSI in combination with assisted oocyte activation (ICSI-AOA) in patients suffering from previous TFF or LF following conventional ICSI (Nasr-Esfahani et al., 2010). Several activation agents, such as calcium ionophore, strontium ions and electrical pulses, are efficient in restoring fertilization and pregnancy rates by artificially provoking calcium rises in the cytoplasm of the oocyte (Yanagida et al., 1999, 2006; Eldar-Geva et al., 2003; Chi et al., 2004; Heindryckx et al., 2005). Others reported the use of a mechanically modified ICSI technique to overcome activation failure (Tesarik et al. 2002; Ebner et al., 2004). Chemical activation using the ionophore ionomycin is the most commonly used method for AOA, resulting in high fertilization rates (Nasr-Esfahani et al., 2010).

In our centre, ICSI-AOA is performed using ionomycin, which enables us to restore fertilization rates to normal in most patients with a history of previously failed or LF (Heindryckx et al., 2005, 2008). The current study focuses on the patients with a normal MOAT result, namely patients in *MOAT Group 3*, who are presenting with TFF or LF probably caused by an oocyte-related activation problems. Our former policy was to perform ICSI-AOA on all available oocytes following the MOAT in most of these patients. Nevertheless, pregnancy results were lower in this group compared with patients in whom a sperm activation deficiency (*MOAT Group 1*) or a diminished oocyte activation capacity (*MOAT Group 2*) was found (Heindryckx et al., 2008). To our knowledge, 'split activation' on part of the available metaphase II (MII) oocytes was firstly mentioned by Yanagida (2004) in a review regarding fertilization failure following ICSI. Yanagida stated that 'split activation' could be performed when fertilization failure is anticipated but did not discussed the validity of this method since, at that time, no data were available on the subject. Therefore, since 2006, we changed our policy for patients in *MOAT Group 3* to verify their real benefit of ICSI-AOA. We strongly advised conventional ICSI and split ICSI-AOA on sibling oocytes. The majority of patients in *MOAT Group 3* are referred to our centre because of a history of LF or TFF following conventional ICSI elsewhere. By the split ICSI-AOA policy, we aim to distinguish between a previous coincidental LF rate or a real oocyte-related activation deficiency, in order to assess the necessity of AOA in these patients. According to the fertilization outcome of the split ICSI-AOA cycle, either conventional ICSI or ICSI-AOA will be advised on all oocytes in the next treatment cycle. The primary objective of this prospective case series is thus to provide further insight into the efficiency of ICSI-AOA compared with conventional ICSI in patients with a suspected oocyte-related activation deficiency. Furthermore, we present a diagnostic and therapeutic approach based on the results of the split ICSI-AOA cycle for this rare but challenging group of patients.

Materials and Methods

Inclusion and exclusion criteria for patients

Couples with a history of failed or LF following conventional ICSI in whom the MOAT revealed a possible oocyte-related activation deficiency (MOAT result >84%) were eligible to be included in this prospective case series. Patients were divided into two separate groups: the *out-house* patients (referred patients) and *in-house* patients (own patients). All patients were treated in our centre from January 2006 until December 2011. Controlled ovarian hyperstimulation was performed in all included patients using the short GnRH agonist protocol. This protocol consisted of daily injections of triptorelin (0.1 mg) starting on Day 3 of the cycle, followed by gonadotrophin stimulation (112.5–300 IU) from Day 5 of the cycle. Gonadotrophin stimulation was performed with recombinant FSH or HMG. In each case ICSI-AOA was performed on half of the MII oocytes, if six or more MII oocytes were available. Patients with less than six MII oocytes available at retrieval were excluded from the analysis, because in such cases the agreement was to perform ICSI-AOA on all of their available MII oocytes. Main end-points were fertilization rate, pregnancy rate and live birth rate.

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

MOAT

MII mouse oocytes were collected from B6D2 F1 hybrid female mice after ovulation induction by 10 IU pregnant mare's serum gonadotrophin (PMSG, Folligon®, Intervet, Boxmeer, The Netherlands), followed 46–48 h later by 10 IU hCG (Chorulon®, Intervet, Boxmeer, The Netherlands). MII oocytes were collected 13–14 h following hCG administration. Culture and handling media were potassium simplex optimized medium (KSOM) and KSOM-HEPES respectively, both containing 0.2 mmol/l glucose and supplemented with 0.4% w/v bovine serum albumin (BSA®, MP Biochemicals, Asse-Relegem, Belgium). Cumulus cells were removed by a brief exposure to 200 IU hyaluronidase/ml. Injection of immobilized frozen-thawed spermatozoa using piezo pulses was performed at 15–17°C in KSOM-HEPES supplemented with 20% fetal bovine serum (FBS®, Gibco BRL, Carlsbad, USA). Four experimental groups were set up: (i) ICSI with patients spermatozoa; (ii) ICSI with donated spermatozoa with proven fertilization capacity (positive control); (iii) sham ICSI (negative control) and (iv) non-manipulated oocytes (medium control) to exclude spontaneous parthenogenetic activation. Next, the oocytes were put in culture and 1 day later the percentage of oocyte activation was determined by examining the number of 2-cell embryos versus the number of surviving injected oocytes.

AOA and outcome

When six or more MII oocytes were available, ICSI-AOA was performed on half of the sibling MII oocytes. When an odd number of oocytes was available, ICSI-AOA was chosen for half plus one of the oocytes. ICSI-AOA was performed at least 6 h after oocyte retrieval, as previously described by Heindryckx *et al.* (2008). Briefly, a spermatozoon was injected into the oocyte using conventional ICSI together with a small amount of 0.1 mol/l $CaCl_2$ (corresponding to the diameter of the oocyte). Thereafter, the oocytes were incubated for 30 min at 37°C in a 6% CO_2 air atmosphere. Next, the oocytes were exposed to a calcium ionophore (10-μM ionomycin, I9657, Sigma-Aldrich, Bornem, Belgium) for 10 min. Following this ionophore exposure, the oocytes were washed with Cook Cleavage medium (Cook Ireland Ltd, Limerick, Ireland) and were incubated again. After another 30 min, the calcium ionophore treatment was repeated for 10 min. Finally, the oocytes were washed and incubated overnight. Fertilization was evaluated 16 h after the ICSI procedure. Normal fertilized oocytes showed two pronuclei and two polar bodies. The fertilization rate was defined by the number of normally fertilized oocytes compared with the total number of injected and surviving MII oocytes. Embryo transfer was carried out on Day 2 or 3. Pregnancy rate was defined by the number of clinical pregnancies (gestational sac with fetal heartbeat at 6–7 weeks) as a proportion of the number of fresh cycles. Birth rates were defined as the number of live born babies compared with the proportion of clinical pregnancies.

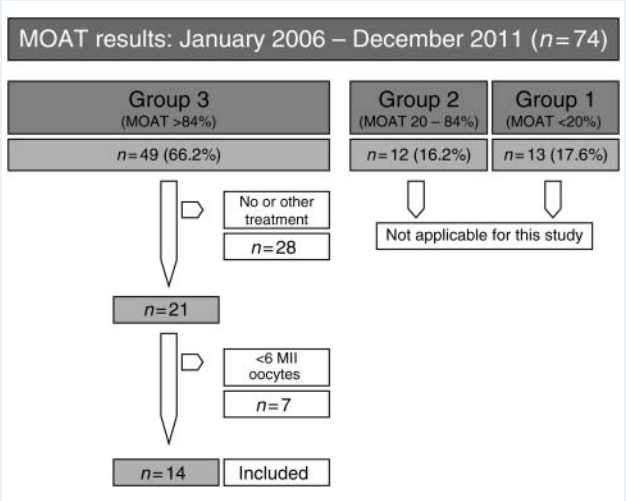

Figure 1 Overview of the MOAT results from January 2006 until December 2011 and subsequent inclusion of study patients.

Statistical analysis

The student's *t*-test, χ^2 test or Fisher's exact test were used for statistical analysis where appropriate using the Statistical Package for the Social Sciences (SPSS® Statistics 19, IBM Corp., NY, USA). A *P*-value < 0.05 was considered statistically significant.

Results

MOAT results and inclusion of patients

From January 2006 until December 2011, 74 MOATs were performed in our centre. *MOAT Group 1* (sperm deficiency confirmed) accounted for 17.6% of the results and *MOAT Group 2* (sperm deficiency suspected) accounted for 16.2%. *MOAT Group 3* (no sperm deficiency) was confirmed in 66.2% of the cases. The reasons for exclusion were the renouncement of any further treatment, signing up for oocyte donation and conventional ICSI or ICSI-AOA on all of the available oocytes (the latter options were taken at the request of the patient; see Fig. 1).

Demographics, fertility history and semen characteristics

The mean age of the included women was 31.6 ± 3.73 years. The age of the two *in-house* patients was 30.0 and 30.2 years. The *out-house* patients were split into two groups regarding (i) a history of TFF ($n = 5$) or (ii) a history of LF ($n = 7$) following conventional ICSI. The mean age in the TFF group was 33.4 ± 3.05 years compared with 31.3 ± 4.23 years in the LF group ($P = 0.365$). Thirteen of the included women were of Caucasian descent and one couple was Arab. Semen characteristics and fertility treatment history are presented in Table I.

Overall fertilization and pregnancy rates

Following conventional ICSI 43.5% of the oocytes were fertilized, whereas following ICSI-AOA the fertilization rate increased to 74.2% ($P < 0.001$). In total, 14 split ICSI-AOA cycles were performed in this study population. As a result of these split ICSI-AOA cycles, five pregnancies were achieved (overall pregnancy rate: 35.7%), of which two ended in the birth of a healthy singleton (overall live birth rate: 14.3%) and one pregnancy is still ongoing. The fertilization history, results and outcome are presented in Table II.

In-house cases with a history of LF rates

Two *in-house* cases (*Cases 1* and *2*) are included in this study (Supplementary Table SI). Both cases showed LF in previous ICSI cycles. ICSI-AOA was shown to be beneficial, with a mean fertilization rate of 75.0% for ICSI-AOA compared with 25.0% for conventional ICSI

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

Table I Semen characteristics and treatment history of the included patients.

Semen characteristics at MOAT	Sperm concentration per ml	Sperm motility A + B (%)	Sperm morphology % normal	Treatment history
In-house cases (both LF)				
Case 1	21.10^6	35	1	IUI, IVF, ICSI
Case 2	58.10^6	71	4	IUI, IVF, ICSI
Out-house TFF				
Case 3	14.10^6	42	5	IUI, IVF, ICSI
Case 4	57.10^6	47	2	IVF, ICSI
Case 5	31.10^6	19	1	ICSI
Case 6	$<0.5.10^6$	0	0	ICSI
Case 7	15.10^6	11	1	ICSI
Out-house LF				
Case 8	30.10^6	56	4	IUI, ICSI
Case 9	8.10^6	7	0	IUI, ICSI
Case 10	1.10^6	4	0	IUI, ICSI
Case 11	29.10^6	4	2	ICSI
Case 12	11.10^6	68	0	ICSI
Case 13	14.10^6	40	7	IUI, IVF, ICSI
Case 14	2.10^6	23	8	IUI, ICSI

TFF, total fertilization failure after previous conventional ICSI; LF, low fertilization after previous conventional ICSI (considered to be 33.3% or less, based on the 5th percentile of mean fertilization rates);
IUI, intrauterine insemination;
MOAT, mouse oocyte activation test.

on sibling oocytes ($P < 0.01$). In one patient (*Case 2*) an ongoing pregnancy was achieved and a healthy singleton was born.

Out-house cases with a history of TFF

Five *out-house* patients (*Cases 3–7*) consulted our centre with a history of TFF in one or more ICSI cycles (Supplementary Table SII). Overall, ICSI-AOA was beneficial, with a mean fertilization rate of 72.7% (24/33) for ICSI-AOA compared with 25.0% (7/28) for conventional ICSI ($P < 0.001$). One of these patients had a miscarriage following the transfer of a single ICSI-AOA embryo.

Out-house patients with a history of LF rates

Seven *out-house* patients (*Cases 8–14*) were referred to our centre with a history of LF in one or more ICSI cycles (Supplementary Table SIII). The previous mean fertilization rate was 21.4%. Overall, ICSI-AOA was not beneficial, with a mean oocyte fertilization rate of 75.0% (39/52) compared with 60.4% (29/48) for conventional ICSI ($P = 0.118$). Three patients became pregnant during this split ICSI-AOA cycle. One pregnancy ended in a miscarriage, one resulted in the birth of a healthy singleton and one pregnancy is still ongoing.

Diagnostic and therapeutic algorithm

Figure 2 represents a diagnostic and therapeutic algorithm based on the outcomes of the above described groups. From 2003 until May 2011, the mean fertilization rate of 13 136 ICSI cycles registered in our centre was 73.7 ± 22.72%. The 5th, 25th, 50th and 75th percentiles were 33.3, 62.5, 76.92 and 90.0%, respectively. Based on this, in our centre, abnormal LF is now considered to be a recurrent fertilization rate of 33.3% or less. A MOAT is thus proposed when the fertilization rate after ICSI is <33.3%. According to the result of the MOAT, patients are classified into three *MOAT groups*. For *MOAT Groups 1 and 2*, ICSI-AOA is performed on all oocytes. For patients in *MOAT Group 3* with a history of TFF, ICSI-AOA is performed on all oocytes. For patients in *MOAT Group 3* with a history of LF, a 'split ICSI-AOA cycle' is proposed. According to the fertilization results of this split cycle, the decision is made to perform conventional ICSI or ICSI-AOA on all available oocytes in future cycles.

Discussion

ICSI-AOA has proved to be a very efficient technique in cases of recurrent TFF or LF rates after ICSI (Nasr-Esfahani *et al.*, 2010). The MOAT is a diagnostic tool which helps to predict the usefulness of ICSI-AOA in a subsequent cycle and helps to diagnose sperm-related activation deficiencies (Rybouchkin *et al.*, 1996; Yanagida *et al.*, 1999; Tesarik *et al.*, 2002; Araki *et al.*, 2004; Heindyckx *et al.*, 2005, 2008). ICSI-AOA with ionophore or electrical pulses is successful in restoring fertilization and pregnancy rates by manipulating the intracellular calcium concentration (Yanagida *et al.*, 1999; Eldar-Geva *et al.* 2003; Murase *et al.*, 2004; Heindryckx *et al.*, 2005). To our knowledge, this is the first study to test the efficiency of ICSI-AOA compared with conventional ICSI on sibling oocytes of patients with a possible oocyte-related activation deficiency, as diagnosed by the MOAT, namely patients in *MOAT Group 3*. As this technique is still considered to be experimental, research to assess the beneficial

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

Table II Fertilization and pregnancy rates before and after the split ICSI-AOA cycle.

	Fertilization rate before ICSI-AOA[a]	Pregnancy rate[b]	Split cycle fertilization rate[a]		Pregnancy rate[b]	Live birth rate[c]	Source of embryos transferred leading to pregnancy
			ICSI-AOA	Conventional ICSI			
In-house LF ($n = 2$)	29.3 (19.2 and 37.5) (17/58)	0 (0/5)	75.0 (57.1–88.9) (12/16)*	25.0 (14.3–33.3) (4/16)*	50.0 (1/2)	50.0 (1/2)	1 × mixed DET[d]
Out-house TFF ($n = 5$)	0 (0–0) (0/54)	0 (0/15)	72.7 (28.6–90.9) (24/33)†	25.0 (0.0–100) (7/28)†	20.0 (1/5)	0.0 (0/5)	1 × AOA SET[d]
Out-house LF ($n = 7$)	21.4 (6.3–35.1) (27/126)	0 (0/8)	75.0 (40.0–100) (39/52)‡	60.4 (33.3–80.0) (29/48)‡	42.9 (3/7)[e]	14.3 (1/7)	2 × mixed DET, 1 × AOA SET
Total	17.7 (0–37.5) (44/248)	0 (0/28)	74.2 (28.6–100) (75/101)$	43.5 (0.0–100) (40/92)$	35.7 (5/14)	14.3 (2/14)	See above

AOA, assisted oocyte activation.
[a]Values are percentage (range) (number of normally fertilized oocytes/number of injected metaphase II oocytes).
[b]Values are percentage (number of clinical pregnancies/number of fresh cycles).
[c]Values are percentage (number of live births/number of fresh cycles).
[d]Mixed DET = double embryo transfer of one ICSI-AOA embryo and one conventional ICSI embryo; AOA SET = single embryo transfer of an ICSI-AOA embryo.
[e]One pregnancy is still ongoing.
*$P < 0.01$ (Pearson χ^2 test).
†$P < 0.001$ (Pearson χ^2 test).
‡$p = 0.118$ (Pearson χ^2 test).
$p < 0.001$ (Pearson χ^2 test).

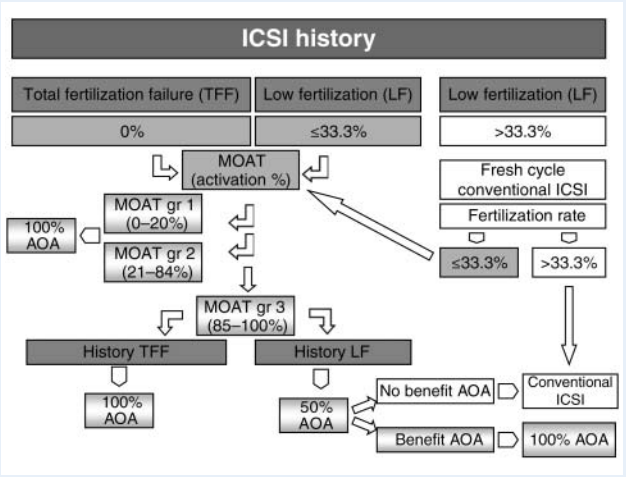

Figure 2 Diagnostic and therapeutic approach of patients with previous total fertilization failure or LF rates following conventional ICSI.

effect of, and necessity for, ICSI-AOA in this specific patient group remains highly important.

Owing to practical considerations, the MOAT was performed using frozen-thawed human sperm. Cryopreservation is widely known to raise impaired sperm motility and decrease fertilization rates through detrimental effects on membranes, acrosomal structure and acrosin activity (Cross and Hanks, 1991). Nevertheless, we consider our MOAT results to be reliable because only motile spermatozoa with the best morphology were used for heterologous ICSI. Moreover, data on fertilization and pregnancy rates after ICSI comparing fresh and frozen-thawed human ejaculated spermatozoa are reassuring: no statically significant differences were found between fresh and frozen-thawed sperm in fertilization rates (Kuczyński *et al.*, 2001). Furthermore, we did not find any differences in calcium releasing ability after heterologous ICSI when fresh sperm were compared with frozen-thawed sperm from the same patient (unpublished data).

The mean age of the 14 included women in this study is 31.6 years. The age did not differ between the *in-house* compared with the *out-house* cases, neither did the age differ between the *out-house* group with TTF in their history compared with the *out-house* group with LF in their history. Several authors have reported on the age-related decline in the success of IVF/ICSI cycles. Most likely this lies in the progressively diminished ovarian reserve, with a decreasing quantity and quality of oocytes (Broekmans *et al.*, 2007; van Loendersloot *et al.*, 2010). However, in this relatively young study population, it is plausible that age did not yet influence oocyte quality.

Two *in-house* patients with previous LF rates were included in this study. It is clearly shown that ICSI-AOA was clinically and statistically (albeit for only two patients) beneficial compared with conventional ICSI for both cases. This emphasizes the presence of a real oocyte-related activation deficiency in these *in-house* patients, rather than a previous technical failure. Unfortunately, in *Case 1*, although ICSI-AOA was efficient in improving the fertilization rate, neither the split ICSI-AOA cycle nor two subsequent 100% ICSI-AOA cycles led to a pregnancy. Even after several oocyte donation cycles, no ongoing pregnancy was achieved. So, it is clear that this patient suffers from severe underlying female factor infertility, and whether

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

this infertility is strictly oocyte related or not remains debatable. *Case 2* did also benefit from ICSI-AOA according to fertilization rate, although not as explicitly as compared with *Case 1*. In *Case 2*, a structural oocyte defect lies at the basis of the history of the LF: as described by Heindryckx *et al.* (2008), a spindle defect was suspected owing to (i) the absence of spindle visualization using polarized microscopy and (ii) commonly seen formation of multiple pronucleii and multinucleated embryos following IVF, conventional ICSI as well as ICSI-AOA. Previous studies on unfertilized oocytes in IVF cycles have revealed the presence of abnormal spindle and interphase microtubules, indicating that deficiencies in ooplasmic components may be a cause of failed fertilization (Kovacic and Vlaisavljevic, 2000; Rawe *et al.*, 2000). A very interesting case was recently reported by Combelles *et al.* (2010). In this case, no fertilization was seen following conventional ICSI. Subsequently, heterologuous ICSI showed a normal ability of the husband's sperm to activate mouse oocytes. Next, ICSI-AOA was performed on all oocytes after ICSI with half of the sperm from the husband and half from a donor. None of the injected oocytes in either group was fertilized. Thus, in contrast to both our in-house patients, the oocyte-related activation failure in the case described by Combelles *et al.* (2010) could not be rescued by ICSI-AOA. Analysis of the cytoskeletal and chromatin organization of the unfertilized oocytes revealed severe cytoplasmic abnormalities across the cohort of oocytes. It is obvious that further research is necessary to find other treatment options for such patients.

The patients from the *out-house* TFF group showed a significant benefit from ICSI-AOA over conventional ICSI. This underlines the probability of an oocyte-related activation deficiency of unknown source in this group, which can be rescued by ICSI-AOA. It is well known that nuclear as well as cytoplasmic maturation is crucial for oocytes before being able to respond properly to the sperm PLCζ during the fertilization process (Swain and Pool, 2008). Thus, successful fertilization depends also on the inherent quality of the oocyte, which correlates strongly with successful oocyte maturation. The ability to generate calcium oscillations requires several cytoplasmic changes: reorganization of the endoplasmic reticulum (ER), an increase in the number of inositol-3-phosphate receptors (IP3Rs), changes in the biochemical properties of the IP3Rs (sensitivity to IP3), an increase in the concentration of Ca^{2+} ions stored in ER and redistribution of Ca^{2+}-binding ER proteins (Goud *et al.*, 1999; Goud *et al.*, 2002; Ajduk *et al.*, 2008; Vanderheyden *et al.*, 2009). As our ICSI-AOA protocol using $CaCl_2$ and ionomycin artificially provokes some calcium rises in the oocyte cytoplasm, it is likely that an underlying cytoplasmic defect (related to the Ca^{2+}-releasing machinery of the oocyte) is the reason for the previous TFF in the *out-house* patients, rather than a structural cytoskeletal-related or nuclear defect.

In contrast to the *out-house* TFF group, the patients from the *out-house* LF group showed no significant benefit from ICSI-AOA. Moreover, *Cases 8*, *9* and *13* had pregnancies, four miscarriages and one ongoing pregnancy, with the same male partner before being referred to our centre. Interestingly, all but one of the *out-house LF* group had acceptable fertilization rates in our centre (considering the >33.3% threshold for normal fertilization) when conventional ICSI was applied as part of the split cycle. This underlines even more that the indication for ICSI-AOA in this group should not be made implicitly and that a split cycle could be advised instead of 100% AOA. The aim of the split ICSI-AOA cycle policy was thus to distinguish between a molecular oocyte-related activation deficiency from a previous technical or other biological failure. Nevertheless, the absence of benefit from ICSI-AOA in this group leads to the assumption that either a previous technical (and neither an oocyte nor a sperm deficiency) or an unidentified temporary biological failure could have led to LF rates elsewhere. Although ICSI is considered to be a routine technique, it remains one of the most demanding techniques for embryologists to master. It is not unusual for any embryologist to experience a dip in performance following the acquisition of proficiency in the technique. A comprehensive investigation of factors that influence fertilization rates after ICSI, found that the ICSI embryologist conducting the procedure was a significant predictor of success, while laboratory conditions, such as incubators or storage of eggs individually versus grouped, did not affect the fertilization rates (Shen *et al.*, 2003).

The factors leading to fertilization failure are complex and may involve either sperm- or oocyte-related activation deficiencies, which can be indicated by the MOAT result and overcome with ICSI-AOA (Heindryckx *et al.*, 2008). It is important to keep in mind that the human PLCζ has a higher activation potential compared with mouse PLCζ on mouse oocytes (Cox *et al.*, 2002; Ito *et al.*, 2008). The latter needs to be considered when interpreting a MOAT result, because the activation rate in mouse oocytes cannot be extrapolated as such to human oocytes. Furthermore, factors leading to fertilization failure may also relate to cycle-specific parameters and the number and quality of the mature oocytes, as well as the availability of normal motile sperm (Liu *et al.*, 1995; Flaherty *et al.*, 1998, Kovacic and Vlaisavljevic, 2000, Esfandiari *et al.*, 2005, Swain and Pool, 2008).

Although ICSI-AOA is considered a very efficient technique to overcome fertilization failure, this is not true for all cases of fertilization failure. A previous study has showed that AOA is more efficient to overcome sperm-related activation deficiencies, as diagnosed by the MOAT, rather than suspected oocyte-related activation deficiencies (Heindryckx *et al.*, 2008). In the study of Heindryckx *et al.*, (2008), patients from all three MOAT groups were included and the main treatment strategy was 100% ICSI-AOA. Since in this patient series only one *MOAT group 3* couple received split ICSI-AOA, the validity of the split ICSI-AOA strategy could not be investigated. Nevertheless, it was demonstrated that although fertilization rates could be restored by ICSI-AOA for all MOAT groups, the clinical pregnancy rates in *MOAT Group 3* patients were lower compared with *MOAT Group 1 and 2* patients (17 versus 34 and 43%, respectively). The latter is confirmed by the current study, with a mean pregnancy rate and birth rate of 35.7 and 14.3%, respectively.

In order to increase the efficiency of AOA in some subgroups of patients (for example, with extreme oligoasthenoteratozoospermia or when an oocyte factor is responsible for the failed ICSI), injection of PLCζ cRNA or recombinant PLCζ protein might offer a better alternative as these provide a more physiological stimulus than ionophore. Furthermore, the use of ionophore is still experimental because of insufficient knowledge about the potential cytotoxic, teratogenic and mutagenic effects on embryos and offspring. In previous animal research, no adverse effects of ionomycin on *in-vitro* or *in-vivo* mouse embryo development were noticed, giving arguments in favour of the use of ionomycin (Heytens *et al.*, 2008). On the

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

contrary, no long-term follow-up studies of children born after ICSI-AOA using ionomycin are yet available. Therefore, ICSI-AOA should not be performed without a proper indication in patients with previous LF, nor should it be used as an ultimate resort when other ARTs have failed. In this respect, it is advisable that patients that might benefit from a treatment with AOA after previously having TFF or LF after ICSI, first seek a diagnostic test, such as the MOAT, to be able to confirm whether a sperm-borne activation deficiency is present (Rybouchkin *et al.*, 1996; Araki *et al.*, 2004). Recent studies have applied ionophore treatment or electrical stimulation for infertile patients with no obvious indication, because in these patients the fertilization and pregnancy rates were comparable with or without AOA (Mansour *et al.*, 2008; Borges *et al.*, 2009b). In one study, AOA was even applied in the first cycle ICSI attempt (Borges *et al.*, 2009a).

The primary objective of this follow-up study was to provide further insight in the necessity of ICSI-AOA in patients in *MOAT Group 3* and its efficiency with respect to fertilization and pregnancy rates. The low sample number and heterogeneity of the study population might interfere with the predictive value of this study. Nevertheless, we are dealing with a very rare but challenging group of patients, for which we hope this study can set guidelines. The proposed diagnostic and therapeutic algorithm based on the outcomes of this study enables the evidence-based counseling of couples suffering from TFF or LF after ICSI. A MOAT, or similar heterologous ICSI test, should always be recommended when the previous ICSI fertilization rate was <33.3% before ICSI-AOA is considered. This threshold is chosen because 33.3% is the 5th percentile of the fertilization rate after ICSI in our centre. Next, if the MOAT shows a normal result, any further treatment should depend on the fertility history. If the previous fertilization rate was 0%, ICSI-AOA is advisable on all available MII oocytes. In contrast, if the previous fertilization rate was <33.3%, but not zero, the best strategy is to perform ICSI-AOA on half of the sibling oocytes to distinguish between a previous coincidental LF rate or a real oocyte-related activation deficiency. When a future cycle is necessary, AOA should be performed on all or none of the available MII oocytes according to the fertilization results of the split cycle. Finally, if any structural oocyte defect is suspected, non-invasive spindle evaluation on fresh MII oocytes by means of polarized light microscopy or invasive spindle evaluation on spare (MI or unfertilized) oocytes by means of immunostaining should be considered. Thus, taking into consideration the history of the couple, a split ICSI-AOA cycle on sibling oocytes, together with additional diagnostic approaches, such as spindle evaluation of the oocytes, is essential to select the proper therapeutic strategy in these cases. Based on the limited data available, we were able to claim that the fertility history of the patients in *MOAT Group 3* plays a critical role in choosing the appropriate diagnostic and therapeutic approach. Furthermore, it was shown that ICSI-AOA is not beneficial for all patients with a suspected oocyte-related activation deficiency. As ICSI-AOA is still an elaborate and experimental technique, it should only be considered in case of a well-diagnosed indication.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

F.V.M. analysed the data, interpreted the results and wrote the manuscript; D.N. contributed to interpreting the results; S.D, V.D. and B.H. performed the assisted oocyte activation; E.V.A and J.G. revised the manuscript; B.H. and P.D.S. designed the study, revised the manuscript and approved the final draft.

Funding

F.V.M. is the holder of an aspirant clinical research mandate by the Flemish Foundation of Scientific Research (FWO-Vlaanderen). P.D.S. is the holder of a fundamental clinical research mandate by the same Flemish foundation of Scientific Research (FWO-Vlaanderen).

Conflict of interest

None declared.

References

Ajduk A, Malagocki A, Maleszewski M. Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca2+ oscillations. *Reprod Biol* 2008;**8**:3–22.

Araki Y, Yoshizawa M, Abe H, Murase Y, Araki Y. Use of mouse oocytes to evaluate the ability of human sperm to activate oocytes after failure of activation by intracytoplasmic sperm injection. *Zygote* 2004; **12**:111–116.

Borges E Jr, de Almeida Ferreira Braga DP, de Sousa Bonetti TC, Iaconelli A Jr, Franco JG Jr. Artificial oocyte activation using calcium ionophore in ICSI cycles with spermatozoa from different sources. *Reprod Biomed Online* 2009a;**18**:45–52.

Borges E Jr, de Almeida Ferreira Braga DP, de Sousa Bonetti TC, Iaconelli A Jr, Franco JG Jr. Artificial oocyte activation with calcium ionophore A23187 in intracytoplasmic sperm injection cycles using surgically retrieved spermatozoa. *Fertil Steril* 2009b;**92**:131–136.

Broekmans FJ, Knauff EA, te Velde ER, Macklon NS, Fauser BC. Female reproductive ageing: current knowledge and future trends. *Trends Endocrinol Metab* 2007;**18**:58–65.

Chi HJ, Koo JJ, Song SJ, Lee JY, Chang SS. Successful fertilization and pregnancy after intracytoplasmic sperm injection and oocyte activation with calcium ionophore in a normozoospermic patient with extremely low fertilization rates in intracytoplasmic sperm injection cycles. *Fertil Steril* 2004;**82**:475–477.

Combelles CM, Morozumi K, Yanagimachi R, Zhu L, Fox JH, Racowsky C. Diagnosing cellular defects in an unexplained case of total fertilization failure. *Hum Reprod* 2010;**25**:1666–1671.

Cox LJ, Larman MG, Saunders CM, Hashimoto K, Swann K, Lai FA. Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca2+ oscillations, activation and development of mouse oocytes. *Reproduction* 2002;**124**:611–623.

Cross NL, Hanks SE. Effects of cryopreservation on human sperm acrosomes. *Hum Reprod* 1991;**6**:1279–1283.

Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA. Globozoospermia revisited. *Hum Reprod Update* 2007;**13**:63–75.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S, Ozil JP. Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev Biol* 2002; **250**:280–291.

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015

Ebner T, Moser M, Sommergruber M, Jesacher K, Tews G. Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. *Hum Reprod* 2004;**19**:1837–1841.

Eldar-Geva T, Brooks B, Margalioth EJ, Zylber-Haran E, Gal M, Silber SJ. Successful pregnancy and delivery after calcium ionophore oocyte activation in a normozoospermic patient with previous repeated failed fertilization after intracytoplasmic sperm injection. *Fertil Steril* 2003; **79**(Suppl. 3):1656–1658.

Esfandiari N, Javed MH, Gotlieb L, Casper RF. Complete failed fertilization after intracytoplasmic sperm injection—analysis of 10 years' data. *Int J Fertil Womens Med* 2005;**50**:187–192.

Flaherty SP, Payne D, Swann NJ, Mattews CD. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1995;**10**:2623–2629.

Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1998; **13**(Suppl. 1):155–164.

Goud PT, Goud AP, Van Oostveldt P, Dhont M. Presence and dynamic redistribution of type 1 inositol 1,4,5-trisphosphate receptors in human oocytes and embryos during in-vitro maturation, fertilization and early cleavage divisions. *Mol Hum Reprod* 1999;**5**:441–51.

Goud PT, Goud AP, Leybaert L, Van Oostveldt P, Mikoshiba K, Diamond MP, Dhont M. Inositol 1,4,5-trisphosphate receptor function in human oocytes: calcium responses and oocyte activation-related phenomena induced by photolytic release of $InsP_3$ are blocked by a specific antibody to the type I receptor. *Mol Hum Reprod* 2002; **8**:912–918.

Heindryckx B, Van der Elst J, De Sutter P, Dhont M. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. *Hum Reprod* 2005;**20**:2237–2241.

Heindryckx B, De Gheselle S, Gerris J, Dhont M, De Sutter P. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod Biomed Online* 2008;**17**:662–668.

Heytens E, Soleimani R, Lierman S, De Meester S, Gerris J, Dhont M, Van der Elst J, De Sutter P. *Reprod Biomed Online* 2008;**17**:764–771.

Ito M, Shikano T, Oda S, Horiguchi T, Tanimoto S, Awaji T, Mitani H, Miyazaki S. Difference in Ca2+ oscillation-inducing activity and nuclear translocation ability of PLCZ1, an egg-activating sperm factor candidate, between mouse, rat, human, and medaka fish. *Biol Reprod* 2008;**78**:1081–1090.

Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 2010;**16**:690–703.

Kovacic B, Vlaisavljevic V. Configuration of maternal and paternal chromatin and pertaining microtubules in human oocytes failing to fertilize after intracytoplasmic sperm injection. *Mol Reprod Dev* 2000;**55**:197–204.

Kuczyński W, Dhont M, Grygoruk C, Grochowski D, Wołczyński S, Szamatowicz M. The outcome of intracytoplasmic injection of fresh and cryopreserved ejaculated spermatozoa—a prospective randomized study. *Hum Reprod* 2001;**16**:2109–2113.

Liu J, Nagy Z, Joris H, Tournaye H, Smitz J, Camus M, Devroey P, Van Steirteghem A. Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. *Hum Reprod* 1995; **10**:2630–2636.

Mansour R, Fahmy I, Tawab NA, Kamal A, El-Demery Y, Aboulghar M, Serour G. Electrical activation of oocytes after intracytoplasmic sperm injection: a controlled randomized study. *Fertil Steril* 2009; **91**:133–139.

Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG, Nyboe Andersen A. Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE. European IVF-monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). *Hum Reprod* 2010;**25**:1851–1862.

Murase Y, Araki Y, Mizuno S, Kawaguchi C, Naito M, Yoshizawa M, Araki Y. Pregnancy following chemical activation of oocytes in a couple with repeated failure of fertilization using ICSI: case report. *Hum Reprod* 2004;**19**:1604–1607.

Nasr-Esfahani MH, Deemeh MR, Tavalaee M. Artificial oocyte activation and intracytoplasmic sperm injection. *Fertil Steril* 2010;**94**:520–526.

Palermo GD, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: where we have been and where we are going. *Semin Reprod Med* 2009;**27**:191–201.

Parrington J, Davis LC, Galione A, Wessel G. Flipping the switch: how a sperm activates the egg at fertilization. *Dev Dyn* 2007;**236**:2027–2038.

Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, Vitullo AD. Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Mol Hum Reprod* 2000;**6**:510–516.

Rybouchkin A, Dozortsev D, Pelinck MJ, De Sutter P, Dhont M. Analysis of the oocyte activation capacity and chromosomal complement of roundheaded human spermatozoa by their injection into mouse oocytes. *Human Reprod* 1996;**11**:2170–2175.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 2002; **129**:3533–3544.

Shen S, Khabani A, Klein N, Battaglia D. Statistical analysis of factors affecting fertilization rates and clinical outcome associated with intracytoplasmic sperm injection. *Fertil Steril* 2003;**79**:355–360.

Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization. *Hum Reprod* Update 2008;**14**:431–446.

Tesarik J, Mendoza C. In vitro fertilization by intracytoplasmic sperm injection. *Bioessays* 1999;**21**:791–801.

Tesarik J, Mendoza C, Greco E. The activity (calcium oscillator?) responsible for human oocyte activation after injection with round spermatids is associated with spermatid nuclei. *Fertil Steril* 2000; **74**:1245–1247.

Tesarik J, Rienzi L, Ubaldi F, Mendoza C, Greco E. Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures. *Fertil Steril* 2002; **78**:619–624.

Vanderheyden V, Wakai T, Bultynck G, De Smedt H, Parys JB, Fissore RA. Regulation of inositol 1,4,5-trisphosphate receptor type 1 function during oocyte maturation by MPM-2 phosphorylation. *Cell Calcium* 2009;**46**:56–64.

van Loendersloot LL, van Wely M, Limpens J, Bossuyt PMM, Repping S, van der Veen F. Predictive factors in in vitro fertilization (IVF): a systematic review and meta-analysis. *Human Reprod Update* 2010; **16**:577–589.

Yanagida K. Complete fertilization failure in ICSI. *Hum Cell* 2004; **17**:187–193.

Yanagida K, Katayose H, Yazawa H, Kimura Y, Sato A, Yanagimachi H, Yanagimachi R. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. *Hum Reprod* 1999;**14**:1307–1311.

Yanagida K, Morozumi K, Katayose H, Hayashi S, Sato A. Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization. *Reprod Biomed Online* 2006;**13**:801–806.

Downloaded from http://humrep.oxfordjournals.org/ at University of Lethbridge on November 14, 2015