Deposition of the spermatozoon in the human oocyte at ICSI: impact on oocyte survival, fertilization and blastocyst formation

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Abstract

Purpose To evaluate whether the deposition of the spermatozoon in the human oocyte at ICSI has any effect on oocyte survival, fertilization, blastocyst development and quality.

Methods In a prospective study, including 78 ICSI cycles, sibling oocytes were injected with “no intention” (group A, standard ICSI, $n=393$) or “intention” to deposit the spermatozoon under the cortex (group B, $n=354$). Outcome parameters were oocyte survival and fertilization, as well as blastocyst formation and quality.

Results Depositing the sperm under the cortex of the oocyte was not always successful for its final position, therefore, group B was divided into three subgroups: B1 successful deposition (119 oocytes, 33.6 % of oocytes in group B); B2 initially successful but spermatozoon spontaneously relocated after 2 min (136 oocytes, 38.4 %); and B3 unsuccessful deposition (99 oocytes, 28.0 %). Group A and B were compared on an intention-to-treat basis. Additionally, A, B1, B2 and B3 were also compared. The oocyte survival and fertilization, blastocyst and top-quality blastocyst developmental rates were not significantly different.

Conclusions The procedure of depositing the spermatozoon intentionally under the oocyte cortex demanded high technical

skills. Successful positioning was only obtained in 34 % of the attempts. We obtained no evidence of improved oocyte survival and fertilization, blastocyst formation and quality when the spermatozoon was permanently positioned under the oocyte cortex. Taken together, depositing the spermatozoon under the oocyte cortex is not recommended for routine ICSI application.

Keywords ICSI · Spermatozoon deposition · Oocyte fertilization · Blastocyst formation

Introduction

Conventional IVF and ICSI are commonly used as standard fertilization techniques in ART, for female and male factor infertility respectively. Between the moment of sperm entry and the appearance of the two pronuclei, a number of events occur in the oocyte: the cytoskeleton undergoes modifications, cortical granules are released into the perivitelline space, mitochondria are translocated, Ca^{2+} is released from the endoplasmic reticulum in a repetitive (oscillatory) way, sperm head decondensation occurs and the second polar body is extruded. It has been shown in mice that repetitive Ca^{2+} oscillations are essential for oocyte activation and embryo development [7]. Ca^{2+} oscillations have also been reported in human oocytes after conventional IVF [27] or after ICSI [28]. They are triggered by the sperm-specific phospholipase C zeta (PLC ζ) that is released in the ooplasm when the spermatozoon fuses with the oolemma and subsequently activates the oocyte [23]. Though essential, Ca^{2+} oscillations might not be sufficient for oocyte activation and are not necessarily indicative of sperm entry. Additionally, whereas PLC ζ may be one component of the so-called sperm factor, it has not been conclusively shown to be the sperm factor by itself.

Capsule Spermatozoon deposition under the human oocyte cortex at ICSI did not improve oocyte survival and fertilization, nor blastocyst formation and quality.

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More recently, cytoplasmic movements triggered by Ca^{2+} oscillations have been observed after sperm entry into mouse oocytes [3]. These cytoplasmic flows, caused by contractions of the actin cytoskeleton, last for several hours until the pronuclei are formed. In order to detect those fast oscillatory cytoplasmic movements, zygotes have to be subjected to rapid time-lapse imaging [2]. Similar cytoplasmic movements have also been observed in activated human eggs [26], however, these have to be distinguished from earlier observations in human zygotes, showing movements in the cytoplasm with a slower cycle time and thus not directly related to activation events [20]. In mice, the pattern of cytoplasmic movements is predictive for preimplantation development and reproductive success [2]. Their predictive potential is currently being tested in human embryos, as mentioned by these investigators [2].

Ajduk and colleagues further characterized the relative contribution made by either the sperm per se or the fertilization cone (FC), i.e., a transient protrusion at the site of sperm entry [11] occurring in respectively about 69 and 76 % of mouse zygotes after natural conception or in vitro fertilization (IVF) [21]. While the FC is not observed after standard intracytoplasmic sperm injection (ICSI) by depositing the spermatozoon arbitrarily in the mouse oocyte, it does appear when the spermatozoon is deposited under the oocyte cortex [3]. In both cases, sperm injection results in Ca^{2+} oscillations and oocyte activation. In the absence of FC, only about 71 % of the zygotes display cytoplasmic movements and their amplitude is lower than in zygotes with FC or naturally fertilized oocytes (97 % showing Ca^{2+} transients accompanied by speed peaks). Interestingly, mouse zygotes displaying high cytoplasmic movements develop better to blastocyst and to a full term pregnancy. Thus, recording of cytoplasmic movements in fertilized oocytes may be a non-invasive tool to predict their developmental capacity.

Whether this technique can be used to predict the clinical outcome in IVF cycles in the human by selecting the zygotes with the best developmental capacity, remains to be proven. The FC has not yet been described in the human, however, Ca^{2+} oscillations after conventional IVF [27] and after ICSI [28] have been reported as well as small cytoplasmic movements after PLC ζ cRNA injection [26]. In conventional IVF the spermatozoon fuses with the oolemma; in the standard ICSI procedure the spermatozoon is arbitrarily put between the cortex and the centre of the oocyte [16]. Based on Ajduk's findings [3], it is tempting to speculate that the position of the spermatozoon after ICSI, under the cortex or arbitrarily in the centre of the human oocyte, may affect the amplitude of the cytoplasmic movements and thus embryonic development.

Therefore, the aim of this study was to compare the effect of injecting the spermatozoon either under the cortex or in the centre of the human oocyte on fertilization and in vitro embryo development.

Materials and methods

This prospective study was approved by the local ethical committee and was performed between September 2012 and April 2013. After informed consent, 78 patients having ICSI cycles with at least 4 mature oocytes and fresh ejaculated sperm (>0.1 million/ml) were included. There was no female age limit. Baseline semen parameters (concentration and motility) were 52.6 ± 70.8 million/ml and 50.0 ± 21.1 % progressive A + B motility ($\pm$ SD). Patients having preimplantation genetic diagnosis were excluded. Sibling oocytes were injected with “no intention” (group A, standard ICSI) or “intention” (group B) to deposit the spermatozoon under the cortex. Embryo transfers were performed on day 5.

Our primary endpoint was development to top-quality blastocyst stage. Our secondary endpoints were oocyte survival, oocyte fertilization and total blastocyst formation rate per fertilized oocyte.

Ovarian stimulation was performed using either a long GnRH-agonist protocol (Suprefact, Aventis Pharma, Frankfurt, Germany) in combination with hMG (Menopur, Ferring Pharmaceuticals A/S) [18] or in combination with recombinant FSH, (Puregon, MSD Organon, Oss, The Netherlands or Gonal-F, Merck-Serono, Overijse, Belgium). Alternatively, using a GnRH-antagonist protocol, Orgalutran (MSD Organon, Oss, The Netherlands) or Cetrotide (Merck-Serono, Overijse, Belgium) was administered in order to inhibit a premature LH surge [14]. Final oocyte maturation was achieved by 5 000 or 10 000 IU hCG (Pregnyl, MSD Organon, Oss, The Netherlands) when at least three follicles with a minimum diameter of 17 mm were present at ultrasound. Intravaginally administered progesterone (Utrogestan, Besins, Brussels, Belgium) was used for luteal phase supplementation.

After oocyte retrieval (36 h after hCG administration) and subsequent oocyte denudation, the cohort of mature oocytes (as evidenced by the presence of the first polar body) was equally divided and either part was assigned to group A or B according to a randomization list. Presence of a first polar body is considered as the best measure for oocyte maturity although it does not mean that all oocytes are at the same level of cytoplasmic maturity. In case of an odd oocyte number, the surnumerary mature oocyte was assigned to group A. In both groups the oocyte was held with a holding pipette at the 9 o'clock position and the first polar body at the 6 o'clock position. The ICSI pipette was intruded deeply into the oocyte at the 3 o'clock position and the oolemma was aspirated as described before [16]. In group A, the spermatozoon was left arbitrarily in the centre of the oocytes. In group B, the pipette was intruded deeper after oolemma aspiration and the spermatozoon was left under the cortex of the oocyte at the 9 o'clock position. All injections were limited to two operators, one operator for all the oocytes in a given cycle. The authors are aware that the position of the metaphase II spindle is not

always predicted exactly by the location of the first polar body [12]; however, spindle damage vulnerability is believed to be equally low in both groups.

The injected oocytes were incubated at 37 °C (6 % CO₂, 5 % O₂ and 89 % N₂) in 25 µl droplets of Quinn's Advantage™ sequential media (SAGE, cleavage and blastocyst medium, Rochford Medical Ltd, Coventry, England) under oil (Ovoil, Vitrolife, Goteborg, Sweden). Embryos were transferred to blastocyst medium on day 3. The embryologists scoring fertilization and blastocyst quality were blinded for the study. Embryo transfer was performed on day 5, transferring one, two or three blastocysts according to Belgian legislation, i.e., depending on female age and the rank of trial.

Oocyte survival was expressed per injected oocyte. The fertilization rate was calculated as the number of oocytes with two pronuclei (2-PN) 16–19 h after ICSI upon the number of injected oocytes. The blastocyst formation rate was defined as the number of good-quality blastocysts on day 5 (BL1; BL2; BL3, BL4 and BL5, AA, AB, BA and BB, according to Gardner and Schoolcraft [9]) upon the number of fertilized oocytes. Top-quality blastocyst rates were calculated in two ways: (a) BL3 to BL5, AA and AB according to Van Landuyt et al. [30]; and (b) BL4 AA and BA as proposed by Ahlström et al. [1].

A clinical pregnancy was defined as the presence of a gestational sac at ultrasound performed at 7 weeks of gestation [35].

To achieve an 80 % power and detect a 10 % difference in the proportion of top-quality blastocysts, the sample size was 250 oocytes in each group (group A, no intention, standard ICSI and group B, intention to deposit the spermatozoon under the cortex). Outcome parameters of interest were summarized as mean percentage (SE, standard error). Group A and B were compared on an intention-to-treat basis using a Student's *t*-test. Given the skewed distribution of the outcome variables, groups A, B1, B2 and B3 were compared using a nonparametric Kruskal-Wallis test. The significance level was 0.05.

Results

Overall, 747 sibling oocytes from 78 patients (mean age 31.4 ±4.9 SD) were injected; 393 oocytes were allocated to group A, and 354 to group B (Table 1). Overall 95.6±0.9 % of the injected oocytes survived the injection procedure and 85.5±1.4 % got fertilized. On day 5, 51.1±3.0 % of the fertilized oocytes developed into blastocysts and 17.4±2.3 % developed into top-quality blastocysts according to Van Landuyt et al. [30] or 9.9±1.6 % according to Ahlström et al. [1].

Putting the spermatozoon under the cortex of the oocyte was not always successful regarding the final position of the spermatozoon, therefore group B was divided into three subgroups (three videos included): B1 successful (119 oocytes, 33.6 % of oocytes in group B); B2 initially successful but the

Table 1 Oocyte survival, fertilization and blastocyst formation

	No intention standard ICSI		Intention-to-treat		As-treated		P-value
	Group A	Total group B	Successful group B1	Relocated group B2	Unsuccessful group B3	P-value	
Number of injected oocytes	393	354	119	136	99		
Oocyte survival rate ^a	96.6±1.0	94.6±1.3	94.2±2.7	95.8±2.1	90.5±3.3	0.460	
Fertilization rate ^a	85.6±2.0	85.6±2.1	86.2±3.3	89.9±2.9	83.2±4.5	0.065	
Blastocyst development ^b							
Blastocyst formation rate	50.0±3.7	52.3±3.8	46.5±6.0	58.3±5.1	48.5±6.9	0.361	
Top-quality blastocyst rate ^c	16.6±2.8	18.2±2.9	23.6±5.2	14.6±3.8	15.2±4.9	0.263	
Top-quality blastocyst rate ^d	10.3±2.2	10.1±2.2	16.7±4.7	6.0±2.7	9.8±4.0	0.070	

Mean percentages per cycle±SE

Group A and B were compared using a Student's *t*-test; groups A, B1 B2 and B3 were compared using a nonparametric Kruskal-Wallis test

^a % per injected oocyte

^b % per 2-PN fertilized oocyte

^c according to Van Landuyt et al. [30]

^d according to Ahlström et al. [1]

spermatozoon was spontaneously relocated (with the length of one sperm head defined as the relocation distance) after 2 min (136 oocytes, 38.4 %); and B3 unsuccessful (99 oocytes, 28.0 %).

A comparison between group A and B on an intention-to-treat basis, did not reveal any significant difference in oocyte survival rate, fertilization rate, blastocyst formation rate and top-quality blastocyst developmental rates (according to [30] or to [1]) (Table 1). The same was true comparing A and B1, B2 or B3 (Table 1).

All 78 ICSI cycles resulted in embryo transfer of one ($n=37$), two ($n=39$) or three blastocysts ($n=2$). Clinical pregnancies were obtained in all groups (Table 2). An ultrasound at 7 weeks of gestation was available for all cycles, showing 38 clinical pregnancies (48.7 % per cycle). Of all transfers, 48 were informative (single embryo transfer or multiple embryo transfer with all transferred embryos resulting from the same injection group). These resulted in a clinical pregnancy rate of 45.8 % per cycle. Successful or unsuccessful deposition of the sperm cell into the oocyte in group B did not influence clinical outcome.

Discussion

Although in the mouse deposition of the spermatozoon under the oocyte cortex influences ICSI outcome [3], the present study in the human showed no evidence of improved oocyte survival, oocyte fertilization, blastocyst development or top-quality blastocyst formation. Obtaining this particular permanent deposition of the spermatozoon revealed to be technically challenging and relocation inside the ooplasm occurred in a high proportion of the oocytes. Therefore, this targeted ICSI technique is not recommended for routine clinical application.

The choice between IVF and ICSI treatment depends on the cause of infertility in the couple. Female infertility problems may be overcome by conventional IVF [24] imitating the natural way of reproduction because the spermatozoon penetrates the oocyte through a physiological process and the spermatozoon is selected in a natural way. Severe male infertility may be overcome by ICSI by injecting one single spermatozoon into the oocyte [17]. When performing ICSI, the embryologist chooses the spermatozoon based on its motility and gross morphology bypassing the three natural barriers of natural conception or IVF, i.e., the cumulus-corona-complex by denudation, the zona pellucida (ZP) by penetration with a pipette and the oolemma by penetration and/or aspiration with a pipette [16]. During the standard ICSI procedure, the oocyte is conventionally positioned with its first polar body at the 6 o'clock position and the oocyte is penetrated with the pipette at the 3 o'clock position [16] in order to avoid damage to the meiotic spindle. After breaking the ZP and oolemma, the

Table 2 Clinical outcome

	Total	No intention Standard ICSI	Intention-to-treat	As-treated		
				Successful group B1	Relocated group B2	Unsuccessful group B3
Number of transfers	78					
Mean age ($\pm$ SD)	31.4 $\pm$ 4.9					
Positive hCG	39 (50.0 %)					
Clinical pregnancy	38 (48.7 %)					
Number of informative transfers	48	29	19	6	8	5
Mean age	31.0 $\pm$ 4.7	31.2 $\pm$ 5.1	30.5 $\pm$ 4.0	29.2 $\pm$ 5.2	31.4 $\pm$ 3.4	31.2 $\pm$ 4.1
Positive hCG	23 (47.9 %)	12 (41.4 %)	11 (57.9 %)	4 (66.7 %)	4 (50.0 %)	3 (60.0 %)
Clinical pregnancy	22 (45.8 %)	12 (41.4 %)	10 (52.6 %)	4 (66.7 %)	3 (37.5 %)	3 (60.0 %)

spermatozoon is arbitrarily put into the oocyte, between the cortex and the centre but never under the cortex.

So far, ICSI injection studies have been focussed on the position of the first polar body, as being associated with the presumed area of the meiotic spindle [16, 29, 4, 25]. The location of the meiotic spindle relative to the site of sperm deposition is indeed an important technical parameter of the ICSI procedure, which can affect fertilization and embryo development. Potential damage to the spindle should be avoided at all price, however, a shorter distance between the sperm and the spindle can benefit undelayed pronuclear syngamy. Clinical practice mainly involves positioning the first polar body at 6 or 12 o'clock (eventually 7 or 11 o'clock) and injection of the spermatozoon into the oocyte at 3 or 9 o'clock. It should however not be neglected that the position of the first polar body is only a crude measure for spindle position, because both do not always coincide as evidenced by immunostaining [12] or Polscope imaging [32].

In this study, we aimed to mimic the natural way of fertilization by intentionally leaving the spermatozoon under the cortex. However, this ICSI procedure demanded high technical skills and great concentration and eventually did not result in a better oocyte survival, fertilization, blastocyst and top-quality blastocyst developmental rates.

A permanent deposition of the spermatozoon under the cortex of the oocyte was only successful in 34 % of the attempts. The spermatozoon was mostly relocated due to the flow inside the ICSI pipette and/or the constitution of the ooplasm. It remained quite close to the cortex in 38 % of the attempts and it finally ended up in the centre of the oocyte in 28 % of the attempts. Most likely, the introduction of a minimal amount of PVP into the oocyte creates a microenvironment of less viscosity or absence of microtubular structures. Less viscosity could promote dislocation towards the centre. Technical difficulties in precisely injecting the spermatozoon at an intended location during ICSI have been described before [33]. Yanaihara et al. [33] also obtained similar fertilization rates with center and left center spermatozoon positions. Fertilization rates significantly dropped when the spermatozoon moved back closer to the 3 o'clock position. Embryo quality on day 3 was similar for center and left center spermatozoon position, consistent with our findings [33].

Spermatozoon dislocation might also be related to the viscosity of the ooplasm itself. Peripheral areas indeed show less viscous cytoplasm than central more granulated areas [20]. Extensive cytoplasmic granularity may be centrally located or homogeneous, affecting the whole gamete. In contrast, slight or moderate granularity has been accepted as a normal feature of oocytes. All oocytes injected in this study belonged to the latter category, although minimal differences in viscosity cannot be excluded, since we did not record injection funnel persistence after withdrawal of the injection pipette. This latter feature has been used as a measure for oocyte

cytoplasmic viscosity, showing that increased viscosity represents a negative prognostic marker of preimplantation development [8].

In mouse oocytes, cytoplasmic movements, as measured by particle image velocimetry (PIV), are dependent on the actin cytoskeleton and influenced by the presence of the sperm and the fertilization cone (FC) [3]. Since FC formation seems to depend on a close interaction between the sperm chromatin and cortical actin, FC formation was only observed when the sperm was injected immediately under the cortex. However, in the absence of the FC, when the sperm was positioned into the centre of the egg, speed peaks still occurred, albeit at a lower frequency (71 % as compared to 97 %) and with a lower amplitude. If the pattern of cytoplasmic movements is indeed predictive of preimplantation development, this would mean that the sperm deposition into the egg plays a role in future embryo development. Yet, our data do not support this hypothesis in the human. There are two major technical differences in the ICSI procedure between mice and humans. One difference is sperm cell immobilization and the second is oolemma breakage. Before injection, mouse sperm cells are decapitated by piezo pulses, allowing injection of the head alone [34]. In contrast, human sperm cells are immobilized by scratching their tail on the bottom of the injection dish using the micropipette [13]. This procedure causes sperm membrane damage [5] and the positional effect of the sperm cell would be disturbed if PLC ζ would be lost by passive release, masking any effect on cytoplasmic movements and future development. However, it has been demonstrated that this tail scratching method does not lead to a release of PLC ζ [6], which is predominantly localized to the equatorial region of the human sperm head [10]. In the absence of immediate passive loss, as we believe, our data are in line with Swann et al. [26], i.e., PLC ζ induced cytoplasmic movements in human oocytes may depend merely on the presence of the sperm cell rather than on its position in the oocyte, which is in contrast to findings in mice [3]. Secondly, the human oolemma is mechanically broken with a micropipette at 37 °C [31], whereas the mouse oolemma needs to be broken using a piezo micropipette at room temperature in order to avoid damage [34]. We do not believe that this latter difference in oolemma breakage could differentially affect PLC ζ triggered cytoplasmic movements.

One other fundamental difference between mice and the large majority of mammalian species, including the human, is the maternal inheritance of the centrosome. [22]. Only one centrosome, maternal or paternal, needs to be active to ensure normal fertilization and development. In the human, this machinery for cell division, located in the neck region of the sperm cell, is transmitted to the egg by the male gamete at fertilization. In contrast, in mice, the centrosome is provided by the oocyte itself. It remains unclear whether this fundamental biological difference between mice and human could help

to explain the observed differences in regard to sperm location. On the other hand, the fact that immobilized human sperm maintains oocyte activating activity longer as compared to mouse sperm heads [15] could offer some additional explanation.

If cytoplasmic movements triggered by fertilization would play a major role during embryogenesis as shown in the mouse, but still to be validated in human embryos [3, 2], IVF embryos are expected to have a better quality than ICSI embryos due to the more natural position of the spermatozoon near the oocyte cortex. However, mouse IVF and ICSI embryos developed at rates comparable to naturally conceived embryos [21]. Likewise, in humans, evidence exists for similar blastocyst development after IVF and ICSI using sibling oocytes and thus excluding any possible influence of the sperm quality on either fertilization or development [30].

The present study did not include PIV recordings of cytoplasmic movements because such equipment was not available. Therefore, it remains unknown whether differentially injected oocytes would display differential developmental patterns. Anyway, it would not be predictive as a tool to select the best embryo since our study does not show a significant difference in blastocyst formation.

Despite all the efforts taken to predict embryo development at an early stage, embryo selection by culturing to the blastocyst stage is currently an efficient tool to select the best embryo for transfer, with highest implantation rates obtained when transferring a top-quality blastocyst [18, 19]. However, day 5 embryo culture and transfer may interfere with crucial epigenetic events occurring during early embryogenesis. In that respect, the search for an early embryo selection tool, avoiding extended in vitro culture, should not be ceased. An early prediction of the best embryo would not only avoid multiple pregnancies through single embryo transfer, but may also reduce the time to pregnancy.

From our RCT in the human, we conclude that deposition of the spermatozoon under the cortex of the oocyte at ICSI, mimicking the natural way of fertilization, does not have any effect on survival, fertilization or blastocyst formation rate and does not improve blastocyst quality.

Furthermore, given the technical difficulty of this targeted ICSI procedure, the routine ICSI technique as described by our group in 1992 [17] still remains the gold standard.

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Conflict of interest The authors declare that they have no conflict of interest.

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