Article

Outcome of ICSI using zona pellucida-bound spermatozoa and conventionally selected spermatozoa

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Abstract

This study aimed to investigate whether the spermatozoa–zona pellucida binding test is able to select spermatozoa with higher fertilization potential and higher rate of successful embryo development. This prospective study was performed with metaphase II (MII) oocytes retrieved from couples undergoing intracytoplasmic sperm injection (ICSI) cycles. For each patient, half of the MII oocytes were injected using a routine ICSI method (control group, $n = 194$) while the other half were injected with previously zona pellucida-bound spermatozoa (ZP-binding group, $n = 194$). Fertilization rate, high-quality embryo rate, and embryo transfer rate were compared between the groups. No significant difference was observed among the groups in the fertilization rate (76.8% versus 77.3% for control and ZP-binding groups, respectively). However, an increased percentage of high-quality embryos was observed when zona pellucida-bound spermatozoa were injected (70.0% versus 83.3% for control and ZP-binding groups, respectively, $P = 0.003$). Moreover, when embryo selection was performed while ignoring experimental group origin, embryos from the ZP-binding group were more commonly selected for transfer (43.6% versus 54.6% for control and ZP-binding groups, respectively, $P = 0.004$). These findings suggest that the spermatozoa–zona pellucida binding test may be an efficient method to identify the most competent spermatozoa for ICSI.

Keywords: *embryo development, fertilization, ICSI, implantation, sperm function, zona pellucida*

Introduction

The interaction between oocyte and spermatozoa implies a series of physiological events involving species recognition, adhesion and fusion between gametes (Ben-Yosef and Shalgi, 2001). The ultimate step is egg activation – the starting point of a developmental programme leading to the formation of a new individual (Ciapa and Chiri, 2000). The first interaction between the spermatozoon and the oocyte is at the zona pellucida. The specific binding of the spermatozoon to the zona pellucida induces the acrosome reaction, leading to fertilization (Ben-Yosef and Shalgi, 2001).

In the process of intracytoplasmic sperm injection (ICSI), a single spermatozoon is selected and delivered into the oocyte. The injected fertilizing spermatozoon therefore bypasses several physiological barriers compared with in-vivo or conventional in-vitro fertilization (IVF). Traditional semen analysis, however, does not provide accurate information regarding the fertilization ability of the spermatozoa. Furthermore, the lower developmental competence of embryos originating from ICSI when compared with those obtained by conventional IVF is still under debate (Griffiths *et al.*, 2000; Miller and Smith, 2001). Liu and Baker (2000) described that defective spermatozoa–zona

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pellucida interaction is the major cause for low fertilization rates in IVF, and that this is usually due to defects in the spermatozoa rather than defective oocytes.

It has indeed been found that oligozoospermic men have a higher frequency of defective spermatozoa–zona pellucida interactions (Van Steirteghem *et al.*, 1993). In addition, only a small proportion of motile spermatozoa from infertile men is capable of binding the zona pellucida *in vitro*. It was observed that sperm morphology was significantly related to the percentage of motile spermatozoa capable of binding to the zona (Liu *et al.*, 2003). Data on hyaluronic acid-bound spermatozoa were also reported. The formation of hyaluronic acid receptors is similar to the spermatozoa–zona pellucida-binding sites. It has been demonstrated that, in immature spermatozoa with cytoplasmic retention, there is a low density of zona pellucida binding sites and also of hyaluronic acid receptors. Moreover it was shown that when the integrity of the acrosomal membrane is disrupted, the spermatozoa is not able to bind to hyaluronic acid (Huszar *et al.*, 2003). Together, these studies suggest that the spermatozoa–zona pellucida binding process plays an important role in the selection of spermatozoa with normal motility and morphology.

Since the conception of the first healthy child via ICSI (Palermo *et al.*, 1992), this technique has becoming increasingly popular as a means of infertility therapy. The main challenge for the success of ICSI is to produce viable embryos that have high implantation potential. Implantation and early post-implantation development are conditioned by the viability of each embryo transferred, which, in turn, depends on the biological quality of the oocyte and the spermatozoon at the given embryo's origin. Consequences of the actions of sperm-derived factors on preimplantation embryo development, referred to as paternal effects, have been shown to be responsible for repeated failures of assisted reproduction attempts (Tesarik, 2005).

The goal for this study was to investigate whether the spermatozoa–zona pellucida binding test is able to select spermatozoa with higher fertilization potential and a higher rate of successful embryo development.

Materials and methods

Experimental design

This prospective study was performed in 388 metaphase II (MII) oocytes retrieved from 40 couples undergoing ICSI for the first time. The patients included in this study were: (i) $\leq$35 years old; (ii) had at least six MII oocytes and one metaphase I oocyte retrieved; and (iii) had semen samples with sperm concentration $\geq 5 \times 10^6$ motile spermatozoa/ml. Informed written consent was obtained in which patients agreed to share the outcomes of their own cycles for research purposes. The study was approved by the local institutional review board.

For each patient, half of the MII oocytes were injected with the study centre's routine ICSI method (control group), which is performed in all cases in this centre and the other half were injected with previously zona pellucida-bound spermatozoa (ZP-binding group). A total of 194 oocytes were included for each group. Successful fertilization and embryo quality were compared between the groups.

Controlled ovarian stimulation

Controlled ovarian stimulation was achieved by long pituitary down-regulation using a GnRH agonist (Lupron Kit; Abbott, Paris, France) followed by ovarian stimulation with recombinant-FSH (Gonal-F, Merck-Serono, Geneva, Switzerland). The follicular dynamic was followed with ultrasound starting on day 4 of gonadotrophin administration. When adequate follicular growth and serum oestradiol concentrations were observed, recombinant human chorionic gonadotrophin (HCG, Ovidrel; Merck-Serono) was administered to trigger the final follicular maturation. Oocytes were collected 34–36 h after HCG administration using transvaginal ultrasound ovum retrieval.

Sperm samples

Ejaculated spermatozoa were obtained by masturbation after 3–5 days of ejaculatory abstinence. For all samples, the sperm concentration and motility were assessed based on the World Health Organization criteria (World Health Organization, 1999). After liquefaction at room temperature, sperm samples were prepared by discontinuous density-gradient centrifugation or swim-up. For discontinuous density-gradients, the bottom fraction was aspirated and washed twice at 300 *g* for 8 min. For swim-up, sperm samples were diluted 1:1 with a HEPES-buffered medium (Irvine Scientific, Santa Ana, USA) and incubated at 37°C for 1 h, allowing spermatozoa to move from the seminal plasma to the overlaid culture medium.

Preparation of oocytes

After retrieval, oocytes were incubated in HTF culture medium (Human Tubal Fluid, Irvine Scientific, Santa Ana, USA) covered with mineral oil (Ovoil; Vitrolife, Kungsbacka, Sweden) and incubated at 37°C in 6% CO_2 for 5 h. Cumulus cells were removed with 30 s exposure to a HEPES-buffered medium containing 80 IU/ml hyaluronidase (Irvine Scientific). Coronal cells were then manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA). Denuded oocytes were then assessed for nuclear status. Oocytes that were observed to have released the first polar body were considered mature and used for ICSI.

Zona pellucida binding test and intracytoplasmic sperm injection

For each patient, a 5 µl sperm sample (concentration: 1×10^6 motile spermatozoa per ml) was incubated with one MI oocyte in 5 µl of buffered medium (HTF-HEPES; Irvine Scientific, Santa Ana, USA) in a 50 × 40 mm glass culture dish (WillCo-dish; New Jersey, USA) covered with

warm mineral oil and kept at 37°C in 5% CO_2. After incubating for 2 h, the oocytes were carefully washed in two 5 µl droplets of buffered medium, to dislodge spermatozoa loosely adhering to the surface of the zona pellucida. The dish was placed under an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a heated stage at 37.0 ± 0.5°C and observed at ×400 magnification. The spermatozoa bound to the MI oocyte zona pellucida were removed with a microinjection needle (ICSI micropipette; Humagen Fertility Diagnostics, Charlottesville, Virginia, USA) and transferred to a central 5 µl droplet of polyvinylpyrrolidone solution (Irvine Scientific). Morphologically normal and motile spermatozoa were immobilized, aspirated into the microinjection needle and injected into the MII oocytes from the ZP-binding group.

Oocytes from the control group were placed individually into 5 µl droplets of buffered medium. Spermatozoa were placed in a central 5 µl droplet of polyvinylpyrrolidone solution in a 5 × 40 mm glass culture dish covered with warm mineral oil. Spermatozoa were retrieved from the central droplet and used for ICSI.

For both groups, only motile and morphologically normal spermatozoa were injected.

Assessment of fertilization, embryo quality and embryo transfer

Fertilization was assessed 18 h after ICSI. Normal fertilization was confirmed when two clearly distinct pronuclei were present. Embryo quality was evaluated under an inverted microscope on the third day of development. The following parameters were recorded: (i) number of blastomeres; (ii) fragmentation percentage; (iii) variation in blastomere symmetry; (iv) presence of multinucleation; and (v) defects in the zona pellucida and cytoplasm. High-quality embryos were defined as those having all of the following characteristics: 8–10 cells, less than 15% fragmentation, symmetric blastomeres, absence of multinucleation, colourless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity and absence of zona pellucida dysmorphism. Embryos lacking any of the above characteristics were considered to be of low quality. Embryo transfer was performed on the third day of development, with one to four embryos for each couple. Embryo selection for transfer was performed blindly and was based on embryo quality.

Statistical analysis

Results are expressed as the mean ± SD for numeric variables and proportions (%) for categorical variables. Mean values were compared by a Student's *t*-test, and proportions were compared by the chi-squared test. Regression models were conducted to study the influence of using ZP-bound spermatozoa on ICSI outcome. Results are expressed as odds ratios (OR), 95% confidence intervals (CI) and *P* values. Results were considered to be significant at the 5% critical level ($P < 0.05$). Data analysis was carried out using the Minitab (version 14) statistical program.

Results

No significant difference was observed in the fertilization rate regardless of whether oocytes were injected with zona pellucida-bound spermatozoa (**Table 1**). Similarly no statistically significant difference was found in pronuclear morphology (data not shown). An increased percentage of high-quality embryos was observed, however, when zona pellucida-bound spermatozoa were injected (**Table 1**). This finding was confirmed using a binary logistic regression, showing that the use of zona pellucida-bound spermatozoa for ICSI was determinant of the likelihood of the embryo quality (OR = 2.23; CI 95% = 1.30–3.81; $P = 0.004$).

In addition, when embryo selection was performed while ignoring the experimental group origin, it was observed that embryos from the ZP-binding group were more commonly selected for transfer when compared with the control group (**Table 1**). This result was also confirmed by the logistic regression model, which demonstrated a nearly twofold increase in embryo transfer rates in embryos derived from the ZP-binding group (OR = 1.94; IC 95% = 1.21–3.13; $P = 0.006$).

The implantation and pregnancy rates among the study group were 30.5% and 37.5%, respectively.

Discussion

Both oocyte and sperm quality have been regarded as variables that influence the implantation potential of derived embryos. Many studies have focused on the identification of biochemical makers of human sperm maturity and function, independent traditional semen criteria of sperm concentration such as motility and morphology (Evensonm

Table 1. Comparison of fertilization, embryo quality and embryo transfer rates in oocytes undergoing routine intracytoplasmic sperm injection (control) or injected with zona pellucida-bound spermatozoa.

Parameter	*Control* (n = *194*)	*ZP-bound spermatozoa* (n = *194*)	P-*value*
Fertilization rate	76.8 (149/194)	77.3 (150/194)	NS
High-quality embryo rate	70.0 (104/149)	83.3 (125/150)	0.003
Embryo transfer rate	43.6 (65/149)	54.7 (82/150)	0.004

Values are percentage (number/total); NS = not statistically significant at the 5% level; ZP = zona pellucida.

et al., 1999; Larson *et al.*, 2000; Larson-Cook *et al.*, 2003; Sergerie *et al.*, 2005).

During fertilization, spermatozoa bind to the zona pellucida, undergo the acrosome reaction, penetrate the zona and fuse with the oolema (Ben-Yosef and Shalgi, 2001). Spermatozoa capable of binding to the zona pellucida represent a subgroup of the sperm population satisfying a necessary condition for fertilization (Liu *et al.*, 2003). The use of spermatozoa-ZP interaction test to improve the diagnosis and management of infertility by assisted reproduction technology has been well studied (Van Steirteghem *et al.*, 1993; Oehninger *et al.*, 2000; Liu de *et al.*, 2007). As far as is known, this is the first study to evaluate the usefulness of the zona pellucida binding test to select more competent spermatozoa for ICSI.

It has been previously demonstrated that the spermatozoa–zona pellucida binding test correlates well with fertilization *in vitro* (Liu *et al.*, 1988). In the present study, improvements in the fertilization rate were not observed. However, embryos derived from oocytes injected with zona pellucida-bound spermatozoa presented a higher developmental capacity. Although it is well recognized that sperm quality plays a key role during fertilization (Swann *et al.*, 2006; Saunders *et al.*, 2007), it has also been shown that preimplantation embryo development can be compromised by deficiencies in both the nuclear genome of the spermatozoa or by sperm-derived cytoplasmic factors (Tesarik, 2005). Expression of the embryonic genome, which is a combination of the sperm and oocyte contribution, starts between the four- and eight-cell stage of human embryo development (Tesarik *et al.*, 1986, 1988). The eventual disruption of sperm-derived genes is therefore unlikely to manifest between fertilization and the four-cell stage. Four-cell stages are routinely observed in the first 50 h of development, and eight-cell stages are usually noted before 72 h (Veeck, 1991). In the present study, embryo quality was evaluated in the third day of development (i.e. 72 h after ICSI), when embryonic genome activation should have occurred. In addition, although it has been suggested that oocyte-derived factors are responsible for the control of preimplantation development up until the activation of embryonic gene expression, Tesarik and Kopecny (1989) demonstrated that the sperm-derived genome is not completely silent in the period between fertilization and early cleavage division stages.

This study's findings raise the question of whether the spermatozoa capable of binding to the zona pellucida may be compromised by other specific characteristics responsible for the positive paternal effects on early embryonic development, and what the mechanism of this effect would be.

In humans, of the 300 million spermatozoa ejaculated, only about 200 reach the site of fertilization in the oviduct (Neill, 2006). There is evidence that chemical signals released by the oocyte attract the spermatozoa to the zona pellucida, but the nature of the chemoattractant molecules is unknown (Alberts *et al.*, 1994). Another hypothesis of mammalian gamete binding postulates a set of proteins on the spermatozoa capable of recognizing specific carbohydrate regions of the zona glycoproteins (Kopf, 1998). The proposed mechanism for this event is based on the specific binding of sperm-surface carbohydrate-binding proteins to glycoconjugates present in the zona pellucida and this sets off the signal transduction pathways that result in the acrosome reaction (Oehninger, 2003). Rosano *et al.* (2007) demonstrated that the D-mannose binding sites participate in spermatozoa–egg interaction and, moreover, that there is an association between the detection of D-mannose binding sites and acquisition of in-vitro sperm fertilizing ability. The hyaluronic acid binding ability of human spermatozoa have also been evaluated (Huszar *et al.*, 2006; Nasr-Esfahani *et al.*, 2008). After a pre-ovulatory gonadotrophin surge, cumulus cells secrete a hyaluronic acid-rich matrix that binds the oocyte and cumulus together, facilitates follicular extrusion and oviductal fimbrial capture, and allows sperm penetration and fertilization (Kimura *et al.*, 2007).

Cayli *et al.* (2003) postulated that there are three sperm populations: (i) spermatozoa that remain permanently bound to hyaluronic acid; (ii) spermatozoa exhibiting no binding; and (iii) a small proportion of spermatozoa that initially binds to hyaluronic acid, is shortly released and then binds again. A significant inverse correlation was observed between hyaluronic acid binding with protamine deficiency, DNA fragmentation and abnormal sperm morphology (Nasr-Esfahani *et al.*, 2008). The hyaluronic acid binding ability of the spermatozoa was also related to sperm maturity and acrosomal integrity (Huszar *et al.*, 2003).

The above-cited studies suggest that normal spermatozoa have a higher chance to bind to hyaluronic acid. Whether the zona pellucida binding ability of the spermatozoa is also related to sperm maturity remains to be elucidated. Nevertheless, the spermatozoa–zona pellucida binding process has been shown to be actively selective with respect to specific characteristics of sperm morphology (Liu and Baker, 1994; Garrett *et al.*, 1997). It was recently described that sperm binding to the zona pellucida is highly selective for double-stranded DNA, suggesting that spermatozoa with abnormal DNA can be eliminated by the spermatozoa–zona pellucida interaction during the process of fertilization under IVF conditions (Liu and Baker, 2007).

It is known that although the direct injection of a single spermatozoon into an oocyte can produce apparently normal offspring in mice and humans (Nagy *et al.*, 1995; Yanagimachi, 2005), it is less successful in many other species. In fact, for species with small acrosomes, injection of the acrosome, which contains powerful hydrolysing enzymes, into an oocyte apparently does not produce serious problems, but for species like the hamster, with very large acrosomes, injection inevitably results in death of the oocyte (Yamauchi *et al.*, 2002; Morozumi and Yanagimachi, 2005). When the spermatozoon binds to the zona pellucida, it is induced to undergo the acrosome reaction, in which the contents of the acrosome are released by exocytosis (Alberts *et al.*, 1994). In this study, no difference was observed among the groups in the fertilization rate, but an increased percentage of high-quality embryos when zona pellucida-bound spermatozoa were injected was noted. This could

be, in part, due to a decreased amount of acrosome enzymes derived into the oocytes.

In conclusion, this study has shown that the spermatozoa–zona pellucida interaction is extremely important in the selection of spermatozoa with a higher embryo developmental capacity, and that the spermatozoa–zona pellucida binding test may be an efficient method to identify the most competent spermatozoa for ICSI.

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