A modified method of intracytoplasmic sperm injection without the use of polyvinylpyrrolidone

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In a controlled study we compared the outcome of intracytoplasmic sperm injection (ICSI) performed by two different methods. The oocytes from 20 patients were equally divided into two groups and injected either by conventional ICSI using polyvinylpyrrolidone (PVP) or by a modified PVP-free ICSI procedure. While in the conventional ICSI method the spermatozoon is aspirated into the injection pipette, in the modified ICSI procedure the spermatozoon is attached to the end of the narrow micropipette by aspirating its tail. The sperm head is never drawn into the pipette. Accordingly, even a fast-moving spermatozoon can be ‘caught’ easily. As a result of such an aspiration the spermatozoon loses its motility. Therefore, PVP is required neither to slow down the movement of the spermatozoon nor to facilitate the movement of the solution in the injection pipette. A total of 230 mature oocytes were injected by both methods and the results were analysed. No differences were observed in survival rate between the two ICSI procedures (89% and 91%, respectively). However, the proportion of normally fertilized oocytes was significantly higher after microfertilization by modified ICSI (74%) when compared with the outcome of the conventional ICSI method (62%). The frequency of abnormal fertilization was not influenced by the method of ICSI used. The cleavage rate and quality of resulting embryos were also comparable. In conclusion, we have identified as crucial to the success of the procedure. Besides the oolemma breakage and mixing of the cytoplasm with injected spermatozoa (Nagy et al., 1995c), initial damage to the sperm plasma membrane is now generally considered to be essential for successful oocyte activation after ICSI (Dozortsev et al., 1994; Antinori et al., 1995; Dozortsev, 1995a,b; Fisel et al., 1995; Gerris et al., 1995). The membrane is damaged by gently touching the tail against the bottom of the dish. In order to facilitate the process of sperm tail breaking, the majority of the laboratories use a solution of 10% polyvinylpyrrolidone (PVP) which slows down the movement of spermatozoa. PVP is also used as a vehicle facilitating smooth movement of the spermatozoon inside the injection pipette and thus PVP is also injected intracytoplasmically. However, PVP should not be considered as a completely inert substance. Dozortsev et al. (1995a) suggested that the presence of PVP in the oocyte caused some delay between sperm injection and the beginning of calcium oscillations observed by some authors after ICSI (Sousa and Tesarik, 1994; Tesarik et al., 1994; Parrington et al., 1996). Another effect of this substance was documented by Dozortsev et al. (1995b) when the role of the damaged plasma membrane in the process of the oocyte activation was studied. Their experiments have shown that PVP stabilizes the sperm plasma membrane even at low concentrations and makes the sperm nucleus inaccessible for agents involved in sperm nucleus decondensation and oocyte activation. It is believed, therefore, that the presence of PVP compromises the fertilization rate after ICSI. Moreover, Feichtinger et al. (1995) suggested that chromosomal abnormalities in pregnancies after ICSI could be related to the injection of PVP into the oocyte. All of these findings suggest that the avoidance of PVP in ICSI procedure seems to be a reasonable choice.

In our work we modified the ICSI procedure and in a controlled study compared the results with the ICSI method as described by Palermo et al. (1992) and Van Steirteghem et al. (1993a,b). Our modification differs in the type of the injection pipette used and in the process of the sperm injection. PVP is not utilized in the proposed method. Here we present our first experience with the technique which may provide an alternative method of ICSI without the use of PVP.

Introduction

Thirty years after the pioneering work of Hiramoto (1962), who successfully activated a sea-urchin egg by injection of the sperm into the cytoplasm, human oocytes were also fertilized by this method and the first births were obtained (Palermo et al., 1992). Soon after introduction, the method of intracytoplasmic sperm injection (ICSI) was applied worldwide in the treatment of mainly male-factor infertility (Palermo et al., 1993; Devroy et al., 1994; Silber et al., 1994; Nagy et al., 1995a,b) Since the first application of ICSI in human assisted reproduction, several steps have been identified as crucial to the success of the procedure. Besides the oolemma breakage and mixing of the cytoplasm with injected spermatozoa (Nagy et al., 1995c), initial damage to the sperm plasma membrane is now generally considered to be essential for successful oocyte activation after ICSI (Dozortsev et al., 1994; Antinori et al., 1995; Dozortsev, 1995a,b; Fisel et al., 1995; Gerris et al., 1995). The membrane is damaged by gently touching the tail against the bottom of the dish. In order to facilitate the process of sperm tail breaking, the majority of the laboratories use a solution of 10% polyvinylpyrrolidone (PVP) which slows down the movement of spermatozoa. PVP is also used as a vehicle facilitating smooth movement of the spermatozoon inside the injection pipette and thus PVP is also injected intracytoplasmically. However, PVP should not be considered as a completely inert substance. Dozortsev et al. (1995a) suggested that the presence of PVP in the oocyte caused some delay between sperm injection and the beginning of calcium oscillations observed by some authors after ICSI (Sousa and Tesarik, 1994; Tesarik et al., 1994; Parrington et al., 1996). Another effect of this substance was documented by Dozortsev et al. (1995b) when the role of the damaged plasma membrane in the process of the oocyte activation was studied. Their experiments have shown that PVP stabilizes the sperm plasma membrane even at low concentrations and makes the sperm nucleus inaccessible for agents involved in sperm nucleus decondensation and oocyte activation. It is believed, therefore, that the presence of PVP compromises the fertilization rate after ICSI. Moreover, Feichtinger et al. (1995) suggested that chromosomal abnormalities in pregnancies after ICSI could be related to the injection of PVP into the oocyte. All of these findings suggest that the avoidance of PVP in ICSI procedure seems to be a reasonable choice.

In our work we modified the ICSI procedure and in a controlled study compared the results with the ICSI method as described by Palermo et al. (1992) and Van Steirteghem et al. (1993a,b). Our modification differs in the type of the injection pipette used and in the process of the sperm injection. PVP is not utilized in the proposed method. Here we present our first experience with the technique which may provide an alternative method of ICSI without the use of PVP.
Materials and methods

Patient selection and semen evaluation

In our in-vitro fertilization (IVF) laboratory patients are treated with ICSI when they experience very low or failed fertilization in the previous standard IVF cycles and when poor sperm parameters are detected. The evaluation of semen density and motility is carried out according to the recommendations of the World Health Organization (1992). The strict Tygerberg’s criteria are used to evaluate sperm morphology (Kruger et al., 1986). In all, 20 cycles treated by ICSI were involved in this controlled comparison. In order to lower the variation in the fertilization rate due to the differences in oocyte quality, only cycles with at least four metaphase II (MII) oocytes were analysed. With respect to their morphological evaluation, the oocytes from each patient were equally divided into two groups and injected by the two different ICSI methods. The protocol was reviewed and approved by the ethical committee of the University Hospital of L.Pasteur.

Ovarian stimulation

Ovarian stimulation was carried out by a desensitizing protocol using a gonadotrophin-releasing hormone agonist (GnRHa; Deca- cyclopentyl depot; Ferring) in association with follicle-stimulating hormone (FSH; Metrodin; Serono). Human choric gonadotrophin (HCG; Profasi; Serono) was administered when the cohort of follicles reached a mean diameter of 20 mm. Oocyte retrieval was performed 36 h after HCG administration by transvaginal ultrasound-guided aspiration.

Sperm preparation

Motile spermatozoa from infertile men were obtained using the direct swim-up method. After 20–30 min of incubation, the supernatant was removed and the spermatozoa concentrated at 300 g for 5 min. The sperm suspension was then placed in drops (50 µl) of tissue culture medium (TCM) 199 with Hanks salts (USOL; Praha, Prague) supplemented with 7.5% of heat-inactivated maternal serum. In cases of extreme oligoasthenoteratozoospermia, the seminal fluid was removed by washing the liquefied semen with TCM. After centrifugation at 300 g for 5–10 min, the supernatant was removed and the procedure repeated. The pellet was resuspended in a small volume of medium and the suspension placed in drops of TCM medium (50 µl). In order to obtain only motile spermatozoa, free of immobile and dead ones or other debris, the drops of TCM containing the sperm suspension were connected through little channels either with a drop of 10% PVP (10 µl) (for the conventional ICSI) or TCM (10 µl) (for the modified ICSI) (Figure 1 A, B). Even from extremely poor samples, the spermatozoa were able to swim out through these channels into surrounding drops of medium after 15–90 min of incubation at 37°C in an atmosphere of 5% CO2 in air. To prevent evaporation, the drops of medium were covered by pre-equilibrated paraffin oil.

Oocyte preparation

The oocytes were freed from their cumulus cells by incubation in 80 IU/ml hyaluronidase (Type VIII; Sigma Chemical Co., St Louis, MO, USA). The process was ended by placing the oocytes in another drop of medium free of hyaluronidase. Denuded oocytes were then rinsed several times and transferred back to the culture medium and incubated until ICSI. Only MII oocytes with an extruded first polar body were selected for microinjection. The abnormal MII oocytes and those with overt signs of degeneration were not used for microfertilization.

ICSI procedures

Micromanipulation was performed with the aid of two micromanipulators and microsyringes (Leitz, Wetzlar, West Germany) mounted on an inverted microscope (Cambridge Instruments, UK) equipped with a heated stage. Two methods of ICSI were involved in this study. The conventional ICSI procedure (sperm non-aspirating-ICSI; A-ICSI) was the same as that described by Palermo et al. (1992) and Van Steirteghem et al. (1993a,b) (Figure 2 A). The modified method of ICSI (sperm non-aspirating-ICSI; NA-ICSI) was different in the type of injection pipette used and in the process of injection (Figure 2 B). A microneedle with a very small opening was used for NA-ICSI, and commercially available injection pipettes (Swemad Lab. International AB, Sweden) for A-ICSI. The microneedles for NA-ICSI were pulled from glass tubes (diameter 0.95–1.05 mm) by a vertical puller (Leitz, West Germany) and bent for convenient manipulation. The needle was opened by breaking the tip against a holder pipette just before ICSI. The opening thus produced was just enough for aspirating the sperm tail (i.e. ~1 µm), and therefore the sperm head was never drawn into the pipette. The external diameter of this non-aspirating pipette was ~2 µm. Before starting ICSI, the oocytes were placed either in a drop of medium containing spermatozoa which had swum out from the sperm suspension through the connecting channels (NA-ICSI) or into droplets situated next to the PVP-sperm suspension (A-ICSI) (Figure 1 A, B).

In our proposed NA-ICSI method, the sperm head is not aspirated into the injection pipette but remains fixed and unprotected at the tip of the micropipette. In order to avoid damage to the spermatozoon during the passage through the investments of the egg, the injection procedure was performed differently. The motile spermatozoon was fixed to the end of the micropipette by aspiration of its tail. After becoming immotile (when necessary, aspiration and release of the spermatozoon from the pipette was repeated until it was immobilized), the spermatozoon was transferred to the vicinity of an oocyte where it was then released. The tail was broken at its tip by the pipette. Following this, the microneedle without the spermatozoon was positioned against the equatorial region of the oocyte, with the polar body at the 6- or 12-o’clock position, and the zona was penetrated.
Figure 2. Diagram showing the basic difference between the conventional sperm aspirating (A) and the modified sperm non-aspirating ICSI method (B).

Figure 3. Diagram representing the basic steps of the proposed modification of ICSI. (A) After zona penetration, the oolemma is separated from the surface of the pipette by expulsion of a small volume of the medium. (B) The oolemma is broken as a result of the aspiration of cytoplasm. (C) The micropipette is moved along its axis to the vicinity of the immobilized spermatozoon by an extra fourth axis movement. (D) After trapping the spermatozoon by aspiration of its tail, the pipette is transferred back to the previous position. (E) The pipette carrying the spermatozoon is inserted deeply into the cytoplasm. (F) The spermatozoon is released from the tip and the pipette is withdrawn from the oocyte.

Then the pipette was moved through the oocyte until close to the holding pipette and a small volume of medium was expelled. This caused the separation of the plasma membrane from the surface of the pipette and prevented the sticking and evagination of membrane during withdrawal of the needle (Figure 3 A). At this point the oolemma was gently aspirated until it was clearly broken and the aspirated ooplasm was reinjected (Figure 3 B). After withdrawal from the oocyte, the micropipette was moved to the immobilized spermatozoon (Figure 3 C) and the tail of the spermatozoon was sucked up into the pipette. Then the pipette with attached spermatozoon was transferred back to the previously performed hole in the zona/oolemma (Figure 3 D) and inserted deeply into the ooplasm (Figure 3 E). The spermatozoon was released from the slowly withdrawing pipette by application of slight pressure (Figure 3 F). The Leitz micromanipulator is equipped with an extra fourth axis movement which is along the axis of an angled micropipette. The place of zona penetration was adjusted by a control knob for vertical movement to the maximal vertical position of this extra fourth axis movement. Relocation of the hole in the zona and oolemma was achieved by simple reposition of the fourth axis movement to its maximal vertical position, and was then corrected by the horizontal movement controls.

Assessment of fertilization and embryo development
About 16–18 h after microinjection the oocytes were observed. Fertilization was considered normal when two distinct pronuclei were detected. The presence of one pronucleus or three and more pronuclei...
was noted. After 24 h of culture, embryo quality was checked. The embryos were classified according to the number of blastomeres and the percentage of anucleate fragments, and assigned to one of four categories (Van Steirteghem et al., 1994).

**Statistical methods**

Comparison between the groups of ICSI experiments was performed using the \( \chi^2 \) test.

**Results**

A total of 230 oocytes from 20 treatment cycles were micro-injected by conventional A-ICSI or modified NA-ICSI methods. The outcome of ICSI was compared between the two different ICSI procedures and the results are summarized in Table I. In this controlled study, no significant differences were noticed with regard to the percentage of intact oocytes after ICSI (91.2 and 88.9 % respectively). However, the effect of the different injection procedures on the formation of two pronuclei showed a significant increase (\( P < 0.01 \)) in the fertilization rate after microinjection by modified NA-ICSI method when compared with conventional A-ICSI (74.4 and 62% respectively). The frequency of one pronucleus or three pronuclei formation was not statistically different in both groups. No differences in the cleavage rate could also be observed between the different ICSI methods. The embryo quality in both studied groups was again similar.

**Discussion**

Since the introduction of ICSI into the treatment of human male infertility by Palermo et al. (1992) and Van Steirteghem et al. (1993a,b), the diameter of the injection pipette (Vandervzel-wmen et al., 1996), the mode of oolemma breakage (Nagy et al., 1995c) and damage to the sperm plasma membrane prior to injection (Dozortsev et al., 1994; Antinori et al., 1995; Dozortsev 1995a,b; Fishel et al., 1995; Gerris et al., 1995) were confirmed as the most important steps in the procedure ultimately influencing the outcome of ICSI. In order to facilitate the handling of the selected spermatozoa and the process of the injection, a substance with high viscosity, PVP, is added to the manipulation medium. The majority of laboratories utilize PVP in their routine work, despite the evidence suggesting that PVP negatively influences intracytoplasmic processes following ICSI. Although some authors (Jean et al., 1996; McDermott et al., 1996; Butler and Masson, 1997) were able to obtain results from PVP-free procedures which were comparable with those from longer-established ICSI programmes using PVP (Van Steirteghem et al., 1995), the extensive application of the PVP-free system is prevented by the fact that the immobilization of the motile spermatozoa and the injection procedure itself have some difficulties without the presence of PVP.

In order to overcome these difficulties in performing ICSI without PVP, we modified the injection procedure. Whereas in the conventional ICSI method the spermatozoon is aspirated into the injection pipette, in the modified ICSI procedure the spermatozoon was attached to the end of the injection microneedle by suction. The pipette aperture is just large enough to aspirate the sperm tail only. Accordingly, even fast-moving spermatozoa can be caught by aspiration of the tail leaving the head sticking out. As a result of this aspiration the spermatozoon loses its motility. Therefore, there is no need to slow down the movement of the spermatozoon by PVP before breaking its tail. Since the spermatozoon is never drawn into the needle, PVP is not necessary even for facilitating the movement of the solution in the injection pipette. The injection of this unnatural and potentially harmful agent into the oocyte can therefore be omitted.

It was demonstrated that the presence of PVP stabilizes the disrupted sperm plasma membrane, which is essential for the onset of sperm nuclear swelling after ICSI (Johnson and Clarke, 1988; Dozortsev et al., 1995b). In the NA-ICSI procedure, the damaged sperm plasma membrane is not compromised by PVP. In consequence, the sperm head is accessible to the sperm nucleus-decondensing factor of the oocyte, and subsequent release of the sperm-associated oocyte-activating factor can induce oocyte activation (Dozortsev et al., 1997). This may have contributed to the significant increase in the fertilization rate after sperm injection by modified PVP-free ICSI method when compared with the conventional procedure.

The smaller external diameter (~2 µm) of the pipette we used in this study for NA-ICSI could be another factor influencing the outcome of the procedure. The amount of the cytoplasm aspirated into the injection pipette before oolemma rupture was found to be directly related to the diameter of the injection pipette. While the mechanical disruption of the ooplasm is a prerequisite for correct intracytoplasmic deposition of the spermatozoon, significant cytoplasmic aspiration may be deleterious for different organelles of the cytoplasm. The sharp ends and small diameter of our non-aspirating micropipette seem to facilitate the process of the oolemma rupture and thus prevent extensive aspiration of the cytoplasm. Similarly, a minimal volume of the fluid is expelled during

### Table I. The fertilization outcome and embryo cleavage rate after intracytoplasmic sperm injection (ICSI) using conventional (sperm-aspiring ICSI: A-ICSI) with polyvinylpyrrolidone (PVP) and modified (sperm non-aspiring ICSI: NA-ICSI) without PVP

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total</th>
<th>Method of ICSI</th>
<th>( \chi^2 ) test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A-ICSI (with PVP)</td>
<td>NA-ICSI (without PVP)</td>
</tr>
<tr>
<td>No. of ICSI cycles</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of injected oocytes</td>
<td>230</td>
<td>113</td>
<td>117</td>
</tr>
<tr>
<td>Pronuclear status (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-PN (%)</td>
<td>157 (68.3)</td>
<td>70 (62)</td>
<td>87 (74.4)</td>
</tr>
<tr>
<td>1-PN (%)</td>
<td>2 (0.9)</td>
<td>1 (0.9)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>3-PN (%)</td>
<td>8 (3.5)</td>
<td>5 (4.5)</td>
<td>3 (2.6)</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% of 2-PN)</td>
<td>151 (96.2)</td>
<td>68 (97.1)</td>
<td>83 (95.4)</td>
</tr>
<tr>
<td>Quality of cleaved embryos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excellent (%)</td>
<td>82 (54.3)</td>
<td>37 (54.4)</td>
<td>45 (54.2)</td>
</tr>
<tr>
<td>Good (%)</td>
<td>48 (31.8)</td>
<td>22 (32.4)</td>
<td>26 (31.3)</td>
</tr>
<tr>
<td>Fair (%)</td>
<td>14 (9.3)</td>
<td>6 (8.8)</td>
<td>8 (9.6)</td>
</tr>
<tr>
<td>Poor (%)</td>
<td>7 (4.6)</td>
<td>3 (4.4)</td>
<td>4 (4.8)</td>
</tr>
</tbody>
</table>

PN = pronuclei; NS = not significant.
sperm deposition into the oocyte. This lowers the total amount of exogenous substances injected into the oocyte. All of these factors may contribute to the significant increase in the fertilization rate using the NA-ICSI.

In the proposed ICSI method, the sperm head is never drawn into the injection pipette but remains fixed and unprotected at the tip of the pipette. In order to avoid sperm damage during the passage through the investments of the egg, an extra step of the injection procedure has been introduced. The zona pellucida and the oolemma are penetrated by the injection pipette prior to sperm deposition. Subsequently, the pipette with a previously immobilized spermatozoon is passed through the original hole in the zona/oolemma and the spermatozoon is released into the cytoplasm. Relocation of the hole in the zona and the oolemma is facilitated by the micromanipulator which is equipped with an extra fourth axis movement and a means for storing co-ordinates. Prolongation of the ICSI procedure by reinsertion of the pipette is minimal since the whole process is performed at the same place (the oocytes and the spermatozoa are in the same drop of medium). When compared with the conventional ICSI method, there is no need to change the position of the injection pipette between PVP-sperm and medium-oocyte droplets. This compensates for the delay caused by the reinsertion of the pipette when modified ICSI is performed. Although the time necessary for the sperm injection was not measured extensively in the two different procedures, in our opinion, the modified ICSI method is quicker and easier to perform (e.g. there are no spermatozoa stuck in the pipette). Moreover, the manufacture of the sperm non-aspirating pipettes is much easier than for those used conventionally.

The frequency of abnormal fertilization was not different between the two methods. The cleavage rate and the quality of resulting embryos similarly did not reveal any significant differences between the two procedures. This suggests that the modified ICSI method has no apparent negative impact upon early events occurring after the intracytoplasmic sperm injection. Since the ability of the embryos produced by NA-ICSI to implant could not be assessed in this study, the two methods will be analysed cycle by cycle in the second series. Nevertheless, it is expected that at least comparable pregnancy rates will be obtained by NA-ICSI due to the omission of PVP in the procedure, the absence of significant cytoplasmic aspiration during the oolemma penetration, and the minimization of the amount of exogenous substances which are injected intracytoplasmically, etc.

Despite these encouraging results, the percentage of damaged oocytes still remained relatively high. Although the incidence of damaged oocytes by our ICSI procedure was within the range reported by the ESHRE ICSI Task Force (Tarlatzis, 1996) and was not significantly different when compared with the conventional ICSI method, nevertheless we expected a substantial decrease in damage rate. The injection pipette used in the modified PVP-free ICSI method is thinner than pipettes that aspirate the spermatozoon and should therefore be less traumatic. However, we observed that, when the zona and oolemma had been penetrated, in some cases the oolemma remained attached to the surface of the injection pipette and exvaginated during its withdrawal. After reinsertion of the pipette with a spermatozoon, the outflow of the cytoplasm caused lysis of the oocyte. In order to prevent sticking of the oolemma to the injection pipette, we added an extra step to the procedure. After penetrating the zona pellucida, the oolemma was depressed and a small volume of medium expelled. This separated the plasma membrane from the surface of the injection pipette before its rupture and prevented its exvagination during the extraction of the pipette. Although this step led to an increase in the survival rate (data not included in this study), some of the oocytes still degenerated more frequently than the others. This was a group of oocytes with a type of the oolemma which is broken immediately after the injection pipette penetrates the zona. These oocytes degenerated more frequently also after microinjection by conventional ICSI, a finding that is in accordance with the observations presented by Nagy et al. (1995c). In spite of this, the first results are promising and it can be expected that further technical modifications of the procedure will increase the proportion of intact oocytes.

In conclusion, we have demonstrated a modified PVP-free ICSI method which does not require PVP for the handling of the spermatozoa. The different type of the injection pipette and the differences in the process of the sperm injection allowed the omission of PVP without making the procedure more laborious. When compared with the conventional ICSI procedure, even better fertilization rates can be achieved. The proposed ICSI modification may provide an alternative procedure for elimination of the potentially harmful effects which may be associated with the conventional ICSI.

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References
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