Comparison of day 2 embryo quality after conventional ICSI versus intracytoplasmic morphologically selected sperm injection (IMSI) using sibling oocytes

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Abstract

Objective: To evaluate whether intracytoplasmic morphologically selected sperm injection (IMSI) could influence early paternal effects by observing embryo quality at day 2.

Study design: The study included 30 couples with at least one of the following criteria: male factor infertility, at least 2 previous failures of implantation or previous miscarriages after IVF/ICSI. Sibling oocytes of each patient were randomly assigned to either the ICSI group or the IMSI group. For IMSI, spermatozoa were selected at 8400 × magnification through an inverted microscope equipped with Nomarski differential interference contrast optics, Uplan Apo 100 × oil/1.35 objective lens and variable zoom lens. For conventional ICSI, spermatozoa were selected at 400 × magnification. An embryo was defined as top quality if there were four identical blastomeres on day 2 with no fragments or multinucleation of blastomeres. Data were analysed using the Wilcoxon and chi-squared tests. The significance level was set at P < 0.05. The variables were analysed in relation to the general population and the subpopulations with or without male factor.

Results: A total of 331 MII oocytes (30 oocyte retrievals) were selected and injected by the ICSI (n: 159) or IMSI (n: 172) procedure. For IMSI, only spermatozoa classified as morphologically normal at high magnification were used. No differences (P > 0.05) in fertilisation rate (ICSI: 70.9%; IMSI: 70.4%), early embryo cleavage rate (ICSI: 66.9%; IMSI: 60.4%) or cleavage rate (ICSI: 99.2%; IMSI: 99.1%) were observed. On day 2, as compared to ICSI, IMSI provided a similar proportion of top quality embryos (ICSI: 57.8%; IMSI: 52.2%; P > 0.05). These results were not influenced by the presence or absence of male factor.

Conclusion: In terms of embryo quality at day 2, IMSI had the same performance as conventional ICSI. However, we cannot exclude the possibility that IMSI effects occur only as a positive later paternal effect. © 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The quality of DNA in spermatozooids evaluated by the presence or absence of fragmentation can influence later embryo development (late paternal effect) [1–3]. Although DNA damage cannot be identified with selection of spermatozooids at high magnification [4], at these resolutions (≥6000×) it is possible to identify sperm with intranuclear vacuoles. These structures may reflect greater probability of molecular defects responsible for DNA damage [5,6] but the vacuoles are not detected at the magnification range commonly employed for conventional ICSI (200–400×). Therefore, it is expected that the exclusion of such spermatozooids could reduce the probability of a spermatozoid with DNA damage being injected into the oocyte [7]. In fact, different reports on blastocyst quality [8,9], implantation, pregnancy and miscarriages rates [7,10–15] and the chance of having a healthy normal child [16], have supported the hypothesis that a late adverse paternal effect on embryo development can be avoided through better selection of spermatozooids at high magnification and intracytoplasmic morphologically selected sperm injection (IMSI).

On the other hand, the spermatozooids also participate in early embryo development (early paternal effect), specifically the centrosome, which tends to provoke a failure of fertilisation or stoppage of embryo development with blockage in stage 2PN, as well as oocyte activation factor capable of inducing delay in the...
first cell cycles [1–3,17–19]. Therefore, an early adverse paternal effect may cause increases in the degree of cleaving embryo fragmentation [1,2,20]. Tesarik et al. [20] have shown that the paternal effect can become manifest as early as the 1-cell zygote stage.

In this context we cannot discard the hypothesis that selection of spermatozoids at high magnification (≥6000×) and the consequent lower probability of DNA damage, would provoke better early embryo development. The aim of this study was to evaluate whether IMSI could influence embryo quality at day 2 when compared to conventional ICSI.

2. Materials and methods

2.1. Study participants

A total of 30 cycles of 30 couples enrolled in the ICSI program of the Centre for Human Reproduction Prof Franco Jr. were admitted into this study. Inclusion criteria were male factor infertility, at the Centre for Human Reproduction Prof Franco Jr. were admitted 2. Materials and methods

evaluate whether IMSI could influence embryo quality at day 2 consequent lower probability of DNA damage, would provoke better early embryo development. The aim of this study was to evaluate whether IMSI could influence embryo quality at day 2 when compared to conventional ICSI.

2.2. Ovarian stimulation, oocyte recovery and culture protocol

All patients were submitted to the same scheme of controlled ovarian stimulation. After pituitary down-regulation with nafarelin acetate at a dose of 400 μg/day (Synarel®; Pharmacia, São Paulo; SP, Brazil) started in the mid-luteal phase, recombinant human FSH (r-FSH/Gonal F®; Serono, SP, Brazil) was administrated at a starting dose of 150–300 IU, depending on the age of the patient and recombinant LH (r-LH/Luveris®, Serono, SP, Brazil) was administered at a dose of 75 IU/day for a period of 7 days. On day 8 of stimulation, follicular development was monitored by 7 MHz transvaginal ultrasound only (Medison Digital Color MT, Medison Co. Ltd, Seoul, Korea) and the FSH dose was adapted according to ovarian response. The r-LH supplementation was increased to 150 IU/day when one or more follicles measuring ≥ 10 mm in diameter were found. When at least three follicles measuring ≥ 17 mm in diameter were observed, recombinant choric gonadotropin (HCG/Ovidrel® 250 mg Serono, SP, Brazil) was administered. Transvaginal, ultrasound-guided oocyte retrieval was performed 36 h after HCG.

2.3. Randomization

A double randomization was carried out. First of all, a table was constructed by computer (first randomization) indicating, for each case, from which wells of a Nunc dish (Nunc Multidishes 176740, Nunc™, Denmark) the oocytes would be chosen to perform conventional ICSI or to perform IMSI (for example: case 1: wells 1 and 2–ICSI/wells 3 and 4–IMSI; case 2: wells 1 and 4–ICSI/wells 2 and 3–IMSI; case 3 wells 2 and 4–ICSI/wells 1 and 3–IMSI; ...). At the moment of the oocyte retrieval, after a sequential distribution of oocytes among the wells (identified numerically from 1 to 4) of the Nunc dish and before maturity analysis, lots were drawn (second randomization) to determine with which case that specific patient would correspond (could be case #1 as well as case #30). In this manner, the sibling oocytes of each patient were randomly allocated into two groups:

- Control group: oocytes to perform conventional ICSI.
- Studied group: oocytes to perform IMSI.

2.4. Semen preparation

Semen samples were collected in sterile containers by masturbation after a sexual abstinence period of 2 to 5 days. The liquefied fresh semen samples were prepared by Isolate (Irvine Scientific, USA) discontinuous concentration gradient. The final pellet was resuspended in 0.2 ml modified HTF medium (Irvine) supplemented with 10% HSA (Irvine Scientific, Santa Ana, CA, USA). Parts of each semen sample were immediately taken for ICSI and IMSI procedures. The remainder of the semen sample was analysed for standard semen quality parameters according to the World Health Organisation [21].

2.5. ICSI procedure

Conventional ICSI was performed utilising a Nikon Eclipse TE 300 inverted microscope equipped with Narishige 231 D–2 (Narishige, Tokyo, Japan) remote control hydraulic micromanipulators and Narishige IM–9B injectors. Spermatozoa were selected at 400× magnification using Hoffman modulation contrast according to a set of previously published guidelines [22].

2.6. IMSI procedure

A 1 μl aliquot of sperm cell suspension was transferred to a 5 μl micro droplet of modified HTF medium containing 7% polyvinylpyrrolidone solution (PVP medium Irvine Scientific, USA). This microdroplet was placed in a sterile glass dish (FluoroDish™, Word Precision Instrument, USA) under sterile paraffin oil (Ovovil 100, Vitrolife, Goteborg, Sweden). The sperm cells, suspended in the microdroplet, were placed on a microscope stage above an Uplan Apo 100× oil/1.35 objective lens previously covered by a droplet of immersion oil. In this manner, suspended motile sperm cells in the observation droplet could be examined at high magnification through an inverted microscope (Eclipse TE 2000 U Nikon, Japan) equipped with high-powered differential interference contrast optics (DIC/Nomarski). The images were captured by a colour video camera containing effective picture elements (pixels) for high quality image production and projected onto a colour video monitor. Morphological evaluation was accomplished on a monitor screen and the total calculated magnification was 8400× (total magnification: objective magnification × 100×, magnification selector × 1.0×, video coupler magnification × 1.0×, calculated video magnification × 84.50×).

The spermatozoa used for IMSI were classified into 5 groups. Grade 1 consisted of spermatozoa free of any morphological abnormality (normal spermatozoa). A spermatozoon was classified as morphologically normal when it exhibited a normal nucleus as well as acrosome, post-acrosomal lamina, neck, tail and mitochondria, besides not presenting a cytoplasmic droplet
or cytoplasm around the head [4]. For the nucleus, the morphological state was defined by the form and content of the chromatin. The criterion for normality of nuclear form was a smooth, symmetric and oval configuration. Normal means for length and width were estimated as 4.75 ± 2.8 and 3.28 ± 0.20 μm [4] respectively, where the form classified as abnormal presented variation of 2 SD in one of the axes (length: ≥5.31 or <4.19 μm, width: ≥3.7 or <2.9 μm). For rapid evaluation of nuclear form, a fixed, transparent, celluloid form of a sperm nucleus fitting the criteria was superimposed on the examined cell (chablon construction based on ASTM E 1951-2 [23]). In the same manner, the nuclear form was considered abnormal if extrusion or invagination of the nuclear chromatin mass has been detected (regional malformation of nuclear form). Chromatin content was considered abnormal if one or more vacuoles were observed to occupy more than 4% of the nuclear area. A nucleus was considered normal if both nuclear form and chromatin content were normal. When no Grade I spermatozoa were available or the number was insufficient for injection, spermatozoa were classified by head morphology. Grade II: large oval: (>5.31 μm), small oval (<4.19 μm), wide (>3.7 μm width) or narrow (<2.9 μm width); Grade III: presence of regional disorders; Grade IV: presence of large vacuoles on 5–50% of head surface and Grade V: presence of large vacuoles on >50% of head surface.

The same technician, blinded to subject identity, performed all sperm selection. The time involved in the selection step was 30–120 min/sample. At least three spermatozoids were selected for each MII oocyte. After the sperm selection, the microinjections were still motile when captured for final selection.

2.7. Oocyte and embryo culture, embryo grading and transfer

Sperm injected oocytes, zygotes and embryos from IMSI and ICSI were submitted to the same culture conditions. Oocytes were examined after 17–20 h to assess fertilisation; zygotes with two distinct equal-sized pronuclei were considered normal. 25 to 27 h after injection, on day 1 of culture, early cleavage was evaluated [24]. Embryos, graded on day 2, were deemed top quality if there were four identical blastomeres (44 h after the sperm injection) with no fragments or multinucleation [25,26]. The same technician performed all oocyte/embryo evaluations. On day 2, the two best-scoring embryos were transferred to the patient’s uterus, independent of origin (ICSI or IMSI).

2.8. Statistical analysis and sample size

Data are reported as means ± SD and were analysed using the InStat 3.0 program (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). The Wilcoxon matched-pairs signed-ranks and chi-squared tests were used where appropriate. The significance level was set at P < 0.05. The primary outcome was the percentage of top quality embryos, defined by the number of top quality embryos obtained from the total number of cleaved embryos. The fertilisation, early cleavage and cleavage rates (secondary outcomes) were also analysed. The variables were analysed in relation to the general population and the subpopulations with male factor and without male factor (with at least 2 previous failures of implantation and/or previous miscarriage after ICSI). The sample size (for the general study) was calculated by planning a comparison between two proportions, control and experimental. Usually, our mean proportion of day 2 embryos considered top quality is 55%. Thus a sample size of 100 embryos in each group has 80% power to detect an increase of 20% with an alpha significance level of 0.05 (two-tailed).

3. Results

Table 1 summarises the characteristics of the general study population and of the subpopulations with and without male factor (≥2 previous failures or previous miscarriages).

A total of 331 MII oocytes were selected and injected by the ICSI (n: 172) or IMSI (n: 159) procedure. For IMSI, only spermatozoa from group I were used. No differences (P > 0.05) in fertilisation rate (ICSI: 70.9%, 122/172; IMSI: 70.4%, 112/159), early embryo cleavage rate (ICSI: 66.9%, 81/121; IMSI: 60.4%, 67/111) or cleavage rate (ICSI: 99.2%, 121/122; IMSI: 99.1%, 111/112) were observed. On day 2, IMSI and ICSI provided similar proportions of top quality embryos (ICSI: 57.8%, 70/121; IMSI: 52.2%, 58/111; P > 0.05). Table 2 summarises the results.

No statistically significant differences were found between ICSI and IMSI in the two subpopulations analysed. In the subpopulation of cases with male factor, a total of 155 MII oocytes were selected and injected by the ICSI (n: 81) or IMSI (n: 74) procedure. No differences (P > 0.05) were determined in fertilisation rate (ICSI: 66.7%, 54/81; IMSI: 70.3%, 52/74), early embryo cleavage rate (ICSI: 79.6%, 43/54; IMSI: 61.5%, 32/52), cleavage rate (ICSI: 100%, 54/54; IMSI: 100%, 54/54) or proportion of day 2 top quality embryos (ICSI: 57.4%, 31/54; IMSI: 50%, 26/52). In relation to the

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<td>Characteristics of the general study population and the subgroups.</td>
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<td>Couples (n)</td>
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<td>Male age (years) (mean ± SD)</td>
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<td>Total sperm count (×10^6/ml)</td>
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<td>Motility (% spermatozoa) (rapid + slow progression)</td>
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<td>Normal spermatozoa [%]</td>
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<td>Vitality [%] (mean ± SD)</td>
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<td>Leukocytes in semen (×10^3) (mean ± SD)</td>
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subpopulation without male factor, a total of 176 MII oocytes were selected and injected by the ICSI (n: 91) or IMSI (n: 85) procedure. Again, the statistical analysis demonstrated no differences (P > 0.05) in fertilisation rate (ICSI: 74.7%, 68/91; IMSI: 70.6%, 60/85), early embryo cleavage rate (ICSI: 56.7%, 38/67; IMSI: 59.3%, 35/59), cleavage rate (ICSI: 98.5%, 67/68; IMSI: 98.3%, 59/60) or proportion of day 2 top quality embryos (ICSI: 58.2%, 39/67; IMSI: 54.2%, 32/59). Table 2 summarises the results from the both subpopulations.

### Table 2
Comparison between the morphologically selected sperm injection (IMSI) and conventional intracytoplasmic sperm injection (ICSI) study groups.

<table>
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<th>Total Retrieved oocytes (n)</th>
<th>With male factor infertility</th>
<th>Without male factor infertility</th>
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<tr>
<td></td>
<td>IMSI (n: 150 oocytes)</td>
<td>ICSI (n: 172 oocytes)</td>
<td>IMSI (n: 74 oocytes)</td>
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<tr>
<td>Fertilisation rate</td>
<td>70.4%</td>
<td>70.8%</td>
<td>ns</td>
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<tr>
<td>Early cleavage rate</td>
<td>60.4%</td>
<td>66.9%</td>
<td>ns</td>
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<tr>
<td>Cleavage rate</td>
<td>99.1%</td>
<td>99.2%</td>
<td>ns</td>
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<tr>
<td>Top quality day 2 8 embryos</td>
<td>52.2%</td>
<td>57.8%</td>
<td>ns</td>
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ns: not significant (P > 0.05).

4. **Discussion**

Currently, ICSI is performed after morphological selection of spermatozoa at 200 × 400 × magnification. In this magnification range, spermatozoa carrying defects of the head, neck or tail can be detected, but not nuclear vacuoles. Therefore, magnification of 6000 × to 12,500 × to select spermatozoa, particularly by virtue of permitting a perfect identification of vacuoles, appears to be a better strategy [5,6,8,11–13,27,28]. Nevertheless, in relation to early embryo development and consequently, early paternal effect, the high magnification of sperm selection does not appear to exert significant influence.

In the literature, there is a controversy about the influence of high magnification sperm selection for ICSI on the initial embryo development and quality. Hazout et al. [7] in a study that included 125 couples (with male infertility and at least 2 previous failed ICSI attempts) found a similar number of good-morphology embryos (P = ns) on days 2–3 between ICSI cycles (4.0 ± 1.2) and previous conventional ICSI cycles (3.1 ± 1.1). However, in a comparative matched study performed on 50 IMSI and 50 matched ICSI couples (with male infertility and at least 2 failed previous ICSI attempts), Bartoov et al. [10] concluded that the IMSI procedure may significantly increase the number of top quality embryos at days 2–3. Another comparative matched study by the same group [12], including a newly enlarged number of studied patients (80 couples who underwent a single IMSI trial matched with 80 couples who underwent a routine ICSI procedure) confirmed a statistical difference in favour of IMSI. Unfortunately, its lack of randomization hinders any definite conclusion. The only randomized prospective work (446 couples with diagnoses of severe oligoasthenoteratozoospermia) comparing IMSI and conventional ICSI did not analyse embryo quality [15]. However, in our randomized study there was no statistical difference (P = ns) in the rate of top quality embryos at day 2 between IMSI (52.2%) and ICSI (57.8%) groups. In addition, this result was not altered by the presence (ICSI: 57.4% vs. IMSI 50% , P = ns) or absence of male factor (ICSI: 58.2% vs. IMSI 54.2% , P = ns). This observation supports the lack of influence of high magnification sperm selection on early paternal effect.

On the other hand, the stage of embryo development must be considered. The early paternal effect was observed before the major activation of embryonic genome expression, which starts between the 4-cell and 8-cell stage of human pre-implantation development [1,2]. Our study is the only one that limited the analysis of embryo quality exclusively to day 2 while the others reported this aspect on days 2 and 3. Thus, depending on the number of embryos considered on each day, the embryo quality analysis may represent more of a late paternal effect (activation of embryonic genome expression) than an early one. Given that the late paternal effect appears to be positively correlated with IMSI, the time of embryo quality analysis can justify the divergent results.

In relation to secondary results, again the analysis found no correlation between the high magnification sperm selection and early paternal effect. The fertilisation rates were similar (P = ns) between IMSI (70.4%) and ICSI (70.9%) groups, a result not influenced by the presence or absence of male factor and that confirms previous findings from other studies that also did not detect any significant difference in the fertilisation rate [4,7,8,10,12]. In addition, in a randomized study, Antinori et al. [15] did not find a statistically significant difference in the number of two-PN zygotes between ICSI and IMSI. In a similar manner, we did not observe any statistical difference in early cleavage rate (ICSI: 66.9%, IMSI: 60.4%, P = ns) or cleavage rate (ICSI: 99.2%, IMSI: 99.1%, P = ns) between the groups. Few studies have attempted to analyse cleavage rate. Only Hazout et al. [7] and Bach et al. [8] made this observation and both reported similar cleavage rate between conventional ICSI attempts and the IMSI cycles.

In conclusion, based on prospective and randomized analysis (sibling oocytes) of all 30 treated cases, IMSI had the same performance with respect to embryo quality at day 2 as conventional ICSI. To the best of our knowledge, this paper is the first randomized study that compared IMSI and conventional ICSI with respect to second-day embryo quality.

### Conflict of interest

None declared.

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### References


