Limiting the number of injected oocytes to three impairs ICSI outcomes in patients with nonobstructive azoospermia

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BACKGROUND: Since March 2004, only a maximum of three oocytes were allowed to be subjected to ICSI at one time in Italy. A previous study failed to show an impact of this restriction on fresh embryo transfer outcomes. The objective of this study was to compare ICSI outcomes before and after this restriction in patients with nonobstructive azoospermia. METHODS: Patients underwent testicular sperm extraction followed by ICSI. Biological (fertilization rate and the percentage of good-morphology zygotes and embryos) and clinical (clinical pregnancy and implantation rates) outcomes of the last 100 ICSI attempts before the restriction and outcomes of the first 100 ICSI attempts after the restriction were compared. RESULTS: Despite comparable fertilization rates (58.8% versus 59.2%; \(P > 0.05\)), there was a significant decrease in the percentage of good-morphology zygotes (41.1% versus 88.4%; \(P < 0.05\)) and embryos (36.7% versus 74.0%; \(P < 0.05\)) in the cohort of embryos transferred, clinical pregnancy rate (22.7% versus 42.4%; \(P < 0.05\)) and cumulative pregnancy rate from fresh and frozen embryo transfers (22.7% versus 42.4%; \(P < 0.05\)) after the restriction. CONCLUSION: The oocyte number restriction reduces dramatically the chance of achieving a clinical pregnancy in cases of nonobstructive azoospermia.

Key words: clinical outcomes/ICSI/nonobstructive azoospermia/oocyte number/testicular sperm

Introduction
Since 10 March 2004, no more than three oocytes were allowed to be fertilized at one time during an IVF treatment in Italy, by application of a new law regulating assisted reproduction technology (Benagiano and Gianaroli, 2004). In practical terms, this restriction means that the number of oocytes that can be treated by ICSI has to be restricted to three and, except for the situations in which transfer of fresh embryos is impossible, no embryos are cryopreserved.

One year after the application of the oocyte restriction, a multicentre Italian study evaluated the impact of this new policy on IVF outcomes (Ragni et al., 2005). Undertaken after the prohibition to freeze embryos, that multicentre study concluded that the rate of success of IVF–ICSI cycles using fresh embryos was not significantly influenced by the restriction of the number of oocytes used in ICSI imposed by this new legislation (Ragni et al., 2005). However, this conclusion was made on the basis of an analysis of a mixed patient population, of whom the overwhelming majority was represented by cases treated with conventional IVF or with ICSI using ejaculated spermatozoa. Only a very small patient subpopulation required the recourse to testicular spermatozoa, and it was not specified what percentages of these interventions were performed in cases of nonobstructive azoospermia. Because several studies have suggested that the developmental potential of embryos resulting from ICSI with testicular spermatozoa from men with nonobstructive azoospermia is lower than that of embryos conceived with ejaculated spermatozoa or with epididymal or testicular spermatozoa from obstructed men even when no restriction on the number of ICSI-treated oocytes is imposed (Mansour et al., 1997; Ghazzawi et al., 1998; Ubaldi et al., 1999), a larger-scale study is needed to evaluate the eventual impact of the oocyte number restriction on ICSI outcomes in this particular patient population.

This study was undertaken to compare biological and clinical outcomes of ICSI in cases of nonobstructive spermatozoa in the last 100 couples treated before the application of oocyte restriction and in the first 100 couples treated after the restriction application in a busy centre specialized in the treatment of severe male infertility.

Subjects and methods

Patients and design
This study involves 200 sequential ICSI attempts performed between June 2003 and April 2005 in patients who met the following criteria:
nonobstructive azoospermia with spermatozoa detected in the testis by previous diagnostic biopsy, normal ovarian reserve, the absence of endometriosis and the female age of <38 years. If the ICSI attempt was repeated in the same couple during this period, only the first attempt was included in the analysis. This patient group consisted of 100 couples complying with the above inclusion criteria and treated before the application of the oocyte-restriction legislation (March 2004) and of 100 similar couples treated after the application of this law restricting the number of oocytes subjected to ICSI in one attempt to three. The ovarian stimulation protocol and laboratory techniques for ICSI and embryo culture remained unchanged throughout the period in question.

**Assisted reproduction techniques**

Ovarian stimulation was performed with the use of recombinant human FSH (Gonal F, Serono, Geneva, Switzerland) after pituitary down-regulation with triptorelin (Decapeptyl, Ipsen Pharma, Paris, France) started in the mid-luteal phase, as described previously (Tesarik et al., 2001). The overall dose of FSH per stimulated cycle varied between 1700 and 3400 IU depending on the individual response. As soon as at least three follicles of >18 mm were detected, ovulation was induced with 10 000 IU of hCG (Gonasi, Serono). Oocytes were recovered by transvaginal ultrasound-guided follicle aspiration 36–37 h later.

Testicular spermatozoa were obtained either by open testicular biopsy or by fine-needle aspiration, as described previously (Ubaldi et al., 1999). After disintegration with sterile microscope slides, the presence of spermatozoa in the wet preparations was assessed under the inverted microscope at ×200 or ×400 magnification. Spermatozoa were selected with the use of an assisted hatching micropipette (Humagen, Charlottesville, VA, USA) and used for ICSI, as described previously (Ubaldi et al., 1999). The use of assisted hatching micropipettes facilitates sperm handling in the dense cell suspension resulting from the seminiferous tubule disintegration. Maximum effort was made to choose motile spermatozoa (first choice) and, if possible, morphologically normal ones (second choice). If no motile and morphologically normal spermatozoa were found, motile morphologically abnormal spermatozoa were preferred to immotile morphologically normal ones. In the absence of motile and morphologically normal spermatozoa, immotile and morphologically abnormal spermatozoa were injected. Only attempts using fresh testicular biopsy samples were included in this study.

Before the application of the oocyte number restriction, all metaphase II (MII) oocytes were subjected to ICSI. After the oocyte-restriction application, the three best oocytes to be used in ICSI were selected from the whole MII oocyte cohort according to previously described criteria of oocyte and polar body morphology and meiotic spindle evaluation using the Poloscope system (Rienzi et al., 2003).

**Oocyte and embryo culture, embryo grading and transfer**

Sperm-injected oocytes, zygotes and embryos were cultured at 37°C in IVF-30 medium (Vitrolife, Göteborg, Sweden) equilibrated with 5% CO₂ in air.

Fertilization was assessed 16–20 h after ICSI. Two-pronucleated zygotes were cultured further, under the same conditions, for an additional 2 days. Zygote and embryos were evaluated on days 1, 2 and 3 after ICSI and graded as good morphology and poor morphology according to previously published criteria (Tesarik and Greco, 1999; Tesarik et al., 2000). On day 3 after ICSI, two or three best-scoring embryos were transferred to a patient’s uterus.

**Statistical analysis**

Differences between groups were assessed by two-tailed χ²-test, with Yates’ correction or Fisher’s exact test for categorical variables, and by Mann–Whitney U-test for continuous variables. All analyses were performed using the Statistica 5.0 package (Statsoft Version 5.1, Hamburg, Germany).

**Results**

The patient groups treated before and after the application of the oocyte-restriction policy did not differ on the basic demographic characteristics and the duration of ovarian stimulation (Table I). However, the gonadotrophin doses chosen for the stimulation of patients after the application of oocyte restriction were lower so as to avoid unnecessary cost and risk to the patients. This resulted in lower peak serum estradiol (E₂) levels and less oocytes recovered per attempt as compared with the pre-restriction group (Table I).

In compliance with the law, significantly less oocytes were subjected to ICSI after the application of the restriction policy, but neither fertilization nor cleavage rate differed between the two groups (Table II). Similarly, the percentage of good-morphology zygotes and embryos, calculated from all zygotes and embryos resulting from the ICSI attempts, was comparable in both groups (Table II). However, the percentage of zygotes and embryos that were graded as good-morphology ones was lower in the cohort of embryos transferred after the application

<table>
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<tr>
<th>Characteristics</th>
<th>Patient group</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
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<tr>
<td>Age (years)</td>
<td>33.2 ± 2.0</td>
<td>34.3 ± 2.3</td>
</tr>
<tr>
<td>Number of previous attempts</td>
<td>1.7 ± 1.1</td>
<td>1.8 ± 1.2</td>
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<tr>
<td>Cycle length (days)</td>
<td>28.5 ± 1.3</td>
<td>28.3 ± 1.4</td>
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<tr>
<td>Duration of stimulation (days)</td>
<td>11.7 ± 0.5</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>Peak serum estradiol (pg/ml)</td>
<td>2536 ± 211</td>
<td>1898 ± 177</td>
</tr>
<tr>
<td>Number of oocytes recovered⁴</td>
<td>15.4 ± 2.5</td>
<td>10.2 ± 2.3</td>
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<tr>
<td>Number of MII oocytes recovered⁴</td>
<td>12.3 ± 2.6</td>
<td>8.1 ± 2.1</td>
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MII, metaphase II. Values are mean ± SD.

⁴Per stimulated cycle.

<table>
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<th>Outcome variable</th>
<th>Patient group</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Number of oocytes injected</td>
<td>1188</td>
<td>291</td>
</tr>
<tr>
<td>Number of embryos transferred</td>
<td>277</td>
<td>158</td>
</tr>
<tr>
<td>Number (%) of 2-PN zygotes⁵</td>
<td>703 (59.2)</td>
<td>171 (58.8)</td>
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<tr>
<td>Number (%) of good-morphology zygotes⁵</td>
<td>263 (37.4)</td>
<td>65 (38.0)</td>
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<tr>
<td>From all 2-PN zygotes</td>
<td>245 (88.4)</td>
<td>65 (41.1)</td>
</tr>
<tr>
<td>For the transferred embryo cohort</td>
<td>653 (92.9)</td>
<td>164 (95.9)</td>
</tr>
<tr>
<td>Number (%) of good-morphology embryos⁶</td>
<td>215 (32.9)</td>
<td>58 (35.4)</td>
</tr>
<tr>
<td>From all cleaved embryos</td>
<td>205 (74.0)</td>
<td>58 (36.7)</td>
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⁵The percentage is calculated from all injected oocytes.

⁶The percentage is calculated from two-pronucleated zygotes.

The percentage is calculated from cleaved embryos.
of the oocyte-restriction policy as compared with the pre-restriction period (Table II).

The number of patients who had embryo transfer tended to be lower after the application of the oocyte-restriction policy, but this difference was not significant for these patient numbers (Table III). However, after the application of oocyte number restriction, fewer embryos were available for transfer, and clinical pregnancy rate was negatively affected (Table III). Notwithstanding, clinical implantation rate was not significantly impaired after the application of oocyte number restriction policy (Table III).

The impairment of clinical efficiency after the application of the oocyte number restriction policy was even more evident when the cumulative clinical pregnancy rate per ovarian stimulation cycle resulting in oocyte recovery (taking into account pregnancies from transfers of both fresh and frozen embryos resulting from the same cohort of oocytes recovered) was evaluated. In the group of patients treated before the application of oocyte number restriction, an additional 11 pregnancies were obtained after a second transfer of supernumerary cryopreserved embryos, resulting in cumulative clinical pregnancy rate of 53.5% (53 clinical pregnancies from 99 ovarian stimulation cycles resulting in successful oocyte recovery). This was significantly more \( (P < 0.05) \) than the final clinical outcome of the treatment cycles performed after the application of the oocyte number restriction policy, which is represented by pregnancy rate from fresh embryo transfers only (22.7%). Three couples from the group treated before the application of the law still have cryopreserved embryos.

### Discussion

The results of this study show clearly that the clinical pregnancy rate of assisted reproduction in patients with nonobstructive azoospermia is impaired if the number of oocytes subjected to ICSI is restricted to three. These data, obtained in this specific group of patients, contrast with those reported in a recent multicentre study for an unselected group of patients in whom those suffering from nonobstructive azoospermia were represented only marginally (Ragni et al., 2005).

Impaired outcomes, as compared with patients with obstructive azoospermia or with spermatozoa in their ejaculate, were described previously in this group of patients even when no restriction of oocyte number was applied (Mansour et al., 1997; Ghazzawi et al., 1998; Ubaldi et al., 1999). It was hypothesized that the impaired reproductive performance of spermatozoa recovered from these patients may be due to functional immaturity or to the basic pathological condition responsible for the disturbances of spermatogenesis in these men (Tesarik and Mendoza, 2003).

This analysis of ICSI biological outcomes before and after the application of oocyte number restriction did not show a significant difference in fertilization rate. However, both the number of embryos transferred and the quality of the transferred embryo cohort were lower after the restriction. The impaired embryo quality in the latter period, evaluated for the transferred cohort group, was evident as early as the pronuclear zygote stage and persisted during cleavage. This was apparently due to the impossibility of choosing the best embryos for transfer from a larger cohort in the restricted conditions, because the percentage of good-morphology zygotes and embryos was the same when the overall cohorts of zygotes and embryos formed before and after the application of oocyte number restriction were compared.

Previous studies have shown that the developmental competence of testicular spermatozoa from men with nonobstructive azoospermia can be compromised by various mechanisms (Tesarik and Mendoza, 2003). For instance, different testicular pathologies predispose spermatozoa to the risk of DNA fragmentation, mainly through a decrease in the efficacy of protection by Sertoli cells (Tesarik et al., 2004a). However, the impairment of Sertoli cell function in these patients can also cause incomplete or defective development of sperm cytoplasmic components which act as important epigenetic factors regulating key processes during fertilization and the early embryonic development, namely the sperm centriole and the sperm-derived oocyte-activating factor (Tesarik and Mendoza, 2003). Deficiencies of these factors have been shown to become manifest very early after fertilization by ICSI, already at the pronuclear stage (Tesarik et al., 2002). Because of this early appearance of embryo morphological impairment, this condition has been called ‘early paternal effect’, as opposed to the ‘late paternal effect’, which is mostly associated with sperm DNA damage and does not compromise embryo development during the early cleavage stages (Tesarik et al., 2004b).

The present finding of impaired zygote morphology among the embryos transferred after the application of oocyte number restriction thus suggests that sperm epigenetic factors may have been at least partly responsible for the decrease in clinical efficacy in this group.

Despite the lower pregnancy rate in the group of patients treated with the restricted number of oocytes, implantation rate was comparable in the pre-restriction and post-restriction group. This seems to reflect the fact that more effort was made to select normal-appearing oocytes for ICSI in the post-restriction period as compared with the pre-restriction one. For instance, meiotic spindle visualization was routinely used only in the post-restriction period to assist the oocyte selection step. In fact, abnormalities of the meiotic spindle were previously shown to be responsible for impaired ICSI outcomes in our setting (Rienzi et al., 2003).

In conclusion, these data show that, despite the possibility of minimizing the risk of using abnormal oocytes by making use of recent oocyte selection methods, the risk of injecting a developmentally incompetent spermatozoon remains high in patients.
with nonobstructive azoospermia. Hence, the restriction of the number of oocytes used in ICSI results in a significant decrease in the availability of viable embryos and, consequently, a significant impairment of clinical pregnancy rate. An exception from the restrictive legislation, currently applied in Italy, is thus highly recommended for this particular indication.

References


Tesarik J, Greco E and Mendoza C (2004b) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum Reprod 19,611–615.


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