OOCYTES

Oocyte yield and dysmorphisms as indicators of biological efficiency in intracytoplasmic sperm injection cycles

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Abstract
The aim of the study was to examine whether oocyte yield could be an indicator of morphological oocyte quality and biological competency in patients younger than 36 years undergoing controlled ovarian stimulation (COS). Three hundred and thirty-five intracytoplasmic sperm injection (ICSI) procedures were arbitrarily subdivided into five groups according to the number of retrieved oocytes. Patients’ demographic characteristics and treatment success were compared among the groups. The influence of the morphological oocyte abnormalities on outcomes was also investigated. The proportion of oocytes that gave rise to viable embryos and high-quality embryos decreased significantly according to oocyte yield. Similarly, the number of foetal heartbeat per retrieved oocyte in fresh embryo transfer cycles was higher in patients with fewer oocytes collected. Finally, a negative correlation was observed between the occurrence of intracytoplasmic oocyte dysmorphisms and the number of foetal heartbeat per oocyte. High oocyte yield may be considered an indicator of low oocyte biological efficiency and intracytoplasmic dysmorphisms may contribute to this biological wastage suggesting that protocols of minimal or mild stimulation should be used.

Keywords: Oocyte dysmorphism, oocyte quality, oocyte competency, oocyte biological efficiency, ICSI, embryo quality

Introduction
Despite remarkable progress in clinical and embryological aspects of assisted reproductive technologies (ART), the number of viable embryos and foetal heartbeats per retrieved oocyte during intracytoplasmic sperm injection (ICSI) cycles are still disappointingly low. As previously described, oocyte and embryo quality can be adversely affected by a number of factors, including maternal age (de Bruin et al., 2004; Keefe et al., 2006), raised basal FSH concentration (Akande et al., 2002), higher anti-Müllerian hormone concentration (Ebner et al., 2006), culture environment (Lonergan et al., 2006), endometriosis (Gupta et al., 2008), and smoking habit (Gruber et al., 2008). However, morphological assessment of oocytes and embryos remains the usual parameter to determine their quality in clinical practice.

One of the most important factors that influences oocyte morphological aspects is the controlled ovarian stimulation (COS) protocol and the ovarian response to COS (Ng et al., 2001; Rashidi et al., 2005). Aggressive protocols of ovarian stimulation performed to maximise the number of retrieved oocytes may compromise their quality resulting in morphological abnormalities.

Ovarian stimulation may directly influence oocyte morphology and quality via the hormonal follicular environment through exposure to high doses of gonadotrophins. This, in turn, may lead to the recruitment of follicles that might have become atretic in vivo (Haaf et al., 2009). As a result, the assumption that more oocytes and thus more embryos improve treatment success is controversial.

Mild ovarian stimulation protocols are being developed in order to minimise adverse effects on in vitro
fertilisation (IVF). Verberg et al., (2009) showed that implantation rates are also positively influenced by a lower number of retrieved oocytes and Haaf et al., (2009) reported that fewer retrieved oocytes were associated with a lower abnormal chromosome error rate. Nevertheless, existing reports do not clearly specify mild stimulation influence oocyte morphology and ICSI outcomes.

The aim of this study was to evaluate whether oocyte yield could be an indicator of oocyte biological competence in patients younger than 36 years undergoing ICSI and whether dysmorphisms could affect oocyte developmental competence.

Material and methods

Experimental design

The analysis included morphological evaluation of oocyte and embryo characteristics in 3408 oocytes retrieved from 335 patients undergoing ICSI cycles performed from January 2007 to April 2008 at a private centre for assisted reproduction. All female patients were younger than 36 years old, in order to exclude possible age-related cycle characteristics. In addition, considering that alterations in sperm parameters could potentially be considered a confounding factor the association between oocyte yield and ICSI outcomes, we excluded all cases using surgically retrieved sperm. Patients were arbitrarily subdivided according to the number of retrieved oocytes: 1 to 5 oocytes (Group 1, N = 56); 6 to 10 oocytes (Group 2, N = 85); 11 to 15 oocytes (Group 3, N = 79); 16 to 20 oocytes (Group 4, N = 39) and > 20 oocytes (Group 5, N = 76). The five groups were compared for normal fertilisation rate, percentage of high-quality embryos, mean number of transferred embryos and pregnancy rate. In order to evaluate the biological efficiency of in vitro treatment, the percentage of viable embryos per oocyte, high-quality embryos per oocyte rate and foetal heartbeat per oocyte rate were compared among the groups. In addition, the influence of morphological oocyte abnormalities was investigated. The study was approved by the local institute review board. Written informed consent, in which patients agreed to share outcomes of their own cycles for research purposes, was obtained.

Controlled ovarian stimulation

COS was achieved by long pituitary down-regulation using the GnRH agonist leuproside acetate (Lupron Kit™, Abbott S.A Société Française des Laboratoires, Paris, France) followed by ovarian stimulation with recombinant-FSH (Gonal-F®, Merk-Serono, Geneva, Switzerland). GnRH agonist was administered subcutaneously from the day 18 from the previous menstrual cycle until ovulation. After 12 to 14 days, ovarian stimulation was commenced with 225 IU of r-FSH administered subcutaneously in a daily basis. Follicular dynamic were followed with ultrasound starting on day 4 of gonadotrophin administration. When at least one follicle ≥17 mm was observed and adequate serum estradiol levels were detected, recombinant human chorionic gonadotrophin (rhCG, Ovidrel™, Merk-Serono, Geneva, Switzerland) was administered to trigger the final follicular maturation. Oocytes were collected 34–36 h after hCG administration using transvaginal ultrasound ovum pick-up.

Sperm samples

Ejaculated spermatozoa were obtained after 3–5 days abstinence. 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 human tubal fluid HEPES-buffered medium (HTF-HEPES, Irvine Scientific, Santa Ana, USA) and incubated at 37°C for 1 hour. Allowing spermatozoa to move from the seminal plasma to the overlayered culture medium.

Preparation of oocytes and morphology assessment

After retrieval, oocytes were incubated in HTF culture medium (Irvine Scientific, Santa Ana, USA) covered with mineral oil (Ovoil™, Vitrolife, Kungsbacka, Sweden) at 37°C and 6% CO2 for 5 h. Cumulus cells were removed with a 30 seconds exposure to Hepes-buffered medium containing 80IU/mL hyaluronidase (Irvine Scientific, Santa Ana, USA), after which coronal cells were manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA). Denuded oocytes were then assessed for nuclear status. Oocytes showing first polar body release were considered mature and used for ICSI. Oocyte morphology was assessed using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon®, Tokyo, Japan) with a Hoffmann modulation contrast system under 400 × magnification, before sperm injection. Normal oocytes showed clear cytoplasm with uniform texture and homogenous fine granularity with no inclusions, a small perivitelline space without granularity, and a single unfragmented first polar body (Veeck, 1991). The following intracytoplasmic and extracytoplasmic morphology abnormalities of the oocyte were recorded: Intracytoplasmic: (i) increased cytoplasmic granularity, (ii) smooth endoplasmic reticulum clusters, (iii) vacuoles in the ooplasm. Extracytoplasmic: (i) large perivitelline space size, (ii) perivitelline space
granularity, (iii) fragmented first polar body. For each intracytoplasmic or extracytoplasmic abnormalities detected a value was to the oocyte in order to give a score related to the number of abnormalities detected and oocyte quality. Score values used for statistical analysis were assigned 1, 2 or 3 according to both intracytoplasmic and extracytoplasmic detected abnormalities.

**Intracytoplasmic sperm injection**

ICSI was performed in all MII oocytes according to the technique described by Palermo et al. (1992). Oocytes were transferred into the micro-injection dish, prepared with 4 μl droplets of buffered medium (HEPES, Irvine Scientific, Santa Ana, USA), and covered with mineral oil on a heated stage at 37.0 ± 0.5°C of an inverted microscope.

**Assessment of fertilisation, embryo quality and embryo transfer**

Approximately 16–18 hours after ICSI, normal fertilisation was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Embryos were maintained in a 50 μl drop of HTF medium supplemented with 10% HSA (Human Serum Albumin, Irvine Scientific, Santa Ana, USA) covered with mineral oil in a humidified atmosphere with 6%CO₂ in air and at 37°C until transfer. Embryo transfer was performed on the third day of development. One to three embryos from each couple were transferred.

Embryo quality was evaluated under an inverted microscope prior to transfer. 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 having all of the following characteristics: Eight to ten cells, less than 15% fragmentation, symmetrical blastomeres, absence of multinucleation, colourless cytoplasm with moderate granularity and absence of inclusions, perivitelline space granularity and zona pellucida dysmorphism. Embryos lacking any of the above characteristics were considered as low-quality embryos. Cleaving embryos with at least 6 cells, less than 15% fragmentation and multinucleation, on the third day of development, were considered viable embryos.

**Clinical follow-up**

Serum β-hCG levels were assessed for the first time 12 days after replacement of the embryos. We assessed the clinical pregnancies, both per oocyte and per embryos transferred, as those with a foetal heartbeat on ultrasound scan, performed 3–4 weeks after embryo transfer. To calculate the implantation rate, the number of gestational sacs was divided by the number of embryos transferred.

**Statistical analysis**

Categorical variables were analysed using Chi-square or Fisher test as appropriate. ANOVA test was employed for numerical variables. Residue normality was tested for all variables. Transformations were performed whenever necessary. Results were described as untransformed mean and standard deviation.

The correlation among oocyte morphology and FSH dose administered was analysed using Pearson correlation and the results were expressed as Pearson coefficient (R) and p value.

To study the influence of cycle characteristics on oocytes morphology, linear regression models were performed and the results expressed as odds ratios (OR), 95% confidence intervals (CI) and p values.

Results were considered to be significant at the 5% level (p < 0.05). Data analysis was carried out using Minitab (version 14).

**Results**

The general characteristics of the ICSI cycles are show in Table I. From 335 patients undergoing ICSI cycles for the first time, 3408 MII oocytes were retrieved. There were no statistically significant differences among the groups regarding maternal age, body mass index, total gonadotropin dose used for COS, number of motile sperm and normal fertilisation and high-quality embryo rates were found among groups. In addition, the mean number of embryos and high-quality embryos transferred did not differ among the groups. However, as expected, the serum estradiol level on hCG day and ovarian response to controlled stimulation were different among the groups.

Pregnancy rates per embryo transfer (35.7% in Group 1; 41.5% in Group 2; 40.0% in Group 3; 35.5% in Group 4 and 43.5% in Group 5) and implantation rates (16.0% in Group 1; 21.6% in Group 2; 19.8% in Group 3; 17.8% in Group 4 and 23.2% in Group 5) did not differ among the groups (p < 0.1011 and p = 0.2145, respectively). On the other hand, the percentage of oocytes that produced viable embryos (60.2% in Group 1; 53.2% in Group 2; 49.5% in Group 3; 40.7% in Group 4 and 40.0% in Group 5) and high-quality embryos (34.7% in Group 1; 27.2% in Group 2; 23.0% in Group 3; 22.0% in Group 4 and 19.7% in Group 5) decreased significantly according to oocyte yield (p < 0.001 and p = 0.0016, respectively), as shown in Figure 1.
The number of foetal heartbeat per retrieved oocyte in fresh embryo transfer cycle (8.5% in Group 1; 7.5% in Group 2; 3.8% in Group 3; 2.8% in Group 4 and 1.8% in Group 5) was also significantly higher in patients with fewer oocytes collected ($p < 0.0001$) (Figure 2).

### Table I. General characteristics of stimulation ICSI cycles.

<table>
<thead>
<tr>
<th>Groups</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles (n)</td>
<td>56</td>
<td>85</td>
<td>79</td>
<td>39</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Maternal age (yrs)</td>
<td>$31.2 \pm 4.5$</td>
<td>$31.0 \pm 4.2$</td>
<td>$31.7 \pm 2.7$</td>
<td>$30.3 \pm 3.3$</td>
<td>$30.2 \pm 3.1$</td>
<td>0.0910</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>$23.0 \pm 3.5$</td>
<td>$24.8 \pm 4.8$</td>
<td>$22.6 \pm 5.6$</td>
<td>$27.3 \pm 3.2$</td>
<td>$25.4 \pm 2.8$</td>
<td>0.6981</td>
</tr>
<tr>
<td>Total gonadotropin dose (IU)</td>
<td>$2296 \pm 975$</td>
<td>$2591 \pm 845$</td>
<td>$2333 \pm 504$</td>
<td>$2475 \pm 605$</td>
<td>$2395 \pm 605$</td>
<td>0.1030</td>
</tr>
<tr>
<td>E2 concentration on hCG day (pg/ml)</td>
<td>$792 \pm 711^a$</td>
<td>$1016 \pm 646^b$</td>
<td>$1612 \pm 1052^a,b$</td>
<td>$2504 \pm 2282^b,c$</td>
<td>$3451 \pm 1956^c$</td>
<td>0.0001</td>
</tr>
<tr>
<td>Aspirated follicles (n)</td>
<td>$7.7 \pm 3.3$</td>
<td>$13.1 \pm 5.3$</td>
<td>$19.1 \pm 6.4$</td>
<td>$26.4 \pm 6.6$</td>
<td>$39.8 \pm 11.1$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Retrieved oocytes (n)</td>
<td>$3.7 \pm 1.2$</td>
<td>$8.3 \pm 1.3$</td>
<td>$12.8 \pm 1.4$</td>
<td>$18.5 \pm 1.2$</td>
<td>$27.5 \pm 6.2$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>MII oocytes</td>
<td>$3.0 \pm 1.2$</td>
<td>$6.2 \pm 1.7$</td>
<td>$8.8 \pm 2.6$</td>
<td>$13.3 \pm 3.7$</td>
<td>$19.3 \pm 6.7$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Number of motile sperm $\times 10^6$/ml</td>
<td>$39.8 \pm 3.8$</td>
<td>$30.6 \pm 3.3$</td>
<td>$34.0 \pm 4.6$</td>
<td>$29.3 \pm 8.8$</td>
<td>$38.4 \pm 8.8$</td>
<td>0.6854</td>
</tr>
<tr>
<td>Normal fertilization rate (%)</td>
<td>$73.2$</td>
<td>$70.4$</td>
<td>$71.7$</td>
<td>$66.3$</td>
<td>$69.0$</td>
<td>0.3481</td>
</tr>
<tr>
<td>Rate of high-quality embryos (%)</td>
<td>$47.0$</td>
<td>$50.1$</td>
<td>$50.5$</td>
<td>$53.9$</td>
<td>$48.0$</td>
<td>0.7201</td>
</tr>
<tr>
<td>Mean no. of embryos transferred</td>
<td>$2.0 \pm 1.3$</td>
<td>$2.6 \pm 1.2$</td>
<td>$2.6 \pm 1.4$</td>
<td>$2.4 \pm 1.4$</td>
<td>$2.5 \pm 1.2$</td>
<td>0.4821</td>
</tr>
<tr>
<td>Mean no. of high quality embryos transferred</td>
<td>$1.46 \pm 1.0$</td>
<td>$2.2 \pm 1.0$</td>
<td>$2.2 \pm 1.3$</td>
<td>$2.3 \pm 1.5$</td>
<td>$2.4 \pm 1.6$</td>
<td>0.0876</td>
</tr>
</tbody>
</table>

Values expressed as mean $\pm$ SD, unless otherwise noted. Different subscripts in the same line differ.

Figure 1. Percentage of oocytes that produced viable embryos and high-quality embryos after ICSI procedure, according to the total number of retrieved oocytes.

The number of foetal heartbeat per retrieved oocyte in fresh embryo transfer cycle (8.5% in Group 1; 7.5% in Group 2; 3.8% in Group 3; 2.8% in Group 4 and 1.8% in Group 5) was also significantly higher in patients with fewer oocytes collected ($p < 0.001$) (Figure 2).

### Oocyte morphology score and administered FSH

No correlation was observed between intracytoplasmic ($R$: 0.065, $p = 0.228$) or extracytoplasmic ($R$: 0.052, $p = 0.331$) dysmorphisms and the FSH dose used for COS.

### Oocyte morphology score and cycles outcomes

Evaluation of oocyte morphology was undertaken in all MII oocytes. No influence of intracytoplasmic or extracytoplasmic dysmorphisms was found on viable embryos per retrieved oocytes rate and on high-quality embryos per retrieved oocytes rate. However, we observed a significant negative effect of the occurrence of oocyte intracytoplasmic dysmorphisms and the foetal heartbeat per oocyte rate (Table II).

### Discussion

Although it is established that the number of mature oocytes is correlated with pregnancy rates, it is still not clear which aspect of the IVF cycle is responsible. Since fertilisation and pregnancy rates are not sensitive enough to indicate oocyte quality, as they can be affected by male factor and the women’s own physiology (Ng et al., 2001), other aspects of in vitro culture and ovarian stimulation may be responsible for the cycles outcomes.

Our data show that fertilisation, the first event of oocyte loss during in vitro fertilisation, is not affected
by the number of oocytes generated. Indeed, previous studies have reached a similar conclusion (De Sutter et al., 1996; Balaban et al., 2008), showing that neither oocyte morphological aspects nor the patients age alone can be the cause for low fertilisation rates (Faber et al., 1998; Klinkert et al., 2004). Our findings were consistent since we found that fertilisation was not influenced by a higher number of retrieved oocytes.

On the other hand, the percentage of high-quality embryos decreased significantly according to oocyte yield; the higher the number of retrieved oocytes, the lower their chance of becoming an eight cell embryo with no fragmentation on the third day of development. Fewer high-quality embryos have been observed by the same authors cited above (Faber et al., 1998; Klinkert et al., 2004).

On the other side, our data show that the retrieval of fewer oocytes leads to a higher number of foetal heartbeats demonstrating that ovarian stimulation can influence ICSI outcomes. There seemed to be a constant number of high-quality oocytes so that excessive ovarian responses presented no beneficial effect, since the surplus oocytes did not add any advantage in ART outcome. Because of this, pregnancy rates were maintained independent of the number of retrieved oocytes, however, the biological efficiency was reduced with more embryos since the remaining low-quality ones were not useful. Our data confirm the initial observations of Inge et al. (2005) and the recent study of Patrizio and Sakkas (2009) revealing that although the total live birth rate has improved with COS for IVF treatment, the efficiency of oocyte utilisation has not improved significantly since the early 1980s demonstrating a very high biological inefficiency during ART cycles, especially for younger patients. In addition, Oocyte wastage in terms of the ability to achieve a live birth was also observed when we arbitrarily divided the patients into larger groups of 1 to 10 oocytes, 11 to 20 oocytes and > 20 oocytes (data not shown).

One hypothesis is that in women possessing a stronger ovarian response, the recruited follicles may have lost or not yet developed the ability to express LH receptors, which will lead to a failure on hCG response. In addition, high LH concentrations during the follicular phase of stimulation may negatively influence oocyte quality (Homburg et al., 1988; Regan et al., 1990).

Aggressive stimulation protocols could be responsible for a lower rate of high-quality embryo (Testart et al., 1989; Katz-Jaffe et al., 2005; Baart et al., 2007). In fact, a deviation on normal oocyte morphology could be related to aggressive protocols of ovarian stimulation that may disrupt cellular events affecting oocyte competence. In a natural cycle, generally only one antral follicle becomes dominant while the remainder of the developing follicles undergoes atresia. In contrast, during ovarian stimulation for ART these follicles are rescued, but their oocytes may be already intrinsically abnormal (Kovalevsky & Patrizio 2005; Patrizio et al., 2007). The ability to reinitiate the meiotic process and undergo preimplantation development is also progressively determined during the antral phase (Bukovsky et al., 2005). These changes involve both the nuclear and cytoplasmic compartments, but the underlying molecular dynamics are still poorly understood. It is therefore likely that ‘forcing’ multiple oocyte recruitment using controlled ovarian hyperstimulation may disrupt these intracellular events.

Protocols of minimal or mild ovarian stimulation should be considered as valid alternatives to those in current use (Edwards, 2007; Nygren, 2007). In addition, minimal stimulation has other associated benefits, including exposing the patient to lower hormone levels, maintaining a more balanced hormonal milieu for the endometrium, reducing the need for embryo storage, and most importantly

Table II. The influence of intra and extracytoplasmic dysmorphisms on the oocyte biological efficiency.

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR</th>
<th>CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracytoplasmic dysmorphisms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable embryo per oocyte rate</td>
<td>1.02</td>
<td>0.99–1.05</td>
<td>0.132</td>
</tr>
<tr>
<td>High-quality embryo per oocyte rate</td>
<td>0.98</td>
<td>0.95–1.01</td>
<td>0.223</td>
</tr>
<tr>
<td>Number of foetal heartbeat per oocyte rate</td>
<td>1.06</td>
<td>0.94–1.20</td>
<td>0.356</td>
</tr>
<tr>
<td><strong>Intracytoplasmic dysmorphisms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable embryo per oocyte rate</td>
<td>1.01</td>
<td>0.98–1.04</td>
<td>0.669</td>
</tr>
<tr>
<td>High-quality embryo per oocyte rate</td>
<td>1.01</td>
<td>0.97–1.04</td>
<td>0.737</td>
</tr>
<tr>
<td>Number of foetal heartbeat per oocyte rate</td>
<td>1.48</td>
<td>1.03–1.36</td>
<td>0.018</td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval.
limiting the risk of ovarian hyperstimulation and multiple pregnancy (Hurst et al., 2002).

Our study demonstrated that the presence of intracytoplasmic abnormalities is significantly correlated with low oocyte efficiency. These findings were apparent in the ICSI outcomes, since oocyte abnormalities directly affected the number of foetal heartbeats in each group. There is increasing evidence that excessive ovarian stimulation may induce detrimental effects on oocyte and embryo quality and endometrial receptivity (Greb et al., 2005; Giorgetti et al., 2007) and that deficient cytoplasmic maturity may be reflected by intracytoplasmic abnormalities (Kahraman et al., 2000). This finding has previously been related to the post-ovulatory age of the oocyte and to an asynchrony between nuclear and cytoplasmic maturation (Eichenlaub-Ritter et al., 1995). The studies of Plachot and Crozet (1992) and Haaf et al. (2009) suggested that a lower oocyte cohort development may represent a more appropriate response to ovarian stimulation, allowing only the most competent follicles and oocytes to develop, increasing the overall oocyte quality and maturity.

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References


