Relationship between granular cytoplasm of oocytes and pregnancy outcome following intracytoplasmic sperm injection

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Couples undergoing intracytoplasmic sperm injection (ICSI) for male infertility using oocytes with centrally located granular cytoplasm (CLCG) were evaluated for fertilization, embryo development, implantation and pregnancy rate. CLCG is a rare morphological feature of the oocyte, that is diagnosed as a larger, dark, spongy granular area in the cytoplasm. Severity is based on both the diameter of granular area and the depth of the lesion. Twenty-seven couples with 39 cycles presenting CLCG in >50% of retrieved oocytes were evaluated. A total of 489 oocytes was retrieved, out of which 392 were at MII. CLCG was observed in 258 of the MII oocytes (65.8%); 66.7% of these oocytes had slight and 33.3% had severe CLCG. The overall fertilization rate was 72.2% and no statistical significant difference was found between normal and CLCG oocytes and between the oocytes representing slight and severe CLCG. The development and quality of embryos was the same in normal and CLCG oocytes. In nine cycles, preimplantation genetic diagnosis was executed to evaluate a possible accompanying chromosomal abnormality. Out of 44 blastomeres biopsied, 23 had chromosomal abnormality (52.3%). Eleven pregnancies were achieved in 39 cycles (28.2%), six pregnancies resulted in abortion (54.5%). The implantation rate was found to be 4.2%. Only five ongoing pregnancies were achieved in 39 cycles (12.8%). Couples with CLCG oocytes should be informed about poor ongoing pregnancy rates even if fertilization, embryo quality and total pregnancy rates are normal. Furthermore, a high aneuploidy rate may be linked to a high abortion rate.

Key words: centrally located cytoplasmic granular oocyte/ICSI/male infertility/preimplantation genetic diagnosis

Introduction

Oocyte quality is an important prognostic factor as the nuclear and cytoplasmic maturity of the oocyte may be directly related to the success rate of intracytoplasmic sperm injection (ICSI). In certain cases, granulation may be observed within the cytoplasm and it may be either homogeneously or centrally localized. Homogeneous granularity may affect the whole cytoplasm. Central granulation is of concern when the granulation is located centrally within the cytoplasm with a clear border, easily distinguishable with a darker appearance than normal cytoplasm (Serhal et al., 1997). It is often difficult to assess the cytoplasmic morphology of the oocyte and the exact stage of maturation, as the oocytes are surrounded by cumulus or corona cells at the time of collection. The nuclear and cytoplasmic maturation and the morphology of an oocyte are assessed clearly before an ICSI procedure, however, as the surrounding cumulus–corona complex has to be cleaned by means of either mechanical stripping or chemical hyaluronidase treatment.

Centrally located granular cytoplasm (CLCG) is a rare morphological feature of the oocyte that can be observed in certain cases. It is diagnosed as a larger, dark, spongy granular area. Cytoplasmic granularity of an oocyte can be homogeneous or centrally located, and slight or severe. The severity of granularity is based on the diameter of the granular area and the depth of the lesion. Little attention has been focused on oocyte morphology in standard assisted reproduction techniques. There is a dearth of data in the literature on the relationship between oocyte morphology and pregnancy rate. It has been reported that oocyte morphology is not related to fertilization rates or embryo quality after ICSI (De Sutter et al., 1996). The purpose of this study is to evaluate the effect of CLCG on fertilization rates, embryo quality and pregnancy results in assisted reproduction cycles in which ICSI was performed for severe male infertility.

Materials and methods

This study was carried out at Sevgi Hospital Embryology and Genetic Laboratories. A retrospective analysis was performed on the data obtained from 1431 assisted reproduction cycles that had been performed between January 1998 and September 1999. CLCG was observed in 113 out of 1431 cycles (7.9%). In 39 cycles (34.5%), CLCG was observed in more than half of the oocytes retrieved; of these, eight cycles had CLCG observed in all oocytes retrieved. Pituitary down-regulation was achieved by using a gonadotrophin-releasing hormone agonist (GnRHa) (buserelin, Suprefact®; Hoechst AG, Frankfurt, Germany), starting either from the 21st day of the menstrual cycle in the long protocol (n = 33) or on the 2nd day of cycle in the co-flare protocol (n = 6). Ovarian stimulation was achieved using a combination of FSH (MetrodinHP; Ares Serono Laboratories Co., Welwyn Garden City, UK) and human menopausal gonadotrophin (HMG) (Pergonal; Ares Serono) in a step-down manner; 10 000 IU of human chorionic gonadotrophin (HCG) (Profasi; Ares Serono) was given to trigger ovulation. Oocytes were retrieved
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36 h later and exposed briefly to 80 IU/ml hyaluronidase (type VII; Sigma Chemical Co., St Louis, MO, USA). They were mechanically cleaned from their surrounding cumulus cells by aspiration through a glass pipette ~200 µm inner diameter. All oocytes were examined under an inverted microscope (Olympus IMT2) at a magnification of ×200 and those with a polar body were selected for micromanipulation.

Oocytes were scored by at least two observers for the presence or absence of cytoplasmic granularity, darkness of cytoplasm, localization of granularity: homogeneous or local, centrally located or laterally located, deep or slight granularity, large perivitelline space, perivitelline debris or accompanying refractile bodies, endoplasmic reticulum, vacuoles (small or large, single or multiple). All these characteristics were recorded during the ICSI procedure by a second observer. The severity of CLCG was classified into slight and severe categories (Figure 1).

The severity of granularity is based on the diameter of the granular area and the depth of the lesion. If more than 50% of oocyte cytoplasm was affected by granulation, exhibiting a crater-like appearance, it was classified as severe central granularity. However, if <50% of oocyte cytoplasm was affected by granulation in which the borders could not be distinguished clearly, it was classified as slight central granularity. Oocyte incubation prior to or following ICSI took classi-

A hand-drawn holding pipette, microneedle and biopsy pipette (Cook, IVF, Queensland, Australia) were used for the purpose of the biopsy. A double pipette holder (Narishige, Tokyo, Japan) was used for microneedle and biopsy pipette on the same side.

The embryo was fixed with the holding pipette and the first PZD was created, bypassing the largest perivitelline space at the 12 o’clock position. To create a V-shape opening, the embryo was rotated until the first slit was visible at the 12 o’clock position. A second cross was performed by entering with a microneedle into the first slit tangentially through the perivitelline space and a second zona dissection was performed (Cieslak, 1999). The embryo was then released and rotated until the V-shape opening was at the 3 o’clock position. The embryo was held by the holding pipette and the biopsy pipette was inserted to remove one or two blastomeres with a visible nucleus.

Preimplantation genetic diagnosis (PGD) was executed in nine cycles with severe CLCG to evaluate a possible chromosomal abnormality that may result in this type of oocyte morphology. Multicolour fluorescence in-situ hybridization (FISH) analyses with five DNA probes were used for the simultaneous detection of chromosomes X, Y, 13, 18 and 21 (Vysis, Illinois, IL, USA). Blastomere biopsy was performed on 36 day 3 embryos with seven or more blastomeres having <20% fragmentation. Embryos were classified as ‘complex abnormal’ when two or more chromosomes had an abnormal count but were not completely polyploid or haploid.

The patients were given 100 mg progesterone i.m. beginning from the day after oocyte retrieval until the serum β-HCG assay, 12 days after the embryo transfer. If pregnancy was achieved, the patients were then instructed to use micronized progesterone tablets vaginally 200 mg three times a day. Clinical pregnancy was defined as the presence of fetal heart beats 21 days after the β-HCG assay.

Statistical analysis of the data was performed by a χ² test with SPSS for Windows.

Results

Twenty-seven couples with 39 ICSI cycles with CLCG oocytes were evaluated. All the men had severe oligoasthenoteratozoospermia. A detailed urological examination, including a genetic evaluation with a cytogenetic test, was performed. The mean age of the women was 33.3 ± 4.27 years (range 24–39). Thirty-three cycles had ovarian stimulation by a long protocol (84.7%) and six cycles had ovarian stimulation by a short protocol (15.4%). A total of 489 oocytes was retrieved in 39 cycles. ICSI was performed on 392 (80.2%) MII oocytes. The mean number of retrieved oocytes and MII oocytes per cycle

![Figure 1. Oocytes exhibiting slight cytoplasmic central granulation, small arrow (a) and severe cytoplasmic central granulation, big arrow (b).](image-url)
was 12.53 ± 4.57 and 10.05 ± 3.39 respectively. Out of 489 oocytes retrieved, 392 were MII and 258 of these had centrally located cytoplasmatic granulation. The severity of CLCG was defined as slight and severe in 172 (66.7%) and 86 (33.3%) oocytes respectively. Fertilization was detected in 279 oocytes and the mean number of pre-embryos developed per cycle was 7.15 ± 3.24. The overall fertilization rate (FR) was 71.2% (279/392). For the CLCG group, it was 72.8% (188/258). In the slight and severe CLCG groups FR was 69.8% (120/172) and 79.1% (68/86) respectively (Table I) (not significant). The grading of transferred embryos on the third day is presented in Table II. Grade I transferred embryos were developed from 60% (274/45) of the oocytes with normal cytoplasmic morphology from 69.5% (37/53) of the oocytes with slight CLCG and from 66.7% (28/42) of the oocytes with severe CLCG. There was no significant statistical difference between the groups in terms of embryo quality.

Only good quality embryos were selected for transfer. The embryos which were not transferred were either slowly developed embryos with four to five cells on day 3, or embryos with uneven blastomeres with ≥25% of acellular fragments. Approximately half of the developed embryos were not selected due to poor embryo quality resulting from poor oocyte and poor sperm quality.

Forty-four blastomeres were biopsied from 36 embryos in poor prognostic factors. Approximately half of the developed embryos were not selected due to poor embryo quality resulting from poor oocyte and poor sperm quality.

Discussion

There were conflicting reports regarding the relationship between the oocyte morphology and fertilization rates or embryo quality (De Sutter et al., 1996, Serhal et al., 1997; Xia, 1997).

Oocyte morphology is thought to be insignificant in terms of fertilization, embryo quality and pregnancy rate (De Sutter et al., 1996). Cytoplasmic granulation of an oocyte may be a poor prognostic factor as it may be a sign of oocyte cytoplasmic immaturity. Our observation was that cytoplasmic granulation may be present in all oocytes from the same patient in repeated cycles. However, some patients may have oocytes that contain either granular or normal cytoplasm. Our study investigated the role of cytoplasmic granulation of oocytes on implantation

### Table I. The comparison of the laboratory results according to the presence of centrally located cytoplasmic granulation

<table>
<thead>
<tr>
<th>Cytoplasmic granulation</th>
<th>Absent</th>
<th>Present</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MII oocytes</td>
<td>134</td>
<td>172</td>
<td>306</td>
</tr>
<tr>
<td>Fertilized oocytes</td>
<td>91</td>
<td>120</td>
<td>211</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>67.9</td>
<td>69.7</td>
<td>71.2</td>
</tr>
<tr>
<td>Transferred embryos</td>
<td>45</td>
<td>53</td>
<td>140</td>
</tr>
</tbody>
</table>

*Not significantly different (χ² test).

### Table II. Comparison of quality of transferred embryos on the third day according to the presence of CLCG prior to ICSI

<table>
<thead>
<tr>
<th>Grade</th>
<th>Absent</th>
<th>Slight</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>27 (60)</td>
<td>37 (69.8)</td>
<td>28 (66.7)</td>
</tr>
<tr>
<td>II</td>
<td>17 (37.8)</td>
<td>12 (22.6)</td>
<td>11 (26.2)</td>
</tr>
<tr>
<td>III</td>
<td>1 (2.2)</td>
<td>4 (7.6)</td>
<td>3 (7.1)</td>
</tr>
</tbody>
</table>

There was no significant difference between the two groups. Values in parentheses are percentages.

### Table III. The pregnancy results for the cycles in which the embryos transferred were developed either from normal or centrally located granular oocytes

<table>
<thead>
<tr>
<th></th>
<th>Both normal and CLCG oocytes</th>
<th>Only CLCG oocytes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>21</td>
<td>18</td>
<td>39</td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>6 (28.6)</td>
<td>5 (27.8)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Abortion</td>
<td>4 (19.0)</td>
<td>2 (11.1)</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>On-going pregnancies</td>
<td>2 (9.5)</td>
<td>3 (16.7)</td>
<td>5 (12.8)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
and clinical pregnancy. The fertilization rate, embryo quality and pregnancy rate were not significantly different between the oocytes with or without granulation. The fertilization and embryo quality were found to be normal, but the implantation and on-going pregnancy rate seemed low in cases with CLCG oocytes. A similarly low rate of pregnancy was reported (Serhal et al., 1997) with CLCG oocytes.

It is not known what factors are responsible for cytoplasmic granulation. Chromosomal abnormality may be a reason for cytoplasmic granulation, but the number of the biopsied blastomeres was very low in our study. Moreover, only five chromosomes (13, 18, 21, X, Y) were studied, as these are the only commercially available probes in Turkey. The possibility of researching abnormalities of more chromosomes, including 1, 16 and 22, would also have been beneficial. Although embryos with normal X, Y, 13, 18 and 21 chromosomes were transferred in these patients, only one pregnancy was established out of seven embryo transfers (14.2%). In two cases, no normal embryos were transferred. In addition, the majority of the day 3 embryos developed from these oocytes were classified as high quality embryos. The fertilization rate and embryo quality were not significantly different between oocytes with and without granulation. The fertilization and embryo development were normal in cases with CLCG oocytes.

A previous cytogenetic study of MII stage human oocytes (Van Blerkom and Henry, 1988; Van Blerkom, 1989b), which restricted chromosomal analysis to oocytes that displayed a grossly normal appearing cytoplasm, demonstrated an overall frequency of aneuploidy of between 15 and 20%. However, in our study, the chromosomal abnormality rate with blastomere biopsy was as high as 52.2% in embryos developed from CLCG oocytes. This high aneuploidy rate in our study can be attributed to dysmorphic oocytes with significant granular cytoplasm as well as the embryos developed from couples with severe male infertility and advanced female age. A similar low rate of pregnancy was described previously (Serhal et al., 1997) with CLCG oocytes. In oocytes with a high degree of centrally localized granulation, cytoplasmic granulation can even be seen under a stereo-microscope.

The role of stimulation protocols may be questioned in the development of cytoplasmic granulation. The question of whether a higher frequency of oocyte dysmorphism and aneuploidy occurs in MII oocytes after ovarian stimulation is difficult to answer at present. A comparable set of data involving MII oocytes derived from unstimulated natural ovulatory cycles is currently not available. It has been observed (Jagiello et al., 1976; Van Blerkom et al., 1989a, 1990; Wojcik et al., 1995) that the germinal vesicle (GV) stage human oocytes aspirated from the small antral follicles of unstimulated ovaries are rarely aneuploid (1–3%). However, these oocytes would not be expected to contribute to the frequency of aneuploidy as they usually do not progress beyond the GV stages (Nayudu et al., 1987). In our study the high chromosomal abnormality rate may be due to poor oocyte and sperm quality.

Couples with CLCG oocytes should be informed about poor on-going pregnancy rates even though fertilization, embryo quality and total pregnancy rates may be normal. The presence of cytoplasmic granulation in the oocytes may affect the implantation rate even if normal oocytes are retrieved from the same cohort. Further studies and more data are needed to elucidate the role of aneuploidy in the high abortion rate observed in this study.

References

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