

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

S.Kahraman^{1,5}, K.Yakin¹, E.Dönmez¹, H.Şamlı², M.Bahçeç³, G.Cengiz¹, S.Sertyel¹, M.Şamlı⁴ and N.İmirzaloğlu²

¹Sevgi Hospital, Reproductive Endocrinology and ART Unit,

²Sevgi Hospital, Genetic Division, ³G.A.T.A. Genetic Division and

⁴Sevgi Hospital Andrology Unit, Turkey

⁵To whom correspondence should be addressed at: İstanbul Memorial Hospital, Reproductive Endocrinology and ART Unit, Piyalepaşa Bulvarı, 80270, Okmeydanı – Şişli/İstanbul, Turkey. E-mail: semrakahraman@hotmail.com

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

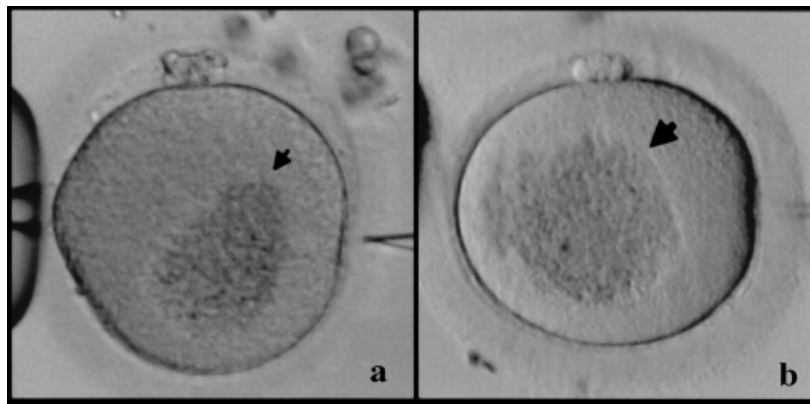


Figure 1. Oocytes exhibiting slight cytoplasmic central granulation, small arrow (a) and severe cytoplasmic central granulation, big arrow (b).

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 $\times 200$ 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 granulation, 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 place in IVF-50 medium (Gamete100; Scandinavian IVF Science, Gothenburg, Sweden) at 37°C in an atmosphere of 5% in air. ICSI was executed as described previously (Kahraman *et al.*, 1999). Assessment of fertilization was made 16–18 h after sperm injection. Further embryonic development was observed up to day 3.

Although the droplet technique used in our practice was time-consuming and created extra work for embryologists handling each oocyte individually, it allowed the strict follow-up of embryo development. Furthermore, it permitted an evaluation of the characteristics of each embryo developed from the corresponding oocytes. Only good quality embryos were selected for transfer. Embryo quality was evaluated using a grading system (Puissant *et al.*, 1987). The embryos that were not transferred were either slow in developing with four to five cells on day 3, or had uneven blastomeres with >25% acellular fragments. Slowly developing embryos may be the result of both poor sperm or oocyte quality. Approximately half of the developed embryos were not selected due to poor embryo quality.

Three-dimensional partial zona dissection (PZD) in a V-shape or diagonal was performed to facilitate the biopsy procedure, as was originally introduced by Verlinsky's group (Cieslak *et al.*, 1999). A V-shaped assisted hatching was performed in most of the embryos to create a triangular flap opening with a diameter of 25–30 μm to allow the replacement of the blastomere biopsy pipette.

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 χ^2 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

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

	Cytoplasmic granulation			Total
	Absent	Present		
		Slight	Severe	
MII oocytes	134	172	86	392
Fertilized oocytes	91	120	68	279
Fertilization rate (%)*	67.9	69.7	79.1	71.2
Transferred embryos	45	53	42	140

*Not significantly different (χ^2 test).

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

Grade	Cytoplasmic granulation		
	Absent	Slight	Severe
I	27 (60)	37 (69.8)	28 (66.7)
II	17 (37.8)	12 (22.6)	11 (26.2)
III	1 (2.2)	4 (7.6)	3 (7.1)

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

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 cytoplasmic 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% (27/45) 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 nine cycles. Of these, 23 (52.3%) had chromosomal abnormalities, while 21 (47.7%) had normal chromosomes. The majority of chromosomally abnormal embryos had simple aneuploidy ($n = 15, 65.2\%$). Monosomy was diagnosed in 11 and trisomy in four blastomeres. Complex aneuploidy with more than

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

	Both normal and CLCG oocytes	Only CLCG oocytes	Total
Number of cycles	21	18	39
Number of pregnancies	6 (28.6)	5 (27.8)	11 (28.2)
Abortion	4 (19.0)	2 (11.1)	6 (15.4)
On-going pregnancies	2 (9.5)	3 (16.7)	5 (12.8)

Values in parentheses are percentages.

two chromosomal abnormalities in the same blastomere was observed in six blastomeres (26%).

The total number of embryos transferred in 39 cycles was 140 and the mean number of embryos transferred was 3.59 ± 1.06 . The number of pregnancies achieved in 39 cycles was 11, thereby giving a pregnancy rate per cycle of 28.2% (Table III). In six cases, the pregnancies resulted in abortion (6/11, 54.5%). Five pregnancies resulted in healthy deliveries, four singletons and one set of twins. The on-going pregnancy rate per cycle was 12.8% (5/39). In 18 cycles, all the embryos transferred were developed from oocytes with CLCG. Pregnancy was obtained in five of these cycles (27.8%); two resulted in abortion (40%) and three ongoing pregnancies were achieved (ongoing pregnancy rate per cycle = 16.7%). In these five cycles, the embryos transferred were developed from oocytes with slight CLCG. In six cycles, all the embryos transferred were developed from oocytes with severe CLCG and no pregnancy was achieved and in the remaining seven cycles, a mixture of slight and severe CLCG was noted. In the remaining 21 cycles, transferred embryos were developed from both normal and CLCG oocytes. Six pregnancies were achieved in these cycles (28.6%). Four pregnancies resulted in abortion. The ongoing pregnancy rate was 9.5% (2/21). Only two pregnancies went to term. The implantation rate per embryo was found to be as low as 4.3% (6/140).

Out of nine cycles with PGD, seven embryo transfers took place (3.4 embryos per patient). A total of 21 embryos with normal chromosomes was transferred and in two cases no embryo transfer was realized. Only one pregnancy was achieved (14.2%) and a healthy boy was born.

Discussion

There are 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

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

- Cieslak J., Ivakhnenko V., Wolf, G. *et al.* (1999) Three-dimensional partial zona dissection for preimplantation genetic diagnosis and assisted hatching. *Fertil. Steril.*, **71**, 308–313.
- De Sutter, P., Dozortsev, D., Qian, C. *et al.* (1996) Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 595–597.
- Jaqiello, G., Ducayen, M. and Fang, J. (1976) Cytogenetic observations in mammalian oocytes. In Pearson, P. and Lewis, K. (eds), *Chromosomes Today*. Wiley, New York, pp. 43–63.
- Kahraman, S., Akarsu, C., Cengiz, G. *et al.* (1999) Fertility of ejaculated and testicular megalohed spermatozoa with intracytoplasmic sperm injection. *Hum. Reprod.*, **14**, 726–730.
- Nayudu, P.L., Gook, D.A., Lopata, A. *et al.* (1987) Follicular characteristics associated with viable pregnancy after in-vitro fertilization in humans. *Gamete Res.*, **18**, 37–55.
- Puissant, F., Van Rysselberge, M., Barlow, P. *et al.* (1987) Embryo scoring as a prognostic tool in IVF treatment. *Hum. Reprod.*, **2**, 705–708.
- Serhal, P.F., Ranieri, D.M., Kinis, A. *et al.* (1997) Oocyte morphology predicts outcome of intracytoplasmic sperm injection. *Hum. Reprod.*, **12**, 1267–1270.
- Van Blerkom, J. (1989a) Developmental failure in human reproduction associated with preovulatory oogenesis and preimplantation embryogenesis. In Van Blerkom, J. and Motta, P. (eds), *Ultrastructure of Human Gametogenesis*. Kluwer, Boston and London, pp. 125–180.
- Van Blerkom, J. (1989b) The origin and detection of chromosomal abnormalities in meiotically mature human oocytes obtained from stimulated follicles after failed fertilization in-vitro. *Prog. Clin. Biol. Res.*, **296**, 299–310.
- Van Blerkom, J. (1990) Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. *J. Electron Microsc. Tech.*, **16**, 324–346.
- Van Blerkom, J. and Henry, G.H. (1988) Cytogenetic analysis of living human oocytes: cellular basis and developmental consequences of perturbations in chromosomal organization and complement. *Hum. Reprod.*, **3**, 777–790.
- Van Blerkom, J. and Henry, G.H. (1992) Oocyte dysmorphism and aneuploidy in meiotically mature human oocytes after ovarian stimulation. *Hum. Reprod.*, **7**, 379–390.
- Wojcik, C., Guerin, J., Pinatel, M. *et al.* (1995) Morphological and cytogenetic observations of unfertilized human oocytes and abnormal embryos obtained after ovarian stimulation with pure follicle stimulating hormone following pituitary desensitization. *Hum. Reprod.*, **10**, 2617–2622.
- Xia, P. (1997) Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. *Hum. Reprod.*, **2**, 1750–1755.

Received on December 30, 1999; accepted on June 27, 2000