Array comparative genomic hybridization screening in IVF significantly reduces number of embryos available for cryopreservation

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This is a copy of the final published version of an article that appeared in Clinical & Experimental Reproductive Medicine, 39 (2). pp. 52-57, 2012. It is available online at:

http://dx.doi.org/10.5653/cerm.2012.39.2.52

ORIGINAL ARTICLE
http://dx.doi.org/10.5653/cerm.2012.39.2.52
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2012;39(2):52-57

CERM

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Objective: During IVF, non-transferred embryos are usually selected for cryopreservation on the basis of morphological criteria. This investigation evaluated an application for array comparative genomic hybridization (aCGH) in assessment of surplus embryos prior to cryopreservation.
Methods: First-time IVF patients undergoing elective single embryo transfer and having at least one extra non-transferred embryo suitable for cryopreservation were offered enrollment in the study. Patients were randomized into two groups: Patients in group A (n = 55) had embryos assessed first by morphology and then by aCGH, performed on cells obtained from trophectoderm biopsy on post-fertilization day 5. Only euploid embryos were designated for cryopreservation. Patients in group B (n = 48) had embryos assessed by morphology alone, with only good morphology embryos considered suitable for cryopreservation.
Results: Among biopsied embryos in group A (n = 425), euploidy was confirmed in 226 (53.1%). After fresh single embryo transfer, 64 (28.3%) surplus euploid embryos were cryopreserved for 51 patients (92.7%). In group B, 389 good morphology blastocysts were identified and a single top quality blastocyst was selected for fresh transfer. All group B patients (48/48) had at least one blastocyst remaining for cryopreservation. A total of 157 (40.4%) blastocysts were frozen in this group, a significantly larger proportion than was cryopreserved in group A ($p = 0.017$, by chi-squared analysis).
Conclusion: While aCGH and subsequent frozen embryo transfer are currently used to screen embryos, this is the first investigation to quantify the impact of aCGH specifically on embryo cryopreservation. Incorporation of aCGH screening significantly reduced the total number of cryopreserved blastocysts compared to when suitability for freezing was determined by morphology only. IVF patients should be counseled that the benefits of aCGH screening will likely come at the cost of sharply limiting the number of surplus embryos available for cryopreservation.

Keywords: Fertilization *in vitro*; Comparative genomic hybridization; Preimplantation genetic diagnosis; Cryopreservation

Introduction

Within the arena of assisted reproduction, several technologies have emerged to address the most important challenge in human fertility medicine: multiple gestation. Single embryo transfer, either elective or mandatory, has been proposed as a way to avoid this problem [1-4]. Selection of an embryo for transfer or cryopreservation is typically done on the basis of morphology [5,6]. However, since suitable morphology by itself cannot negate the potential for chromosomal error in the selected embryo, the transfer or cryopreservation of apparently "normal looking" embryo carries considerable risk [7]. Experience with IVF has shown that aneuploidy is the most common abnormality in human embryos [8-15] and this characteristic contributes substantially to poor reproductive outcomes observed in advanced fertility treatments [16]. As other investigators have noted [17-21], screen-

Received: Apr 21, 2012 · Revised: May 9, 2012 · Accepted: May 14, 2012
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ing embryos by fluorescence in situ hybridization was a reasonable first answer to this challenge, but the approach was too limited because it could not screen all chromosomes at the same time. More recently, single nucleotide polymorphism array and array comparative genomic hybridization (aCGH) have been used to achieve comprehensive chromosome screening to improve efficiency of embryo transfer [22-30]. These techniques enable subsequent frozen embryo transfer so that only euploid embryo(s) are used therapeutically, thus improving implantation and reducing risk of miscarriage. However, because experience using these molecular cytogenetic tests in reproductive medicine remains limited, there is a critical need to validate embryo selection techniques before such technology enters the clinical mainstream [31]. Yet while the optimal method of determining the chromosomal composition of the human embryo remains unsettled, there has been little or no discussion specifically focused on how such technologies might affect the total number of embryos available for cryopreservation. Accordingly, this study compared the impact of aCGH on cryopreservation yield when this molecular cytogenetic technique was applied to young, low-risk patients undergoing their first IVF cycle.

Methods

1. Patient sample

To study the impact of aCGH on embryo cryopreservation rate in IVF, patients at our clinics in Los Angeles and Beijing were offered enrollment in this prospective, single-blind investigation. Institutional Review Board approval was obtained before recruitment and written informed consent was obtained from all study participants. All patients received pre-treatment counseling about aCGH and how this testing technique might affect the number of embryos cryopreserved. Patients were eligible for this study if (female) age was <35 years, if there was a history of regular ovulation, if etiology of infertility was tubal factor or male factor (or both), and if no prior IVF treatment had been initiated. Additionally, all study subjects were required to have a normal 46,XX karyotype, two ovaries, a normal endometrial contour, and basal serum FSH and estradiol on d2-3 at <10 IU/L and <60 pg/mL, respectively. IVF patients whose treatment incorporated donor gametes or frozen/thawed embryos were excluded. A random number table was used to determine patients embryo cryopreservation protocol as either 1) traditional morphology assessment plus aCGH (group A, n=55), or 2) conventional morphology assessment only (group B, n=48). Patients (but not laboratory or clinical staff) were blinded with regard to their randomization group, and clinical features of the two study groups were similar. The two cohorts were mutually exclusive; no study patient had embryos assigned to both laboratory groups.

2. Ovarian stimulation and fertilization

Baseline transvaginal ultrasound evaluation was performed before starting ovulation induction medications, with re-measurement of serum FSH, LH and estradiol on d3 of the index cycle. GnRH-agonist administered on d21 of the cycle immediately preceding treatment was used to attain pituitary downregulation, as described previously [32]. Periodic transvaginal ultrasound and serum estradiol measurements were used to monitor follicular growth and thickness of endometrial lining. When ≥ 3 follicles reached 19 mm mean diameter, periovulatory hCG was administered by subcutaneous injection of recombinant hCG (250 µg Ovidrel, Merck Serono, Geneva, Switzerland) with oocyte retrieval performed under transvaginal ultrasound guidance 35-36 hours later. Following removal of all cumulus cells, ICSI was performed with a 30° needle, and normal fertilization was verified 16-18 hours after injection by presence of two pronuclei and two polar bodies.

3. Embryo culture and trophectoderm biopsy

Sequential media (Vitrolife, Göteborg, Sweden) was used to support all embryos to blastocyst stage. On d3 when embryos were at the 6-8 cell stage, a noncontact 1.48 µm diode laser (OCTAX Microscience GmbH, Bruckberg, Germany) was used to create a circular 6-9 µm diameter opening in the zona pellucida (ZP). For embryos randomized to the aCGH group, this lacuna facilitated biopsy of trophectoderm (TE) on d5. Between 3-5 herniated TE cells were gently aspirated by pipette and, when necessary, freed from the blastocyst by application of several laser pulses. Harvested TE cells were washed in phospate buffered solution (PBS) and placed within a polymerase chain reaction tube with 2.5 µL $1\times$PBS as previously described [33]. The same assisted hatching protocol was used for all embryos irrespective of subsequent TE biopsy or conventional microscopic assessment alone.

4. aCGH protocol

The SurePlex DNA amplification system (BlueGnome Ltd., Cambridge, UK) was used on-site for whole genome amplification in accordance with manufacturer's guidelines [33,34]. Briefly, samples and control DNA (8 µL for each) were labeled with Cy3 and Cy5 fluorophores (BlueGnome Ltd.). Labeling time was approximately 3 hours with DNA resuspended in dexsulphate hybridization buffer and hybridized overnight under cover slides. After washing 1×10 minutes in saline sodium citrate (SSC)/0.05% Tween-20 at room temperature, an additional irrigation in SSC 1×10 minutes was completed at room temperature. Slides were washed in SSC 1×5 minutes at 60°C and again for 1 minute at room temperature (in SSC). Vacuum centrifuge was used to dry microarray slides over 3 minutes, followed by laser scanning at 10 µm (Agilent Technologies, Santa Clara, CA, USA). Blue-

Fuse software (BlueGnome, Ltd.) was used for analysis of microarray data on chromatin loss/gain across all 24 chromosomes. Aberrations were considered non-artifact if ≥15 probes deviated from normal limits as defined by the 24 sure platform. The published accuracy rate for this aCGH technique when applied to TE cells is 95% [34].

5. Selection criteria for cryopreservation

In both aCGH and control groups, blastocysts were graded [35] on a 1 to 6 scale determined by degree of expansion and hatching status, as follows: grade 1 (early blastocyst): blastocoele <1/2 of total embryo volume; grade 2 (intermediate blastocyst): blastocoele ≥1/2 of total embryo volume; grade 3 (full blastocyst): blastocoele fully occupies the embryo; grade 4 (expanded blastocyst): blastocoele is larger than early blastocyst and ZP demonstrates thinning; grade 5 (hatching blastocyst): herniation of TE cells from the ZP; and grade 6 (hatched blastocyst): blastocyst has escaped the ZP. For blastocysts at grades 3 to 6, the inner cell mass (ICM) and TE were also graded. The ICM was graded as follows: A (many ICM cells packed together tightly); B (several ICM cells grouped loosely) and C (very few ICM cells). TE was graded as follows: A (numerous TE cells forming multiple epithelial layers); B (few TE cells consisting of a loose epithelium) and C (scarce large TE cells). For embryos in the aCGH group only one euploid blastocyst was selected for transfer based on aCGH data and surplus euploid blastocysts were vitrified for later use [35]. In the non-aCGH (control) group, a single blastocyst was selected for fresh transfer based on morphological criteria only (i.e., no aCGH evaluation) and those blastocysts with good morphology (grade 3BB or above) were vitrified the same day.

6. Outcome measures and statistical analysis

The number of cryopreserved blastocysts was recorded and compared for IVF patients where aCGH was utilized and for those where cryopreservation was determined by morphology alone. Differences between groups were assessed by chi-square test. A difference of $p<0.05$ was considered statistically significant.

Results

A total of 188 IVF patients met inclusion criteria and 112 volunteered for enrollment (59.6%) during the four-month investigation. Of 56 patients randomized to each group, some did not initiate IVF due to failure to complete required pre-IVF testing, they postponed treatment until a later date, or for other reasons. For group A (morphology + aCGH) and group B (morphology only) 55 and 48 IVF patients completed the study, respectively. There were no cancellations or complications for any patient in either study group.

For patients in group A, 425 of 457 blastocysts were biopsied and analyzed via aCGH (7.7 blastocysts/patient). Trophectoderm biopsy could not be completed in 7% of blastocysts (n=32) due to indistinct TE (poor morphology) or because they degenerated after biopsy. This evaluation revealed aneuploidy in 191/425 (44.9%) of blastocysts. 'No signal' due to amplification failure occurred in 8 blastocysts. Among aneuploid blastocysts, 68/191 (35.6%) had single chromosome loss (monosomy) and 20.9% displayed single chromosome gain (trisomy). Approximately 43% of aneuploid blastocysts were chromosomally abnormal due to a severe, compound genetic defect where two or more chromosomes were affected. While chromosomal abnormalities were detected in all chromosomes, disruptions involving chromosomes 15, 16, 21, 22, and X were most frequently observed. Errors of chromosomes 4 and 6 were relatively uncommon. All patients in group A had at least one euploid blastocyst available for transfer on d6. For patients in group B, 389 blastocysts were microscopically examined (8.1 blastocysts/patient).

Among biopsied embryos in group A (n=425), euploidy was confirmed in 226 (53.1%). After fresh single embryo transfer, 64 (28.3%) surplus euploid embryos were cryopreserved for 51 study patients (92.7% of patients in this group). In group B, 389 good morphology blastocysts were identified and a single top quality blastocyst was selected for fresh transfer. As shown in Table 1, the cryopreservation yield for group B patients was 100% (48/48), because patients in this group all had at least one blastocyst remaining for cryopreservation. A total of 157 (40.4%) blastocysts were frozen in this group, a significantly larger proportion than was cryopreserved in group A ($p=0.017$, by chi-squared analysis).

Discussion

Although the role of embryo cryopreservation will doubtless hold its essential niche in the modern IVF unit, it is not yet known exactly

Table 1. Summary of IVF study patient data for blastocyst cryopreservation following conventional morphology assessment plus comprehensive chromosomal screening via array CGH (group A), or conventional morphology assessment only (group B)

Parameters	A	B
Patients enrolled	55	48
Patients with cryopreserved blastocysts	51	48
Blastocysts available for bx	457	n/a
Bx completed	425	n/a
Euploid blastocysts	226	n/a
Blastocysts remaining after ET	171	341
Cryopreserved blastocysts[a]	64	157

Demographic and clinical parameters were similar for both randomized groups.
CGH, comparative genomic hybridization; Bx, biopsy; n/a, not applicable; ET, embryo transfer.
[a]A vs. B, $p=0.017$ (by chi-square test).

http://dx.doi.org/10.5653/cerm.2012.39.2.52

how newer molecular testing techniques will affect this aspect of assisted fertility treatment. As more practice jurisdictions move toward a mandatory single embryo transfer policy, it may be that pre-transfer assessment of embryos by aCGH (or some other screening method) will become a regular procedure in IVF. Should fresh transfers be limited only to single embryos which undergo routine chromosomal screening, then the number of surplus cryopreserved embryos may be expected to rise. But thus far, clinical research has focused on application of comprehensive chromosomal screening specifically for patients with known translocation, multiple implantation failure or recurrent pregnancy loss [7,19,29]. To our knowledge, a prospective study of cryopreservation yield following aCGH screening of embryos from IVF patients without such history (i.e., first cycle IVF, age <35 and good prognosis) has not yet been conducted. The present investigation was undertaken to address this gap in the literature by providing measurements on rate of blastocyst cryopreservation as a function of aCGH inputs in this setting.

Among young patients with normal karyotype undertaking their first IVF cycle, with no miscarriage history or other predictors of poor prognosis, the present study found euploidy in only 226 of 425 blastocysts (53.1%) as determined by aCGH testing. Although most (92.7%) patients in this group had at least one euploid blastocyst for cryopreservation after fresh transfers, just 67 blastocysts remained for cryopreservation in this group (29.6%). When embryo cryopreservation was performed on the basis of standard morphology assessment alone, all study 48 patients had at least one blastocyst available for cryopreservation. For this group, a total of 154 (39.6%) blastocysts were frozen. Since all study patients were <35 years of age, a very good chance to achieve surplus blastocysts for cryopreservation was expected regardless of randomization group (100% vs. 92.7%). However, a significantly lower ($p=0.017$) proportion of surplus blastocysts were available for cryopreservation when aCGH testing was performed (39.6% vs. 29.6%). Interestingly, when cryopreservation yield is calculated on the basis of blastocysts remaining after fresh transfers rather than absolute (total) number of blastocysts, this difference between study groups persists. Such findings underscore the need for careful counseling for IVF patients throughout the treatment sequence, particularly as the critical time for embryo cryopreservation nears [36,37].

Our work extends prior observations to younger, lower-risk IVF population and finds conventional morphological criteria alone to be insufficiently accurate to select blastocysts for cryopreservation. Focusing on a good-prognosis IVF patient population, the current study provides further evidence of substantial genetic abnormality (including monosomy and complex aneuploidy [7,25,38]) in apparently normal blastocysts which otherwise would have been destined for cryostorage. We believe IVF patients should be counseled that the benefits of aCGH screening are likely to come at the cost of sharply limiting the number of surplus embryos available for cryopreservation. While we favor use of comprehensive chromosomal screening during IVF, these data place the shortcomings of standard embryo morphology –even for good prognosis IVF patients– in sharp relief. From this background an important clinical question emerges: If aCGH were to be become routinely integrated into the IVF treatment sequence, then what is the expected impact on the number of embryos available for cryopreservation?

Several limitations of our investigation should be acknowledged. Because blastocysts in our control group were selected by morphology alone, they were cryopreserved without any genetic testing and therefore have an uncertain reproductive potential. This study was not designed to measure reproductive outcomes after thaw and transfer. Additionally, we were not confident to include a power analysis prior to this investigation because the actual incidence of embryo aneuploidy in first-time IVF patients with no risk factors is unknown (i.e., if no significant difference had been identified, type II error could not have been excluded).

A changing public perception of better prognosis from IVF specifically when frozen embryos are thawed and transferred [39] has further increased general awareness of cryopreservation's central role in the advanced reproductive technologies [38,40,41]. Moreover, recent advancements in whole genome amplification and comprehensive chromosomal screening have permitted levels of human embryo surveillance that were not possible only a few years ago. These developments offer opportunities to verify an embryo's genetic composition before cryopreservation. The present study suggests that designating an embryo for freezing without the benefit of information gained from aCGH would entail the preservation and storage of a reproductively incompetent –albeit morphologically normal– embryo. A multi-center randomized clinical trial with a larger sample will be helpful to validate these preliminary findings.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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