Total Cytoplasmic Volume as Biomarker of Fragmentation in Human Embryos

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Purpose: To use computer-controlled, multilevel embryo morphology analysis to quantify the reduction in cytoplasmic volume from the zygote stage to the combined volume of the individual blastomeres as an expression for the degree of fragmentation.

Methods: Zygotes and their corresponding embryos from patients referred to ICSI treatment were analyzed. Sequences of digital images were taken focussing at 5- μ m intervals through the zygotes and embryos. Assessment of cytoplasmic volumes and fragmentation were based on these sequences.

Results: The mean cytoplasmic reduction of the embryos were significant linear increasing with increasing degree of fragmentation assessed by traditional evaluation ($P < 0.001$). Embryos scored to be highly fragmented had on average a cytoplasmic reduction of 63% larger than embryos assessed to have no fragmentation. In total, 68% of the embryos had a cytoplasmic reduction lying within their allocated fragmentation group.

Conclusion: Assessment of embryonic fragmentation based on computer-controlled, multilevel embryo analysis of the blastomere volumes expressed in relation to the volume of the preceding zygote may allow a more precise and standardized evaluation of fragmentation than the traditional fragmentation assessment.

KEY WORDS: Computer-controlled multilevel morphometric analysis; cytoplasmic reduction; embryo fragmentation; embryo quality; total blastomeric volume.

INTRODUCTION

Selection of embryos for transfer after IVF or ICSI (intracytoplasmic sperm injection) treatment is mainly based on subjective light microscopic morphology analysis. Several studies have shown that morphological structures in the embryo can be used as biomarkers of embryonic quality (1–5). Despite the fact that markers of embryo competence like genome activation may not yet be expressed, it has been demonstrated that early embryo selection based on morphology assessment improves implantation and pregnancy rates (3,6–8).

Evaluation of fragmentation is included in all embryo scoring systems and the presence of high amounts of fragmentation have been shown to correlate negatively with implantation and pregnancy rates (1,3,4,6–10). However, embryo quality score as well as blastomere characterization is based on the subjective assessment by the observer (11), resulting in only a rough estimate and in marked interobserver variation. Furthermore, time is a limiting factor when analyzing the embryo without compromising its quality. This makes it difficult to investigate embryo morphology in detail.

The lack of objective and standardized methods to assess embryonic fragmentation remains a concern in relation to define *embryo quality*. Previous studies have shown that the total cytoplasmic volume remains constant during early embryonic development (11–13). Based on computer-controlled multilevel

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morphometric analysis, our previous study showed a significant linear decrease in blastomere volume with increasing degree of fragmentation in the early embryo (14). Fragments are by definition anucleate structures of blastomeric origin. Therefore the blastomere volume may be affected by the degree of fragmentation. This suggests that the degree of embryonic fragmentation may be quantified as the reduction in the total blastomeric volume compared to the cytoplasmic volume of the zygote.

In this study we used a computer-controlled system for multilevel and noninvasive embryo morphology analysis. This system is based upon analysis of sequential sequences of digital images of the oocytes and embryos which allowed us to overcome any time limitations as well as perform a more precise and detailed analysis of the embryonic structures.

The aim of this study was to quantify the reduction in cytoplasmic volume from the zygote to the total blastomeric volume of the corresponding embryo as an expression for the degree of embryonic fragmentation and to compare these findings to the traditional evaluation of fragmentation.

MATERIALS AND METHODS

Selection of Embryos

This study includes all zygotes from 16 patients undergoing ICSI that had developed to embryos 48 h after aspiration. Overall, 151 oocytes were aspirated in the included patients. Nineteen oocytes were atretic or damaged at aspiration and 19 oocytes were immature (GV or MI-stage). Thus, in the remaining 113 oocytes, ICSI procedure was performed. After ICSI, two oocytes had three pronuclei and were discarded. Twenty oocytes were not fertilized and another 10 oocytes did not cleave. Thus, in total 81 zygotes and embryos consisting of 282 blastomeres were included. Nineteen embryos were at the two-cell stage, 17 at the three-cell stage, 34 at the four-cell stage, 9 at the five-cell stage, and 2 embryos consisted of six blastomeres. Digital microscopy image sequences were taken of all included zygotes and of all their resulting day 2 embryos. All further analysis was performed on the basis of these images.

Patients

Fifteen patients referred to ICSI because of male factor and one patient referred to ICSI because of

unexplained infertility were included. The average age of the women was 32.2 years with a range from 24 to 39 years. Patients were treated with the long protocol, using gonadotropin-releasing hormone agonist for downregulation and recombinant follicle stimulation hormone (r-FSH) (Gonal-F[®], Serono or Puregon[®], Organon) for ovarian stimulation. HCG (Profasi[®], Serono) was given 36 h before oocyte retrieval. The average number of oocytes aspirated per patient was 9.4.

ICSI Procedure

ICSI was performed according to the clinical routine procedures. Briefly, oocytes were aspirated 36 h after hCG injection and the ICSI procedure was performed 4–6 h later. The oocytes and embryos were cultured individually in four-well dishes in Universal IVF Medium (MediCult, Cat. No. 100310060). On the following morning (18 h after insemination) the oocytes were examined for fertilization and cultured for further 24 h. Embryo transfer was carried out 48–51 h after aspiration. Immediately prior to transfer, embryos were selected for transfer evaluating cleavage stage and quality score in accordance with the normal procedures at our clinic. The selection of embryos for transfer and cryopreservation was done independently of the study. Subsequently all included embryos were reevaluated on the basis of the recorded sequences.

Recording of Digital Images

Using the FertiMorph computer system for multilevel embryo morphology analysis (Image House Medical A/S, Copenhagen, Denmark), image sequences were recorded of all included zygotes 18 h after insemination and of all the subsequent day 2 embryos 48 h after aspiration. Each sequence consisted of 26 images of the same oocyte/embryo with the FertiMorph System automatically focusing in 5- μ m intervals through the embryo. The automatically controlled image recording and storing took approximately 15 s per sequence. All recordings were performed at 400 times magnification with Hoffman modulation contrast illumination.

Measurements on the images are done in pixels and converted to actual physical units knowing the distance between two adjacent pixels. The calibration is done by taking an image of a micrometer slide with the same magnification as the embryo images. A line is drawn on the micrometer slide. Knowing the outlined

distance on the slide, the system calculates the physical distance between two pixels.

Computer-Controlled Morphometric Analysis of Embryo Morphology

On the basis of the digital image sequences, blastomere sizes were analyzed in a semiautomatic manner using the morphology analysis software of the Fertimorph System. All images of one sequence could be viewed in detail enabling us to select the pictures where the different blastomeres or the oocyte were in focus. For all zygotes the outer border of the cell were outlined on the particular digital image. For all embryos, the outer border of all morphological structures that were considered to be a blastomere was outlined on the particular digital images and all visible nuclear structures within the blastomeres were registered. To differentiate between blastomeres and fragments, we defined the property of the outlined structures in accordance to the criteria by Johansson *et al.* (15).

1. If at least one nuclear structure was detected, it was considered a blastomere regardless of its size.
2. If no nuclear structures could be detected, it was considered a blastomere when the diameter was $\geq 45 \mu\text{m}$ and a fragment when the diameter was $< 45 \mu\text{m}$.

Morphometric values describing the size of the blastomeres (area, diameter, and volume) were calculated automatically. The system calculated the area of the blastomere. A radius was calculated from a circle with the equivalent area and the cell volume was computed on the basis of the assumption that blastomeres were spherical. Therefore we used the equation $V = 4/3\pi r^3$.

Further, the reduction of the total blastomeric volume compared to the volume of the corresponding zygote was calculated for all embryos. This was done using the following abbreviations and equation:

TCV = Total Cytoplasmic Volume = Zygotic Volume

TBV = Total Blastomeric Volume

CR = Cytoplasm Reduction

(equation) $\text{CR} = \text{TCV} - \text{TBV}$ (CR is assumed equal to the total volume of the fragments)

CRs were compared to the outcome of the traditional evaluation of embryo fragmentation.

On the basis of the digital image sequences, the embryonic fragmentation was evaluated by normal morphology assessment allocating each embryo to one of the following five groups: (i) group I, 0% fragmentation; (ii) group II, $\leq 10\%$ fragmentation; (iii) group III, $> 10 - \leq 20\%$ fragmentation; (iv) group IV, $> 20 - \leq 50\%$ fragmentation; (v) group V, $> 50\%$ fragmentation. This evaluation of embryo fragmentation was performed independently by three different observers viewing the image sequences in detail. If there were disagreement between the three different observers evaluation, the embryo was allocated to a particular group of fragmentation based on the following criteria:

1. If at least two of the three observers scored a particular embryo to the same group, the evaluation by the third observer was disregarded; and
2. If all three observers scored a particular embryo in different groups, the embryo was allocated to the middle group.

The observers were told which *structures* were defined as blastomeres on the basis of the morphometric measurements to ensure that any differences between evaluation of fragmentation and CR were not due to disagreement in regard to what was considered a blastomere.

When comparing CR to fragmentation assessed by traditional light microscopic analysis, a CR of $\pm 1\%$ was considered to be in accordance with the no-fragment group.

Statistical Analysis

Analysis of variance (ANOVA) was done followed by Tukey's test for all pairwise multiple comparisons when evaluating differences between the average CRs of the embryos allocated to different groups of fragmentation, respectively. Linear regression were performed when evaluating relations between the groups of increasing degree of fragmentation and the average CRs of the embryos allocated to these different groups, respectively. Differences were considered significant when $P < 0.05$.

RESULTS

In average the zygotes had a diameter of $111.6 \mu\text{m} \pm 3.7$ and a volume of $0.728 \times 10^6 \mu\text{m}^3 \pm 0.07$. The average total blastomeric volume was $0.598 \times 10^6 \pm$

Fig. 1. Percentage of mean cytoplasmic reduction of the embryos allocated to different groups of fragmentation. There were significant differences between the mean cytoplasmic reduction of the embryos allocated to different fragmentation groups ($P < 0.001$). All pairwise comparisons showed significant differences in the cytoplasmic reduction ($P < 0.05$). The mean cytoplasmic reduction increased linearly with increasing degree of fragmentation assessed by traditional light microscopic evaluation ($P < 0.001$).

$0.13\mu\text{m}^3$ and the average cytoplasm reduction was $18.1\% \pm 17.0$.

The mean CR increased linearly with increasing degree of fragmentation assessed by traditional evaluation (Fig. 1) as R^2 were 0.987 with $P < 0.001$ and a slope coefficient of $\alpha = -15.3$. There were significant ($P < 0.001$) differences in the mean CR for the embryos allocated to different fragmentation groups (Fig. 1) and all pairwise multiple comparison showed significant difference in the cytoplasmic reduction for all comparisons ($P < 0.05$).

Comparing CR with the traditional light microscopic assessment of fragmentation, 73% of the embryos showing fragmentation had a CR lying within the interval of the respectively allocated groups (Fig. 2). In group 2, 56% of the embryos showed accordance between their cytoplasmic reductions and the degree of fragmentation assessed by the observers. In group 3, this accordance was 63% and in both groups 4 and 5 the corresponding accordance was 100%.

Twenty-five percent of the embryos in the no-fragment group had a CR within the definition of this group. Including group 1, a total of 68% of the embryos had a cytoplasmic reduction lying within their allocated fragmentation group.

DISCUSSION

The data from this study showed a significant linear correlation between the cytoplasmic reduction and the degree of fragmentation assessed by normal mor-

Fig. 2. For all embryos the cytoplasmic reduction is shown in relation to fragmentation score made by the observers.

phology evaluation in the cleaved day 2 embryos. We have previously shown that the mean blastomere volume of 2, 3, and 4 cell embryos is decreasing significantly with increasing degree of fragmentation in the embryo (14). However, in that previous study the blastomere volumes of the particular embryos were not related to the volume of their corresponding zygote. The data from the present study suggest that the reduction in the cytoplasmic volume from the zygote stage to the cleaved embryo may be used to quantify the degree of fragmentation in the early embryo stages.

A precondition for fragmentation assessment based on the cytoplasm reduction is that the total cytoplasmic volume remains constant during early embryonic development. Previous morphometric measurements of zygotes and their corresponding embryos showed that the total blastomeric volume in good-quality embryos remained constant at least up to the four-cell stage compared to the volume of their zygotes (12). Further, it was shown that the perivitelline space as well as the volume enclosed by the inner limit of the zona pellucida in these good-quality embryos remained constant from the zygote to at least the four-cell stage. These findings indicate that the total cytoplasmic volume does remain constant in the early embryo compared to the zygotic volume as also suggested by Roux *et al.* (11) and Hardy *et al.* (13). In addition, Goyanes *et al.* (12) showed that the total blastomeric volume decreased significantly in fragmented embryos, which supports the idea that the reduction of the cytoplasm may reflect the degree of embryonic fragmentation. However, Goyanes *et al.* (12) did not distinguish between different degrees of fragmentation within the group of fragmented embryos nor did

they define threshold sizes for blastomeres and fragments, making a detailed comparison of our findings to that study impossible.

When evaluating the correlation between the cytoplasmic reduction of each individual embryo with the traditional fragmentation assessment 73% of the embryos displaying fragmentation had a cytoplasmic reduction lying within the interval of the allocated fragmentation groups.

Group 1 does not include an interval of fragmentation as it represents embryos assessed to have no fragmentation. Thus, the individual cytoplasmic reductions for the embryos allocated to group 1 were expected to be close but not equal to 0. Considering cytoplasmic reductions ranging from +1% to -1% being in accordance with fragmentation group 1, 25% of the embryos allocated to this group showed a cytoplasmic reduction within this range of accordance.

However, for two of the eight embryos allocated to fragmentation group 1, the total blastomeric volume was calculated to be 22.0 and 23.4% larger than the volume of their corresponding zygotes. Interestingly, these two embryos were the only two-cell stage embryos within the group of nonfragmented embryos. As previously shown by Goyanes *et al.* (12), two-cell stage blastomeres are slightly ellipsoid cells, that are flattened where they are in contact with each other. The deformation from spherical towards ellipsoid may be most pronounced in nonfragmented two-cell embryos as they consist of blastomeres of maximum possible size. Thus, one possible explanation for our findings in these two nonfragmented embryos is that the assumption that the blastomeres are spherical, which were used when estimating the volume of the blastomeres, may have resulted in an overestimation of the blastomeric volume. The blastomeres of these embryos were in average $92.4 \mu\text{m} \pm 3.5$ in diameter, thus 15.3% larger than the average diameter of two-cell stage blastomeres found in our previous study (14). The remaining six embryos in fragmentation group 1 were at the four- to six-cell stage. The cytoplasmic reductions of these embryos were in average 0.02%, ranging from +4.5% to -5.9%.

One of the problems with traditional morphology assessment of fragmentation is huge intra- and interobserver variation. This means that traditional fragmentation score is difficult to use as reference material when evaluating whether the cytoplasmic reduction can be used as biomarker for the degree of embryonic fragmentation. This underscores the need for more standardized methods of fragmentation assessment. A key parameter in using cytoplas-

mic reduction as an expression for the degree of fragmentation is the correct differentiation between blastomeres and fragments. Our criteria for distinguishing between a blastomere and a fragment are based on the findings by Johansson *et al.* (15) that suggest that day 2 cells $45 \mu\text{m}$ in diameter should be classified as blastomeres and those $<45 \mu\text{m}$ as fragments. However, the precise minimum sizes for biologically competent blastomeres at different cleavage stages need still to be clarified, to achieve a more objective assessment of fragmentation based on the cytoplasmic reduction.

As fragments are structures of blastomeric origin, they may extract considerable amounts of cytoplasm from the blastomeres resulting in blastomeres with a quantitative deficiency of important cytoplasmic contents such as cell organelles, m-RNA, or proteins as suggested by Johansson *et al.* (15) and Alikani *et al.* (16). The question therefore is whether it is the fragments themselves that compromise embryo quality, or rather the reduction in blastomere sizes that is associated with high degree of fragmentation. Blastomeres in highly fragmented embryos may be too small to be biologically competent. However, different fragmentation patterns may affect blastomere sizes to different extent. Localized fragmentation, which appears to be a result of complete fragmentation of at least one blastomere, is suggested to be less detrimental for the developmental potential of the embryo as the remaining blastomeres would be presumably unaffected. In contrast, a scattered pattern of the same amount of fragmentation may affect the sizes of up to all blastomeres (16). Thus, one problem with using cytoplasmic reduction alone is that if the biological relevance of different fragmentation patterns is true, this would not be reflected in the degree of cytoplasmic reduction. Additionally, Van Blerkom *et al.* (17) and Hardarson *et al.* (18) both have demonstrated that fragmentation in some of the early embryos disappeared at later stages by resorption or lysis. However, in other embryos their fragmentation was still apparent during late cleavage (17). These findings stress, in general, that fragments may have different aetiologies and effects on embryo competence. However, for a single observation, correlations between degree of fragmentation with the embryo quality and clinical outcome are well documented at least for days 2 and 3 embryos and light microscopic morphology assessment is an important noninvasive tool when selecting good-quality embryos for transfer (1,3,4,6-10). Additionally, the use of a computer-assisted multilevel embryo morphology analysis may

be superior to conventional light microscopic analysis allowing a more precise and objective measurement of the morphological structures. This may enable implementation of more standardized procedures in future clinical embryo quality assessment. In addition, by using computer-assisted morphology analysis, the time factor normally limiting embryo assessment could be overcome.

In conclusion, our data indicate that the assessment of embryonic fragmentation based on the reduction of the cytoplasmic volume may allow a more precise and standardized evaluation of fragmentation than by traditional fragmentation estimation.

Further, this study indicates that combining non-invasive morphology analysis with computer-assisted morphometric measurements may improve the analysis of morphological structures in the embryo and thus improve the selection of embryos for transfer.

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