There Is a Cutoff Limit in Diameter Between a Blastomere and a Small Anucleate Fragment

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Purpose: To document the DNA content of blastomeres/fragments from early human preembryos and to determine if there is a “cutoff” diameter at which a cell should be considered an anucleate fragment rather than a blastomere.

Methods: Surplus embryos from in vitro fertilization were used. Individual cells were measured, fixated, and stained for DNA.

Results: In day 2 preembryos, only 2% of cells with a diameter $<45\ \mu\text{m}$ contained DNA, compared with 67% of those $\geq 45\ \mu\text{m}$. In day 3 preembryos, 3% of cells $<40\ \mu\text{m}$ contained DNA, compared with 66% of those $\geq 40\ \mu\text{m}$.

Conclusions: It is suggested that cells $<45\ \mu\text{m}$ in day 2 preembryos, and $<40\ \mu\text{m}$ in day 3 preembryos should be classified as fragments, and cells larger than this, as blastomeres. This may influence the embryo scoring system for in vitro fertilization. We therefore recommend that cells within this critical range should be measured when scoring preembryos for embryo transfer.

KEY WORDS: Blastomere; embryo scoring; fragment; multinucleation; uneven cells.

INTRODUCTION

The aim of in vitro fertilization (IVF) treatment is to achieve a normal pregnancy that will result in the birth of a healthy baby. To fulfil this obligation, it is important to learn how to select preembryos with high implantation potential.

During the first mitotic divisions, not all preembryonic cells cleave into two equal daughter cells. On the contrary, cellular fragmentation and/or uneven cleavage are common, and have been correlated with lower pregnancy and implantation rates (1–7). Accordingly, fragmentation is used as a scoring factor in almost all embryo scoring systems currently used in IVF. However, it is often unclear whether a cell should be regarded as a blastomere (which contains a nucleus and has the minimal

requirements to sustain itself) or as an anuclear fragment (which cannot be self-sustaining). The cellular material usually referred to as “fragments” is nonhomogenous, especially with regard to size. The aim of this study was to document the DNA content of cells of various sizes from early human preembryos, and to determine a “cutoff” size at which they should be considered as fragments or as blastomeres.

MATERIALS AND METHODS

The excess preembryos used in this project were donated by patients treated for infertility at the IVF unit, Reproductive Medicine, Sahlgrenska University Hospital. Ovarian stimulation was carried out by a desensitizing protocol, using a short-acting gonadotropin-releasing hormone agonist preparation (Suprecur or Suprefact, Hoechst, Frankfurt, Germany). Downregulation was followed by stimulating with recombinant follicle-stimulating hormone,

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FSH (Gonal-F, Serono, Geneva, Switzerland or Puregon, Organon, Oss, Netherlands). Follicular aspiration was performed 36–38 h after hCG administration using transvaginal ultrasound-guided puncture. The oocytes either underwent intracytoplasmic sperm injection (ICSI), or were inseminated in our regular IVF programme, after which they were cultured in IVF-20 (Vitrolife, Gothenburg, Sweden) for 2, or sometimes, 3 days. On the day of embryo transfer, the preembryos were scored according to the following grading system:

Grade 1: 4–6 cells at day 2, or 6–8 cells at day 3, evenly sized blastomeres without cellular fragments and smooth cytoplasm without vacuoles.

Grade 2: 4–6 cells at day 2, or 6–8 cells at day 3, <20% fragmentation and/or unevenly sized blastomeres and/or slightly granulated cytoplasm.

Grade 3: >20% but <50% fragments and/or blastomeres/cells of all sizes and/or heavily granulated cytoplasm or vacuoles.

Grade 4: >50% fragments.

Only preembryos evaluated as Grade 1 or 2 were transferred or cryopreserved. Embryo transfer was usually performed at 48 h, or sometimes 72 h, after oocyte retrieval. Preembryos of grade 3 are normally discarded, and some of these preembryos were instead included in this study.

Measurement of the Cells

The preembryos were transferred to culture medium containing 0.5% pronase (IVF-20, Vitrolife, Gothenburg, Sweden) for about 1 min to dissolve the zona pellucida. To separate the cells/fragments, they were then incubated for 1–3 min in Mg^{+} / Ca^{2+} -free medium (EB-10, Vitrolife, Gothenburg, Sweden).

The cells were transferred to separate droplets of IVF-20 medium under mineral oil and the diameter of each cell was measured using a millimetre scale in an inverted microscope at 200 $\times$ magnification. Cells/fragments with a minimum diameter of 20 μm were fixated.

Fixation and DNA Staining

The individual cells/fragments were fixated separately in Tween-20 (0.1%) in 0.01N HCl (8). Tween-20/HCl medium was aspirated into a thin, pulled Pasteur pipette. This medium, together with a cell, was transferred onto a poly-L-lysine-coated slide, using an inverted phase contrast microscope. As soon as the oolemma started to break, the slide was left

to dry. To locate the fixated cell, a circle was drawn around the cytoplasm remnants, using a diamond objective. The slide was washed in PBS, followed by milli-q water, then dehydrated in a series of EtOH and dried at room temperature. The DNA was stained with 4'6-diamino-2-phenylindole (DAPI). To identify the presence or absence of DNA, a fluorescence microscope was used.

To examine whether the cells remained the same size after the zona was removed, a control group of five preembryos was included in the study. Each cell was first measured in the inverted microscope at 200 $\times$ magnification before the zona was removed. The zona was thereafter dissolved in pronase and the cells were separated and transferred to droplets of medium, as described previously. The individual cells ($n = 24$) were measured again and their size before and after zona removal was compared. In fact, there was no change in cell size before and after zona removal (mean values $\pm$ SD = 17.2 ± 4.5 and 17.3 ± 4.2 , respectively, $p = 0.57$).

RESULTS

The diameter of the cells/fragments varied from 20 to 75 μm . Because of the decreasing size of cells during cell cleavage and embryo development, preembryos were divided into two groups, those fixated on day 2 (22 preembryos with 175 cells) and those fixated on day 3 (22 preembryos with 183 cells) (Table I). The cells/fragments were further divided into five groups: 1) no DNA, 2) mononucleated, 3) multinucleated, 4) DNA fragments only, and 5) both nucleus/nuclei and DNA fragments. DNA fragments were defined as all DNA-labelled material not constituting an interphase nucleus.

Cells with a diameter <45 μm (day 2) or <40 μm (day 3) never contained a nucleus and only very seldom DNA fragments (Fig. 1). In contrast, 67% of cells $\geq 45 \mu m$ (day 2) and 66% of cells $\geq 40 \mu m$ (day 3) contained DNA in some form, most frequently as a single nucleus. In total, 34% of cells $\geq 45 \mu m$ (day 2) and 38% of cells $\geq 40 \mu m$ (day 3), showed no nuclear abnormalities. Nuclear abnormalities (more than one nucleus or a fragmented nucleus) occurred in 33% of cells $\geq 45 \mu m$ (day 2) and 28% of cells $\geq 40 \mu m$ (day 3).

DISCUSSION

In a day 2 preembryo, at the four-cell stage, the blastomeres should be of equal size, approximately

Table 1. Relationship Between Cell Size in Day 2 and 3 Preembryos and the Presence of Nuclei and/or DNA Fragments

	Day 2		Day 3	
Number of preembryos	22		22	
Number of analysed cells	175		183	
	<45 μm	$\geq 45 \mu\text{m}$	<40 μm	$\geq 40 \mu\text{m}$
Number of fixated cells	$n = 93$	$n = 82$	$n = 74$	$n = 109$
Cells with no DNA (%)	91 (98)	27 (33)	72 (97)	37 (34)
Cells with 1 nucleus (%)	0	28 (34)	0	41 (38)
Cells with >1 nucleus (%)	0	9 (11)	0	6 (5)
Cells with only DNA fragments (%)	2 (2)	7 (9)	2 (3)	14 (13)
Cells with DNA fragments + nucleus/nuclei (%)	0	11 (13)	0	11 (10)

65–70 μm (own unpublished observations). In a day 3 preembryo, the blastomeres are expected to be smaller, around 55–60 μm . The main finding of this study is that there is a “cutoff” size of 45 μm for cells originating from day 2 preembryos and 40 μm for day 3 preembryos. Below these sizes hardly any DNA was found, and if found, was only in the form of DNA fragments. Above this size, most cells (67% for day 2 and 66 % for day 3), contained at least one nucleus, DNA fragments, or both (Fig. 1).

These results may have implications for the scoring systems commonly used in IVF-laboratories. Embryos with unevenly sized blastomeres are reported to have lower implantation rates, which could be related to chromosomal abnormalities (5). It may be that, in some instances, two apparently “unevenly cleaved” blastomeres of different sizes are in reality one blastomere, plus a large anucleate fragment (Fig. 2). This would, in turn, affect how we evaluate, both the degree of fragmentation and the number of

Fig. 1. The distribution of cells according to cell diameter and DNA content for preembryos fixed on day 2 (a) and day 3 (b).

Fig. 2. Depending on the size of the fourth cell, the embryo may be considered as an unevenly cleaved embryo (a) or as a fragmented embryo (b).

cells (i.e., the cleavage rate). Cellular fragments are commonly found in human early preembryos following IVF. Several studies have shown that the presence of fragments, even at high levels, is not always detrimental to the preembryo. However, a relationship between the degree and pattern of the fragmentation seems to exist (2,3,9). The presence of up to 10–20% of small, scattered fragments does not appear to impair further development. Furthermore, time-lapse photography (10) has shown that small fragments can be reabsorbed into newly cleaved blastomeres. Thus, a low degree of fragmentation may be normal, and perhaps even a sign of viability, a hypothesis that is supported by several studies (5,7).

Large fragments, on the other hand, result in the loss of large volumes of cytoplasm, which may be detrimental to the cellular function of the preembryo. The release of large fragments at an early stage can deplete blastomeres of essential organelles, such as mitochondria, mRNA and proteins, and the cell may therefore arrest.

Four basic patterns of fragmentation have been described (9), in which certain patterns result in partial or total loss of critical regulatory proteins from specific blastomeres. If this loss occurs early, i.e., during the 1- or 2-cell stages, the preembryo may be severely compromised. On the other hand, if fragmentation occurs in cell regions that do not contain these protein domains, or if the loss of cytoplasm occurs at later cleavage stages, the preembryo seems to be able to continue development. These observations may explain why some fragmented preembryos may develop normally and implant, while others do not survive. The process of fragmentation might not have a common origin, but may reflect various underlying disorders such as chromosomal abnormalities, levels of ATP generation, instability within the cortical microfilament network, or apoptosis/necrosis (9). Another mechanism that has been discussed (11,12) is

oncosis, the formation of plasma membrane blebs by certain cell types when deprived of oxygen. It has been speculated that this may be due to disproportionate distribution of mitochondria within the blastomeres during early embryo cleavage, where regions of cortical cytoplasm may be permanently or temporarily devoid of mitochondria (12).

The appearance of two or more nuclei in a cell, or a fragmented nucleus (18% of all blastomeres analysed in this study) may be caused by the dissociation of karyokinesis from cytokinesis, partial fragmentation of the nucleus, or defective migration of chromosomes at mitotic anaphase. It is known to correlate with chromosomal abnormalities in the embryo (5,13,14). However, it was shown by Staessen *et al.* (1998) that among embryos in which both blastomeres were bi- or multinuclear at the 2-cell stage, 30% had only mononuclear blastomeres after the following cleavage (14). Thus, some multinucleate cells still seem to be able to cleave normally. In addition, fragmented nuclei might also indicate apoptosis, possibly suggesting origin from atretic follicles (15). In our study, approximately 98% of cells <40 μm on day 2 and 97% of those <45 μm on day 3 contained no DNA. These cells, unless reabsorbed into a blastomere, will presumably not be able to contribute to further development of the preembryo, and thus represent a loss of cytoplasm.

It is important to note that the preembryos available for inclusion in this study originated from couples with fertility problems. Furthermore, they were donated because of their suboptimal quality. The preembryos might therefore be expected to show more nuclear and other abnormalities than those chosen for transfer. It should be noted that the stated cut-off values do not mean that all cells above that value are normal. Indeed, the high frequency of multinucleation and DNA fragments/lack of nucleus indicate that many larger cells will be unable to contribute to the further development of the embryo.

In conclusion, our results indicate that cells with a diameter $\geq 45 \mu\text{m}$ in day 2 and $\geq 40 \mu\text{m}$ in day 3 preembryos should be classified as blastomeres. Cells smaller than this are always anucleate and should thus be considered as fragments. These findings may influence scoring criteria; some preembryos previously scored as preembryos with uneven-sized blastomeres might now be scored as partially fragmented embryos (Fig. 2). Given that preembryos with uneven-sized blastomeres have a higher aneuploidy rate and a lower implantation rate than preembryos with even-sized cells, regardless of the degree of fragmentation (5), cell size is an important factor in the preembryo scoring system.

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