Human Reproduction Vol.16, No.2 pp. 313–318, 2001

Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation

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Uneven blastomere cleavage in human embryos of 'good morphology', i.e. those normally used for transfer, is a phenomenon which has been poorly investigated. The main objective in this study was to probe deeper into the aetiology behind previous findings that embryos with uneven cell cleavage have a lower developmental capacity in comparison with evenly cleaved embryos. Our hypothesis was that uneven cleavage may result in embryos with a higher degree of aneuploidy and/or multinuclear rate, which in turn might help to explain their low implantation rate. In the first part of the study, 378 embryo transfers performed over a 3-year period were analysed retrospectively, where all the transferred embryos in each cycle were of identical morphology score and cleavage stage. In the second part of the study, multicolour fluorescence in-situ hybridization (FISH) analyses on good quality embryos, representing the uneven (n = 11) and even (n = 13) study groups were performed. When comparing day 2 transfers between 4-cell embryos, it was found that unevenly cleaved embryos had significantly lower implantation (23.9 and 36.4%) and pregnancy rates (37.6 and 52.9%) compared with evenly cleaved embryos. A significantly higher degree of aneuploidy (29.4 and 8.5%) and multinuclear rate (21.1 and 2.1%) in blastomeres from uneven embryos was also found. It is concluded that uneven blastomere cleavage has a negative effect on both pregnancy and implantation rates in human IVF, and that this can partly be explained by a higher degree of aneuploidy/multinuclear rate. In the light of the results obtained, a new approach in the current embryo scoring system, placing more emphasis on blastomere size, is recommended.

Key words: aneuploidy/embryo/fluorescence in-situ hybridization/FISH/IVF

Introduction

In IVF, the selection of pre-embryos for uterine transfer is still based predominantly on morphological appearance according to various scoring systems. These scoring systems have in common that the main parameters judged are blastomere uniformity, fragmentation and cytoplasmic appearance. Of these three morphological parameters, the uniformity of blastomere size has been thought to be of less importance than the others. Uneven embryo cleavage is frequently observed in transferred human embryos, and these embryos may or may not contain fragments. The possibility that uneven distribution of cellular material and/or genetic material could have a detrimental effect on the survival of human embryos has been poorly investigated, especially in morphologically 'good quality' embryos, i.e. those embryos normally used for uterine transfer or freezing.

The study of a single parameter is very valuable when trying to understand better which parameters are most important in an embryo scoring system that includes multiple factors (Steer *et al*., 1992; Scott and Smith, 1998). Based on previous observations (Hardarson *et al*., 1998) indicating that uneven embryo cleavage negatively affected pregnancy rates, the objective of the present study was to compile results from a database in which information regarding embryo morphology and cleavage rates has been collected for many years. Comparison was then made between transfers where only embryos with evenly cleaved blastomeres were replaced, and those where only embryos with unevenly cleaved blastomeres were replaced. Furthermore, in a small group of good quality embryos that were donated by IVF patients, it was investigated whether a difference in the degree of multinuclear content and/or aneuploidy could be found between these groups, as both of these genetic aberrations have been reported to reduce embryo development (Plachot *et al*., 1988; Hardy *et al*., 1993). It was postulated that uneven cellular cleavage may result in an uneven

distribution of genetic material, and thereby negatively affect both pregnancy and implantation rates in human IVF.

Materials and methods

IVF procedure

The clinical data used in this study were collected from IVF/intracytoplasmic sperm injection (ICSI) cycles from 1996 to 1999. Ovarian stimulation was carried out using a desensitizing protocol with a short-acting gonadotrophin releasing hormone agonist preparation in combination with recombinant FSH. Follicular aspiration was performed 36–38 h after human chorionic gonadotrophin (HCG) administration, using vaginal ultrasonography and follicle puncture. The oocytes either underwent ICSI or were inseminated in our regular IVF programme, after which they were cultured in IVF-50 medium (Scandinavian IVF Science, Gothenburg, Sweden) for 2 days. On the day of embryo transfer, the embryos were scored according to a grading system that was modified from a previously published scheme (Steer *et al*., 1992). The new scheme comprised four main grades, with grade I considered to be the 'optimal' embryo, with no fragments, even-sized blastomeres and light, homogeneous cytoplasm. Grade II is subdivided into three categories: <20% fragments (grade IIA); uneven-sized blastomeres (grade IIB); and non-homogeneous cytoplasm (grade IIC). All possible mixtures (e.g. grade IIAB) were also registered. An embryo was considered as having uneven blastomeres if there was at least a 20% difference in diameter between the smallest and the largest blastomere. Finally, remaining embryos fell into the third and fourth main groups, mainly due to too high cytoplasmic fragmentation (grade III >20%, grade IV >50%). As the objective of this study was to examine the differences between even and uneven cleavage, the evenly cleaved embryos (4:I and 4:IIA), were pooled into one group, 'Even', and the unevenly cleaved embryos (4:IIB and 4:IIAB), into another, 'Uneven' (Figure 1). In the present study, only embryo transfer data from women who received one or two 4-cell embryos of the same scoring grade on day 2 after oocyte retrieval were included. Each patient was included only once in the study. In addition, a novel indicator of early embryo cleavage rate was documented, measured by noting if, at 24–26 h after insemination, the embryos were at the 1- or 2-cell stage. In this study, pregnancy was defined as a positive HCG test on day 19 post embryo transfer, and implantation rate as the number of fetal sacs per number of transferred embryos.

Fixation and fluorescence in-situ hybridization (FISH) analysis

Embryos were obtained after receiving informed consent from patients undergoing IVF between 1998 and 1999 at the Sahlgrenska University Hospital, Gothenburg, Sweden. These were good quality embryos that could not be frozen, mainly due to hepatitis, and were donated. This material is very valuable, hence the low number of embryos included in the FISH part of this study. In order to assess the ploidy of each blastomere, the embryos were stripped of their zona pellucida by brief exposure to 0.5% pronase (1–2 min). After 5 min incubation in a Ca^{2+}/Mg^{2+}-free medium (EB-10; Scandinavian IVF Science), the blastomeres were separated by gently pipetting the embryos up and down a thin glass pipette. The diameter of each blastomere was measured, and they were then fixed individually on poly-L-lysine-coated glass slides using 0.01 mol/l HCl + 0.1% Tween 20 (Coonen *et al*., 1994). Care was taken to remove as much of the cytoplasm as possible from the nuclei in order to improve FISH probe penetration. The glass slides were dried and stored at –20°C until the FISH analysis was performed. For the FISH analyses, the slides were washed for 5 min in phosphate-buffered saline (PBS) followed by an ethanol series of 70, 85 and 99.5% for 1 min in each bath. For simultaneous detection of five chromosomes, a multicolour kit was used (PGT®; Vysis Inc., Illinois, USA) which includes chromosomes 13 (spectrum red®), 18 (spectrum aqua®), 21 (spectrum green®), Y (spectrum gold®) and X (spectrum blue®). As the default protocol from the manufacturer did not produce sufficiently reliable results, it was necessary to develop our own protocol. A small amount of the PGT probe was pipetted onto the nucleus, a cover-slip applied and glued, and the preparation denatured at 78 ± 1°C for 5 min on a heating plate. The slides were incubated at 37 ± 1°C for 4 h, after which the cover-slips were removed and the slides washed at 42 ± 1°C in 50% formamide + 2× SSC (15 min) followed by 2× SSC (10 min) and 2× SSC + Igepeal (5 min) to remove unspecific nuclear staining. The slides were then air-dried, an antifade solution was applied, and the preparation sealed with a cover-slip. The nuclei were observed with a Nikon epifluorescence microscope equipped with appropriate filters, and the number of chromosomes was counted. Images were transferred via a video camera using a data imaging program (Applied Imaging, Scotswood Road, Newcastle upon Tyne, UK) and stored in a computer. For the purpose of clarity, the colour of the X chromosome was changed to white.

For statistical analyses, the χ^2 and Student's *t*-tests were used. A P value < 0.05 was considered to be significant. The volume of each blastomere was calculated using the average radius (r), according to the following formula: $4/3\ r^3\pi$.

Results

The database search included results from all IVF/ICSI cycles performed during the 4-year period 1996–1999. Among 17 981 normally fertilized oocytes (2PN), 40% were at the 4-cell stage on day 2. Of all embryos transferred on day 2 in our programme, 75% were at the 4-cell stage. Of these transferred 4-cell embryos, 27% were grade I embryos, 40% grade IIA, 14% grade IIB, and 15% grade IIAB, while the remainder were different combinations of 4:IIA, B and C.

The clinical results in this study were based on 378 embryo transfers. When comparing the pregnancy and implantation results from grades I, IIA, IIB and IIAB, it was found that there was no significant difference within the even groups (I + IIA) or the uneven (IIB + IIAB) groups. No significant difference was seen in maternal age or number of aspirated oocytes between the groups.

It was found that the unevenly cleaved group of embryos had statistically significant lower pregnancy ($P = 0.013$) and implantation rates ($P = 0.003$) than the evenly cleaved group (Table I).

There was also a significant difference between the two study groups with regard to the early cleaving rate, i.e. the percentage of embryos that had undergone the first mitotic cleavage at 24–26 h post insemination. In the even group this was 68.8%, compared with only 37.5% in the uneven group ($P = 0.001$) (Table I).

For the multicolour FISH analyses, a total of 24 unevenly and evenly cleaved human embryos was fixed. It was observed that the unevenly cleaved embryos had a higher aneuploidy rate when compared on the blastomere level, but not per embryo. In contrast, the multinuclear rate was significantly higher, both per blastomere and per embryo (Table II). Furthermore, the rate of anucleate blastomeres in the uneven group

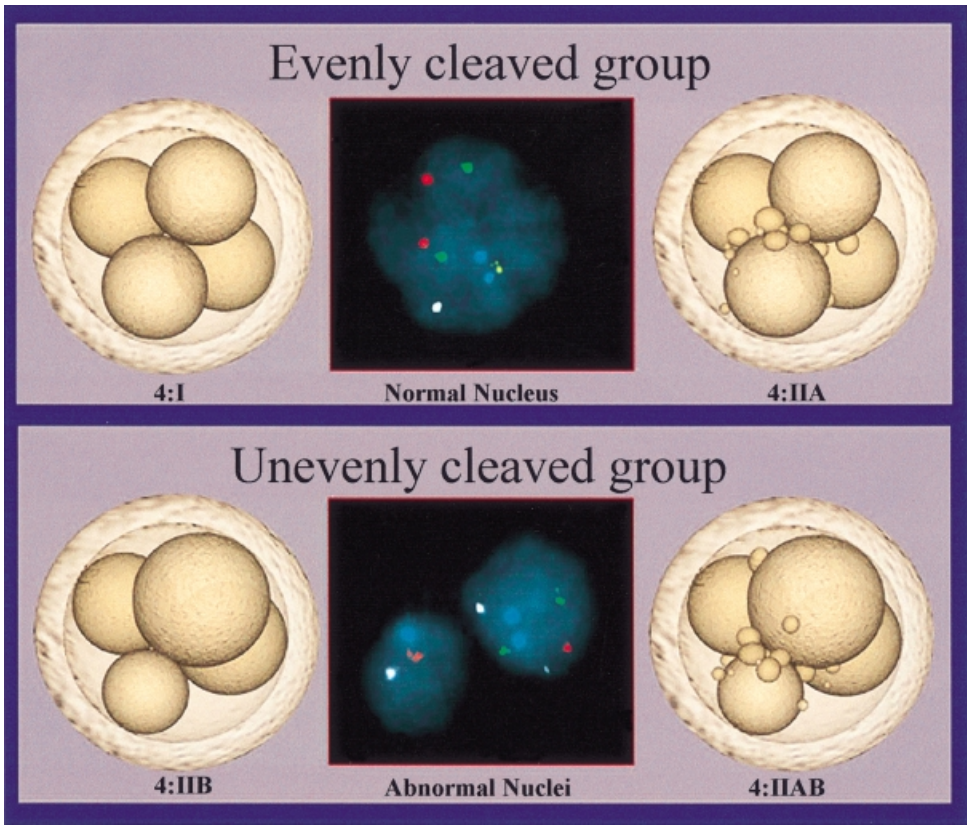

Figure 1. Schematic illustration of the embryo grades and their groupings along with representative fluorescence in-situ hybridization (FISH)-stained nuclei. Normal nucleus fixed from a single even blastomere (top), abnormal nuclei fixed from a single uneven blastomere (bottom). The nuclei were stained with probes specific for chromosomes 13 (red), 18 (aqua), 21 (green), X (white) and Y (gold). (Illustration by G. Coull.)

Table I. Implantation and pregnancy rates in the two embryo groups studied

Parameter	Embryo group		
	Even cleavage	Uneven cleavage	*P*
No. of embryo transfers	293	85	–
No. of embryos/transfer	1.98 ± 0.43	1.92 ± 0.47	NS
Maternal age (years)	32.6 ± 4.1	32.9 ± 4.3	NS
Implantation rate (%)	211/579 (36.4)	39/163 (23.9)	0.003
Pregnancies/embryo transfer (%)	155/293 (52.9)	32/85 (37.6)	0.013
Early cleavage (%)	96/140 (68.6)	12/32 (37.5)	0.001

[a]Values are mean ± SD.
NS = not significant.

Table II. FISH results from the two embryo groups

Parameter	Embryo group		
	Even cleavage	Uneven cleavage	*P*
Embryo aneuploidy[a] (%)	4/13 (30.8)	6/11 (54.5)	0.24
Blastomere aneuploidy (%)	4/47 (8.5)	10/34 (29.4)	0.014
Embryo multinuclear rate (%)	1/13 (2.1)	5/11 (45.5)	0.005
Blastomere multinuclear rate (%)	1/47 (2.1)	8/38 (21.1)	0.033

[a]An embryo was considered aneuploid/multinuclear if one or more cells were affected.

(11%) was found to be higher when compared with the even group (2%) (Table III), though this difference did not reach statistical significance ($P = 0.057$). The percentage of nuclei not analysed by FISH as a result of failure during fixation and/or the FISH procedure was similar in the even (9%) and uneven groups (11%). It was also noted that all the multinuclear blastomeres but one had nuclei with an abnormal chromosomal number (Table III).

Discussion

Today, a majority of embryo transfers are still performed at the cleavage stage, i.e. at day 2 or 3 after insemination/microinjection. The scoring system used at day 2–3 consists mainly of cell number, degree of fragmentation and blastomere size. Although until now the number of cells and grade of fragmentation have been considered to be the most important scoring factors, in the present study it was found that uneven embryo cleavage negatively affects both implantation and pregnancy rates, while slight fragmentation does not. Furthermore, it was found—albeit in a limited number of samples—that unevenly cleaved embryos have a higher rate of aneuploidy and multinuclear rate than do embryos with evenly sized blastomeres.

The clinical results in the present investigation are partly supported by those of a previous study in which a significant difference was found when comparing embryos displaying

Table III. FISH demographics

			Chromosome					
			X	Y	13	18	21	Blastomere classification
Even cleavage group								
Embryo 1:	cell 1–3		2	0	1	2	2	Normal[a], XX
	cell 4		2	0	1	2	2	Monosomy 13
Embryo 2:	cell 1 and 2		1	1	2	2	2	Normal, XY
	cell 3		–	–	–	–	–	FISH failure
	cell 4	(nucleus a)	1	1	1	2	2	Trisomy 21[b]
		(nucleus b)	0	0	2	0	1	
Embryo 3:	cell 1–3		1	1	2	2	2	Normal, XY
	cell 4		1	1	2	1	2	Monosomy 18
Embryo 4:	cell 1		2	0	1	2	2	Monosomy 13
	cell 2–4		2	0	2	2	2	Normal, XX
Embryos 5–10:			1	1	2	2	2	Normal, XY
Embryos 11–13:			2	0	2	2	2	Normal, XX
Uneven cleavage group								
Embryo 1:	cell 1		–	–	–	–	–	Anuclear
	cell 2	(nucleus a)	1	0	1	2	2	Tetrasomy, 18, 21[b]
		(nucleus b)	1	0	1	2	2	
	cell 3	(nucleus a)	2	0	2	1	1	Trisomy X, 13, 21[b]
		(nucleus b)	0	0	0	1	1	
		(nucleus c)	1	0	1	0	1	
	cell 4	(nucleus a)	1	0	1	1	0	Trisomy 13[b]
		(nucleus b)	1	0	2	1	2	
Embryo 2:	cell 1		–	–	–	–	–	Anuclear
	cell 2	(nucleus a)	0	2	1	1	1	YYY[b]
		(nucleus b)	0	1	1	1	1	
	cell 3		–	–	–	–	–	Anuclear
	cell 4		–	–	–	–	–	FISH failure
Embryo 3:	cell 1 and 2		1	1	2	2	2	Normal, XY
	cell 3	(nucleus a)	1	1	2	2	2	Tetraploid[b]
		(nucleus b)	1	1	2	2	2	
	cell 4		–	–	–	–	–	Anuclear
Embryo 4:	cell 1		–	–	–	–	–	Anuclear
	cell 2	(nucleus a)	2	0	2	2	2	Tetraploid[b]
		(nucleus b)	0	0	1	1	0	
		(nucleus c)	1	0	1	0	1	
		(nucleus d)	1	0	0	1	1	
	cell 3		–	–	–	–	–	FISH failure
	cell 4		2	0	2	2	2	Normal, XX
Embryo 5:	cell 1 and 2		2	0	2	2	2	Normal, XX
	cell 3		1	0	2	4	2	Monosomy X, Tetrasomy 18
	cell 4		–	–	–	–	–	Blastomere lost
Embryo 6:	cell 1 and 2		2	0	2	2	2	Normal, XX
	cell 3	(nucleus a)	1	0	2	0	0	XXY[b]
		(nucleus b)	1	1	0	2	2	
	cell 4		0	0	2	2	2	Nullisomy XY
Embryos 7–10			2	0	2	2	2	Normal, XX
Embryo 11			1	1	2	2	2	Normal, XY

[a]As far as can be determined by the probes used in the present study.
[b]More than one nucleus present (multinuclear).
FISH failure = nucleus observed during and/or after fixation, but not found after the FISH procedure;
Anuclear = nucleus neither observed during nor after fixation, and not found after the FISH procedure.

inconsistencies in size and/or abnormalities in shape (Giorgetti *et al*., 1995). The latter authors, however, did not distinguish between irregularity in shape and size. Also, in line with our results, it has been shown previously (Ziebe *et al*., 1997) that uneven embryo cleavage results in a lower implantation rate. However, in that study embryos at different cleavage stages were pooled (2 to >4 cells), while only embryos at the 4-cell stage were included in the present study. Four-cell embryos are expected to have equally sized blastomeres, whereas 3- or 5-cell embryos might well be expected to have one or two larger or smaller cells.

It is interesting to note that slight fragmentation (<20%) does not seem to affect human embryonic development. Using time-lapse photography (own unpublished data), it has been shown that fragments can be reabsorbed into newly cleaved blastomeres, indicating that a slight fragmentation may indeed be normal. The degree of fragmentation observed may thus vary with the time of embryo scoring in the IVF laboratory.

A significant difference was found between the two embryo groups with regard to early embryo cleavage (Table I), this being another indicator of embryo quality and developmental capacity (Shoukir *et al*., 1997; Lundin and Söderlund, 1999). A connection between delayed cell cycle and aneuploidy has been documented in human somatic cells where both monosomic (Nielsen, 1976; Nielsen and Krag-Olsen, 1980) and trisomic (Paton *et al*., 1974) cells have been shown to cleave at a slower rate than their euploid 'counterparts'. This indicates that these embryos have a slightly slower cell cycle already at the zygote stage, possibly due to aneuploidy/multinuclear rate.

It was found that blastomeres originating from embryos with uneven cleavage are more affected by numerical chromosomal aberrations and multinuclear rate than embryos with evenly cleaved blastomeres (Tables II and III). This pattern was not obvious when the overall degree of embryo aneuploidy was compared between the groups, but on examining aneuploidy at a blastomere level it was found not only that there was a higher number of blastomeres affected in each uneven embryo but also that the severity of the aneuploidy and multinuclear rate was higher. Both aneuploidy and multinuclear rate have been shown to have a negative effect on the developmental capacity of the human embryo (Plachot *et al*., 1988; Jackson *et al*., 1998; Pelinck *et al*., 1998). Bi- or abnormal nuclei contribute to cleavage arrest, and these cells are expected to be developmentally incompetent (Hardy *et al*., 1993), with only 30% of them cleaving (Pickering *et al*., 1995). Possible mechanisms of multinucleation include karyokinesis in the absence of cytokinesis (Hardy *et al*., 1993), partial fragmentation of nuclei, or defective migration of chromosomes at the mitotic anaphase (Tesarik *et al*., 1987; Winston *et al*., 1993), and these might be induced either by changes in temperature or by suboptimal culture conditions (Winston *et al*., 1991). It has been suggested (Munné *et al*., 1995) that cytoplasmic impairment may produce both mosaicism and polyploidy, through cytoskeletal and spindle malfunction, cellular division block or other mechanisms. It has also been shown that impairment of the spermatozoa, where abnormal zygote centrioles were generated, could produce chromosome abnormalities (Palermo *et al*., 1994). Furthermore, aneuploidy has been shown by many investigators to be detrimental to embryo development (Angell *et al*., 1986; Bongso *et al*., 1991; Munné *et al*., 1994, 1995; Pellestor *et al*., 1994). It is therefore suggested that at least part of the explanation as to why uneven embryos implant at a lower rate than evenly cleaved ones lies in the severity of the aneuploidy of the embryo. That is, when aneuploidy is found in an uneven embryo it is more severe, and thus the embryo might be less likely to continue with normal development. Favouring the evenly cleaved embryos when performing the selection for embryo transfer might therefore reduce the possibility of replacing a chromosomally abnormal embryo.

In this study, a total aneuploidy rate of 41.7% (10/24) was found, which supports previous observations that human embryos have a relatively high degree of aneuploidy (Harper *et al*., 1995; Delhanty *et al*., 1997; Munné *et al*., 1998; Gianaroli *et al*., 1999; Iwarsson *et al*., 1999). The fact that such a high percentage (30.8%) of morphologically 'good looking' embryos have only one aneuploid blastomere, while the remainder are normal diploids (as far as can be determined by the probes used in the present study), is important to keep in mind when applying methods that try to ascertain the normality of an embryo by single blastomere biopsy, for example in preimplantation genetic diagnosis (Bahce *et al*., 1999). A single aneuploid blastomere in a 4-cell embryo (Table III) could be the result of blastomere fragmentation. If a fragment that is not reabsorbed contains a chromosome, it will be lost when the fragment degenerates, or it may be that a chromosome-containing fragment fuses with another blastomere, leading to trisomy in that cell.

It can be speculated that when a blastomere divides unequally, the two resulting sister cells will receive unequal amounts of, for example, proteins, mRNA, mitochondria and/or different cell organelles. In addition, recent studies suggest that certain proteins and gene products are not evenly distributed, but polarized in the oocyte/embryo (Antczak and Van Blerkom, 1997, 1999; Edwards and Beard, 1997), possibly amplifying the effect of such an uneven cleavage. Despite the difficulty in determining whether or not uneven distribution has a negative effect on either the bigger and/or the smaller cell, it seems plausible that a blastomere needs a certain amount of cytoplasmic constituents to sustain itself.

In conclusion, it has been found that when scoring human embryos for embryo transfer, blastomere size is of more importance than slight fragmentation, and that aneuploidy and multinuclear rate may partially explain why unevenly cleaved embryos have lower implantation and pregnancy rates than embryos cleaving evenly. It is suggested therefore that current scoring systems should be modified, perhaps by a reduction to four scoring categories: grade I, embryos with evenly sized blastomeres, with or without fragments ($<$20%); grade II, embryos with unevenly sized blastomeres with or without fragments ($<$20%); grade III, embryos with 20–50% fragmentation and/or non-homogeneous cytoplasm and/or multinucleate blastomeres; and grade IV, embryos with $>$50% fragmentation.

Acknowledgements

The authors would like to thank Graham Coull, both for valuable comments and for his excellent illustration. We would also like to thank the patients and the laboratory staff at the IVF laboratory, Sahlgrenska University Hospital for participating and/or assisting with this study. This study was supported by the European Commission (Marie Curie research training grant to T.Hardarson), The Hjalmar Svensson Foundation and the Swedish Medical Research Council (11606).

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Received on July 10, 2000; accepted on October 9, 2000