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Treatment-related chromosome abnormalities in human embryos

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Mosaicism was studied in good quality embryos from four different centres in order to assess the effects of follicular induction and exposure to laboratory conditions on chromosomal status. The donated embryos were fully biopsied and analysed by fluorescence in-situ hybridization using probes for chromosomes X, Y, 13, 18 and 21, simultaneously. The number of abnormal cells present indicated the division at which mosaicism first occurred (4/4 cells at first division, 2/4 cells at second, 2/8 at third). The rate of mosaicism in embryos from different centres varied greatly ($P < 0.001$). Most of the mosaic embryos were obtained before 1991. In one clinic increased mosaicism was found in embryos obtained before 1991 when compared to embryos obtained thereafter. The results suggest that certain culture conditions and/or hormonal stimulation protocols may induce chromosomal abnormalities and partly explain differences in pregnancy rates between in-vitro fertilization centres.

Key words: chromosome abnormalities/embryo/in-vitro fertilization

Introduction

Wide disparities in degree of chromosome abnormality have been reported in cleaved human embryos. This may be explained by differences between populations, but could also be the result of a bias caused by the limited use of classical karyotyping in single cells. Another issue is related to the definitions of embryonic normality and viability. For instance, it is well known that aneuploidy increases with maternal age in clinically recognized pregnancies and cleavage-stage embryos (Hassold and Chiu, 1985; Warburton *et al.* 1986; Munné *et al.*, 1995), while polyploidy and mosaicism are more common in arrested and morphologically abnormal embryos (Bongso *et al.*, 1991; Zenzes and Casper, 1992; Munné *et al.*, 1994a; Pellestor *et al.*, 1994; Harper *et al.*, 1995). However, little is known about the effect of in-vitro fertilization (IVF) techniques on chromosome abnormalities. Although studies in the mouse do not suggest a detrimental effect (Santaló *et al.*, 1986), differences between IVF centres regarding hormonal stimulation, gamete collection and embryo culture are profound. It is known that these parameters may have an impact on oocyte and embryo morphology as well as developmental rate. It can be postulated that there may be a relationship between early chromosomal disorders and specific reproductive technologies.

Several factors affecting gametes and embryos have been found to be related to an increase in chromosome abnormalities. Some examples are: (i) changes in temperature during oocyte culture and handling (Pickering *et al.*, 1990; Almeida and Bolton, 1995); (ii) ageing of gametes (Badenas *et al.*, 1989; Munné and Estop, 1993); (iii) use of a 20% oxygen tension instead of 5% (Pabon *et al.*, 1989; McKiernan and Bavister, 1990; Dumoulin *et al.*, 1995); (iv) hormonal stimulation in some mouse strains (Maudlin and Fraser, 1977; Hansmann and El-Nahass, 1979); and (v) sub-optimal stimulation in humans (Sundstrom and Nilsson, 1988; Hammit *et al.*, 1993). Also, oocyte morphological abnormalities, which are correlated with chromosomally abnormal embryos, have been found to be more common in stimulated than non-stimulated women (Van Blerkom and Henry, 1992).

Other studies have found no relationship between chromosomal abnormalities and stimulation regimes (Plachot *et al.*, 1988; Tejada *et al.*, 1991). However, these were performed before the use of down-regulation. Factors that may cause spindle errors, but which have not yet been studied at the chromosome level, are exposure or sensitivity to specific fertility drugs, exposure to volatile organic compounds, sub-optimal pH or heavy metal ions and visible light exposure.

The purpose of this study was to determine whether embryos from different IVF centres, of different chronologies or subjected to different procedures, have different rates of chromosomal abnormalities. Human embryos derived from bi-pronucleated zygotes which were morphologically and developmentally normal were analysed by fluorescence in-situ hybridization (FISH). Other grades of embryos were not included because they are routinely excluded from IVF replacement.

In the present investigation, only chromosome abnormalities that could be generated after zygote formation (such as mosaicism) were considered, in order to determine the effects of intra- and extra-follicular conditions, as well as subsequent in-vitro conditions, upon chromosome composition.

Table I. Average centre characteristics at the time of embryo freezing

	A1	A2	B	C	D
Hormonal stimulation (no. embryos):					
Clomiphene citrate:					
clomiphene–pergonal	0	2	8	2	0
clomiphene–metrodin–pergonal	0	0	7	0	0
Gonadotrophins:					
metrodin (FSH)	5	0	0	0	0
metrodin–pergonal (FSH/HMG)	4	0	0	0	0
Down-regulation:					
lupron–pergonal	0	0	0	16	0
lupron–metrodin–pergonal	0	3	6	0	69
buserelin–metrodin–pergonal	5	1	0	0	0
decapeptyl (3,5 R)–metrodin–pergonal	0	10	0	0	0
Culture conditions*					
Ratio of retrievals/incubator per annum	104	167	91.7	256.0	198.6
Continuous temperature control on microscope stage?	no	yes	yes	yes	yes
Embryo cultured under oil (yes/no, or fraction yes)?	no	yes	yes	no	yes
Culture media	T6	T6	Earle's	HTF	HTF
Pre-heated medium wash used for egg collection?	yes	yes	yes	yes	yes
Follicular fluid kept in heated stage during collection?	no	yes	yes	yes	yes
Embryos produced, 1986–90	14**	0	21	7	0
Embryos produced, 1991–95	0	16	0	11	69***
No. of patients used	9	11	10	9	46
Other factors					
Average maternal age	31.8	35.3	32.5	35.7	34.5
Centre results with fresh embryos					
Cycles/year	272	411	307	NA	1182
Retrievals/year	209	335	233	622	993
Transfers/year	176	311	185	512	866
% success rate (delivery/retrieval)	16.7	19.5	16.9	14.6	30.2

*When embryos were frozen in different years from the same centre, each embryo was characterized according to the year of freezing.
**Includes seven embryos rejected for transfer after PGD.
***Includes 47 embryos with asynchronous nuclear development that could not be frozen, so were fixed on day 4 of development.

Materials and methods

Embryo and centre characteristics

Embryos were obtained from four centres. The characteristics of each centre regarding method of embryo culture, hormonal stimulation and IVF results are shown in Table I. Embryos were recruited by each centre at random, except for centre A, where the embryos were selected *a priori* to have two subgroups of embryos: series 1, which were embryos produced before 1991, and series 2, which were embryos produced in 1991 or later. This division was chosen because after that year, down-regulation became the method of choice for hormonal stimulation in the USA and Europe. All embryos used in the present study were obtained from patients following written consent. The embryos were studied at Cornell University Medical College according to approved guidelines from the ethical and research review board. There were three embryo sources: (i) those frozen at the cleaved embryo stage; (ii) fresh embryos that could not be frozen due to nuclear asynchrony and (iii) embryos rejected after pre-implantation genetic diagnosis. The first group of embryos were fixed immediately after thawing to preclude post-thaw effects, and the second group of embryos on day 4 of development. Only morphologically and developmentally normal embryos were used for this study. All embryos had developed from bipronucleated zygotes, and were at the four-cell stage on day 2 or at the six- to eight-cell stage on day 3, had less than 15% fragmentation, were not multinucleated, and did not show other morphological abnormalities.

Thawing, biopsy, fixation, and FISH analysis

Embryos were thawed using standard procedures and biopsied immediately. All the cells of each embryo were biopsied and fixed individually as described previously (Cohen *et al.*1992; Munné *et al.*, 1994a).

FISH analysis of all the embryos was performed in only one laboratory. The technician performing the analyses had no prior knowledge of each sample embryo's characteristics. After fixation, the slides were analysed with FISH using probes for X, Y, 18, 13 and 21 chromosomes, simultaneously (Munné *et al.*, 1993). The scoring criteria followed in this analysis were also defined previously (Munné *et al.*, 1994a).

Chromosome abnormalities produced during embryo culture

Aneuploidy, which occurs before syngamy, and haploidy and polyploidy, which occur during syngamy due to abnormal fertilization, were not considered to be affected by embryo culture and, although scored, they were not taken into consideration. Only mosaicism was considered.

The cell division that caused chromosome mosaicism was determined by assessing the number of blastomeres of each cell kind (Munné *et al.*, 1994b). This could only be accomplished when a majority of embryonic cells were analysed. Mosaicism had arisen at the first, second or third division, when respectively all, half or quarter of the cells were abnormal. Since most embryos were frozen on day 2 of development, only those abnormalities considered to have arisen during the first embryonic division (100% abnormal cells) or during the second division (50% abnormal cells) were included in the analysis. Because the percentages of abnormal cells were not always precise, we used the following ranges: 75–100% abnormal cells for the first division, and 33–70% for the second division. Embryos frozen on day 3 or 4 of development were scored in the

Table II. Mosaicism according to cellular division and hormonal stimulation

	A1	A2	B	C	D	Total (%)
Mosaicism and hormonal stimulation:						
Clomiphene citrate	0	0/2	9/15	1/2	0	10/19 (53)[a]
Gonadotrophins	4/9	0	0	0	0	4/9 (44)
Down-regulation	5/5	3/14	2/6	1/16	9/69	20/110 (18)[b]
Mosaicism by cellular division:						
1st division mosaic	8/14[h]	1/16	4/21[i]	0/18	2/69	
2nd division mosaic	1/14	2/16	7/21	2/18	7/69	
Total	9/14[c]	3/16[d]	11/21[e]	2/18[f]	9/69[g]	

Significance: a versus b: $P < 0.025$; c versus g, e versus f: $P < 0.001$; c versus d, c versus f: $P < 0.01$; (c + d) versus g, and h versus i: $P < 0.05$.

same way as day 23 embryos. However, mosaicism occurring on day 3 or 4 was disregarded, both to allow better comparison between embryos and because it has been previously considered less detrimental for embryo survival (Munné *et al*., 1994b).

Statistical analysis

For each patient in the study, the numbers of embryos with and without mosaicism were noted. The association between the incidence of mosaicism and factors such as 'Fertility Centre', and type of 'Hormonal Stimulation', was investigated by carrying out analysis on the relevant proportions of mosaics using Generalized Linear Modelling (GLM) methods and employing the algorithm GENSTAT (1988). This analysis is conceptually similar to conventional analysis of variance, but with accommodation for the difficult response variable; proportions based on very low numbers so that 0% and 100% are frequent occurrences. The analysis attempts to disentangle the effects of the factors in the study in what is, inevitably, a non-orthogonal arrangement. The linear model with a function of the proportion as the dependent variable, and with factors such as centre etc., as explanatory variables, was fitted by the method of maximum likelihood. The association between cellular division (1st, 2nd) and mosaicism was investigated using the same methods.

Results

FISH efficiency

A total of 904 blastomeres from 138 monospermic embryos were biopsied. The analysis failure (19.0%) can be assessed as the number of nucleated blastomeres lost during thawing or biopsy (11.9%), plus the number of nucleated blastomeres not analysable (2.0 %) or with false results after FISH (5.1%).

Chromosome abnormalities

Of the 138 embryos analysed, 34 were found to be mosaics (Table II). According to our criteria, 15 of the mosaic embryos arose at the first embryonic division, and 19 at the second. Regarding the type of mosaicism, most of the embryos were chaotic mosaics ($n = 19$), followed by mitotic non-disjunction ($n = 9$) and mixoploid mosaics ($n = 7$). In most mosaics the abnormality affected all chromosomes probed ($n = 24$), while in the rest, it affected only chromosomes 13/21 ($n = 6$), chromosome 18 ($n = 2$) or chromosome X ($n = 1$) alone.

The differences in mosaicism ratios between centres were statistically significant ($P < 0.001$), ranging from 11% (centre C) to 52% (centre B). Total mosaicism rate, and first and second division mosaicism rates for each centre are shown in Table II. Centres with some or all their embryos obtained before 1991 (centres A, series 1 and B) had higher levels of mosaicism. In addition, the embryos from centre A produced between 1986 and 1990 (series 1) showed a significantly higher mosaicism rate than the embryos produced between 1991 and 1995 (64 versus 19%, $P < 0.001$). No other intra-centre comparisons could be done because the embryos analysed belonged mostly to a single time period.

There was also evidence of inter-centre effects in the proportion of mosaics associated with first cellular division. The estimated proportions from the fitted model were 0.89 for centre A, series 1 (SE 0.105), 0.33 for centre A, series 2 (SE 0.272), 0.30 for centre B (SE 0.145), 0.00 for centre C (SE 0.010), and 0.22 for centre D (SE 0.139). Whereas nearly 90% of mosaics were first division at the first centre (A, series 1), the figure was a good deal lower at the other centres ($P = 0.01$).

Most embryos were frozen on day 2 or 3 of development, with two exceptions. The first concerned 47 embryos from centre D, which were embryos with asynchronous nuclear phase that could not be frozen, and which were fixed on day 4 of development. The rest of centre D embryos were frozen/thawed, and the mosaicism rates between these two embryo sources were not statistically different (17% or 8/47 for the asynchronous and 5% or 1/22 for the freeze/thawed). The other exception was seven embryos from centre A, series 1 that were rejected for transfer after PGD. The remainder of centre A, series 1 embryos were frozen/thawed, and the mosaicism rates between these two embryo sources were not statistically different (0% or 0/7 for the rejected after PGD and 33% or 3/9 for the freeze/thawed).

In patients with two or more embryos analysed ($n = 17$), seven patients had no mosaic embryos (20 embryos in total), four patients had normal and mosaic embryos (7/14 were mosaic) and four patients had only mosaic embryos (nine embryos in total).

Overall, 12 embryos were aneuploid. Per centre, none was found in centre A, series 1, one (3.3%) was found in centre A, series 2, three (14%) in centre B, one (5.6%) in centre C, and seven (10.1%) in centre D.

Regime differences

The hormonal stimulation and culture conditions of the four centres at the time of freezing of the embryos are presented in Table I. The major difference was the period during which the embryos were produced, and this was correlated in general with the mode of hormonal stimulation. Temperature optimiza-

Table III. Incidence of mosaicism for the 85 patients in the study; by centre and by hormonal stimulation

Centre	Hormonal stimulation		
	Clomiphene	Gonadotrophins	Down-regulation
A1	–	0/1, 0/1, 1/1, 0/1 3/4, 0/1	2/2, 1/1, 2/2
A2	0/1, 0/1	–	1/2, 0/1, 1/1, 0/1 1/1, 0/2, 0/1, 0/2 0/3
B	1/1, 0/1, 1/3, 2/2 3/3, 2/2, 0/1, 0/1 0/1	–	2/6
C	1/2	–	0/2, 0/1, 0/2, 0/1 0/1, 0/7, 0/1, 1/1
D	–	–	0/1, 0/1, 0/3, 0/4 0/1, 0/1, 0/2, 0/1 0/2, 1/2, 0/2, 0/1 0/1, 0/1, 0/1, 0/1 1/1, 0/1, 1/2, 0/1 0/1, 1/1, 1/1, 1/1 0/1, 1/1, 0/2, 0/1 0/1, 0/1, 0/1, 0/1 0/1, 0/1, 0/1, 0/4 0/2, 0/1, 0/1, 0/2 1/1, 1/4, 0/1, 0/2 0/4, 0/1

tion when handling oocytes and embryos also varied between centres. The centres with more chromosome abnormalities, centre A, series 1 and centre B, had provided most of their embryos from before 1991, when they used mostly clomiphene citrate or gonadotrophins for ovulation induction. In the case of centre A, series 1, warmed stages were not always used to handle oocytes and embryos, and interestingly, this centre showed the highest rate of mosaicism occurring at first embryonic division, significantly higher than the rate for centre B ($P < 0.05$). Conversely, the lowest rates were from centre A, series 2 and centres C and D, which provided embryos produced in 1991 or later, when the main regimen used was GnRH agonist down-regulation, and when appropriate temperature control was applied.

Table III displays quotients which for each patient give the number of mosaics and the total number of embryos. Thus the quotient 3/4 denotes that four embryos were observed for the patient, of which three were mosaics. The patients have been classified by centre and also by hormonal stimulation regime (clomiphene citrate, gonadotrophins, down-regulation). The very severe non-orthogonality of Table III suggests it would be difficult to disentangle the effects of centre and hormonal stimulation. It should be noted that no centre used more than one method of stimulation to any appreciable extent. The GLM analysis provided overwhelming evidence ($P < 0.001$) of systematic variation in the incidence of mosaics over the patient groups. The effect of centre was highly significant ($P < 0.001$), whereas the effect of hormonal stimulation was a good deal less emphatic ($P < 0.05$). Table IV provides estimates of the proportion of mosaics as generated from the fitted model, where the estimates for centre have been corrected for imbalance in the hormonal stimulation regime, and *vice versa*. No evidence was obtained that the culture medium used by centre A caused mosaicism.

Table IV. Estimates of the mean proportion of mosaics, by fertility centre and by hormonal stimulation regime. In each case the estimates have been corrected for imbalance of the 'other' factor

Centre	Proportion of mosaics	SEM
A1	0.96	0.013
A2	0.18	0.091
B	0.36	0.135
C	0.10	0.071
D	0.14	0.048
	$P < 0.001$	
Hormonal stimulation	**Proportion of mosaics**	**SEM**
Clomiphene citrate	0.40	0.131
Gonadotrophins	0.05	0.018
Down-regulation	0.25	0.034
	$P < 0.05$	

Discussion

The analysis presented above provided evidence of systematic variation in mosaicism attributable to 'fertility centre' and/or 'hormonal stimulation'. The structure of the data available for study (see Table III) displayed severe imbalance so that each fertility centre was largely associated with a particular hormonal regime. In order to clarify the two effects, a more balanced arrangement would be advisable where each centre employed the various stimulation regimes to a greater extent. However, this comparison cannot be repeated because gonadotrophin stimulation is now only used for a specific subset of patients that do not respond appropriately to down-regulation. Although the statistical analysis provided clear evidence that both centre and hormonal stimulation affected the incidence of mosaicism, it is difficult to distinguish their individual effects. In view of the evidence that environmental factors influence the incidence of mosaicism, further investigation is clearly necessary.

Most of the factors affecting embryonic mosaicism may be related to the learning process that has taken place during recent years of IVF practice, where improvements in hormonal stimulation and culture conditions have been reflected in an increase in pregnancy rates. Overall, these results suggest that less optimal hormonal stimulation and embryo culture resulted in more chromosome abnormalities. Most of the chromosome abnormalities detected probably gave rise to arrested embryonic development or were lethal. Transfer of such embryos may have contributed to embryo wastage and the reduced pregnancy rates that were observed a decade ago.

Mosaicism occurring at the first embryonic division from bipronuclear zygotes must be produced by impairment of the cytoskeleton and/or mitotic spindle, indicating that at least in centre A, series 1, mosaicism was already induced in the zygote. Unsuitable temperature control during culture and oocyte isolation could produce this kind of damage, such as that shown by centre A, series 1. The lack of temperature control in that centre is the only difference with centre B, and the differences in mosaicism at the first embryonic division between these two centres are statistically significant. This finding corroborates previous studies on human oocytes which showed a significant increase in spindle disassembly and

chromosomal dispersion (Pickering *et al.*, 1990; Almeida and Bolton, 1995).

It cannot be argued that cryopreservation was partially responsible for the abnormalities detected, although several authors have indicated an association between chromosomal abnormalities and embryo freezing (Bongso *et al.*, 1988; Shaw *et al.*, 1991). However, in our study, we biopsied and fixed the blastomeres before any post-cryopreservation cellular division could have taken place; therefore the detected chromosome abnormalities had to have been present at the time of cryopreservation.

An inconsistency with the assumption that higher mosaicism rates would cause lower pregnancy rates, is the fact that centres A, B, and C had very different mosaicism rates but similar pregnancy rates, while centres C and D had similar mosaicism rates but different pregnancy rates. This is probably due to differences between embryo transfer procedures, hormonal dosage and administration, and other factors beyond the scope of this study.

In conclusion, significantly different mosaicism rates were found between embryos obtained from different IVF centres of different chronologies and subjected to procedures within the same centre. This indicates that the way in which IVF is performed, then and now, affects certain chromosome distributions in the embryo. Fortunately, most abnormalities produced at the first mitotic division will result in death of the embryo, and hence babies produced by IVF still have similar chromosome abnormality rates when compared to the general population (SART & ASRM, 1995). The present technique could be used in the future to identify which factors contribute to higher rates of mosaicism in IVF-generated embryos, as well as to serve as a more appropriate quality control for embryo culture systems across different centres than embryo morphology or clinical pregnancy rates alone.

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