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Higher Degree of Chromosome Mosaicism in Preimplantation Embryos from Carriers of Robertsonian Translocation t(13;14) in Comparison with Embryos from Karyotypically Normal IVF Patients

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***Purpose*:** To compare the frequency and the degree of mosaicism in human embryos from Robertsonian translocation (RT) t(13;14) carriers, with embryos from karyotypically normal IVF patients.

***Methods*:** FISH analysis of embryos from PGD cycles for RT t(13;14), with probes for chromosomes 13, 14, and 18 (Group I) and of embryos from karyotypically normal IVF patients with probes for chromosomes 13, 18, 21, X, and Y (Group II).

***Results*:** The incidence of abnormal mosaic embryos was significantly higher in group I (38/51) as compared with group II (6/45) (χ^2: $P < 0.01$). Furthermore, in group I the percentage of diploid cells per embryo was lower for chromosome 13 and 14 in comparison with 18, while in group II no differences were observed between the five chromosomes analyzed.

***Conclusions*:** RT induces a high frequency of mosaicism specifically for the chromosomes implicated in the translocation; the analysis by FISH of two blastomeres is strongly recommended for these patients.

KEY WORDS: Human embryos; mosaicism; preimplantation genetic diagnosis; Robertsonian translocation.

INTRODUCTION

Since the advent of preimplantation genetic diagnosis (PGD) (1) and its application to detect chromosome anomalies on human preimplantation embryos by FISH analysis (2,3), many studies revealed a high frequency of chromosome mosaicism (4–12). This largely documented phenomenon has many practical implications. In particular, the coexistence on the same embryo of different cell lines with different chromosome arrangements produces a risk of misdiagnosis with PGD by FISH analysis performed on one or two blastomeres. PGD by FISH was largely applied to detect abnormal embryos from carriers of chromosome reciprocal translocations and Robertsonian translocations (RTs) (13–18). In a previous study (18) the incidence of mosaicism and the frequency of abnormal chromosome segregation were analyzed in human embryos from translocation carriers. A higher frequency of mosaicism and a higher degree of abnormal segregation were detected for the chromosomes implicated in the translocation in comparison with other autosomal chromosomes. These findings, if confirmed, involve a higher risk of misdiagnosis when PGD is performed on embryos from patient carriers of translocations. We present here a study performed on spare embryos from carriers of RT t(13;14) that underwent a PGD cycle, in which

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frequency and degree of mosaicism for the chromosomes implicated in the translocation were compared with the not-involved chromosome 18 of the same embryos, and with chromosomes 13, 21, 18, X, and Y of embryos from karyotypically normal IVF patients. Couples signed an informed consent to allow FISH analysis of spare embryos. The study was approved by the Ethical Board of our hospital with protocol number 99/121.

MATERIAL AND METHODS

Stimulation Protocol and Oocyte Recovery

Ovarian stimulation was performed using gonadotrophin releasing hormone analogue (Buserelin acetate: Suprefact spray; Hoechst Inc., Frankfurt, Germany), human menopausal gonadotrophin (hMG; Humegon, Organon Inc., Oss, The Netherlands), and human chorionic gonadotrophin (hCG; Pregnyl, Organon Inc.). Oocyte retrieval was performed through vaginal puncture under ultrasound guidance. In vitro oocyte culture and preparation for ICSI have been described elsewhere (10).

FISH Analysis

Control Lymphocytes. The efficiency of the FISH technique and the specificity of the probes were previously tested on interphase nuclei and metaphase chromosome spreads of karyotypically normal individuals, prepared from lymphocyte cultures.

FISH Analysis of Embryos from PGD Cycles for RT t(13;14) (Group I). Fifty-one spare embryos from five couples that underwent seven PGD cycles at our fertility clinic were donated to research in science after informed consent. The mean age of the patient ranged from 32 to 39 years (average = 35.4, SD = 2.9). Only normally fertilized embryos (presenting two pronuclei 16–20 h after the insemination) and embryos with a percentage of fragmentation <50% were included in the study. Embryos were cultured until Day 5 in a "Homemade" sequential medium. For FISH analysis they were spread on slides by HCl-Tween 20 method, as described elsewhere (19) and were then hybridized with a probe mixture containing a locus-specific probe (LS13q14), spectrum green (Vysis Inc., Downers Grove, Illinois), for chromosome 13, a telomeric probe (Tel 14q), spectrum orange, for chromosome 14 (Vysis Inc.) and a centromeric probe (CEP 18), spectrum green + spectrum orange, for chromosome 18 (Vysis Inc.) for ploidy control. Counterstaining of nuclei was performed by adding 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in antifade solution (Vectashield; Vector Laboratories) on the slides. The slides were analyzed at ×1000 magnification using a Zeiss Axioskop fluorescent microscope equipped with 100-W epifluorescent illumination and a single-band pass filter set (Vysis Inc.) for DAPI, fluorescein isothiocyanate, and spectrum orange. By this approach the embryos carrying normal or balanced chromosomes (displaying even spots for each probe) can be differentiated from embryos carrying unbalanced chromosomes (displaying uneven spots for one or two probes) (13).

FISH Analysis of Embryos from IVF Patients (Group II). Forty-five spare embryos were donated to research in science after signed informed consent from 24 couples with normal karyotype, undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) for infertility at our fertility clinic. The maternal age ranged from 25 to 42 years (average 33.8, SD = 4.2). The difference between the age of the patients in group I and II was not significant (*t* Student $P = 0.5$). Only normally fertilized embryos (presenting two pronuclei 16–20 h after the insemination) and embryos with a percentage of fragmentation <50% were included in the study. Embryos were cultured up to day 5 in a "Homemade" sequential medium and were then spread and fixed on slides with 0.01 N HCl 0.1% Tween 20 for FISH analysis, as described elsewhere (19). Five directly labeled DNA probes were used in a two-round hybridization protocol for the simultaneous detection of chromosomes X, Y, 13, 18, and 21. All the probes were obtained from Vysis Inc. (Downers Grove, Illinois). The first round was performed using centromeric probes specific for chromosomes X (CEP X, locus DXZ1, spectrum green), Y (CEP Y, locus DYZ3, spectrum orange), and 18 (CEP 18, locus D18Z1, 1:1 spectrum green + spectrum orange mixture). The second round was performed with locus-specific probes for chromosomes 13 (LSI 13, locus 13q14, spectrum green) and 21 (LSI 21, locus 21q22.13-q22.2, spectrum orange). In the first round, FISH was performed as previously described (20). Counterstaining of nuclei was performed by adding DAPI (Sigma) in antifade solution (Vectashield; Vector Laboratories) on the slides. After analysis of results from the first FISH round slides were rinsed in 1× PBS for 5 min, dehydrated through an ethanol series, and left to air-dry. The second set of probes (chromosomes 13 and 21) was applied on the slides and co-denatured at 73°C for 5 min prior to be left to hybridize in a humidified chamber

at 37°C overnight. The slides were analyzed at ×1000 magnification using a Zeiss Axioskop fluorescent microscope equipped with 100-W epifluorescent illumination and a single-band pass filter set (Vysis Inc.) for DAPI, fluorescein isothiocyanate, and spectrum orange. The scoring criteria previously described (21,22) were applied for chromosome evaluation.

Embryo Chromosome Pattern Classification. Chromosome patterns were classified according to criteria previously proposed (4,11).

Normal (N): ≥90% of diploid cells;
Abnormal (A); ≥90% of uniformly abnormal cells;
Mosaic:
Diploid or moderate mosaic (DM): <90% and ≥62% of diploid nuclei;
Abnormal or extended mosaic (AM): <62% of diploid cells for at least one chromosome analyzed.
Chaotic (C): all nuclei showing randomly different chromosome patterns.

Statistical Analysis. Statistical analysis was performed by χ^2 test, Mann-Whitney test and Kruskall–Wallis test. $P < 0.05$ was considered as the significant level.

RESULTS

Control Lymphocytes.

One hundred male lymphocytes were scored for optimization of FISH signal patterns with the same probe mixture used for the embryo FISH analysis. An average of 96.5% of interphase nuclei displayed the expected number of signals for all the probes.

Embryos from PGD Cycles for RT t(13;14) (Group I). Fifty-one spare embryos were analyzed by FISH on Day 5: 15 embryos at 2–15 cell stage, 12 morulae, and 24 blastocysts. All the embryos analyzed, except one, displayed a mosaic chromosome constitution, and the coexistence of up to 19 different cell lines in the same embryo were observed. Twelve embryos were diploid mosaics and only one was normal. Thirty-eight embryos were abnormal mosaics for at least one chromosome (13 early-cleavage embryos, 9 morulae, and 16 blastocysts). Thirty-seven of them were abnormal mosaics for one or both chromosomes involved in the RT, in nine cases they were additionally abnormal mosaics for chromosome 18, and in one case they were abnormal mosaics only for chromosome 18. In Fig. 1 are shown the average percentages of diploid cells per embryo for each chromosome analyzed at the three developmental stages. The mean percentages of

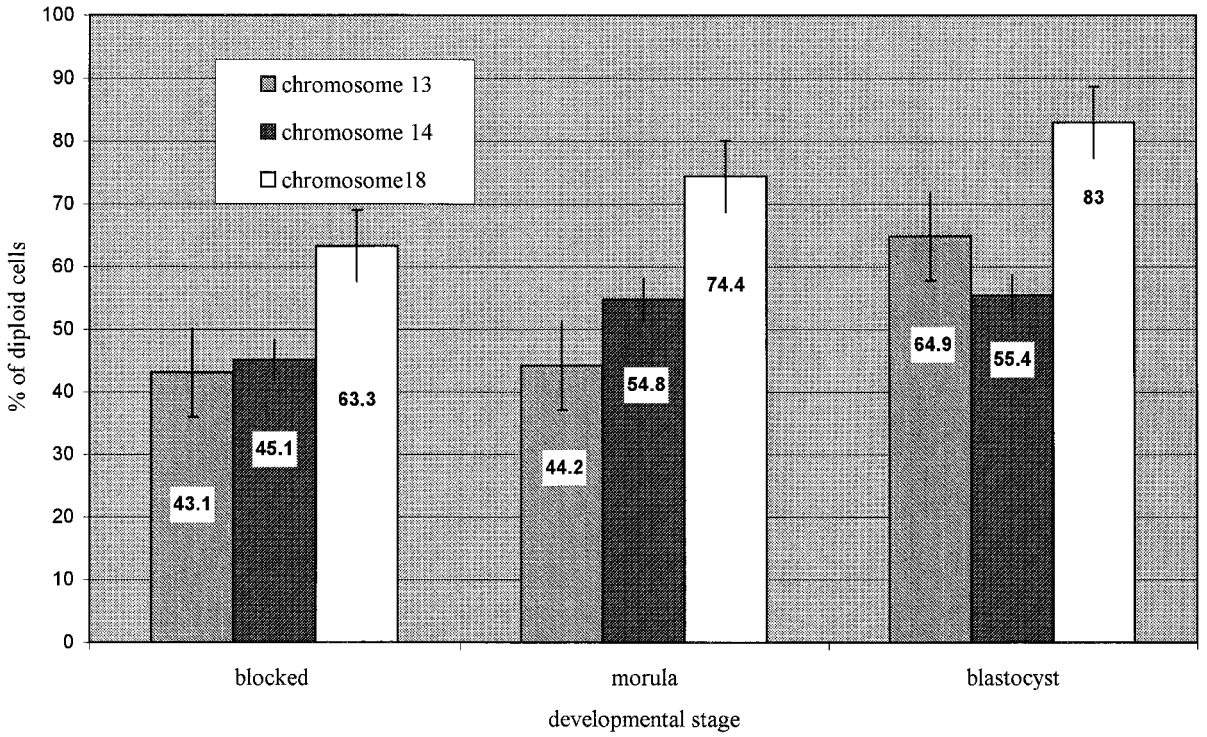

Fig. 1. Percentages of diploid cells for each chromosome analyzed in embryos from Group I (patient carriers of RT t(13;14).

diploid cells per embryo were significantly higher for chromosome 18 in comparison with chromosome 13 and 14, at the three developmental stages analyzed (Mann-Whitney test: $P < 0.01$). The average percentage of tetraploid cells per embryo was 11.7 ± 13.3 and it was 14.2 ± 13.8, 10.0 ± 9.6, and 7.4 ± 8.5 in blocked embryos, morulae, and blastocysts.

Embryos from IVF Cycles (Group II). Forty-five spare embryos were analyzed by FISH on Day 5: 16 embryos at (4–10) cell stage, 14 morulae, and 15 blastocysts. Nine embryos were normal, 30 were diploid mosaics and 6 were abnormal mosaics for at least one of the five chromosomes analyzed (5 early-cleavage embryos and 1 blastocyst). In the embryos displaying a mosaic chromosome constitution the coexistence of up to 23 different cell lines was observed. The principal diploid cell line was associated with a proportion of tetraploid cells and with further completely chaotic chromosome arrangements. In Fig. 2 are shown the average percentages of diploid cells/per embryo for each chromosome analyzed at the three developmental stages observed on Day 5. No statistical differences were observed in the percentage of diploid cells between the five chromosomes analyzed at each developmental stage (Kruskall–Wallis test). The mean percentage of tetraploid cells per embryo was 5.8 ± 10.8, 3.4 ± 6.7, and 3.8 ± 4.2 for blocked embryos, morulae, and blastocysts, respectively. The average percentage of tetraploid cells for all the embryos analyzed was 4.5 ± 7.9.

DISCUSSION

In a previous study on embryos from patient carriers of translocations, a higher incidence and a higher degree of mosaicism were detected for the chromosomes involved in the translocation in comparison with not-involved chromosomes from the same embryos (18). In the present study we observed that the frequency of diploid cells was lower in carriers of RT t(13;14) than those in karyotypically normal embryos (see Figs. 1 and 2). This held true for the chromosome involved in the translocation (13;14) and the results were less impressive in chromosome 18. Presumably, this nonspecific interchromosomal arrangement reflects three-dimensional perturbations of segregation

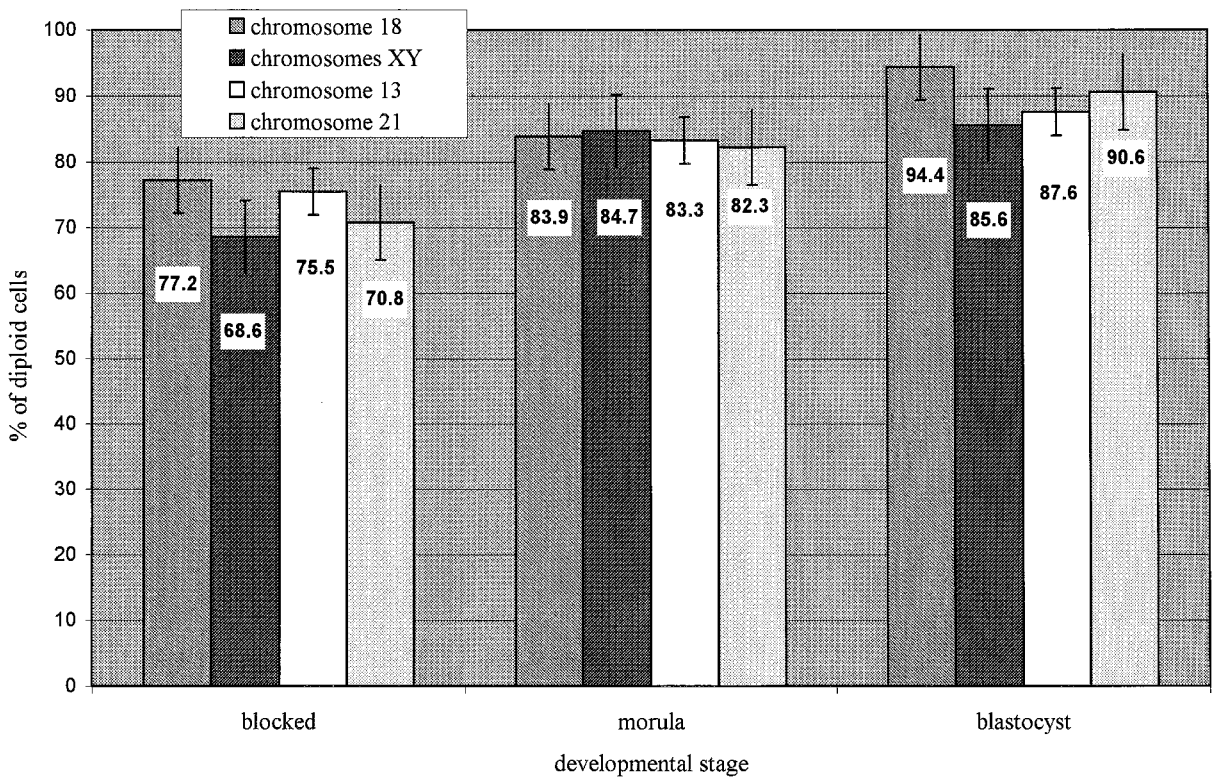

Fig. 2. Percentages of diploid cells for each chromosome analyzed in embryos from Group II (karyotypically normal IVF patients).

distortion because of the chromosome being present in a trivalent. Embryos from group I were more frequently abnormal mosaic (<62% of diploid cells) for the chromosomes 13 and 14 as compared with the control chromosome 18. Moreover, we compared the mosaicism patterns of these embryos with embryos from karyotypically normal couples that underwent IVF cycles (Group II), in which FISH analysis of chromosomes 13, 21, 18, X, and Y, more often implicated in aneuploidies producing abnormal live-born or recurrent miscarriages, was performed. Mosaicism is a largely documented phenomenon observed in human preimplantation embryos (5–12). Several reasons have been proposed to explain the high incidence of chromosome mosaicism observed in preimplantation IVF embryos, for example, in vitro culture conditions (23) or the missing of a cell-cycle checkpoint mechanism in early-cleavage embryos (24). Recently the results on the changes in mosaicism pattern during the development of human embryos from 2-cell stage to blastocysts were presented (12). In this study an increased number of mosaic embryos and an increased percentage of diploid cells were observed during the embryo development up to the blastocyst stage. These results are in agreement with our findings on IVF embryos (Group II). In fact, non-mosaic embryos were found only at early-cleavage stages while all blastocysts were mosaics for the five chromosomes analyzed. At the same time, in mosaic embryos, the percentage of diploid cells per embryos was progressively increased from early-cleavage to the blastocyst stage and all the blastocysts, except one, were diploid mosaic. These results could demonstrate that the probability that chromosomes malsegregate is increased during in vitro culture (20) but, at the same time, a mechanism of cell elimination can progressively reduce the percentage of abnormal cells per embryo during its development up to the blastocyst stage. In fact some apoptotic mechanisms, largely documented in preimplantation embryos, could be evocated as responsible of programmed cell-death of chromosomally abnormal blastomeres (25,26). On the other hand, in group I, 16 out of 24 blastocysts were abnormal mosaic for one or both chromosomes 13 and 14, while only one blastocyst was abnormal mosaic solely for chromosome 18. Furthermore, while in group I the percentage of diploid cells per embryo for chromosomes 13 and 14 was lower, as compared with the control chromosome 18, in group II no differences were observed in the percentage of diploid cells per embryo for the five chromosomes analyzed at the three developmental stages. FISH artefacts were evocated to partially explain the phenomenon of mosaicism (27), but following our observations there are no obvious reasons for which translocations could be more frequently implicated in artefact phenomena as compared with other not-involved chromosomes. Contrary to a previous study (28), in which tetraploidy was the most common chromosomal arrangement detected in blastocysts, we found in both groups a less frequent detection of tetraploid cells during the embryo development up to blastocyst stage. Some hypothetical explanations were evocated to explain the increased incidence of mosaicism in embryos from carriers of translocations like, for example, an higher incidence of acrocentric chromosomes to malsegregate during mitosis or a predisposition by the translocation itself to malsegregate (18). That the first hypothesis can be excluded is indicated by our results for the control group in which two acrocentric chromosomes were analyzed, the 13 and 21, and the same incidence of abnormal segregation was observed as compared with the other three nonacrocentric chromosomes (18, X, Y). Whatever is the reason of embryo chromosome mosaicism it has some practical implications. The principal is that it increases the risk of misdiagnosis in case of PGD, which is normally performed on one or two blastomeres. Some cases of misdiagnosis following PGD performed by FISH were recently reported (29) and the debate if the analysis of two blastomeres can reduce the risk of mistakes without reducing the embryo implantation rate is a matter of discussion (30). FISH analysis is actually largely applied to PGD in case of carriers of reciprocal and RTs (13–18,31–33). What is evident is that the increased incidence of mosaicism observed for the chromosomes implicated in the translocations, as compared with other not-involved chromosomes, increases the probability of misdiagnosis especially when PGD by FISH is performed on one blastomere.

In conclusion, we observed a higher incidence of abnormal mosaic embryos and of abnormal chromosome segregation patterns for the chromosomes implicated in RT t(13;14) as compared with other chromosomes, from either the same embryos or from embryos from karyotypically normal couples. We strongly recommend performing PGD by FISH analysis on two blastomeres in case of PGD for RT t(13;14).

REFERENCES

1. Handyside AH, Kontogianni EH, Hardy K, Winston RML: Pregnancies from biopsied human preimplantation embryos

sexed by Y-specific DNA amplification. Nature 1990;344:768–770
2. Lissens W, Sermon K: Preimplantation genetic Diagnosis: Current status and new developments. Hum Reprod 1997;12:1756–1761
3. Geraedts J, Handyside A, Harper J, Libaers I, Sermon K, Staessen C, Thornhill A, Viville S, Wilton L: ESHRE Preimplantation Genetic Diagnosis Consortium (PGD): Preliminary assessment of data from January 1997 to September. Hum Reprod 1998;14:3138–3148
4. Delhanty JDA, Harper JC, Ao A, Handyside AH, Winston RM: Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. Hum Genet 1997;99:755–760
5. Conn CM, Harper JC, Winston RML, Delanthy JD: Infertile couples with Robertsonian translocations: Preimplantation genetic analysis of embryos reveals chaotic cleavage divisions. Hum. Genet 1998;102:117–123
6. Munné S, Grifo J, Cohen J, Weier HUG: Chromosome Abnormalities in Human Arrested Preimplantation Embryos: A multiple-probe FISH study. Am J Hum Genet 1994;55:150–159
7. Munné S, Weier HUG, Grifo J, Cohen J: Chromosome mosaicism in human embryos. Biol Reprod 1994;51:41–49
8. Evsikov S, Verlinsky Y: Mosaicism in the inner cell mass of human blastocysts. Hum Reprod 1998;13:3151–3155
9. Veiga A, Gil Y, Boada M, Carrera M, Vidal F, Boiso I, Ménézo Y, Barri PN: Confirmation of diagnosis in preimplantation genetic diagnosis (PGD) through blastocyst culture: Preliminary experience. Prenat Diagn 1999;19:1242–1247
10. Emiliani S, Gonzalez-Merino E, Van den Bergh M, Abramowicz M, Vassart G, Englrt Y, Delneste D: Re-analysis by fluorescence *in situ* hybridisation of spare embryos cultured until Day 5 after preimplantation genetic diagnosis for a 47, XYY infertile patient demonstrates a high incidence of diploid mosaic embryos: A case report. Prenat Diagn 2000;20:1063–1066
11. Sandalinas M, Sadowy S, Alikani M, Calderon G, Cohen J, Munné S: Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. Hum Reprod 2001;16:1954–1958
12. Bielanska M, Lin Tan S, Ao A: Chromosomal mosaicism throughout human preimplantation development in vitro: Incidence, type, and relevance to embryo outcome. Hum Reprod 2002;17:413–419
13. Scriven PN, Handyside AH, Mackie Ogilvie C: Chromosome translocations: Segregation modes and strategies for preimplantation genetic diagnosis. Prenat Diagn 1998;18:1437–1449
14. Conn CM, Harper JC, Winston RML, Delhanty JD: Infertile couples with Robertsonian translocations: Preimplantation genetic analysis of embryos reveals chaotic cleavage divisions. Hum Genet 1998;102:117–123
15. Munné S, Scott R, Sable D, Cohen J: First pregnancies after preconception diagnosis of translocations of maternal origin. Fertil Steril 1998;69:675–681
16. Evsikov S, Cleslak MLT, Verlinsky Y: Effect of chromosomal translocations on the development of preimplantation human embryos in vitro. Fertil Steril 2000;74:672–677
17. Escudero T, Lee M, Carrel D, Blanco J, Munne S.: Analysis of chromosome abnormalities in sperm and embryos from two 45,XY,t(13;14) (q10;q10) carriers. Prenat Diagn 2000;20:599–602
18. Iwarsson E, Malmgren H, Inzunza J, Ahrlund-Richter L, Sjoblom P, Rosenlund B, Fridstrom M, Hovatta O, Nordenskjold M, Blennow F: Highly abnormal cleavage divisions in preimplantation embryos from translocation carriers. Prenat Diagn 2000;20:1038–1047
19. Coonen E, Dumoulin JCM, Ramaekers FCS, Hopman AH: Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence in-situ hybridization. Hum Reprod 1994;9:533–537
20. Staessen C, Van Assche E, Joris H, Bonduelle M, Vandervorst M, Liebaers I, Van Steirteghem AC: Clinical experience of sex determination by fluorescent *in-situ* hybridization for preimplantation genetic diagnosis. Mol Hum Reprod 1999;5:382–389
21. Hopman AH, Ramaekers FC, Raap AK, Beck JL, Devilee P, Van der Ploeg M, Vooijs GP: *In situ* hybridization as a tool to study numerical chromosome aberrations in solid bladder tumours. Histochemistry 1998;89:307–316
22. Munné S, Marquez C, Magli C, Morton P, Morrison L: Scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes X, Y, 13, 16, 18 and 21. Mol Hum Reprod 1998;4:863–870
23. Munné S, Magli C, Adler A, Wright G, De Boer K, Mortimer D, Tucker M, Cohen J, Gianaroli L: Treatment-related chromosome abnormalities in human embryos. Hum Reprod 1997;12:780–784
24. Harrison RH, Kuo HC, Scriven PN, Handyside AH, Makkie Ogilvie C: Lack of cell cycle check points in human cleavage stage embryos revealed by a clonal pattern of chromosomal mosaicism analyzed by sequential multicolour FISH. Zygote 2000;8:217–224
25. Hardy K, Spanos S, Becker D, Iannelli P, Winston RM, Stark J: From cell death to embryo arrest: Mathematical models of human preimplantation embryo development. Proc Natl Acad Sci USA 2001;13:1655–1660
26. Chen HW, Jang WS, Tzeng CR: Nitric oxide as a regulator in preimplantation embryo development and apoptosis. Fertil Steril 2001;75:1163–1171
27. Munné S, Dailey T, Finkelstein M, Weier HUG: Reduction in signal overlap results in increased FISH efficiency: Implications for preimplantation genetic diagnosis. J Assist Reprod Genet 1996;13:149–156
28. Ruangvutilert P, Delhanty JDA, Serhal P, Simopolou M, Rodeck CH, Harper JC: FISH analysis on day 5 post-insemination of human arrested and blastocyst stage embryos. Prenat Diagn 2000;20:552–560
29. ESHRE PGD Consortium Steering Committee: ESHRE Preimplantation Genetic Diagnosis Consortium: Data collection III (May 2001). Hum Reprod 2002;17:233–246
30. Van de Velde H, De Vos A, Sermon K, Staessen C, De Rycke M, Van Asshe E, Lissens W, Vandervorst M, Van Ranst H, Libaerts L, Van Steirteghem A: Embryo implantation after biopsy of one or two cells from cleavage-stage embryos with a view to preimplantation genetic diagnosis. Prenat Diagn 2000;20:1030–1037
31. Munné S, Escudero T, Sandalinas M, Sable D, Cohen J: Gamete segregation in female carriers of Robertsonian translocations. Cytogenet Cell Genet 2000;90:303–308
32. Munné S, Sandalinas M, Escudero T, Fung J, Gianaroli L, Cohen J: Outcome of preimplantation genetic diagnosis of translocations. Fertil Steril 2000;73:1209–1218
33. Munné S: Preimplantation genetic diagnosis of structural abnormalities. Mol Cell Endocrinol 2001;183(Suppl 1):S55–58