Published in final edited form as:

Fertil Steril. 2010 May 15; 93(8): 2545–2550. doi:10.1016/j.fertnstert.2009.06.040.

Incidence of chromosomal mosaicism in morphologically normal non-human primate preimplantation embryos

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Abstract

Objective—To establish the exact rates of chromosomal mosaicism in morphologically normal rhesus macaque embryos by determining the chromosomal complement of all blastomeres.

Design—Retrospective rhesus monkey IVF study.

Setting—Academic laboratory and Primate Research Center.

Patients—Young fertile rhesus macaque females.

Interventions—Morphologically normal, *in vitro* produced rhesus macaque embryos were dissociated and cytogenetically assessed using a 5-color fluorescent *in situ* hybridization assay developed for rhesus macaque chromosomes homologous to human chromosomes 13, 16, 18, X and Y.

Main Outcome Measure(s)—The incidence and extent of chromosomal mosaicism in rhesus macaque preimplantation embryos.

Result(s)—Seventy-seven preimplantation embryos, displaying normal morphology and development, from 17 young rhesus macaque females were analyzed. Overall, 39 embryos (50.6%) were normal, 14 embryos (18.2%) were completely abnormal and 24 embryos were mosaic (31.2%). Of the 226 blastomeres analyzed in the mosaic group, 110 blastomeres (48.7%) were normal.

Conclusion(s)—The observed rate of mosaicism in good quality rhesus embryos resembles previously-documented frequencies in poor-quality human preimplantation embryos. A high incidence of mosaicism may limit the diagnostic accuracy of preimplantation genetic diagnosis.

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Keywords

Mosaicism; embryos; monkey; non-human primate; aneuploidy; FISH

INTRODUCTION

The incidence of aneuploidies in *in vitro* produced (IVP) human preimplantation embryos ranges between 19.3 and 81% (1–7). While some of these aneuploidies arise during meiosis (8), most chromosomal segregation errors occur during mitotic divisions following fertilization (9). As a result, many human IVP preimplantation embryos possess blastomeres with dissimilar chromosomal compositions and are classified as chromosomally mosaic. While some researchers use this primary definition to define chromosomal mosaicism in embryos (4, 10, 11), others employ the term more selectively to embryos possessing a mixture of normal euploid and aneuploid blastomeres (9, 12–15). Using the latter definition, reports suggest that between 25 and 62% of human ‘day 3’ preimplantation embryos are mosaic (4, 9, 11–13, 15–17) and that the average percentage of aneuploid blastomeres within these mosaic embryos ranges between 40 and 52% (9, 15). Most of these mosaicism rates are derived, due to ethical and practical limitations, from whole embryo analyses performed on embryos that were not suitable for transfer or from embryos undergoing preimplantation genetic diagnosis (PGD). As a result, the true incidence of mosaicism in human preimplantation embryos of good quality is not precisely known.

A detailed assessment of mosaicism from day 3 embryos could provide a better overview of mechanistic origins of mosaicism and provide information that is relevant to the accuracy of PGD. Since rhesus macaque and human IVP preimplantation embryos are comparable in vulnerability to chromosomal anomalies (18), the rhesus macaque is a good model to address this issue. In this retrospective study, cytogenetic data from previously analyzed morphologically normal day 3 rhesus macaque embryos (18, 19), which had at least 70% of their blastomeres analyzed, were used to provide insights into the mechanistic origins of mosaicism and implications for PGD and human infertility treatment in general.

MATERIALS AND METHODS

Unless otherwise stated, chemicals and reagents were procured from Sigma-Aldrich (St Louis, MO, USA). All procedures have been described previously (18, 19) and will only be briefly mentioned.

Controlled hormonal ovarian stimulation

All of the procedures at the Oregon National Research Primate Center (ONPRC) and the Caribbean Primate Research Center (CPRC) in Puerto Rico were performed according to protocols approved by their respective institutional animal care and animal use committees (IACUCs). Ovarian follicular growth in female rhesus macaques (age range between 4 and 13 years old) was supported using sequential administrations of recombinant human follicle stimulating hormone (rhFSH, Organon, Oss, The Netherlands) and recombinant human luteinizing hormone (rhLH, EMD Serono, Rockland, MA, USA) (18, 19). On the final day of the rhFSH administration, a single dose of recombinant human chorionic gonadotropin (rhCG; 750–1000 IU, i.m.) was injected.

Oocyte and sperm collection, insemination and embryo culture

The procedures for oocyte recovery, sperm collection, insemination and embryo culture have been described previously (18–25). Fluid from ovarian follicles was aspirated 33 to 36

hours after the rhCG administration. Follicular fluid aspirates collected in TALP-Hepes containing 0.3% bovine serum albumin (BSA) were passed through a cell filter (Becton-Dickinson, Franklin Lakes, NJ; Falcon, 70 μ m pore size) and the oocytes were collected by washing the filter. After removal of the cumulus cells using hyaluronidase (0.03%), the oocytes were rinsed in BSA-supplemented TALP-Hepes and subsequently placed in BSA-supplemented TALP-medium culture drops in a 5% CO₂ in air atmosphere at 37°C (25). Semen recovery, seminal plasma removal and insemination of the cumulus-free oocytes were performed using standard procedures (20, 23, 24). The following day, presumptive zygotes were transferred to amino acid-supplemented HECM-9 culture drops (26) and cultured under mineral oil in a 5% CO₂ in air atmosphere at 37°C.

Embryo collection and blastomere fixation

A cohort of embryos, having reached at least the 5-cell stage after 54 to 78 hr of *in vitro* culture after insemination, with no or minor fragmentation, were collected. The selected embryos were dissociated after removal of the zona pellucida (18, 19) and blastomere nuclei were fixed on a slide using 0.01M HCl/0.1% Tween20 (27). Samples were dehydrated in an ethanol series and subsequently held at -20°C until required for analysis.

DNA Probes

Mixtures containing directly labeled DNA probes for macaque chromosomes X, Y, 17, 18, and 20 (homologous to human chromosomes X, Y, 13, 18, and 16 respectively) (28) were created as described in previous studies (18, 19, 29, 30).

Fluorescence In Situ Hybridization (FISH) and FISH analysis

The five-probe FISH assay, carried out in two successive hybridization procedures, visualization and scoring were all performed as described previously (18, 19). The embryos were classified as normal, aneuploid, chaotic, mosaic, haploid or polyploid after the analysis of each blastomere (Table 1). In addition, mosaic embryos were classified as diploid-polyploid if the abnormal blastomeres were haploid and/or polyploid and categorized as diploid-aneuploid if the abnormal blastomeres were simply aneuploid. The mechanistic origin of post-zygotic segregation errors in diploid-aneuploid embryos was determined according to the anomalies in the mosaic embryos (Figure 1).

Statistical analysis

The dataset only included embryos in which at least 70% of their blastomeres were successfully analyzed. False negative (diagnosed normal when abnormal) rates were estimated after the biopsy of one or two blastomeres according to formulas represented in Table 2, using values from Table 3. Briefly, the false negative rate was calculated by dividing the number of mosaic embryos likely to be classified as normal by the total number of normal embryos plus the number of mosaic embryos likely to be classified as normal. The probability that the first blastomere biopsied from a mosaic embryo would be normal was calculated by dividing the total number of normal blastomeres in all mosaic embryos by the total number of blastomeres in all mosaic embryos. To calculate the false negative rate after the biopsy of two blastomeres, the formula was modified by taking into account the removal of a normal blastomere from each mosaic embryo after the first blastomere biopsy.

RESULTS

Only embryos that displayed good morphology (no or minor fragmentation) and normal development (at least 5-cell stage on 'day 3') and in which at least 70% of the blastomeres were successfully analyzed were included in this evaluation. The total number of embryos

considered was 80; 3 were excluded because the amount of analyzed blastomeres was insufficient (< 70% of total embryo), and a total of 77 preimplantation embryos from 17 young rhesus macaque females met the criteria and were analyzed. The average age of the females was 8 years (age range 4 to 13 years of age) and at the time of stimulation no female had more than 5 previous hormonal stimulations (range 1 to 5). Overall, 39 embryos (50.6%) were normal, 4 were aneuploid (5.2%), 5 were chaotic (6.5%), 5 were triploid (6.5%) and 24 were classified as mosaic after the analysis of rhesus macaque chromosomes X, Y, 17, 18 and 20 (31.2%; Table 3).

Within the mosaic group, 236 blastomeres were available of which 229 were successfully fixed. Of these, 226 blastomeres yielded a result for human homologous chromosomes X (Alexa488), Y (Alexa488-Cy3) and 13 (Cy3) after the first FISH hybridization and 206 of those provided results for human homologous chromosomes 16 (Cy3) and 18 (Alexa488) after the second hybridization. From a total of 226 blastomeres analyzed in the mosaic group, 110 blastomeres (48.7%) were normal (Table 3). The number of blastomeres analyzed in completely normal and entirely abnormal embryos is shown in Table 3.

Mosaic embryos were classified as diploid-aneuploid in 75% (18/24) of the cases, followed by diploid-polyploid embryos (17%; 4/24) and embryos with aneuploid as well as polyploid blastomeres (8%; 2/24). Within the diploid-aneuploid group (18 embryos) only seven rhesus macaque embryos clearly possessed mitotic non-disjunction errors (7/17). However, only three of these seven embryos presented no other post-zygotic errors originating through other mechanisms. This is consistent with the conclusion that splitting and anaphase lagging was the most prevalent origin for errors in diploid-aneuploid mosaic embryos.

False negative rates after a single or double blastomere biopsy amounted to 23.1% and 11.3%, respectively.

DISCUSSION

In this retrospective study, 77 *in vitro* produced (IVP) rhesus macaque preimplantation embryos with good morphology and normal development on 'day 3' were chromosomally analyzed. The results show that after the analysis of 5 chromosomes, 31.2% of 'morphologically good day 3' IVP rhesus monkey preimplantation embryos contain both chromosomally normal and abnormal blastomeres (Table 2). The percentage of chromosomally anomalous blastomeres within the mosaic rhesus macaque embryos mostly ranged between 25 and 75% with an average of 51.3% aneuploid blastomeres (Table 3). The range of mosaicism in human day 3 preimplantation embryos varies between 25 and 62% (4, 9, 11–13, 15–17) with an average of 40 to 52% blastomeres having an abnormal chromosomal complement (9, 15). While the incidence of mosaicism (30%) in rhesus macaque preimplantation embryos is in the lower range of the rates reported in human IVP preimplantation embryos, the average number of aneuploid blastomeres in mosaic rhesus monkey embryos (average of 51.3%) is at the high end in comparison to frequencies reported in human IVP preimplantation embryos. The analysis of a wider range of chromosomes, however, could increase the present reported abnormality rates in 'day 3' rhesus macaque embryos. Since most of the mosaicism rates in humans were established using surplus embryos or embryos not suitable for transfer, it is evident that the current rates resulting from morphologically normal rhesus monkey embryos from fertile animals may better represent human IVP preimplantation embryos that might be selected for transfer.

While the severity of the impact of mosaicism on embryo viability and offspring health is unknown, mosaicism has significant consequences for the diagnostic accuracy of PGD. During PGD, single blastomeres biopsied from preimplantation embryos are genetically

assessed and the results are used to classify embryos as genetically normal or abnormal. Because of mosaicism and technical errors, the genetic results from a biopsied blastomere may not be representative of the remaining blastomeres. Therefore, chromosomally normal embryos may sometimes be discarded and chromosomally abnormal embryos may be transferred. Several misdiagnoses identified after miscarriage or during prenatal screens have been reported (31–37). Although the reports of these errors are scarce, the number of transferred misdiagnosed embryos must be much higher. Indeed only a small proportion of chromosomally abnormal embryos, carrying specific aneuploidies, are able to implant or progress through development (38, 39).

In order improve PGD accuracy, two blastomeres may be biopsied (40, 41). Although it has been reported that the development and implantation after biopsy of two cells is not adversely affected (42), other studies challenge this claim (43). The rate of misdiagnosis after biopsy of one or two blastomeres has been estimated by reassessing human biopsied embryos later during development. There are multiple biases using this approach. Firstly, chromosomal errors identified in more advanced stages may not represent the aneuploidies at the time of biopsy. It has been suggested that aneuploid blastomeres may be outcompeted by normal diploid blastomeres or eliminated through programmed cell death (44). Additionally, new aneuploidies may arise during mitotic divisions subsequent to the biopsy (9, 45), which is consistent with reports that most IVP human blastocysts possess some aneuploid blastomeres (15, 46–49). Secondly, misdiagnosis estimates are severely biased because most reanalyzed embryos are categorized as chromosomally abnormal on day 3 or morphologically abnormal on the day of transfer. The percentage of false positive results in human day 3 embryos after the biopsy of one or two blastomeres ranges between 3.3 and 46% (41, 42), while the frequency of false negatives ranges between 0 and 100% (41, 42). Thus, exact rates of mosaicism in morphologically normal embryos can improve estimates of misdiagnosis.

The rhesus macaque data were used to evaluate false negative and false positive rates after the hypothetical biopsy of one or two blastomeres. Since no technical artifacts were taken into consideration, false positive results were considered non-existent in the misdiagnosis calculations. Hypothetically, the biopsy of a single euploid blastomere would be obtained from a mosaic embryo in 23.5% of the cases (false negative). Although a significant number of these mosaic embryos would be identified after a second blastomere biopsy, 11.1% of the mosaic embryos would still remain undetected (false negative). These false negative rates reflect the problems encountered during PGD of human day 3 preimplantation embryos.

Mosaic embryos are usually classified as diploid-polyploid or diploid-aneuploid depending on whether they respectively possess haploid-polyploid or simply aneuploid blastomeres. The majority of mosaic embryos in the present study were classified as diploid-aneuploid (75%), followed by diploid-polyploid embryos (17%) and embryos with aneuploid as well as polyploid blastomeres (8%). Although the mechanistic origin of diploidy-polyploidy in embryos is unclear, diploid-aneuploid embryos are believed to arise through mitotic non-disjunction, anaphase lagging of chromosomes or nuclear splitting (50). Mitotic non-disjunction in embryos is generally identified by the presence of blastomeres with a trisomy compensated by a monosomy in another blastomere (Figure 1a). In contrast, anaphase lagging is diagnosed when monosomies in certain blastomeres are not compensated by trisomies in other blastomeres (Figure 1b). Mechanistically, anaphase lagging is caused by the delayed movement of one or more chromosomes, thereby preventing the incorporation of these chromosomes in the newly formed nucleus (Figure 1b). A third cause of diploid-aneuploid embryos is an error occurring after division of blastomeres that failed to replicate (50) (Figure 1c). These nuclei with split complements, however, most likely arise through major fragmentation of the nucleus and blastomere (Figure 1d). There is a possibility that

mosaic embryos containing monosomies may produce these through minor fragmentation of the nucleus and blastomere instead of anaphase lagging (Figure 1e). Indeed some undersized blastomeres (fragments) that were observed in the present study contained small nuclei that possessed a chromosome that was missing from adjacent blastomeres that presented a monosomy. Mitotic non-disjunction errors were observed in seven rhesus macaque embryos of which only three embryos presented no additional post-zygotic errors originating through other mechanisms. Consequently, splitting and anaphase lagging were the most prevalent sources of error in diploid-aneuploid mosaic embryos. Since splitting and anaphase lagging may alternatively originate through minor or major fragmentation of the blastomere and nucleus, it is possible that certain aneuploid blastomeres in mosaic embryos are blastomeres that are undergoing a form of programmed cell death and will consequently not divide further. In addition, certain aneuploidies originating through anaphase lagging or mitotic non-disjunction may be outcompeted by euploid blastomeres. As a result, certain embryos categorized as abnormal after PGD could potentially yield a normal pregnancy.

In conclusion, the evaluation of mosaicism in morphologically normal rhesus macaque preimplantation embryos resembles the lower reported rates of mosaicism in human embryos. The observed anomalies in this study suggest that many abnormalities in mosaic embryos arise as a result of blastomere degeneration. It has been argued that mosaicism is a direct result of hormonal ovarian stimulation or poor *in vitro* culture conditions because embryos from different human IVF clinics display very dissimilar aneuploidy rates (51). Since rhesus macaque and human IVP preimplantation embryos display similar rates of chromosomal anomalies (18, 19), studies conducted using the rhesus macaque appear to be a useful model to address these issues.

Acknowledgments

This study was supported by the National Institutes of Health (1R01HD045966-01A2 and 5R24RR015395-04 awarded to B.D. Bavister, 1R03HD046553-01A1 and 1R21RR021881-01 awarded to C.A. Brenner and RR03640 awarded to the Caribbean Primate Research Center). We thank Dr. L. Froenicke for his technical advice. Additionally, we would like to thank Organon, The Netherlands for their generous support by providing recombinant FSH.

Financial support Supported by the National Institutes of Health (1R01HD045966-01A2 and 5R24RR015395-04 awarded to B.D. Bavister, 1R03HD046553-01A1 and 1R21RR021881-01 awarded to C.A. Brenner, RR03640 awarded to the Caribbean Primate Research Center and the Reproductive Biology and Medicine Branch, NICHD).

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Figure 1.

Mechanistic models for post-zygotic chromosomal errors in preimplantation embryos. 1A; mitotic non-disjunction, identified by the presence of blastomeres with a trisomy compensated by a monosomy in another blastomere. 1B and 1E; anaphase lagging or minor fragmentation of the nucleus and blastomere, identified when monosomies in certain blastomeres are not compensated by trisomies in other blastomeres. 1C and 1D; splitting or major fragmentation of the nucleus and blastomere, identified by the presence of blastomeres with split or fragmented chromosomal complements.

Table 1

Definition of Embryo Aneuploidy Classifications

A. Normal	All blastomeres normal diploid
B. Abnormal	
Haploid	Most blastomeres with a haploid complement (haploid-normal, haploid-aneuploid, haploid-mosaic or haploid-chaotic)
Diploid	
· Aneuploid	All blastomeres abnormal because of a prezygotic division error
· Mosaic	Some blastomeres normal diploid - some abnormal / not excluding a prezygotic division error
· Chaotic	All blastomeres abnormal with uncontrolled division involving all chromosomes / not excluding a prezygotic division error
Polyploid	Most blastomeres with a polyploid complement (polyploid-normal, polyploid-aneuploid, polyploid-mosaic or polyploid-chaotic)

Table 2

Formulas to calculate false negative rates after the biopsy of one or two blastomeres

After biopsy single blastomere	$[M \times (n/t)] / [N + (M \times (n/t))]$
After biopsy of two blastomeres	$[M \times (n/t) \times ((n-M)/(t-M))] / [N + (M \times (n/t) \times ((n-M)/(t-M)))]$

The symbols are defined as: N = number of normal embryos, M = number of mosaic embryos, n = number of normal blastomeres in all mosaic embryos, t = total number of blastomeres in all mosaic embryos

Table 3

Rhesus macaque preimplantation embryos classified according to their chromosomal complement

Embryo Classifications	No. of Embryos	No. of blastomeres		
		Abnormal	Normal	Total
Normal embryos	39 (50.6%)	-	290	290
Abnormal embryos				
Completely abnormal *	14 (18.2%)	99	-	99
Partially abnormal (mosaic)	24 (31.2%)	116	110	226

* Completely abnormal embryos represent haploid, polyploid, aneuploid and chaotic embryos

Table 4

Mosaic Embryo Classifications according to the numbers of abnormal cells

Mosaic Embryo Classifications (% Abnormal Blastomeres)	No. of embryos	No. of blastomeres		
		Abnormal	Normal	Total
$0 < x^* \leq 25$	4	7	31	38
$25 < x^* \leq 50$	10	35	49	84
$50 < x^* \leq 75$	6	44	23	67
$75 < x^* < 100$	4	30	7	37
Totals	24	116	110	226

*
× represents % abnormal blastomeres in a mosaic embryo