Human Embryos Derived from In Vitro and In Vivo Matured Oocytes: Analysis for Chromosomal Abnormalities and Nuclear Morphology

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Purpose: To determine whether embryos resulting from oocytes matured in vitro have a higher incidence of nuclear and/or genetic abnormalities compared to embryos resulting from oocytes matured in vivo.

Methods: Fluorescence in situ hybridization analysis for chromosomes X, Y, and 18 was used to compare the rates of aneuploidy, mosaicism, and nuclear abnormalities in embryos derived from oocytes that were prophase I at aspiration (immature group) to that observed in embryos resulting from oocytes that were metaphase I or II at aspiration (mature group).

Results: Based on nuclear morphology, significantly more embryos in the mature group (23%) were classified as normal compared to embryos in the immature group (3%). No difference was found in the rate of aneuploidy or in the incidence of mosaicism involving these chromosomes.

Conclusions: These findings suggest that few embryos derived from prophase I oocytes collected following ovarian stimulation are morphologically normal.

KEY WORDS: fluorescence in situ hybridization; in vitro fertilization; in vitro maturation; oocyte.

INTRODUCTION

Reproductive endocrinologists have developed controlled ovarian hyperstimulation (COH) protocols that utilize the exogenous administration of gonadotropic hormones to increase the number of mature oocytes available for assisted reproductive techniques. By arti-

ficially increasing the serum level of follicle-stimulating hormone (FSH), the cohort of developing follicles can avoid the usual "selection" phase, allowing all of the recruited follicles to achieve growth and maturation. Following aspiration for in vitro fertilization (IVF), the collected oocytes sometimes demonstrate various levels of nuclear maturity. Preovulatory, or "mature" oocytes, technically those in metaphase I or metaphase II of meiosis, are inseminated shortly after collection and assessed for fertilization the following day. Prophase I oocytes, also called germinal vesicle-bearing or "immature" oocytes, must be cultured for 24–36 hr to permit germinal vesicle breakdown and first polar body extrusion before being exposed to spermatozoa. Despite the fact that the majority of such in vitro matured oocytes do fertilize and appear to cleave normally, the resulting embryos demonstrate an extremely low incidence of pregnancy following intrauterine transfer when compared to embryos derived from oocytes that were mature at collection (1,2).

The purpose of this study was to determine whether the reduced developmental potential of embryos derived from prophase I oocytes could be explained in part by an increase in nuclear and/or genetic abnormalities. Fluorescence in situ hybridization (FISH) analysis with DNA probes specific for chromosomes X, Y, and 18 was used to compare rates of aneuploidy, mosaicism, and morphological nuclear abnormalities in embryos derived from oocytes that were prophase I at aspiration to that observed in embryos derived from oocytes that were metaphase I or II at aspiration.

MATERIALS AND METHODS

Human Embryos

Prior to initiation of the study, the Institutional Review Board (IRB) of Eastern Virginia Medical

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School approved all protocols and consent forms. Embryos for this study were obtained from two sources. The first source was cryopreserved-thawed (3) embryos ($n = 65$) donated by 17 IVF patients for IRB-approved research. This source provided the majority of embryos resulting from oocytes matured in vivo (metaphase I or II at aspiration). One patient was an oocyte recipient and the remaining were infertility patients using their own oocytes. The types of infertility included tubal factor ($n = 4$), luteal phase defects ($n = 3$), male factor ($n = 3$), immunologic factors ($n = 2$), idiopathic ($n = 2$), endometriosis ($n = 1$), and ovulatory defect ($n = 1$). Twelve (71%) of the patients received luteal lupron, two patients (12%) received follicular lupron, and three patients (18%) received no lupron.

The second source of embryos resulted from prophase I oocytes donated by consenting non-male factor IVF patients ($n = 2$) and oocyte donors ($n = 21$). Of the two infertility patients, one was diagnosed with polycystic ovaries and the other with tubal infertility. All patients and donors received luteal lupron. Donated oocytes were incubated overnight to allow for maturation. Following extrusion of the first polar body, the oocytes ($n = 159$) were inseminated, resulting in 61 embryos for analysis in this study. Sperm for insemination was obtained from the spouse in the case of IVF patients, or from consenting sperm donors in the case of oocyte donors.

Ovarian Stimulation and Oocyte Retrieval

The majority of patient cycles were suppressed with leuprolide acetate (LA) (Lupron, TAP Pharmaceuticals, Abbot Park, IL) 0.5 mg subcutaneously (SC) daily starting in the midluteal phase. Patients were classified into high, moderate, and low responders based on prior ovarian stimulation and basal serum levels of FSH, luteinizing hormone (LH), and estradiol (E_2) and ovarian stimulation performed as previously described (4). Human chorionic gonadotropin, 10,000 IU intramuscularly (IM) (Profasi, Serono Laboratories, Randolph, MA) was administered when three or more follicles were at least 16 mm in largest diameter. Transvaginal follicular aspiration was performed 35–36 hr later.

IVF and Embryo Culture

Culture conditions for oocytes and embryos, as well as IVF procedures were as previously described for the Norfolk program (5). Culture of oocytes and embryos occurred in organ culture dishes kept at 5% CO_2 in

air under humidified conditions. Incubation medium consisted of modified Ham's F-10 (GIBCO Laboratories, Grand Island, NY) supplemented with human fetal cord serum, human serum albumin (Irvine Scientific, Santa Ana, CA), or synthetic serum substitute (SSS) (Irvine Scientific). Sperm samples were prepared by either standard swim-up methods or after Percoll gradient centrifugation. Insemination concentration was dependent on sperm morphology. Oocytes were classified according to nuclear maturity at aspiration and inseminated as follows: metaphase II oocytes, 3–5 hr after aspiration; metaphase I oocytes, 3–5 hr after first polar body extrusion; prophase I oocytes, 24–29 hr after aspiration. Oocytes were evaluated for the presence of pronuclei 12 to 19 hours after insemination. When prophase I oocytes were utilized, they were first incubated overnight in Ham's F-10 supplemented with 15% SSS to allow for maturation prior to insemination. Embryos were evaluated daily for cleavage status and their morphology graded using criteria previously published (5).

Blastomere Isolation and Fixation

The fresh two pronuclear and viable postthaw embryos were cultured in Hams F-10 medium supplemented with 15% SSS for 48 hr. Each embryo was placed briefly (2–3 sec) in acidified Tyrodes solution (6) to effect zona pellucida removal, and then washed and transferred to a culture dish containing calcium and magnesium-free Dulbecco's phosphate-buffered saline (GIBCO) for 10–15 min to decrease cell-to-cell contacts. Blastomere separation was facilitated by gentle pipeting through a fine-drawn glass pipet. Individual blastomeres were placed in a hypotonic solution consisting of 1% sodium citrate and 6 mg/ml bovine serum albumin for 5 min, during which time they were evaluated for nuclear status under an inverted microscope. Although the nuclear status before fixation was recorded, this was sometimes found to be incorrect, especially in cases where the cytoplasm demonstrated excessive granularity. For this reason, all blastomeres were fixed, even if a nucleus could not be identified under the inverted microscope. The absence of a nucleus in a blastomere was not a failure of the fixation procedure, since cytoplasm was present and could be readily identified on the slide. Also, some blastomeres lacked a distinct, membrane-bound nucleus but a clear, lightened area was present within the cytoplasm. Some of these blastomeres were found to contain metaphase chromosomes.

Following the 5-min incubation in hypotonic solution, individual blastomeres were transferred to a glass microscope slide. All slides were previously cleaned with methanol and marked with a small circle on the bottom using a diamond tip pen, indicating the approximate location of the nucleus. Immediately before the microdroplet dried, 8.5 μ l of fixative (methanol:acetic acid 3:1) was dropped on top of the blastomere, lysing the cell membrane and fixing the nucleus. The fixative was prepared fresh and stored on ice during the procedure. Gentle blowing across the slide was used to free the nucleus of remaining cytoplasm. The fixed nucleus was immediately located using a phase-contrast microscope. The location of the nucleus (or nuclei) was recorded with a Field Finder microscope slide (Fisher Scientific, Pittsburgh, PA). All slides were stored at 8°C until the FISH procedure was performed.

Fluorescence In Situ Hybridization

FISH was performed using a mixture of DNA probes (Vysis, Downer's Grove, IL) specific for chromosomes X, Y, and 18 directly labeled with Spectrum Green, Spectrum Orange, and Spectrum Aqua fluorochromes, respectively. The probes were purchased predenatured and premixed with appropriate hybridization buffer. Each nucleus, fixed onto an individual glass microscope slide, was denatured in the following way: 100 μ l of denaturing solution, consisting of 70 μ l formamide (United States Biochemical, Cleveland, OH), 10 μ l 20X SSC (Vysis), and 20 μ l water, was placed on top of the nucleus and covered with a 22 mm $\times$ 40 mm coverslip. The microscope slide was then placed on a glass plate in an oven at 80°C for 5 min. After removing the coverslip, the denatured nucleus was dehydrated in an ethanol series (70%, 85%, 100%) for 1 min in each dilution. Each slide was allowed to air dry before being placed on a slide warmer at 50°C for 2–3 min. Two microliters of the probe mixture were applied to the target area and covered with a small coverslip prepared by quartering a 22 mm $\times$ 22 mm coverslip using a diamond-tip pen. After excluding air bubbles, the coverslip was sealed with rubber cement. Hybridization was allowed to proceed overnight in a moist chamber at 37°C. The posthybridization washes used to remove excess probe consisted of a 2-min wash in 0.4X SSC/0.3% NP-40 at 73°C followed by a 1-min wash in 2X SSC/0.1% NP-40 at room temperature. After allowing the slides to air dry protected from direct light, 10 μ l DAPI (Vysis) counterstain was applied to the target area and covered with a 22 mm $\times$ 22 mm coverslip.

Fluorescence microscopy for evaluation of the nuclei was performed on a Nikon epifluorescent microscope (Garden City, NY) equipped with four single bandpass filter sets: a Nikon filter set for viewing ultraviolet light was used to locate the DAPI-counterstained nuclei; a filter set (Nikon) for viewing green fluorochromes was used to identify chromosome X; a Nikon filter set for viewing red fluorochromes was used to visualize chromosome Y; and a Vysis filter set for viewing the aqua fluorochrome was used to identify chromosome 18. In addition, a Vysis triple bandpass filter set that allows simultaneous viewing of all three fluorochromes was used to photograph the nuclei. A computerized image analysis system was not used at any time. Initial location of the nucleus was performed using the Field Finder microscope slide and the coordinates recorded during the fixation procedure.

Scoring Criteria

The scoring criteria described by Hopman and co-workers (7) were followed. Signals of low intensity, most likely the result of cross-hybridization to nontarget DNA, were not scored. Double signals, two signals found close together or interconnected, which may represent sister chromatids or split signals due to the nature of the target alphoid sequences, were scored as one signal.

In addition, since all or most of the blastomeres from each embryo were analyzed, the criteria described by Munne *et al.* (8) were used to distinguish FISH failure from true mosaicism:

- (a) blastomeres with one signal per chromosome analyzed were considered haploid cells;
- (b) blastomeres with three or more signals per chromosome analyzed were considered polyploid cells;
- (c) embryos in which all of the blastomeres analyzed contained the same abnormality, whether aneuploidy, haploidy, or polyploidy, were considered genetically abnormal;
- (d) embryos in which sibling blastomeres had extra or missing signals compensating for those missing or extra in another blastomere were considered "compensated mosaic" embryos; and
- (e) all other blastomeres with more or less than two signals for each chromosome analyzed were considered FISH failures. In addition, blastomeres with a nucleus observed under phase microscopy following fixation, but either not found or covered by debris following FISH analysis, were considered FISH failures.

Lymphocyte Controls

Lymphocyte control slides, prepared using standard cytogenetic technique, were used to determine the efficiency of the FISH procedure. A total of 300 nuclei obtained from a healthy male were scored.

Embryo Classification

The following definitions, based on the nuclear morphology, were used to classify the embryos:

(a) *normal*—an embryo in which all blastomeres analyzed contain a single nucleus with the same normal diploid complement;

(b) *mosaic*—an embryo containing blastomeres with two or more different genetic complements, indicating the presence of two or more different cell lines. In a diploid mosaic, one of the cell lines is a normal diploid complement. Also included in this group are all embryos with one or more multinucleated blastomere and/or blastomere(s) with a fragmented nucleus;

(c) *aneuploid*—an embryo in which all of the blastomeres analyzed contain the same genetic abnormality;

(d) *abnormal nuclear morphology*—an embryo in which 50% or more of the blastomeres demonstrated abnormal nuclear morphology, i.e., they were either multinucleated or contained a fragmented nucleus. Figure 1 illustrates the difference between a multinucleated blastomere (A) and one containing nuclear fragments (B). In a multinucleated blastomere, the nuclei, which usually number two but occasionally number three or more, are roughly the same size and round in shape. In a blastomere with a fragmented nucleus, many (>5) smaller, irregularly shaped pieces of nuclear material are present.

Statistical Analysis

Data were collected on two groups of embryos. One group (immatures) consisted of 61 embryos derived from oocytes that were prophase I at collection, and the other group (matures) consisted of 65 embryos derived from oocytes that were either metaphase I or II at collection. Patient information was compared between the two groups using unpaired *t*-test for the following continuous variables: age at retrieval, number of amps of hMG administered, number of amps FSH administered, number of mature oocytes aspirated, number of mature oocytes that fertilized normally, and the number of embryos transferred.

Comparisons between the two groups were made with contingency table analysis (χ^2) for categorical variables: stimulation protocol, highest transfer grade, establishment of a fresh pregnancy, and establishment of a cryopregnancy.

Comparisons between the two groups with respect to embryo information were made as follows. An unpaired *t*-test was used to compare the mean number of blastomeres per embryo. A χ^2 test was used to compare the number of embryos in each group classified as normal, mosaic, aneuploid, and containing abnormal nuclear morphology according to the classification system outlined above. Chi-square tests were used to compare embryo classification with the following variables: patient age, type of infertility, stimulation protocol, highest grade of embryo(s) transferred, and fresh and cryopregnancy status. In addition, a χ^2 test was used to compare the number of blastomeres in each group that were lost during fixation, were anuclear, contained a single nuclei, contained multiple nuclei, contained a fragmented nucleus or nuclei, and contained metaphase spreads, independent of the embryos from which they came. A nonparametric rank test was used to compare the number of blastomeres in each group that were lost during the FISH procedure, were lost during fixation, were anuclear, contained a single nuclei, contained multiple nuclei, contained a fragmented nucleus or nuclei, and contained metaphase spreads.

Photography

Embryos and individual blastomere were evaluated and photographed using a Nikon inverted microscope equipped with Hoffman optics (Hoffman Modulation Contrast, Greenvale, NY) and Kodak Technical Pan film (ASA 100). The fixed nuclei were evaluated and photographed using an Olympus phase-contrast microscope (Olympus America, Inc, Melville, NY) and Kodak Technical Pan film (ASA 100). Fluorescent signals were evaluated and photographed using a Nikon epifluorescent microscope and Kodak Gold film (ASA 400).

RESULTS

Comparison of Two Patient Groups

Comparisons were made between the two groups, designated as mature and immature based on the nuclear status of the oocyte at aspiration, to compare

Fig. 1. Photographs illustrating the difference between a multinucleated blastomere (A) and a blastomere containing fragmented nuclei (B). The blastomeres were obtained from two different embryos. Original magnification, $\times 600$.

the patient's responses to stimulation. The average age of patients in the mature group was 35.5 ± 2.7 years compared to 28.0 ± 3.8 years for patients in the imma-

ture group. This difference was highly significant ($P < 0.0001$). The fertilization rate was significantly higher in the mature group than in the immature group (88%

Table I. IVF Parameters for Mature Oocytes in the Mature and Immature Groups

Group	No. Mature oocytes aspirated	Fertilization rate (%)	No. Embryos transferred	Embryo grade 1 or 2 (%)	Fresh pregnancy rate (%)	Cryopregnancy rate (%)
Mature	15.5 ± 10.9	231/264 (88) ^a	3.4 ± 1.4	24/27 (89)	6/15 (40)	5/8 (63)
Immature ^b	16.1 ± 6.8	291/436 (67) ^a	3.7 ± 1.3	13/15 (89)	14/27 (53)	4/5 (80)

^a $P = 0.00001$.^b Information on fertilization, transfer, and pregnancy outcome refer to mature oocytes received by recipient(s).

vs. 67%, $P = 0.00001$). No other differences were statistically significant (Table I). No significant differences were noted in the number of mature oocytes aspirated, rate of fertilization, number of embryos transferred, or fresh or cryopreserved pregnancy rates between the two groups. In the case of oocyte donors, some parameters will pertain to their respective recipient(s). Twenty-two oocyte donor cycles had one recipient and three had two recipients.

Nuclear Status of Individual Blastomeres

A total of 331 blastomeres from 65 embryos were analyzed in the mature group. A total of 342 blastomeres from 61 embryos were analyzed in the immature group. The mean number of blastomeres per embryo was 5.1 ± 2.3 and 5.6 ± 2.6 for the mature and immature groups, respectively (nonsignificant). Table II presents the nuclear status of the individual blastomeres independent of the embryos from which they came. Also included are those blastomeres lost during the fixation procedure. The mature group contained a significantly greater number of blastomeres with a single nucleus compared to the immature group (56% vs. 33%, $P < 0.0001$). The immature group had both a significantly greater number of blastomeres with a fragmented nucleus (19% vs. 3%, $P < 0.0001$) or anuclear blastomeres (33% vs. 25%, $P = 0.0181$) compared to the mature group. No other differences were statistically significant (Table II).

Oocyte Maturity and Embryo Classification

Table III presents the classification of each of the embryos analyzed according to the system outlined in the methods section. A significantly greater percentage of embryos in the mature group were classified as normal compared to embryos in the immature group (23% vs. 3%, $P = 0.0012$). A significantly greater percentage of embryos in the immature group were classified as abnormal compared to matures (64% vs. 35%, $P = 0.0014$). No other differences were significant (Table III). In addition, if the mosaic embryos in each group are reevaluated disregarding multinucleated blastomeres and blastomeres containing a fragmented nucleus, 15 of 65 (23.1%) embryos in the mature group are mosaic compared to 13 of 61 (21.3%) embryos in the immature group (nonsignificant).

The relationship between oocyte maturity and embryo classification for embryos with less than four blastomeres and embryos with four or more blastomeres was also evaluated. There were no significant differences between the mature and immature groups when only those embryos with less than four blastomeres were considered. However, when only those embryos with four or more blastomeres were considered, a significantly greater percentage of matures were normal compared to immatures (30% vs. 4%, $P = 0.0009$). Also, a significantly greater percentage of immatures were classified as having abnormal nuclear morphology compared to matures (64% vs. 28%, $P = 0.0006$).

Table II. Nuclear Status of Individual Blastomeres in the Mature and Immature Groups

Group	Single nucleus (%)	Multiple nuclei (%)	Fragmented nucleus (%)	Anuclear (%)	Metaphase spreads (%)	Lost during fixation (%)	Total
Mature	185 (56) ^a	44 (13)	11 (3) ^b	82 (25) ^c	2 (1)	7 (2)	331
Immature	114 (33) ^a	37 (11)	65 (19) ^b	113 (33) ^c	9 (3)	4 (1)	342

^a $P < 0.0001$.^b $P < 0.0001$.^c $P = 0.0181$.

Table III. Oocyte Maturity and Embryo Classification

Group	Normal (%)	Aneuploid (%)	Mosaic (%)	Abnormal nuclear morphology (%)	Did not cleave (%)	Technical failure (%)
Mature	15/65 (23) ^a	0 (0)	22/65 (34)	23/65 (35) ^b	3/65 (5)	2/65 (3)
Immature	2/61 (3) ^a	2/61 (3)	14/61 (23)	39/61 (64) ^b	3/61 (5)	1/61 (2)

^a $P = 0.0012$.^b $P = 0.0014$.

Patient Variable and Embryo Classification

No significant relationships were observed between the following patient variables and embryo classification for either matures or immatures: patient age, highest embryo grade at transfer, and establishment of a pregnancy. In the mature group, there was no significant relationship between the following types of infertility and embryo classification: idiopathic, immunologic, luteal-phase defect, male factor, and tubal infertility. There were too few embryos in the remaining groups (endometriosis, ovulatory, polycystic ovarian disease) for statistical analysis. Also, in the mature group there was no significant relationship between stimulation protocol and embryo classification.

Lymphocyte Controls

Of the 300 lymphocyte nuclei scored, 297 contained one signal for chromosome X (99%) and the remaining 3 nuclei contained no signal for X (1%); 293 nuclei contained one signal for chromosome Y (98%), 4 contained no signal for Y (1%), and 3 contained 2 signals for Y (1%); and 286 nuclei contained two signals for chromosome 18 (95%), 9 contained one signal for 18 (3%), and 5 contained 3 signals for 18 (2%).

Efficiency of the FISH Procedure

In the mature group, 7 of 331 (2%) blastomeres were lost during the fixation procedure and 2 of 234 (0.6%) nuclei were either covered with cytoplasm or were without clear signals following the FISH procedure. In the immature group, 4 of 342 (1.2%) were lost during the fixation procedure, and 5 of 338 (1.5%) nuclei were either covered with cytoplasm or were without clear signals following FISH.

DISCUSSION

In the present study, FISH analysis using DNA probes specific for chromosomes X, Y, and 18 found

that only two (3%) of the embryos resulting from in vitro matured oocytes were normal. This was significantly lower ($P = 0.0012$) than the percentage of normal embryos (23%, Table III) resulting from the fertilization of in vivo matured oocytes. This study also evaluated nuclear morphology and found that 64% of the embryos in the immature group were abnormal, compared to 35% in the mature group (Table III). While the incidence of multinucleated blastomeres was the same in the two groups, a significantly greater percentage of blastomeres in the immature group (19%) contained a fragmented nucleus compared to their counterparts in the mature group (3%, $P < 0.0001$, Table II).

The presence of nuclear fragmentation in the human embryo has been reported by other investigators (9,10), and it is speculated to represent a form of programmed cell death (PCD) via apoptosis, a process that may also result in anuclear blastomeres and cleavage arrest (10,11). Hardy *et al.* (9) reports that 2.4% of normally fertilized embryos contain a fragmented nucleus, a finding similar to our reported rate of 3.0% in embryos resulting from in vivo matured oocytes.

Hardy and co-investigators (9) also noted the presence of binucleate blastomeres that occurred in 17% of normally fertilized embryos at the 2- to 4-cell stage and in 65% of those at the 9- to 16-cell stage. The authors speculate that binucleate blastomeres most often arise through a failure of cytokinesis between the second and fourth cleavage divisions. In the present study, there was no difference in the incidence of multinucleated blastomeres between the groups (Table II), with the rate of binucleate nuclei being 80% and 63% in the mature and immature groups, respectively (data not shown). Our analysis suggested that acytokinesis and nuclear amitotic splitting contributed to the formation of binucleate blastomeres in human embryos. We observed binucleated blastomeres in which each nucleus contained the same number of signals for chromosomes X, Y, and 18 as sibling mononucleated cells and others in which the sum of the signals in the two nuclei was the same as that observed in sibling

mononucleated cells. However, the number and distribution of chromosomes in the nuclei of binucleate blastomeres varied greatly, suggesting that other mechanisms may be involved as well. Our results are comparable to that reported by Munne and co-workers (12) who found that 11.5% of blastomeres from arrested human embryos were multinucleated. They also reported that a vast majority (79.6%) of the multinucleated blastomeres contained two nuclei.

Another abnormality observed in preimplantation embryos is the presence of anuclear blastomeres. Munne *et al.* (12) reported the incidence of anuclear blastomeres in human embryos as follows: 24.8% of blastomeres in arrested embryos; 11% of blastomeres in slow and/or fragmented embryos; and 4.4% of blastomeres in normally developing embryos. In the present study, 25% of the disaggregated blastomeres in the mature group were anuclear and 33% of their counterparts in the immature group were anuclear. One possible explanation for the higher incidence of anuclear blastomeres in the study reported here is that all blastomeres, even those without a visible nucleus under the inverted microscope, were fixed and recorded. Some of these may have been cytoplasmic fragments. In later stages of development (8–12 cells), it is difficult to distinguish an anuclear blastomere from a cytoplasmic fragment.

The incidence of mosaicism in human embryos also may be related to in vitro culture conditions. Mosaicism is defined as the presence of two or more different cell lines within a single embryo. In a study in which normally developing human embryos obtained from four IVF centers were evaluated using FISH with DNA probes specific for chromosomes X, Y, 13, 18, and 21, Munne and co-investigators (13) found that the rate of mosaicism differed greatly between the centers, ranging from 11% in one center to 52% in another. They concluded that in vitro culture conditions and/or hormonal stimulation protocols may affect the incidence of mosaicism in the resulting embryos, perhaps explaining in part the differences in success rates between IVF centers. In the study reported here, 34% of embryos in the mature group were mosaic compared to 23% in the immature group. This difference was not statistically significant, perhaps due to the fact that the embryos in both groups were cultured under identical conditions. As outlined in the methods section, embryos with one or more multinucleated blastomeres and/or blastomeres with a fragmented nucleus were automatically classified as mosaic. We acknowledge that this classification system may result in an overestimation of the incidence of chromosomal mosa-

icism in preimplantation embryos. In an attempt to address this issue, we reevaluated all the mosaic embryos in each group considering only those blastomeres with a single nucleus (i.e., disregarding multinucleated blastomeres and blastomeres with a fragmented nucleus). Considering mononucleated blastomeres only, seven of the mosaic embryos in the mature group and one of the mosaic embryos in the immature group would be classified as normal, changing the incidence of mosaicism in the mature group from 34% to 23%, and in the immature group from 23% to 21%. Although this difference is still not statistically significant, it did make the difference in the incidence of normal embryos in the two groups more apparent (34% in the mature group vs. 5% in the immature group).

We also investigated the incidence of aneuploidy for chromosomes X, Y, and 18 in the two groups of embryos. Aneuploidy, defined as a chromosomal constitution different from the normal diploid constitution by loss or duplication of one or more chromosomes or chromosome segments, was found to be low in both the mature (0%) and immature (3%) groups. This is in contrast to a rate of 5.3% reported by Munne *et al.* (14). There are several possible explanations for this discrepancy between the two studies. First, the average age of patients in the study presented here was low, 35.5 ± 2.7 years and 28.0 ± 3.8 years in the mature and immature groups, respectively. While our data include only one embryo from a patient over 38 years of age, more than a third of the embryos in Munne's study were from patients 40 years of age or older. In addition, although Munne reported a significant increase in aneuploidy with maternal age, this increase was mostly due to an increase in aneuploidy for chromosomes 13 and 21. Indeed, the trend toward increasing aneuploidy for gonosomes and chromosome 18 in Munne's study, which began only after 40 years of age, did not reach statistical significance. Last, according to Munne's classification system, embryos are both mosaic and aneuploid when the average of their cells, corrected for error in ploidy, are aneuploid for a specific chromosome. In the present study, such embryos were classified as mosaic only.

We acknowledge several differences in the two groups of embryos in our study that may have contributed to the results we observed. For example, all the embryos in the mature group were cryopreserved and subsequently thawed before analysis. In contrast, all but seven of the embryos in the immature group were fresh. In addition, embryos in the immature group, by virtue of the fact that they were derived from prophase I oocytes that required a 24- to 36-hr incubation period

to complete nuclear maturation before insemination, were in culture one day longer than embryos in the mature group. It is possible that this extra day in culture conferred instability to the nuclear membrane in blastomeres from these embryos, observed as nuclear fragmentation in our study. If so, perhaps a change in culture conditions would correct this problem. Indeed, Lanzendorf and co-workers (15) demonstrated that embryos derived from prophase I oocytes differ in culture requirements than embryos derived from metaphase I or II oocytes in the cynomolgus monkey.

We also caution that our findings may not apply to all prophase I oocytes obtained following COH. In a study performed at the Jones Institute, Moffitt and co-investigators (16) found that prophase I oocytes from large cohorts and cohorts with a greater percentage of prophase I oocytes may have a greater developmental potential than those from smaller cohorts and cohorts with a greater percentage of metaphase I and II oocytes. In addition, the results presented here may not apply to oocytes matured in vitro following collection during an unstimulated cycle.

In summary, the present findings suggest that few embryos derived from prophase I oocytes are normal, perhaps explaining in part why they rarely establish pregnancies in our IVF program. Comparison of the incidence of nuclear abnormalities observed in blastomeres in the two groups revealed that embryos derived from prophase I oocytes had a significantly higher incidence of both anuclear blastomeres and blastomeres with a fragmented nucleus than their counterparts derived from mature oocytes. Because nuclear fragmentation is a hallmark of programmed cell death via apoptosis, which has been implicated in the processes of follicular atresia in vivo and cleavage arrest in vitro, we speculate that prophase I oocytes obtained following COH may originate from follicles in early stages of atresia.

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