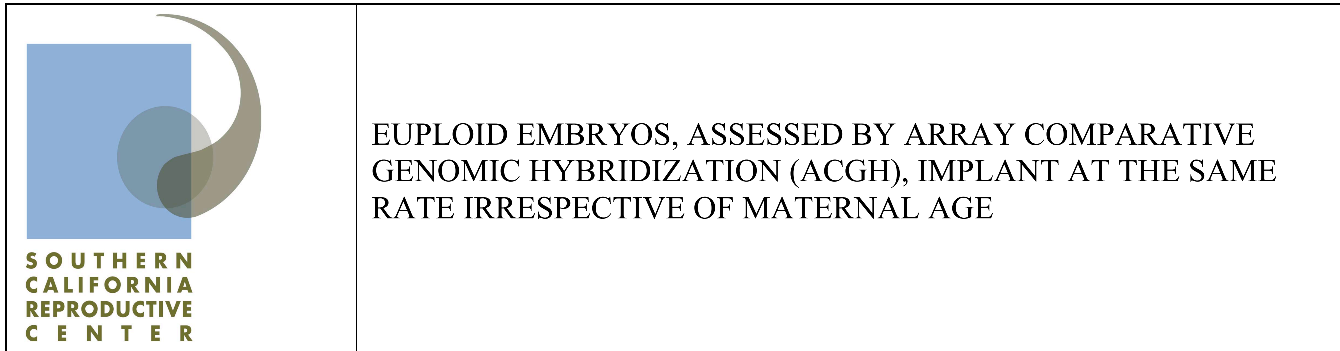

EUPLOID EMBRYOS, ASSESSED BY ARRAY COMPARATIVE GENOMIC HYBRIDIZATION (ACGH), IMPLANT AT THE SAME RATE IRRESPECTIVE OF MATERNAL AGE

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ABSTRACT

Objective: To assess the relationship between maternal age, chromosome abnormality, embryo implantation and pregnancy loss.
Design: A multi-center retrospective study.
Material and Methods: Only patients with follow up data were included in the study. Patients from several infertility clinics around the United States underwent stimulated IVF cycles with the aim of having embryo biopsy and preimplantation genetic screening (PGS). Embryo biopsy was performed on either day-3 (cleavage stage) or day 5-6 (blastocyst stage) of embryo development. Cleavage stage analysis involved biopsy of a single cell, followed by microarray comparative genomic hybridization (aCGH) and transfer of euploid embryos on day-5. Analysis of blastocysts involved biopsy of approximately 3-7 cells from the trophectoderm followed by aCGH, cryopreservation/vitrification and transfer of euploid embryos in a subsequent hormone replacement cycle or fresh transfer of euploid blastocysts.
Results: Aneuploidy rates for day-3 biopsy for maternal age groups 30-34, 35-39 and 40-42 were respectively 49.5 %, 66.8 % and 77.0% ($p<0.001$). Aneuploidy rates for blastocyst biopsy for these same age groups were, respectively, 29.8 %, 46.6 % and 67.1 % ($p<0.001$). The implantation rates for euploid embryos tested at the cleavage stage were 40.7 %, 41.7 % and 38.7 % for the three age classes, respectively, while for euploid blastocysts implantation rates were 51.6 %, 50.2 % and 44.9 %. The differences in implantation rates related to age were not significant for cleavage stage or blastocyst stage biopsy, a very important finding. The ongoing pregnancy rates, per transfer, for day-3 biopsy were 49.3%, 50.4% and 42.1%, respectively. For blastocyst biopsy the ongoing pregnancy rates, per transfer were 59.2%, 53.4%, and 57.1%, respectively. In both cases there was no significant difference in the ongoing pregnancy rate per transfer as maternal age increased. Pregnancy loss rates ranged between 3.9%-2.3% per cycle on day-3 biopsies, and 6%-1.9% in blastocyst biopsies, again with no significant increase with maternal age.
Conclusions: Across all ages, better outcomes were achieved for euploid blastocyst transfer following day-5 biopsy rather than blastocyst transfer subsequent to day-3 biopsy. This may be due to less

damage to the embryo during trophectoderm biopsy, higher accuracy of day-5 PGS and/or better uterine receptivity in some cases where embryos were cryopreserved following biopsy and transferred in a controlled endometrium development (CED) cycle. Interestingly, implantation rates and ongoing pregnancy rates per transfer for euploid embryos (cleavage stage and blastocyst stage biopsy) were not significantly different between young and old patients. This is in stark contrast to data from cycles in which embryos are transferred without chromosome screening, which show a rapid decline in implantation rates associated with advancing female age. Mounting data from our group and others suggests that the dramatic decline in IVF treatment success rates with female age is primarily caused by aneuploidy. Although further randomized trials are needed for conclusive proof, it is possible that embryo biopsy and chromosome screening may allow for better IVF outcomes in all patient age populations. This opens up the prospect of applying elective single embryo transfer (eSET) in virtually all cases regardless of maternal age and achieving excellent implantation and pregnancy rates.

Key Words: preimplantation genetic screening, embryo biopsy, aneuploidy, elective single embryo transfer

INTRODUCTION

Aneuploidy in spontaneous abortions has been reported to be as high as 70 % in some studies (1, 2, 3, 4, 5, 6). Large studies on human embryos have shown much higher rates of chromosome abnormalities than seen in established clinical pregnancies suggesting an elimination of aneuploidies takes place (7, 8, 9, 10, 11). Indeed, a recent study shows a decrease in chromosome abnormalities from cleavage stage to blastocyst stage (11), indicating that the loss of chromosomally abnormal embryos begins during preimplantation development. The high frequency of aneuploid preimplantation embryos may provide an explanation for the low implantation and birth rates achieved by assisted reproductive treatments (ART), especially for women of advanced reproductive age. For example, of 7753 embryos analyzed by microarray comparative genome hybridization (aCGH), between 30% (maternal age <35) and 87% (>42 years) of blastocysts analyzed harbored chromosome abnormalities, most of which are considered incompatible with establishment of a viable pregnancy (11).

Preimplantation genetic diagnosis (PGD) for aneuploidy, also known as preimplantation enetic screening (PGS), was first attempted by Munne et al (12). By screening embryos for chromosome abnormalities and transferring only those found to be euploid, the expectation was that increased implantation and pregnancy rates and reduced pregnancy loss rates would be observed. The first techniques used for PGS were polar body biopsy or cleave-stage biopsy followed by fluorescence *in situ* hybridization (FISH) analysis. This version of PGS can be called first generation PGS. Initial studies with first generation PGS suggested that implantation rates increased while loss rates decreased (13, 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23). However, other studies, including several randomized controlled trials (RCT), showed no benefit (24, 25, 26, 27, 28, 29, 30, 31) or, worse yet, a negative impact on implantation, pregnancy or loss rates (32, 33).

The discrepancies between these studies have been debated extensively, and are probably due to technical differences (34). Specially, the Mastenbroek et al. (32) manuscript showed that in their hands, 60% of implantation potential was lost solely due to the performance of cleavage-stage biopsy in their laboratory. Later studies by Treff et al. (35) have shown that cleavage-stage biopsy has the potential to reduce implantation rates, a negative affect which is not seen for blastocyst biopsy. Thus, the selection of euploid embryos may or may not compensate for the damage caused by the biopsy,

depending on the center performing the biopsy. In addition, the use of FISH suffers from a number of issues inherent in the technique, including a limited number of unique fluorescent colors allowing only a handful of chromosomes to be tested in each case (5-12), but worse, cell fixation and FISH interpretation are not standard techniques and are also technician dependent. For example, FISH error rates reported have ranged from 50% (36) to 3.7% (37). In the best hands, first generation PGS may have provided some benefits, but it was unable to convincingly prevent the deterioration in IVF outcomes, especially implantation rate, seen with advancing maternal age.

It became clear that a new version of PGS was needed. An ideal technique would allow for the simultaneous analysis of all 24 chromosomes (autosomes 1-22, X and Y), would be robust and less prone to technical issues that could lead to errors and misdiagnosis than were earlier FISH methods, and would incorporate a biopsy type less detrimental to embryo development.

The first comprehensive analysis technique appearing after FISH was comparative genomic hybridization (CGH) (38, 39, 40), but it was challenging to put into clinical practice because it needed at least three full days for the analysis to be completed. This meant that application to cleavage stage embryos had to be combined with cryopreservation. However, the slow freezing methods available at the time were detrimental to biopsied day-3 embryos (41). Blastocyst biopsy was perfected clinically by McArthur et al. (42). Finally, the advent of vitrification, which permitted the safe cryopreservation of biopsied embryos, (43) allowed all the components for second generation PGS to be assembled: complete chromosome screening (via CGH); less damaging embryo biopsy (at the blastocyst stage); and enough time to carry out the test (afforded by vitrification). The first clinical complete chromosome analysis of blastocysts was published by Schoolcraft et al. (44), achieving exceptional implantation rates in a poor prognosis patient group (advanced reproductive age, multiple IVF failures). CGH was later displaced by more automated techniques such as aCGH (45, 46, 47, 48) single nucleotide polymorphism (SNP) arrays (49, 50, 51), and quantitative fluorescent PCR (52). Of these techniques, aCGH (53) and qPCR (54) have been shown in randomized controlled trials to improve pregnancy rates.

The current study aims to determine if aCGH has the potential to arrest the decline in implantation rates associated with advancing maternal age. If so, the underlying hypothesis of PGS would be finally proven, that is, the bulk of implantation failure and pregnancy loss with advancing maternal age is due to chromosome abnormalities and it can be prevented by replacing euploid embryos.

MATERIALS AND METHODS

This data set includes patients that underwent PGS by aCGH at a number of fertility clinics around the United States and for which it was possible to obtain follow up information. Excluded were oocyte donor cycles as well as patients 43 and older. This last group was excluded because the majority of patients did not have euploid embryos for transfer.

Briefly, following initial infertility testing, patient's ovaries were stimulated using gonadotropins to produce multiple mature oocytes, which were retrieved using ultrasound guidance. Oocytes were inseminated using conventional IVF techniques or using intracytoplasmic sperm injection (ICSI) (55). Approximately 18 hours after insemination, fertilization was assessed by microscopic evaluation. Normally fertilized embryos were further cultured until either day-3 or day-5/6 of embryo development, usually with sequential media, however, some laboratories employed a single culture medium for all phases of embryo growth.

Embryo biopsy took place either on the morning of day-3 of embryo development or at blastocyst stage, usually on day-5 but on day-6 for some embryos growing at a slower rate. Biopsy was performed using laser assisted hatching followed by removal of a single cell (cleavage stage biopsy) or a clump of trophectoderm tissue (blastocyst biopsy). The biopsied material was washed in clean biopsy wash medium (supplied by Reprogenetics, Livingston, NJ). Following washing, the cell or tissue was placed in the supplied transport tube labeled with the patient initials and embryo number and kept on ice until being shipped to the genetics lab for testing. All samples were shipped by custom courier or commercial shipping companies and delivered to the genetics laboratory either the same day or early the next morning for testing.

Microarray-CGH was performed as previously described (46). Briefly, following sample receipt in the lab, each tube was opened in a dedicated DNA amplification clean-room, under laminar flow conditions, and the amplification reagents were added (SurePlex, Rubicon Genomics Inc, Ann Arbor, MI, USA/BlueGnome LTD, CPC4, Capital Park, Fulbourn, Cambridge, UK). Following amplification according to the manufacturer's instructions, each sample was loaded onto an agarose gel to check for amplification. A smear of DNA, observed on the gel following electrophoresis, is indicative of positive amplification. All samples that were positive for DNA amplification were taken to the fluorescent labeling steps. Labeling was performed using manufacturer's recommendations with Cy3 dye for test DNA and Cy5 dye for reference male DNA (BlueGnome LTD). After labeling, embryo biopsy samples and reference DNA samples were separately denatured at 74 degrees C prior to being mixed together and added to each microarray. For this study, Version 2 of 24sure microarrays were used (BlueGnome LTD). Microarrays were hybridized at 47 degrees C for at least 4 hours or overnight in a humidified chamber. Following hybridization, each microarray was washed as follows: 10 minutes in 2xSSC/0.05% Tween 20 at room temperature, 10 minutes in 1xSSC at room temperature, 5 minutes in 0.1xSSC at 60 degrees C and 2 minutes in 0.1xSSC at room temperature.

Each microarray was then scanned for green fluorescence at 632 nm and for red fluorescence at 587 nm. Raw images were loaded automatically into BlueFuse software (BlueGnome LTD) allowing for automated evaluation of fluorescent signals. Each sample was scored by a trained technologist who assessed all 24 chromosomes, noting all gains and losses, as well as determining the gender of each sample. A second technologist then scored the sample blindly, with no knowledge of the initial score by technologist number one. A final score for each sample was assigned by comparing the score of technologist one with technologist two. Any discrepancies were noted and were adjudicated by a third technologist and/or the laboratory supervisor or director. Once results for all samples from each patient were finalized, a diagnostic report was prepared and shared with the referring physician ahead of embryo transfer.

Transfer of euploid embryos typically took place on the morning of day-5 for cleavage stage biopsy. For samples biopsied on day-5 or day-6 of embryo development, resulting blastocysts were cryopreserved using either vitrification or slow-cooling techniques. For blastocyst biopsy cases, euploid embryos were transferred in a CED cycle into a uterus free of gonadotropin stimulation. Clinical decisions were left with the individual IVF centers including which embryos to transfer and how many embryos would be transferred. Follow-up information regarding these clinical decisions was shared with the genetics laboratory through secure email exchanges and/or phone calls to individual IVF centers.

The goal of this study was to determine if implantation rates across different maternal ages changed after PGS with embryo biopsy on day-3 (cleavage stage) or blastocyst biopsy, followed by aCGH.

Within each biopsy group, the data was further broken down into three age groups, 30-34, 35-39, and 40-42 years of age at the time of cycle start. Within each group, implantation rate as defined by detection of a fetal sac on ultrasound, ongoing pregnancy rate per transfer and aneuploidy rate (as defined as an abnormal number of chromosomes in the sample) were assessed. In addition, pregnancy loss rate, as defined by loss of a clinical pregnancy following a positive pregnancy test, was also assessed.

RESULTS

A total of 392 cycles of cleave-stage biopsy and 421 cycles of blastocyst biopsy were included in the study, with 3204 and 2388 analyzed embryos, respectively.

As can be seen in Table 1, the implantation rate for the youngest age group, 30-34 year old patients was 40.7% for day-3 biopsy and 51.6% for blastocyst biopsy. For the 35-39 year old patient groups, the most common patient at most IVF centers, the implantation rate for biopsy on day-3 was 41.7%, while for blastocyst biopsy was 50.2%. For the last patient group, 40-42 year old patients, the implantation rate for biopsy on day-3 was 38.7% while the implantation rate for blastocyst biopsy was 44.9%. There was no significant change in implantation rates with increasing maternal age either for biopsy at the cleavage stage or for blastocyst stage. Implantation rates were lower across all age groups when embryos were biopsied at day-3 compared to embryos biopsied at day-5/6 ($p<0.001$).

While implantation and pregnancy rates are a very important factor in infertility treatment, an often overlooked goal of IVF treatment is that the pregnancy must be maintained. Toward this end, we also looked at pregnancy loss rates in each age group and time of biopsy to determine if there were any differences (Table 2). No significant difference was noted between loss rates at any age group or time of biopsy, on average 3% for day-3 and 4.5% for blastocyst biopsy. It is important to note that the loss rates across all age groups were quite low compared to loss rates typically quoted following natural pregnancy and pregnancy following IVF treatment, and that after second generation PGS it did not increase with advancing maternal age, as it does in natural cycles. Of note, it was possible to analyze the chromosomes of three of the 16 pregnancy losses. One was 46XX, one was a trisomy 16, and one was a trisomy 22. The trisomy 22 was determined to be caused by human error, exacerbated by a software design problem, since then corrected.

Since implantation rates and miscarriage rates did not change with advancing maternal age, we would expect that pregnancy rates per transfer would not change either. Indeed, provided that there were euploid embryos for transfer, ongoing pregnancy rate per transfer did not decrease with advancing maternal age (Table 3). Ongoing pregnancy rate per transfer in the day-3 biopsy group was 49.3%, 50.4% and 42.1% for patients 30-34, 35-39 and 40-42, respectively. Ongoing pregnancy rate per transfer in the blastocyst biopsy group was 59.2%, 53.4%, and 57.1% respectively. Again, no statistically significant difference in ongoing pregnancy per transfer was seen in this group.

As expected, aneuploidy rates increased as maternal age advanced (Table 1), no matter the day of biopsy ($P < 0.001$). For day-3 biopsy, aneuploidy rates for patients in the 30-35 year range were 50.8 %, increasing to 66.8 % for the 35-39 year old patients and to 77.0 % for the 40-42 year old patients. For biopsy at the blastocyst stage, aneuploidy rates were 29.8 % for the 30-34 year old patients, 46.6 % for 35-39 year old patients and 67.1 % for patients aged 40-42 years old. Comparing aneuploidy rates between day-3 and day-5/6 biopsy for each patient age, rates for each age group were lower in the blastocyst biopsy group than for the day-3 biopsy group, across the board.

Although implantation rates and pregnancy rates per transfer did not decrease with advancing maternal age, the number of cycles with no transfers due to lack of euploid embryos was increased. When cycles without a transfer are included, it resulted in a decreasing ongoing pregnancy rate per cycle started with advancing maternal age. As can be seen in Table 3, ongoing pregnancy rate per cycle for the day-3 biopsy group were 42.9%, 39.1% and 24.1%, respectively, a statistically significant drop ($P<0.01$). For blastocyst biopsy, ongoing pregnancy rates per cycle started were 50.6%, 42.2% and 33.3 % respectively. Again, a statistically significant decrease in ongoing pregnancy per cycle started was seen in this group ($P<0.001$).

DISCUSSION

The main conclusion of this study is that implantation rates of euploid embryos are constant at any maternal age, no matter the day of biopsy. Essentially, second generation PGS abrogates the maternal age effect on implantation rates observed in ART. This finding confirms that the main cause of decreasing implantation rates with advancing maternal age is chromosome abnormality.

Overall, blastocyst biopsy produced better outcomes than day-3 biopsy across all ages when looking at implantation rate and ongoing pregnancy rate per transfer. This may be due to less damage to the embryo during trophectoderm biopsy (35). These differences were less stark than expected from previous studies (32, 35), perhaps due to the fact that not all centers use identical biopsy techniques (day-3 or blastocyst) nor do they have a homogeneous patient population or apply PGS to the same groups of patients. The lack of standardization of biopsy methodology is a limitation of this study. However, since data was obtained from multiple independent IVF clinics, it is likely that the results are, in general, representative of what can be expected when these methods are clinically applied.

The majority of cycles using blastocyst biopsy in this study involved cryopreservation and transfer in an unstimulated cycle. While cryopreservation may entail some risk to the embryo, it also has the potential advantage that uterine receptivity may be enhanced in comparison with stimulated cycles. This may go some way to explaining the superior results obtained for embryos biopsied at the blastocyst stage. However, recent studies using blastocyst biopsy and PGS in conjunction with fresh embryo transfer have shown significant improvement in ongoing pregnancy rates (53, 54), and thus enhanced receptivity might not be a key advantage of the blastocyst biopsy approach. On the other hand, not all laboratories attempting fresh transfer have had great success and it seems that the timing of fresh day-6 transfer is critical (personal communication). This may be further proof that the transfer window in stimulated cycles is quite small and easily missed. As with first generation PGS using cleavage-stage biopsy, a lack of standardization concerning blastocyst biopsy and transfer could lead some clinics to obtain suboptimal results and therefore conclude that the new PGS techniques are ineffective. Consequently, it is important that second generation PGS is standardized quickly and best practice guidelines established.

In addition to achieving pregnancy following IVF treatment, maintaining pregnancy to delivery is vitally important. Loss rates in patients over 35 years old are reported in the 20%-25% range (56) meaning that in this group 1/4-1/5 of all recognized pregnancies are lost before delivery. The pregnancy loss rate in this study was 4.4% overall. Clearly, the transfer of euploid blastocysts following aCGH is having a positive effect on pregnancy loss in this population, leading to higher live birth rates following PGS testing.

Clinicians that offer chromosome testing have to make their patients aware that embryo transfer will not happen in every cycle, even in younger patients. While the improved embryo selection afforded by

methods such as aCGH can increase implantation rates across all age groups, it cannot improve pregnancy rates in patients that fail to produce at least one chromosomally normal embryo. Unfortunately, such patients become increasingly common with advancing maternal age, a problem that currently has no solution other than oocyte donation. As shown by Ata et al. (11), depending on cohort size and number of blastocysts available, 61% of women 40-42 with four or fewer blastocysts will have at least one euploid embryo available for replacement, this number increases with cohort size and decreasing maternal age. However, as seen in the Yang study, in good-prognosis patients 35 and under, 100 % of patients in the testing arm (aCGH at the blastocyst stage) achieved transfer of one euploid embryo.

After advanced maternal age and low implantation rates, the next great problem in assisted reproductive technology is multiple pregnancies. The use of second generation PGS to achieve improved embryo selection and high implantation rates and the ability to reliably vitrify blastocysts mean that there is now little reason to replace more than one euploid blastocyst. In that respect, it is important to point out the excellent study of Yang et al. (53), a recent RCT using aCGH, which simultaneously demonstrated improved ongoing pregnancy rates and an absence of any multiple pregnancies.

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Table 1: Comparison of Implantation Rate and Aneuploidy Rate Between Biopsy at Day 3 or Day 5/6 by SART Age Group

Day 3 Biopsy			Day 5/6 Biopsy		
Age Group	Implan. (+ Sac)	Aneuploidy Rate	Age Group	Implan. (+ Sac)	Aneuploidy Rate
30-34	40.7 (48/118)	50.8 (337/664)	30-34	51.6 (63/122)	29.8 (143/480)
35-39	41.7 (78/187)	66.8 (808/1209)	35-39	50.2 (101/201)	46.6 (418/897)
40-42	38.7 (43/111)	77.0 (725/941)	40-42	44.9 (40/89)	67.1 (342/510)
P Value	NS	<0.001	P Value	NS	<0.001

Implan. (+ Sac): Implantation rate as measured by the presence of a fetal sac by ultrasound

NS: not significant

Table 2: Comparison of Pregnancy Loss Rate Between Biopsy at Day 3 or Day 5/6 by SART Age Group

Day 3 Biopsy		Day 5/6 Biopsy	
Age Group	Loss Rate	Age Group	Loss Rate
30-34	3.9 % (3/77)	30-34	6.0 % (5/83)
35-39	3.3 % (5/151)	35-39	5.4 % (9/166)
40-42	2.3 % (3/133)	40-42	1.9 % (2/108)
P Value	NS	P Value	NS

NS: Not significant

Table 3: Comparison of Ongoing Pregnancy Rate per Cycle Start and per Transfer Between Biopsy at Day 3 or Day 5/6 by SART Age Group

Day 3 Biopsy			Day 5/6 Biopsy		
Age Group	OP/Cycle Start	OP/Transfer	Age Group	OP/Cycle Start	OP/Transfer
30-34	42.9 (33/67)	49.3 (33/67)	30-34	50.6 (42/83)	59.2 (42/71)
35-39	39.1 (59/151)	50.4 (59/117)	35-39	42.2 (70/166)	53.4 (70/131)
40-42	24.1 (32/133)	42.1 (32/76)	40-42	33.3 (36/108)	57.1 (36/63)
P Value	<0.01	NS	P Value	<0.001	NS

OP: ongoing pregnancy as determined by the presence of a fetal sac at ultrasound investigation

NS: not significant