Assessment of Chromosomal Aneuploidy in Day 3 Cleavage-Stage Embryos and Day 5/6 Blastocysts from Indigenous Black Africans

Ajayi AB¹, Ajayi VD¹, Atiba AA¹, Oyetunji IO¹, Ehichioya J¹, Ayelehin IB¹, Adeoye AT¹, Adesida TE¹ and Afolabi BM²*

¹Nordica Fertility Centre, Ikoyi, Lagos, Nigeria
²Health, Environment and Development Foundation, Yaba, Lagos, Nigeria

Abstract

The objectives of this study were to examine the prevalence and to characterize the types of chromosomal aberrations of aneuploidy in cleavage-stage Day 3 human embryos and in Day 5/6 human blastocysts in indigenous Black African women aged ≤ 35 and >35 years. Of the 312 oocytes fertilized for Day 3 embryos and 269 for Day 5/6 blastocysts, 230 blastomeres and 122 blastocysts were biopsied respectively and then subjected to genetic analysis using the array CGH and next generation sequencing methods, genotyping to characterize chromosomal error types of aneuploidy across all 24 chromosomes. A total of 562 oocytes with 486 MII oocytes, were fertilized from which 230 Day 3 embryos and 122 Day 5/6 blastocysts were biopsied. Euploidy rates in Day 3 embryo group and in Day 5/6 blastocyst group were 24.3% and 75.0% respectively while aneuploidy rates were 75.7% and 25.0% respectively. Women aged ≤ 35 years were approximately thrice as likely to have euploid Day 3 embryos (Fisher’s χ²=1.18, p-value=0.29, OR=2.79, 95% CI: 0.61, 12.67). Blastocyst stage biopsy was more efficient in PGS. Blastocyst culture had some level of aneuploidy selection. Higher diagnosis rate was observed as there was lower rate of embryos with no diagnosis.

Keywords: Aneuploidy; Pre-implantation genetic diagnosis; Cleavage-stage; Indigenous Black Africans

Introduction

In recent times, there has been considerable advancement in the technology of in-vitro fertilization (IVF) not only for infertility but also for pre-implantation genetic diagnosis (PGD). The process of PGD was initially applied to diagnose sex-linked disorders, monogenic disorders or chromosomal structural abnormalities and also to test for HLA compatibility. However, genetic testing in IVF evolved to include the screening of numerical chromosomal abnormalities which is referred to as PGD aneuploidy screening or simply PGS (Pre-implantation genetic screening) [9]. Aneuploidy has been regarded as one of the main reasons for failure of implantation [1-4]. Aneuploidy is also regarded as the leading cause of spontaneous abortion and mental retardation in women of older age [5,6] and the study of chromosomal aberrations in embryos is because, it is believed that, these aberrations contribute significantly to spontaneous abortion and recurrent implantation failure in women, irrespective of age, who experience unexplained infertility [7,8]. Studies have reported that early-stage embryos often suffer from chromosomal abnormalities [9-12] and that over 50% of human embryos produced through IVF contain aneuploidy cells [9-11]. The undesirable outcomes of chromosomal aberration include failure to implant, spontaneous abortion and foetus with trisomic condition [13-17]. Pre-implementation screening (PGS) to detect aneuploidy within embryos is progressively performed in IVF, with the goal of improving the efficacy of infertility management [18]. Pre-implementation genetic screening allows investigation of ploidy across all 24 chromosomes, but this technology is relatively new in sub-Saharan Africa. The incidence of spontaneous abortion in sub-Saharan Black Africa is unknown. Much more surprising is that the cause of spontaneous abortion or babies born with chromosomal disorders such as Trisomy 21 is hardly reported in studies of children with such disorders. There is a lacuna regarding data on chromosomal abnormalities among indigenous Black Africans. However, in the past decade or so, probably due to more awareness, women now come forward to request for pre-implantation genetic screening due to having previous abnormal babies, recurrent miscarriages, having babies with congenital abnormalities, genotype or sex selection. Even if these women do not request for it but their medical history indicates any of the above, except for sex selection, they are advised to consider PGD to which they almost always acquiesce. Nordica Fertility Centre follows the policy that it is ethically wrong to transfer an abnormal embryo.

The first study describing successful biopsy of a human embryo for PGS was performed in 3-day-old embryos, which consisted of 6–8 cleavage-stage cells [19-21]. Currently, biopsies of 8-cell blastomeres or blastocyst trophoderm obtained on day 3 or 5/6 are performed in IVF laboratories worldwide [21-23]. A European study on pre-implementation genetic aneuploidy screening in 11 embryos found four (36.4%) to be aneuploid [24]. Part of the conclusion from the study of Rabinowitz and colleagues in California was that chromosome gains were predominantly maternal in origin whereas chromosome losses were not biased in terms of parental origin of chromosome [25]. While a study from Florida, USA, stated that the availability of euploid embryos is associated with high ongoing pregnancy and implantation rates and the absence of euploid embryos for transfer predicts poor reproductive outcome [26], another from Houston, Texas reported that aneuploidy rates in day 7 human blastocysts produced by IVF are very high [27]. Chromosomal abnormalities are categorized into two types: numerical and structural. Numerical abnormalities involve the addition (trisomy, tetrasomy, etc) or deletion (monosomy) of an entire chromosome which is called aneuploidy, and the addition or
deletion of an entire set of chromosomes is referred to as polyploidy and haploidy, respectively. Aneuploidy has been reported to occur in approximately 20% of cleavage-stage human embryos [28] with an increase to 45% of cleavage-stage embryos taken from patients with advanced maternal age (AMA; ≥36 years) [29]. Whereas, polyploidy and haploidy occur much less frequently (in 7% and 3% cleavage-stage embryos, respectively) [29] on the other hand, Structural chromosomal abnormalities refer to when the structure of a chromosome is altered and can occur spontaneously or as a result of external forces such as radiation. An embryo can present with both numerical and structural abnormalities concurrently. Although, aneuploidies are naturally prevented as most are incompatible with life however, Some aneuploidies have been seen to persist through implantation resulting in a live birth for trisomy 21 (Down syndrome), trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), monosomy X (Turner syndrome) and trisomy XXX (Klinefelter syndrome). Trisomies 13, 18, and 21 have been implicated in the etiology of early pregnancy loss. The occurrence of these aneuploidies in human embryos poses to be a major drawback in IVF, compromising the efficiency in achieving healthy live birth, hence the advent of PGS aneuploidy screening in IVF. Advancement in media culture permitted the growth of embryos in-vitro up to the blastocyst stage which has facilitated the evolution of PGS to include trophectoderm cell biopsy [30]. Here, several trophectoderm cells are removed and screened for aneuploidy whereas cleavage stage biopsy involves one or two blastomeres with cleavage-stage biopsy [30,31]. McArthur et al. [32] reported the first routine use of blastocyst biopsy with florescent in situs hybridization FISH in human pre-implantation embryos to produce successful pregnancies and live births.

Indications for PGS

The main indications for the use of PGS in IVF treatments include advanced maternal age (AMA), repeated implantation failure (RIF), and recurrent pregnancy loss (RPL). It is well known that the rate of chromosome abnormalities is higher in patients with AMA and RPL. Also, PGS has been used in women with previous trisomic conceptions [33], women who have partners with male factor infertility [34-36], and sometimes in egg donor cycle [37]. Today, the use of PGS for healthy patients with no indications for the purpose of improving IVF outcomes is on the rise however, in this part of the world, the major indication for PGS has remained for gender balancing.

Correction mechanism

It has been established that Mitotic errors are common in human preimplantation embryos occurring highest during the first three cleavages after fertilization resulting in about three quarters of human preimplantation embryos affected by aneuploidies and mosaicism at day three of development. However, at later developmental stages such as the blastocyst stage, the mitotic aneuploidy rate is lower which could be explained by the fact that apoptotic mechanisms which could deselect against aneuploid cells are very low in cleavage stage with a significant increase at the blastocyst stage.

In regard to self-correction mechanisms, it is assumed that during blastocyst development, the embryo experiences a stringent self-correction probably based on cell cycle checkpoint control mechanisms such as cell arrest, apoptosis, active correction of the aneuploidies and preferential allocation of the aneuploid cells to the extra-embryonic tissues. Thus, to form a blastocyst, the embryo must successfully undertake the first cellular differentiation and epigenetic modification to form the trophectoderm (TE) and inner cell mass (ICM); this process may be hampered by the inappropriate gene expression that inevitably accompanies aneuploidy. In the case of mosaic embryos, aneuploid cells could arrest development in favor of euploid ones [38]. We can therefore assume that blastocyst formation from cleavage stage is a form of selection of euploid embryos involving some level of self-correction. Li et al. [39] did a comparison of sequential chromosomal data on the two developmental stages, day-3 and subsequently on day-5 with both stages tested with five-chromosome FISH, they observed 40% aneuploid embryos on day-3 were euploid on day-5. In 2008, Barbash-Hazan et al. [40] re-analyzed 83 abnormal day-3 embryos out of which 27 embryos (32.6%) underwent self-normalization. More interestingly, 41% of the abnormal embryos diagnosed as trisomic underwent trisomic rescue (which is the loss of a chromosome in trisomic cells). Another recent study by Northrop et al. [41] demonstrated similar results with re-analysis of blastocyst-stage embryos using SNP microarray-based 24 chromosome aneuploidy screening which revealed 65% of the monosomic, 47% of the trisomic, and 63% of the complex aneuploid embryos were euploid at the blastocyst stage. Hence, Northrop et al. [41] found a significant number of euploid blastocyst-stage embryos that were previously diagnosed as aneuploid on day 3. This self-correction mechanism may also be due to monosomic or trisomic rescue as suggested by some studies [40-42].

Day 3 versus day 5 biopsy

This important aspect of preimplantation embryo screening needs to be considered in order to prevent possible erroneous disposal of euploid blastocysts that were previously diagnosed as abnormal at the cleavage stage [41]. Hence the birth of the ESHRE report on PGD/PGS which recommends a shift from cleavage stage biopsy towards trophectoderm biopsy [43] due to the high degree of mosaicism of cleavage stage embryos [44]. Blastocyst biopsy is rapidly becoming the more preferred biopsy method for aneuploidy screening. Biopsy at the blastocyst stage has been demonstrated to be more desirable since embryos at this stage have a smaller risk of aneuploidy (38.8%) than embryo biopsy at the cleavage stage (51%) mostly since aneuploid cells in cleavage stage embryos are more likely not to develop into blastocyst stage involving some sort of de-selection of aneuploid embryos. Furthermore, the major limiting factor trophectoderm biopsy which is the length of time of genetic analysis has been solved with the optimization of blastocyst cryopreservation by vitrification especially for IVF clinics that send their biopsy samples to an external genetic lab for analysis. Some studies have concluded that there is a better implantation rates across all ages for euploid blastocyst transfer following Day 5/6 biopsy rather than blastocyst transfer subsequent to day 3 biopsy. This could be influenced by varying factors such as may be due to less damage to the embryo during trophectoderm biopsy, higher accuracy of day 5/6 PGS due to a larger amount of genetic material can be retrieved from biopsy and better uterine receptivity when the blastocysts are cryopreserved following biopsy and transferred in a controlled endometrium development (CED) cycle [45]. Therefore, the main objective of this study was to compare the prevalence and characteristics of chromosomal aberrations of aneuploidy in cleavage-stage Day 3 human embryos and in Day 5/6 human blastocysts among indigenous Black Africans. The main hypothesis is that euploid embryos are more prevalent in women 35 years or less than in older women or vice versa.

Materials and Methods

Ethical statement

Patients undergoing IVF, egg donation and PGS signed written
consents for all kinds of laboratory and clinical procedures. All egg donors were anonymous in the present study. The data was retrospectively collected from the medical records at the clinic from September 2012 to August 2014 and the study was approved by Nigerian Institute for Medical Research Institutional Review Board (NIMR-IRB 18–006).

We retrospectively evaluated an analysis of 352 embryos from 45 ICSI/PGS cycles including oocyte donor cycles from 35 cycles for aneuploidy screening and gender selection at our clinic from June 2014 to August 2017. We assigned the couples into two groups according to biopsy day: day 3 (230 embryos, 29 cycles) and day 5/6 (122 blastocysts, 16 cycles). Within each biopsy group, the data was further broken down into two age groups, ≤ 35 and >35 years of age at the time of cycle start. All couples presenting for chromosome aneuploidy testing and gender selection had one or two blastomeres biopsied from their embryos, providing that the embryo had ≥ 8 blastomeres with less than 10% fragmentation rate on day 3 and 2-5 trophectoderm cells from day 5/6 blastocysts post-oocyte collections. Egg source were aged 29.6 ± 9.4 years and 28.9 ± 7.1 years, respectively. All patients signed an informed consent form for ICSI/PGS that included counselling on the IVF program, risk of ovarian hyperstimulation syndrome, probability of pregnancy, risk of pregnancy complications, risk of misdiagnosis, necessity of a prenatatal diagnosis, and possible cryopreservation of supernumerary embryos obtained during the program and risk of no suitable embryos for transfer. Embryo transfer was cancelled in cycles without embryos reaching blastocyst stage for day 3 biopsy or without euploid embryos in both groups. Chaotic embryos (complex abnormal) were defined as those showing a complex pattern of aneuploidies, involving more than six chromosomes.

Patient stimulation and egg retrieval

Patients were subjected to ovarian stimulation with the use of gonadotropins (follicle-stimulating hormone or human menopausal gonadotropin), and gonadotropin-releasing hormone analogues or antagonists were used for controlled ovarian hyperstimulation. Patients received human chorionic gonadotropin when the diameter of the three leading follicles was >18 mm. Ultrasound-guided oocyte retrieval was performed 34-36 hours depending on protocol used after the human chorionic gonadotropin injection, and luteal support consisting of progesterone injections were provided. Only Oocytes at MII were microinjected with ejaculated spermatozoa by incotytoplasmic sperm injection (ICSI). Fertilization check was carried out approximately 18 hours after the procedure to confirm the presence of two pronuclei and extrusion of the second polar body. Normally fertilized oocytes were further cultured until either day-3 or day-5/6 of embryo development before and after biopsy by using standard embryo culture conditions in our laboratory. Embryo culture was done at 37°C in a humidified atmosphere of 5% CO2 in a one-step single culture medium under oil for all phases of embryo development (SAGE 1-Step, ORIGIO, Denmark). From July 2016, the PGS screening process was updated in our clinic to involve biopsy at the blastocyst stage as well as use of NGS. Therefore, in total 45 PGS cycles were included in the study comprising of 29 cycles of day 3 analysis using array CGH and 16 cycles of day 5/6 biopsy using NGS. Day 3 biopsy was performed on 126 embryos from 18 cycles, and day 5/6 biopsy was performed on 150 embryos from 20 cycles. In all cases, a Leica DM IRB inverted microscope (Wetzlar, Germany), equipped with a Narishige NT-88 3D hydraulic micromanipulator (Tokyo, Japan) and Origio microtools were used for micromanipulation. A total of eight (8) cycles with day 3 biopsy ended with no embryo transfer as there were no euploid embryos for transfer while all cycles with day 5/6 had euploid embryos to transfer. Oocytes were inseeded using conventional IVF techniques. 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 one or two blastomere cell (cleavage stage biopsy) or 2-5 cells of trophectoderm tissue (blastocyst biopsy). The biopsied material was washed in clean biopsy wash medium (supplied by Igenomix, Spain). Following washing, the cell or tissue was placed in the supplied transport tube labelled with the patient initials and embryo number and kept on ice until being shipped to the genetic lab for testing. All samples were shipped by custom courier or commercial shipping companies and delivered to the genetic laboratory within two days for testing.

Day 3 biopsy

Embryos were de-compacted in Ca2+/Mg2+-free biopsy media. For the cases of day-3 embryo biopsy, only embryos with five or more nucleated blastomeres and less than 25% fragmentation degree were biopsied. Day-3 embryo biopsy can be summarized as follows: embryos were placed on a droplet containing Ca2+/Mg2+-free medium (LifeGlobal, Guilford, CT), the zona pellucida was perforated by pulses of laser using the ZILOS-tkTM laser system (Hamilton Thorn Bioscience Inc., MA, USA), and one or two blastomere (s) was withdrawn from each embryo, then individual blastomeres were placed in 0.2 mL PCR tubes containing 2 μL PBS. For blastomere washing and handling, 1% polyvinylpyrrolidone (PVP) was used. PCR tubes were immediately frozen at -20°C and kept in the freezer until transportation to the genetic analysis laboratory. Embryo culture was continued till day 5/6 for results and euploid embryos were candidates to be transferred on day 5/6 while excess euploid embryos were vitrified either on day 5 or on day 6.

Day 5 biopsy

Blastocyst biopsy was performed on TE cells at days 5 and 6 depending on blastocyst development. At day 3, a hole about 20 μm was opened in the zona pellucida to facilitate blastocyst hatching using the ZILOS-tkTM laser system (Hamilton Thorn Bioscience Inc., MA, USA). On day 5/6, embryos for biopsy were examined with an inverted microscope, and if the embryos developed to a full blastocyst stage and some trophectoderm (TE) cells started to hatch from the opening in the zona pellucida, some hatched TE cells (3 ~ 10) were biopsied using a 20 μm polished biopsy pipette with assisted cutting by the laser. All biopsy procedures were performed in droplets of buffered medium (HEPES, Sage in-vitro fertilization, Inc., Trumbull, CT, USA) overlaid with mineral oil on the heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools. After biopsy, the embryo was cultured in SAGE one-step medium for 1-3 hours before cryopreservation. The biopsied cells were washed with a washing buffer provided and placed in tubes with cell lysis buffer and were then frozen at −20°C before being shipped to the genetic laboratory. For samples biopsied on day-5 or day-6 of embryo development, resulting blastocysts were cryopreserved using the cryotop vitrification method after which euploid embryos were transferred in a frozen embryo transfer cycle (FET) into a uterus free of gonadotropin stimulation.

Microarray comparative genomic hybridization (aCGH)

Microarray-CGH was performed as stated previously by Rodrigo et al., [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 labelling steps. Labelling was performed using manufacturer’s recommendations with Cy3 dye for test DNA and Cy5 dye for reference male DNA (BlueGnome LTD). After labelling, embryo biopsy samples and reference DNA samples were separately denatured at 74°C prior to being mixed together and added to each microarray. Microarrays were hybridized at 47°C for at least 4 hours or overnight in a humidified chamber. Following hybridization, each microarray was washed as follows: 10 minutes in 2x SSC/0.05% Tween 20 at room temperature, 10 minutes in 1x SSC at room temperature, 5 minutes in 0.1x SSC at 60°C and 2 minutes in 0.1x SSC 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 that of the second technologist. The Cryotop method was used to vitrify embryos as previously described by Kuwayama et al. [30].

Results

The means (±sd) of age and Body Mass Index of all egg sources of Day 3 embryos were 29.8 (4.3) years and 25.3 (5.3) kg/m² respectively while the mean (±sd) age and Body Mass Index of all egg sources of Day 5/6 blastocyst were 28.9 (3.5) years and 23.6 (6.5) kg/m² respectively (Table 1). The ICSI/PGS day 3 and day 5/6 groups did not differ significantly in age; egg source were aged 29.6 ± 9.4 years and 28.9 ± 7.1 years, respectively. Of the Day 3 embryos, there were 25 (86.2%) egg sources aged 35 years or younger while there were 4 (14.8%) older than 35 years, respectively. Of the Day 3 embryos, there were 25 (86.2%) egg sources aged 29.6 ± 9.4 years and 28.9 ± 7.1 years, respectively.

Table 1: Socio-demographic characteristics of egg source and recipients.

| Variables          | Items                  | Statistics | Day 3 Embryo | Day 5 Blastocyst |
|--------------------|------------------------|------------|--------------|-----------------|
|                    |                        |            | Egg source   | Recipient       | Egg source   | Recipient       |
|                    |                        |            | Total        | Donor | Own | Total        | Donor | Own | Total        | Donor | Own |
|                    |                        | Freq. (%)  | Freq. %      | Freq. % | Freq. % | Freq. (%)   | Freq. % | Freq. % | Freq. (%)   | Freq. % | Freq. % | Freq. % | Freq. % | Freq. % | Freq. % | Freq. % |
| Age (years) <= 35 |                        |            | 29 (100.0)   | 19     65.5  | 10   34.5  | 29 (100.0) | 16     100.0 | 12   75.0 | 4   25.0 | 16     100.0 |
|                    | Mean (± SD)            | 29.6 ± (4.3)| 27.1 ± (1.7) | 34.5 ± (3.4) | 40.8 ± (6.7) | 28.9 ± (3.5) | 27.3 ± (1.7) | 33.8 ± (2.9) | 40.9 ± (5.5) |
| Age (years) >35   |                        |            | 25 (86.2)    | 19     76.0  | 6   24.0  | 6   20.7  | 14     87.5  | 12   100.0 | 2   50.0 | 2   12.5 |
|                    | Mean (± SD)            | 28.7 ± (3.3)| 27.1 ± (1.7) | 32.7 ± (3.4) | 32.7 ± (3.4) | 27.9 ± (2.3) | 27.3 ± (1.7) | 31.5 ± (2.1) | 31.5 ± (2.1) |
| BMI (Kg/m²)       | <18.5                  |            | 29 (100.0)   | 19     65.4  | 10   34.6  | -               | 15   100.0  | 11   73.3  | 4   26.7  | -               |
|                    | Mean (± SD)            | 25.3 ± (5.3)| 22.2 ± (2.8) | 31.1 ± (3.7) | -               | 23.6 ± (6.5) | 20.7 ± (1.6) | 31.5 ± (8.7) | -               |
| BMI (Kg/m²)       | 18.5-24.5              |            |              | -               | -               | -               | -               | -               | -               |
|                    | Mean (± SD)            | 19 ± (65.5) | 18     94.7  | 1   5.3   | -               | 11   73.3  | 10   90.9  | 1   25.0  | -               |
| BMI (Kg/m²)       | 25.0-29.9              |            |              | -               | -               | -               | -               | -               | -               |
|                    | Mean (± SD)            | 21.6 ± (1.5)| 21.6 ± (1.5)| -               | -               | 20.9 ± (1.4)| 21.0 ± (1.4)| 20.3 ± (0.0) | -               |
| BMI (Kg/m²)       | ≥30                    |            |              | -               | -               | -               | -               | -               | -               |
|                    | Mean (± SD)            | 26.8 ± (0.4)| 26.8 ± (0.4)| -               | -               | 0.0 ± (0.0) | -               | -               | -               |
| Reason for PGD    | Sex selection          | Freq. (%)  | 27 (93.1%)   | -               | -               | 13 (81.2%) | -               | -               |
|                  | Genotype and sex selection | Freq. (%) | 2 (6.9%)     | -               | -               | 2 (12.5%) | -               | -               |
|                  | Aneuploidy screening   | Freq. (%)  | 0 (0.0%)     | -               | 1 (6.3%) | -               | -               |
|                  | Others (Advanced age)  | Freq. (%)  | 0 (0.0%)     | -               | 0 (0.0%) | -               | -               |
| Statistics        | χ²=0.51; P-value=0.47; Odds Ratio=3.12; 95% Confidence Interval: 0.46, 20.99 | -           | -               | -               | -               | -               | -               |

The Cryotop method was used to vitrify embryos as previously described by Kuwayama et al. [30].
35 years of age; while 14 (87.5%) egg sources of Day 5/6 blastocysts were aged 35 years and younger with 2 (12.5%) aged over 35 years. In general, the mean age of recipients of Day 3 embryos (40.8 years) was significantly different (t=-10.5, df=33.3, P-value = 0.00001) from that of the donors used in the donor cycles (27.1 years). Likewise, there was a noteworthy disparity (t=-9.32, df=18.7, P-value = 0.00001) in the mean age of recipients of Day 5/6 blastocysts (40.9 years) and that of the donors (27.3 years).

As depicted in Figure 1, 562 and 486 MII oocytes from 29 cycles in the Day 3 embryo study group and 16 cycles in Day 5/6 blastocyst study group respectively, were obtained. In the day 3 embryo study group 312 oocytes were fertilized compared to 269 in the Day 5/6 blastocyst study group producing 297 Day 3 embryos out of which 230 embryos were biopsied and for Day 5/6 group, 122 blastocysts were formed and biopsied from 237 embryos for chromosomal anomalies. Sixty-one embryos and 6 blastocysts had no results, giving an overall euploidy rate of 24.3% and aneuploidy rate of 75.7% in Day 3 embryo group and euploidy rate of 75.0% and aneuploidy rate of 25.0% in Day 5/6 blastocyst study group.

Of the 562 MII oocytes retrieved from Day 3 embryo group, 506 (90.0%; mean [± sd] = 20.2 [8.9]) were from women aged ≤ 35 years and 56 (10.0%; mean [± sd] = 14.0 [3.4]), signifying a statistically significant difference (t=10.3, df=160.0, P-value=0.00001) (Table 2). Likewise, of the 486 MII oocytes retrieved from Day 5/6 blastocyst group, 422 (86.8%; mean [± sd] = 30.1 [12.2]) were from women aged ≤ 35 years and 64 (13.2%; mean [± sd] = 32.0 [1.4]) from their older counterparts, also signifying a statistically significant difference (t=--

| Variables            | Day 3 embryos (PGD cycles=29) | Day 5 Blastocysts (PGD cycles=16) |
|----------------------|-------------------------------|-----------------------------------|
|                      | Total | Mean (± SD) | Median | Range  | Total | Mean (± SD) | Median | Range |
| Oocyte retrieval     |       |             |       |        |       |             |        |       |
| Age ≤ 35 y (n=25)    | 44    | 1.5 (0.6)   | 1     | 2 (1-3) | 22    | 1.4 (0.6)   | 1      | 1-3   |
| Age >35 y (n=4)      | 36    | 1.4 (0.6)   | 1     | 2 (1-3) | 18    | 1.3 (0.6)   | 1      | 1-3   |
| Oocyte retrieved     |       |             |       |        |       |             |        |       |
| Age ≤ 35 y (n=25)    | 562   | 19.4 (8.6)  | 16    | 30 (7-37) | 486   | 30.4 (11.4) | 29     | 38 (17-55) |
| Age >35 y (n=4)      | 506   | 20.2 (8.9)  | 18    | 30 (7-37) | 422   | 30.1 (12.2) | 26.5   | 38 (17-55) |
| Oocyte fertilized    |       |             |       |        |       |             |        |       |
| Age ≤ 35 y (n=25)    | 312   | 10.8 (4.4)  | 9     | 5-23   | 269   | 16.8 (8.6)  | 14.5   | 10-45 |
| Age >35 y (n=4)      | 275   | 11.0 (4.7)  | 9     | 18 (5-23) | 228   | 16.3 (9.1)  | 14     | 35 (10-45) |
| Embryos/Blastocysts  |       |             |       |        |       |             |        |       |
| Age ≤ 35 y (n=25)    | 297   | 10.2 (3.8)  | 9     | 5-20   | 237   | 14.8 (8.7)  | 13     | 4-42  |
| Age >35 y (n=4)      | 262   | 10.5 (4.0)  | 9     | 15 (5-20) | 214   | 15.3 (8.7)  | 13     | 8-42  |
| Fertilization rate   |       |             |       |        |       |             |        |       |
| Age ≤ 35 y (n=25)    | 2772.4| 59.3 (17.7) | 100   | 25-100 | 1429.3| 88.3 (22.2) | 100    | 19-100|
| Age >35 y (n=4)      | 1450.5| 58.0 (18.4)| 53.8  | 75 (25-100) | 1315.3| 94.0 (12.8)| 100    | 53.3-100.0 |
| Embryos/Blastocyst biopsied |       |             |       |        |       |             |        |       |
| Biopsy rate          |       |             |       |        |       |             |        |       |
| No. of embryos/blastocysts with no results | 269.9 | 67.5 (9.8) | 67.9 | 21.5 (56.3-77.8) | 114 | 57.0 (53.7) | 57.0 | 19-95 |
| No. of cycles with at least one normal | 230 | 8.0 (2.7) | 7 | 12 (3-15) | 122 | 7.5 (1.3) | 7.5 | 6-9 |
| Total No. of cycles  | 2401.1| 80.9 (20.9) | 85.7  | 79 (35.3-114.3) | 950.8 | 86.4 (10.7) | 57.2 | 75-100.0 |
| Cancellation rate    |       |             |       |        |       |             |        |       |
| There was a significant difference in the proportion of Day 3 cancellation rate compared to Day 5 (χ²=4.42, P-value=0.04)
likely to have 1 trisomy ($\chi^2=0.03$, P-value=0.86, OR=1.13, 95% CI: 0.25, 3.97), indicating a higher prevalence of euploidy in Day 3 embryos and in Day 5 blastocysts in different age groups.

Table 3: Prevalence of euploidy in day 3 embryos and in Day 5 blastocysts in different age groups.

| Variables | Day 3 Embryo | Blastoctyst |
|-----------|--------------|-------------|
|          | ≤ 35 | >35 | All | ≤ 35 | >35 | All |
| Screened |       |     |     |       |     |     |
| Frequency | 151  | 18  | 169 | 104   | 12  | 116 |
| %         | 89.3 | 10.7| 100.0| 89.7   | 10.3| 100.0|
| Euploid   |       |     |     |       |     |     |
| Frequency | 39   | 2   | 41  | 78     | 9   | 87  |
| %         | 25.8 | 11.1| 24.3| 75.0   | 75.0| 75.0|
| Aneuploid |       |     |     |       |     |     |
| Frequency | 112  | 16  | 128 | 28     | 3   | 29  |
| %         | 74.2 | 88.9| 75.7| 25.0   | 25.0| 25.0|

| Statistics | Item | ≤ 35 | >35 | All | ≤ 35 | >35 | All |
|------------|------|------|-----|-----|------|-----|-----|
| $\chi^2$   | 1.18*| -    | -   | 0.12*| -    | -   |
| P-value    | 0.29 | -    | -   | 0.72 | -    | -   |
| Odds ratio | 2.79 | -    | -   | 1.0  | -    | -   |
| 95% CI     | 0.61, 12.67 | - | 0.25, 3.97 |

All-in-all, in Day 3 embryos, there were 23 (20.5%) monosomies, 31 (27.7%) trisomies, 23 (20.5%) combinations of monosomies and trisomies and 35 (31.3%) complex aneuploidy observed in women aged ≤ 35 years compared to only 1 (100.0%) monosomy in women aged >35 years (Table 5 and Figure 2).

The percentages of monosomies and trisomies seen in all Day 3 embryos are illustrated in Figure 3. The bulk (34.4%) of the aneuploidy in Day 3 embryos comprised of complex aberration, followed by 1 trisomy (17.2%), 1 monosomy (12.5%) and 1 monosomy and 1 trisomy (9.4%). Other aneuploidy in lower prevalence included 2 trisomy (4.7%), 2 monosomies (3.9%), 4 trisomies (3.1%).

The prevalence of various monosomies and trisomies, and the combinations of these in Day 5/6 blastocysts are shown in Figure 4. The largest prevalence was in 1 monosomy (50.0%) followed by 1 trisomy (28.0%). Complex aneuploidy and 5 trisomies had a prevalence of 6.0% respectively while the combination of 1 monosomy and 1 trisomy as well as 2 monosomies and 1 trisomy each had a prevalence of 5.0%.

Table 6 and Figures 5 and 6 indicate that, in the Day 3 embryos, a very low prevalence (0-3.99) of aneuploidy was observed in chromosomes 3, 7 and 8; low prevalence of 4-9.99 was observed in chromosomes 11, 12, 17 and Y; a moderate prevalence of 6-7.99 in chromosomes 4, 13 and X indicating that chromosome X was more affected in aneuploidy than chromosome Y. High aneuploidy prevalence of 8-9.99 was recorded in 5 chromosomes: 2, 5, 10, 18 and 22; very high aneuploidy prevalence of 10-14.99 observed in 7 chromosomes: 1, 6, 9, 14, 15, 16 and 19 and extremely high aneuploidy prevalence of 15-20.99 was observed in 2 chromosomes – 20 and 21. This indicates that, among indigenous Black Africans, very low aneuploidy prevalence occurred in 2 chromosomes – 20 and 21.
| Variables | Item | ≤ 35 | >35 | χ² | P-value | OR | 95% CI | Total (n=128) |
|-----------|------|------|-----|-----|--------|----|--------|----------------|
|           | Freq. | %    | Freq. | %  |        |     |        | Freq. | % |
| 1 Monosomy| 16    | 14.3 | 0    | 0  | 1.47   | 0.22| -      | 16    | 12.5 |
| 1 Trisomy | 19    | 17.0 | 3    | 18.8 | 0.03  | 0.86| 1.13   | 0.29, 4.35 | 22  | 17.2 |
| 1 Monosomy & 1 Trisomy | 12 | 10.7 | 0    | 0  | 0.84  | 0.34| -      | 12    | 9.4  |
| 1 Monosomy & 2 Trisomy | 1   | 0.9  | 0    | 0  | 1.30  | 0.25| -      | 1     | 0.8  |
| 2 Monosomy | 5    | 4.5  | 0    | 0  | 0.03  | 0.86| -      | 5     | 3.9  |
| 2 Trisomy | 5    | 4.5  | 1    | 6.3 | 0.10  | 0.75| 1.43   | 0.16, 13.06 | 6   | 4.7  |
| 2 Monosomy & 2 Trisomy | 2  | 1.8  | 1    | 6.3 | 0.05  | 0.83| 3.67   | 0.31, 42.93 | 3   | 2.3  |
| 3 Monosomy | 1    | 0.9  | 0    | 0  | 1.30  | 0.25| -      | 1     | 0.8  |
| 3 Trisomy | 2    | 1.8  | 0    | 0  | 0.29  | 0.59| -      | 2     | 1.6  |
| 3 Monosomy & 1 Trisomy | 1  | 0.9  | 1    | 6.3 | 0.29  | 0.59| 7.49   | 0.44, 124.62 | 2   | 1.6  |
| 3 Monosomy & 3 Trisomy | 0   | 0.0  | 1    | 6.3 | 1.30  | 0.25| -      | 1     | 0.8  |
| 3 Trisomy & 1 Monosomy | 3  | 2.7  | 0    | 0  | 0.05  | 0.82| -      | 3     | 2.3  |
| 4 Monosomy | 1    | 0.9  | 0    | 0  | 1.30  | 0.25| -      | 1     | 0.8  |
| 4 Trisomy | 4    | 3.6  | 0    | 0  | 0.00  | 1.00| -      | 4     | 3.1  |
| 4 Monosomy & 1 Trisomy | 1  | 0.9  | 0    | 0  | 1.30  | 0.25| -      | 1     | 0.8  |
| 4 Monosomy & 2 Trisomy | 2   | 1.8  | 0    | 0  | 0.29  | 0.59| -      | 2     | 1.6  |
| 5 Monosomy & 2 Trisomy | 1   | 0.9  | 0    | 0  | 1.30  | 0.25| -      | 1     | 0.8  |
| 6 Trisomy | 1    | 0.9  | 0    | 0  | 1.30  | 0.35| -      | 1     | 0.8  |
| Complex   | 35    | 31.2 | 9    | 56.3 | 3.88  | 0.04| 2.83   | 0.97, 8.21 | 44  | 34.4 |
| Total     | 112   | 87.5 | 16   | 12.5 | -     | -    | -      | 128   | 100.0 |

Fisher's exact. Only one (1) aneuploidy was observed in blastocysts of women aged >35 years compared to 17 among those aged ≤ 35 years.

Table 4: Types (not chromosome specific) of aneuploidies found in different age groups.

| Type of aneuploidy | Day 3 embryo | Blastocyst |
|--------------------|--------------|------------|
|                    | Age group ≤ 35 years | Age group >35 years | Age group ≤ 35 years | Age group >35 years |
|                    | Freq. | %    | Freq. | %  | Freq. | %    | Freq. | %  |
| Monosomy           | 23    | 20.5 | 0    | 0  | 8     | 47.0 | 1     | 100.0 |
| Trisomy            | 31    | 27.7 | 4    | 25.0 | 6    | 35.3 | 0     | 0.0  |
| Combination of monosomy and trisomy | 23  | 20.5 | 3    | 18.8 | 2    | 11.8 | 0     | 0.0  |
| Complex            | 35    | 31.2 | 9    | 56.2 | 1    | 5.9  | 0     | 0.0  |
| All                | 112   | 100.0 | 16  | 100.0 | 17  | 100.0 | 0.0  | 0.0  |

Table 5: Aggregate number of aneuploidy in day 3 embryos and day 5 blastocysts among women of different age groups.

Figure 2: Percent distribution of various types of aneuploidy in age groups ≤ 35 years and >35 years.

Figure 3: Percentage of monosomies and trisomies seen in biopsied day 3 embryos in Nigeria.
Day 3 embryo Blastocyst

| Chromosome number | No. of embryos with aneuploidy | Prevalence %± no. Aneu/84 | No. of blastocysts with aneuploidy | Prevalence %± no. Aneu/84 |
|-------------------|-------------------------------|---------------------------|-----------------------------------|---------------------------|
| 1                 | 11                            | 13.09                     | 2                                 | 11.76                     |
| 2                 | 7                             | 8.33                      | 3                                 | 17.65                     |
| 3                 | 3                             | 3.57                      | 1                                 | 5.88                      |
| 4                 | 6                             | 7.14                      | 1                                 | 5.88                      |
| 5                 | 7                             | 6.33                      | 0                                 | 0.00                      |
| 6                 | 9                             | 10.71                     | 3                                 | 17.65                     |
| 7                 | 3                             | 3.57                      | 0                                 | 0.00                      |
| 8                 | 3                             | 3.57                      | 0                                 | 0.00                      |
| 9                 | 12                            | 14.28                     | 0                                 | 0.00                      |
| 10                | 7                             | 8.33                      | 2                                 | 11.76                     |
| 11                | 4                             | 4.76                      | 2                                 | 11.76                     |
| 12                | 4                             | 4.76                      | 0                                 | 0.00                      |
| 13                | 6                             | 7.14                      | 0                                 | 0.00                      |
| 14                | 9                             | 10.71                     | 0                                 | 0.00                      |
| 15                | 9                             | 10.71                     | 2                                 | 11.76                     |
| 16                | 10                            | 11.90                     | 1                                 | 5.88                      |
| 17                | 4                             | 4.76                      | 0                                 | 0.00                      |
| 18                | 7                             | 8.33                      | 2                                 | 11.71                     |
| 19                | 11                            | 13.09                     | 2                                 | 11.76                     |
| 20                | 16                            | 19.05                     | 0                                 | 0.00                      |
| 21                | 17                            | 20.23                     | 1                                 | 5.88                      |
| 22                | 8                             | 9.52                      | 1                                 | 5.88                      |
| X                 | 6                             | 7.14                      | 2                                 | 11.76                     |
| Y                 | 5                             | 5.95                      | 0                                 | 0.00                      |

Table 6: Aneuploidy prevalence for different chromosomes in day 3 embryos and blastocysts (excluding number of complex abnormal).

Discussion

This study, as far as we know, is the first study to analyze and characterize aneuploidy formation in Day 3 human embryos in comparison to Day 5 human blastocysts produced by IVF among indigenous Black African women. It is a well-known fact that the quality and appropriate selection of embryo are critical standards for transfer and eventual achievement of the goal of assisted reproductive technologies (ART). Also, the transfer of good quality embryos is associated with increased implantation, pregnancy and live birth rates and also decreased pregnancy loss and prenatal complications in comparison with transfer of embryos with impaired quality.

Previous studies have shown that the success of IVF is drastically reduced when an aneuploid embryo is transplanted, if such embryos are not screened for defect. Thus, it is suggested that transfer of aneuploid embryo could be minimized if there is a screening process to select euploid embryos for transfer. The overall Day 3 embryo euploidy rate of 24.3% observed in this study is similar to the 27.7% earlier reported in USA [25] and the overall Day 5 blastocyst euploidy rate of 75.0% in this study is higher than the 36.7% reported by Yu Su et al. [31] and the 60.9% reported by Haddard et al., [31] after pre-implantation genetic...
screening. This suggests that as in similar studies of pre-implantation of another racial origin, the culture of embryos to blastocysts in black African pre-implantation embryos eliminates aneuploidy in cleavage stage embryos to a certain extent through some sort of self-correction mechanism. However, it is important to note that blastocyst culture alone is not a total eradication of aneuploidy as some studies have shown that the attrition of embryos from day 3 to day 5 is largely due to aneuploidy [50].

Considering age and aneuploidy rate, many studies have observed significant decreasing euploidy rate with advancing age. [51] observed a proportion of euploid embryos in women ≤ 35 years as 35% in day 3 embryos and rapidly declined to 0% by age 44. Harton et al., [52] also showed a similar trend of euploidy rate with increasing age. Similarly, we also observed a decrease in day 3 euploidy rate with advancing age, women aged ≤ 35 had Day 3 euploidy rate of 25.8% while those >35 had euploidy rate of 11.1%. Interestingly also, it was discovered that although the aneuploidy rate of women >35 was significantly higher compared to aneuploidy of younger women in the cleavage group, the situation was different in the blastocyst group as women in the two age groups has similar euploidy rates. These evidences can be useful in the assisted reproductive treatment of older women using own eggs to consider the combination of blastocyst culture and PGS to select euploid embryos for transfer and improve the efficiency of having not just an offspring but healthy offspring at an advanced age.

Some have suggested that aneuploidies in chromosomes 1 to 12 is more common in cleavage stage embryos as these do not persist to the blastocyst stage. [53] however, aneuploidies in chromosomes 13, 18, 21 are able to persist throughout development and are also compatible with life. Another study suggests at the cleavage stage, aneuploidies affect chromosomes 15, 16, 21 and 22 most frequently and chromosomes X and Y least frequently [54] similar to our study with high prevalence of 10.71%, 11.90%, 20.23%, 9.52% for chromosomes 15, 16, 21, 22 and a lower prevalence of 7.14% and 5.95% for X and Y respectively.

Furthermore, we observed a significant decrease in prevalence of chromosome 13 (0.00%) and chromosome 21 (5.88) aneuploidies at the blastocyst stage compared to 7.14% and 20.23% observed respectively at the cleavage stage, supporting suggestions that embryos to blastocyst stage may genetically select those that are competent among them.

**Conclusion**

As evidenced by our study, blastocyst stage biopsy appears to be the most efficient stage of biopsy in PGS, by retrieving 3 – 10 trophoblast cells; more DNA material is available thus improving the accuracy rate as seen with significantly lower rate of embryos with no results in the blastocyst group compared to the cleavage stage group. Another efficiency of the blastocyst stage biopsy can be evidenced as seen in this study with significantly higher euploidy rates in the blastocyst group compared to the cleavage stage group attesting to the fact that blastocyst culture is a form of abnormality screening for pre-implantation embryos. This study was also able to show that in indigenous Black Africans, blastocyst culture and PGS can be more effective in the ART treatment of women of advanced age to achieve pregnancy and live birth of a healthy child.

**Study Limitations**

Although the research has reached its aims, there was some unavoidable limitation. First, because PGS is a relatively new technology amongst indigenous Black Africans, this research was conducted only on a small sample size of population of those attending our IVF unit mainly for the purpose of sex selection by PGS. Therefore, to generalize the results for larger groups, the study should have involved more participants at different ages.

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**Conflicts of Interest**

None.

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