The first live birth in India after the transfer of vitrified, warmed, single euploid blastocyst screened within 24 hours in-house using next generation sequencing technology: A case study

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Abstract

Preimplantation genetic screening (PGS) is now considered as a feasible strategy for the selection of chromosomally normal (euploid) embryos for transfer in IVF programs with the intention of improving live birth rates. Chromosomal abnormalities may cause early pregnancy loss, poor IVF outcomes, recurrent implantation failures (RIF) and miscarriage. A higher rate of these abnormalities are observed in patients with advanced maternal age, women having recurrent pregnancy loss and in couples having chromosomal aberrations. The present case describes a healthy live birth following a single vitrified, warmed euploid blastocyst transfer which underwent PGS using next generation sequencing (NGS).

Conclusion: The implementation of PGS using modern technologies like NGS represents a new era of infertility treatment for couples with the higher chance of a healthy pregnancy as early as within the first cycle. It saves time and the patient's ovarian reserve. Additionally, it avoids the mental burden associated with repeated miscarriages and IVF failures.

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Introduction

PGS has been validated recently and has been used to select chromosomally normal (euploid) embryos for transfer in IVF programs in order to improve live birth rates (Handyside, 2013; Ma et al., 2016). It has been well documented that IVF embryos are prone to age-dependent chromosomal segregation errors at the time of meiosis I and II which can lead to chromosomal abnormalities or aneuploidies (Xu, 2016; Battaglia, 1996). Chromosomal abnormalities may cause early pregnancy loss, poor IVF outcomes, or RIF.

It is now understood that on Day 5 or 6 of the blastocyst trophectoderm (TE) biopsy, the implantation potential may improve in PGS cycles when compared with the biopsy done on Day 3 of the cleavage-stage embryos (Munne, 2010). However, advanced genetic tools may not always be able to provide the PGS report in a turnaround time of less than 24 hours and in

those cases, embryos need to be frozen. The array comparative genomic hybridizattion (aCGH)-based PGS protocol usually takes at least two to three days (analysis time not included) to report from referral PGS laboratories (Ma et.al., 2016). NGS-based PGS may offer several advantages over aCGH in copy number assessment and has shown a high level of accuracy (Treff et al., 2013; Yin et al., 2013). Recently, it has been reported that a combination of trophectoderm biopsy with PGS and blastocyst vitrification has improved the live birth rate in older women with single embryo transfer in a frozen cycle (Schoolcraft et al., 2011).

In this case report, we report a live birth from a single euploid blastocyst transfer in a Frozen Embryo Transfer (FET) cycle. To the best of our knowledge, this is the first report of a live birth from vitrified warmed euploid blastocyst with PGS using NGS technology in a turn around time of 24 hours.

Case report

Prior to reporting this case, approval was obtained from both the Institutional Review Board of the Gunasheela IVF Centre, and Gunasheela Surgical and Maternity Hospital along with a written consent from the couple. The patient was 34 years of age, when she first came for consultation with us in January 2013 with a married life of 19 years. She had irregular cycles of 45-60 days, with polycystic ovarian syndrome (PCOS) as evidenced by high antral follicle count and a high level of Antimullerian hormone (AMH) of 15.42ng/ml. Her husband was 42 years old and his semen analysis report indicated oligoasthenoteratozoospermia. The first IVF cycle was started in June 2013, when she underwent straight stimulation with the antagonist protocol. A total of seven oocyte cumulus complexes were collected in which five were Metaphase II and all were injected with the husband's sperm. Four blastocysts were formed and three embryos were transferred on patient's request. She conceived with dichorionic diamniotic twins but unfortunately had a premature rupture of membranes at 17 weeks and aborted the twins in November 2013. The second cycle was started in October 2014, and the patient opted for the Time Lapse Monitoring System (TMS) EmbryoScope™ (Vitrolife, Sweden) & NGS-based PGS . A total of 35 oocytes were obtained, of which, 24 were M II. All 24 oocytes were fertilised with the husband's sperm using ICSI resulting in 24 embryos. 12 of the 24 embryos were cultured in the embryoscope and the remaining 12 in a trigas incubator (ORIGIO® / PLANER Benchtop Incubator BT37, Denmark). Keeping in mind the risk of ovarian hyperstimulation syndrome, a fresh transfer was not carried out and the embryos were vitrified on Day 4 at the morula stage.

The patient underwent a FET cycle in November 2014 and three embryos from the embryoscope cohort were warmed. Two of the expanding blastocysts were transferred. The patient conceived in the same cycle but miscarried at six weeks. The patient underwent APLA and thrombophilia screening prior to the next FET cycle. Patient was found to be Lupus anticoagulant positive and DRVVT was mildly raised, 1.33 (normal-0.8-1.3). They were counseled regarding PGS which they consented to.

The second FET cycle was started in January 2016 with straight HRT with Progynova 2 mg (Estradiol valerate, Zydus German remedies). In this cycle six morula stage embryos were warmed of which three strictly met the selection criteria of the Embryoscope.An opening was made in the zona of all the embryos using Saturn5 Active™ laser System (Research Instruments, UK) and they were cultured for 24 hours. Five embryos reached blastocyst stage. Trophectoderm biopsy was performed on all the five herniating blastocysts. Aspirated trophectoderm cells from individual blastocysts were transferred into individually marked thin-walled 0.2 mL PCR tubes of which each tube contained 1ul wash buffer solution and were sent to our in-house PGS laboratory.

NGS protocol applied for PGS

The biopsied samples were whole genome amplified and libraries prepared according to and using the ION single Seq kit (Thermo Fisher Scientific).The libraries obtained were then quantified using the Qubit-3 Assay kit (Invitrogen-Life Technologies), bar coded according to manufacturer's protocol and subsequently pooled. Samples were purified using Agencourt Ampure XP beads and quantified using the Qubit-3 Assay kit. They were then diluted to 1nM concentration using low TE buffer and further diluted to 10pM using

nuclease free water. The DNA samples were then subjected to Isothermal Amplification using the Ion PGM Template IA reaction 500kit, where the DNA is amplified on ION sphere particles (ISP's)at a constant temperature of 40^{o}C for 25 minutes.The template positive ISP's were recovered using the Ion PGM Template IA recovery solution.

Enrichment of the Template positive ISPs was carried out on the Ion One touch ES for 35 minutes using 'Dyna BeadsMyOne Streptavidin C1' beads. The enriched template ISP's were annealed using specific primers and loaded onto an'Ion 316 chipV2' and sequenced on the Ion Torrent Personal Genome Machine. Analysis was carried out using the Ion Reporter and IGV Softwares with Low-Coverage Whole-Genome workflow.

Of the five embryo samples that were analysed, two samples did not generate enough data for analysis. As the number of reads were insufficient to provide any conclusions, (a minumum number of reads are required for data analysisand degraded DNA could result in the absence of or insufficient number of reads). Embryos were evaluated on the basis of their IGV data profile which indicates chromosome gains or losses along with a Median Average Pairwise Differences (MAPD) value. Internal confidence scores were based on the Median Average MAPD value, number of reads obtained and the coverage value. Due to a 'Low' confidence value, one embryo sample could not be analysed, one embryo had an aneuploidy of Trisomy 19 and one embryo was normal and was recommended for implantation (Table 1). This embryo was in a hatched blastocyst stage (6AA) when it was transferred (Figure 1).

The serum beta hCG was done on the Day 14 and post transfer and was positive (1456 mIU/mL). Post embryo transfer (ET), the patient was prescribed low molecular weight heparin 0.2 ml (Lonopin, Bharat Serum & Vaccines Pvt Ltd, India), Ecospirin 75 mg, Prednisolone 15 mg along with other progesterone supportives.

The antenatal course was uneventful except for the appearance of Gestational Diabetes Mellitus (GDM) at 11weeks which was managed with Insulin and Oral Hypoglycemic Agents (OHA). All Antenatal scans were normal. Non invasive prenatal testing at 18 weeks was found to be negative for trisomy 13,18 and 21. At 34 weeks patient presented to the ER with preterm labor pains. Tocolytic ritodrine drip was started and 2 doses of steroid prophylaxis with betamethasone 12mg was administered, following which, a decision to proceed for emergency LSCS was taken. A live preterm female baby with a weight of 2.5kg was extracted on September 14, 2016 at 5.30AM. No external abnormalities were observed.

Discussion

The implementation of PGS in IVF programs were first reported as successful in the UK & USA in 1990 (Handyside, 1990). Initially fraught with complications and associated with a high risk of errors, advances in the field of Artificial Reproductive Technology (ART) has grown by leaps and bounds which is evident in the success rates obtainable at present. The indications to enroll in an IVF with PGS program are based on the following criterions and can include one or more of the following; advanced maternal age, RIF, recurrent miscarriages with normal karyotypes, recurrent miscarriages with balanced translocations and severe male infertility. As aneuploidies are usually the causal factors for failed pregnancies it has been indicated that screening embryos before implantation for aneuploidies increases the chance of a favorable outcome in IVF. Concurrently, many studies also prove that a Day 5 embryo biopsy and transfer have increased pregnancy rates as compared to Day 3 biopsy and transfer (Mastenbroek, 2007).

Many methods have been used to enable PGS,with an aim to select euploid as opposed to aneuploid embryos for transfer, the first of which was Fluorescent*in situ*Hybridization (FISH). However, this technique only screened for a few chromosomes and did not contribute much in increasing the outcome rate while it did have an error rate of up to 15% (Yang et al., 2015). Studies employing FISH as a method of PGS did not observe a significant benefit in including the procedure as a means to increase pregnancy rates (Hardarson et al., 2008; Schoolcraft et al., 2009).

This changed with the advent of more intricate screening techniques like Comparative Genomic Hybridization (CGH) and aCGH, as,when these were employed as methods of PGS, a significant increse in positive outcomes were observed that indicated the advocacy of

PGS. These techniques screened all 24 chromosomes and aCGH has been the choice of PGS for a few years now and has proved successful in many studies (Hardarson, 2008; Gutiérrez-Mateo et al., 2011).

The introduction of NGS techniques has now further advanced the method of PGS and promises to improve the PGS and IVF procedure (Handyside 2013; Treff et al., 2013). NGS has many advantages over the older techniques as it is capableof detecting single gene mutations, de novo mutations and mitochondrial mutations (Liss, 2016). Its ability to generate high throughput data and especially the fact that samples can be multiplexed makes this a highly desirable and cost effective technology. It also enables the fresh or frozen transfer as the results can be obtained within 24 hours using NGS.

Studies comparing the results of PGS employed in conjunction with Single Nucleotide polymorphism(SNP) array, FISH, aCGH are now quite a few. The study by Tan et al. (2014) was one of the first to compare SNP arrays and NGS in PGS. Of the 150 blastocysts tested by both methods, SNP analysis failed to pick up aneuploidies in seven embryos which was picked up by the NGS method and confirmed by Quantitative PCR. Though this study did not observe a significant difference in the clinical outcome, it did indicate the reliability of the NGS method over SNP analysis in weeding out both false positives as well as false negatives. Similarly, studies have compared aCGH with NGS, a recent study by Yang et al. (2015) , observed that the NGS method was as consistent and comparable to the much more validated aCGH method. Though there were no significant differences between the two methods in terms of outcome, the percentages of aneuploid embryos detected, miscarriage rates and ongoing pregnancies (at the time of writing), there was however, a noted difference in the ability of NGS to detect partial aneuploidies and segmental changes as compared to a CGH.

A study by Lukaszuk (2015) compared the application of NGS to carry out PGS with IVF as opposed to only IVF. The study showed twice the pregnancy rate in the study group as opposed to that of the control group, 84.4% versus 41.5%.

Conclusion

In the present case report, we carried out PGS on vitrified warmed embryos at blastocyst stage with a 24 hours turn around time, which is yet in the initial stages of establishment and of which, not many reports are available. We were successful in accomplishing this as we have established an in-house PGS laboratory associated with our ART centre. This is the first case report from India of a live birth using PGS with NGS to transfer a single euploid embryo from a set of embryos screened. More studies are definitely needed to compare outcome rates as this is still a newly adopted technology in India and more data needs to be generated and analyzed for actualities to be established. It is however quite apparent based on current worldwide studies that NGS holds promise as a PGS technology and would be advantageous technique to choose a viable embyo to transfer, especially when one includes goals like picking up segmental and partial aneuploidies, turnaround time, ability to multiplex samples and costs involved.

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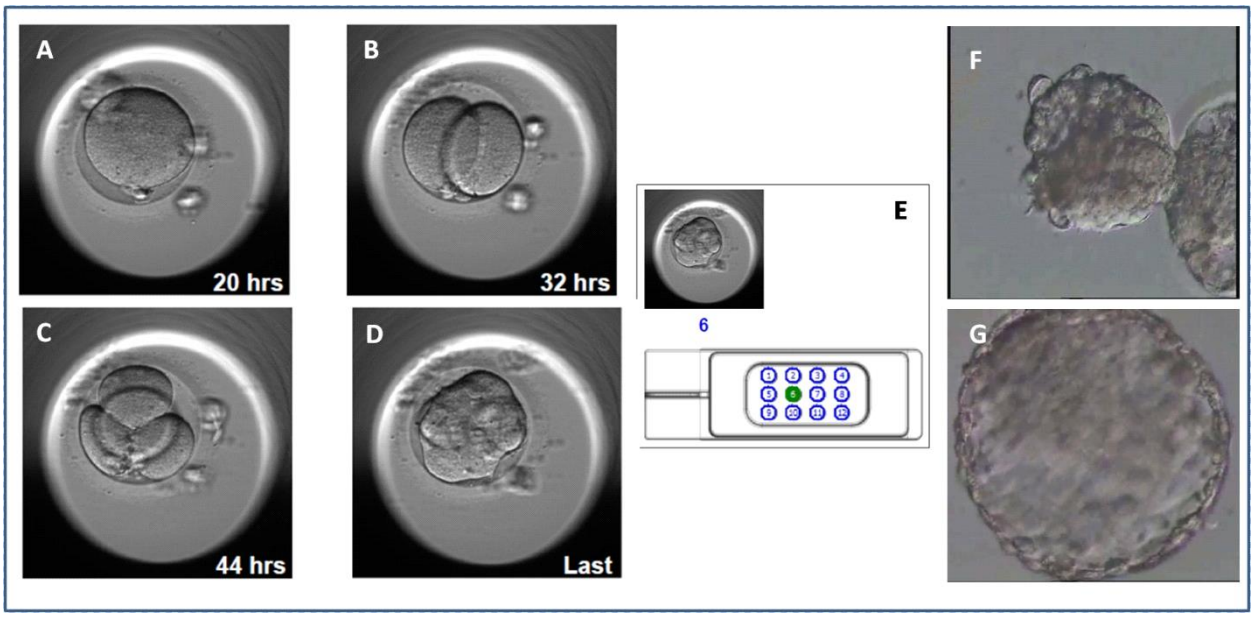
Table 1: NGS-based PGS analysis report of 5 blastocyst stage embryos

	PGS Results	Transfer Recommendation From PGS Laboratory
Embryo 1	Normal with low confidence value	No recommendation
Embryo 2	Normal	YES
Embryo 3	Aneuploid - (Trisomy 19)	NO
Embryo 4	Not enough data	No recommendation
Embryo 5	Not enough data	No recommendation

Figure 1: EmbryoScope embryo development till day 4 morulla stage (A-D), Morulla stage embryo and embryo position on EmbryoSlide (E), thawed and cultured embryo on Day 5 blastocyst at the time of embryo biopsy (F), and fully hatched euploidy embryo on day 6 used for single embryo transfer which was led to a live birth (G)



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