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Non-invasive chromosome screening of embryos fertilized *in vitro*: A single center, retrospective study

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ABSTRACT

Objective: To evaluate the center's experience with non-invasive preimplantation genetic testing for aneuploidy (NIPGT-A), focusing on embryo aneuploidy assessment, euploidy-linked pregnancy outcomes, and the relationship between embryo morphology and chromosomal status.

Methods: A retrospective study was conducted on couples undergoing *in-vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles that yielded day-5 blastocysts eligible for NIPGT-A. Spent culture media from 159 blastocysts were analyzed using multiple annealing and looping-based amplification cycles (MALBAC)-based whole genome amplification followed by next-generation sequencing with a 10 Mb resolution threshold for copy-number variation detection. Embryo ploidy was categorized as euploid, low mosaic, high mosaic, or aneuploid based on cell-free DNA (cfDNA) abnormality levels. Euploid embryos were transferred in subsequent frozen embryo transfer (FET) cycles. Clinical outcomes included biochemical pregnancy, clinical pregnancy, ongoing pregnancy, and live birth rates.

Results: Fifty couples were included in this study. Among 213 blastocysts formed, 159 (74.7%) underwent NIPGT-A. Of these, 43.4% were euploid, 19.5% aneuploid, 23.3% mosaic, and 13.8% uninterpretable. A total of 82 embryos were transferred across 45 FET cycles, including euploid (69), mosaic (7), and uninterpretable (6) embryos. The clinical pregnancy rate was 53.3%, with a biochemical loss rate of 6.7% and early pregnancy failure of 4.4%. Ongoing pregnancy occurred in nearly half of the participants, and several successful outcomes were documented even among mosaic and uninterpretable embryos. Embryo morphology did not correlate significantly with euploidy, reaffirming the limited predictive value of morphologic grading alone.

Conclusion: NIPGT-A is a feasible, safe, and clinically meaningful alternative to invasive PGT-A, offering favorable pregnancy outcomes while avoiding embryo manipulation. Although technical limitations such as cfDNA variability and uninterpretable results persist, NIPGT-A enhances embryo selection, reduces miscarriage

risk, and is particularly beneficial in low-yield or high-risk IVF populations. Continued refinement of laboratory protocols and long-term outcome studies are essential for broader clinical adoption.

Keywords: NIPGT-A; Spent culture media; Non-invasive genetic testing; IVF; Embryo selection; Aneuploidy; cfDNA; MALBAC; Next-generation sequencing

Key Points

Question: Does non-invasive pre-implantation genetic testing of embryos fertilized *in-vitro* improve pregnancy outcome?

Findings: In this retrospective study involving 50 couples undergoing NIPGT-A, 213 day 5 blastocysts were obtained, of which 159 blastocysts were tested for NIPGT-A. Of the embryos, 82 (51.6%) were transferred, predominantly euploid (69 embryos), 24 couples (53.3%) achieved an ongoing pregnancy, reflecting a promising success rate and good pregnancy outcome of 21 live births among the study population containing 38% recurrent pregnancy loss and 20% recurrent implantation failure rate.

Meaning: NIPGT-A-guided embryo transfer may improve clinical pregnancy outcomes by enabling a more individualized and targeted approach to *in vitro* fertilization for women.

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1. Introduction

Assisted reproductive technology (ART), particularly *in-vitro* fertilization (IVF)/Intracytoplasmic sperm injection (ICSI), has revolutionized the management of infertility. One of the recent innovations in this field is preimplantation genetic testing (PGT), which aims to improve pregnancy outcomes by identifying embryos with genetic abnormalities[1]. Traditional PGT techniques involve invasive embryo biopsies, including polar body biopsy, cleavage-stage biopsy, blastomere biopsy, and Day 5/6 trophectoderm biopsy[2]. While effective, these approaches carry risks such as potential harm to the embryo, increased costs and technical complexity[3].

To overcome these limitations, a novel approach, non-invasive preimplantation genetic testing for aneuploidy (NIPGT-A) has emerged. NIPGT-A analyzes cell-free DNA (cfDNA) released by embryos into the culture medium or blastocoel fluid, offering a safer and less invasive alternative[4]. This cfDNA originates from normal physiological cell turnover or apoptosis and reflects the embryo's genetic status[5].

Recent advancements in whole genome amplification (WGA) and next generation sequencing (NGS) have further improved the accuracy and reliability of NIPGT-A[6]. Key advantages include avoidance of embryo injury, the ability to use higher sample volumes, high amplification rates, and minimal technical expertise requirements, making NIPGT-A a promising alternative to conventional methods[7]. This study represents a detailed analysis of NIPGT-A outcomes from a tertiary fertility center, focusing on its clinical utility, accuracy, and reproductive outcomes.

1.1. Aim

To analyze and describe the center's experience with the adoption of NIPGT-A.

1.2. Objectives

1. To assess the aneuploidy status of embryos tested using NIPGT-A.
2. To evaluate the euploidy status and corresponding clinical pregnancy rates.
3. To examine the association between embryo morphological grades and euploidy rates.

2. Methods

This observational study was conducted at a tertiary fertility center in strict accordance with ethical principles. Informed consent was

obtained from all participants prior to sample collection.

2.1. Inclusion criteria

Couples with advanced maternal age, long-standing infertility, recurrent implantation failures, recurrent pregnancy loss, previous IVF failures, male factor infertility requiring ICSI, or undergoing aneuploid screening with elective single euploid blastocyst transfer using Day 5 blastocysts were included, irrespective of embryo transfer status.

2.2. Exclusion criteria

Patients who did not provide consent for testing or were carriers of structural chromosomal abnormalities or monogenic diseases were excluded.

2.3. Population

A total of 50 patients meeting the inclusion and exclusion criteria and achieving blastocyst formation *via* ICSI cycles were included. From these patients, 213 Day 5 blastocysts were obtained, of which 159 blastocysts were tested for NIPGT-A.

2.4. Oocyte retrieval

Individualized ovarian stimulation protocols were applied based on age and ovarian reserve, using a gonadotropin-releasing hormone (GnRH) antagonist protocol (Cetrorelix, CETNOSURGE, Gufic Biosciences LTD, Gujarat, India). The starting dose of recombinant FSH (Newmon-R, Gufic Biosciences LTD, Gujarat, India) ranged from 150–450 IU, with HMG (CipHMG150, SANZYME Private LTD, Telangana, India) added later as needed.

When three or more follicles ≥ 18 mm in diameter were observed ultrasonographically, ovulation was triggered using either leuprolide 2 mg (GnRH agonist, Eurolide, SAMARTH Life Sciences Pvt LTD, Himachal Pradesh, India) or human chorionic gonadotropin (hCG) 6500 IU (r-Hucog, Bharat Serums and Vaccines Ltd, Maharashtra, India), depending on ovarian response and serum estrogen (E_2) levels. Oocyte retrieval was performed under general anesthesia 36 hours after trigger.

Oocytes were retrieved 36 hours after ovulation trigger, and cumulus cells were removed using hyaluronidase (V-HYADASE, Langenfeld, Germany). ICSI was performed 3–4 hours post-retrieval. Fertilized oocytes were cultured individually in one-step culture medium (Vitromed, Langenfeld, Germany) until the Day 5 blastocyst stage, with all embryos maintained in 30 μ L micro-droplets[8].

2.5. Spent culture medium analysis

Embryos were morphologically graded on Day 5. Based on the number, quality, grade of embryos per patient and also after logistical considerations, 159 embryos were selected for NIPGT-A testing. For each patient, spent culture medium (SCM) was collected on Day 5. The embryos in the medium that was collected for testing were incubated under identical conditions and subsequently frozen without exposure for testing.

cfDNA from the spent culture medium was subjected to pre-PCR and WGA using multiple annealing and looping-based amplification cycles (MALBAC) technology. MALBAC provides high sensitivity, uniformity, and genome coverage for DNA amplification.

Following WGA, library preparation and library quality assessment were performed. cfDNA was incorporated into NGS libraries using target capture enrichment (TACS) technology, optimized for low-abundance and fragmented cfDNA[9]. Sequencing was carried out using the Thermo Fisher NGS platform, generating over 200,000 raw reads per sample.

The resulting FASTQ and BAM files were analyzed using ChromGo™ software for data visualization and copy number variation (CNV) analysis, with GRCh38 used as the reference genome. For reliable CNV/aneuploidy detection from cfDNA in spent culture media using NGS, a minimum resolution of 10 Mb (megabases) was used as the standard cutoff[10]. Samples generating <150 000 reads were considered uninterpretable, and chromosomal analysis could not be performed.

The ploidy status of embryos/SCM was classified as follows:

- <30% abnormality: Euploid
- 30%–50% abnormality: Low mosaic

- 51%–80% abnormality: High mosaic
- >80% abnormality: Aneuploid

2.6. Frozen embryo transfer and pregnancy

Embryos with a euploid SCM report were thawed and transferred in subsequent frozen embryo transfer (FET) cycles. Transfers were performed after endometrial synchronization with a Day 5 blastocyst. Hormonal replacement protocols were used for endometrial preparation, and all transfers were performed under ultrasound guidance.

Pregnancy testing was performed 16 days post-transfer. A pregnancy was considered positive if serum beta-human chorionic gonadotropin (β -HCG) ≥ 100 IU/L. All the ongoing pregnancies are beyond 20 weeks of gestation.

2.7. Statistical analysis

All categorical variables are presented as frequencies. Associations within groups were analyzed for significance using Fisher’s exact test, with P -value < 0.05 considered statistically significant. All statistical analyses were performed using STATA 14.2 software.

2.8. Ethics statement

The study was approved by the Iswarya Fertility Centre Ethical Committee, affiliated with Iswarya Fertility Centre and Hospital. Ethical clearance was obtained under approval number: IFC/24/1/4 dated 20-12-2024. Written consent was taken from all the participants who met the inclusion criteria regarding the purposes

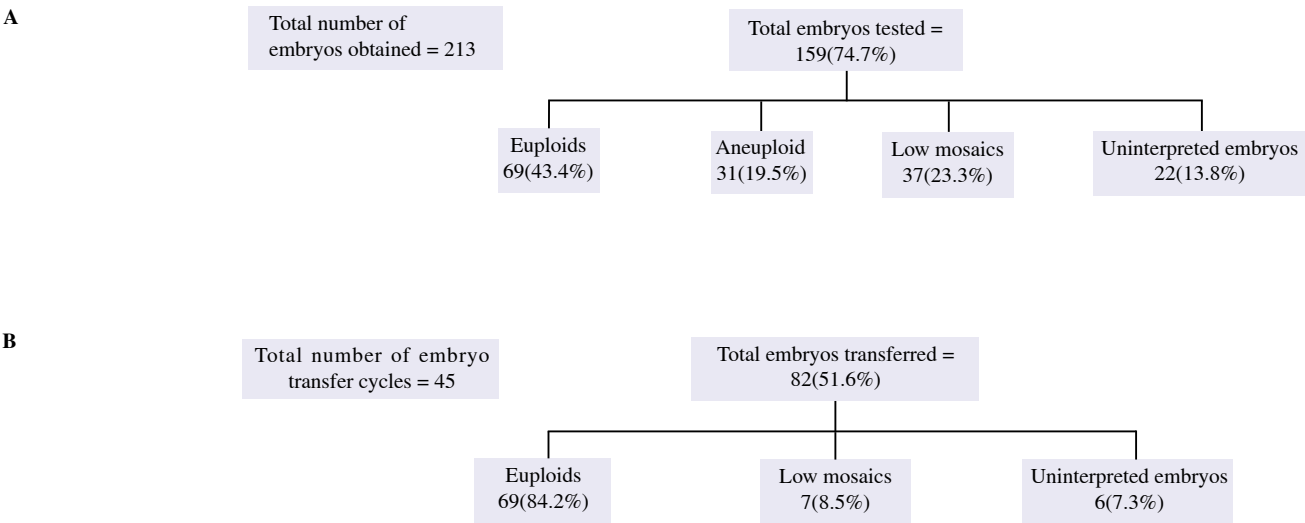


Figure 1. Embryos tested and their ploidy status & transfer status. A) Total number of embryos tested and their ploidy status; B) Total number of embryos transferred and their ploidy status.

and method of the study. All participants were also guaranteed that their information was kept confidential and anonymous and that the research results would be used and published only for research purposes. All study procedures adhered to the principles outlined in the Declaration of Helsinki and the Indian Council of Medical Research (ICMR) ethical guidelines for biomedical research.

3. Results

The majority of the female participants were aged 31–40 (66%) and male participants 36–45 (70%). Eighty percent of couples had infertility for over five years. Main causes of infertility were recurrent pregnancy loss (38%) and poor ovarian reserve (24%). The main indication for NIPGT-A testing was recurrent pregnancy loss (38%), followed by recurrent implantation failure (20%). 64% had undergone prior IVF treatments and only 2 achieved live births. Obstetric history revealed that 78% of the participants had a history of abortions. 58% had Sr. AMH levels between 1.5–4.0 ng/mL. Karyotyping results showed that 92% of participants had normal genetic profiles and in the rest 8% at least one of the partners had a heterozygous chromosomal abnormality. Among male partners, 70% had normal semen parameters, while abnormalities such as oligozoospermia, teratozoospermia, and OATS were present in the remaining cases. Data on various medical comorbidities in the female partners have been demonstrated (Table 1).

A total of 213 embryos were obtained from the 50 study participants, out of which 159 (74.7%) were tested for chromosomal status. The distribution of embryo nature revealed that 43.4% were euploid (chromosomally normal), 19.5% were aneuploid, and 23.3% were mosaic embryos. Around 13.8% of embryos could not be interpreted due to insufficient DNA reads. Of the embryos, 82 (51.6%) were transferred, predominantly euploid (69 embryos), followed by 7 mosaic embryos and 6 uninterpreted embryos in 45 transfer cycles (45 patients) (Figure 1). Additionally, 5 participants did not undergo embryo transfer, due to various clinical, logistical, and personal factors. Among the 50 study participants, the distribution of outcomes following embryo transfer showed that nearly half (53.3%) achieved an ongoing pregnancy, reflecting a promising success rate in this cohort. However, 6.7% experienced a biochemical loss and 4.4% had early pregnancy failure after clinical confirmation. A negative outcome, where pregnancy was not achieved, was reported in 35.6% of participants (Figure 2). In comparison to embryos with grade 3 morphology, there is no significant difference in the probability of euploids in blastocysts with morphological grade 1 ($OR=1.50$, $P=0.27$) and grade 2 ($OR=0.77$, $P=0.50$) (Figure 3).

Table 1. Baseline demographic and clinical characteristics of the study group.

Characteristics	N=50
Age distribution of female patients, n(%)	
<25 Years	1(2%)
25-30 Years	6(12%)
30-35 Years	15(30%)
35-40 Years	18(36%)
40-45 Years	10(20%)
>45 Years	0(0%)
Age distribution of male patients, n(%)	
<25 Years	0(0%)
25-30 Years	0(0%)
30-35 Years	10(20%)
35-40 Years	19(38%)
40-45 Years	16(32%)
>45 Years	5(10%)
Duration of couples infertility, n(%)	
<2 Years	1(2%)
2-5 Years	9(18%)
5-10 Years	21(42%)
10-15 Years	16(32%)
15-20 Years	3(6%)
Cause of infertility, n(%)	
Unexplained	7(14%)
B/L tubal block	1(2%)
B/L salpingectomy	4(8%)
Male factor	3(6%)
PCO	3(6%)
POR and male factor	6(12%)
POR	6(12%)
Endometriosis	1(2%)
RPL	6(12%)
RPL with male factor	6(12%)
RPL with advanced maternal age	7(14%)
Indication for NIPGT-A, n(%)	
RPL	12(24%)
RPL with advanced maternal age	7(14%)
Recurrent implantation failure	10(20%)
Previous aneuploidy	4(8%)
Advanced maternal age	7(14%)
Long years of infertility with advanced maternal age	3(6%)
Long years of infertility	7(14%)
Comorbidities of the female partners in the study group, n(%)	
Nil	27(54%)
Hyperthyroid	3(6%)
Hypothyroid	6(12%)
Diabetes mellitus	7(14%)
DM and hypothyroid	4(8%)
Hypertension and hypothyroid	1(2%)
Kidney failure and renal transplant	1(2%)
DM and hypertension	1(2%)

Note: RPL: recurrent pregnancy loss; POR: poor ovarian reserve; PCO: polycystic ovaries; NIPGT-A: non invasive preimplantation genetic testing for aneuploidy; DM: diabetes mellitus.

4. Discussion

Preimplantation genetic testing for aneuploidy (PGT-A) was initially introduced to reduce the incidence of aneuploid pregnancies by screening embryos for chromosomal abnormalities. Over time, its role has expanded significantly, contributing to improved

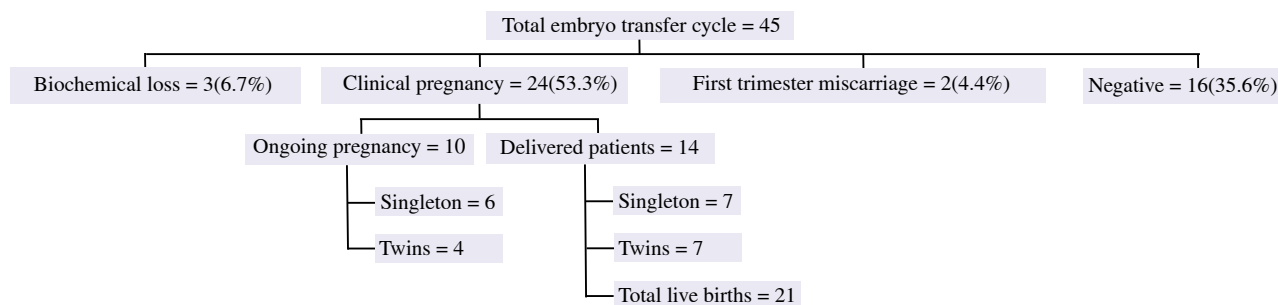


Figure 2. Pregnancy outcomes.

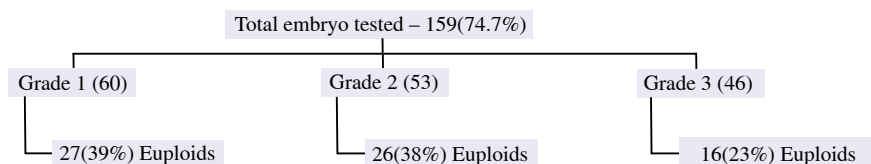


Figure 3. Ploidy status vs morphology of embryos.

outcomes in ART through better embryo selection, reduction of pregnancy loss, and duration of infertility, which minimizes risks associated with multiple pregnancies. Technological advancements such as vitrification, NGS, and refined biopsy methods have further enhanced its clinical utility[11]. Despite widespread use, the utility of PGT-A remains controversial, especially concerning its predictive value from a single trophectoderm (TE) biopsy. Studies suggest inconsistent concordance between TE and inner cell mass (ICM) and highlight the limitations posed by embryo mosaicism and segmental aneuploidies. Moreover, the appropriateness of PGT-A is influenced by patient-specific factors such as maternal age, infertility history, and clinic expertise.

Our study supports the growing clinical evidence on the feasibility and outcome relevance of NIPGT-A. The observed euploidy rate of 43.4% mirrors findings by Kim *et al* who reported similar rates using spent culture media[12]. Notably, the aneuploidy rate (19.5%) in our cohort is significantly lower than in traditional PGT-A studies, such as those by Bolton *et al* which reported rates above 40% in advanced maternal age populations[13]. This difference may arise from limitations in cfDNA quantity, allele dropout, or underrepresentation of abnormal DNA fragments in non-invasive samples[14].

Despite this, the clinical pregnancy rate (53.3%) in our study is consistent with NIPGT-A literature, such as Fang *et al* who reported a 58% rate[15]. Our miscarriage rate (4.4%) was notably lower than the 10%–15% range often reported for euploid embryo transfers, possibly due to reduced embryo manipulation and stress.

One important observation is the lack of significant correlation between embryo morphological grading and ploidy status. This echoes findings by Fang *et al* who demonstrated that visual grading does not reliably predict chromosomal normalcy. Morphologically high-grade embryos were not consistently euploid, suggesting the stochastic nature of chromosomal errors during meiosis. These

findings advocate for the integration of molecular diagnostics into embryo selection strategies, especially in low-yield cycles where biopsy risks outweigh benefits[16].

The study population was demographically diverse, including patients with long-standing infertility, advanced maternal age, and multiple IVF failures. Among 32 couples with prior IVF attempts, only two had achieved live births, underscoring the challenging nature of our cohort. Notably, male factor infertility was implicated in 30% of cases, with 8% showing Oligoasthenoteratozoospermia (OATS) and another 8% displaying sperm morphological abnormalities. This aligns with Kakourou *et al*'s findings on the influence of male factors on blastocyst quality and cfDNA release[17].

NIPGT-A is based on analyzing cfDNA released into culture media by developing blastocysts. This cfDNA likely originates from apoptotic cells of both the TE and ICM[18], which could account for mixed or mosaic signals in sequencing data. The lower-than-expected aneuploidy detection might reflect mosaicism or suboptimal cfDNA yields. However, the consistency of the euploidy rate with age-related expectations indicates reliable detection within current technical constraints.

The WGA process, a key component of cfDNA analysis, introduces technical challenges including allele dropout and amplification bias, leading to uninterpreted or inaccurate results. Our uninterpreted rate of 13.8% could be mitigated by optimizing DNA input and refining WGA protocols. Innovations such as single-cell sequencing and high-fidelity amplification can further improve reliability[19].

The live birth rate per cycle was 46.7%, and 7 of the 21 live births were twins. While twin births can be seen as a success in some clinical settings, they raise maternal and fetal risks, necessitating careful embryo transfer policy considerations.

Importantly, embryos with uninterpreted or low-mosaic results

showed successful implantation and live births, reinforcing the need for clinical discretion rather than automatic rejection of ambiguous cases. This supports recommendations for multidisciplinary review in such scenarios.

Technical aspects such as cfDNA origin, WGA fidelity, and media contamination remain limitations. The low miscarriage rate suggests reduced embryo stress, supporting non-invasive testing advantages[20]. Male factor infertility in 30% of cases also highlights the potential paternal influence on cfDNA yield.

These findings align with global literature suggesting that while not yet a complete substitute for invasive PGT-A, NIPGT-A is effective, especially in select populations.

5. Conclusions

Our center's experience with NIPGT-A supports its feasibility and potential to improve reproductive outcomes in selected infertility populations. Despite technical limitations inherent in cfDNA-based testing, NIPGT-A provided reliable identification of euploid embryos, yielding a clinical pregnancy rate of 53.3% and a live birth rate of 46.7% per transfer cycle. The method also minimized embryo manipulation and reduced miscarriage incidence, thereby contributing to safer and more efficient ART practices. The lack of a significant correlation between morphology and ploidy status underscores the importance of molecular screening over visual assessment. Furthermore, the comparable outcomes achieved in embryos classified as low mosaic or uninterpreted suggest that clinical judgment, in conjunction with laboratory findings, should guide transfer decisions in ambiguous cases. Given the inclusion of challenging cases such as RPL, advanced maternal age, and male factor infertility, the outcomes are promising and reflect the real-world applicability of NIPGT-A in diverse clinical settings.

5.1. Recommendations

Selectively in low-risk populations: Given its non-invasive nature and clinical reliability, NIPGT-A shall be reserved for aneuploidy testing and low risk groups.

Standardize cfDNA collection protocols: Protocols must be optimized for media collection, timing, and media volume used so that maximum DNA yield and minimum contamination and more reliability is achieved.

Interpret uninterpreted or low mosaic results with caution: A multidisciplinary embryo assessment committee should review cases with ambiguous results before determining clinical disposition.

Invest in molecular validation techniques: Integration of NGS platforms with improved WGA protocols and use of orthogonal validation (*e.g.* trophoectoderm rebiopsy when possible) can enhance

diagnostic accuracy.

Encourage long-term follow-up studies: Data on neonatal and long-term health outcomes from NIPGT-A-conceived pregnancies are essential for establishing its safety profile.

5.2. Clinical implications

Reduced embryo trauma: The non-invasive nature of NIPGT-A obviates the need for trophoectoderm biopsy, making it safer for embryos.

Enhanced embryo selection: Euploidy detection facilitates more informed embryo selection and improves clinical pregnancy and live birth outcomes. **Applicable in low-yield cycles:** NIPGT-A may be preferable in cases with limited blastocyst numbers, where biopsy might compromise embryo viability.

Decreased miscarriage risk: The lower observed miscarriage rate suggests that NIPGT-A can reduce early pregnancy loss, particularly in high-risk groups. **Supports precision medicine:** NIPGT-A contributes to the personalisation of ART protocols, enhancing success rates through tailored embryo selection.

5.3. Strengths

The primary strength of this study is its inclusion of a clinically diverse population reflective of real-world ART practice, encompassing patients with prolonged infertility, advanced maternal age, and male factor infertility. The sample size of embryos ($n=213$) and transfer cycles ($n=45$) provides substantial data to assess trends and outcomes.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Data availability statement

The raw data files will be available on request to the corresponding

author.

Authors' contributions

Dr. Gayathri K, consultant gynaecologist designed the study, analysed clinical and experimental data, and wrote the manuscript. Dr. Manipriya R, senior consultant gynaecologist evaluated the experimental data and revised the manuscript. All authors approved the final manuscript.

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