# CLINICAL STUDY PROTOCOL

## Clinical Outcomes of Non-invasive Embryo Implantation Potential Assessment Versus Conventional Morphological Selection for Single Blastocyst Transfer Following Conventional IVF: A Multicenter Randomized Controlled Trial

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| Sponsor | Sun Yat-sen Memorial Hospital, Sun Yat-sen University |
| Official Title | Clinical Outcomes of Non-invasive Embryo Implantation Potential Assessment Versus Conventional Morphological Selection for Single Blastocyst Transfer Following Conventional IVF: A Multicenter Randomized Controlled Tria |
| ClinicalTrials.gov Identifier (NCT Number) | Not yet assigned |
| Protocol ID / Version | NICSAI20260107 |
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| Principal Investigator | Ping Yuan |

### Compliance Statement

This study will be conducted in accordance with Good Clinical Practice (GCP), the Administrative Measures (Trial) for investigator-initiated clinical research in medical institutions, and the Declaration of Helsinki. All study staff will be trained prior to participation. The study will be initiated only after written approval by the Ethics Committee and written informed consent from each participant. Any protocol amendment must be re-submitted and approved before implementation.

## 1. Protocol Synopsis

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| Study design | Multicenter, prospective, randomized controlled clinical trial; participant-blinded. |
| Objective | To evaluate reproductive effectiveness and safety of NICS-AI-guided embryo selection in women of advanced maternal age and/or with recurrent pregnancy loss undergoing conventional IVF (c-IVF). |
| Population | Women aged 35-43 years and/or with >=2 pregnancy losses before 28 gestational weeks (including biochemical pregnancy), undergoing c-IVF. |
| Sample size | 520 participants (1:1 randomization; 260 per arm). |
| Primary endpoint | First live birth rate after the first frozen-thawed single blastocyst transfer. |
| Secondary endpoints | First clinical pregnancy rate; first early miscarriage rate (<12 weeks, excluding biochemical pregnancy); first ongoing pregnancy rate (>=12 weeks); cumulative clinical pregnancy, early miscarriage, ongoing pregnancy, and live birth rates within 1 year after randomization (up to 3 single-blastocyst transfers). |
| Safety endpoints | Fetal malformation rate; prenatal screening/diagnostic results; delivery and neonatal outcomes (sex, weight, length, birth defects). |
| Statistical analysis | Primary analysis by intention-to-treat (ITT) with supportive per-protocol and safety analyses; chi-square/Fisher tests for proportions; t-test or nonparametric tests for continuous variables; multivariable logistic regression as appropriate; two-sided alpha=0.05. |
| Planned outputs | Peer-reviewed publications. |

## 2. Introduction

Embryo selection remains a key determinant of clinical pregnancy and live birth in in vitro fertilization (IVF). Conventional morphological assessment (e.g., Gardner grading) is widely used, but is subjective and cannot directly reflect chromosomal status. Aneuploidy rates remain substantial even among morphologically high-quality blastocysts, and increase markedly with maternal age, thereby limiting pregnancy outcomes.

Preimplantation genetic testing (PGT) provides direct genetic assessment but is invasive, requires biopsy, has restricted indications, and often necessitates intracytoplasmic sperm injection (ICSI), limiting broad implementation in routine IVF practice.

Non-invasive chromosome screening (NICS) analyzes cell-free DNA (cfDNA) in spent embryo culture medium as a non-invasive source for chromosomal assessment. While NICS can avoid biopsy-related

risks, its diagnostic performance may be affected by contamination and biological factors. Artificial intelligence (AI) approaches that integrate developmental day, morphology, and NICS-derived signals may improve the prediction of euploidy and implantation potential. Existing evidence has largely been generated in ICSI cohorts; robust multicenter randomized controlled trial (RCT) evidence in conventional IVF (c-IVF) is needed.

## 3. Risk/Benefit Assessment

Study samples are collected from spent blastocyst culture medium that would otherwise be discarded after embryo cryopreservation; therefore, the NICS-AI procedure does not introduce direct physical risk to participants or embryos beyond routine IVF procedures.

Potential benefits include improved embryo prioritization for transfer, potentially increasing implantation and live birth rates and reducing miscarriage risk through non-invasive selection.

Potential risks primarily relate to (i) blastocyst culture and cryopreservation (including the possibility of no usable blastocysts and rare suboptimal post-thaw survival), and (ii) limitations of non-invasive testing (e.g., contamination, test failure, false-positive/false-negative results). NICS-AI results are intended for prioritizing transfer order only and should not be used as the sole indication for embryo disposal. Routine prenatal care and diagnostic testing (e.g., amniocentesis when indicated) are recommended following a successful pregnancy.

## 4. Study Objectives and Endpoints

### 4.1 Primary objective

To determine whether NICS-AI-guided embryo selection increases the first live birth rate after the first single blastocyst transfer compared with conventional morphology-based selection.

### 4.2 Primary endpoint

First live birth rate: number of deliveries resulting in at least one live-born infant after the first embryo transfer cycle divided by the number of randomized participants x 100%.

### 4.3 Secondary endpoints

- First clinical pregnancy rate: ultrasound confirmation of one or more gestational sacs (including intrauterine pregnancy, ectopic pregnancy, or heterotopic pregnancy) after the first transfer / number randomized x 100%.
- First early miscarriage rate: spontaneous pregnancy loss before 12 gestational weeks after the first transfer (excluding biochemical pregnancy) / number of clinical pregnancies x 100%.
- First ongoing pregnancy rate: pregnancy continuing to 12 gestational weeks after the first transfer / number randomized x 100%.
- Cumulative clinical pregnancy rate within 1 year after randomization: clinical pregnancy achieved during up to three single-blastocyst transfers in one oocyte retrieval cycle / number randomized x 100%.

- Cumulative early miscarriage rate within 1 year: early miscarriage (<12 weeks) during the transfer period / number of clinical pregnancies during the transfer period x 100%.
- Cumulative ongoing pregnancy rate within 1 year: ongoing pregnancy to 12 gestational weeks during the transfer period / number randomized x 100%.
- Cumulative live birth rate within 1 year: deliveries with at least one live-born infant during the transfer period / number randomized x 100%.

### 4.4 Safety endpoints

- Fetal malformation rate: total malformations (including those identified in miscarriages and deliveries) / total intrauterine live fetuses x 100%.
- Prenatal screening/diagnostic results (e.g., chorionic villus sampling, amniocentesis, cord blood genetic testing where available).
- Delivery and neonatal outcomes (mode of delivery, gestational age, sex, birthweight, length, and birth defects).

### 4.5 End of study

The study ends 35 months after the last participant's oocyte retrieval, allowing observation of transfers within 1 year after oocyte retrieval and follow-up of offspring to 1 year postpartum.

## 5. Study Population

### 5.1 Inclusion criteria

(1) Conventional IVF (c-IVF) insemination.

(2) Either (a) advanced maternal age: 35-43 years, or (b) recurrent pregnancy loss: >=2 pregnancy losses before 28 gestational weeks, including biochemical pregnancy (serum hCG >25 IU).

(3) Agreement to culture all Day-3 embryos to blastocyst in the fresh cycle, or to culture >=6 embryos (with >=1 embryo having >=7 cells on Day 3), and to cryopreserve all embryos as single blastocysts.

(4) Agreement to undergo frozen-thawed single blastocyst transfer.

(5) At least two Day-5/Day-6 blastocysts derived from 2PN fertilization, with morphology grade >=4BC/4CB.

(6) Signed written informed consent.

### 5.2 Exclusion criteria

(1) Any fertilization approach involving intracytoplasmic sperm injection, including but not limited to ICSI, TESA-derived ICSI, or PGT-related procedures.

(2) Known monogenic disorder or chromosomal disorder meeting PGT indications at enrollment.

(3) Use of donor oocytes to achieve pregnancy.

(4) Untreated conditions affecting uterine cavity anatomy or endometrial receptivity (e.g., uterine malformations such as septate/unicornuate/didelphys uterus; untreated hydrosalpinx).

(5) Contraindications to pregnancy or to assisted reproductive technology.

(6) Any other condition judged by investigators to make the participant unsuitable for the study.

### 5.3 Screening failure

Participants may be considered screening failures if they withdraw consent, deviate from the protocol, decline to continue study procedures, or conceive naturally before randomization. Reasons will be documented in the case report form (CRF).

### 5.4 Recruitment and retention

Potential participants will be screened in outpatient clinics by designated physicians. Contact information (telephone and WeChat) will be collected and updated at each visit to facilitate reminders and follow-up.

## 6. Study Design and Procedures

This is a multicenter, prospective RCT conducted in eight reproductive centers in China. Eligible couples undergoing c-IVF will be enrolled. All embryos will be cultured to the blastocyst stage (or >=6 Day-3 embryos will be cultured), and all blastocysts will be vitrified as single blastocysts. Participants will be randomized after confirmation that inclusion/exclusion criteria are met and that >=2 eligible Day-5/Day-6 blastocysts are available.

### 6.1 Study visits and follow-up

- Screening (baseline): demographic data, medical history, physical exams, and routine laboratory and imaging assessments.
- Oocyte retrieval: ovarian stimulation and retrieval details; assessment of adverse events and concomitant medications.
- Embryo culture period: fertilization and embryo development; assessment of adverse events and concomitant medications.
- Randomization: review eligibility, record blastocyst availability/quality, and assign treatment arm.
- Frozen-thawed single blastocyst transfer: record embryo selection and endometrial preparation; assess adverse events and concomitant medications.
- Biochemical pregnancy test (ET +12-14 days): serum hCG and clinical data recording.
- Clinical pregnancy visit (ET +28-30 days): transvaginal ultrasound, pregnancy status, and follow-up planning.
- Telephone follow-up at 12 gestational weeks: ongoing pregnancy and early miscarriage status; adverse events and concomitant medications.
- Telephone follow-up at 28 gestational weeks: anomaly scan findings; adverse events and concomitant medications.
- Telephone follow-up 2 weeks postpartum: delivery information and neonatal outcomes.
- Telephone follow-up 1 year postpartum: confirmation of any birth defects.

### 6.2 Participating centers

- Sun Yat-sen Memorial Hospital, Sun Yat-sen University
- Peking University Third Hospital
- Shenzhen Maternity & Child Healthcare Hospital
- Shenzhen Luohu People's Hospital
- Hainan Women and Children's Medical Center
- Shunde Women and Children's Hospital of Guangdong Medical University

- Yuebei People's Hospital
- Yulin Maternity and Child Health Hospital

## 7. Randomization and Blinding

Participants will be randomized 1:1 to the NICS-AI arm (experimental) or the morphology arm (control) using stratified block randomization by center. Block sizes of 4 and 6 will be used. A central electronic randomization system will allocate participants; the randomization list will be generated using SAS 9.4 (RcPlan).

This is a single-blind trial: participants will remain blinded to allocation until the primary endpoint occurs or the trial is completed.

## 8. Sample Size and Statistical Analysis

Based on the previous data from other single-center NICS studies and clinical data from our hospital, the expected first live birth rate within 1 year is approximately 40% in the morphology arm and 52.9% in the NICS arm. Assuming a 12.9% absolute difference, two-sided alpha=0.05 and 80% power, PASS calculations indicate that at least 234 participants per arm (468 total) are required. Allowing for about 10% attrition, 520 participants (260 per arm) will be enrolled.

### 8.1 Analysis sets

- Full Analysis Set (FAS): all randomized participants with post-intervention outcome data; analyzed under ITT principles.
- Per-Protocol Set (PPS): participants meeting inclusion criteria, receiving at least one study intervention, completing the first live birth follow-up, and without major protocol violations.
- Safety Analysis Set (SAS): all participants receiving at least one treatment with at least one safety assessment.

### 8.2 Handling of missing data

For the primary endpoint, missing outcomes will be treated as negative. Secondary and safety endpoints will not be imputed unless otherwise specified in the statistical analysis plan.

### 8.3 Statistical methods

Normality will be assessed as specified in the source protocol. Continuous variables will be summarized as mean +/- SD (normal) or median (IQR) (non-normal) and compared using t-tests or nonparametric rank-sum tests. Categorical variables will be summarized as counts and percentages and compared using chi-square tests or Fisher's exact tests. Bonferroni correction will be applied for multiple comparisons. Univariable logistic regression will screen potential risk factors; multivariable logistic regression will adjust for confounders. Two-sided $p<0.05$ will be considered statistically significant.

## 9. Study Interventions

### 9.1 NICS-AI arm (experimental)

(1) Collection of spent blastocyst culture medium and embryo cryopreservation: after thorough removal of granulosa cells from the zona pellucida, Day-4 embryos are washed and the medium is refreshed. When the embryo reaches an expanded stage-4 blastocyst (about 180 um), pre-freeze collapse is performed and the culture medium is collected. Samples are stored at -20 C pending testing; blastocysts are vitrified.

(2) Testing and scoring: samples undergo whole-genome amplification, library preparation, and sequencing. An AI-assisted model integrates sequencing results (including CNV resolution, mosaicism, euploidy/sex chromosome status, number of abnormal chromosomes), morphology grade, and day of blastulation to generate a composite implantation potential score (reported as <10 or 10-66).

(3) Frozen-thawed single blastocyst transfer: embryos are prioritized for transfer from highest to lowest composite score. If scores are identical, embryos are prioritized by cryopreservation order (e.g., straw number).

(4) For enrolled participants, enrolled blastocysts are prioritized for transfer.

### 9.2 Morphology arm (control)

(1) Embryo cryopreservation: after thorough removal of granulosa cells from the zona pellucida, when the embryo reaches an expanded stage-4 blastocyst (about 180 um), pre-freeze collapse is performed and blastocysts are vitrified.

(2) Frozen-thawed single blastocyst transfer based solely on blastulation day and morphology (Istanbul Consensus update): Day 5 blastocysts are prioritized over Day 6. Within the same blastulation day, embryos are ranked as follows: 6AA > 6BA > 6AB > 5AA > 5BA > 5AB > 4AA > 4BA > 4AB > 6BB > 5BB > 4BB > 6CA > 5CA > 6CB > 5CB > 4CA > 4CB > 6AC > 5AC > 6BC > 5BC > 4AC > 4BC. If day and grade are identical, prioritize by cryopreservation order (e.g., straw number).

(3) For enrolled participants, enrolled blastocysts are prioritized for transfer.

### 9.3 Concomitant treatments

Routine pregnancy supplements (e.g., folic acid, calcium, iron, multivitamins) are permitted. For this high-risk population, standard-of-care medications for threatened miscarriage or obstetric complications may be used as clinically indicated, including tocolytics, antispasmodics, hemostatic agents, immunoglobulin, progesterone formulations, and low-molecular-weight heparin. All concomitant treatments must be recorded in the CRF.

## 10. Discontinuation, Withdrawal, and Loss to Follow-up

Participants may withdraw at any time without affecting their standard clinical care. Investigators may also withdraw a participant for safety concerns, poor compliance, serious adverse events, intercurrent illness, or other reasons compromising study integrity. Reasons for withdrawal and relevant follow-up will be documented.

Retention measures include minimizing visit burden, providing flexible scheduling and transportation support where appropriate, maintaining consistent communication with reminders, and promptly contacting participants and emergency contacts if follow-up is missed.

## 11. Adverse Events and Serious Adverse Events

An adverse event (AE) is any unfavorable and unintended sign (including abnormal laboratory findings), symptom, or disease temporally associated with study procedures, whether or not considered related. AE severity will be graded according to CTCAE v5.0 (mild, moderate, severe). Causality will be assessed as definitely related, probably related, possibly related, possibly unrelated, or unrelated.

All AEs and serious adverse events (SAEs) will be recorded in source documents and CRFs, including onset, duration, severity, management, outcome, and causality. Potential study-related AEs may include culture medium contamination, equipment failure, or sample mix-up.

A SAE is defined as any event resulting in death, life-threatening condition, hospitalization or prolongation of hospitalization, persistent or significant disability/incapacity, congenital anomaly/birth defect, or other important medical events. A 24-hour emergency response team (reproductive physician, ethics officer, data safety officer) and standard operating procedures will be in place to manage unexpected SAEs and foreseeable risks (e.g., OHSS, ectopic pregnancy).

## 12. Data Collection and Management

Data will be collected using electronic case report forms (eCRFs) with password-controlled access. Data entry will occur contemporaneously with clinical data collection. Queries generated by the data system will be resolved by investigators in a timely manner. All enrolled participants must have complete CRFs; CRFs may not be disclosed to third parties without written permission from the sponsor.

After data verification and confirmation, the database will be locked jointly by the lead and collaborating centers. Locked data files may not be modified. The statistical analysis plan must be finalized before database lock.

## 13. Ethical Considerations and Confidentiality

The study will be initiated only after ethics approval. Protocol amendments require re-review and approval before implementation. Written informed consent is required for all participants.

Clinical data and personal information will be used for scientific research only. All study staff and data analysts will sign confidentiality agreements and will not disclose personal or disease-related information to any non-study individual or institution. Data will be centrally managed to prevent privacy breaches.

## 14. References

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