The Impact of Assisted Hatching on Pregnancy Outcomes After Vitrified-Warmed Embryo Transfer in Advanced-age Patients

1. Protocol Summary

Study Title	The Impact of Assisted Hatching on Pregnancy Outcomes After Vitrified-Warmed Embryo Transfer in Advanced-age Patients
Version No.	V2.1
Sponsoring and Participating Institutions	Reproductive Medicine Center, Shanghai Jiao Tong University School of Medicine Affiliated Ninth People's Hospital
Study Nature	Randomized Controlled Clinical Trial
Study Objective	To investigate whether laser assisted hatching (LAH) of thawed embryos prior to frozen-thawed embryo transfer can improve the live birth rate in advanced age patients.
Study Population	Advanced age women undergoing non-donor IVF/ICSI cycles and planning vitrified-warmed embryo transfer.
Study Method	Single-center, parallel-group, open-label RCT with 1:1 stratified block randomization.
Inclusion Criteria	1. Female age ≥35 years.2. First or second frozen-thawed embryo transfer cycle.3. Transferred embryos meet the quality criteria: Grade I, Grade II, or CP and above (cleavage-stage embryos); 4BC/CB and above (blastocysts).4. Provided written informed consent.
Exclusion Criteria	1. Use of donor oocytes or donor sperm; or scheduled for preimplantation genetic testing (PGT).2. Severe immune or chromosomal abnormalities.3. Uterine cavity abnormalities (i.e., adenomyosis, submucous fibroids, hydrosalpinx, uterine septum, and endometrial polyps).4. Embryos with abnormal zona pellucida.5. Complicated with severe underlying diseases (such as uncontrolled hypertension/diabetes, active malignant tumor).6. History of recurrent implantation failure (≥2 cycles) or recurrent spontaneous abortion (≥2

	episodes).
Study Progress Plan	Subject enrollment will last for 18 months, with monitoring every quarter, and the entire study is expected to be completed within 36 months.
Statistical Analysis Methods	Analysis Population: ITT analysis includes all randomized patients (primary analysis), missing data is handled by multiple imputation (MICE); PP analysis includes patients who comply with the protocol (sensitivity analysis).Continuous Variables: Normally distributed data are expressed as mean ± standard deviation, and t-test is used for inter-group comparison; non-normally distributed data are expressed as median (interquartile range), and Mann-Whitney U test is used for inter-group comparison.Categorical Variables: Expressed as frequency (percentage), and χ^2 test or Fisher's exact test (when expected frequency < 5) is used for inter-group comparison.Subgroup Analysis: Preset subgroup analysis by age (<40 years and ≥40 years) and embryo stage (cleavage-stage embryos, blastocysts).Statistical Software: SAS 9.4 and R 4.3.0 software are used, with a two-sided $P<0.05$ considered statistically significant.
Forms of Study Outcome Publication	The study results will be published in peer-reviewed journals and presented at international conferences. A plain language summary of the study results will be provided to participants (sent during offline follow-up or via email).

Principal Investigator	Jiang Shutian	**Title**	Attending Physician	**Date of Birth**	October 1991
Key Members of the Research Team	**Name**	**Date of Birth**	**Title**	**GCP Certificate**	**Research Duties**
	Jiang Shutian	October 1991	Attending Physician	Yes	Principal Investigator
	Li Wenzhi	May 1989	Senior Technician	Yes	Clinical Execution (Embryologist)
	Guo Haiyan	October 1981	Associate Chief Physician	Yes	Clinical Execution (Clinician)

	Lü Qifeng	September 1970	Researcher	Yes	Quality Control
	Jiang Xueyi	January 1999	Physician	No	Statistical Analysis
	Li Danjun	February 1995	Physician	No	Data Management
	Mi Yan	August 1999	Graduate Student	No	Data Management/Research Coordinator

2. Research Background

Hatching is a key step in a series of physiological events leading to final implantation. Failure of hatching caused by intrinsic abnormalities of the zona pellucida (ZP) may be one of the many factors limiting human reproductive efficiency. Assisted hatching (AH) refers to the artificial thinning or disruption of the zona pellucida to promote embryo hatching and implantation into the endometrium, and is considered an auxiliary technology to improve embryo implantation and pregnancy outcomes after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Assisted hatching was first described by Cohen et al. in the 1980s [3], and the current implementation methods mainly include mechanical method [4], acidification method, laser method [8], and piezoelectric micromanipulator method [9], among which the laser method is the most commonly used clinical assisted hatching method [10]. At present, the safety of assisted hatching has been verified to a certain extent.

To date, there is no consensus on whether assisted hatching can improve pregnancy outcomes after IVF/ICSI [1]. Some studies have reported a significant increase in the clinical pregnancy rate of patients, and pointed out that assisted hatching is beneficial to patients with repeated failures or advanced age. However, other studies have shown that the clinical pregnancy rate may not increase after assisted hatching, and assisted hatching may not affect the live birth rate. There is also some uncertainty about whether assisted hatching can improve the embryo implantation rate, and the research conclusions have not reached a consistent conclusion. However, since cryopreserved embryos must undergo in vitro culture and freeze-thaw processes, which cause changes in the glycoprotein matrix and lead to zona pellucida hardening [27], assisted hatching is recommended for use in FET cycles to improve pregnancy outcomes. A recent randomized controlled trial (RCT) study confirmed that the use of AH on previously cryopreserved embryos has higher clinical pregnancy rate and implantation rate, but failed to prove a significant effect on live birth rate. Unfortunately, the sample size of the included study was small, and it focused on cleavage-stage embryos. In fact, the effect of AH on frozen embryos is inconclusive based on existing data. Current studies are focused on retrospective studies, lacking RCT studies. Moreover, these studies have high heterogeneity, making it impossible to draw any definite conclusions about the actual effect of AH.

Considering the possible benefits of AH for advanced age patients, studying whether the use of AH on cryopreserved embryos of advanced age patients can improve the live birth rate may have more important clinical significance. To fill the gaps in current research, we have developed this protocol for a randomized trial to compare the effect of laser-mediated AH on the ZP of vitrified/warmed embryos with non-AH control on pregnancy outcomes after transfer in advanced age patients.

3. Study Objectives

3.1 Primary Objective

To investigate whether laser assisted hatching (LAH) of thawed embryos prior to frozen-thawed embryo transfer can improve the live birth rate in advanced age patients.

3.2 Secondary Objectives

Secondary outcomes include pre-transfer embryo morphological characteristics, implantation rate, biochemical pregnancy rate, clinical pregnancy rate (ultrasound-visible gestational sac), ectopic pregnancy rate, ongoing pregnancy rate, miscarriage rate, multiple pregnancy rate, preterm birth (gestation <37 weeks) rate, obstetric and neonatal complications, and congenital anomaly rate.

4. Study Methods

4.1 Overall Study Design

This study is a single-center prospective study adopting a parallel randomized controlled design, aiming to investigate whether laser-mediated AH of thawed embryos prior to frozen-thawed embryo transfer can improve the live birth rate in advanced age patients after transfer. Eligible advanced age patients (age ≥35 years) will be randomly assigned to the study group (AH group) and the control group (non-AH group) at a ratio of 1:1. Stratified block randomization (variable block size of 4 or 6) will be used according to age (<40 years, ≥40 years) and embryo stage (cleavage-stage embryos and blastocysts). Subject enrollment will last for 18 months, with monitoring every quarter, and the entire study is expected to be completed within 36 months.

4.2 Sample Size

Sample size calculation is based on the assumption that the live birth rate in the non-AH group is 25% and the live birth rate in the AH group is increased by 10% (i.e., 35%), with a test power of 90%, α=0.05, and a dropout rate of 10%. A total of 916 patients need to be enrolled (458 patients in each group).

4.3 Randomization

All participants will be divided into two groups at a ratio of 1:1 through online software

according to the following strata:

1. Age <40 years and ≥40 years.
2. Embryo stage: cleavage-stage embryos and blastocysts.

Block randomization will be used to reduce bias and achieve allocation balance. Variable block sizes (4 or 6) will be used to avoid allocation prediction. Investigators involved in the randomization process will not know the block size. SAS 9.4 software will be used to generate the random allocation sequence.

Randomization will be performed before embryo thawing. The coordinator will inform the patient, clinician and embryologist of the grouping result, all of whom will know the grouping information on the day of transfer (to facilitate the implementation of AH and arrangement of subsequent transfer work).

4.4 Control and Blinding

4.4.1 Control Selection

Allocation concealment will be achieved through a third-party hosted central randomization system. On the day of transfer, the study center coordinator will log in to the system, enter the patient information (age, embryo stage), and obtain the grouping result (AH/non-AH).

4.4.2 Blinding Implementation

An open-label design will be adopted, and the patient, clinician and embryologist will all know the grouping information to manage and arrange AH and transfer surgery (unblinded). The outcome assessor (independent obstetrician and gynecologist) will be unaware of the grouping throughout the study to ensure the objectivity of outcome judgment. There will be no foreseeable circumstances allowing unblinding.

4.4.3 Unblinding Method and Emergency Unblinding Procedure

Unblinding Method: Data analysis will be unblinded uniformly by an independent statistician after data locking.

Emergency Unblinding: Only in the case of a serious adverse event that requires clarification of the grouping information to implement rescue, the researcher can log in to the "Emergency Unblinding" module of the central randomization system for operation after approval by the PI. The system will automatically record and notify the PI and the Ethics Committee at any time, and a written report will be submitted within 24 hours. The unblinded subject will withdraw from the study treatment but continue follow-up, and the data will be included in the safety analysis. If the cumulative unblinding rate >5%, the DMC will evaluate whether to terminate the study.

4.5 Follow-up Time Points

Subject enrollment will last for 18 months, which is expected to be completed from March 2026 to September 2027. Follow-up will be carried out in stages of "post-transfer follow-up,

pregnancy follow-up, and post-delivery follow-up".

Follow-up Phase	Follow-up Time Point	Follow-up Examinations
Post-embryo Transfer Follow-up 1	12-15 days after embryo transfer	Serum β-hCG test to determine biochemical pregnancy
Post-embryo Transfer Follow-up 2	28 days after embryo transfer	Transvaginal ultrasound examination to determine pregnancy type (clinical pregnancy [intrauterine gestational sac]/ectopic pregnancy, number of gestational sacs)
Pregnancy Follow-up 1	12 weeks ±7 days of gestation	Ultrasound to confirm intrauterine viable fetus and its number (ongoing pregnancy/early miscarriage)
Pregnancy Follow-up 2	28 weeks ±7 days of gestation	Ultrasound to confirm intrauterine viable fetus and its number (ongoing pregnancy/mid-trimester miscarriage)
Post-delivery Follow-up	1 month ±7 days after delivery	Delivery mode, gestational age, neonatal vital signs and health status, birth weight, obstetric complications (live birth outcome, neonatal outcome)

5. Selection, Withdrawal and Management of Subjects

5.1 Diagnostic Criteria

Undergoing non-donor IVF/ICSI cycles and planning vitrified-warmed embryo transfer.

5.2 Inclusion Criteria

1. Female age ≥35 years.

2. First or second frozen-thawed embryo transfer cycle.

3. Transferred embryos meet the quality criteria: Grade I, Grade II, or CP and above (cleavage-stage embryos); 4BC/CB and above (blastocysts).

4. Provided written informed consent.

5.3 Exclusion Criteria

1. Use of donor oocytes or donor sperm; or scheduled for preimplantation genetic testing (PGT).

2. Severe immune or chromosomal abnormalities.

3. Uterine cavity abnormalities (i.e., adenomyosis, submucous fibroids, hydrosalpinx, uterine septum, and endometrial polyps).

4. Embryos with abnormal zona pellucida.

5. Complicated with severe underlying diseases (such as uncontrolled hypertension/diabetes, active malignant tumor).

6. History of recurrent implantation failure (≥2 cycles) or recurrent spontaneous abortion (≥2 episodes).

5.4 Exclusion and Withdrawal Criteria

5.4.1 Exclusion Criteria

1. Violation of important inclusion criteria;

2. Subject did not receive the study treatment;

3. No observation data after randomization.

5.4.2 Withdrawal Criteria

1. During the trial, the subject developed certain comorbidities, complications or special physiological changes that made it inappropriate to continue receiving the trial;

2. Voluntary withdrawal by the subject.

5.4.3 Data Processing of Withdrawn Subjects

Regardless of the reason, for subjects who withdraw from the trial, their complete clinical data should be retained. All withdrawn subjects should fill in the trial conclusion form and the reason for withdrawal in the case report form. Generally, there are 6 reasons, namely occurrence of adverse events (including adverse drug reactions and allergic reactions), lack of efficacy (disease deterioration or occurrence of complications), violation of the trial protocol (including poor compliance), loss to follow-up (including voluntary withdrawal of the patient from the trial), termination by the sponsor, or others.

5.5 Conditions for Terminating the Trial

1. The clinical trial has not ended according to the protocol and is stopped entirely in the middle.

2. Serious safety issues occur during the trial, which should be terminated in a timely manner;

3. Major mistakes in the trial protocol or major deviations in implementation are found during the trial, making it difficult to evaluate the drug effect;

4. Other circumstances requiring termination.

5.6 Subject Management

5.6.1 Subject Recruitment Methods

The identification of study candidates will be carried out in cooperation with laboratory personnel and clinical staff. All couples planning to undergo frozen-thawed embryo transfer will be considered for inclusion. Women who agree to participate will be required to sign a written informed consent form, and they will receive a copy signed by the researcher. Any waste or exclusion due to failure to meet the inclusion criteria will be recorded. On the day of thawing, laboratory staff will assess whether a signed consent form exists, and if so, randomization will be performed. Subjects participating in the study will receive the same procedures except for LAH treatment.

5.6.2 Informed Consent Process

Adequate information will be provided, and a written informed consent form will be signed.

6. Study Process

6.1 Introduction to Study Drugs/Devices

1. Cryotop system (Kitazato Biopharma Co Ltd, Japan);

2. 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulfoxide, and 0.5M sucrose as cryoprotectants;

3. Thawing: stepwise dilution of cryoprotectants using 1M, 0.5M, and 0M sucrose solutions;

4. G2 medium (G-2™ PLUS, Vitrolife Group).

6.2 Drug/Device Administration Methods

(1) Fertilization, Embryo Culture, Vitrification and Thawing

According to semen parameters, the collected oocytes will undergo in vitro fertilization or intracytoplasmic sperm injection according to routine laboratory procedures. Cleavage-stage embryo quality will be observed on the third day after in vitro fertilization or intracytoplasmic sperm injection, and the number and regularity of embryo blastomeres and the degree of embryo fragmentation will be checked and graded according to Cummins criteria. Grade I

and II embryos (≥6 cells) are defined as high-quality embryos and will be vitrified. Other high-quality cleavage-stage embryos and poorer-quality (Grade III and IV) cleavage-stage embryos will be cultured into blastocysts. Blastocysts will be graded according to Gardner criteria on the fifth day. If blastocysts on day 5 or 6 are at stage 4 or above, and at least one of the inner cell mass or trophoblast cell grades is grade B, they will be vitrified. Blastocysts on day 7 must be at least 4CC to be vitrified. These blastocysts are frozen for subsequent FET cycles. Vitrification and thawing are performed according to routine methods, as described in previous studies. The Cryotop system (Kitazato Biopharma Co Ltd, Japan) is used for vitrification, with 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulfoxide, and 0.5M sucrose as cryoprotectants. For warming, 1M, 0.5M, and 0M sucrose solutions are used to dilute the cryoprotectants stepwise. All vitrification and thawing steps are performed at room temperature except for the first thawing step at 37°C.

(2) Laser Assisted Hatching

After thawing, cleavage-stage embryos and blastocysts will be transferred to G2 medium (G-2™ PLUS, Vitrolife Group) for culture for 10~20 minutes. For cleavage-stage embryos, the zona pellucida will be thinned using a microlaser continuous puncher; for blastocysts, 1/4 to 1/3 of the zona pellucida circumference will be removed using the same method. The embryos will then be transferred to fresh G2 medium droplets for transfer. Each viable embryo will be transferred within 3 hours after assisted hatching.

(3) Endometrial Preparation and Frozen-Thawed Embryo Transfer

Endometrial preparation and frozen-thawed embryo transfer protocols are performed according to the center's protocol, as described in previous studies. Endometrial preparation is performed under natural cycle, ovulatory cycle, or hormone replacement cycle according to the patient's condition. For all three protocols, day 2, 3, or 5~6 embryos are thawed on the second, third, or fifth day of endometrial transformation for transfer. Transfer is performed under ultrasound guidance using a Cook catheter according to the department's standard procedures. According to Chinese law, one to two embryos will be transferred to the middle of the uterine cavity. All embryos are thawed on the day of transfer, and embryos with ≥50% blastomeres after thawing are considered viable. Non-viable embryos will not be transferred. In this case, there are two options: (1) if there are additional vitrified blastocysts, a second embryo will be thawed and transferred according to the initially assigned random group; (2) if there are no additional vitrified blastocysts, the patient will not undergo embryo transfer.

6.3 Treatment Course and Follow-up Points

Subject enrollment will last for 18 months, which is expected to be completed from March 2026 to September 2027. Follow-up will be carried out in stages of "post-transfer follow-up, pregnancy follow-up, and post-delivery follow-up". On-site monitoring will be conducted every 6 months to verify data integrity. The entire study is expected to be completed within 36 months.

7. Evaluation Indicators

7.1 Baseline Indicators

1. Demographic data: Subject's unique identification number, gender, age, BMI, infertility duration, cause of infertility, ovarian reserve status, etc.;

2. General clinical data: Endometrial preparation protocol, endometrial thickness, hormone level on the day of transfer, comorbid diseases and concurrent medications, etc.;

3. Embryological data: Embryo stage (cleavage-stage/blastocyst), embryo grade, number of embryos, etc.

7.2 Efficacy Evaluation

7.2.1 Primary Evaluation Indicator

The primary outcome examined in this study will be the live birth rate, defined as the delivery of at least one neonate with heartbeat and respiration at ≥28 weeks of gestation, which will be recorded at delivery.

7.2.2 Secondary Evaluation Indicators

Secondary outcomes include pre-transfer embryo morphological characteristics, implantation rate, biochemical pregnancy rate, clinical pregnancy rate (ultrasound-visible gestational sac), ectopic pregnancy rate, ongoing pregnancy rate, miscarriage rate, multiple pregnancy rate, preterm birth (gestation <37 weeks) rate, obstetric and neonatal complications, and congenital anomaly rate.

7.3 Safety Evaluation

7.3.1 Vital Signs

Changes in blood pressure, pulse, body temperature, respiration, and heart rate before and after treatment.

7.3.2 Laboratory Examinations

Laboratory examination results, changes in normal/abnormal status before and after the trial, and the relationship between abnormal changes and the trial drug. For those with abnormal results of the above examinations after drug administration, close follow-up and observation should be conducted until they return to normal, stable levels or pre-treatment levels.

7.3.3 Adverse Events

Serious adverse events (such as ectopic pregnancy) must be reported to the DMC, Ethics Committee, and Sponsor within 24 hours; all adverse events shall be recorded in the EDC system.

7.3.4 Specific Risks Related to Laser-Assisted Hatching (LAH)

The study intervention LAH may have the following specific risks, and corresponding prevention and control measures have been formulated in the study protocol:

(1) Risk of embryo damage: LAH operation may cause damage to embryonic blastomeres.

Countermeasures: The operation shall be performed by senior embryologists with more than 5 years of experience and special training in this study;

(2) Risk of multiple pregnancy: LAH may increase the risk of multiple pregnancy, thereby causing maternal and infant complications such as preterm birth and low birth weight.

Countermeasures: Strictly follow the principle of single embryo transfer (SET) for the first transfer in this center, and the twin pregnancy rate will be used as a key safety indicator for inter-group comparison.

The above risks will be highlighted in plain and easy-to-understand language in the informed consent form, and subjects will be clearly informed that participation in the study is voluntary and they can withdraw unconditionally at any time.

8. Data Quality Assurance

8.1 Quality Control and Assurance

Dual data entry is adopted, and original data are verified through medical record review.

8.2 Data Management

(1) Data Collection: An Electronic Data Capture (EDC) system (such as MedPro) is adopted. All variables related to the study protocol will be recorded and stored in a digital database, mainly including baseline data (such as age), embryo data (embryo stage, embryo grade, number of embryos, etc.), transfer cycle data (endometrial thickness on the day of transfer, P on the day of transfer, endometrial preparation plan, etc.), and outcome data (live birth rate, clinical pregnancy rate, etc.). Original data are verified through medical record review.

(2) Data Management: Dual data entry, range check, and query resolution mechanisms are adopted. Data are stored on an encrypted server, and only authorized personnel can access them.

(3) Trial Monitoring: On-site monitoring is conducted every 6 months to check data integrity; the DMC reviews Adverse Event (AE) reports every quarter.

(4) The processing of personal data shall comply with relevant national regulations.

9. Statistical Analysis

9.1 Analysis Datasets

The ITT analysis includes all randomized patients (primary analysis), and missing data are handled by the Multiple Imputation by Chained Equations (MICE) method; the PP analysis includes patients who comply with the protocol (sensitivity analysis).

9.2 Statistical Methods

(1) Continuous Variables: Normally distributed data are expressed as mean ± standard deviation, and t-test is used for inter-group comparison; non-normally distributed data are expressed as median (interquartile range), and Mann-Whitney U test is used for inter-group comparison.

(2) Categorical Variables: Expressed as frequency (percentage), and χ^2 test or Fisher's exact test (when the expected frequency < 5) is used for inter-group comparison.

(3) Subgroup Analysis: Preset subgroup analysis will be conducted by age (<40 years and ≥40 years) and embryo stage (cleavage-stage embryos, blastocysts).

(4) Statistical Software: SAS 9.4 and R 4.3.0 software are used, with a two-sided $P < 0.05$ considered statistically significant.

10. Ethical Requirements and Informed Consent Form

10.1 Ethics Committee Review

This protocol, written informed consent form, and materials directly related to subjects must be submitted to the Ethics Committee, and the study can be officially carried out only after obtaining written approval from the Ethics Committee. Investigators must submit an annual study report to the Ethics Committee at least once a year (if applicable). When the study is suspended and/or completed, the investigator must notify the Ethics Committee in writing; the investigator must promptly report all changes occurring in the study work (such as revisions to the protocol and/or informed consent form) to the Ethics Committee, and shall not implement these changes unless they are made to eliminate obvious and direct risks to subjects. In such cases, the Ethics Committee will be notified.

10.2 Informed Consent

Investigators must provide subjects or their legal representatives with an easy-to-understand informed consent form approved by the Ethics Committee, and give subjects or their legal representatives sufficient time to consider this study. Subjects shall not be enrolled until a signed written informed consent form is obtained from them. During the subjects' participation, all updated versions of the informed consent form and written information will be provided to the subjects. The informed consent form shall be retained as an important document of the clinical trial for inspection.

11. Insurance

No insurance is required.

12. References

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