Research protocol

Protocol Title: Microfluidic Sperm Selection Versus Density Gradient Centrifugation in ICSI Cycles With Abnormal Semen Parameters: A Randomized Sibling-Oocyte Study.

Project Summary

This prospective, randomized sibling-oocyte study was conducted to compare embryologic outcomes between microfluidic sperm selection (MSS) and density gradient centrifugation (DGC) in couples undergoing intracytoplasmic sperm injection (ICSI). Although DGC is widely used for sperm preparation, centrifugation may increase reactive oxygen species (ROS) generation and contribute to sperm DNA fragmentation (SDF). MSS is a centrifugation-free alternative designed to mimic physiological sperm selection and may enrich for sperm with superior functional and genomic integrity. Men with abnormal semen parameters often have higher baseline DNA damage and may therefore particularly benefit from MSS; however, direct comparative evidence in this population remains limited.

A total of 55 infertile couples were enrolled at the Center of Excellence in Assisted Reproductive Technology, Ramathibodi Hospital. Eligible participants included couples undergoing ICSI in whom the male partner had abnormal semen parameters according to the World Health Organization (WHO) 2021 criteria, and the female partner was aged 42 years or younger with at least four dominant follicles on the trigger day. Within each treatment cycle, sibling metaphase II (MII) oocytes were randomly allocated in a 1:1 ratio to undergo ICSI using sperm prepared by either MSS or DGC.

The primary outcome was blastocyst formation rate per two-pronuclear embryo (2PN). Secondary outcomes included fertilization rate (2PN/MII), cleavage rate (cleaved embryos/2PN), good-quality blastocyst rate, and embryo euploidy rate in cycles undergoing preimplantation genetic testing for aneuploidy (PGT-A). The study was designed to determine whether MSS influences fertilization and subsequent embryo development compared with DGC in ICSI cycles involving abnormal semen parameters.

General Information

Trial Registration: Thai Clinical Trials Registry (TCTR20250130004), registered on 30 January 2025.

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Background and Rationale

Density gradient centrifugation (DGC) is a widely used method for sperm preparation in intracytoplasmic sperm injection (ICSI). However, centrifugation may increase reactive oxygen species (ROS) generation and contribute to sperm DNA fragmentation (SDF), potentially affecting reproductive outcomes. Microfluidic sperm selection (MSS) is a centrifugation-free technique designed to mimic physiological sperm selection and may enrich for sperm with superior motility, functional competence, and genomic integrity.

Men with abnormal semen parameters frequently exhibit elevated baseline DNA damage and may particularly benefit from MSS. Nevertheless, direct comparative evidence between MSS and DGC in this specific population remains limited. A randomized sibling-oocyte design allows within-cycle comparison under identical laboratory conditions, thereby minimizing confounding from female factors and cycle-level laboratory variability. This study was therefore conducted to compare embryologic outcomes following MSS and DGC in ICSI cycles involving abnormal semen parameters.

Study goals and objectives

Study Goal: To determine whether microfluidic sperm selection (MSS) influences embryo development and genetic competence compared with density gradient centrifugation (DGC) in ICSI cycles with abnormal semen parameters.

Primary Objective: To compare blastocyst formation rate per 2PN embryo between sibling oocytes injected with sperm prepared by MSS and those injected with sperm prepared by DGC.

Secondary Objectives: To compare the following outcomes between the two sperm preparation methods:

- Fertilization rate (2PN/MII)
- Cleavage rate (cleaved embryos/2PN)
- Good-quality blastocyst rate per 2PN
- Good-quality blastocyst rate per total blastocysts
- Euploidy rate per biopsied blastocyst in cycles undergoing PGT-A

Study design

Study Design: This was a prospective, randomized sibling-oocyte study conducted between February 2025 and February 2026 at the Center of Excellence in Assisted Reproductive Technology, Ramathibodi Hospital. The study population consisted of infertile couples undergoing ICSI.

Inclusion Criteria:

- Infertile couples undergoing ICSI
- Male partner with abnormal semen parameters according to WHO 2021 criteria
- Female partner aged ≤ 42 years
- Presence of ≥ 4 dominant follicles on the trigger day

Exclusion Criteria:

- Retrieval of <4 metaphase II (MII) oocytes
- Semen volume <1.4 mL on the day of oocyte retrieval
- Pre-preparation total motile sperm count (TMSC) <5 million
- Inability to provide written informed consent

Methodology

Ovarian stimulation and oocyte retrieval

Controlled ovarian stimulation (COS) was performed using individualized protocols at Ramathibodi Hospital, including GnRH antagonist, GnRH agonist, or progestin-primed ovarian stimulation (PPOS) regimens. Follicular development was supported with recombinant FSH or highly purified human menopausal gonadotropin (hMG), with doses adjusted according to ovarian response monitored by transvaginal ultrasonography (TVUS).

Final oocyte maturation was triggered when at least three leading follicles reached ≥17 mm in diameter, using human chorionic gonadotropin (hCG), a GnRH agonist, or a dual trigger. Oocyte retrieval was performed 34–36 hours after triggering under TVUS-guided follicular aspiration.

Retrieved cumulus–oocyte complexes (COCs) were collected in FertiCult® Flushing medium (FertiPro, Beernem, Belgium) and transported to the embryology laboratory. Approximately 2 hours after retrieval, oocytes were denuded enzymatically and mechanically to assess nuclear maturity. Only MII oocytes, identified by the presence of the first polar body, were included for subsequent randomization.

Oocyte Allocation and Randomization

Eligible MII oocytes were randomly allocated in a 1:1 ratio to either the MSS group or the DGC group using simple randomization at the oocyte level within each treatment cycle. Randomization was performed by an embryologist after denudation and confirmation of MII status.

In cycles with an odd number of MII oocytes, the final unpaired oocyte was assigned according to a prespecified alternating rule between treatment groups to maintain near-equal allocation within cycles.

Because randomization occurred at the oocyte level after confirmation of maturity and required immediate implementation within the embryology laboratory workflow, allocation concealment was not performed.

Semen collection and preparation

Semen samples were obtained by masturbation on the day of oocyte retrieval after 2–7 days of sexual abstinence. After liquefaction at 37 °C for 20–30 minutes, sperm concentration and motility were assessed in accordance with the WHO 2021 recommendations using a Makler counting chamber. Sperm morphology was not reassessed on the day of oocyte retrieval to preserve the maximum number of viable spermatozoa for clinical use, as morphology assessment requires staining and cannot be performed on sperm intended for ICSI.

Following the initial assessment, each sample was divided into two portions: an 850 µL aliquot was allocated for MSS, while the remaining volume was processed using DGC.

Microfluidic sperm selection

For the MSS group, an 850 µL aliquot of liquefied semen was loaded into the inlet port of a sperm separation device (ZyMōt® Multi, 850 µL; ZyMōt Fertility Inc., USA) according to the manufacturer's instructions.

The outlet port was first primed with approximately 50 µL of sperm washing medium (FertiCult®; FertiPro, Beernem, Belgium) until the medium reached the separation membrane. Subsequently, an additional 700 µL of washing medium was gently added to cover the membrane surface.

The device was incubated horizontally at 37 °C for 30 minutes, allowing motile spermatozoa to migrate through the membrane into the upper collection chamber. After incubation, the separated motile fraction (up to 500 µL) was gently aspirated and used for ICSI.

Density gradient centrifugation

For the DGC group, semen samples were processed using a two-layer density gradient (45%/90%) with Sil-Select Plus™ density gradient medium (FertiPro, Beernem, Belgium). Samples were centrifuged at 1250 rpm (~140×g; rotor radius 8 cm) for 10–12 minutes. The resulting sperm pellet was washed twice with FertiCult® medium (FertiPro, Beernem, Belgium) by centrifugation at 1000 rpm (~89×g; rotor radius 8 cm) for 6 minutes per wash. The final 0.4 mL sperm suspension was subsequently used for ICSI.

Intracytoplasmic sperm injection and embryo culture

ICSI was performed on all MII oocytes using spermatozoa prepared according to the randomized allocation.

Due to the distinct technical nature of the sperm preparation methods, embryologists were not blinded to the treatment allocation during the ICSI procedure. However, to minimize inter-

operator variability, all oocytes within a single treatment cycle were injected by the same embryologist.

Following ICSI, oocytes were cultured in Global® Total® LP medium (CooperSurgical, Inc., Trumbull, CT, USA) under mineral oil in a conventional incubator maintained at 37 °C with 6% CO_2 and 5% O_2.

Fertilization was assessed 16–18 hours post-injection by the presence of two pronuclei (2PN).

Embryo assessment

Embryo development was evaluated on Day 3 for cleavage-stage embryos and on Days 5–6 for blastocyst development. Blastocysts were graded according to the Gardner and Schoolcraft grading system, based on the degree of blastocyst expansion and the quality of the inner cell mass (ICM) and trophectoderm (TE).

Good-quality blastocysts were defined as those graded ≥3BB on Day 5 or Day 6.

Because the sperm preparation techniques were visually distinguishable during laboratory processing, the embryologists performing embryo assessments were not blinded to the method.

Embryo Biopsy and preimplantation genetic testing for aneuploidy

For cycles undergoing preimplantation genetic testing for aneuploidy (PGT-A), TE biopsy was performed on Day 5 or Day 6 blastocysts. Genetic analysis was performed using next-generation sequencing (NGS) to assess the copy number status of all 24 chromosomes.

Embryos were classified based on chromosomal status as euploid, mosaic, or aneuploid as follows:

- Euploid: Blastocysts with no detectable chromosomal gains or losses across all 24 chromosomes.
- Aneuploid: Embryos with whole-chromosome or segmental copy number abnormalities.

- Mosaic: Embryos demonstrating intermediate copy number changes suggestive of a mixture of euploid and aneuploid cell lines.

Sample Size Calculation

Sample size was calculated based on prior sibling-oocyte studies. Assuming a mean difference of 0.12 in blastocyst formation rate and a standard deviation of paired differences of 0.30, a sample size of 52 cycles provides 80% power at a two-sided α of 0.05. To account for potential attrition, 55 cycles were included.

Data Management and Statistical Analysis

Data Management

All data were collected in de-identified form using unique study codes and were stored securely in compliance with the Thai Personal Data Protection Act (PDPA) 2019. Digital data were encrypted and accessible only to authorized study personnel. All study records will be retained for at least 10 years in accordance with institutional policy.

Statistical Analysis

Baseline characteristics were summarized using descriptive statistics. Continuous variables were presented as mean ± standard deviation (SD) or median with interquartile range (IQR), as appropriate according to data distribution, whereas categorical variables were presented as counts and percentages.

Paired comparisons of sperm parameters obtained after MSS and DGC were performed using paired t-tests or Wilcoxon signed-rank tests, as appropriate.

Embryo-level outcomes were analyzed using generalized linear mixed models (GLMMs) with a binomial distribution and logit link function. Outcomes were modeled as binomial trials with the

number of events specified relative to the appropriate denominator for each endpoint. To account for clustering of embryos within treatment cycles and the paired sibling-oocyte design, cycle identification was included as a random intercept. Results were reported as odds ratios (ORs) with 95% confidence intervals (CIs).

Sensitivity analyses were additionally performed at the cycle level to evaluate the robustness of embryo-level findings. For each cycle, outcome proportions were calculated separately for each sperm preparation method and compared using paired statistical tests, as appropriate according to data distribution. All analyses were performed using Stata version 18 (StataCorp, College Station, TX, USA).

Ethics and Dissemination

The study was approved by the Human Research Ethics Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (Approval No. MURA2025/109). Written informed consent was obtained from all participants before study enrollment. All methods were performed in accordance with relevant guidelines and regulations. Results will be disseminated through peer-reviewed publications and academic presentations.

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