

Article

Intracytoplasmic Sperm Injection Using Polyvinylpyrrolidone Versus Hyaluronic Acid: A Prospective Sibling-Oocyte Study

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Abstract

Background/Objectives: Hyaluronic acid (HA) has been proposed as a physiological alternative to polyvinylpyrrolidone (PVP) for sperm immobilization during intracytoplasmic sperm injection (ICSI). This prospective sibling-oocyte study aimed to compare embryological outcomes and morphokinetic parameters between HA and PVP. **Methods:** A total of 811 sibling-oocytes from 51 ICSI cycles were allocated to the HA group (SpermCatch; $n = 377$) or the PVP group (10% PVP Solution; $n = 434$). Fertilization outcomes, cleavage, blastocyst formation, and good-quality embryo development were assessed. Embryo morphokinetic parameters (t2–tB) and an AI-derived embryo score were analyzed in a subset of blastocysts with available time-lapse data. **Results:** The fertilization rates were comparable between the HA and PVP groups (80.9% vs. 85.3%, $p = 0.25$), as were the cleavage rates (99.0% vs. 97.0%, $p = 0.27$). However, the HA group had significantly lower rates of good-quality cleavage-stage embryos (33.4% vs. 47.9%, $p < 0.01$), blastocyst formation (52.3% vs. 69.9%, $p < 0.01$), and good-quality blastocysts (49.4% vs. 64.1%, $p < 0.01$). Morphokinetic timings did not differ significantly, whereas embryos in the HA group showed lower AI scores than those in the PVP group ($p = 0.04$). **Conclusions:** In this prospective sibling-oocyte cohort, HA-based sperm immobilization did not improve embryological outcomes compared with conventional PVP-assisted ICSI. Differences observed at the blastocyst stage should be interpreted cautiously and require confirmation in larger, randomized studies with clinical follow-up.

Keywords: intracytoplasmic sperm injection; polyvinylpyrrolidone; hyaluronic acid; blastocyst development; embryo morphokinetics



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1. Introduction

Intracytoplasmic sperm injection (ICSI) is a widely used method in assisted reproductive technology (ART). Initially developed to overcome severe male factor infertility, ICSI is now applied across a broad range of clinical indications and accounts for approximately two-thirds of fresh ART cycles worldwide [1]. The success of ICSI depends not only on biological compatibility between gametes but also on micromanipulation techniques that may

directly influence oocytes and subsequent embryo development. Even subtle variations in laboratory handling procedures can therefore have downstream effects on embryogenesis.

A critical technical step during ICSI is sperm immobilization prior to injection. This process facilitates sperm handling and is traditionally performed using polyvinylpyrrolidone (PVP), a synthetic viscous polymer that slows sperm motility and improves operator control [2]. Although PVP has been incorporated into routine practice for decades and has demonstrated acceptable clinical safety, it is not a physiological component of the female reproductive tract. Experimental studies have suggested that prolonged exposure to PVP may be associated with increased sperm DNA strand breaks [3], and the presence of PVP within the ooplasm has been reported to delay calcium oscillation initiation after ICSI and potentially influence fertilization dynamics [4]. Consequently, it has been recommended that sperm exposure to PVP be minimized, ideally limited to less than 15 min during the ICSI procedure [5]. These observations have led to ongoing discussions regarding whether the use of non-physiological viscous media—particularly when small volumes may be introduced into the oocyte—could influence subsequent embryonic development. In addition, inter-laboratory variations in exposure time and handling techniques may contribute to heterogeneous outcomes.

Hyaluronic acid (HA), also referred to as hyaluronan, represents a biologically relevant alternative for sperm handling and selection. HA is a naturally occurring, non-sulfated glycosaminoglycan widely distributed within the extracellular matrix of mammalian tissues. Structurally, it is a linear polysaccharide composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked via alternating β (1 $\rightarrow$ 3) and β (1 $\rightarrow$ 4) glycosidic bonds [6]. In the female reproductive tract, HA is a major component of the cumulus–oocyte complex and plays an essential role in cumulus expansion and extracellular matrix organization during fertilization [7,8]. Due to its high molecular weight, negative charge, and strong hydrophilicity, HA contributes to clinical needs in obstetric and gynecological fields and assisted reproduction [9,10].

In the ICSI setting, HA-based systems have been developed to provide a more physiological microenvironment for sperm selection and immobilization [11,12]. Spermatozoa are chosen according to their capacity to bind immobilized hyaluronan, a characteristic proposed to indicate functional maturity. Because the oocyte is naturally surrounded by an HA-rich cumulus matrix, HA binding has been proposed to mimic physiological sperm selection. Spermatozoa capable of HA binding have been reported to demonstrate reduced DNA fragmentation and lower rates of chromosomal abnormalities compared with unbound sperm populations [13]. These findings provide a biological rationale for incorporating HA binding into ICSI practice.

However, the translation of these mechanistic observations into consistent clinical benefit remains uncertain. While some studies have reported improved fertilization and embryo development when HA is used [14], others have described differences in cleavage-stage or blastocyst quality [11], and some have concluded that physiological ICSI does not improve embryo outcomes [15]. The largest multicenter randomized controlled trial evaluating HA-based sperm selection did not demonstrate a significant improvement in live birth rates in the overall study population [16]. Furthermore, systematic reviews have highlighted substantial heterogeneity in the reported effects of HA-assisted ICSI, reflecting variability in study design, patient characteristics, and laboratory protocols [17,18].

Embryo culture and assessment systems, including those incorporating machine learning algorithms, have evolved substantially in recent years [19]. Conventional evaluation relies on static morphological assessment at predefined time points, typically on Day 3 and Day 5, providing only limited snapshots of a dynamic developmental process. In

contrast, time-lapse incubation systems enable continuous monitoring under stable culture conditions and allow the precise measurement of morphokinetic parameters.

Whether replacing PVP with HA-based sperm immobilization influences not only embryological outcomes but also morphokinetic timing patterns remains insufficiently characterized. Although artificial intelligence-derived embryo scores integrated within time-lapse platforms have been introduced as adjunctive tools, their role in detecting subtle differences related to sperm immobilization strategies has not been clearly established.

To address these gaps, we conducted a prospective sibling-oocyte study comparing HA-based sperm immobilization with conventional PVP-based ICSI. Outcomes included fertilization, cleavage-stage development, blastocyst formation, embryo quality, and morphokinetic parameters. By accounting for the clustering of embryos within patients, this study sought to provide an evaluation of whether the choice of sperm immobilization medium influences embryo developmental competence in contemporary IVF practice.

2. Materials and Methods

This prospective sibling-oocyte study was conducted at Hung Vuong Hospital from December 2025 to February 2026. The study protocol was approved by the institutional review board (approval number: CS/HV/25/38; approval date: 24 December 2025), and written informed consent was obtained from all participants prior to inclusion.

Consecutive patients undergoing ICSI during the study period were invited to participate. Within each cycle, mature oocytes (metaphase II, MII) were allocated to sperm immobilization using either HA or PVP, allowing intra-patient comparison under a sibling-oocyte design. The allocation of sibling MII oocytes to the HA or PVP group was performed sequentially during the ICSI procedure according to standard laboratory workflow. Because allocation occurred within the same cycle and during the same injection session, both groups were exposed to identical culture conditions and operator handling. All the procedures were performed according to institutional clinical protocols.

Patients were eligible if they underwent ICSI treatment and had sufficient MII oocytes to allow sibling allocation to both study conditions within the same cycle with complete embryological follow-up data available. Cycles were excluded in cases of absence of oocytes after oocyte pick-up, retrieval of only one oocyte, or use of donor oocytes, vitrified oocytes, frozen sperm, or surgically retrieved sperm.

Baseline demographic and clinical characteristics were retrieved from medical records. Collected variables included male age and female age (years), anti-Müllerian hormone (AMH, ng/mL), body mass index (BMI, kg/m²), the number of previous IVF attempts, duration of infertility (months), and primary IVF indications. Laboratory baseline parameters included the total number of retrieved oocytes, the number of immature oocytes (germinal vesicle and metaphase I), the number of abnormal oocytes, and the number of MII oocytes. Abnormal oocytes were defined at denudation and pre-ICSI assessment as oocytes exhibiting dysmorphic features, including abnormal shape, dark or granular cytoplasm, cytoplasmic vacuoles, enlarged perivitelline space or excessive perivitelline debris, abnormal zona pellucida, and fragmented or enlarged first polar body.

Controlled ovarian stimulation was performed using an antagonist protocol. Final oocyte maturation was triggered when appropriate follicular development was achieved. Oocyte retrieval was performed 34–36 h after triggering. Retrieved cumulus–oocyte complexes were cultured in standard culture media under controlled temperature, humidity, and gas conditions. Denudation was performed prior to ICSI to assess nuclear maturity, and only MII oocytes were injected.

Semen samples were prepared using discontinuous density gradient centrifugation. Briefly, liquefied semen was layered onto a two-layer gradient system (Isolate Medium and

Sperm Washing Medium, Irvine Scientific, Santa Ana, CA, USA) and centrifuged according to the manufacturer's instructions. The resulting pellet was collected and washed in Sperm Washing Medium before use for ICSI.

For sperm immobilization in the HA group, SpermCatch (Nidacon, Mölndal, Sweden) was prepared according to the manufacturer's instructions. A 10 µL droplet of SpermCatch was placed in a 35 mm culture dish, surrounded by microdroplets of the injection medium, and overlaid with oil. The dish was equilibrated at 37 °C in a CO₂ incubator for 30 min. Subsequently, 1 µL of prepared sperm suspension was added to the SpermCatch droplet and incubated for 10 min at 37 °C before sperm selection. Motile spermatozoa were identified within the hyaluronic acid medium and immobilized by tail nicking prior to aspiration into the injection pipette. According to the manufacturer's product documentation, SpermCatch contains HA, human serum albumin, and physiological salts, with a reported pH of 7.0–8.5 and osmolality of 290–320 mOsm/kg. However, the manufacturer's publicly available documentation does not specify the concentration or molecular weight of the HA component.

In the PVP group, sperm immobilization was performed using 10% PVP solution (Irvine Scientific, Santa Ana, CA, USA). Motile spermatozoa were selected and immobilized in the PVP droplet prior to injection. All ICSI procedures were performed using standard micromanipulation techniques under inverted microscopy by experienced embryologists. Following ICSI, injected oocytes were cultured under standard conditions using Continuous Single Culture- NX Complete (Irvine Scientific, Santa Ana, CA, USA).

Fertilization was assessed 16–18 h after ICSI. Normal fertilization was defined as the presence of two pronuclei (2PN), whereas abnormal fertilization included 1PN or ≥3PN. Oocyte degeneration was recorded when oocytes exhibited cytoplasmic darkening, membrane rupture, or other morphological signs of non-viability following ICSI.

The fertilization rate was calculated as the number of normally fertilized oocytes divided by the total number of injected oocytes. The abnormal fertilization rate was calculated as the number of abnormally fertilized oocytes divided by the total number of injected oocytes. The degeneration rate was defined as the number of degenerated oocytes divided by the total number of injected oocytes.

Cleavage was evaluated on Day 3. The cleavage rate was calculated as the number of cleaved embryos divided by the number of normally fertilized oocytes (2PN). Good-quality Day 3 embryos were defined according to established morphological criteria, including appropriate cell number, symmetrical blastomeres, and minimal fragmentation [20]. The good-quality cleavage rate was calculated as the number of good-quality Day 3 embryos divided by the total number of cleaved embryos.

Blastocyst development was assessed on Day 5 and Day 6. The blastocyst formation rate was calculated as the number of blastocysts divided by the number of cleaved embryos. Good-quality blastocysts were defined based on expansion stage and inner cell mass and trophectoderm grading according to standard laboratory criteria [20]. The good-quality blastocyst rate was calculated as the number of good-quality blastocysts divided by the total number of blastocysts.

Morphokinetic parameters, including t₂, t₃, t₄, t₅, and tB (expressed as hours post insemination, hpi), were recorded using a time-lapse imaging system in a subset of cycles (EmbryoScope time-lapse incubator, Vitrolife, Gothenburg, Sweden). AI scores ranging from 0 to 10 were generated by the integrated algorithm of the time-lapse system and were used as an adjunctive indicator of embryo developmental potential. According to the manufacturer, the score is derived from the analysis of time-lapse developmental information, but the exact algorithmic weighting of individual features is not publicly disclosed.

Morphokinetic analyses were restricted to patients with available time-lapse data. At our center, time-lapse culture was performed only in a single EmbryoScope system and depended on patient registration for this additional service as well as equipment capacity; consequently, only a limited number of oocytes could be monitored per cycle.

Statistical analyses were performed using R (R version 4.5.2 Foundation for Statistical Computing, Vienna, Austria). Embryological outcomes were analyzed using generalized estimating equations (GEEs). Odds ratios with 95% confidence intervals were reported. Sensitivity analyses for embryological outcomes were conducted using paired Wilcoxon signed-rank tests based on patient-level rates. Morphokinetic parameters and AI scores were analyzed using linear mixed-effects models. A *p*-value < 0.05 was considered statistically significant.

3. Results

A total of 51 ICSI cycles were included during the study period, yielding 811 injected oocytes. Of these, 377 oocytes were allocated to the HA group and 434 to the PVP group. Table 1 describes the baseline characteristics of included patients. The median female age was 32 years, and the median male age was 34 years. Most patients were undergoing their first IVF attempt. The main indications for IVF included male factor infertility, tubal factor, and PCOS. The mean infertility duration was approximately 26 months.

Table 1. Baseline characteristics of included patients.

Parameters	
No. of included patients	51
Female age (year), median (IQR)	32 (29–34)
Male age (year), median (IQR)	34 (32–36)
BMI (kg/m ²), mean ± SD	22.14 ± 3.17
AMH (ng/mL), mean ± SD	4.45 ± 3.05
IVF indication, n (%)	
1/Male factor	12 (23.5%)
2/Diminished ovarian reserve	3 (5.9%)
3/Uterus factor	6 (11.8%)
4/PCOS	8 (15.7%)
5/IUI failed	7 (13.7%)
6/Tubal factor	9 (17.6%)
7/Other reasons	1 (2%)
8/Unexplained	5 (9.8%)
Infertility duration (month), mean ± SD	26.16 ± 18.22
Previous IVF attempts:	
1/None	44 (86.2%)
2/One	6 (11.8%)
3/Two	1 (2%)
Number of retrieved oocytes, mean ± SD	19.67 ± 9.21
Number of immature oocytes, mean ± SD	2.22 ± 1.98
Number of abnormal oocytes, mean ± SD	1.57 ± 1.8
Number of MII oocytes, mean ± SD	15.9 ± 7.68

IQR: interquartile range; BMI: body mass index; SD: standard deviation; AMH: anti-Müllerian hormone; IVF: in vitro fertilization; PCOS: polycystic ovary syndrome; IUI: intrauterine insemination.

On average, nearly 20 oocytes were retrieved per cycle, with a mean of approximately 16 MII oocytes available for ICSI. The numbers of immature and abnormal oocytes were low relative to the total number of retrieved oocytes. Because of the sibling-oocyte design, demographic and cycle characteristics were inherently balanced between the HA and PVP groups within each patient.

Fertilization outcomes are summarized in Table 2. No statistically significant differences were observed between the HA and PVP groups in the normal fertilization rate, the abnormal fertilization rate, or the degeneration rate after adjustment for intra-patient clustering using GEEs. Sensitivity analyses using paired Wilcoxon signed-rank tests yielded consistent findings.

Table 2. Fertilization outcomes per method.

Outcome	HA (<i>n</i> = 377)	PVP (<i>n</i> = 434)	OR (95% CI)	<i>p</i> -Value
Fertilization rate	305/377 (80.9%)	370/434 (85.3%)	0.73 (0.43–1.25)	0.25
Fertilization failure rate	46/377 (12.2%)	47/434 (10.8%)	0.85 (0.51–1.43)	0.55
Abnormal fertilization rate	8/377 (2.1%)	13/434 (3.0%)	0.71 (0.27–1.87)	0.49
Oocyte degeneration rate	18/377 (4.8%)	16/434 (3.7%)	1.33 (0.66–2.69)	0.42

p-values derived from GEE models accounting for the sibling-oocyte design. Sensitivity analyses using the Wilcoxon signed-rank test at the patient level yielded consistent results for all the outcomes (all *p* > 0.05).

Cleavage-stage outcomes are presented in Table 3. While the overall cleavage rates were comparable between the groups, the proportion of good-quality Day 3 embryos was significantly lower in the HA group compared with the PVP group. After adjustment, the odds of obtaining a good-quality Day 3 embryo were significantly reduced in the HA group relative to PVP. Sensitivity analyses using paired Wilcoxon signed-rank tests yielded consistent findings.

Table 3. Cleavage embryo outcomes per method.

Outcome	HA (<i>n</i> = 377)	PVP (<i>n</i> = 434)	OR (95% CI)	<i>p</i> -Value	Wilcoxon Paired <i>p</i>
Cleavage rate	302/305 (99.0%)	359/370 (97.0%)	3.08 (0.41–23.10)	0.27	0.49
Good-quality cleavage rate	101/302 (33.4%)	172/359 (47.9%)	0.55 (0.39–0.77)	<0.01	0.01

p-values derived from GEE models accounting for the sibling-oocyte design. The Wilcoxon signed-rank test was used for the sensitivity analyses.

Blastocyst-stage outcomes are shown in Table 4. The blastocyst formation rate was significantly lower in the HA group compared with the PVP group. Similarly, the good-quality blastocyst rate was reduced in the HA group. These differences remained statistically significant after accounting for within-patient clustering. Sensitivity analyses using paired Wilcoxon signed-rank tests yielded consistent findings.

Table 4. Blastocyst outcomes per method.

Outcome	HA (<i>n</i> = 377)	PVP (<i>n</i> = 434)	OR (95% CI)	<i>p</i> -Value
Blastocyst formation rate	158/302 (52.3%)	251/359 (69.9%)	0.47 (0.35–0.65)	<0.01
Good-quality blastocyst rate	78/158 (49.4%)	161/251 (64.1%)	0.49 (0.29–0.80)	<0.01

p-values derived from GEE models accounting for the sibling-oocyte design. Sensitivity analyses using the Wilcoxon signed-rank test at the patient level yielded consistent results for all the outcomes (all *p* < 0.05).

Time-lapse morphokinetic outcomes are summarized in Table 5. No statistically significant differences were observed between HA and PVP in early cleavage timings (*t*₂, *t*₃, *t*₄, *t*₅) or time to blastocyst formation (*t*_B). However, AI scores were significantly lower in embryos derived from the HA group compared with those from the PVP group (5.8 [4.1–7.4] vs. 6.7 [5.1–8.1], respectively; β = 0.64, 95% CI 0.01 to 1.28; *p* = 0.04).

Table 5. Time-lapse morphokinetics outcomes.

Outcome	HA (n = 54)	PVP (n = 136)	β (95% CI)	p-Value
t2 (hpi)	25.9 (24.4–27.7)	25.6 (24.2–28.2)	0.28 (−0.86 to 1.42)	0.63
t3 (hpi)	36.0 (33.2–39.2)	36.7 (34.1–39.2)	0.60 (−0.98 to 2.19)	0.46
t4 (hpi)	38.4 (35.6–40.7)	38.0 (35.5–41.1)	0.09 (−1.87 to 2.04)	0.93
t5 (hpi)	50.5 (44.3–53.9)	50.1 (46.1–54.3)	0.77 (−1.96 to 3.50)	0.58
tB (hpi)	104.3 (98.5–109.2)	104.8 (98.8–110.7)	−0.91 (−4.15 to 2.33)	0.58
AI score (0–10)	5.8 (4.1–7.4)	6.7 (5.1–8.1)	0.64 (0.01 to 1.28)	0.04

hpi: hours post insemination. Linear mixed-effects models with a random intercept for patient were used to compare morphokinetics outcomes.

4. Discussion

In this prospective sibling-oocyte study, we evaluated whether HA-based sperm immobilization during ICSI is associated with embryological advantages compared with conventional PVP-assisted immobilization. Our findings demonstrate that HA-based immobilization did not improve fertilization outcomes. The fertilization rate, fertilization failure rate, abnormal fertilization, and oocyte degeneration were comparable between the groups.

Similarly, the overall cleavage rate did not differ between the groups. However, the proportion of good-quality cleavage-stage embryos was significantly lower in the HA group. This divergence became more evident at the blastocyst stage, where both the blastocyst formation rate and the good-quality blastocyst rate were significantly higher following PVP-assisted ICSI. These results were robust across GEE models and confirmed by patient-level sensitivity analyses.

Time-lapse morphokinetic parameters (t2–tB) were comparable between the groups, suggesting similar developmental kinetics. Nonetheless, AI-derived embryo scores were modestly higher in the PVP group. Taken together, our findings indicate that, within this cohort, HA-based sperm immobilization did not confer embryological advantages over conventional PVP-assisted ICSI. However, because this study did not assess implantation, clinical pregnancy, or live birth, the lower blastocyst development rates observed in the HA group should not be over-interpreted as evidence of inferior clinical effectiveness.

The development of HA-based sperm selection techniques was supported by biological plausibility. Hyaluronic acid binding has been linked to sperm maturity and membrane integrity. However, these molecular characteristics have not consistently translated into improved clinical outcomes. The existing literature on HA-based sperm selection demonstrates a heterogeneous pattern of findings.

Several investigations align with our observation that HA-based selection does not improve embryological outcomes. Importantly, even in male factor infertility—where HA binding is theoretically most relevant—clinical benefit has not been uniformly demonstrated. A systematic review focusing on male factor infertility concluded that pooled analyses did not show statistically significant improvements in key reproductive outcomes [21]. Although our cohort included mixed indications, male factor infertility represented the largest subgroup. In this context, the absence of a positive effect in our study is consistent with broader evidence suggesting that HA-based selection does not consistently translate into improved embryological outcomes. However, the present study was not designed or statistically powered for subgroup analyses by infertility indication, and detailed baseline sperm parameters were not incorporated into the comparative analysis. Future studies should stratify by male factor infertility and include sperm concentration, motility, and morphology to better define subgroups that may benefit from HA-based sperm selection.

Similarly, another meta-analysis reported no significant improvement in fertilization or pregnancy rates and did not support the routine clinical use of HA-based techniques [17].

Sibling-oocyte studies provide particularly valuable insight because they minimize inter-patient confounding. One such study concluded that PICSI did not improve overall embryo quality compared with conventional ICSI [15]. A time-lapse analysis in a sibling-oocyte setting demonstrated comparable embryo development and morphokinetic parameters between HA-ICSI and PVP-ICSI [22], paralleling our findings of similar fertilization, cleavage, and kinetic profiles. In a matched case-control study of advanced maternal age patients, PICSI failed to improve fertilization, embryo utilization, or ongoing pregnancy rates [23], reinforcing the notion that HA-based selection does not enhance outcomes even in higher-risk populations. Furthermore, studies evaluating hyaluronate-containing media as alternatives to PVP have shown improvements in selected sperm characteristics without corresponding increases in live birth rates [24], suggesting that the replacement of PVP does not inherently enhance developmental competence.

Collectively, these neutral or negative studies support the interpretation that HA-based sperm immobilization does not consistently improve embryological performance under controlled conditions. Our findings—demonstrating comparable fertilization but significantly lower blastocyst formation and good-quality blastocyst rates in the HA group—therefore align with a broader pattern of heterogeneous outcomes.

Conversely, several cohort studies and selected meta-analytic interpretations have reported the potential benefit of HA-based selection. Scaruffi et al. reported that the injection of HA-bound sperm significantly improved the cleavage rate compared with standard ICSI [11]. A prospective study suggested that PICSI should be considered for patients with altered nuclear sperm quality undergoing ICSI [25]. In addition, a retrospective sibling-oocyte study also reported improvements in fertilization and transfer-eligible embryo rates with PICSI [14]. Interestingly, a recent meta-analysis indicated that cohort studies demonstrated a significant advantage of HA-ICSI in embryo and pregnancy outcomes [18]. Notably, these analyses also highlighted a discrepancy between observational and randomized evidence. While pooled cohort data sometimes suggest improved intermediate endpoints, randomized controlled trials typically demonstrate attenuated or non-significant effects. This pattern raises the possibility that effect sizes may be overestimated in non-randomized settings or diluted under stricter methodological control.

Among sibling-oocyte studies, the only investigation reporting a clear positive effect of PICSI was retrospective and conducted in a selected population largely composed of patients with previous IVF failure [14]. In contrast, prospective sibling-oocyte studies—including ours—have generally reported neutral findings [15,22]. Differences in patient selection, allocation procedures, laboratory workflow, and analytical strategies may partially explain these discrepancies.

In our study, HA-based sperm immobilization did not confer embryological advantages over conventional PVP-assisted ICSI. The divergence between theoretical plausibility and the observed outcomes warrants careful consideration. Several explanations may account for these findings.

Firstly, although HA binding has been associated with markers of sperm maturity, such as HspA2 expression and reduced chromosomal aneuploidy [26], these molecular features may not directly translate into improved blastocyst competence across all clinical contexts. Blastocyst development is a multifactorial process. Early human embryogenesis—particularly compaction and blastocyst formation—depends critically on centrosome integrity and cytoskeletal reorganization, processes that may not be fully reflected by HA affinity alone [27].

Secondly, beyond sperm selection, embryo development *in vitro* is influenced by a complex interplay of chemical and physical factors within the laboratory environment [28]. Human embryos cultured *in vitro* are exposed to conditions including fluctuations in pH and temperature, atmospheric oxygen tension, mechanical manipulation, and the potential release of volatile compounds from cultureware. When multiple stressors coexist, synergistic effects may amplify developmental vulnerability. In this context, even subtle procedural differences—such as extended observation for HA binding or additional handling steps—could increase cumulative microenvironmental stress. While such differences may not affect fertilization, later developmental events such as compaction and blastocyst formation, which rely heavily on cytoskeletal reorganization and centrosome-driven microtubule dynamics, may be more sensitive to environmental perturbations.

Finally, procedural factors may also play a role. PVP has been incorporated into routine ICSI practice for decades, and laboratory workflows are largely optimized around its physical properties. In contrast, HA-based systems differ in viscosity, sperm handling characteristics, and immobilization dynamics. Even minor variations in manipulation time or microinjection workflow may alter oocyte exposure outside controlled culture conditions.

Interestingly, morphokinetic timing parameters did not differ significantly between the groups. Growing evidence suggests that embryo morphokinetics primarily reflect intrinsic gamete quality and patient-related factors rather than isolated technical modifications in sperm selection. Indeed, time-lapse studies have shown that oocyte morphological abnormalities may influence the cleavage patterns of embryos in ICSI cycles [29].

Studies in donor-oocyte cycles further demonstrate that severe oligospermia is associated with alterations in Day 3 morphokinetic events, underscoring the influence of baseline sperm quality [30]. A recent meta-analysis similarly reported that while early cleavage events were not significantly affected by the sperm quality, reduced sperm concentration and motility were associated with delays in later developmental stages and with implantation and live birth outcomes [31].

Maternal characteristics also play a role. For example, body weight has been associated with differences in morphokinetic timing, with embryos from underweight women reaching the eight-cell stage more rapidly than those from normal-weight or obese women [32]. Collectively, these findings suggest that morphokinetics are sensitive to both the intrinsic biological conditions of gametes and parental physiology. In our sibling-oocyte design, embryos in the HA and PVP groups originated from the same semen samples and maternal environments within each cycle, thereby sharing identical baseline biological characteristics. The absence of significant morphokinetic differences between the groups is therefore biologically plausible.

The principal strength of this study lies in its prospective sibling-oocyte design, which inherently controls for patient- and cycle-level confounders. The application of GEE and linear mixed-effects models appropriately accounted for intra-patient clustering. Moreover, the integration of conventional embryological endpoints with time-lapse morphokinetic assessment and AI-derived scoring enabled a multidimensional evaluation of developmental competence. Nonetheless, our findings should be interpreted within the context of current laboratory workflows, and it remains possible that HA-based selection may demonstrate benefit in specific clinical subgroups or under alternative protocol conditions.

Several limitations should be acknowledged. The primary limitation of this study is that oocyte allocation to the HA or PVP group was performed according to routine laboratory workflow rather than formal randomization, which may have introduced procedural variability. No formal sample size calculation was performed prior to study initiation, and although the overall sibling-oocyte sample was sufficient for primary embryological

outcomes, the study may have been underpowered for secondary analyses, particularly morphokinetic assessments, because these were restricted to a smaller subset of embryos with available time-lapse data. Accordingly, the morphokinetic subset was not randomly selected and may have been subject to selection bias. Furthermore, this study focused on embryological and morphokinetic outcomes and did not assess implantation, clinical pregnancy, or live birth, which remain the ultimate measures of ART success. Therefore, the observed differences in blastocyst development should be interpreted cautiously and cannot be assumed to translate into differences in clinical outcomes.

Our results emphasize the importance of evaluating minor laboratory modifications before widespread implementation. For laboratories contemplating the routine adoption of HA-based immobilization, these data underscore the necessity of internal validation. Future multicenter randomized sibling-oocyte studies with larger sample sizes and linkage to clinical outcomes are needed to clarify whether specific patient subgroups may derive benefit from HA-assisted ICSI.

In this prospective sibling-oocyte cohort, HA-based sperm immobilization was not associated with differences in fertilization-related outcomes, but it was associated with lower blastocyst formation and lower good-quality blastocyst rates compared with conventional PVP-assisted ICSI. These findings suggest less favorable later embryological development in the HA group. However, the present study was not designed to determine whether this difference was attributable to the immobilization medium itself or to unmeasured procedural factors; therefore, any mechanistic interpretation remains speculative.

5. Conclusions

Our findings suggest that replacing PVP with HA did not improve embryological outcomes in this prospective sibling-oocyte cohort. Although HA-based sperm selection is supported by a plausible biological rationale, previous studies have reported heterogeneous results, with some suggesting potential benefit and others showing no clear advantage over conventional ICSI. However, because no clinical follow-up was available, the clinical significance of the lower blastocyst development rates observed in the HA group remains uncertain and should not be over-interpreted. Further well-designed studies incorporating clinical endpoints are required to clarify the role of HA-based immobilization in ICSI practice.

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