Archives of Gynecology and Obstetrics (2019) 299:247–258
https://doi.org/10.1007/s00404-018-4937-8

Effect of maternal and embryonic factors on frozen-thawed IVF-ET outcome after pre-equilibration with hyaluronan

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Received: 16 February 2018 / Accepted: 12 September 2018 / Published online: 7 December 2018

Abstract

Purpose To systematically evaluate the effect of maternal and embryonic factors on in vitro fertilization and embryo transfer (IVF-ET) outcomes among Chinese patients after using hyaluronan-enriched transfer medium (HETM).

Methods This retrospective study included 637 frozen-thawed ET cycles. Patients were divided into subgroups based on their maternal or embryonic status or treatment procedures. The implantation, clinical pregnancy, delivery, and abortion rates were compared between the HETM and control groups. In addition, the implantation and clinical pregnancy rates were used to analyze the reciprocal effect of HETM and Preimplantation genetic screening (PGS) assessment.

Results Maternal risk factors, especially maternal aging and a low number of retrieved oocytes, have a significant adverse impact on the efficacy of HETM usage. Endometrial preparation with artificial and natural cycles but not stimulated cycles showed a satisfying outcome after IVF-ET treatment. Compared with cleavage embryos, blastocyst stage embryo transfer showed more prominent improvement when using HETM. Prolonged pre-equilibration treatment with HETM notably compromised the IVF-ET outcome. PGS-based preselection could further facilitate the HETM-induced beneficial effect on IVF-ET outcomes. The body weight, length, and sex ratio of the neonate did not significantly differ between the HETM and control groups.

Conclusion Both the maternal and embryonic status or treatment procedures affected the IVF-ET outcomes after using HETM. HETM had a beneficial effect on advantaged IVF cycles but did not improve the outcomes of disadvantaged IVF cycles. Endometrial preparation with stimulated cycles is not recommended when using HETM. Prolonged pre-equilibration treatment must be avoided.

Keywords IVF · Embryo transfer · Hyaluronan · Pregnancy rate · Implantation rate · Frozen-thawed embryos

Introduction

Implantation is of prime importance for pregnancy establishment and subsequent embryonic development. Successful implantation involves the fully-achieved pluripotency of blastocysts and well-controlled reciprocal interaction between embryo and receptive uterus [1]. Defects in embryos and uterine, due to either genetic abnormalities or other pathological disorders, may lead to implantation failure [2–4], which usually result in a low pregnancy rate in both natural conception and in vitro fertilization and embryo transfer (IVF-ET) [5, 6].

Infertility is a condition that affects approximately 15% of couples worldwide. IVF is one of the most effective and successful assisted reproductive technologies that has been routinely used to treat infertility [7]. Despite this, its success

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rates remain disappointingly low, owing primarily to frequent implantation failure [8–10]. There is a continuing need to enhance the implantation potential of IVF embryos. Over the past decades, numerous efforts have been made to overcome IVF-associated implantation failure, including improving endometrial preparation, optimizing the in vitro culture medium, and preselecting potential embryos before transfer [6, 11, 12]. However, the implantation and subsequent pregnancy outcome remains dissatisfying. Notably, during the last decade, researchers have investigated the effects of optimizing the transfer medium with macromolecules such as hyaluronan (hyaluronic acid, HA), which has provided a promising strategy for improving implantation after IVF-ET in both animals and humans.

Hyaluronic acid is a non-sulfated glycosaminoglycan polymer that naturally occurs in many mammalian tissues and fluid secretions. It is one of the most abundant macromolecules in the female reproductive tract [13–16]. Early studies found that pre-equilibration of rodent and bovine embryos using high levels of HA before transfer for IVF significantly increased the subsequent implantation rates [17, 18]. In accordance with these findings, both clinical studies and meta-analysis showed improved implantation and clinical pregnancy rates using HA-enriched transfer medium (HETM, EmbryoGlue; Vitrolife, Sweden) [19–21]. However, the clinical outcomes with HETM remain controversial due to contradictory reports [22, 23].

In addition to increasing embryo attachment to the endometrium, HA also improves the embryonic implantation potential via its principal receptor CD44, which is located on the embryonic cell surface. [13, 14]. These facts suggest that the beneficial effect of HETM on IVF-ET outcomes should depend on both the embryonic and maternal status. However, no studies have systematically explored how maternal and embryonic factors affect IVF-ET outcome after using HETM. In particular, the effect of technical procedures of IVF-ET treatment, such as the duration of pre-equilibration treatment and the methods of endometrial preparation, on the efficacy of HETM usage, has never been tested. Therefore, we evaluated the effect of maternal and embryonic factors on the outcome of IVF-ET after HETM usage among Chinese patients undergoing IVF, with a focus on IVF risk factors and IVF-ET treatment procedures. Moreover, we also focused on how HETM usage affected IVF-ET outcomes at birth. Compared with the implantation and pregnancy rates, the live birth rate and other neonatal characteristics are of greater importance for both patients and clinicians, but have not been fully analyzed after using HETM in previous reports [12].

Finally, we evaluated outcomes of embryo undergoing preimplantation genetic screening (PGS) after HETM. PGS biopsy and assessment are frequently used techniques that help improve the outcome of specific groups of IVF patients, particularly for those suspected to have a high proportion of aneuploid or genetic defect embryos [24]. Thus far, no studies have examined the reciprocal effect of HETM usage and PGS assessment.

Materials and methods

Patients

The records of patients who underwent IVF-ET at the Chinese PLA Naval General Hospital, Beijing, China, from June 2015 and October 2017 were retrospectively reviewed. This study was approved by the Institutional Review Board of the Chinese PLA Naval General Hospital. Before accepting IVF-ET procedures, all patients were extensively counseled before standard informed consent was obtained. However, due to the retrospective nature of the study, as well as the fact that both transfer mediums are commercially available and routinely used in IVF laboratories, specific informed consent was waived for data collection and analyses. There was no special criterion for deciding the usage of HETM. For the study group ($n = 161$), the ET medium was EmbryoGlue (Vitrolife, Sweden) containing recombinant human albumin and a high concentration of HA. For the control group ($n = 476$), the ET medium was G2™ PLUS medium (Vitrolife, Sweden) containing lower concentration of HA. To exclude potential effects of clinicians, the clinicians performing IVF and ET procedures were randomly allocated to two groups of patients. Oocyte donation cycles were not included in the study.

Ovarian stimulation

Following regimens were used for ovarian stimulation: (1) gonadotrophin-releasing hormone agonist (GnRH-a) long-term regimen: 0.1 mg GnRH-a (Decapeptyl, Ferring, Germany) was administered per day from days 2–4 of the menstrual or mid-luteal phases. After 14 days, 75–300 U/day Gn (Gonal-F, Merck Serono, Germany) was administered until the hCG (Ovidrel®, Merck Serono, Germany) injection day. (2) GnRH-a short-term regimen: 0.1 mg/day GnRH-a and 150–300 U/day Gn were administered on day 2 of the menstrual cycle until hCG day. (3) GnRH-ant (Cetrorelix acetate, Merck Serono, Germany) protocol: after administration of 150–225 IU/day Gn at days 2–3 of the menstrual cycle when the dominant follicle diameter was ≥ 14 mm, 0.25 mg/day GnRH-ant was administered until the hCG injection day. When the diameter of at least one follicle was ≥ 18 mm or at least two follicles were ≥ 17 mm, the patients received 250 μg hCG injections in the evening. Follicles were aspirated using transvaginal ultrasound guidance 34–36 h after hCG administration.

IVF/ICSI procedure and embryo culture

Fertilization was performed using conventional IVF [25] or ICSI [26] according to a previous reports. In the cases undergoing IVF, semen was produced by masturbation and, after washing, motile sperm were separated using a 15–30 min swim-up period. For in vitro fertilization, approximately 5 oocytes were incubated with $50–100 \times 10^3$ motile sperm within 4–6 h of oocyte collection. In the cases that IVF cannot be carried out due to male-factor infertility (poor sperm morphology and/or motility), ICSI was performed. At 16–18 h after either insemination or ICSI, the oocytes were examined for fertilization using a dissecting microscope. The presence of two pronuclei with extrusion of the second polar body was considered as evidence of successful fertilization. The day the pronuclei appeared, the oocytes/embryos were individually cultured in 10-μL droplets of cleavage medium (CM, COOK, Australia) with mineral oil (Ovoil, Vitrolife, Sweden) at 37 °C in a tri-gas incubator containing 6% CO_2 and 5% O_2 to support embryonic development from the pronuclear stage until day 3. On day 3, the embryos were transferred to blastocyst medium (BM, COOK, Australia) until days 5–6.

Embryo grading

The number of blastomeres and embryo morphology were graded on days 2 and 3 after fertilization. The embryo morphology was scored based on their quality as follows. Cleavage stage embryos were divided into four levels: grade I, ≥ 8 blastomeres, uniform cytoplasm, no vacuoles, ≤ 5% fragments; grade II, ≥ 6 blastomeres, slightly uneven blastomeres, uniform cytoplasm, no vacuoles, 5–15% fragments; grade III, ≥ 6 blastomeres, obviously uneven blastomeres, few vacuoles in the cytoplasm, 15–30% fragments; grade IV, < 6 blastomeres, obviously uneven blastomeres or containing a large number of vacuoles, 30–50% fragments. Of these, embryos categorized under grades I and II were defined as good-quality embryos. Grades I, II, and III embryos could be used for ET and as frozen-thawed embryos. Embryos were evaluated on day 5 or 6 of culture by assessing their morphology and developmental stage. These embryos were also classified as grades I, II, III, and IV Grade I, embryos are any cavitating blastocysts containing a discrete compact inner cell mass (ICM) with many trophectoderm cells forming a cohesive layer; Grade II, embryos are any cavitating blastocysts with a lower cell number and/or a nondiscrete ICM and/or minor degeneration or an early blastocyst with an indiscreet ICM. Grade III, blastocyst stage embryos with a small degree of cavitation with very low cell number and/or a nondiscrete ICM. Grade IV, embryos are not used for transfer or freeze–thaw cycles. Likewise, blastocyst stage embryos of grades I and II were defined as good-quality embryos.

Endometrial preparation

Endometrial preparation methods: (1) Natural cycle: Patients with regular menstruation and normal ovulation underwent vaginal ultrasound monitoring of follicular growth and endometrial thickness until ovulation and endometrial transformation on the ovulation day. Thawing and transfer of embryo should be performed 3–5 days after ovulation depending on the stage at which the embryo was frozen. (2) Stimulation cycle: Patients with poor follicular development and ovulation disorders received 75–150 U/day HMG on days 3–5 of their menstrual cycle until mature follicles appeared, and then received hCG injection. They were monitored to detect ovulation day in accordance with the natural cycle of endometrial transformation after ovulation. (3) Artificial cycle: patients with irregular menstruation, poor natural follicular growth, and no ovulation received 4–8 mg estradiol valerate per day (Progynova, Bayer, Guangzhou) from days 3–4 days after menstruation. Endometrial transformation was performed when the endometrium grew to ≥ 8 mm, and frozen-thawed ET was then carried out on day 3 after ovulation.

Preparation of HETM and embryo transfer

Commercially available medium (EmbryoClue, Vitrolife, Sweden) was used as the HETM. On the day before ET, 1.5 mL of EmbryoGlue was prepared for transfer, and 5 × 20-μL drops were placed into a 3.5-cm culture dish, covered with mineral oil for equilibration, and incubated at 37 °C in air containing 6% CO_2 and 5% O_2 for 6–18 h. All ETs were carried out on day 3 cleavage embryos or days 5 or 6 blastocysts in freeze–thaw cycles. For the control groups, selected embryos were thawed on the day before ET, then placed in pre-equilibrated G2™ PLUS medium (Vitrolife, Sweden) overnight, and cultured at 37 °C in air containing 5% CO_2 before transfer. Embryos from the study group were placed in drops of pre-equilibrated EmbryoGlue medium and incubated for at least 30 min at 3 °C in air containing 5% CO_2 on the day of transfer. Embryos from the control group were transferred without using additional transfer medium. EmbryoGlue contains a higher concentration of HA (0.5 mg/mL) than G-2™ PLUS medium (0.125 mg/mL). Furthermore, EmbryoGlue contains 2.5 mg/mL of recombinant human serum albumin solution (HSA, Vitrolife, Sweden) while G-2™ PLUS contains 10 mg/mL HAS [25, 28]. ET was routinely performed using a Guardia™ Access Embryo Transfer Catheter with Internal Support Cannula (G02346, COOK, Australia). The catheter was washed with EmbryoGlue medium before embryo loading. For luteal phase support, the patients received three doses of 200 mg micronized

progesterone per day (Utrogestan; Besins Iscovesco, France) intravaginally.

Outcome measures

The fertilization rate was calculated as the number of normally fertilized zygotes divided by the number of MII oocytes; however, only injected metaphase II (MII) oocytes were taking into consideration for the ICSI treatments. Pregnancies were confirmed by the presence of elevated serum β-hCG concentrations (> 30 IU/mL) measured 12 and 14 days after ET. The clinical pregnancy rate was calculated as the number of patients with at least one gestational sac detected via transvaginal ultrasound 5–6 weeks after ET divided by the total number of patients who underwent ET. A biochemical pregnancy was defined as a positive β-hCG test (> 30 IU/mL) that was not followed by a clinical pregnancy. The implantation rate was calculated as the number of gestational sacs divided by the number of transferred embryos. The abortion rate was defined as the number of patients with loss of pregnancy before 22 weeks of gestation divided by the total number of chemical pregnancies. Pregnancies were followed up until delivery and the number of live newborns, and their genders, birth weights, and heights were also recorded. The delivery rate was calculated as the ratio of the number of patients who delivered and the total number of patients who underwent ET cycles.

Data analysis

Statistical analysis was performed using Statistica 8.0 software (StatSoft Inc., USA). Student's *t* test was used to compare mean values and the Chi squared test was used to compare proportional values. Statistical significance was set at $P < 0.05$. The main outcome measures were clinical pregnancy rate, implantation rate, and delivery rate. Secondary outcome measures were abortion rate, birth weight, and height. Subgroup analysis was also performed in patients < 35 years of age, in patients with previous IVF failure, in cycles with ≤ 5 oocytes, and in cycles where at least one good-quality embryo was available for ET.

Results

Overall outcome

A total of 637 frozen-thawed ET cycles were analyzed in our study (Table 1). The clinical parameters, including the mean maternal age, total (mature) oocytes retrieved, number of transferred embryos, average IVF-ET attempts, proportion of IVF/ICSI case, were similar between the HETM and control groups. Likewise, the embryonic developmental parameters, such as the mean number of oocytes fertilized, two-pronuclear fertilization rate, and the percentage of good-quality embryos transferred, were similar (Table 1).

In general, the HETM group was observed to have a statistically higher implantation rate (41.7% vs. 30.9%, $P < 0.01$) and clinical pregnancy rate (57.1% vs. 46.4%, $P < 0.05$) compared to the control group. Further analysis showed that the improved pregnancy establishment in the HETM group can result in a higher delivery rate (43.5% vs. 35.1%, $P > 0.05$). The abortion rate did not differ significantly between the two groups (23.9% vs. 24.4%, $P > 0.05$). Similarly, the number of newborns evaluated in this study did not significantly differ between the two groups (Table 2).

Table 1 Patients characteristics

	Control group	HA group	*P* value
No. of frozen-thawed ET	476	161	–
Patient's age	34.29 ± 5.32	33.52 ± 4.99	0.107
No. of oocytes collected	12.86 ± 9.16	13.84 ± 8.97	0.234
No. of metaphase II (MII) oocytes	11.55 ± 8.34	12.41 ± 8.17	0.293
No. of embryos transferred	1.88 ± 0.54	1.83 ± 0.52	0.350
Average ET attempts	1.82 ± 1.17	2.00 ± 1.54	0.128
Method of fertilization			
IVF	68.07% (324/476)	68.94% (111/161)	0.836
ICSI	25.63% (122/476)	20.50% (33/161)	0.189
No. of oocytes fertilized	8.47 ± 6.16	9.29 ± 6.05	0.210
Fertilization rate (all cycles)	73.28% (4031/5501)	74.60% (1495/2004)	0.250
Fertilization rate following IVF	72.37% (2876/3974)	74.20% (1139/1535)	0.170
Fertilization rate following ICSI	75.64% (1155/1527)	75.91% (356/469)	0.906
Good-quality embryos transferred	84.56% (756/894)	85.42% (252/295)	0.721

Table 2 Comparison of clinical outcome of IVF-ET cycles using HETM and control embryo transfer medium

	Control group	HA group	*P* value
No. ET cycles	476	161	
Clinical pregnancy	46.43% (221/476)	57.14% (92/161)	0.019
Implantation	30.87% (276/894)	41.69% (123/295)	0.0006
Delivery rate	35.08% (167/476)	43.48% (70/161)	0.057
Abortion rate	24.43% (54/221)	23.91% (22/92)	0.875

Impact of maternal factors on the efficacy of HETM usage

To test the effect of maternal risk factors and maternal treatments on the efficacy of HETM usage, we divided the tested patients into subgroups according to maternal age, previous implantation failure (PIF), number of retrieved oocytes, and endometrial preparation methods, followed by comparisons between the control and HETM groups.

In the subgroup of young patients (< 35 years of age), HETM resulted in an improved outcome, revealed by a significant increase in the implantation rate (49.3% vs. 37.5%, $P < 0.01$), clinical pregnancy rate (65.8% vs. 53.8%, $P < 0.05$), and delivery rate (52.5% vs. 41.7%, $P < 0.05$). The abortion rates are similar between young patients in the HETM and control groups (20.3% vs. 22.4%, $P > 0.05$). However, the beneficial effect of HETM was not observed in the older group (≥ 35 years of age): the implantation rate (22.0% vs. 19.6%, $P > 0.05$), clinical pregnancy rate (31.7% vs. 33.1%, $P > 0.05$), and delivery rate (17.1% vs. 23.1%, $P > 0.05$) of older patients seems to be similar between the HETM and control groups (Fig. 1a).

In the subgroup of patients without PIF, HETM was associated with a significantly increased implantation rate (49.3% vs. 33.7%, $P < 0.01$) and clinical pregnancy rate (64.7% vs. 48.6%, $P < 0.05$). Although the delivery rate was approximately 9% higher in the HETM group than in the control group, the difference did not reach statistical significance (48.2% vs. 39.0%, $P > 0.05$). In contrast, HETM did not facilitate the pregnancy and subsequent outcomes in the subgroup of patients with PIF: the implantation rate (34.0% vs. 28.2%, $P > 0.05$), clinical pregnancy rate (48.7% vs. 44.0%., $P > 0.05$), and delivery rate (38.2% vs. 30.8%, $P > 0.05$) for this subgroup were similar between the HETM and control groups (Fig. 1b).

Next, we compared subgroups of patients with different number of retrieved oocytes. 5 oocytes at retrieval was used as the criterion for evaluating ovarian response, as reported previously [27, 28]. In patients with > 5 oocytes collected, HETM resulted in a significant increase in the implantation rate (47.0% vs. 35.9%, $P < 0.01$) and clinical pregnancy

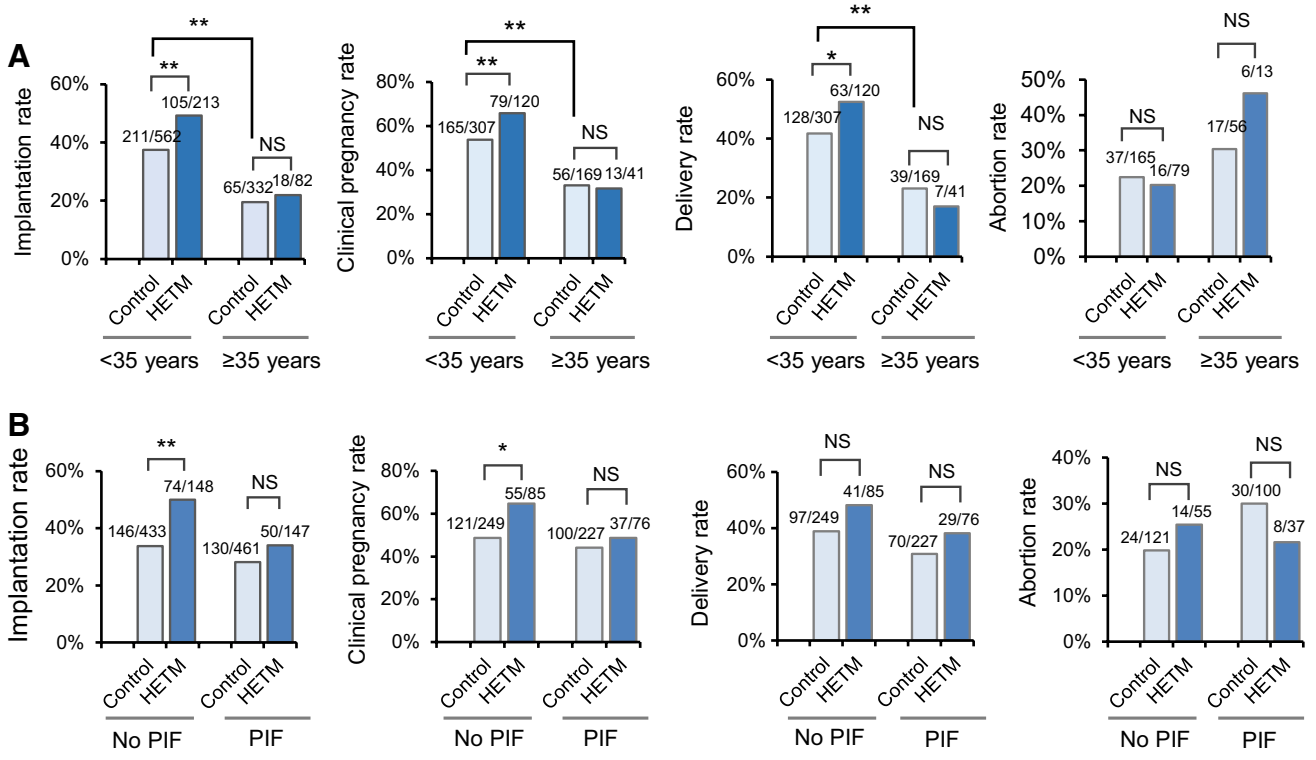

Fig. 1 Comparison of clinical outcome of IVF-ET cycles using HETM and control embryo transfer medium based on the subgroups of different maternal ages (**a**) and occurrence of previous implantation failure (PIF) (**b**). *NS* not significant; *$P < 0.05$, **$P < 0.01$

rate (64.0% vs. 51.7%, $P < 0.05$). However, the increase in delivery rate did not reach statistical significance (48.5% vs. 39.1%, $P > 0.05$). In contrast, for patients with ≤ 5 oocytes collected, HETM did not exhibit any beneficial effects on the IVF-ET outcome (Fig. 2a). Unexpectedly, we observed a lower outcome of patients with ≤ 5 oocytes after HETM usage, might be due to the limited sample size in this subgroup.

Having evaluated the influence of maternal risk factors, we further detected the effect of different endometrial preparation methods on the efficacy of HETM. Patients in artificial cycles showed significantly improved implantation and clinical pregnancy rates (44.5% vs. 33.6%, $P < 0.05$; 63.8% vs. 48.5%, $P < 0.05$), but not delivery rate. However, patients undergoing natural cycles exhibited a significant improvement in the implantation (43.2% vs. 28.8%, $P < 0.05$) and delivery (48.9% vs. 31.4%, $P < 0.05$) rates following HETM usage. There were no significant benefits in patients with stimulated cycles associated with HETM use (Fig. 2b). Besides, it should be mentioned that when using conventional transfer medium, three methods of endometrial preparation produced comparable IVF-ET outcomes without any significant difference. However, when using HETM, patients undergoing artificial and natural cycles appear to have an advantaged outcome. Of note, these conclusions should be drawn with caution because of the limited sample size in these subgroups of HETM usage.

Impact of embryonic factors on the efficacy of HETM usage

To test the impact of embryonic status or treatment procedures on the efficacy of HETM usage, we divided patients into subgroups according to the developmental stages at which embryos were transferred, duration of pre-equilibration, and quality of transferred embryos.

Here, we analyzed 431 cleavage stage and 206 blastocyst stage ET cycles. When using conventional transfer medium, blastocyst stage ET had a significantly higher implantation rate (48.0% vs. 25.3%, $P < 0.01$), clinical pregnancy rate (56.9% vs. 41.5%, $P < 0.01$), and delivery rate (43.1% vs. 31.2%, $P < 0.05$) compared to cleavage stage ET. In the cleavage stage transfer subgroup, only the implantation rate (34.8% vs. 25.3%, $P < 0.01$) was significantly better in the HETM group, but the clinical pregnancy, delivery, and abortion rates were not significantly altered following HETM treatment. In the blastocyst stage transfer subgroup, HETM showed significantly more benefits compared to the control group, and the implantation (62.2% vs. 48.0%, $P < 0.05$) and clinical pregnancy (75.5% vs. 56.9%, $P < 0.05$) rates increased by approximately 14% and 18%, respectively. However, the increase in the delivery rate (56.6% vs. 43.1%, $P > 0.05$) following HETM use did not reach statistical significance (Fig. 3a), maybe due to the limited sample size. This fact led us to the question of whether cleavage stage

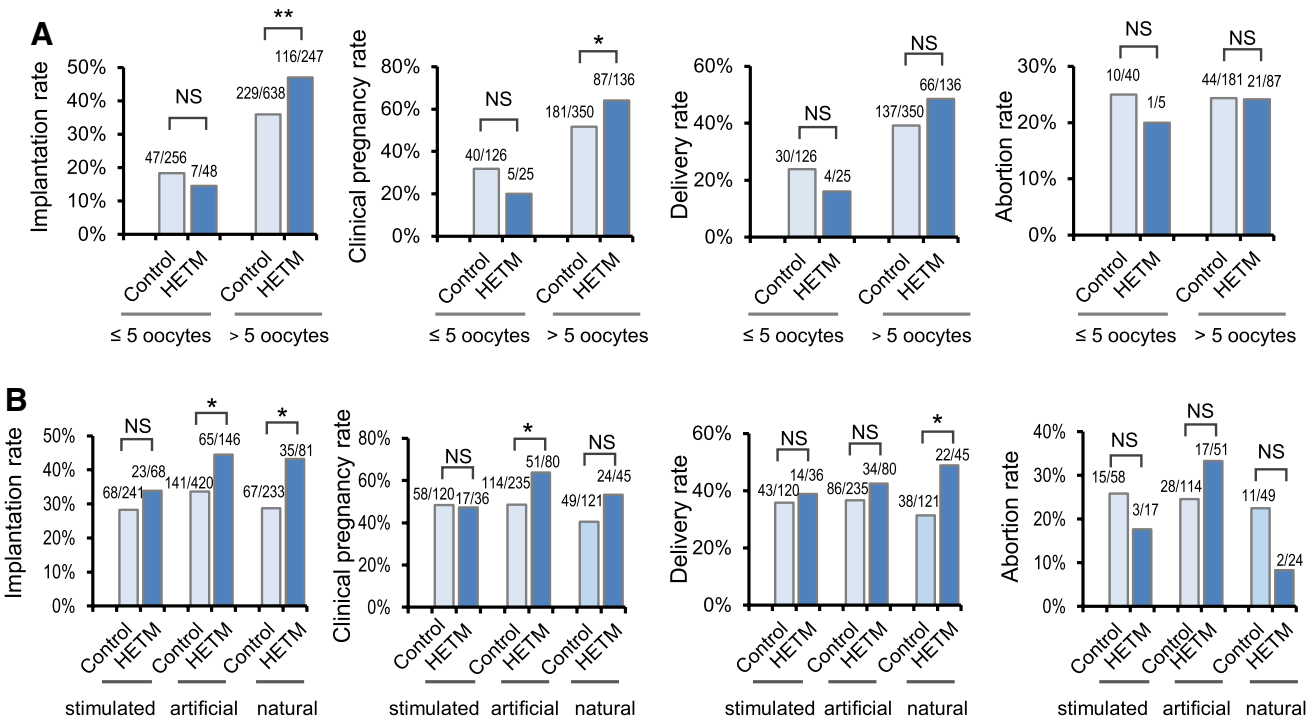

Fig. 2 Comparison of clinical outcome of IVF-ET cycles using HETM and control embryo transfer medium based on the subgroups of number of retrieved oocytes (**a**), and endometrial preparation methods (**b**). *NS* not significant; *$P < 0.05$, **$P < 0.01$

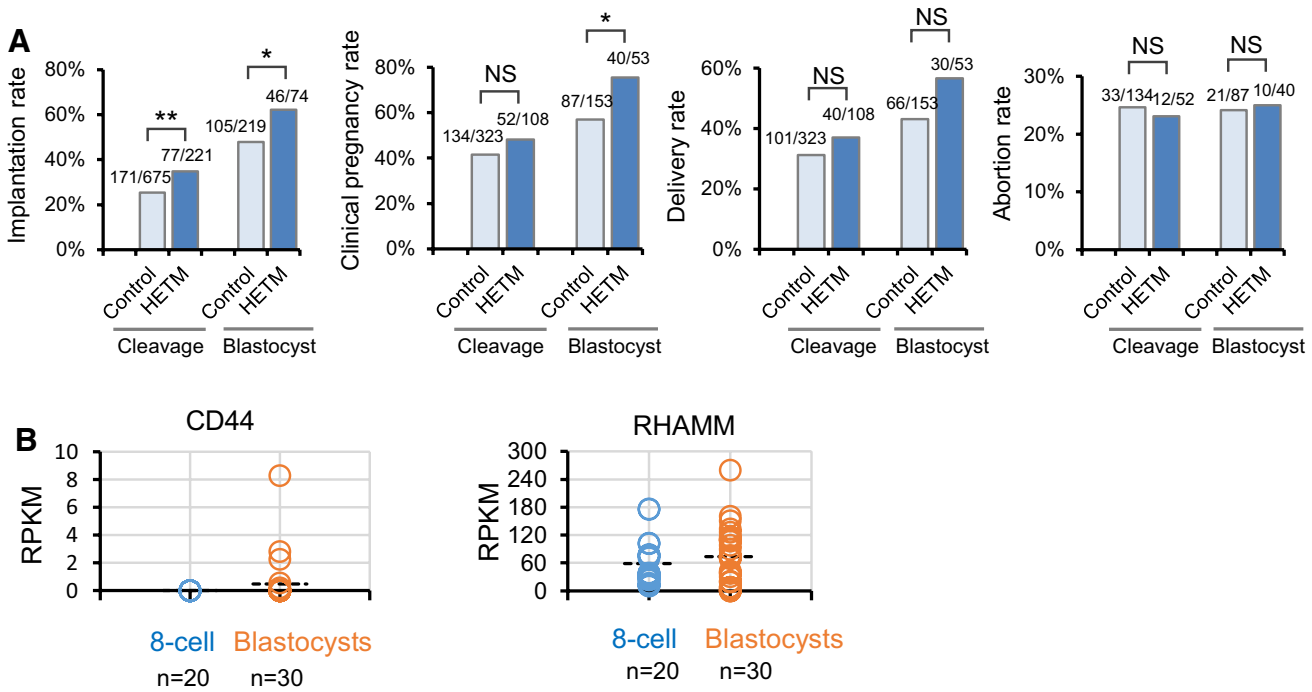

Fig. 3 Comparison of clinical outcome of IVF-ET cycles using HETM and control embryo transfer medium based on the subgroups of developmental stages at which embryos were transferred (**a**). Expression levels of hyaluronan receptors CD44 and RHAMM in human 8-cell embryos and blastocysts (**b**). Expression levels are measured by RPKM (reads per kilobase per million reads) from single-cell RNA-Seq data of human preimplantation embryos. *N* the number of tested blastomeres at each stage. *NS* not significant; $^{*}P<0.05$, $^{**}P<0.01$

and blastocyst stage embryos had different responses to HA. To address this question, we reanalyzed the previously published single-cell RNA-Seq data of human preimplantation embryos [29]. By comparing the expression levels of two well-known HA receptors in cleavage stage and blastocyst stage embryos, we found that both *CD44*, the primary HA receptor, and the receptor for hyaluronan-mediated motility (*RHAMM,* also known as intracellular hyaluronic acid-binding protein, *IHABP*), showed higher expression level in blastocysts (Fig. 3b).

Next, we asked if the quality of the transferred embryo affected the efficacy of HETM usage. We divided patients into subgroups according to number of good-quality embryos transferred. In the patients in whom ≥ 2 good-quality embryos (G1 or G2 embryos) were transferred, the implantation rate (42.1% vs. 30.9%, $P<0.01$) was significantly enhanced in the HETM group, but the improvement in clinical pregnancy rate and delivery rate was not significant. In patients in whom < 2 good-quality embryos were transferred, HETM use did not result in significant improvements (Fig. 4a).

In our clinical practice, we noticed that the duration of pre-equilibration treatment may be critical for the efficacy of HETM application. Compared with the recommended procedure (0–60 min), prolonged pre-equilibration treatment (> 60 min) led to a significant decrease in the implantation rate (23.9% vs. 52.8%, $P<0.01$), clinical pregnancy rate (34.5% vs 69.9%., $P<0.01$), and delivery rate (25.9% vs. 53.4%, $P<0.01$), all of which were comparable to those using conventional transfer medium (Fig. 4b).

Effect of HETM on neonatal characteristics

Sex ratio at birth, which is defined as proportion of males in all live births [30, 31], is an important indicator of reproductive health, and its skewing reflects disturbed embryonic development. In our study, there were no significant differences in the sex ratio at birth in both the HETM (46.1%) and control (51.7%) groups, with the expected natural ratio of 50% (Fig. 5a).

In addition, neonatal body length was similar between the HETM and control groups (49.5 ± 2.5 cm vs. 49.4 ± 2.95 cm, $P>0.05$) (Fig. 5b). Neonatal body weight was also comparable between the HETM and control groups (3122.5 g vs. 3109.5 g, $P>0.05$) (Fig. 5c).

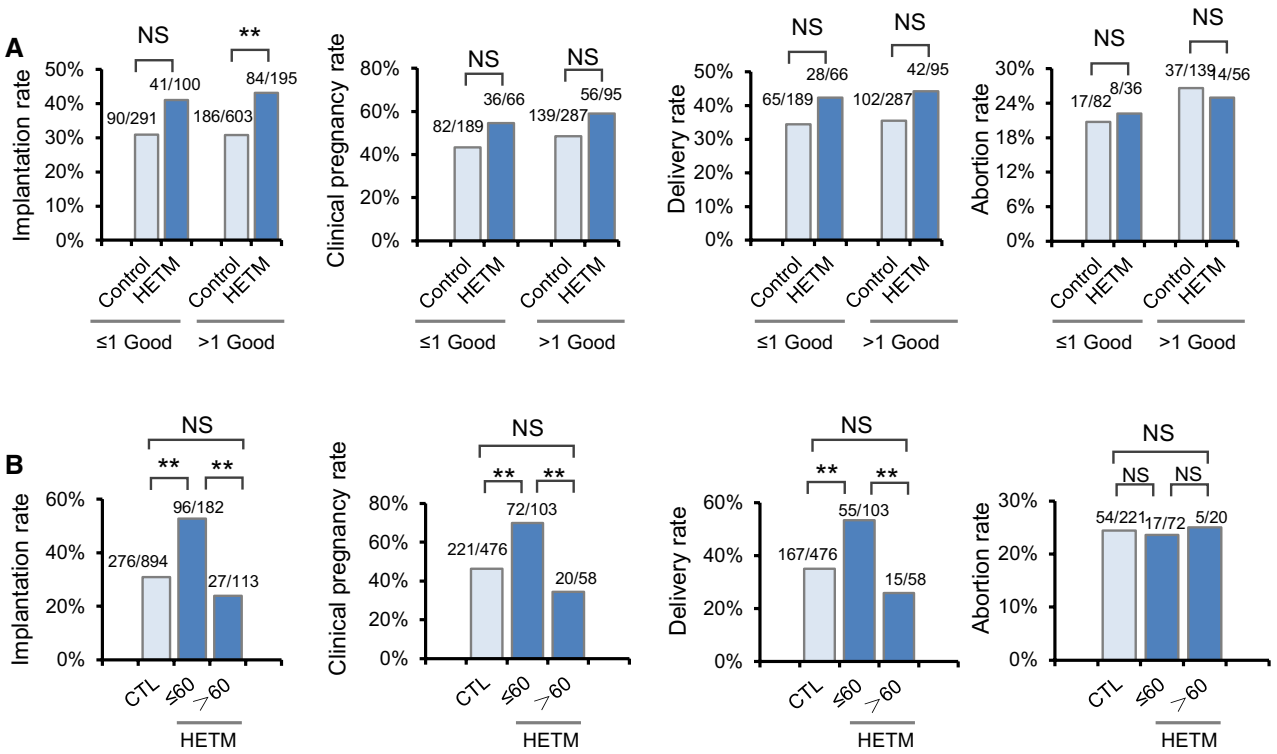

Fig. 4 Comparison of clinical outcome of IVF-ET cycles using HETM and control embryo transfer medium based on the subgroups of quality of transferred embryos (**a**), and duration of pre-equilibration (**b**). *NS* not significant; $*P<0.05$, $**P<0.01$

Effect of HETM application on the outcome of PGS assessment

Finally, we tested the reciprocal effect of HETM usage and PGS assessment on the implantation and pregnancy rates after IVF-ET. Our data showed that pre-equilibration with HETM did not significantly affect the implantation and pregnancy rates of PGS-coupled IVF-ET cycles. On the other hand, our data suggested that PGS-based preselection can facilitate the HETM-induced beneficial effects on the implantation rate but not on the pregnancy rate (Fig. 6). These observations suggest that PGS assessment and HETM pre-equilibration do not have any synergistic effects on the IVF outcome.

Discussion

Until now, implantation remains to be a major limiting factor for success rate after IVF-ET. It is generally accepted that HETM offers several advantages improving the IVF outcome; however, the efficacy of IVF after using HETM remains largely inconclusive, especially in specific populations. Together with our own data, results from previous studies suggest that the benefits from HETM usage may largely depend on the maternal and embryonic status or on treatment procedures. These factors, which are associated with embryonic potential or uterine receptivity, are systematically evaluated in the present study.

Our data demonstrated that maternal factors essentially influence the efficacy of HETM usage. Among the tested maternal factors, maternal aging is thought to be a convincing predictor of poor IVF-ET outcome [32]. Aging is a well-accepted risk factor for both natural and IVF conceptions, and it is well known to compromise oocyte/embryo development and endometrial receptivity. In our study, when conventional transfer medium was used, maternal aging was associated with the most significant adverse effects on IVF-ET outcome, including the implantation, clinical pregnancy, and delivery rates, which decreased by approximately 20% in aging patients. In the present study, HETM usage had evident benefits on the IVF outcome in young patients, but did not show any positive effect in aging populations. Our observation is in accordance with previously published results [22, 23], but absolutely contradicts with the report of Urman et al., in which it was found that the beneficial effect from HETM was specific to aging patients but not to young patients [19].

In the present study, approximately 24% of our tested patients had ≤ 5 collected oocytes, which is an indicator of poor ovarian response [33, 34]. Fewer retrieved oocytes is thought to be highly associated with poor IVF outcome [35].

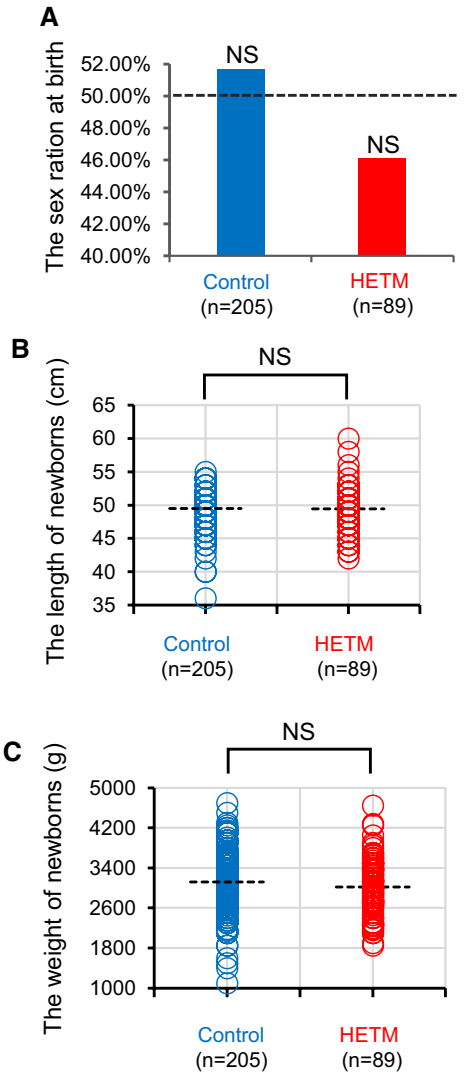

Fig. 5 Effect of HETM on neonatal characteristics. The sex ration at birth is reveal by male birth rate (**a**), NS means not significant with expected natural ratio of 50% (dashed line). The length (**b**) and weight (**c**) of newborns were revealed by scatter plots (blue or red cycles) and average values are presented as dashed lines. *NS* not significant

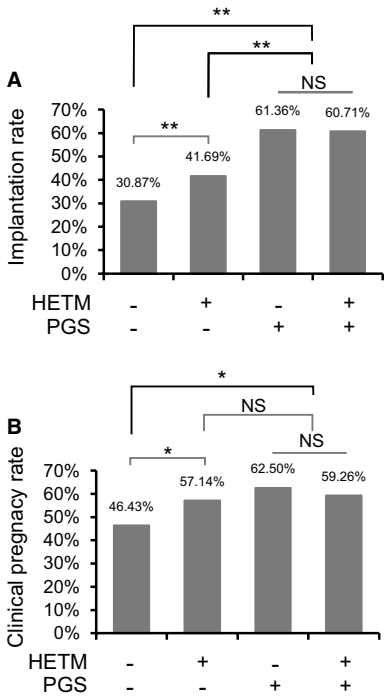

Fig. 6 Analysis of reciprocal effect of HETM usage and preimplantation genetic screening (PGS) assessment on the implantation (**a**) and pregnancy rates (**b**) after IVF-ET. *NS* not significant; $*P<0.05$, $**P<0.01$

Our data also showed that when using conventional transfer medium, the implantation, clinical pregnancy, and delivery rates decreased significantly by approximately 18, 20, and 16%, respectively, when fewer oocytes were retrieved in comparison to when a higher number of oocytes were retrieved. HETM facilitated implantation and pregnancy establishment in patients with > 5 collected oocytes, but not in patients with ≤ 5 collected oocytes. This result is similar to those reported by Fancsovits et al., in which HETM in patients with ≤ 3 oocytes was associated with no significant benefits [23]. Further, it must be noted that in the ≤ 5 collected oocytes group, HETM usage even appeared to show an adverse effect. Despite the lack of statistical significance, decreases in the clinical pregnancy, implantation, and delivery rates of 10, 12, and 7%, respectively in the ≤ 5 collected oocyte group following HETM treatment can be regarded as clinically notable.

In our study, approximately 50% of the patients had PIF after IVF-ET. At least in our study population, unlike maternal aging and few retrieved oocytes, PIF did not appear to be a severe risk factor for IVF outcome. However, the efficacy of HETM usage in patients with or without PIF was distinct: HETM usage obviously enhanced the implantation, clinical pregnancy, and delivery rates (increased by 10% but not statistically significant) in patients without PIF, but HETM usage did not increase any of these parameters in patients with PIF. In previous studies, the efficacy of HETM usage in patients with PIF is largely inconclusive. In the studies of Urman et al. and Nakagawa et al., HETM significantly improved the IVF outcome in patients with PIF, even in patients with a history of multiple (≥ 4) implantation failures [19, 25, 36]. In particular, a more recent study based on a large population, demonstrated that the beneficial effect of HETM usage is specific to patients with repeated PIF (≥ 3); unexpectedly, HETM treatment lowered the IVF outcomes among patients with first or second PIF [37]. However, similar with our finding, other studies reported no any advantages of using HETM in patients with PIF [23, 38, 39]. We speculate that this large diversity in results may be

due to the basic IVF outcome in patients with PIF, wherein the implantation and clinical pregnancy rates varied from approximately 10–30% and 15–48% among studies, respectively. In addition, synergistic effect among the presence/absence of PIF, and other uncertain factors that differed among different studies, may contribute essentially to the IVF outcome. Thus, our data of patients with PIF, together with results from subgroups of different maternal age and number of retrieved oocytes, implies that maternal IVF risk factors, especially those considered severe, may significantly attenuate the beneficial effects of HETM.

Cycle regimens used for endometrial preparation are critical for the outcome of frozen-thawed ET cycles. Currently, no single method of endometrial preparation has been identified as being more effective for cryopreserved-thawed ET [40]. Accordingly, our data showed that when using conventional transfer medium, three methods of endometrial preparation produced comparable IVF-ET outcomes. This observation is in accordance with that reported by previous studies [41–43]. In contrast, when using HETM, the IVF-ET outcome by different endometrium preparation methods is more distinct: both artificial and natural cycles offer several advantages; however, the HETM usage for the patients with stimulated cycles should be done with caution. Finally, highlighting the effect of different endometrial preparation methods on the efficacy of HETM, our results suggested that benefits of HETM usage seems to be associated with artificial or natural cycles, but not stimulated cycles. To the best of our knowledge, no previous studies have studied whether endometrial preparation methods influence the efficacy of HETM treatment on IVF outcomes. Collectively, our analyses suggest that maternal factors contribute essentially to the IVF-ET outcomes after using HETM, especially the advantaged maternal factors, such as younger age, absence of PIF, higher ovarian response, would be associated a satisfying outcome after IVF-ET treatment.

In addition to maternal factors, embryonic status or treatments also substantially influence the efficacy of HETM usage. Our data reveal that various developmental stages at which ET is carried out is a critical factor influencing IVF outcome: blastocyst stage ET displayed a notable advantage over cleavage stage ET with both conventional transfer medium or HETM. In addition, HETM was capable of improving the implantation, clinical pregnancy, and delivery rates following blastocyst transfer, to 62.2, 75.5 and 56.6%, respectively, which was the highest level in all tested subgroups. In contrast, when HETM was used in cleavage stage ET, no benefits were observed. This finding, together with similar results by previous studies [19, 22, 44], further supports the idea that the developmental stage of ET is a critical determinant of the outcome after HETM. One possible explanation for this finding may be that blastocysts express the HA receptors CD44 and RHAMM at higher levels, as a result of which they are more responsive to HA stimulation. Moreover, the number of transferred high-quality embryos did not appear to be a limiting factor for the efficacy of HETM treatment. It is possible that the potential disadvantage of poor-quality embryos may be compensated by increasing the number of embryos transferred per patient.

The outcomes associated with HETM treatment are more or less dependent on the duration of pre-equilibration treatment. Clinical outcomes after prolonged pre-equilibration appear to be lower than those when using conventional transfer medium. We did not find any published evidence supporting the occurrence of adverse effects following high concentrations or prolonged use of HA treatment in any cell types, and a previous study also indicated that HA is not cytotoxic and shows good biocompatibility [45]. Despite this, our data suggested that prolonged pre-equilibration treatment must be avoided in the clinical usage of HETM. To the best of our knowledge, our data is the first report to evaluate the reciprocal effect of HETM and PGS-based preselection on IVF outcomes. HETM does not improve the IVF-ET outcome after PGS assessment. However, on the other hand, when using HETM, embryos preselected by PGS had a higher implantation potential than control group. This fact suggests that HETM may help improve the implantation rate among the patients prone to aneuploidy or embryos with genetic defects. Together, these observations imply that similar with maternal factors, advantaged embryonic factors, such as blastocyst transfer, genetic preselection, may benefit the IVF-ET outcomes.

Thus far, the exact mechanism underlying HA-facilitated embryo implantation remains undetermined. In addition to improving embryo apposition and attachment by increasing high viscosity environments, HA was thought to facilitate embryo implantation via its receptors CD44 [46] and RHAMM [47]. Furthermore, the interaction between CD44 and receptors of other growth factors such as TGFβ [48] and EGF [49] may be also involved. Notably, the maternal factors tested in our study, such as maternal aging and low number of retrieved oocytes, will affect both embryo potential and uterine receptivity, which are likely to adversely affect HETM efficacy. These results imply that the HA-induced beneficial effect may be facilitated by improving embryo–endometrium cross-talk but not by improving the embryo or endometrium alone.

Taken together, the results from our tested populations suggest that HETM application further improves the outcome of advantaged IVF cycles, e.g., young maternal age, absence of PIF, >5 collected oocytes, higher number of good-quality embryos transferred, and preselection by PGS assessment. In addition, we also demonstrated the treatment procedures that influence IVF-ET outcomes, such as selection of developmental stage for transfer, endometrial preparation, and the pre-equilibration period, which are of prime

importance in improving IVF-ET outcomes. In contrast, our study indicates that HETM should be used with caution in IVF cycles with disadvantaged conditions. Finally, it must be mentioned that some conclusions in the present study should be drawn with caution because of the limited sample size in these subgroups of HETM usage, and the possible confounders cannot be excluded for the comparisons in the subgroups with limited sample size.

Acknowledgements This work was supported by grants from the PLA Key Research Program (no. 17JS004).

Author contribution WW and LR: Data collection, analysis, and manuscript writing; DW: Data collection and analysis; YS, BL, XW, FC: IVF/ICIS procedures; ML, LY: Embryo transfer and PGS; WS and ZF: Project development and experimental design.

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