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RESEARCH

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Second ejaculation produces good quality sperm and blastocyst and decreases the rate of unexpected ICSI cycle: a propensity score-matched analysis

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Abstract

Background Second ejaculation can influence sperm quality which may define the first-line treatment. The purpose of this study was to evaluate the effectiveness of a second ejaculation in decreasing the unexpected intracytoplasmic sperm injection (ICSI) rate by a propensity score-matched (PSM) analysis.

Methods Patients who were projected to undergo IVF were included between January 2016 and November 2021 in this monocentric, retrospective analysis. 2782 patients included in the study, 143 and 2639 patients were non-randomized in the unexpected ICSI and IVF groups, respectively. One hundred fourteen patients with unexpected ICSI produced two semen samples on the day of ovum pick-up. After 1:4 PSM, we matched 61 patients in the second ejaculation IVF group to 238 patients in the conventional IVF group. Outcomes of sperm quality, fertilization rate, embryo quality, and pregnancy were compared.

Results Second ejaculation significantly improved sperm concentration, progressive motility before and after sperm swim-up, total progressive motility sperm count after swim-up, and decreased sperm DNA fragmentation (SDF). Sixty-one of 114 (53.5%) unexpected ICSI couples had enough total progressive motility sperm for IVF with the second ejaculation. There were no differences in basic clinical characteristics between couples in second ejaculation IVF and matched-conventional IVF group. For the two groups, no differences were observed in IVF outcomes. However, a significant increase in good-quality blastocyst rate was observed for second-ejaculation IVF couples. Univariate and multivariate linear regression analysis also confirmed that the second ejaculation was an independent risk factor for the good quality blastocyst rate.

Conclusion Second ejaculation could be an economical and secure alternative to get good quality sperm, and blastocyst and decrease the rate of unexpected ICSI. Multicenter studies should be conducted to confirm the potential advantages of using second ejaculation IVF in effectively reducing the rate of ICSI.

Keywords Second ejaculation, ICSI, Sperm quality, PSM, Good quality blastocyst

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Background

The semen quality can be affected considerably by abstinence times and will define the first-line treatment options for infertility couples undergoing assisted reproductive technologies. Studies have evaluated that the duration of abstinence affects both conventional sperm

parameters (volume, sperm concentration, motility, and morphology) and SDF [1–3]. It is well-accepted that semen volume and sperm concentration will increase with prolonged abstinence, but such abstinence can have a negative impact on motility and viability. Likewise, ejaculates from men with oligozoospermia exhibited a significant improvement in sperm motility, progression, and morphology when a second ejaculate was produced within only 40 min of the first [4]. Additionally, when a second ejaculation was collected within 1 to 3 h of the first ejaculation, the second ejaculation of men showed a significant enhancement in terms of total motility sperm count [4, 5]. Second ejaculation may reduce the use of ICSI by providing more available good-quality spermatozoa. Although many studies have found the impact of second ejaculation on sperm quality, further research is still needed to determine whether second ejaculation can provide sufficient progressive motility sperm for IVF in unexpected ICSI patients.

ICSI was originally applied to overcome the most severe forms of male factor infertility [6]. Nowadays, the use of ICSI for patients with borderline or even normal semen parameters has become more common [7]. Despite the widespread use of ICSI, evidence to support the use of ICSI in different infertility cases is still debatable. Recently, studies found that ICSI did not improve the overall clinical outcomes compared with IVF in the treatment of non-severe male and non-male factor infertility [8, 9]. As an invasive operating technique, ICSI may affect the decondensation of spermatozoa and may disrupt the oocyte meiotic spindle during the spermatozoon injection into the oocyte cytoplasm [10–12]. Furthermore, ICSI may be associated with increased risk of autism, intellectual impairment and genitourinary malformation in offspring than IVF-conceived children [13, 14]. In addition, compared with conventional IVF, ICSI may need the increased required laboratory experience, resources, effort, and time [7]. Given the effectiveness, safety, and costs of ICSI, we should follow the sperm characteristics of Chinese expert consensus on ICSI patients [15].

Moreover, it is noteworthy that SDF can be significantly diminished by short-term ejaculation [16]. Compromised spermatozoa quality varies with different sexual abstinence and may influence SDF as well as pregnancy outcomes [2, 17]. A recent sperm proteome study has proposed that the molecular events occurring in sperm proteins may play an important role in sperm quality and reproductive potential after reduced periods of male ejaculatory abstinence [18]. Therefore, how to correctly evaluate the outcomes of second ejaculation IVF would contribute to reasonably judge the effectiveness of second ejaculation IVF in reducing the use of ICSI.

Second ejaculation was requested from patients who presented unexpected ICSI on the day of treatment. We compared semen parameters between the first and second samples with the aim of verifying if the second ejaculation could be a good strategy for obtaining enough progressive motile sperm for IVF. Furthermore, outcomes of fertilization, embryo quality, and pregnancy in second ejaculation IVF patients, were compared with propensity score matched-conventional IVF patients who produced a single semen sample, in order to evaluate the effectiveness of second ejaculation IVF.

Methods

Study design and participants

In this retrospective single-center study, we obtained data from 2782 infertile couples who were projected to undergo IVF at our hospital between January 2016 and November 2021. Patients with severe oligozoospermia (concentration < 1×10^6/mL), severe asthenozoospermia (progressive motility < 1%), and other ICSI indications [15] were excluded. All procedures were performed in accordance with the ethical standards of the ethics committee of our hospital (No: LL-KY-ZC-2021-02) and with the 1964 Declaration of Helsinki and its later amendments. The first ejaculation after swim-up was checked on the day of ovum pick-up, 143 unexpected ICSI patients were found with the sperm under the conventional ICSI criteria (progressive motility < 1 × 106). 114 of them refused to undergo ICSI and provided a second semen sample 1–3 h after the first semen collection. Patients with a total progressive motile sperm count enough for IVF of the second ejaculation were included in the second ejaculation IVF group (n = 61). Patients with traditional IVF were assessed as the conventional IVF group (n = 2639). A flow chart for patient recruitment is shown in Fig. 1.

PSM

As the second ejaculation was not randomized, to reduce the differences in baseline characteristics of the patient, a propensity score was used to adjust for potential selection or predisposition bias. We calculated PSM analyses using a logistic regression model by R version 4.3.1 software. These covariates included female age, male age, number of oocytes retrieved, ovarian stimulation protocol, infertility types, concentration after swim-up, progressive motility after swim-up and total progressive motile sperm count after swim-up based on prior knowledge. After the propensity scores were estimated, patients were selected by 1:4 nearest neighbor matching including replacement and a caliper of 0.2.

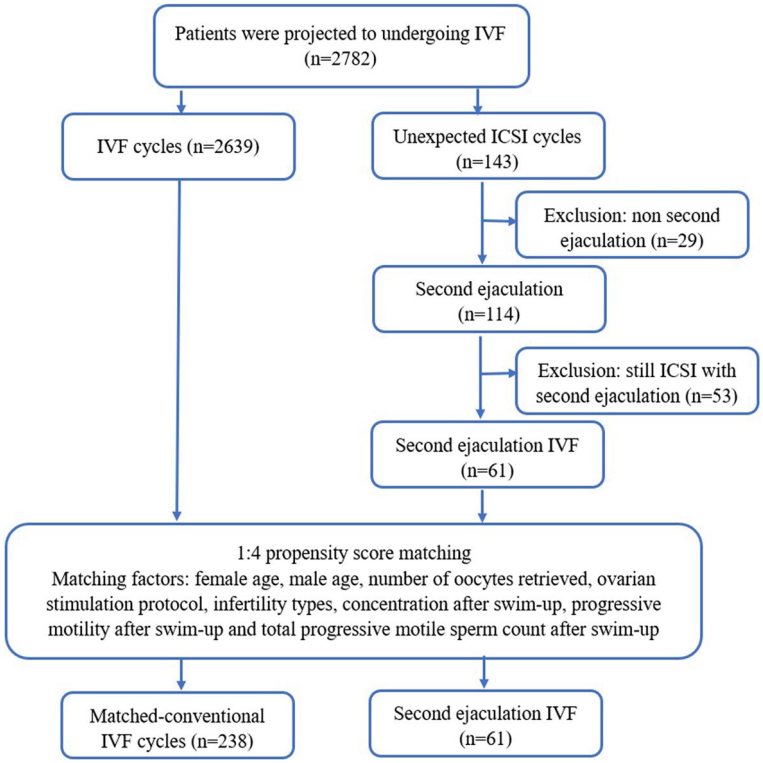

Fig. 1 A flow chart for patient recruitment

Semen handling and analysis

Semen samples were obtained in a private room by masturbation into a sterile wide-mouthed plastic container. After liquefaction at 37 °C for 30 min, the samples were assessed under an inverted microscope at × 200 magnification. Semen analysis was performed according to the fifth edition of the World Health Organization guidelines and included sperm concentration and motility. A Sperm Class Analyzer CASA system (Microptic S.L., Barcelona, Spain) was used to assess sperm concentration and motility. Motile sperm fractions were isolated after density gradients and the "swim-up" process. Morphology was assessed by smearing 10 μL of the semen samples and subsequently washed and fixed using MGG Quick Stain (Bio-optica, Milan, Italy). The SDF was assessed by the sperm chromatin dispersion test using the Halosperm kit (Halotech DNA S.L., Madrid, Spain).

Ovarian stimulation protocols

When more than three follicles were larger than 18 mm in diameter, a dose of 5000–10,000 IU human chorionic gonadotropin was injected intramuscularly. After 36–40 h oocytes were retrieved under ultrasonographic guidance. According to clinical indications, the patient underwent conventional IVF 3–5 h after oocyte retrieval. When the amount of second polar bodies released from mature oocytes was less than 30%, the culture was continued for more than 2 h and then the second PB exposure was checked again. When < 50% of the mature oocytes were exposed to the second PB, rescue ICSI was carried out immediately for the oocytes with only one PB. The operation of rescue ICSI was like that of conventional ICSI.

Embryo assessment and embryo transfer protocols

All oocytes were cultured in a culture medium with 6% CO_2, 5% O_2, and 89% N_2 at 37 °C. Conventional morphology, cell count, cleavage patterns, and degree of fragmentation scores of the embryos were determined. To minimize operator-dependent variations, especially in blastocyst annotation, two embryologists were specifically trained, and annotation was performed according to the published guidelines. Good quality embryos were defined as having 8 to 10 cells, no embryo fragmentation, and cells that were very even, regular, and similarly sized.

Fresh embryo transfer was performed on day 3 or day 5 based on the number of embryos available and their quality according to clinic protocol. Intramuscular progesterone, 50 mg per day, was begun on the day after oocyte retrieval for those undergoing a fresh transfer and continued until 10 weeks of gestation if conception occurred.

Confirmation of pregnancy

If β-hCG was detected in the blood 14 days after egg collection, the trace test after 1 week showed a continuous increase, and vaginal ultrasonography performed at 6 to 7 weeks of pregnancy showed a gestational sac, then clinical pregnancy was verified. The implantation rate was calculated as the percentage of embryonic sacs implanted in the uterus that became implanted embryos. Miscarriage was defined as a fetal loss prior to 12 weeks of gestation despite the presence of a gestational sac in the first-trimester ultrasound.

Statistical analysis

The results are expressed as mean ± standard deviation or proportions (%). Student's *t* test was used to compare the clinical baseline data of continuous variables between the two groups, and the Mann–Whitney *U* test was used to compare continuous variables with a skewed distribution. The chi-square test was used to compare categorical data between groups. Univariate and multivariate linear regression were performed to identify independent risk factors for clinical outcomes. All statistical tests were two-sided, and values were considered significant at $P < 0.05$. All statistical analyses were performed using the statistical package SPSS V.22.0 (IBM Co., Armonk, NY, USA).

Results

One hundred fourteen patients were treated with two ejaculates of unexpected ICSI couples. The sperm parameters of two ejaculates were compared. There were significant differences in semen volume, concentration, total sperm count, progressive motility, SDF, and both concentration, progressive motility, total progressive motility sperm count, SDF after swim-up between the two ejaculates (all $P < 0.05$), but no significant differences were found in total progressive motile sperm count, sperm morphology, and sperm morphology after swim-up ($P = 0.588$, $P = 0.355$, $P = 0.112$, respectively). Sixty-one of 114 (53.5%) unexpected ICSI couples had enough total progressive motility sperm with second ejaculation for IVF (Table 1).

Comparisons of the basic characteristics between the second ejaculation IVF and conventional IVF before and after PSM are shown in Table 2. The PSM analysis was performed to reduce the inter-group imbalances in the baseline data (Fig. 2). Before PSM, the concentration ($P < 0.001$) and total progressively motile sperm count after swim-up ($P < 0.001$) were significantly lower in second ejaculation IVF group than in conventional IVF group. After 1:4 PSM, we matched 61 cases in the second ejaculation IVF group to 238 controls in the matched-conventional IVF. There were no significant differences in any of the baseline characteristics between the two groups (all $P > 0.05$).

The clinical outcomes between the second ejaculation IVF group and the matched-conventional IVF group are shown in Table 3. No significant differences in terms of 2PN fertilization rate, total fertilization rate, rescue ICSI rate, cleavage rate, good quality embryo rate on day 3, blastocyst formation rate, and clinical outcomes were observed between the two groups (all $P > 0.05$). There was a higher good-quality blastocyst rate in the second ejaculation IVF group compared with matched-conventional IVF groups (35.1% *vs.* 26.1%, $P = 0.026$).

To analyze the risk factors associated with the good quality blastocyst rate, univariate and multivariate regression linear analyses were conducted (Table 4). The univariate analysis showed that second ejaculation (coefficient, 0.173; SE, 0.054; $P = 0.014$) and secondary

Table 1 Comparison of sperm parameters between the two ejaculates of unexpected ICSI couples

Parameters	1st	2nd	*P*
Volume	2.00 (1.50,3.00)	1.0 (0.80,2.00)	<0.001
Concentration (million/mL)	14.65 (10.00,24.70)	21.70 (10.15,39.25)	0.038
Total sperm count (million)	35.05 (16.45,60.13)	22.50 (14.68,39.10)	0.009
Progressive motility (%)	13.00 (5.05,24.30)	21.55 (11.75,32.85)	0.001
Total progressive motile sperm count (million)	3.99 (1.34,8.19)	4.32 (1.45,9.50)	0.588
Morphology (%)	1.00 (1.00,2.00)	1.00 (1.00,3.00)	0.355
SDF	39.50 (26.00,59.00)	28.00 (17.00,42.00)	0.014
Concentration after swim-up (million/mL)	1.70 (0.95,2.50)	2.90 (1.05,5.40)	0.003
Progressive motility after swim-up (%)	69.10 (58.30,78.70)	73.10 (59.00,86.80)	0.003
Total progressive motile sperm count after swim-up (million)	0.67 (0.31,1.08)	1.04 (0.38,2.38)	< 0.001
Morphology after swim-up (million) (%)	1.0 0 (1.00,2.00)	1.50 (1.00,2.00)	0.112
SDF after swim-up (million)	8.00 (5.00,12.00)	5.00 (3.00,8.00)	0.005
Total progressive motile sperm count enough for IVF (%)	0/114 (0.0)	61/114 (53.5)	/

Table 2 Baseline patient characteristics in second ejaculation IVF and conventional IVF before and after propensity score matching

Baseline characteristics	Second ejaculation IVF $n = 61$	Conventional IVF $n = 2639$	*P* value	Matched-conventional IVF $n = 238$	*P* value
Female age	33.50 ± 5.09	34.31 ± 7.71	0.413	33.26 ± 4.56	0.712
Male age	36.10 ± 5.56	35.92 ± 5.94	0.823	35.68 ± 5.87	0.617
Number of oocytes retrieved	10.00 (4.00, 13.00)	9.00 (5.00,13.00)	0.591	8.00 (5.00, 12.00)	0.710
Ovarian stimulation protocol *n* (%)					
Long-acting protocol	11 (18.0)	509 (19.3)	0.096	59 (24.8)	0.307
Short-acting protocol	29 (47.5)	913 (34.6)		89 (37.4)	
Others	21 (34.4)	1217 (46.1)		90 (37.8)	
Primary infertility (%)	25 (41.0)	988 (37.4)	0.572	92 (38.7)	0.740
Secondary infertility (%)	36 (59.0)	1651 (62.6)		146 (61.3)	
Concentration after swim-up (million/mL)	5.25 (3.75, 8.60)	20.40 (9.90, 37.40)	< 0.001	5.20 (3.30, 9.90)	0.904
Progressive motility after swim-up (%)	86.65 (76.40, 93.15)	88.90 (79.50, 94.20)	0.109	83.85 (72.30, 91.30)	0.203
Total progressive motile sperm count after swim-up (million)	2.24 (1.52, 2.94)	8.67 (4.06, 16.32)	< 0.001	2.13 (1.30, 3.55)	0.777

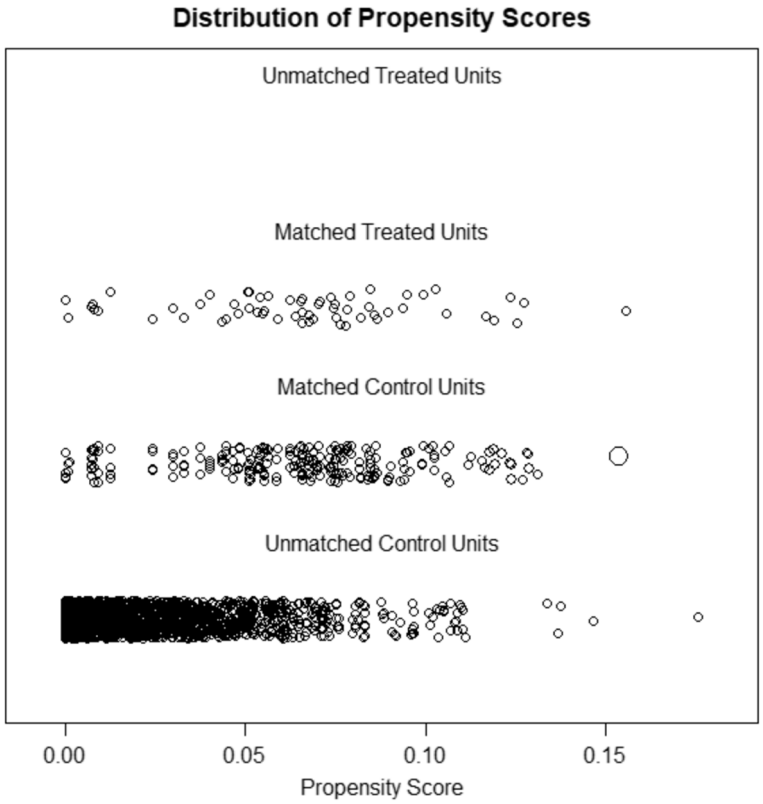

Fig. 2 Distribution of propensity scores in the second ejaculation IVF, conventional IVF, and matched-conventional IVF groups

infertility (Coefficient, 0.152; SE, 0.043; $P = 0.031$) were related to the good quality blastocyst rate. Further multivariate analysis confirmed that second ejaculation (coefficient, 0.168; SE, 0.055; $P = 0.020$) was an independent risk factor for the good quality blastocyst rate.

Discussion

Despite the wide use of ICSI, its effectiveness in patients with non-severe male infertility is still debatable. The semen fluid characteristics on the day of ovum pick-up with male infertility patients create an increase in

Table 3 Embryological and clinical outcomes comparison between the second ejaculation IVF and matched-conventional IVF

Variable	Second ejaculation IVF	Matched-conventional IVF	*P* value
2PN fertilization rate	324/559 (58.0)	1318/2158 (61.1)	0.180
Total fertilization rate	434/559 (77.6)	1659/2158 (76.9)	0.703
Rescue ICSI rate	3/61 (4.9)	24/238 (10.1)	0.316
Cleavage rates	423/434 (97.5)	1632/1659 (98.4)	0.208
Good-quality embryo rate on day 3	165/320 (51.6)	595/1298 (45.8)	0.066
Blastocyst formation rate	148/229 (64.6)	652/936 (69.7)	0.141
Good-quality blastocyst rate	52/148 (35.1)	170/652 (26.1)	0.026
Biochemical pregnancy	17/23 (73.9)	68/126 (31.0)	0.168
Clinical pregnancy rate	19/46 (41.3)	78/252 (52.2)	0.382
Implantation rate	16/23 (66.7)	62/126 (49.7)	0.117
Live birth rate	13/23 (56.5)	51/126 (40.5)	0.153
Miscarriage rate	3/23 (13.0)	8/126 (6.3)	0.259

Table 4 Univariate and multivariate linear regression analyses of risk factors for good quality blastocyst rate

Factor	Univariate Coefficient	SE	*P* value	Multivariate Coefficient	SE	*P* value
Second ejaculation	0.173	0.054	0.014	0.168	0.055	0.020
Female age (years)	− 0.088	0.005	0.218	0.019	0.007	0.854
Male age (years)	− 0.092	0.004	0.196	-0.094	0.005	0.333
Number of oocytes retrieved	− 0.012	0.004	0.863	-0.038	0.004	0.606
Ovarian stimulation protocol)						
Long-acting protocol						
Short-acting protocol	0.111	0.052	0.203	0.085	0.053	0.335
Others	0.098	0.057	0.259	0.071	0.058	0.423
Primary infertility						
Secondary infertility	0.152	0.043	0.031	0.124	0.047	0.108
Concentration after swim-up	− 0.004	0.002	0.958	0.772	0.025	0.473
Progressive motility after swim-up	− 0.080	0.002	0.260	-0.017	0.003	0.893
Total progressive motile sperm count after swim-up	− 0.013	0.004	0.851	-0.771	0.056	0.479

unexpected ICSI. Most studies have demonstrated an improvement in sperm quality following second ejaculation [1, 2, 4, 16, 17]. However, only a few studies have focused on whether a second ejaculation can offer enough spermatozoa with progressive motility for IVF [19]. We compared the sperm quality between the two ejaculates. Then we comprehensively analyzed the clinical outcomes of the second ejaculation IVF and matched-conventional IVF. We showed that the second ejaculation could produce good quality sperm and blastocyst with a lower rate of application of ICSI in unexpected ICSI couples.

In the present study, we performed semen analysis between the first and second ejaculations. There was higher concentration, progressive motility, total progressive motility sperm count after swim-up, and lower SDF in the second ejaculation. Interestingly, about 53.5% of unexpected ICSI patients with second ejaculation were able to obtain sufficient progressive motility sperm for IVF. As is well known, ICSI cannot improve postfertilization reproductive outcomes compared with conventional IVF [20, 21]. Second ejaculation would allow us to increase the number of motile spermatozoa available to undergo IVF in cases of poor sperm for unexpected ICSI patients. Similar to our study, studies have indicated that an increase in motility and a decrease in SDF are associated with second ejaculation [16, 17]. It is not clear why the second ejaculation with a short abstinence period was associated with higher-quality sperm. Human spermatozoa are produced in the seminiferous

tubules and are stored in the epididymis. One reasonable explanation might be that the second ejaculation is associated with shorter storage time in the epididymis and lower oxidative stress. Shortening exposure to reactive oxygen species arising from dead spermatozoa and leukocytes in the epididymis [22, 23] will lead to an increase in sperm quality and a decrease in SDF with second ejaculation. During epididymal transit, a series of elaborate interactions between spermatozoa and epididymal secretions occurs [24], which may influence flagellar beating and sperm motility [25]. Moreover, a bioinformatics analysis showed the differentially expressed proteins were enriched in sperm motility [18]. It may also be explained that spermatozoa play a major role in this process to improve sperm motility after a reduced period of male abstinence.

We detected lower concentration after swim-up and total progressive motile sperm count after swim-up between the ejaculation IVF group and conventional IVF before PSM. Given that sperm concentration and total progressive motile sperm count after swim-up may directly affect fertilization rate and cleavage rate as well as embryo quality, they are often correlated with negative pregnancy outcomes [26–29]. Moreover, many factors can influence the outcomes of IVF. Previous studies showed that female age, male age, number of oocytes retrieved, ovarian stimulation protocol, and infertility types were associated with the terms of fertilization rate, embryo quality rate, embryonic aneuploidy risk, and pregnancy outcomes [30–32]. In order to minimize the selection bias of baseline characteristics for conventional IVF and evaluate the effectiveness of second ejaculation IVF, we carried out PSM to balance all the baseline characteristics.

The current study found no difference in 2PN fertilization, total fertilization, rescue ICSI, cleavage, good quality embryo on day 3, and blastocyst formation rates between the second ejaculation IVF group and the matched-conventional IVF group. The good-quality blastocyst rate was significantly higher in the second ejaculation IVF group. Furthermore, our data showed that biochemical pregnancy, clinical pregnancy, implantation, live birth, and miscarriage rates were still not significantly different in the two groups. ICSI was originally applied to overcome the risk of low or total failed fertilization [33]. Patients with very poor semen characteristics on the day of ovum pick-up usually need to change to ICSI treatment. Although 53 out of 114 men in our study still need to undergo ICSI treatment. However, the second ejaculation had improved the total progressive motility of sperm, 53.5% of them within the range appropriate for IVF treatment. Moreover, the fertilization rate and rescue ICSI of second-ejaculation IVF patients were not different from those of matched-conventional IVF. This means that second ejaculation IVF can serve as an effective fertilization method without the increasing of unexpected ICSI. Furthermore, when evaluating the success of two kinds of IVF techniques, data showed no difference in clinical outcomes between second ejaculation IVF and matched-conventional IVF. These may suggest that second ejaculation is of greatest benefit in unexpected ICSI patients to decrease the application of ICSI, considering the high cost and additional required laboratory experience.

In this study, compared with the matched-conventional IVF group there was an increase in the good-quality blastocyst rate in the second ejaculation IVF group. In addition, both the univariate and multivariate analysis also confirmed that the second ejaculation was a risk factor for the good quality blastocyst rate. Second ejaculation can not only improve sperm quality by reducing the rate of unexpected ICSI but also provide higher-quality blastocysts for patients who have enough progressive sperm to undergo IVF. Our previous time-lapse sibling oocyte study found that significantly higher rate of high-quality blastocysts in the second ejaculation group compared with in the first ejaculation group [34]. A study on 106 sibling biopsied blastocysts also found that sperm with second ejaculation could reduce aneuploidy rate in blastocysts [35]. These studies are based on comparing the clinical outcomes of the first and second ejaculation of sibling oocyte study. Although sibling oocyte studies could minimize the bias caused by female factors, the difference in sperm progressive motility between two ejaculations could affect the outcome of embryonic development. In this study, we applied PSM to reduce the impact of basic parameters on blastocyst development and verified again with univariate and multivariate analysis. Interestingly, despite no difference being found in the progressive motility between the second ejaculation IVF group and matched-conventional IVF, we still found a significant increase in good-quality blastocyst obtained in the second ejaculation IVF group. On the one hand, the epididymis is exposed to a higher oxidative stress environment than elsewhere in the reproductive tract, and the second ejaculation with lower oxidative stress by storing in the epididymis for a shorter time. Many studies have shown that oxidative stress can lead to poor embryonic development, so lower oxidative stress of second ejaculation may be associated with good-quality embryos [36, 37]. On the other hand, the lower SDF in the second ejaculation may result in a good-quality blastocyst, supported by current studies that oocytes fertilized with damaged sperm DNA may exhibit a lower good-quality blastocyst rate [38,

39]. Therefore, second ejaculation IVF can improve the good quality blastocyst in unexpected ICSI patients.

Our study was limited by the small number of patients in the second ejaculation IVF group, which was more likely to produce research bias. Otherwise, the potential confounding factors of retrospective analysis are not all available, although PSM was performed to reduce biases between the two groups.

Conclusions

This study showed that a second ejaculation could be a simple, low-cost, and effective way to improve sperm, and blastocyst quality and also to decrease the rate of unexpected ICSI. However, further studies are needed to validate these findings.

Abbreviations

ICSI	Intracytoplsmic sperm injection
PSM	Propensity score-matched
IVF	In vitro fertilization
SDF	Sperm DNA fragmentation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43043-024-00165-x.

Additional file 1.

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Authors' contributions

Conceived and designed the study: XHZ, SKW, DWL and LTX. Performed the experiments: XHZ, YYH, XBM, ZDL, PPW and LSC. Analyzed the data: XHZ. Wrote the manuscript: XHZ. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the ethical standards of the ethics committee of our hospital (No: LL-KY-ZC-2021-02) and with the 1964 Declaration of Helsinki and its later amendments.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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