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*Research Article*

# High-DNA Stainability Is Not Related to Embryonic Development and Clinical Outcomes on *In Vitro* Fertilization Cycles: A Retrospective Study Using a Propensity Score-Matching Analysis

**Mingzhe Song**,[1] **Wensi Huang**,[1,2,3] **Lijun Ye**,[1,2,3] **Zhiqiang Liu**,[1,2,3] **Weiqiang Xiao**,[1] **Feng Xiong**,[1] **Yueqin Peng**,[1] **Jing Cai**,[1,2,3] **and Yong Zeng**[1,2,3]

[1]*Department of Urology and Fertility Center, Shenzhen Zhongshan Urology Hospital, Shenzhen 518045, China*
[2]*Shenzhen Key Laboratory of Reproductive Immunology for Peri-Implantation, Shenzhen Zhongshan Institute for Reproduction and Genetics, Shenzhen Zhongshan Urology Hospital, Shenzhen 518045, China*
[3]*Guangdong Engineering Technology Research Center of Reproductive Immunology for Peri-Implantation, Shenzhen 518045, China*

Correspondence should be addressed to Yong Zeng; zengyong1966@gmail.com

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The high-DNA stainability (HDS) might be an indicator for the detection of sperm chromatin decondensation structure resulting from the incomplete histone-to-protamine exchange. The aim of our study was to investigate the association of HDS with embryonic development and clinical outcomes after *in vitro* fertilization (IVF) treatment. Couples underwent IVF cycles from January 2016 to December 2020 were retrospectively studied, including a total of 2,604 target couples undergoing IVF treatment and 628 couples undergoing fresh IVF-embryo transplantation (IVF-ET) treatment. Couples were divided into HDS > 15% group and HDS ≤ 15% group according to HDS levels. After controlling the bias between groups using the propensity score-matching method, data of embryonic development, and clinical outcomes were analyzed. No significant differences were observed between HDS > 15% group and HDS ≤ 15% group regarding fertilization rate (83.33% vs. 84.62%, $P = 0.349$), two pronuclei rate (81.82% vs. 83.33%, $P = 0.613$), cleavage rate (100.00% vs. 100.00%, $P = 0.172$), and high-quality embryo rate (60.00% vs. 60.00%, $P = 0.961$). Linear regression analysis showed that HDS was negatively associated with fertilization rate ($B$ value = −0.094, 95% CI: −0.184 to −0.005, $P = 0.039$), whereas no correlation (adjusted $B$ value = −0.081, 95% CI: −0.170 to 0.008, $P = 0.074$) was observed after adjusting potential confounding factors. The clinical pregnancy rate (62.11% vs. 60.39%, $P = 0.699$), ongoing pregnancy rate (52.17% vs. 53.10%, $P = 0.838$), early miscarriage rate (9.94% vs. 7.28%, $P = 0.283$), late miscarriage rate (0.62% vs. 2.14%, $P = 0.305$), and live birth rate (51.55% vs. 50.96%, $P = 0.838$) were not significantly different between groups. Binary logistic regression analysis showed that HDS levels did not affect clinical outcomes after fresh IVF-ET treatments. HDS was not significantly associated with embryonic development and clinical outcomes of IVF. Our findings suggested that HDS evaluation before IVF treatment might be with limited potential to predict embryo development and clinical outcomes.

## 1. Introduction

Infertility affects a high percentage of reproductive-age couples worldwide. Exhaustive literature review about infertility demography from 2000 to 2014 has shown that 8%–12% of reproductive-age couples in the world could not have their own babies through natural pregnancies, in some regions, the proportion reported was as high as 30% [1]. In addition, a US National Survey of Family Studies conducted from 2006 to 2010 showed that the prevalence of infertility was 6% among all married women aged 15–44, whereas the proportion of infertile men aged 25–44 was up to 11.5% [2]. Assisted reproduction techniques (ART), especially *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), brought hope for infertile couples to give birth. As of 2019, ART has resulted in over 8 million births globally [3].

In recent decades, medical scientists mainly focused on the correlation of female factors with ART outcomes, whereas less attention was paid to male factors. In the future, thus, ascertaining the underlying cause of male infertility will help to improve ART outcomes, because approximately 50% of a couple's infertility is partly related to the male [4].

During late spermiogenesis, sperm chromatin undergoes a process involving histone-to-protamine exchange (also known as protamination) in which approximately 90% of the histone are replaced by protamine [5]. Protamination facilitates normal chromatin condensation, resulting in a 10–20-fold reduction in nuclear volume to obtain the smallest sperm for fast movement [6, 7]. Furthermore, chromatin condensation is necessary for protecting genetic material from damage and subsequently ensures the transmission of paternal genetic information to offspring with both high fidelity and efficiency [6, 8]. Abnormal protamination with defective protamine-1/protamine-2 ratio or increased histone retention was associated with poor semen quality, elevated sperm DNA fragmentation, as well as reduced fertilization capability, poor embryo quality, and low-pregnancy rates during IVF cycles [9, 10].

Protamination can be assessed by various techniques, such as aniline blue staining testing histones specifically, chromomycin A3 staining assessing protamine deficiency, and Sperm Chromatin Structure Assay (SCSA) evaluating DNA fragmentation and chromatin condensation [11]. SCSA, a more widely used method with adequate sensitivity and repeatability to detect sperm chromatin maturity in clinic, could be used to evaluate high-DNA stainability (HDS, also known as the decondensation index) and DNA fragmentation index (DFI) at the same time [12]. DFI as a well-established indicator for the detection of sperm DNA damage was significantly negative correlated with ART outcomes such as lower fertilization, worse pregnancy, and higher miscarriage [13, 14]. The HDS parameter might be associated with loosening and weakening of chromatin condensation due to the lack of full protamination [12, 15]. Using Feulgen staining and Comet test, additionally, Evenson [15] found that the population of HDS sperm sorted by flow cytometry was exhibited rounder morphology compared to normal sperm and few DNA strand breaks. Several studies have shown that elevated HDS might be negatively correlated with sperm quality, such as sperm morphology, motility, and concentration [16, 17]. Moreover, some studies suggested that semen with elevated HDS was related to poor IVF fertilization rate [18, 19], decreased embryo development capacity [19, 20], and increased early miscarriage rates [16, 21] during IVF and ICSI.

The results from above mentioned studies suggested that HDS parameter had clinical implications in ART, but our clinicians did not pay attention to it. The aim of our study was to analyze the HDS data from our fertility center to elucidate the potential significances of HDS, and determine whether it has a significant value for both clinicians and couples seeking IVF treatment. We analyzed the association of HDS with embryonic development of IVF rather than ICSI. This was chosen because either mature or immature sperm has an equal opportunity to make contact with the oocyte in IVF cycle, whereas during ICSI treatment mature sperm with a better morphology and motility have a higher chance to be selected for injection [16]. As mentioned above, HDS were negatively correlated with sperm morphology and motility, which meant that sperm with elevated HDS were less likely to be selected. In addition, we focused on the impact of HDS on clinical outcomes in the fresh IVF-ET cycles, which might minimize the intervention of preserved and thawed embryos.

## 2. Materials and Methods

*2.1. Participants.* This retrospective study initially included 7,795 couples that underwent IVF treatment in Shenzhen Zhongshan Urology Hospital, China, from January 2016 to December 2020. Male partners had undergone semen analysis, including HDS, DFI, sperm number, sperm motility, and sperm morphology before IVF treatment. All couples were tested for chromosomal karyotypes. We excluded couples of IVF cycles with the following conditions, including chromosome abnormality, female age over 40 years old, female body mass index (BMI) beyond 30 $kg/m^2$, and the date of oocyte recovery beyond 12 months after semen analysis. Additionally, we excluded couples who did not undergo fresh IVF-ET treatment, or have no clinical outcomes, or suffered ectopic pregnancy, or lost to follow-up. After exclusions, 5,607 target couples with embryo information were investigated for the effect of HDS on fertilization and embryo quality, and 1,178 couples undergoing fresh IVF-ET treatment were the target population for investigating the effect of HDS on the clinical outcomes (shown in Figure 1). Couples were classified into HDS > 15% group and HDS ≤ 15% group based on the threshold of HDS recommended by the manufacturer's instructions and other studies [18, 21].

This retrospective study was approved by the Ethics Committee of Shenzhen Zhongshan Urology Hospital, Shenzhen, China (Approval number: SZZSECHU-F-2021012).

*2.2. Propensity Score Matching (PSM).* PSM is a useful method to match treated and untreated subjects so that covariate values are the same or similar between matched pairs. Therefore, the PSM method can reduce the influence of these covariates like confounding covariates affecting both the outcome and consequently the variability and potential bias in matching-based estimators [22]. To control the bias between groups, the PSM method was applied R version 4.2.1 software to screen the counterparts of patients in the HDS > 15% group from the HDS ≤ 15% group according to previous study [23]. The caliper and matching ratio were 0.2 and 1 : 3, respectively. The following variables, including paternal age, sperm concentration, percentage of progressive sperm motility, percentage of morphologically normal spermatozoa, DFI, maternal age, maternal BMI, endometrial thickness, infertility years, and infertility causes (female factors including ovulatory disorders, endometriosis, immune factors, uterine and tubal factor, male factor including oligozoospermia, teratozoospermia, asthenozoospermia, both female and male factor, and unexplained factor) were included as matched factors in the PSM. After achieving a balanced cohort by

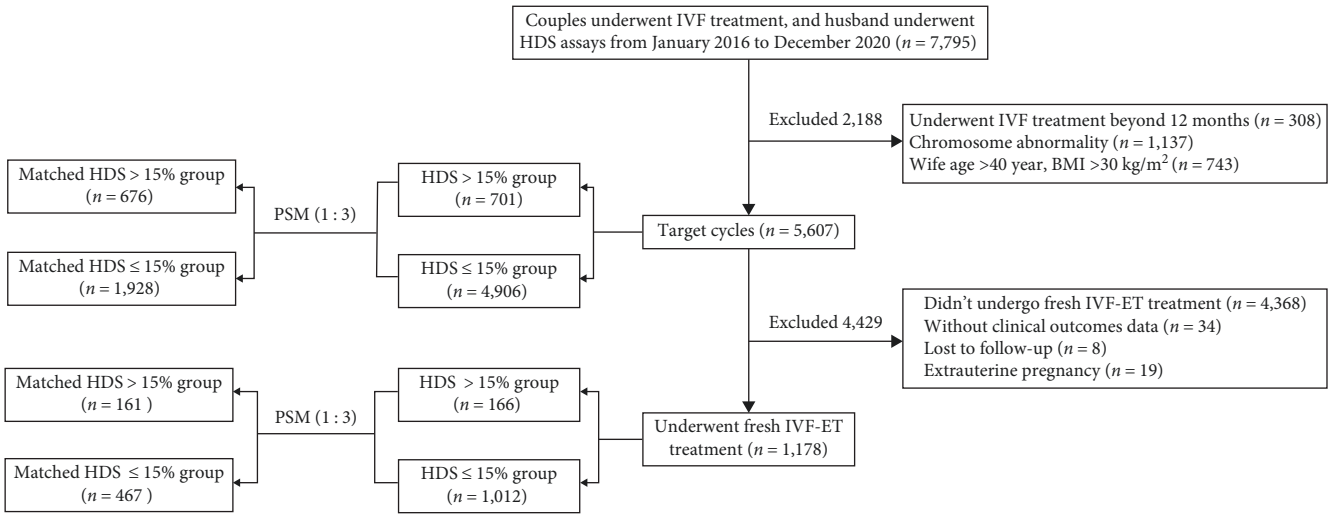


Figure 1: Flowchart of cycles included in this study. IVF, *in vitro* fertilization; IVF-ET, *in vitro* fertilization-embryo transplantation; DFI, DNA fragmentation index; HDS, high-DNA stainability; BMI, body mass index; PSM, propensity score matching.

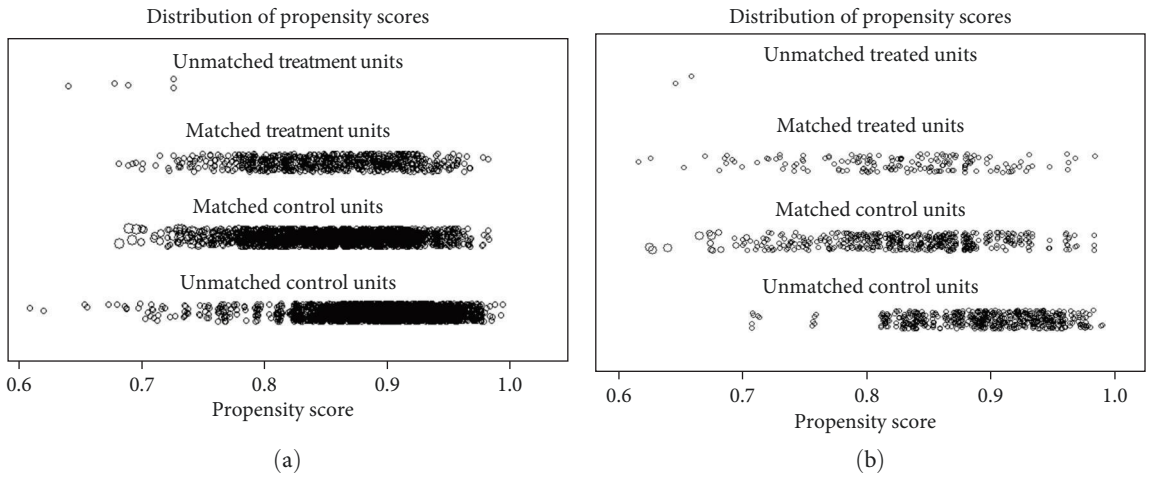


Figure 2: Propensity score matching for HDS > 15% group and HDS ≤ 15% group. The distribution of propensity scores of (a) IVF treatment cycle and (b) fresh IVF-ET treatment cycle after matching.

PSM, 676 couples in the HDS > 15% group matched with 1,928 couples in the HDS ≤ 15% group. Furthermore, in the HDS > 15% group, 20 couples with missing data were excluded, and five couples did not match any counterpart. The matching results are displayed in Figures 1 and 2(a). Next, a similar method was used to match the data of fresh IVF-ET cycles between the two groups. After matching, 161 couples undergoing fresh IVF-ET treatment in the HDS > 15% group matched with 467 couples in the HDS ≤ 15% group. A total of three couples were excluded because of missing data, and two couples did not match any counterpart in the HDS > 15% group. The matching results are displayed in Figures 1 and 2(b).

*2.3. Semen Collection and Analysis.* Semen samples were collected by masturbation after 2–7 days of sexual abstinence and allowed to liquefy at 37°C for 30–60 min prior to analysis. Sperm concentration and motility were determined through computer-aided sperm analysis system (MICROPTIC, Barcelona, Spain) according to the World Health Organization 2010 guidelines [24]. Semen smears were stained using a staining kit (HUAKANG BIOMEDICAL, Shenzhen, China) according to the Diff-Quik staining method. Then, the sperm morphology was assessed by a bright light microscope (NIKON, Tokyo, Japan) using oil immersion at ×1,000 magnification. After semen analysis, 500 $\mu$L of the raw semen samples were frozen at −80°C for subsequent SCSA tests.

*2.4. Sperm Chromatin Structure Assay (SCSA).* SCSA is a flow cytometric test where DNA breaks in sperm can be indirectly evaluated through acridine orange staining. Acridine orange, a fluorescent dye, inserts into dsDNA and fluoresces green ($F$ between 515 and 530 nm) when exposed to 488 nm light. It stacks on single-stranded nucleic acids that collapse into a crystal that produces a metachromatic shift to red fluorescence emission ($F$ > 630 nm). In our study, the assay was

performed through a Sperm Nuclear Integrity Staining Kit (Cellpro Biotech used before September 2020, Ningbo, China; and ANKEBIO used from September 2020, Anhui, China). In brief, sperm were pretreated with acid exposure to induce DNA denaturation in situ, followed by acridine orange staining. By using a flow cytometer (BD Biosciences, New York, USA), 5,000 sperm cells can be analyzed within a few seconds. Through specific SCSA software (DFIView), the DFI was defined as the ratio of sperm with red fluorescence to red and green fluorescence sperm, and HDS was the ratio of intensive green fluorescence sperm to red and green fluorescence sperm.

*2.5. In Vitro Fertilization (IVF) Procedure.* Clinicians at our reproductive center will formulate a personalized controlled ovarian stimulation protocol according to the individual ovarian reserve and response. Patients were administered a human chorionic gonadotropin (hCG) or GnRH agonist injection when two-thirds of all follicles had reached a mean diameter of 18 mm. The endometrial thickness was measured on the day of hCG administration. Oocyte recovery was achieved by means of follicular aspiration under ultrasound guidance at 36–48 hr after administration of hCG. At the time of oocyte recovery, semen samples were prepared by discontinuous gradient centrifugation using the double-layer density gradient media (SAGE BioPharma, Cambridge, USA). Sperm were cultured at 37°C with 5% $CO_2$ before IVF treatment.

Oocytes were fertilized through traditional IVF at 4–6 hr after oocyte recovery. The culture of cleavage-stage embryos was performed in Quinn's Advantage Sequential Media (SAGE BioPharma, Cambridge, USA) and blastocyst culture was in Quinn's Advantage Blastocyst Medium (SAGE BioPharma, Cambridge, USA) based on the manufacturer's instructions. Embryo quality was determined under a light microscope on Day 3 after fertilization according to the number and evenness of blastomeres as well as the percentage of fragmentation [25]. Fresh cleavage-stage embryos or blastocysts were selected to transfer.

*2.6. Outcome Evaluation.* The development of embryos was evaluated by the fertilization rate, two pronuclei rate, cleavage rate, and high-quality embryo rate, respectively. Fertilization was determined to be normal if two pronuclei and two polar bodies were identified at 16–18 hr after insemination. High-quality embryos represented Grade 1 and two embryos on Day 3 after fertilization. The fertilization rate was the ratio of fertilized oocytes to retrieved oocytes. The two pronuclei rate was the ratio of two pronuclei zygotes to fertilized oocytes. The cleavage rate was the ratio of cleaved zygotes to fertilized oocytes. The high-quality embryo rate was the ratio of high-quality embryos to cleaved zygotes.

The quantitative detection of maternal serum hCG was performed on Day 11, followed by a transvaginal ultrasound test to confirm pregnancy at 1 month after embryo transfer. At least one intrauterine gestational sac in the uterus was defined as a clinical pregnancy. The ongoing pregnancy rate was defined as the ratio of cycles of pregnancy beyond 3 months' gestation to the total number of transferred cycles. The early miscarriage rate was calculated as cycles losing a fetus within the first 12 weeks of gestation per transferred cycle, while the late miscarriage rate was calculated as cycles losing the fetus within 12–28 weeks of gestation per transferred cycle. The live birth rate was defined as the ratio of cycles that resulted in the delivery of a live baby after 22 weeks of gestation to the total number of transferred cycles.

*2.7. Statistical Analysis.* Statistical analyses were performed by Statistical Package for Social Sciences (SPSS) version 23.0 software (SPSS Inc., Chicago, IL, USA). Owing to all continuous variables were abnormal distribution, the Mann-Whitney $U$ test was applied, and the results were presented as median (interquartile range). For categorical variables, the $\chi 2$ test was used to determine the intergroup difference, and the results were shown as number and frequency.

Linear regression analyses were used to assess the correlations of HDS with fertilization rate, two pronuclei rates, cleavage rate, and high-quality embryo rate. In addition, binary logistic regression was adopted to assess the impact of HDS levels on clinical pregnancy, ongoing pregnancy, early miscarriage, late miscarriage, and live birth. The variables including the above matched factors and the number of oocytes retrieved were included as potential confounding factors in the multivariate linear regression analyses. In addition to the above-mentioned variables, other variables, including the number of fertilized oocytes, the number of two pronuclei zygotes, the number of cleaved zygotes, the number of high-quality embryos on Day 3, the number of transferred embryos, the number of high-quality embryos transferred, and the type of embryo transferred were also included as potential confounding factors in the binary logistic regression analyses.

The significance level of statistical analysis was set to $P < 0.05$.

## 3. Results

*3.1. Demographic Data.* When compared the baseline characteristics of couples in the HDS > 15% group and the HDS ≤ 15% group before and after matching, the data of IVF cycles and fresh IVF-ET cycles are shown in Table S1 and Table 1, Table S3 and Table S4, respectively. After matching, there was no significant difference of the baseline data between the HDS > 15% group and the HDS ≤ 15% group ($P > 0.05$).

*3.2. Correlation between HDS and Embryonic Development.* As shown in Table 2, the fertilization rate between groups was not significantly different. Linear regression analysis showed that HDS was negatively associated with fertilization rate ($B$ value = −0.094, 95% CI: −0.184 to −0.005, $P$ = 0.039). However, after adjusting the potential confounding factors, HDS was not related to fertilization rate (adjusted $B$ value = −0.081, 95% CI: −0.170 to 0.008, $P = 0.074$). two pronuclei rate, cleavage rate, and high-quality embryo rate did not differ between groups. Linear regression results showed that HDS had no association with these parameters of embryonic development.

Table 1: Baseline characteristics of IVF cycles of the HDS > 15% group and the HDS ≤ 15% group after matching.

| | HDS > 15% group ($n = 676$) | HDS ≤ 15% group ($n = 1928$) | $P$-value |
|---|---|---|---|
| HDS (%) | 19.01 (16.54, 24.32) | 8.04 (6.15, 10.40) | <0.001 |
| Male age (year) | 34.00 (31.00, 38.00) | 34.00 (30.75, 38.00) | 0.564 |
| Sperm concentration (million/ml) | 55.95 (35.90, 91.83) | 59.60 (37.80, 94.48) | 0.175 |
| Progressive sperm motility (%) | 43.60 (33.48, 53.82) | 45.20 (33.30, 56.90) | 0.140 |
| Morphologically normal spermatozoa (%) | 5.25 (4.10, 7.20) | 5.50 (4.20, 7.60) | 0.264 |
| DFI (%) | 9.38 (6.15, 14.31) | 9.02 (5.65, 14.24) | 0.102 |
| Female age (year) | 32.00 (29.00, 36.00) | 32.00 (29.00, 35.00) | 0.760 |
| Female BMI (kg/m$^2$) | 21.34 (19.64, 23.24) | 21.23 (19.63, 23.23) | 0.527 |
| Endometrial thickness on the day of ovulatory dose of hCG (mm) | 10.00 (8.00, 12.00) | 10.00 (8.00, 12.00) | 0.695 |
| Duration of infertility (year) | 3.00 (1.50, 4.00) | 3.00 (1.00, 4.00) | 0.974 |
| Cause of infertility | | | |
| Female factor, $n$ (% per included cycle) | 359 (53.11) | 1023 (53.06) | 1.000 |
| Male factor, $n$ (% per included cycle) | 102 (15.09) | 268 (13.90) | 0.443 |
| Female and male factor, $n$ (% per included cycle) | 61 (9.02) | 182 (9.44) | 0.818 |
| Unexplained, $n$ (% per included cycle) | 154 (22.78) | 455 (23.60) | 0.712 |
| Retrieved oocytes ($n$) | 12.00 (7.00, 19.00) | 13.00 (8.00, 19.00) | 0.454 |

*Note*: DFI, DNA fragmentation index; BMI, body mass index.

Table 2: Intergroup differences of fertilization and embryo quality, and linear regression analysis on the relation of HDS with embryonic development after matching.

| | HDS > 15% group ($n = 676$) | HDS ≤ 15% group ($n = 1928$) | $P$-value | $B$ value (95% CI) | $P$-value | $AB$ value (95% CI) | $P$-value |
|---|---|---|---|---|---|---|---|
| Fertilization rate (%) | 83.33 (70.83, 94.67) | 84.62 (72.22, 95.83) | 0.349 | −0.094 (−0.184–−0.005) | 0.039 | −0.081 (−0.170–0.008) | 0.074 |
| Two pronuclei rate (%) | 81.82 (66.67, 93.33) | 83.33 (69.68, 94.12) | 0.613 | −0.036 (−0.135–0.064) | 0.483 | −0.041 (−0.140–0.059) | 0.424 |
| Cleavage rate (%) | 100.00 (100.00, 100.00) | 100.00 (95.24, 100.00) | 0.172 | 0.010 (−0.029–0.049) | 0.604 | 0.011 (−0.028–0.050) | 0.592 |
| High-quality embryo rate (%) | 60.00 (40.00, 78.52) | 60.00 (40.00, 77.78) | 0.961 | 0.024 (−0.119–0.168) | 0.740 | 0.011 (−0.132–0.154) | 0.876 |

*Note*: AB, adjusted $B$ value.

*3.3. The Effect of HDS on Clinical Outcomes of Fresh In Vitro Fertilization-Embryo Transplantation (IVF-ET) Cycle.* The clinical outcomes of the HDS > 15% group and the HDS ≤ 15% group after transfer are shown in Table 3. Compared with the HDS ≤ 15% group, the HDS > 15% group was not significantly different regarding clinical pregnancy rate and ongoing pregnancy rate. Furthermore, no effect was observed regarding HDS levels on clinical pregnancy and ongoing pregnancy using binary logistic regression analysis. The occurrence of early miscarriage and late miscarriage were similar between the two groups. These findings were confirmed by binary logistic regression analysis using the nonabortion population as a reference. The live birth rate was similar between the HDS > 15% group and the HDS ≤ 15% group. Binary logistic regression analysis also showed that HDS levels did not affect live birth.

## 4. Discussion and Conclusion

The HDS parameter measured through SCSA might be related with the decondensation of sperm chromatin. In the 1999 year, the flow cytometry data showed that HDS populations were above the upper boundary of DNA staining of normal sperm [26]. Subsequently, gel electrophoresis performed in the semen sample with high HDS by Evenson et al. [27] showed an increased ratio of histone to protamine and a significant amount of unprocessed protamine two precursor, which might result in less chromatin condensation. After treating spermatozoa with dithiothreitol to induce decondensation, Zini et al. [17] found a substantial increase in HDS (from 2.7% to 35.4%).

Since this interesting indicator was proposed, a number of studies had begun to analyze the clinical significance of HDS in ART. Several previous investigations suggested that increased HDS was related to poor embryonic development after either IVF or ICSI [18–20]. Data reported by Lin et al. [16] and Jerre et al. [21] showed that elevated HDS was related to increased early miscarriage rates after IVF or ICSI treatment. Although the biological background for increased HDS is not yet fully understood, there are three plausible explanations for the negative effect of HDS on ART outcomes. First, the structural or biochemical defect in

Table 3: Intergroup difference of clinical outcomes, and logistic regression analysis on the contribution of HDS levels to clinical outcome after matching.

| | HDS > 15% group ($n = 161$) | HDS ≤ 15% group ($n = 467$) | $P$-value | OR (95% CI) | $P$-value | AOR (95% CI) | $P$-value |
|---|---|---|---|---|---|---|---|
| Clinical pregnancy rate (%) | 62.11 (100/161) | 60.39 (282/467) | 0.699 | 1.000 (0.977–1.025) | 0.968 | 0.997 (0.973–1.023) | 0.837 |
| Ongoing pregnancy rate (%) | 52.17 (84/161) | 53.10 (248/467) | 0.838 | 1.000 (0.977–1.023) | 0.998 | 0.995 (0.971–1.019) | 0.682 |
| Early miscarriage rate (%) | 9.94 (16/161) | 7.28 (34/467) | 0.283 | 1.001 (0.957–1.042) | 0.946 | 1.009 (0.963–1.052) | 0.698 |
| Late miscarriage rate (%) | 0.62 (1/161) | 2.14 (10/467) | 0.305 | 0.946 (0.826–1.042) | 0.338 | 0.929 (0.796–1.033) | 0.259 |
| Live birth rate (%) | 51.55 (83/161) | 50.96 (238/467) | 0.897 | 1.003 (0.980–1.026) | 0.799 | 0.999 (0.975–1.024) | 0.957 |

*Note*: OR, odds ratio; AOR, adjusted odds ratio.

sperm chromatin packaging during spermatogenesis might contribute to the failure of decondensation [28]. The incapacity of sperm chromatin decondensation was related to the failure of fertilization and pronucleus formation after IVF [29]. Data published by Flaherty et al. [30] and Dozortzev et al. [31] showed that sperm chromatin exhibited a completely unsuccessful decondensation in about 11% and up to 38% of unfertilized oocytes after ICSI, respectively. Second, lacking proper chromatin structure might disturb appropriate gene expression and then lead to early embryo death and miscarriages [21]. Third, although oocytes possess the capacity to repair sperm chromatin damage, it is unable to repair sperm chromatin perturbations when deterioration is severe [32, 33].

In contrast to the above studies, however, our study showed that HDS level >15% was still compatible with the fertilization rate, two pronuclei rate, cleavage rate, and high-quality embryo rate of IVF cycles. Additionally, HDS had no significant correlation with the parameters of embryonic development after adjusting the potential confounding factors. Moreover, we found that there was no significant influence of HDS in the clinical pregnancy, ongoing pregnancy, early miscarriage, late miscarriage, and live birth with fresh IVF-ET cycles, which was in accordance with the several reported literatures [21, 34, 35]. Larson-Cook et al. [34] observed that HDS was not a significant predictor for pregnancy. Additionally, Bungum et al. [35] demonstrated that HDS did not seem to be a predictive value for the biochemical pregnancy, clinical pregnancy, and live birth with IVF or ICSI cycles, neither alone nor in combination with DFI. Jerre et al. [21] proposed that high-HDS levels were not a risk of early miscarriage after IVF treatment. Similar ineffectiveness of HDS on predicting clinical outcomes had been reported in ICSI cycles [36].

The contradictory conclusions from above-mentioned studies might be at least in part resulted from the heterogeneous nature of the study design, such as sample size, criteria of included participants, and treatment of bias. Although our results are not entirely new, it can be considered as reliable because 2,604 IVF cycles and 628 fresh IVF-ET cycles were included and subsequently controlled the bias among the two groups through PSM. DFI was also included as a matched factor in PSM to reduce its interference in HDS, because the parameter had a significantly positive correlation with HDS (Table S2) and a definitely negative effect on ART outcomes [13, 14]. After matching, the baseline characteristics including DFI between the two groups were similar, which would help to analyze the effect solely caused by HDS. Univariate linear regression analysis showed that fertilization rate was the only parameter among IVF outcomes significantly but weakly affected by HDS, while there was no correlation between fertilization rate and HDS after adjusting. This might be because the effect in univariate model was caused by female factors rather than HDS, given the small contribution of HDS to the equation in which the coefficient of determination ($R^2$) was 0.02 (data not shown) and the large and well-established role of female factors (such as advanced age, obesity, and poor ovarian reserve) in fertility [37–39]. Thus, the actually prognostic value of sperm HDS on fertilization was observed after adjusting. The other plausible explanation for the little influence of HDS on IVF outcomes might be that we prepared the semen samples before IVF through discontinuous gradient centrifugation, which might impact the level of HDS. After gradient centrifugation, the proportion of sperm with condensed chromatin would be significantly increased, and the corresponding decrease was observed in the decondensed sperm population [40, 41].

Furthermore, it is factually difficult to interpret the controversial results from the current studies because of the ambiguous relationship between HDS and nuclear proteins exchange with chromatin condensation and the lack of underlying mechanisms regarding HDS level on ART outcomes. Recently, some researchers argued that HDS was not a reliable indicator in evaluating sperm chromatin immaturity. Lu [42] considered the hypothesis was doubtful that HDS populations represented immature sperm with less condensed chromatin due to incomplete protamination, because decreased chromatin condensation was observed in all spermatozoa after acid treatment. According to the principle of acridine orange staining, increased DNA stainability might be the result of more intact double-stranded DNA. It was supported by the other studies that the levels of HDS decreased linearly with increasing age [43, 44], which

was in accordance with the evidence that sperm with more intact double-stranded DNA decrease with age [42]. Other authors, Mohammadi et al. [11] and Amor et al. [45] found that HDS have a weak correlation with the results evaluated by aniline blue staining, toluidine blue staining, and chromomycin A3 staining. Moreover, although Evenson et al. have found an increased amount of retained histones in the semen sample with high HDS [27], it lacked a directly experimental evidence to confirm the correlation of elevated HDS with retained histones and deficient protamine. Thus, to confirm whether HDS was related to ART outcomes, the specific correlation of HDS with sperm chromatin structure and the actual significance of HDS on sperm function should be first explored. Additionally, to research whether the elevated HDS impacts clinical outcomes, animal experiments in which HDS sperm were selected to inject into normal oocytes should be performed.

In summary, our data suggested that HDS was poorly related to IVF outcomes, including fertilization, two pronuclei, cleavage, high-quality embryo on Day 3, clinical pregnancy, ongoing pregnancy, early miscarriage, late miscarriage, and live birth. HDS could not be recommended as a reliable indicator for the prediction of embryonic development and clinical outcomes before IVF treatment. Thus, it is unnecessary for clinicians to pay too much attention to this parameter. The main weakness of our study was the nature of the retrospective design in which patients were not randomized. Further, since the semen samples used to evaluate HDS were not the samples used for fertilization, the level of HDS might not truly reflect the level at the time of fertilization. Therefore, it might be more persuasive to analyze the clinical implication of HDS tested in the semen samples used for fertilization. Additionally, animal experiments are needed to directly study the relation between HDS with IVF outcomes, which may help to deeply understand the effect of HDS on male fertility.

## Data Availability

Readers can access the data underlying the findings of this study by contacting authors through email (zengyong1966@gmail.com).

## Conflicts of Interest

All authors declare that there is no conflict of interest regarding the publication of this paper.

## Authors' Contributions

Mingzhe Song and Wensi Huang have contributed equally to this work and share first authorship. Yong Zeng, Mingzhe Song, and Wensi Huang substantially contributed to the design of this study. Feng Xiong and Yueqin Peng carried out laboratory work. Mingzhe Song, Weiqiang Xiao, and Feng Xiong collected the data. Jing Cai, Wensi Huang, Zhiqiang Liu, and Lijun Ye analyzed and interpreted data. Mingzhe Song and Wensi Huang mainly drafted the manuscript. Jing Cai, Mingzhe Song, Lijun Ye, Yueqin Peng, and Yong Zeng critically revised. All authors agreed to be an author, and approved the final manuscript.

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## Supplementary Materials

Table S1: baseline characteristics of IVF cycles of HDS > 15% group and HDS ≤ 15% group before matching. Table S2: correlations between HDS with male age and semen parameters. Table S3: baseline characteristics of fresh IVF-ET cycles of HDS > 15% group and HDS ≤ 15% group before matching. Table S4: baseline characteristics of fresh IVF-ET cycles of HDS > 15% group and HDS ≤ 15% group after matching. (*Supplementary Materials*)

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