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Research Article

Number of Available Embryos: A Prediction Tool for Analyzing the Effect of Sperm DNA Fragmentation on Pregnancy Outcome of Patients Undergoing IVF Cycles from Embryology

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Sperm DNA fragmentation (SDF) can affect the pregnancy outcome of assisted reproduction technology. The cumulative live birth rate (CLBR), a new parameter of pregnancy outcome, attracts the attention of researchers to study the effect of SDF on CLBR. This study will focus on whether the number of available embryos (AEN) can be used as a predictor to investigate the effect of SDF on CLBR of in vitro fertilization (IVF) cycles. Our study included 1,347 couples who underwent IVF cycles and detected their SDF results by the sperm chromatin structure assay. Subsequently, CLBR and AEN on Day 3 were examined and compared between the SDF $\leq 30\%$ and SDF $> 30\%$ group. The finding by the correlation analysis showed a strong correlation in CLBR and AEN, suggesting that AEN can be used as a predictor of CLBR. When comparing the two groups, significant differences were noticed in CLBR (81.1% vs. 71.5%, $P = 0.009$) and AEN (8.10 ± 4.43 vs. 7.21 ± 4.27, $P = 0.021$), revealing that SDF has an influence on both indicators. The results of the covariance analysis suggested that the available embryo rate per maturity oocyte was higher in the SDF $\leq 30\%$ group, and the embryologic parameters showed a significant difference in the available embryo formation rate on Day 3, but not in the fertilization and cleavage rates, suggesting that SDF reduced the quality of embryos, which did not reach the morphological standard of the available embryos, and further reduced AEN and CLBR in the IVF cycles.

1. Introduction

Sperm DNA fragmentation (SDF) can affect pregnancy outcome in both natural and assisted reproduction. Multiple factors like heat, radiation, smoking, and oxidative stress [1] can contribute to its occurrence. Moreover, SDF can adversely affect fertilization and embryo growth. Apoptosis is induced when the severity of DNA damage surpasses the cells' repair capacity [2], although oocytes can repair minor DNA damages in assisted reproductive technology [3, 4].

Numerous studies have focused on understanding the correlation between SDF and the results of pregnancy, particularly in individuals undergoing the process of in vitro fertilization (IVF). However, most studies involve the outcome only in the fresh embryo transfer cycles [5], so there may be bias in determining the pregnancy outcome. Currently, cumulative live birth rate (CLBR), a more comprehensive indicator to assess pregnancy outcome, is widely used [6]. It refers to the probability of live birth occurring when all embryos (including fresh and frozen embryos) generated during a fresh ovarian stimulation cycle are transferred multiple times until live birth occurs or when the embryos are depleted. Since CLBR includes both fresh and frozen embryos transferred cycles, it is suitable to assess the IVF success rate, so researchers have started to concentrate on the impact of SDF on CLBR [7].

Several studies [8–10] have reported on the correlation between the available embryo number (AEN) and CLBR, leading us to consider if AEN can be used as a predictor for CLBR. The aim of this research was to utilize AEN as a predictor of CLBR to examine the impacts of SDF on both indicators and explain the effect of SDF on AEN from embryo development.

2. Materials and Methods

2.1. Study Design. The data set was of patients who visited the Reproductive Medicine Center between January 2012 and December 2017 for IVF cycles. The criteria for study inclusion were: (1) 35-year-old female age or younger undergoing cycles of retrieved oocyte and (2) ovarian stimulation with the downregulated protocol of the gonadotrophin-releasing hormone agonist (GnRH-a). The exclusion criteria were as follows: (1) diminished ovarian reserve, as defined by Zhang et al. [11], if patients reach any of the following three: (i) basal follicle-stimulating hormone (FSH) level ≥10 mIU/ml, (ii) antral follicle count <5, and (iii) poor ovarian response: the number of retrieval oocytes <5 or cancelation of the cycle due to poor response to ovarian stimulation; (2) cycles of frozen oocytes; (3) cycles of testicular sperm aspiration or percutaneous epididymal sperm aspiration; and (4) complete treatment failed to constitute: no live birth record was found in 2 years, but frozen embryos that are subordinate to the oocyte collection cycle were available for transfer.

2.2. Controlled Ovarian Hyperstimulation. The GnRH-a long protocol [12] was utilized for ovarian stimulation. During the middle luteal phase, patients received a daily injection of 0.1 mg of triptorelin acetate (Decapeptyl; Ferring, Saint-Prex, Switzerland). Recombinant FSH (GONAL-f, Merck KGaA, Darmstadt, Germany) was injected around 16–20 days following the administration of GnRH-a, once the levels of serum estradiol and luteinizing hormone dropped below 50 pg/ml and 3 IU/L, respectively. The dosage of GONAL-f were personalized based on age, body mass index, and levels of anti-Müllerian hormone. B-scan ultrasound was used to monitor the development of follicles, while the levels of serum hormones were measured. Patients were administered with 5,000–10,000 IU of human chorionic gonadotrophin (HCG) when either a follicle reached a diameter of 18 mm or three follicles reached a diameter of 17 mm. The oocytes were collected 34–36 hr after the administration of HCG.

2.3. In Vitro Fertilization and Embryo Culture. After being rinsed with Quinn's HTF Medium (CooperSurgical Corp., Målov, Denmark), the oocytes were then co-incubated with optimized spermatozoa at a temperature of 37°C in a humid environment consisting of 95% air and 5% CO_2 for IVF. The assessment of fertilization was conducted 18 hr later. After 3 days of fertilization, the quality of the embryo was assessed using the ESHRE classification system [13]. The evaluation criteria for available embryos were as follows: (1) normal fertilization on Day 1, that is, fertilized eggs with two pronuclei (2PN); and (2) embryo with 6–12 blastomeres and fragmentation ≤25% on Day 3. Embryos with 7–9 equal size blastomeres, and fragmentation <20% were considered top-quality embryos. If the number of embryos was fewer than 4, embryo transfer occurred on Day 3; otherwise, it took place on Day 5, and any extra embryos were cryopreserved by vitrification (Kitazato Corporation, Yanagishima, Fuji, Japan).

2.4. Frozen Embryo Transfer. To transfer the frozen embryo, the endometrium was prepared using a natural or stimulated protocol. On the day of transfer, the frozen embryos were thawed in Kitazato thawing medium (Kitazato Corporation, Yanagishima, Fuji, Japan) and transferred according to the manufacturer's protocol. Luteal support was provided after embryo transfer.

2.5. Semen Analysis. The patients collected their samples by masturbation after abstaining for 3–5 days, and semen analysis was conducted on the same day. Semen volume, sperm concentration, and motility were assessed in accordance with the WHO guidelines (2010) [14].

2.6. Sperm Chromatin Structure Assay. Following the liquefaction of semen, the sperm cells were diluted to a concentration of $(1–2) \times 10^6$ cells/ml using a solution containing 0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA (pH 7.4). Spermatozoa were subjected to denaturation using a denaturing solution containing 0.08 M HCl, 0.15 M NaCl, and 0.1% Triton X-100 (pH 1.2) for a duration of 30 s. Subsequently, the stained the spermatozoa were stained with acridine orange (Sigma–Aldrich, St. Louis, MO, USA) following the guidelines provided by the manufacturer. Five thousand events were detected by flow cytometry (Novocyte D2040R, Agilent Technologies, Beijing, China). Intact double-stranded DNA was denoted by green fluorescence, while fragmented DNA was denoted by red fluorescence. The SDF measurement was calculated by dividing the red fluorescence intensity by the total sperm count.

2.7. Outcome Measure. According to the definition of CLBR for complete treatment [15], patients who had live birth records or used all their embryos resulting from one episode of ovarian stimulation were considered to have undergone a complete treatment. CLBR was defined as the proportion of women having at least one live birth to those who finished their first complete treatment during the course of 2 years.

2.8. Statistical Analysis. The descriptive characteristics of the high (SDF > 30%) and low (SDF ≤ 30%) SDF groups were compared using Student's *t*-test, provided that the requirements of normal distribution and homogeneity of variance were satisfied; otherwise, the Mann–Whitney *U* test was employed. The χ^2 test was utilized to compare categorical variables. The comparison of regression coefficients between the AEN and the oocyte number in two groups was conducted using analysis of covariance (ANCOVA). In this study, a generalized linear mixed model (GLM) with a binary logistic regression link function was employed to compare the rates of fertilization, cleavage, and embryo development. The aim was to reduce the intra-subject effect attributed to data with a two-level nested structure, specifically, the data set consisted of multiple oocytes resulting from one oocyte retrieval cycle. Statistical analysis was performed using IBM's SPSS 25.0 Software (Armonk, NY, USA), and statistical significance was determined by considering *P* values less than 0.05.

3. Results

3.1. AEN Was Strongly Related to CLBR. As shown in Figure 1, CLBR in the first treatment was elevated with increasing AEN on Day 3, suggesting that more AEN offered more

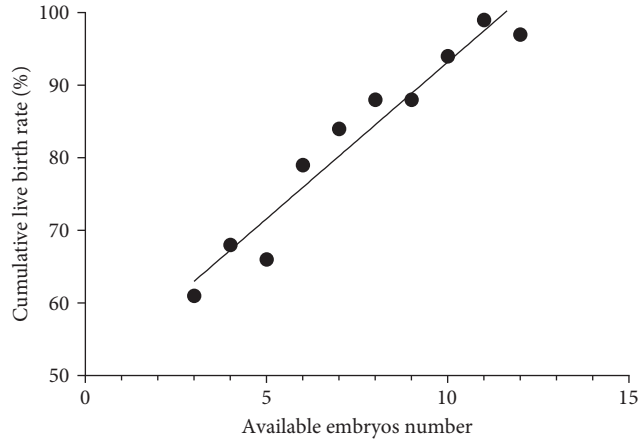

Figure 1: Correlation analysis of the CLBR and AEN ($r = 0.97$, $P < 0.01$).

Table 1: Baseline characteristics of female.

	SDF ≤ 30% ($n = 1{,}206$)	SDF > 30% ($n = 129$)	P-value
Age of female (years)	29.75 ± 3.01	29.92 ± 3.17	0.611
Duration of infertility (years)	3.89 ± 2.58	3.83 ± 2.58	0.803
BMI (kg/m^2)	21.72 ± 3.20	21.21 ± 2.90	0.114
Basal FSH level (mIU/ml)	5.49 ± 1.63	5.49 ± 1.73	0.668
Basal LH level (mIU/ml)	4.90 ± 3.77	4.57 ± 3.61	0.114
Basal estradiol level (mIU/ml)	55.19 ± 30.97	56.51 ± 32.51	0.964
Estradiol level at HCG trigger (mIU/ml)	6,405 ± 3,416	6,850 ± 3,708	0.362
Progesterone level at HCG trigger (mIU/ml)	1.21 ± 0.80	1.22 ± 0.57	0.121
Oocyte retrieval number	14.85 ± 6.26	14.66 ± 6.41	0.667

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; HCG, human chorionic gonadotropin.

opportunities for transfer and a greater probability of success. Furthermore, a strong positive correlation was found between the two outcome variables, suggesting that AEN can be used as a predictor of CLBR.

3.2. Sperm DNA Fragmentation-Reduced CLBR by AEN. Age, FSH level, body mass index, and oocyte retrieval number exhibited comparable traits among female patients in both groups (SDF ≤ 30% and SDF > 30%), as depicted in Table 1. Among males, the two groups exhibited no disparities in age and seminal parameters, except for the percentage of sperm with progressive motility (PR%) (Table 2). Table 3 presents the impact of SDF on CLBR and various other indicators of outcomes. There were no notable disparities observed in the rates of clinical pregnancy, miscarriage, and live birth (per transfer cycles) among the two groups, except for a significant distinction in CLBR. Based on the consideration that the CLBR is the probability of patients who obtain a live birth in their initial complete treatment, we speculate that each patient has a different number of transfer cycles within the complete cycle. To verify this speculation, we conducted a comparison of the overall transfer cycles between the two groups. The results showed a significant reduction in transfer cycles within the high-SDF group compared to the low-SDF group. (Table 3). Simultaneously, consistent with the relationship between CLBR and AEN demonstrated above, it was found that the number of embryos with high SDF reduced significantly, which may infer that the high-SDF group has fewer embryos that can be transferred, resulting in fewer transfer cycles, thus reducing the cumulative success rate.

In the semen parameters, we noticed a correlation between SDF and PR. To find the main parameters that affect the outcome, we included the relevant variables in the multivariate regression model and observed the factors that affect CLBR (Table 4). The results show that SDF is the semen parameter that affects CLBR in our data set.

3.3. The Embryo Formation Rate Is the Main Factor in the Decreased Number of Embryos. In order to demonstrate the reason for the decrease in embryo numbers, a further study was carried out. It is known that embryo numbers are related to the number of oocytes, which has become a factor that must be considered when studying the impact of SDF on AEN [16–19]. Despite our efforts to control the conditions of women, there persist variations among individuals, even though the average count of oocytes is similar in both groups. Hence, regression analysis is employed to examine AEN and the quantity of mature oocytes, and to compare the disparity in their regression coefficients within the two groups

Table 2: Baseline characteristics of male.

	SDF ≤ 30% ($n = 1,206$)	SDF > 30% ($n = 129$)	P-value
Age of male (years)	32.41 ± 4.24	31.81 ± 3.58	0.385
Sperm concentration	57.13 ± 41.77	55.38 ± 38.30	0.977
Progressive of sperm (%)	51.60 ± 19.78	46.08 ± 19.35	0.002
Seminal volume (ml)	3.69 ± 1.38	3.59 ± 1.46	0.243
Total number of sperm ($\times 10^6$/ml)	197.70 ± 147.32	181.67 ± 130.68	0.335
Total number of progressive sperm ($\times 10^6$/ml)	114.83 ± 115.26	94.79 ± 89.72	0.112

Table 3: Parameter of pregnancy outcome.

	SDF ≤ 30% ($n = 1,206$)	SDF > 30% ($n = 129$)	P-value
Clinical pregnancy rate per cycle (%)	59.93 (1,101/1,837)	56.67 (102/180)	0.394
Live birth rate per cycle (%)	53.62 (985/1,837)	51.67 (93/180)	0.616
Miscarriage rate (%)	5.26 (58/1,101)	5.88 (6/102)	0.791
Cumulative live birth rate (%)	81.67 (985/1,206)	72.1 (93/129)	0.009
Number of transferred cycles patient experienced in the treatment	1.52 ± 0.75	1.40 ± 0.63	0.033
Available embryo number on Day 3	8.10 ± 4.45	7.22 ± 4.28	0.026
Embryo transferred number	1.96 ± 0.33	1.98 ± 0.29	0.578

Table 4: Analysis of related factors of CLBR in a complete treatment set.

	P-value	OR (95%CI)
Age of female	0.500	0.980 (0.923–1.040)
Duration of infertility	0.027	0.941 (0.891–0.993)
BMI	0.033	1.054 (1.004–1.107)
Basal FSH level	0.031	0.907 (0.830–0.991)
Basal LH level	0.180	1.032 (0.985–1.081)
Basal estradiol level	0.328	1.003 (0.997–1.009)
Estradiol level at HCG trigger	0.001	1.000 (1.000–1.000)
Progesterone level at HCG trigger	0.001	0.682 (0.559–0.831)
Age of male	0.656	0.991 (0.951–1.032)
Sperm concentration	0.938	1.000 (0.996–1.004)
Seminal volume	0.682	1.022 (0.920–1.137)
Progressive motile of sperm	0.961	1.000 (0.992–1.008)
SDF	0.012	4.575 (1.400–14.954)

(Figure 2). In IVF cycles, the high-SDF group had a significantly lower regression coefficient, indicating a reduced rate of embryo formation per oocyte. This suggests that patients with high-SDF had fewer embryos formed, despite having the same number of oocytes.

To further study the cause of the reduction in the rate of embryo per oocyte, we compared the rates of fertilization, cleavage, and embryo formation between the two groups, as the process of IVF from oocyte retrieval to embryo formation involves several stages, including fertilization, cleavage, and early embryo formation stages. The results showed that SDF mainly affect the rate of available embryos in the IVF cycle (Table 5), suggesting that SDF reduced the quality of embryos, which failed to reach the morphological standards for transferring, although the fertilization and cleavage stages had been successful. Additionally, we also observed a notable reduction in the blastocyst rate in high-SDF group.

4. Discussion

CLBR is a more comprehensive outcome parameter that is widely used for evaluating the success rate of IVF. Several studies have reported the relationship between CLBR and AEN when they included some variables in the multivariate regression model to analyze the influence factors of CLBR [8, 10]. However, when variables were included in the multivariate model, the independence between variables should be taken into account, because AEN is also related to the sperm and oocyte factors and embryo culture conditions, and should also be considered as an outcome variable, similar to CLBR. From this perspective, correlation analysis may be suitable for the analysis of the relationship instead of regression analysis.

In this study, we noticed a strong positive correlation between CLBR and AEN, suggesting that more AEN offered more opportunities for transfer and a greater probability of success. Moreover, the strong positive correlation also revealed that AEN can be used as an early predicting indicator for CLBR [9]. Thus, our finding provided a tool to predict the cumulative pregnancy outcome by AEN when patients are still in the process of oocyte retrieval for IVF and have remaining embryos that have not yet been transferred. Our findings also provide a new perspective to analyze the CLBR from an embryological standpoint.

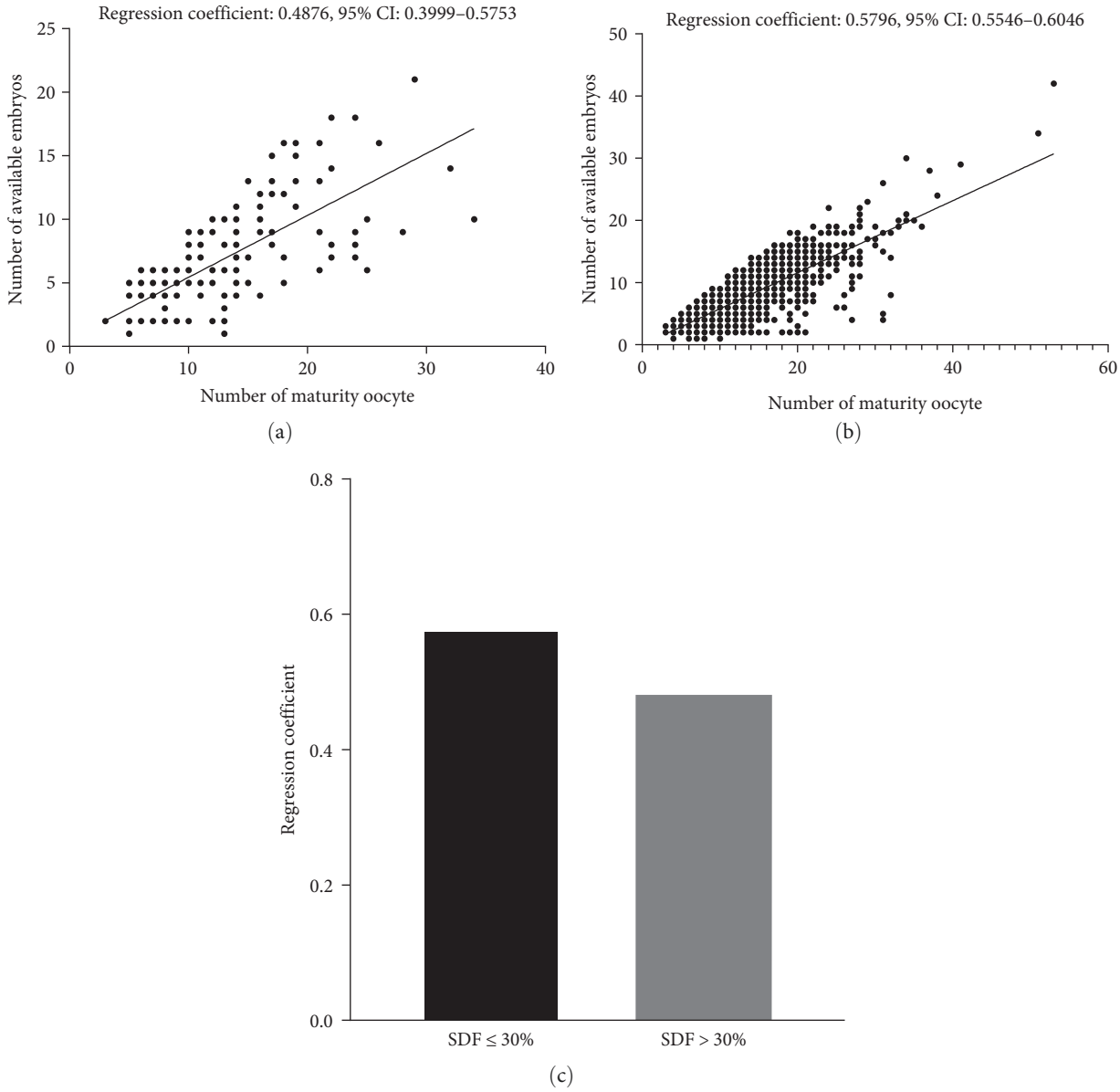

Figure 2: Comparison of the slope of regression lines between the number of available embryos and maturity oocyte. (a) Regression line in the SDF > 30% group (coefficient: 0.4876, 95% CI: 0.3999–0.5753). (b) Regression line in the SDF ≤ 30% group (coefficient: 0.5796, 95% CI: 0.5546–0.6046). (c) Coefficient comparison of each regression line (0.4876 vs. 0.5796) by ANCOVA ($P = 0.0268$).

Table 5: Embryologic parameters between the two groups.

	SDF < = 30% ($n = 1{,}206$)	SDF > 30% ($n = 129$)	P-value
Maturity oocyte number	13.98 ± 6.11	13.45 ± 5.87	0.376
Number of fertilized eggs with 2PN	10.20 ± 5.00	9.66 ± 4.96	0.245
Fertilization rate (%)	73.6 (72.7–74.4)	72.3 (69.5–74.9)	0.383
Number of cleaved embryos	10.04 ± 4.94	9.57 ± 4.90	0.315
Cleavage rate (%)	96.5 (96.2–96.8)	96.7 (95.5–97.5)	0.778
Available embryo rate (%)	82.5 (81.4–83.5)	77.6 (73.6–81.1)	0.008
Number of top-quality embryos	5.31 ± 3.67	4.71 ± 3.53	0.079
Top-quality embryo rate (%)	52.8 (51.3–54.3)	48.8 (44.2–53.3)	0.099
Blastocyst formation rate (%)	78.7 (75.3–79.6)	65.8 (60.5–69.6)	<0.001

Fertilization rate = fertilized oocytes with 2PN/maturity oocytes, cleavage rate = cleaved embryos on Day 2/fertilized oocytes with 2PN, available embryo rate = available embryos on Day 3/cleaved embryos on Day 2, Top-quality embryo rate = Top-quality embryos on Day3 /cleaved embryos on Day 2.

SDF caused by DNA damage can lead to genomic changes and poor embryo quality that affect the pregnancy outcome [20–22]. In this study, we showed that excessive SDF could reduce CLBR of IVF patients in the first treatment, suggesting that SDF influenced pregnancy outcome. Consistent with the results of Vončina et al. [7], this study may provide another support to reveal the impact of SDF on pregnancy outcomes from a reliable indicator. Utilizing the strong association between CLBR and AEN, we employed AEN as a prognostic instrument, while simultaneously noting the decrease in AEN caused by SDF during IVF cycles. At the same time, we noticed that patients with high SDF have lower numbers of transfer cycles within a complete treatment. In this regard, the reduction of CLBR may be attributed to the decreasing number of embryos by SDF, thereby reducing the chances of embryo transfer for patients undergoing IVF cycles.

Previously, the influence of sperm DNA damage on the stage of embryonic development was widely discussed [23]. It is generally believed that after sperm penetration and sperm-oocyte fusion, the zygote undergoes pronuclear formation, pronuclear migration, and nuclear fusion processes under the influence of relevant factors in the cytoplasm of oocytes. During the 4–8 cell stage of cleavage, the zygote genome of the embryo starts to activate, and the regulation of embryonic development gradually shifts from maternal to zygotic, also known as maternal to zygotic transition [24]. It is speculated that in the 4–8 cell stage, with the activation of paternal genes, severely damaged sperm DNA may gradually start to affect embryonic development. Some previous studies have investigated the correlation between DNA damage of sperm and growth of embryos, and suggested that the impact of SDF gradually began to appear in the late stage of embryonic development, but not in the stage of pronuclear formation [25, 26]. Consistent with their research, when we further studied the cause of AEN reduction, our study revealed that the available embyo formation rate per oocyte was significantly lower in the patients with high SDF, and by further comparing embryologic parameters found that SDF affected the formation of available embryos on Day 3 and blastocyst, but not in fertilization stage, which suggested the existence of the late paternal effect [26, 27]. This may provide some clues for further research regarding the impact of SDF on outcomes.

5. Conclusions

In conclusion, we identified a strong positive correlation between CLBR and AEN, indicating that AEN can be used as an early indicator of CLBR. On this basis, our study demonstrated that SDF mainly affected the embryo formation rate, which decreased the number of embryos and reduced CLBR in IVF cycles. Our findings provide a new prediction tool for assessing the impact of SDF on CLBR.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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