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ARTICLE

IVF/ICSI cumulative live birth rates per consumed oocyte remain comparable regardless of sperm DNA fragmentation by TUNEL

BIOGRAPHY

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KEY MESSAGE

The cumulative probability of live birth was not negatively affected by fragmented paternal chromatin when calculated per embryo transfer, replaced embryo or oocyte used in IVF and ICSI cycles with autologous oocytes. This information is useful for advising patients individually on their likelihood of achieving a live birth.

ABSTRACT

Research question: Does sperm DNA fragmentation (SDF) affect reproductive success of IVF and intracytoplasmic sperm injection (ICSI) cycles measured as cumulative live birth rates (CLBR) in unselected couples?

Design: Clinical data from 1339 couples undergoing 2759 IVF/ICSI cycles using autologous oocytes with a SDF test by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay on their ejaculated spermatozoa were retrospectively evaluated. Main outcomes were calculated according to two different analyses: using 15% SDF as cut-off point (low ≤15% and high >15%); and categorizing participants based on four SDF ranges (<10%, 10– <20%, 20–30% and >30%). Live birth rate and CLBR per number of embryo transfers, per number of embryos replaced and consumed oocytes required to achieve the first live birth according to level of SDF were the main outcomes assessed.

Results: No significant difference was found in clinical pregnancy rate and miscarriage rate between both groups. No differences in LBR per embryo transfer were found for the first or for all embryo transfers when comparing ≤15% and >15% sperm DNA fragmentation or by SDF ranges. The CLBR according to the number of embryo transfers and the number of embryos replaced showed no statistically significant differences between different SDF groups. When the same number of oocytes were inseminated, similar CLBR were obtained regardless of the degree of male sperm DNA fragmentation.

Conclusions: High SDF did not impair live birth rates of unselected males undergoing IVF/ICSI cycles with autologous oocytes per transfer or the cumulative probability of a live birth.

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KEYWORDS

Cumulative live birth rate
IVF/ICSI
Male infertility
Sperm DNA fragmentation
TUNEL assay

INTRODUCTION

Spermatozoa play a critical role for successful reproduction, and poor seminal quality can cause fertilization failure, impaired embryo development and miscarriage (*Lewis and Aitken, 2005*). Male factor is responsible for nearly 30% of infertility cases (*Mélodie and Christine, 2018*), but its physiological cause is often unexplored. About 15% of men with normal seminal parameters according to World Health Organization guidelines (*World Health Organization, 2010*) are unable to achieve pregnancy and live birth (*Hamada et al., 2012*). To alleviate this problem, new diagnostic tools may help determine male fertility potential.

One such approach evaluates sperm genomic integrity via sperm DNA fragmentation (SDF). The origin of DNA fragmentation can be due to different factors, but the presence of oxidative radicals is one of the major causes (*Aitken and Krausz, 2001*; *Esteves et al., 2014*; *Lewis, 2015*; *Majzoub et al., 2016*; *Panner Selvam et al., 2020*). Moreover, these breakages can occur in one or both DNA strands (single strand break and double strand break) (*Ribas-Maynou et al., 2012*; *Casanovas et al., 2019*), of which the effect on fertility potential may be different.

Among couples attending an assisted reproductive technology centre, high SDF is found in the ejaculate of 30% of men undergoing IVF and intracytoplasmic sperm injection (ICSI) (*Bungum et al., 2007*), whether they present normal (*Saleh et al., 2002*; *Hamada et al., 2012*) or abnormal (*Evgeni et al., 2015*; *Samplaski et al., 2015*) seminal parameters according to the *World Health Organization (2010)* standard assessment. As DNA integrity is important for paternal genetic transmission (*Lewis et al., 2013*; *Gosálvez et al., 2015*), the relationship between SDF and poor clinical outcomes has been extensively evaluated. The findings, however, continue to be controversial.

On the one hand, high levels of SDF have been associated with lower fertilization and pregnancy rates, poorer embryo quality (*Avendaño et al., 2010*; *Borges et al., 2019*), impaired embryo kinetics (*Esbert et al., 2018*) and decreased live birth rates in IVF patients compared with those with no DNA fragmentation (*Simon et al., 2013*). Conflicting evidence from prospective studies, however, indicates that sperm DNA damage may not be directly related to IVF and ICSI pregnancy outcomes (*Muriel et al., 2006*; *Esbert et al., 2011*; *Green et al., 2020*).

This lack of consensus may reflect bias in measuring the effect of SDF on reproductive outcomes. Success rates in ART are commonly calculated per embryo transfer, considering only the contribution of the embryos from the first transfer. Without considering the potential contribution of all the embryos available for transfer if the first attempt fails, outcome measures may be subject to selection bias.

A more reliable and accurate approach to measure the effect of SDF, or any factor, on the chances of achieving a live birth, is through cumulative live birth rates (CLBR). The CLBR per attempt, equivalent to the statistical time-to-event approaches, represents a new strategy to measure the likelihood of success of any assisted reproductive technology (ART) as each embryo is considered a single opportunity for live birth (*Garrido et al., 2011*). Previously, our team determined the likelihood of a couple achieving a live birth according to the number of embryos transferred (*Garrido et al., 2011*; *2012*) and the number of oocytes consumed (*Cobo et al., 2015*; *Cozzolino et al., 2021*; *Gil Juliá et al., 2021*). The CLBR according to the number of embryo transfers captures the additional probability of live birth as the couple undergoes consecutive transfers. The effect of sperm quality on embryos obtained, however, cannot be assessed as the number of embryos replaced in a single transfer is not known. The CLBR according to the number of embryos replaced provides more detailed information on the contribution of fresh and frozen embryos that were consecutively transferred until live birth was achieved.

Importantly, this measurement does not include embryos that were blocked during embryo development, which may underestimate the overall effect on the IVF/ICSI cycle. Therefore, CLBR per consumed oocyte averts this bias and evaluates the cycle's yield. This measurement per oocyte is useful in assessing how spermatozoa might influence ART success. Indeed, it indicates how many oocytes a patient needs to inseminate depending on the degree of paternal chromatin damage until a high probability of obtaining a live birth is achieved.

To date, no report has been published evaluating how sperm DNA fragmentation affects the cumulative probability of couples undergoing ART achieving a live birth. With this approach, the effect of SDF on the entire embryo cohort can be better addressed, providing a realistic estimation. The aim of the present study was to determine the influence of elevated SDF on live births rates by measuring the success of IVF/ICSI cycles per embryo transfer along with CLBR per number of embryo transfers, the number of transferred embryos and the number of oocytes required to obtain at least one live birth in couples with sperm chromatin damage.

MATERIALS AND METHODS

Study design

A retrospective multicentre cohort study was conducted using clinical data from couples who had a sperm DNA fragmentation test carried out between January 2000 and March 2019 at IVIRMA clinics in Spain.

Inclusion criteria were males with a SDF test via TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay who underwent ART (conventional IVF or ICSI) with their own fresh or frozen ejaculated spermatozoa using autologous oocytes. Indications for SDF measurement were males with very low sperm motility (genital tract infection discarded); males with long-term infertility with no known cause; couples with implantation failure; and couples who have experienced multiple miscarriages.

Analyses were conducted using two methods: in one approach, two groups were created for comparisons using 15% SDF as cut-off point (low ≤15% and high >15%) fragmentation based on published research (*Agarwal et al., 2020b*; *Green et al., 2020*). Participants were also categorized based on four SDF ranges: <10%, 10– <20%, 20–30% and, >30%, which were arbitrarily selected.

Semen analysis and preparation

Semen samples were collected in a sterile container by masturbation after

3–5 days of sexual abstinence. After 30 min of liquefaction (37°C, 5% CO_2), samples were evaluated for volume, sperm concentration and motility. Semen samples were prepared before IVF or ICSI using density gradient centrifugation (*Esbert et al., 2018*) or by swim-up (*Romany et al., 2014*) technique.

Sperm DNA fragmentation analysis

Sperm DNA fragmentation analysis was determined using an In-situ Cell Death Detection Kit (Roche Diagnosis, Barcelona, Spain) based on TUNEL assay at IVIRMA Madrid following the manufacturer's instructions. First, fresh samples were washed with phosphate-buffered saline (PBS) (Gibco; Invitrogen, Barcelona, Spain) and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate in PBS) for 2 min at 4°C. Second, samples were incubated for 1 h at 37°C with TUNEL reaction-mixture label solution and terminal transferase solution. A positive control was used for each experiment incubated with 3 IU/ml DNAase I recombinant in 50 mM Tris-HCl, pH 7.5 (Roche Diagnostics, Basel, Switzerland) for 15 min at 25°C to induce DNA fragmentation before labelling. A negative control without the enzyme solution was also used. Samples were washed twice by resuspension in PBS and centrifugation for 3 s at 7200xg, and PBS was added to bring the final volume to 1 ml. DNA breakup quantification was carried out on a minimum of 10,000 sperm cells. The proportion of spermatozoa with fragmented DNA was measured using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) cytometer until 2015, and then using a MACSQuant (Miltenyi Biotec, Bergisch Gladbach, Germany) cytometer (excitation: 488 nm; emission 510–520 nm).

IVF procedures

Ovarian stimulation procedures were carried out following protocols previously reported (*Garrido et al., 2004*; *Esbert et al., 2018*). After follicular aspiration, oocytes were inseminated by conventional IVF (drops containing capacitated spermatozoa and the cumulus–oocyte complex) or by ICSI introducing spermatozoa into the metaphase II oocyte as previously described (*Esbert et al., 2018*). All inseminated oocytes were incubated in a highly controlled environment (37°C, 5% CO_2, 6% O_2). Metaphase II oocytes that were not inseminated were vitrified following protocols previously described (*Martinez et al., 2014*) until subsequent warming for fertilization by ICSI.

Embryo evaluation and embryo transfer

Embryos were cultured until they reached the blastocyst stage on day 5. Day-3 cleavage-stage embryos were evaluated considering the following morphological parameters: cell number, symmetry, percentage of fragmentation and multinucleation. Embryos were classified into four categories (A–D) according to Association for the Study of Reproductive Biology (ASEBIR) guidelines (*Asebir, 2015*). All embryos classified as type A or B were considered optimal. Optimal cleavage-stage embryos rate was defined as the number of A and B embryos divided by the total number of correctly fertilized oocytes (two pronuclei [2PN]).

Blastocyst rate was defined as the number of embryos that reached blastocyst stage divided by the total number of 2PN. Blastocyst morphology was evaluated according to ASEBIR guidelines (2015), considering trophectoderm and inner cell mass quality. Blastocysts were scored from A to D according to embryologist criteria; type A and B embryos were considered optimal for transferring or for cryopreservation. The rate of good-quality blastocysts was calculated as the total number of A and B blastocysts divided by the total number of 2PN.

Embryo transfer took place between day 2 and day 7 of embryo development (fresh embryo transfer or frozen–thawed embryo transfer) based on patient need. Transferred embryos were chosen based on morphological quality criteria. The number of transferred embryos always complied with Spanish law.

Data collection

Medical data were obtained from patient clinical charts and included demographics and cycle characteristics. Biochemical pregnancy was confirmed if serum beta-HCG level was greater than 10 UI/l 14 days after fertilization. Implantation rate was calculated as the number of gestational sacs concerning the number of transferred embryos. Clinical pregnancy was considered positive with the detection of fetal heartbeat by transvaginal ultrasound at 5–7 weeks of gestation. Ongoing pregnancy was diagnosed by ultrasound in the first trimester (<week 12). Miscarriage was defined as the absence of intrauterine pregnancy after a positive beta-HCG. Clinical pregnancy loss was determined by spontaneous loss of pregnancy before gestational week 12. All clinical outcomes were measured per IVF/ICSI cycle. Live birth rate was determined by a delivery with at least one live newborn.

Statistical analyses

R Software (4.02 version. R Core Team, 2020) was used for statistical analysis. R is a language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was considered when $P < 0.05$. Means and proportions were calculated with corresponding 95% confidence intervals and compared using student's t-test, chi-squared test and Fisher's exact test. The odds ratio (95% CI) of the LBR was calculated in terms of SDF level, always compared with the lowest value, using Fisher's exact test.

Kaplan–Meier method was used to calculate CLBR according to the overall number of embryo transfers, embryos replaced and oocytes required to achieve live birth. Survival curve comparisons were made with the Mantel-Cox test. All embryo transfers carried out, and the total number of transferred embryos, including fresh and vitrified–warmed embryos consecutively transferred, if applicable, were included in the count. Further, the number of consumed oocytes that were correctly inseminated and resulted in transfer or non-viable embryos were summed until at least a live birth was achieved or patients declined further transfer (either no more embryos were available or patients abandoned treatment without achieving a live birth). Oocytes and embryos that were vitrified at the time of export or attempts to have a second child were not included in the final embryo count.

Multivariate regression analysis using a Cox proportional-hazards model was carried out to identify parameters associated with CLBR as the dependent variable and the number of embryo transfers, embryos replaced and inseminated oocytes as the independent variables. The following covariates were assessed: SDF, female and male ages, body mass index, semen state, insemination method, ovarian stimulation protocol and day of embryo transfer.

Receiver operating characteristic curve analysis was conducted to determine the predictive value of 15% SDF on the live births rates in IVF/ICSI cycles.

Ethical approval

Approval from the Ethical Committee of Instituto Valenciano de Infertilidad (IVI) Valencia, which regulates and approves database analysis for research at IVI, was obtained for this study (IRB reference number 1902-FIVI-027-NG) on 24 November 2020. Additionally, the project complies with the Spanish law governing assisted reproduction.

RESULTS

The cohort included 1339 couples who underwent a total of 2759 IVF/ICSI cycles involving 10,193 oocytes and 2850 transferred embryos (1786 were fresh and 1064 were cryopreserved embryos). The mean number of embryo transfers per patient was 1.4 and a mean of 1.4 embryos were replaced per embryo transfers. In total, 685 babies were delivered with at least one live birth (n = 820 newborns). The mean sperm DNA fragmentation in the study population was 8.8% (95% CI 8.5 to 9.1) (range 1–62%); 857 patients had low SDF, whereas 198 males were found to have high SDF.

Clinical characteristics of the cycles are presented in TABLE 1. The cause of female infertility was poor responders (42.1%), advanced maternal age

TABLE 1 BASELINE CHARACTERISTICS AND CLINICAL PARAMETERS OF THE STUDY POPULATION PER CYCLE ACCORDING TO SPERM DNA FRAGMENTATION GROUPS (≤15% VERSUS >15%)

Variable	SDF ≤15% (n = 2287)		SDF > 15% (n = 472)		*P-value*
	Mean or proportion (n)	95% CI	Mean or proportion (n)	95% CI	
SDF level, %	5.8 (2287)	5.7 to 5.9	23.7 (472)	23.0 to 24.4	<0.001[a]
Male age, years	39.1 (2238)	38.9 to 39.3	39.2 (453)	38.8 to 39.7	0.58[a]
Male BMI, kg/m^2	22.5 (2016)	22.4 to 22.7	23.0 (386)	22.7 to 23.3	0.01[a]
Female age, years	37.1 (2287)	37.0 to 37.2	37.1 (472)	36.8 to 37.4	0.82[a]
Female BMI, kg/m^2	22.6 (1628)	22.4 to 22.7	22.9 (301)	22.5 to 23.2	0.12[a]
AMH, ng/ml	2.5 (354)	2.3 to 2.8	1.8 (75)	1.5 to 2.1	<0.001 [a]
Antral follicle count	12.2 (1475)	11.9 to 12.5	12.2 (285)	11.4 to 13.0	0.95 [a]
Length of stimulation, days	10.9 (2276)	10.8 to 11.0	11.2 (469)	10.8 to 11.5	0.14 [a]
FSH total administered, IU	1639.0 (1548)	1610.6 to 1667.5	1630.5 (343)	1573.6 to 1687.4	0.79 [a]
LH total administered, IU	757.7 (443)	730.4 to 785.0	698.9 (91)	642.6 to 755.2	0.06 [a]
HMG total administered, IU	1329.1 (1360)	1292.4 to 1365.8	1348.5 (271)	1269.8 to 1427.3	0.65 [a]
Oestradiol level at HCG trigger, IU	1858.2 (2193)	1810.2 to 1906.1	1935.7 (457)	1833.7 to 2037.7	0.18 [a]
Progesterone level at HCG trigger, IU	0.6 (2116)	0.6 to 0.6	0.7 (413)	0.6 to 0.7	0.02 [a]
Days of endometrial preparation	8.2 (2112)	7.8 to 8.6	8.0 (445)	7.2 to 8.8	0.68 [a]
Endometrial preparation protocol, n (%)	–	–	–	–	0.61[b]
Stimulated cycle	1489/2277 (65.4)	63.5 to 67.4	313/468 (66.9)	62.6 to 71.2	–
Natural cycle	156/2277 (6.9)	5.9 to 7.4	35/468 (7.5)	5.1 to 9.9	–
Hormone replacement therapy	632/2277 (27.8)	26.0 to 29.6	120/468 (25.6)	21.7 to 29.6	–
Final endometrial thickness, mm	9.9 (2159)	9.8 to 10.0	9.8 (450)	9.6 to 10.0	0.25 [a]
Retrieved oocytes	10.8 (2287)	10.5 to 11.0	11.0 (472)	10.5 to 11.6	0.47 [a]
Oocyte state, n (%)	–	–	–	–	0.52[b]
Fresh	1905/2193 (86.9)	85.5 to 88.3	367/429 (85.5)	82.2 to 88.8	–
Mixed	168/2193 (7.6)	6.5 to 8.7	40/429 (9.3)	6.6 to 12.1	–
Vitrified	120/2193 (5.5)	4.6 to 6.5	22/429 (5.1)	3.0 to 7.2	–
Semen state, n (%)	–	–	–	–	0.03[b]
Fresh	2198/2278 (96.5)	95.8 to 97.3	440/467 (94.2)	96.6 to 99.2	–
Frozen	80/2278 (3.5)	2.8 to 4.3	27/467 (5.8)	3.7 to 7.9	–
Insemination method, n (%)	–	–	–	–	<0.001[b]
Conventional IVF	30/2287 (1.3)	0.8 to 1.8	9/472 (1.9)	0.7 to 3.1	–
ICSI	2008/2287 (87.8)	86.5 to 89.1	445/472 (94.3)	92.2 to 96.4	–
Conventional IVF + ICSI	249/2287 (10.9)	9.6 to 12.2	18/472 (3.8)	2.1 to 5.5	–

Values are mean or proportion (n) with 95% confidence interval, unless otherwise indicated.

P-value was calculated by [a] Student t-test or [b]Chi-squared test.

BMI, body mass index; HMG, human menopausal gonadotrophin; ICSI, intracytoplasmic sperm injection; IU, international units; SDF, sperm DNA fragmentation.

TABLE 2 SEMINAL PARAMETERS FROM THE NEAT EJACULATE PER CYCLE ACCORDING TO SPERM DNA FRAGMENTATION LEVEL

Variable	≤15% SDF (*n* = 2287)	95% CI	>15% SDF (*n* = 472)	95% CI	*P-value*[a]
SDF level, %	5.8 (2287)	5.6 to5.9	23.7 (472)	23.0 to 24.4	<0.001
Seminal volume, ml	2.9 (1542)	2.8 to 2.9	2.9 (316)	2.7 to 3.1	0.7
Seminal concentration, x10^6/ml	38.6 (1541)	26.9 to 40.3	25.2 (317)	22.2 to 28.2	<0.001
Total sperm progressive, %	37.8 (1404)	37.0 to 38.6	31.4 (266)	29.2 to 33.1	<0.001
Total sperm non progressive, %	11.4 (1402)	10.9 to 11.8	10.8 (266)	9.8 to 11.8	0.3
Total sperm immobile, %	50.8 (1404)	50.0 to 51.7	58.1 (266)	55.8 to 60.3	<0.001
Total progressive sperm count	46.7 (1531)	44.0 to 49.5	24.0 (309)	20.1 to 28.0	<0.001

Values are mean (*n*) with 95% confidence interval.

[a] Calculated by student's T-test.

SDF, sperm DNA fragmentation.

(15.8%), endometriosis (15.8%) and polycystic ovary syndrome (15.8%). Male infertility was diagnosed as follows: normozoospermia (32%), asthenoteratozoosermia (20%), oligoasthenozoospermia (16%), asthenozoospermia (8%), oligoasthenoteratozoospermia (8%), oligoteratozoospermia (8%) and teratozoospermia (8%). Seminal parameters are presented in TABLE 2.

Laboratory and clinical outcomes according to SDF category are presented in TABLE 3. Live birth rate per first embryo transfer was not statistically different between ≤15% and >15% SDF groups: 38.2% (95% CI 34.5 to 41.9; *n* = 665) versus 41.9% (95% CI 34.2 to 49.7; *n* = 155; OR 1.2, 95% CI 0.8 to 1.7; *P* = 0.4); LBR for all embryo transfers was 36.5% (95% CI 34.1 to 38.9; *n* = 1525) versus 39.9% (95% CI 34.6 to 45.3; *n* = 323; OR 1.2; 95% CI 0.9 to 1.5; *P* = 0.3), respectively.

Live birth rate considering SDF ranges were not significantly different for the first embryo transfer (*P* = 0.56): 38.2% (95% CI 34.1 to 42.4) in <10% (*n* = 526), 40.2% (95% CI 33.5 to 46.8) in 10–<20% (*n* = 209, OR 1.1; 95% CI 0.8 to 1.5; *P* = 0.6), 44.8% (95% CI 32 to 57.6) in 20–30% (*n* = 58; OR 1.3; 95% CI 0.7 to 2.4; *P* = 0.3), 29.6% (95% CI 12.4 to 48.9) in >30% (*n* = 27; OR 0.7; 95% CI 0.3 to 1.7; *P* = 0.4). Moreover, for all embryo transfers (*P* = 0.5): 36.4% (95% CI 34.5 to 38.3; *n* = 2435) 37.5% (95% CI 32.8 to 42.1; *n* = 415; OR 1.1, 95% CI 0.8 to 1.3; *P* = 0.7), 43.6% (95% CI 34.6 to 52.6; *n* = 117; OR 1.4, 95% CI 0.9 to 2; *P* = 0.1), 36.9% (95% CI 25.2 to 48.7; *n* = 65; OR 1.0, 95% CI 0.6 to 1.8; *P* = 1.0) for each group.

Cumulative live birth rates

When calculating CLBR according to the number of embryo transfers carried out until live birth was achieved (Supplementary Figure 1), no statistically significant differences were found between high or low fragmentation groups (*P* = 0.08): 40.7% (95% CI 37.3 to 43.9) versus 46.9% (95% CI 39.5 to 43.5) at first embryo transfer, 59.1% (95% CI 55 to 62.9) versus 66.4% (95% CI 56.9 to 73.9) at second embryo transfer, and 70% (95% CI 65.1 to 74.2) versus 77% (95% CI 65.9 to 74.2) with three embryo

TABLE 3 LABORATORY AND CLINICAL OUTCOMES PER CYCLE BASED ON ≤15% SPERM DNA FRAGMENTATION AND >15% SPERM DNA FRAGMENTATION

Variable	≤15% SDF (*n* = 2287)	95% CI	>15% SDF (*n* = 472)	95% CI	*P*-value
Laboratory outcomes					
Optimal cleavage embryo rate	24.7	21.5 to 28.0	26.7	19.1 to 34.2	0.6
Blastocyst rate	55.7	54.3 to 57.1	55.4	52.2 to 58.5	0.9
Good-quality blastocyst rate	27.7	26.5 to 28.9	27.4	24.6 to 30.2	0.9
Clinical outcomes					
Implantation rate	84 (744)	82.1 to 85.9	82.8 (175)	78.7 to 86.8	0.61
Mean number of sacs	1.2 (744)	1.2 to 1.2	1.2 (175)	1.2 to 1.3	0.64
Biochemical pregnancy rate	52.5 (1558)	50.0 to 55.0	59.0 (332)	53.7 to 64.3	**0.03**
Clinical pregnancy rate	46.5 (1558)	44.1 to 49.0	52.4 (332)	47.0 to 57.8	0.05
Ongoing pregnancy rate	37.7 (1551)	35.3 to 40.1	41.6 (332)	36.2 to 46.9	0.20
Miscarriage rate	5.1 (1538)	4.4 to 6.4	6.6 (332)	3.9 to 9.3	0.31
Clinical pregnancy loss rate	8.3 (1551)	6.9 to 9.6	10.8 (332)	7.5 to 14.2	0.17
Live birth rate	36.5 (1522)	34.1 to 39.0	40.0 (323)	34.6 to 45.3	0.26
Singleton	29.3 (1522)	27.0 to 31.6	32.5 (323)	27.4 to 37.6	–
Twins and triplets	7.3 (1522)	6.0 to 8.6	7.4 (323)	4.6 to 10.3	–

Values are presented as % (total *n*) unless otherwise indicated. *P*-values were calculated by Student's t-test.

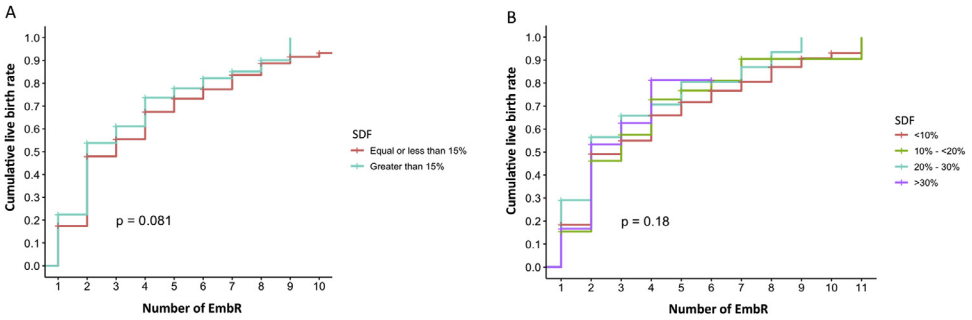

FIGURE 1 Kaplan–Meier curves for cumulative live birth rates (CLBR) according to the total number of embryos replaced in consecutive embryo transfer until live birth was achieved categorized by (A) low or high sperm DNA fragmentation (SDF) or by (B) SDF ranges. EmbR, embryos replaced.

transfers, reaching a CLBR of 86.7% (95% CI 78.9 to 91.6) versus 100% with five embryo transfers (FIGURE 1A).

If computed by SDF ranges, no statistically significant differences were found between groups (Supplementary Figure 1B). A Cox proportional-hazard model did not reveal a significant association between live birth rate and SDF grade (HR 1.2, 95% CI 1.0 to 1.6; $P = 0.1$). Receiver operator characteristic curve assessment showed non-relevant predictive power of SDF on live birth rate (AUC 56.7%, 95% CI 53.3 to 60.1; $P < 0.001$).

The CLBR according to the number of embryos replaced increased with the number of embryos transferred. After three embryos were replaced, the cumulative rate reached a value more than 50% (55.5%, 95% CI 51.5 to 59.2) in the ≤15% group and 61.2% (95% CI 52.3 to 68.5) in the >15% group. An increase of approximately 10% in both groups was seen with four embryos replaced (67.5% [95% CI 63.1 to 71.4] versus 73.7% [95% CI 63.8 to 80.9]). With eight embryos replaced, CLBR was 88.7% (95% CI 82.4 to 92.8) compared with 90.1% (95% CI 72.9 to 96.4) in lower and higher SDF groups (FIGURE 1A).

Results were similar when comparing CLBR by SDF ranges (<10% versus >30%), and CLBR reached 49.1% (95% CI 44.9 to 53) and 53.3% (95% CI 32 to 68) and 66% (95% CI 61 to 70.3) and 81.3% (95% CI 52.4 to 92.7) with two and four embryos replaced, respectively. These outcomes were not statistically significant (FIGURE 1B). Accordingly, no significant relationship between SDF and the live birth rate was observed with Cox model assessment (HR 1.2, 95% CI 0.9 to 1.5; $P = 0.3$). Receiver operator characteristic curve assessment showed an area under the curve of 56.8% (95% CI 53.5 to 60.2; $P < 0.001$) of SDF effect on live birth rate.

The cumulative probability of live birth based on the number of consumed oocytes required is presented in FIGURE 2, Supplementary Table 1 and Supplementary Table 2. The CLBR of the ≤15% and >15% fragmentation groups were: 43.3% (95% CI 39.6 to 46.9) versus 46% (95% CI 37.8 to 53) with five oocytes, 67.3% (95% CI 62.9 to 71.2) versus 68.3% (95% CI 59.2 to 75.3) with 10 oocytes, 74.3% (95% CI 69.8 to 78.3) versus 73.8% (95% CI 64.1 to 80.9) with 12 oocytes, 81.9% (95% CI 77.1 to 85.7) versus 87.3% (95% CI 75.9 to 93.3) with 15 oocytes, and 89.6% (95% CI 84.1 to 93.2) versus 96.6% (95% CI 79.6 to 99.4) with more than 20 oocytes.

The CLBR was analogous when plotted by SDF ranges, i.e. in <10% versus >30% groups: 43.5% versus 49.2% with five oocytes, 68.3% versus 77.4% with 10 oocytes, and 81.6% versus 88.7% with 15 oocytes. The Cox model analysis showed no significant relationship between live birth rate and SDF (HR 1.1, 95% CI 0.9 to 1.5; $P = 0.3$), consistent with the univariate analysis. The receiver operating characteristic curve assessment showed a low predictive power of SDF on live birth rate (AUC 52.3%, 95% CI 50.5 to 54.2; $P = 0.007$).

DISCUSSION

New sperm markers, such as sperm DNA integrity, are being evaluated as diagnostic tools to complement male fertility diagnosis owing to the low predictive value of basic semen analysis. Although these markers do not have a clear threshold able to discriminate between fertile and infertile males or successful or unsuccessful ART procedures, they may aid in predicting reproductive success. Evidence is insufficient to

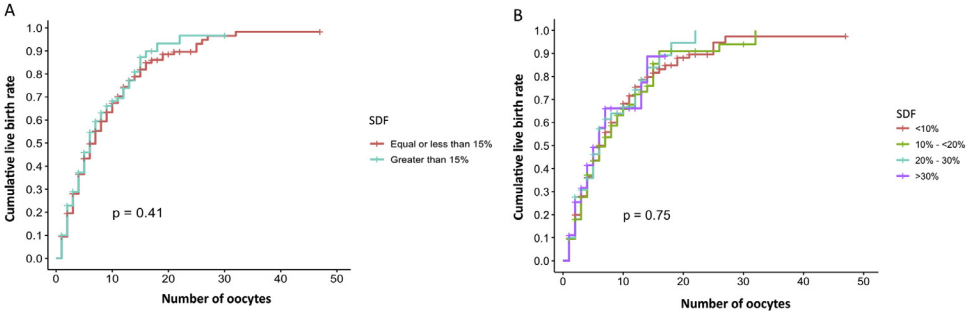

FIGURE 2 Kaplan–Meier curves for cumulative live birth rate based on the total number of consumed oocytes until live birth was achieved categorized by a (A) low or high Sperm DNA fragmentation (SDF) or by (B) SDF ranges.

establish SDF as a routine clinical tool when evaluating male factor infertility in couples undergoing ART (*Cissen et al., 2016*), but SDF is used to help determine why IVF and ICSI cycles are unsuccessful despite good semen quality. Our evaluation of SDF as a marker of sperm potential revealed that SDF above 15% measured by TUNEL assay did not reduce live birth rate or affect the cumulative probability of live birth in non-selected patients undergoing IVF/ICSI cycles using autologous oocytes. To our knowledge, this is the first report evaluating the effect of SDF by means of cumulative LBR from patients undergoing ART.

In the present study, the population was divided into two groups using a cut-off value of 15%. Comparing the seminal parameters according to SDF groups, higher seminal values of sperm concentration, motility and total sperm count were found in couples with low SDF. This finding is in line with previous reports suggesting that SDF is related to suboptimal semen parameters (*Belloc et al., 2014*; *Green et al., 2020*). Notwithstanding, both groups are within the normal standard semen parameters (*World Health Organization, 2010*).

The sperm cell plays an important role in the correct embryonic development at fertilization (*Amdani et al., 2015*), in the subsequent cell divisions (*Avidor-Reiss et al., 2020*) and in embryo competence (*Castillo et al., 2018*). If the spermatozoon presents fragmented chromatin, however, its effect might be evident after activation of the embryonic genome from the eight-cell stage and onwards, known as late paternal effect (*Tesarik et al., 2004*). Therefore, SDF has been correlated with poor blastocyst development (*Seli et al., 2004*; *Casanovas et al., 2019*) and lower quality rates (*Avendaño et al., 2010*; *Borges et al., 2019*) in IVF and ICSI cycles. Some investigators have related the presence of double stranded SDF to the impairment of embryonic development after ICSI, although this effect was not associated in the case of single stranded SDF (*Casanovas et al., 2019*). In this study we did not detect the deleterious effect of SDF on embryo formation and their quality at days 3 and 5 of development. Ambiguous data were also found in a systematic review and meta-analysis compiling 3226 IVF/ICSI cycles (*Zini et al., 2011*); 17 studies showed no relationship between SDF and embryo quality, development, or both, whereas 11 studies revealed an association between an elevated SDF and impaired embryo development. In these studies, however, the type of SDF was not differentiated (and specifically in this study) because the TUNEL assay does not allow distinguishing between single strand SDF and double strand SDF); therefore, the results may be underestimated.

Clinical studies and meta-analyses have evaluated the relationship between SDF and ART outcomes. One review (*Esteves et al., 2020*) described the discrepancy between studies that found a deleterious effect of SDF on pregnancy rates with an increased risk of miscarriage in IVF/ICSI cycles and others that reported no correlation between SDF and pregnancy outcome. Nevertheless, it is worth noting that heterogeneity in the design of studies and among the study population may be responsible for the different conclusions.

In our study population, no clinically relevant differences were identified between two groups in the IVF/ICSI outcomes evaluated. Notwithstanding, the presence of SDF has been strongly correlated with greater miscarriage rates (*Zini et al., 2008*; *Robinson et al., 2012*; *Zhao et al., 2014*) as a result of defective embryo development, although the underlying mechanism(s) are not known. This association was observed in the couples with >15% of SDF, but in a non-significant fashion. Moreover, it has recently been reported that the type of SDF, particularly double strand SDF, may differentially affect the probability of pregnancy loss in couples undergoing ICSI (*Ribas-Maynou et al., 2012*). Nevertheless, evidence is still limited on this issue, which should be extensively studied. Furthermore, the >15% group also showed higher pregnancy rates as well (significantly higher biochemical pregnancy rate), which likely may account for greater pregnancy losses. A slightly higher LBR than their counterparts with lower DNA damage was also found. Therefore, the SDF effect on miscarriages is weakened.

The effect of SDF on live births was appraised, as this is the primary objective of ART. A negative association was found between SDF and LBR in 230 IVF patients and was particularly enhanced in men with idiopathic infertility. This effect was not observed in ICSI cycles among 136 couples (*Simon et al., 2013*). *Osman et al. (2015)* were the first to evaluate this in a meta-analysis, reporting a significantly better LBR in men with low SDF compared with those with a high SDF in IVF cycles (RR 1.27, 95% CI 1.1 to 1.5; $P = 0.01$). In contrast, only a minor significant relationship was found after ICSI (RR 1.11, 95 CI 1 to 1.2; $P = 0.04$) (*Osman et al., 2015*). The meta-analysis by *Deng et al. (2019)* (10 studies, 1785 couples) showed that LBR was not statistically different between the low and high SDF group (RR 0.87, 95% CI 0.8 to 1.1; $P = 0.2$), although couples with high SDF had a higher risk of miscarriage, lower rate of good-quality embryos and lower clinical pregnancy rate. A plausible explanation of the different effect size of SDF on IVF or ICSI cycles, and which can also be involved in the results of this study, may be due to the prior choice of spermatozoa to be microinjected according to their motility and morphology for ICSI (associated with a reduction in single strand SDF (*Lara-Cerrillo et al., 2021*), and the gametes are not subjected to extended in-vitro culture time during conventional IVF reducing the exposure to oxidative stress damage (*Lewis, 2013*).

In the present study, live birth rates per first embryo transfer or per all embryo transfers were not impaired in couples with SDF >15% but they were similar between both groups. Importantly, we used CLBR as a novel approach with the aim of evaluating sperm performance instead of just comparing the best embryos obtained that were transferred. We propose that knowing how many embryos and oocytes a couple needs to use if they have high or low SDF will aid in the clinical management of these couples and improve their chances of achieving a live birth in the long term. In both approaches, we did not observe reduced probability of achieving a live birth in patients with elevated SDF. Moreover, these couples presented a higher CLBR compared with men with lower SDF when using an equal number of embryos or oocytes. This suggests that these men with sperm DNA fragmentation have the same likelihood of success as a couple without SDF.

This finding is reinforced by multiparametric analysis, adjusted for potentially confounding variables, which corroborated the limited effect of high SDF on live births. In this case,

controlling for significant confounders, like the insemination method (IVF or ICSI), female and male age, and day of embryo transfer (day 3 or day 5), is imperative when evaluating the SDF influence on reproductive outcomes after IVF/ICSI for their determinant. On the other hand, a threshold of 15% by TUNEL assay presented a scarce predictive capacity of live birth by TUNEL assay in the receiver operator characteristic curve analyses carried out (*Esbert et al., 2011*). It has been reported that a more sensitive and specific cut-off value to discriminate male infertility by TUNEL assay would be between 17% and 20%, and different cut-off values need to be established to determine the reproductive success of ART (*Esteves et al., 2020*).

In the present study, a 15% of SDF threshold was chosen to for a balanced statistical analysis in both groups owing to the average value of the male population evaluated (around 9%). Moreover, this threshold is within the range of intervals (10–36%) that have been previously published for the TUNEL assay for ART (*Esteves et al., 2020*), although at present this cut-off value may not be the most predictive of reproductive success. Nevertheless, limiting the analysis to a single cut-off point may not reveal the real effect of SDF, as it would be over-or underestimated in some cases. In addition, it is difficult to define the fertility or infertility status from a simple universal numerical value because reproductive biology is a complex process in which a multitude of parameters are involved. For this reason, the study population was also divided into four groups to enable comparison of the degree of SDF effect on the cumulative live birth rate in a more representative manner, from very low values (<10%) to higher values (>30% SDF).

Other factors that should be considered to understand the controversy around SDF's performance on reproductive outcomes includes the different methods to measure SDF (*Ribas-Maynou et al., 2013*) and lack of universal threshold value for discriminating male infertility and determining reproductive success (*Duran, 2002*; *Henkel et al., 2004*; *Sergerie et al., 2005*; *Avendaño et al., 2010*). The TUNEL assay is a direct and objective method with high sensitivity and reliability to measure DNA fragmentation in a large number of cells using flow cytometry or fluorescent microscopy despite a lack of standardized universal protocols (*Agarwal et al., 2020b*; *Ribeiro et al., 2017*). This test is capable of detecting single strand SDF and double strand SDF indistinctively, giving the global SDF (*Sharma et al., 2020*). This could, however, lead to a disadvantage compared with alkaline comet assay, which is the only test capable of discerning specifically the two types of SDF that could be contributing to the failure of IVF/ICSI cycles differently (*Ribas-Maynou and Benet, 2019*; *Agarwal et al., 2020a*), and possess the highest predictive value of male infertility (*Ribas-Maynou et al., 2013*).

In addition, the effect of damage from fragmented paternal chromatin depends in part on the quality of the oocyte, although this capacity is reduced by older age (*Setti et al., 2021*). Female age plays a key role in human reproduction and is one of the major confounders that should be considered in ART evaluation, and, indeed, whenever the effect of SDF is clinically assessed. The mean female age was 37 years and was the same in both groups, so oocyte quality was assumed to affect reproductive success equally. We considered it in the Cox regression model applied that evaluates the effect of SDF over the probability of obtaining a live birth after adjusting for other significant variables. The results showed no significant risk in conjunction with the degree of sperm fragmentation assessed (>15%) in the CLBR per consumed oocyte.

Despite the novelty of this study, it is not without limitations. The retrospective design of our study allows us to analyse a large amount of clinical data; however, uncontrolled biases have arisen from clinical practice over the years. One of them is the sperm preparation technique used: density gradients or swim up. The effect that both procedures could have on the proportion of spermatozoa with SDF after preparation is still controversial, with references to both swim up (*Muratori et al., 2019*; *Zini et al., 2000*) and density gradients (*Amiri et al., 2012*; *Xue et al., 2014*) reducing damage to sperm chromatin integrity.

Another important point to consider is that, in the present study, the outcomes have not been analysed separately according to the ART used (conventional IVF or ICSI technique). Despite the evidence that the effect of SDF is different in IVF outcomes than in ICSI outcomes (*Simon et al., 2013*; *Osman et al., 2015*; *Lara-Cerrillo et al., 2021*), our intention was to understand how both treatments were affected depending on the degree of SDF over many years of clinical practice. The multivariate analysis of the CLBR, however, was adjusted for the insemination technique used as an important confounder that may be conditioning the relationship between SDF and CLBR. Oocyte preservation protocols (both slow freezing and vitrification) have varied during the study time evaluated. The possible negative effect of slow freezing until the incorporation of vitrification on reproductive outcomes should be considered as a limitation as this has not been assessed in the multivariate analysis.

Finally, another limitation is that SDF was measured on a semen sample taken before and not in the IVF/ICSI cycle, considering the given SDF degree as the one that men would have present on the day of insemination, despite knowing that the SDF may vary over time as a result of several factors (*Erenpreiss et al., 2006*). We usually carry out the sperm DNA fragmentation testing in unfractionated sperm samples because it is a preliminary diagnostic tool that provides additional information about semen quality after poor results are attained in a previous ART or based on individual patients' requirements. In our centres, SDF is not routinely measured in the semen sample before IVF/ICSI treatment, although the time elapsed between the SDF analysis and the start of the cycle is approximately between 1 and 3 months. Nonetheless, because these techniques are invasive for the sperm cells, the levels of DNA damage in the fertilizing spermatozoa used in ART cannot every be known. Therefore, the SDF level is the representative mean of the given sperm population evaluated. Nevertheless, SDF was measured under the same protocol and by the same technical methods, avoiding inter-laboratory variations.

In conclusion, we observed that sperm DNA fragmentation (measured by TUNEL assay) neither disrupts live birth rates nor reduces the cumulative probabilities of obtaining a live birth among unselected patients undergoing IVF/ICSI cycles with autologous oocytes. This innovative approach allows us to follow up patient outcomes over time according to the

number of embryo transfers, transferred embryos and consumed oocytes, which is measured as opportunities to achieve a live birth in the long term, depending on the sperm DNA fragmentation grade. The systematic and generalized evaluation of SDF in all infertile males is not suitable for predicting a live birth, but these results are useful for clinicians who need to inform patients accurately and individually on their likelihood of achieving a live birth after an ART. Studies on patients suspected of suffering from poor reproductive outcomes related to sperm DNA fragmentation should be critically and carefully conducted.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.02.010.

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