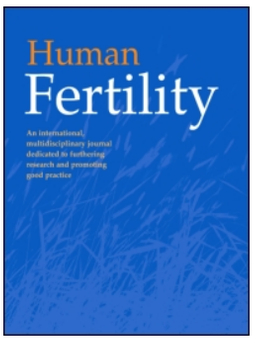

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ORIGINAL ARTICLE

Sperm DNA fragmentation on the day of fertilisation is not associated with assisted reproductive technique outcome independently of gamete quality

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ABSTRACT

The evaluation of sperm DNA fragmentation has been postulated as a predictive molecular parameter of the semen fertilising potential, as well as the ability to give rise to a healthy embryo and an ongoing pregnancy. However, there are controversial results due to oocyte quality, the use of different measurement techniques and interpretation criteria. Our objective is to investigate if sperm DNA fragmentation on the day of fertilisation influences *in vitro* fertilisation (IVF) outcome in a prospective double-blind study. Three groups of patients were defined: (i) 68 couples undergoing intracytoplasmic sperm injection (ICSI) due to severe male factor with normal ovarian response (NOR); (ii) 113 couples undergoing conventional in vitro fertilisation (IVF) in our oocyte donation programme due to ovarian failure; and (iii) 150 low ovarian response (LOR) patients undergoing ICSI or IVF. TUNEL assay was performed from an aliquot of each capacitated semen sample to detect DNA fragmentation. There was no relationship between blood serum β-hCG positive test, clinical pregnancy and first trimester miscarriage with DFI levels in NOR ($p = 0.41$, $p = 0.36$, $p = 0.40$), recipient ($p = 0.49$, $p = 0.99$ and $p = 0.38$) and LOR ($p = 0.52$, $p = 0.20$, $p = 0.64$) groups of patients, respectively. Therefore, ART outcomes are not affected by sperm DNA fragmentation independently of gamete quality.

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KEYWORDS
Sperm DNA fragmentation; TUNEL; in vitro fertilisation; Donor oocyte; Low ovarian response; Male infertility

Introduction

Infertility affects approximately one out of six couples who try to conceive (Zhao et al., 2014). While the essential function of a human spermatozoa is to deliver the entire paternal genome to the oocytes, a prerequisite for ensuring normal fertilisation (Agarwal & Said, 2003), it is also attributed an important role in embryonic development (Álvarez-Sedó et al., 2017; Lewis et al., 2013). Therefore, sperm DNA integrity is indispensable in the success of human reproduction (Agarwal et al., 2017).

In the last few years, sperm integrity tests have been developed to assess DNA damage in addition to poorly predictive standard seminal parameters (sperm concentration, motility and morphology) used in the evaluation of male infertility (Evenson & Wixon, 2006; Lewis et al., 2008; Li et al., 2006; Simon et al., 2016). Furthermore, it has been reported that sperm DNA fragmentation levels are significantly higher in infertile couples (Santi et al., 2018), even in those which do not present male factor (Borges et al., 2019).

Although these diagnostic tests are widely used, there are no standardised protocols that can lead to universally accepted clinical thresholds (Majzoub et al., 2017). In fact, there is no strong or direct correlation between DNA damage determined by terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and sperm parameters defined by the World Health Organisation (Cohen-Bacrie et al., 2009; World Health Organisation, 2010). Moreover, the association between pregnancy in conventional *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) cycles and sperm DNA fragmentation is not strong enough to provide a clinical indication for routine use of these tests in male infertility evaluation (Pacey, 2018).

Poor inclusion criteria for recruited couples, different sperm population used for DNA fragmentation tests (unprocessed semen or selected sperm) and different techniques used to evaluate DNA damage (Simon et al., 2016) are some of the main causes ascribed to this controversy (Muratori et al., 2008). However, nine factors have been described as representing parameters involved in the predictive value of DNA fragmentation testing (Sakkas & Alvarez, 2010), taking special importance in the type of DNA damage occurred. New studies are needed in order to identify

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possible subgroups in which such diagnostic tests might be more valuable (Cunha-Filho, 2017).

Therefore, we tried to ascertain if there is a real effect of sperm DNA fragmentation detected by the TUNEL assay on the day of fertilisation on assisted reproductive outcomes in three specific groups of patients: (i) couples undergoing ICSI due to poor sperm parameters with normal ovarian response (NOR); (ii) couples undergoing conventional IVF with oocyte donation due to ovarian factor; and (iii) couples undergoing ICSI or IVF with low ovarian response (LOR).

Materials and methods

Study population

From April 2011 to July 2014, a total of 331 couples were included in our prospective double-blind study (Figure 1). The work received approval from our Ethics Committee (ref:03/2011). Patients were informed and signed a consent form.

Sixty-eight couples underwent ICSI due to severe male factor (oligo and/or astheno and/or teratozoospermic men (WHO, 2010). Inclusion criteria were: (i) >1 million spermatozoa/mL in the semen sample in order to assure a sufficient number of spermatozoa to carry out the TUNEL technique; (ii) maternal age <38 years; and (iii) at least 6 mature oocytes (metaphase II, MII) to perform ICSI.

In addition, 113 couples underwent conventional IVF within our oocyte donation program due to ovarian failure related to advanced maternal age. Inclusion criteria were: (i) normal uterine cavities after ultrasound; (ii) nomozoospermic semen samples (World Health Organisation, 2010); (iii) at least 6 good oocyte-corona-cumulus (OCC) complexes to perform insemination; and (iv) endometrial thickness in the day of IVF ≥ 7 mm.

Finally, our third study group included 150 LOR patients according to the Bologna criteria, where at least two of the following three features must be present: (i) advanced maternal age or any other risk factor for LOR; (ii) a previous LOR; or (iii) an abnormal ovarian reserve test (Ferraretti et al., 2011). These patients were included regardless of the indication for the assisted reproduction technique, conventional IVF or ICSI.

Nevertheless, frozen seminal samples, testicular biopsies, donor seminal samples or cases with preimplantation genetic diagnosis (PGD) were not included in any of the study groups.

Ovarian stimulation and endometrial preparation

For controlled ovarian hyperstimulation (COH), conventional protocols with gonadotropin-releasing hormone (GnRH) agonist or antagonist were used in NOR and LOR patients. In donors, only antagonist protocol and agonist triggering were used according with the usual protocol in our institution. All the embryo transfers evaluated in the study were performed in fresh cycles. In recipients, conventional hormonal replacement was used to prepare the endometrium (Bernabeu et al., 2006).

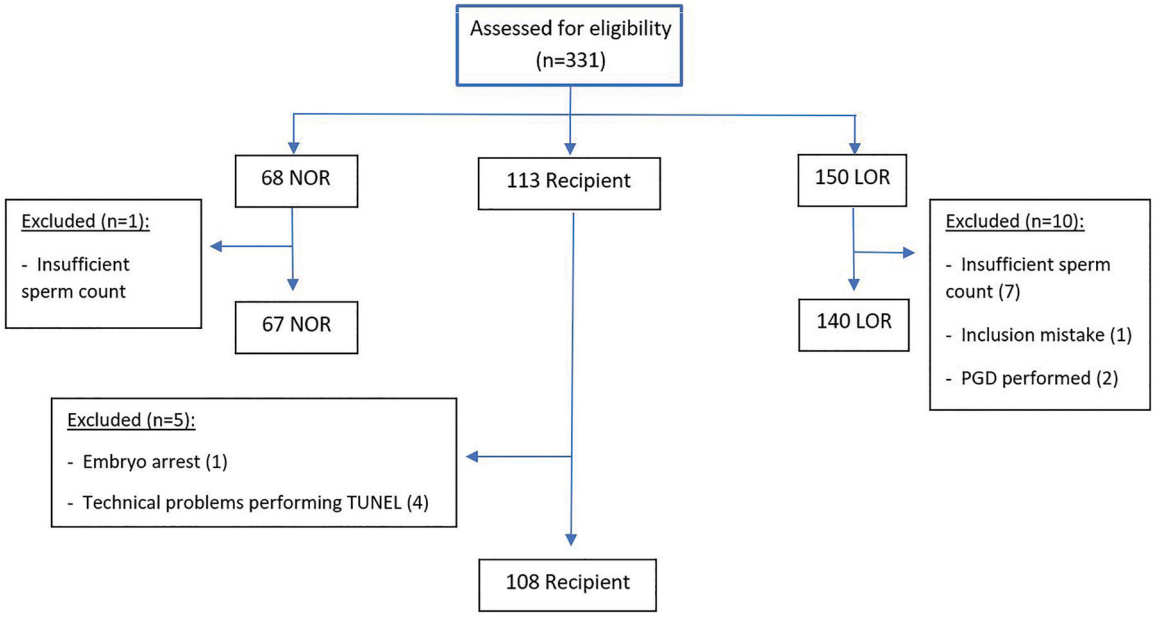

Figure 1. Flow chart for study participants in each patient group: Normal Ovarian Response (NOR), donor oocyte recipient and Low Ovarian Response (LOR).

Sperm and oocyte preparation

Semen samples were collected by masturbation, after a recommended period of 3–5 days of sexual abstinence. Semen analyses (concentration, sperm motility and morphology) were performed according to World Health Organization (2010). The semen samples were prepared at room temperature with a discontinuous density gradient method using two 1-mL layers of PureSperm® (Nidacom International AB, Goteborg, Sweden): 80% and 40%. A total of 1 mL of semen was deposited over the 40% layer. The gradient was then centrifuged at 300 g for 20 min. After centrifugation, the 80% layer was collected and washed with 5 mL of Sperm Rinse® (Vitrolife, Göteborg, Sweden) at 400 g for 10 min. The sperm pellet was suspended in IVF medium (IVF®; Vitrolife, Göteborg, Sweden) at 37 °C in an atmosphere of 6% CO_2 and 5% O_2.

Following retrieval, all oocyte-corona-cumulus (OCC) complexes were washed in buffer (G-MOPS®; Vitrolife, Göteborg, Sweden) and deposited in IVF medium at 37 °C in an atmosphere of 6% CO_2 and 5% O_2. In case of oocytes undergoing ICSI, surrounding cumulus and corona radiata cells were removed by a brief exposure to 80 IU/ml of hyaluronidase (Hyase®; Vitrolife, Göteborg, Sweden) followed by gentle pipetting, and then oocytes were rinsed in G-MOPS. Oocytes with a first polar body (MII) were selected for microinjection.

In the IVF group, the OCC complexes were distributed in IVF four-well dishes (Nunc™, Thermo Fisher Scientific, Roskilde Site, Denmark), with a maximum of four complexes per well.

ICSI and IVF procedures

MII oocytes were microinjected between 3–5 h after oocyte retrieval. Immediately after ICSI, oocytes were cultured in IVF medium individually in 30 µL drops covered with 3 mL of sterile equilibrated mineral oil (Ovoil®; Vitrolife, Göteborg, Sweden) at 37 °C in an atmosphere of 6% CO_2 and 5% O_2.

For IVF, donor oocytes were inseminated 3–5 h after recovery with 150,000 progressive motile spermatozoa (Grade a) per well. After insemination, each well was covered with 300 µL of mineral oil.

Fertilisation and embryo development

Fertilisation was assessed 16–18 h after insemination or microinjection (day 1). Oocytes were considered fertilised when they contained two pronuclei. Fertilisation rate was defined as the ratio between fertilised oocytes and the number of mature oocytes inseminated or microinjected.

Zygotes were cultured in G1.3™ medium (Vitrolife AB, Kungsbacka, Sweden) individually in 30 µL drops, with a maximum of 6 per dish, covered with 3 mL of mineral oil at 37 °C in an atmosphere of 6% CO_2 and 5% O_2. Embryo morphology was evaluated under an inverted microscope after 67–71 h (day 3) and 112–120 h (day 5, long culture), according to the recommendations of the Spanish Association for the Study of the Reproductive Biology (ASEBIR) (Ardoy et al., 2008). For long cultures, on day 3, the embryos were transferred to 30 µL drops of medium CCM (Vitrolife, Göteborg, Sweden) under mineral oil at 37 °C in an atmosphere of 5.5% CO_2 and 5% O_2. The rate of blastocyst formation was calculated dividing the total number of blastocysts formed on day 5 by the total number of embryos transferred to CCM medium on day 3.

Embryo quality classification was established in four categories (A-D) according to Ardoy et al. (2008). Type A and B embryos were considered good quality embryos, and, if possible, were transferred to the uterus on days 3 or 5 (long culture) using ultrasound guidance and a soft catheter (Rocket Medical, Washington, USA). After transfer, the remaining embryos exhibiting good quality (types A and B) were cryopreserved.

Sperm preparation for the study of DNA fragmentation

For both IVF and ICSI procedures, an aliquot (30 µL) of each capacitated and incubated sperm suspension was dropped onto slides and air-dried in the IVF laboratory. Then, a code was assigned to each sample, and the slides were given to our molecular and genetic laboratory in order to perform the TUNEL assay.

Measurement of DNA fragmentation by TUNEL assay

DNA fragmentation was measured by TUNEL assay using the *in situ* Cell Death Detection kit with fluorescein isothiocyanate (FITC)-labelled dUTP (Roche). Briefly, the slides were incubated in TUNEL reaction mixture in the dark at 37 °C for 1 h. Spermatozoa with fragmented DNA were detected in an epifluorescence microscope with a x100 oil immersion objective. For quantitative evaluation, a minimum of 500 spermatozoa were evaluated for each sample. The percentage of TUNEL-positive spermatozoa was referred to as the DNA fragmentation index (DFI). Sperm capacitated samples were considered as normal when DFI was under 15% (Lewis et al., 2008). The slides were analysed in

duplicate and blindly by two molecular biologists, to ensure the level of reproducibility of the TUNEL assay.

Double-blind study

The embryologists correlated the code of each slide with the assisted reproductive techniques (ART) information (semen parameters, fertilisation, embryo characteristics, β-hCG positive test, clinical pregnancy and first trimester miscarriage). They were blind to the TUNEL assay results. This database was sent to the clinical coordinator of the study (JLL).

Conversely, the molecular biologists correlated the cited codes with the DFI results. They were blind to the ART information. They sent these data to JLL, and once all the information was sent to the coordinator, statistical analysis was undertaken.

Clinical outcome variables

Our main clinical outcomes were: β-hCG positive test, clinical pregnancy and first trimester miscarriage. Serum β-hCG positive test was defined as the blood determination of >5 mIU/mL of beta human chorionic gonadotropin after 10–15 days post embryo-transfer. Clinical pregnancy was defined as the occurrence of at least one ultrasound-confirmed gestational sac in the uterus 4–6 weeks after the ART. First trimester miscarriage was defined as the loss of a clinical pregnancy before completing 20 weeks of gestational age (18 weeks after fertilisation).

Statistical analysis

Analysis of the correlation between a continuous variable (DFI) with fertilisation and blastocyst characteristics was performed in those groups of patients that were transferred on day 5, NOR and recipients, using the Pearson correlation coefficient. We performed natural log (ln) transformation of DFI to normalise its asymmetric distribution.

In each group of patients (NOR, Recipients and LOR), the Mann-Whitney test was performed between a continuous and independent variable (DFI) and categorical variables such as β-hCG positive test (yes/no), clinical pregnancy (yes/no) and first trimester miscarriage (yes/no). In the LOR group the relationship between our variables and the DFI was also analysed using an ANOVA test, establishing different DFI thresholds. In addition, the relationship between the DFI and an ordinal variable such as embryo quality was analysed using the Spearman correlation coefficient.

All tests were two-tailed and the level of statistical significance was set at 0.05. Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL).

Results

DFI and NOR patients after ICSI

Data from 67 couples were included in the analysis. In one sample, it was impossible to count a sufficient number of spermatozoa in the TUNEL assay (minimum of 500), so the data was not included. Semen samples of all subjects were found to be abnormal according to the World Health Organisation (2010) criteria. The mean maternal age was 33.7 ± 3.7 and the mean number of embryos transferred was 2.1 ± 0.5. Fifty patients were transferred on day 3. The other 17 cycles underwent long cultures with embryo transfers on day 5. The median DFI value of the subjects of the study was 3.5%, with a minimum of 0 and a maximum of 33.5%. In addition, the samples had an average concentration of 14.2 ± 10.5 million spermatozoa/mL and a total of 9.6 ± 7.3 million/mL progressively motile sperm. Regarding laboratory results, the fertilisation rate was 54.4% and the blastocyst formation rate was 50.8%. Finally, the global clinical pregnancy rate was 55.9%, with a first trimester miscarriage of 7.9%. These results are shown in Table 1.

Fertilisation rate, the number of good quality embryos (A plus B), number of embryos transferred and blastocyst formation rate on day 5 were not affected by DFI (Table 2). There was no relationship

Table 1. Descriptive and clinical data of the normal ovarian response (NOR) group of patients ($n = 67$).

Parameter	Value
Maternal age (mean ± SD)	33.7 ± 3.7
Paternal age (mean ± SD)	37.3 ± 4.8
Median DFI (%)	3.5
Sperm count (millions/mL) (mean ± SD)	14.2 ± 10.5
Sperm motility (millions/mL) (mean ± SD)	9.6 ± 7.3
Number of oocytes retrieved (mean ± SD)	13.3 ± 6.7
Fertilisation rate (%)	54.4
Blastocyst formation rate (BFR) (%)	50.8
Number of embryos transferred (A + B) on day 5 (mean ± SD)	2.1 ± 0.5
Clinical pregnancy rate (%)	55.9
1st trimester miscarriage rate (%)	7.9

Table 2. Correlation coefficients between DNA fragmentation index (DFI) and semen quality, fertilisation, embryo characteristics and implantation in patients undergoing ICSI (univariate analysis, $n = 67$).

	DFI	
	r	*p* Value
Fertilisation rate	0.11	0.39
Total embryos grade A + B	0.08	0.54
Number of transferred embryos	0.04	0.76
Rate of blastocyst formation on day 5[a]	−0.05	0.86

[a] $n = 17$ subjects.

Table 3. Differences between DFI level (mean ± SD) for the clinical variables according to each study group.

	β-hCG positive test			Clinical pregnancy			First trimester miscarriage		
DFI level	Yes	No	p^*	Yes	No	p^*	Yes	No	p^*
NOR ($n = 67$)	4.68 ± 2.87	3.08 ± 1.15	0.41	3.91 ± 2.85	3.13 ± 1.23	0.36	3.85 ± 2.48	2.91 ± 1.96	0.40
Recipient ($n = 108$)	2.98 ± 2.01	4.22 ± 2.24	0.49	3.07 ± 1.96	2.62 ± 2.70	0.99	2.42 ± 1.34	4.75 ± 2.24	0.38
LOR ($n = 140$)	4.68 ± 3.35	6.89 ± 2.49	0.52	5.11 ± 2.63	5.74 ± 3.93	0.20	4.14 ± 3.44	6.64 ± 2.67	0.64

*Mann–Whitney U test.

between serum β-hCG positive test (yes/no) ($p = 0.41$), clinical pregnancy (yes/no) ($p = 0.36$) or first trimester miscarriage (yes/no) ($p = 0.40$) and DFI levels (Table 3).

DFI and recipient patients after IVF

Data from 108 couples were included in the analysis. Embryo transfer was cancelled in one patient due to an embryo arrest. Another four samples were not processed for TUNEL assay due to technical problems. The mean paternal age was 42.7 ± 5.9 and semen samples of all their partners were found to be normozoospermic according to WHO (2010) criteria. Mean maternal age was 40.0 ± 5.1, with a mean donor age of 25.3 ± 3.3. The mean number of embryos transferred was 1.9 ± 0.4. Fifty-four patients were transferred on day 3, and the others ($n = 54$) on day 5. The median DFI value of the subjects of the study was 3.5%, with a minimum of 0 and a maximum of 52.0%. In addition, the samples had an average concentration of 71.1 ± 41.0 million spermatozoa/mL and a total of 54.6 ± 11.2 million/mL progressive motile sperm. Regarding laboratory results, the fertilisation rate was 69.9% and the blastocyst formation rate was 70.5%. Finally, the global clinical pregnancy rate was 53.2% with a first trimester miscarriage rate of 25.4%. These results are shown in Table 4.

Fertilisation rate, the number of good quality embryos (A plus B), number of embryos transferred and blastocyst formation rate on day 5 were not affected by DFI (Table 5). There was no relationship between serum β-hCG positive test (yes/no) ($p = 0.49$), clinical pregnancy (yes/no) ($p = 0.99$) or first trimester miscarriage (yes/no) ($p = 0.38$) and DFI levels (Table 3).

Table 4. Descriptive and clinical data of the donor oocyte recipient group of patients ($n = 108$).

Parameter	Value
Maternal age (mean ± SD)	40.0 ± 5.1
Oocyte donor age (mean ± SD)	25.3 ± 3.3
Paternal age (mean ± SD)	42.7 ± 5.9
Median DFI (%)	3.5
Sperm count (millions/mL) (mean ± SD)	71.1 ± 41.0
Sperm motility (millions/mL) (mean ± SD)	54.6 ± 11.2
Number of oocytes donated (mean ± SD)	12.2 ± 4.1
Fertilisation rate (%)	69.9
Blastocyst formation rate (BFR) (%)	70.5
Number of embryos transferred (A + B) on day 5 (mean ± SD)	1.9 ± 0.4
Clinical pregnancy rate (%)	53.2
1st trimester miscarriage rate (%)	25.4

Table 5. Correlation coefficients between DFI and semen quality, fertilisation, embryo characteristics and implantation in patients undergoing IVF with oocyte donation (univariate analysis, $n = 108$).

	DFI	
	r	p Value
Fertilisation rate	−0.16	0.09
Total embryos grade A + B	−0.07	0.50
Number of transferred embryos	−0.08	0.41
Rate of blastocyst formation on day 5[a]	−0.07	0.62

[a]$n = 54$ subjects.

Table 6. Descriptive and clinical data of the low ovarian response (LOR) group of patients ($n = 140$).

Parameter	Value
Maternal age (mean ± SD)	37.4 ± 4.7
Paternal age (mean ± SD)	39.2 ± 6.3
Median DFI (%)	5.8
Sperm count (millions/mL) (mean ± SD)	37.1 ± 21.2
Sperm motility (millions/mL) (mean ± SD)	22.6 ± 13.4
Number of oocytes retrieved (mean ± SD)	4.4 ± 3.1
Fertilisation rate (%)	66.5
Number of progressive embryos on day 3 (mean ± SD)	2.9 ± 1.9
Number of embryos transferred (A + B) on day 3 (mean ± SD)	1.8 ± 1.2
Clinical pregnancy rate (%)	28.3
1st trimester miscarriage rate (%)	2.9

DFI and LOR patients after ICSI/IVF

Data from 140 couples were included in the analysis. It was not possible to assess the DFI using the TUNEL technique in seven couples, two cycles had PGD and one was a donation cycle included by mistake, so these data were not analysed. Mean paternal age was 39.2 ± 6.3. In contrast to the two previous patient groups, the quality of the semen samples was not taken into account as an inclusion criteria. Semen samples had an average concentration of 37.1 ± 21.2 million spermatozoa/mL and a total of 22.6 ± 13.4 million/mL progressive motile sperm. The median DFI value of the subjects of the study was 5.8%, with a minimum of 0 and a maximum of 63.8%. Mean maternal age was 37.4 ± 4.7, the average number of oocytes obtained was 4.4 ± 3.1, the fertilisation rate was 66.5% and the mean number of embryos transferred was 1.8 ± 1.2 on day 3 of embryo development. The global clinical pregnancy rate was 28.3%, with a first

trimester miscarriage of 2.9%. The results are shown in Table 6.

There was no relationship between serum β-hCG positive test (yes/no) ($p = 0.52$), clinical pregnancy (yes/no) ($p = 0.20$) or first trimester miscarriage (yes/no) ($p = 0.64$) and DFI levels (Table 3). It was also not possible to find statistically significant differences between mean DFI value in the group of patients with a positive pregnancy test (mean DFI = 4.95%) and those where embryo implantation did not occur (DFI = 6.02%) ($p = 0.52$) (data not shown).

Moreover, a DFI threshold of 15% was established to distinguish between normal samples, with DFI <15% ($n = 113$) and altered DFI ≥ 15% ($n = 10$). However, using this cut off there were no significant differences between the two groups and our study variables (Table 7).

In addition, two groups were established according to the reference threshold (DFI mean) of the LOR patients group (DFI = 5.8%). We differentiated between normal samples with DFI < 5.8% ($n = 63$) and altered samples with DFI ≥ 5.8% ($n = 60$). As in the previous case, there were not statistically significant differences between these two groups using our variables (Table 8). Both groups were comparable in terms of maternal age, number of collected oocytes, fertilisation and embryo quality.

Finally, and using the Spearman correlation coefficient, it was also not possible to establish any significant association between the TUNEL value for sperm DNA fragmentation and embryo quality in the *in vitro* fertilisation laboratory ($p = 0.78$) (data not shown).

Discussion

In this prospective cohort study, sperm DNA fragmentation values from aliquots of the same spermatozoa used for IVF or ICSI were not associated with any significant variable related to ART, mainly pregnancy and miscarriage outcomes.

Table 7. Clinical results in cases with normal DFI vs. pathological (DFI> 15) in the LOR group of patients.

Variable	Normal ($n = 113$)	Pathogenic ($n = 10$)	p Value
β-hCG positive (%)	35.4	50	0.495
Clinical pregnancy (%)	26.5	50	0.144
1st trimester miscarriage (%) %)	3.5	0	0.853

Table 8. Clinical results in cases with normal DFI vs. pathological (DFI> 5.8) in the LOR group of patients.

Variable	5.8< ($n = 63$)	≥5.8 ($n = 60$)	p Value
β-hCG positive (%)	39.7	33.3	0.575
Clinical pregnancy (%)	30.2	26.7	0.686
1st trimester miscarriage (%) (%)	4.8	0	0.588

However, whether or not we can truly compare these results with other scientific publications remains difficult to ascertain. In fact, there are few studies and those with a low number of cases. In addition, the study characteristics are heterogeneous (Zini, 2011; Zini & Sigman, 2009). According to some systematic reviews and meta-analysis published recently in the literature, our data are not consistent with almost any of them (Osman et al., 2015; Zhao et al., 2014; Simon et al., 2016), except from one (Cissen et al., 2016).

Simon et al. (2016) observed that studies using the SCSA ($n = 23$) and SCD ($n = 8$) assays showed a detrimental effect of sperm DNA damage on clinical pregnancy. In contrast, they made an analysis of studies using the TUNEL ($n = 18$) assay and demonstrated the negative effect of sperm DNA damage on clinical pregnancy, suggesting that a direct method of DNA damage measurement may be a better predictor of pregnancy outcome. On the other hand, Osman et al. (2015) observed a small but significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles. Following this trend, Zhao et al. (2014), reported that sperm DNA damage was significantly associated with an increased risk of pregnancy loss after IVF and ICSI. Finally, Cissen et al. (2016) do not support this idea and suggest that current evidence of sperm DNA fragmentation presents a limited ability to discern between couples who have low or high probability of conceiving after medically assisted reproduction.

However, a weakness of a meta-analysis is the highly variable study characteristics: data collection (prospective or retrospective), population characteristics, female inclusion/exclusion criteria and sperm DNA test type and sperm DNA test cut off (Zini et al., 2008). As advised by many authors (Barratt et al., 2010; Sakkas & Alvarez, 2010; Zini & Sigman, 2009), larger, more properly designed and controlled prospective studies are absolutely necessary to confirm the aforementioned results and before carrying out a clinical indication for routine use of these tests in patient management.

Due to this, we decided to perform our study in three characteristic groups of patients with clear inclusion criteria: (i) couples undergoing ICSI due to poor sperm parameters with normal ovarian response; (ii) couples undergoing conventional IVF with oocyte donation due to an ovarian factor; and (iii) couples undergoing ICSI or conventional IVF with low ovarian response. In the first group we truly evaluated the

effect of the sperm DNA fragmentation in pathological samples, responsible for the infertility problem. In the second group, we assessed this effect in normozoospermic samples that were potentially fertile. The use of oocytes from donors with proven fertility eliminates the female factor. In this group, the recipients received correct endometrial preparation and had normal uterine cavities after ultrasound on the days of oocyte retrieval and embryo transfer. In the third group, we evaluated the effect of the sperm DNA fragmentation in a poor prognosis group, patients with low ovarian response, where oocyte quality is clearly reduced.

In relation to the sperm DNA fragmentation test employed, the SCSA and TUNEL methods are the most frequently used in published infertility studies (Cui et al., 2015). Nowadays, TUNEL assay is a routine diagnostic tool that measures DNA breaks by introducing fluorochrome labelled molecules. We decided to use TUNEL assay because this test measures DNA damage directly, without a denaturation step, such as the SCSA test (use of severely acid conditions) or Comet assay (use of alkaline conditions), and it is preferentially recommended (Panner-Selvam & Agarwal, 2018), showing the real fragmentation present in the DNA. In fact, the full nature of how these pH-exposures affect the sperm chromatin is not completely known at present (Barratt et al., 2010). Unfortunately, due to economic and technical reasons, flow cytometer was unavailable in our study and it was impossible to undertake with the TUNEL method.

The nature of the sperm DNA damage and the ability of the oocyte to repair DNA damage in the fertilising spermatozoon are, probably, the most important aspects to study further (Sakkas & Alvarez, 2010). An example of this fact is that, in their study, Casanovas et al. (2019) reported that only double-stranded sperm DNA fragmentation caused a delay in embryo development and impaired implantation.

Another important question in relation to an elected DFI cut off level is the characteristics of the patients and the reproductive variables that we want to analyse in the study. Collins et al. (2008), where results were available for more than one cut off point, chose that one recommended by the investigators or the cut off point nearest to the most frequently reported cut off point (SCSA DFI $\leq$30% and TUNEL $\leq$4%). However, Zini et al. (2008) argued that it is not known whether a clinically relevant cut off (that is based on fertile population) is optimal for the evaluation of pregnancy loss (their study variable) after IVF or ICSI. We obtained the same median DFI value in the first two groups of study (3.5%) and a higher median DFI value in the last one (5.8%).

The correlation coefficients between DFI and semen quality (concentration, % forward motility and morphology) in the first two groups of patients were not significant. This means that the quality of the seminal samples and its fertile/infertile potential are independent of the result of the DNA fragmentation test. Nevertheless, an increase in DNA fragmentation is observed in exclusively teratozoospermic samples with atypical sperm forms (Mehdi et al., 2009) and in those with poor motility. In our study, only 3 samples from NOR group were exclusively teratozoospermic, with a mean DFI of 4.4 ± 2.8.

In relation to the fertilisation rates, our data are consistent with that obtained by Esbert et al. (2011) but differ from others (Jin et al., 2015; Velez de la Calle et al., 2008). Also, controversial results are found in the publications with respect to embryo development and quality. While some authors found a positive association (Borges et al., 2019; Mangoli et al., 2018; Simon et al., 2014; Zheng et al., 2018), others (in agreement with our data) did not find any relationship (Gat, Tang, et al., 2017; Green et al., 2020; Tavalaee et al., 2009; Zini et al., 2011). As commented above, this could be due to the characteristics of the patients and the study design, as in the study from Borges et al. (2019) where sperm DNA damage was measured using a sperm chromatin dispersion (SCD) test, which has a higher interobserver and interexam variability in comparison to TUNEL assay.

There is hardly any information regarding blastocyst formation. In the study from Álvarez-Sedó et al. (2017), when 15% was used as a cut off for TUNEL positivity in sperm capacitated samples before IVF, the percentage of blastocyst development decreased from 59.2% ($<$15% TUNEL positivity) to 37.5% ($\geq$15% TUNEL positivity). In contrast, we did not find any relationship between DFI and blastocyst formation. In fact, in the NOR group we had a total of 50.8% blastocyst formation rate (BFR) (96 blastocysts from 189 cultured embryos); with a DFI $\geq$ 15 ($n = 2$), the BFR was 50.0% while with a DFI $<$ 15 ($n = 15$) BFR was 50.9%. In the IVF recipient group, we had a total BFR of 70.5% (316 blastocysts from 448 cultured embryos), with 65.0% BFR when DFI $\geq$ 15 ($n = 6$) and 71.1% BFR when DFI $<$ 15 ($n = 48$) (data not shown in results).

Some authors (Borges et al., 2019; Khadem et al., 2014; Leach et al., 2015; Robinson et al., 2012) have correlated pregnancy loss with increasing sperm DNA fragmentation. As commented above, our results do not show any relationship between the rate of first

trimester miscarriage and DFI levels in the three groups of patients. We found more miscarriage in the recipient group compared to the NOR and LOR groups (25.4% vs 7.9% and 2.9%, respectively), but this result was not associated to the sperm DNA damage. It could be a sporadic or temporary result.

The female gamete has been described as having reparative capacity over the spermatozoa DNA (Meseguer et al., 2011). This characteristic is compromised by oocyte quality although, compared to our data, we did not notice statistically significant associations between the DFI and the study variables in any of the three groups. Our results agreed with those of Gat, Li et al. (2017) for the group of recipients and with Coughlan et al. (2015) for the NOR group with own oocyte. However, in the LOR group they differentiated between those of Jin et al. (2015), where the implantation and live child born rate decreased significantly for a threshold of the DFI of 27.3%, and of those of Choi et al. (2017), where if the DFI was higher than 13%, the probability of miscarriage increased in the first trimester.

The selection of spermatozoa before IVF/ICSI with low levels of DNA fragmentation can be another important strategy. In fact, the introduction of confocal light absorption scattering spectroscopy (Sakkas & Alvarez, 2010), sperm selection by MACS, hyaluronic acid binding assay (PICSI), IMSI technique and microfluidic sorting (Erberelli et al., 2017; Knez et al., 2011; Quinn et al., 2018) may allow selecting spermatozoa with the intact chromatin to be microinjected by ICSI.

Despite the relevance of the results presented in this research work, we are aware of the limitations of this study. In addition to the small population that showed a high DFI percentage, it was not possible to include a long-term follow-up of the children conceived after ART and, therefore, we were not able to confirm if any postnatal developmentdisorder related to high sperm DNA fragmentation exists. The statistical power of the study was not determined because it was originated as a pilot study, in order to assess the results obtained and propose a larger study.

In summary, in this study we did not find any association between sperm DNA fragmentation on the day of fertilisation, as measured by TUNEL assay, and seminal parameters, fertilisation, embryo quality, blastocyst formation, pregnancy, clinical pregnancy and first trimester miscarriage neither in the NOR group nor in the recipient group nor in the LOR group. It seems that ART outcomes are not affected by sperm DNA fragmentation, independently of semen quality and oocyte origin. Further future research is needed, following the recommendations cited previously.

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Ethical approval

All procedures performed involving human participants were in accordance with the ethical standards of the Instituto Bernabeu research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from every participant.

Disclosure statement

The authors report no conflict of interest.

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References

Agarwal, A., Cho, C., Majzoub, A., & Esteves, S. (2017). The Society for Translational Medicine: clinical practice guidelines for sperm DNA fragmentation testing in male infertility. *Translational Andrology and Urology*, *6*(Suppl 4), S720–S733. https://doi.org/10.21037/tau.2017.08.06

Agarwal, A., & Said, T. M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. *Human Reproduction Update*, *9*(4), 331–345. https://doi.org/10.1093/humupd/dmg027

Álvarez-Sedó, C., Bilinski, M., Lorenzi, D., Uriondo, H., Noblía, F., Longobucco, V., Lagar, E. V., & Nodar, F. (2017). Effect of sperm DNA fragmentation on embryo development: Clinical and biological aspects. *JBRA Assisted Reproduction*, *21*(4), 343–350. https://doi.org/10.5935/1518-0557.20170061

Ardoy, M., Calderon, G., Cuadros, J., Figueroa, M. J., Herrer, R., Moreno, J. M., Ortiz, A., Prados, F., Rodríguez, L., Santaló, J., Ten, J., & Torelló, M. J. (2008). *Criterios ASEBIR de valoración morfológica de oocitos, embriones tempranos y blastocistos humanos: Cuadernos de Embriología Clínica* (2nd ed.). ASEBIR Press.

Barratt, C. L. R., Aitken, R. J., Björndahl, L., Carrell, D. T., De Boer, P., Kvist, U., Lewis, S. E., Perreault, S. D., Perry, M. J., Ramos, L., Robaire, B., Ward, S., & Zini, A. (2010). Sperm DNA: organization, protection and vulnerability: From basic science to clinical applications-a position report.

Human Reproduction (Oxford, England), *25*(4), 824–838. https://doi.org/10.1093/humrep/dep465

Bernabeu, R., Roca, M., Torres, A., & Ten, J. (2006). Indomethacin effect on implantation rates in oocyte recipients. *Human Reproduction (Oxford, England)*, *21*(2), 364–369. https://doi.org/10.1093/humrep/dei343

Borges, E., Zanetti, B., Setti, A., Braga, D., Provenza, R., & Iaconelli, A. (2019). Sperm DNA fragmentation is correlated with poor embryo development, lower implantation rate, and higher miscarriage rate in reproductive cycles of non–male factor infertility. *Fertility and Sterility*, *112*(3), 483–490. https://doi.org/10.1016/j.fertnstert.2019.04.029

Casanovas, A., Ribas-Maynou, J., Lara-Cerrillo, S., Jimenez-Macedo, A., Hortal, O., Benet, J., Carrera, J., & García-Peiró, A. (2019). Double-stranded sperm DNA damage is a cause of delay in embryo development and can impair implantation rates. *Fertility and Sterility*, *111*(4), 699.e1–707.e1. https://doi.org/10.1016/j.fertnstert.2018.11.035

Choi, H., Kim, S., Kim, S., Choi, Y., & Jee, B. (2017). Impact of sperm DNA fragmentation on clinical in vitro fertilization outcomes. *Clinical and Experimental Reproductive Medicine*, *44*(4), 224–231. https://doi.org/10.5653/cerm.2017.44.4.224

Cissen, M., Wely, M., Scholten, I., Mansell, S., Bruin, J., Mol, B., Braat, D., Repping, S., & Hamer, G. (2016). Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: A systematic review and meta-analysis. *PLoS One*, *11*(11), e0165125. https://doi.org/10.1371/journal.pone.0165125

Cohen-Bacrie, P., Belloc, S., Ménézo, Y. J. R., Clement, P., Hamidi, J., & Benkhalifa, M. (2009). Correlation between DNA damage and sperm parameters: A prospective study of 1,633 patients. *Fertility and Sterility*, *91*(5), 1801–1805. https://doi.org/10.1016/j.fertnstert.2008.01.086

Collins, J. A., Barnhart, K. T., & Schlegel, P. N. (2008). Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertility and Sterility*, *89*(4), 823–831. https://doi.org/10.1016/j.fertnstert.2007.04.055

Coughlan, C., Clarke, H., Cutting, R., Saxton, J., Waite, S., Ledger, W., Li, T., & Pacey, A. A. (2015). Sperm DNA fragmentation, recurrent implantation failure and recurrent miscarriage. *Asian Journal of Andrology*, *17*(4), 681–685. https://doi.org/10.4103/1008-682X.144946

Cui, Z. L., Zheng, D. Z., Liu, Y. H., Chen, L. Y., Lin, D. H., & Feng-Hua, L. (2015). Diagnostic accuracies of the TUNEL, SCD, and Comet based sperm DNA fragmentation assays for male infertility: A metaanalysis study. *Clinical Laboratory*, *61*(5–6), 525–535. https://doi.org/10.7754/clin.lab.2014.141005

Cunha-Filho, J. S. (2017). Evidence based sperm DNA fragmentation. *Translational Andrology and Urology*, *6*(Suppl 4), S527–S528. https://doi.org/10.21037/tau.2017.04.39

Erberelli, R., Salgado, R., Pereira, D., & Wolff, P. (2017). Hyaluronan-binding system for sperm selection enhances pregnancy rates in ICSI cycles associated with male factor infertility. *JBRA Assisted Reproduction*, *21*(1), 2–6. https://doi.org/10.5935/1518-0557.20170002

Esbert, M., Pacheco, A., Vidal, F., Florensa, M., Riqueros, M., Ballesteros, A., Garrido, N., & Calderón, G. (2011). Impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes. *Reproductive BioMedicine Online*, *23*(6), 704–710. https://doi.org/10.1016/j.rbmo.2011.07.010

Evenson, D. P., & Wixon, R. (2006). Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology*, *65*(5), 979–991. https://doi.org/10.1016/j.theriogenology.2005.09.011

Ferraretti, A., La Marca, A., Fauser, B., Tarlatzis, B., Nargund, G., & Gianaroli, L. (2011). ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Human Reproduction (Oxford, England)*, *26*(7), 1616–1624. https://doi.org/10.1093/humrep/der092

Gat, I., Li, N., Yasovich, N., Antes, R., Kuznyetsov, V., Zohni, K., Weizman, N. F., & Librach, C. (2017). Sperm DNA fragmentation index does not correlate with blastocyst euploidy rate in egg donor cycles. *Gynecological Endocrinology*, *34*(3), 212–216. https://doi.org/10.1080/09513590.2017.1379500

Gat, I., Tang, K., Quach, K., Kuznyetsov, V., Antes, R., Filice, M., Zohni, K., & Librach, C. (2017). Sperm DNA fragmentation index does not correlate with blastocyst aneuploidy or morphological grading. *Plos One*, *12*(6), e0179002. https://doi.org/10.1371/journal.pone.0179002

Green, K., Patounakis, G., Dougherty, M., Werner, M., Scott, R., & Franasiak, J. (2020). Sperm DNA fragmentation on the day of fertilization is not associated with embryologic or clinical outcomes after IVF/ICSI. *Journal of Assisted Reproduction and Genetics*, *37*(1), 71–76. https://doi.org/10.1007/s10815-019-01632-5

Jin, J., Pan, C., Fei, Q., Ni, W., Yang, X., Zhang, L., & Huang, X. (2015). Effect of sperm DNA fragmentation on the clinical outcomes for in vitro fertilization and intracytoplasmic sperm injection in women with different ovarian reserves. *Fertility and Sterility*, *103*(4), 910–916. https://doi.org/10.1016/j.fertnstert.2015.01.014

Khadem, N., Poorhoseyni, A., Jalali, M., Akbary, A., & Heydari, S. (2014). Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. *Andrologia*, *46*(2), 126–130. https://doi.org/10.1111/and.12056

Knez, K., Zorn, B., Tomazevic, T., Vrtacnik-Bokal, E., & Virant-Klun, I. (2011). The IMSI procedure improves poor embryo development in the same infertile couples with poor semen quality: a comparative prospective randomized study. *Reproductive Biology and Endocrinology: RB&E*, *9*, 123. https://doi.org/10.1186/1477-7827-9-123

Leach, M., Aitken, R. J., & Sacks, G. (2015). Sperm DNA fragmentation abnormalities in men from couples with a history of recurrent miscarriage. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, *55*(4), 379–383. https://doi.org/10.1111/ajo.12373

Lewis, S. E., Agbaje, I., & Alvarez, J. (2008). Sperm DNA tests as useful adjuncts to semen analysis. *Systems Biology in Reproductive Medicine*, *54*(3), 111–125. https://doi.org/10.1080/19396360801957739

Lewis, S. E., Aitken, R. J., Conner, S. J., Iuliis, G. D., Evenson, D. P., Henkel, R., Giwercman, A., & Gharagozloo, P. (2013). The impact of sperm DNA damage in assisted conception and beyond: Recent advances in diagnosis and treatment. *Reproductive Biomedicine Online*, *27*(4), 325–337. https://doi.org/10.1016/j.rbmo.2013.06.014

Li, Z., Wang, L., Cai, J., & Huang, H. (2006). Correlation of sperm DNA damage with IVF and ICSI outcomes: A systematic review and meta-analysis. *Journal of Assisted*

Reproduction and Genetics, *23*(9–10), 367–376. https://doi.org/10.1007/s10815-006-9066-9

Majzoub, A., Agarwal, A., & Esteves, S. C. (2017). Sperm DNA fragmentation: Overcoming standardization obstacles. *Translational Andrology and Urology*, *6*(Suppl 4), S422–S424. https://doi.org/10.21037/tau.2017.07.10

Mangoli, E., Khalili, M., Talebi, A., Ghasemi-Esmailabad, S., & Hosseini, A. (2018). Is there any correlation between sperm parameters and chromatin quality with embryo morphokinetics in patients with male infertility? *Andrologia*, *50*(5), e12997. https://doi.org/10.1111/and.12997

Mehdi, M., Khantouche, L., Ajina, M., & Saad, A. (2009). Detection of DNA fragmentation in human spermatozoa: correlation with semen parameters. *Andrologia*, *41*(6), 383–386. https://doi.org/10.1111/j.1439-0272.2009.00953.x

Meseguer, M., Santiso, R., Garrido, N., García-Herrero, S., Remohí, J., & Fernandez, J. (2011). Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertility and Sterility*, *95*(1), 124–128. https://doi.org/10.1016/j.fertnstert.2010.05.055

Muratori, M., Marchiani, S., Tamburrino, L., Tocci, V., Failli, P., Forti, G., & Baldi, E. (2008). Nuclear staining identifies two populations of human sperm with different DNA fragmentation extent and relationship with semen parameters. *Human Reproduction (Oxford, England)*, *23*(5), 1035–1043. https://doi.org/10.1093/humrep/den058

Osman, A., Alsomait, H., Seshadri, S., El-Toukhy, T., & Khalaf, Y. (2015). The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. *Reproductive Biomedicine Online*, *30*(2), 120–127. https://doi.org/10.1016/j.rbmo.2014.10.018

Pacey, A. (2018). Is sperm DNA fragmentation a useful test that identifies a treatable cause of male infertility? *Best Practice & Research. Clinical Obstetrics & Gynaecology*, *53*, 11–19. https://doi.org/10.1016/j.bpobgyn.2018.09.003

Panner-Selvam, M., & Agarwal, A. (2018). A systematic review on sperm DNA fragmentation in male factor infertility: Laboratory assessment. *Arab Journal of Urology*, *16*(1), 65–76. https://doi.org/10.1016/j.aju.2017.12.001

Quinn, M., Jalalian, L., Ribeiro, S., Ona, K., Demirci, U., Cedars, M. I., & Rosen, M. P. (2018). Microfluidic sorting selects sperm for clinical use with reduced DNA damage compared to density gradient centrifugation with swim-up in split semen samples. *Human Reproduction (Oxford, England)*, *33*(8), 1388–1393. https://doi.org/10.1093/humrep/dey239

Robinson, L., Gallos, I., Conner, S., Rajkhowa, M., Miller, D., Lewis, S., Kirkman-Brown, J., & Coomarasamy, A. (2012). The effect of sperm DNA fragmentation on miscarriage rates: A systematic review and meta-analysis. *Human Reproduction (Oxford, England)*, *27*(10), 2908–2917. https://doi.org/10.1093/humrep/des261

Sakkas, D., & Alvarez, J. G. (2010). Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertility and Sterility*, *93*(4), 1027–1036. https://doi.org/10.1016/j.fertnstert.2009.10.046

Santi, D., Spaggiari, G., & Simoni, M. (2018). Sperm DNA fragmentation index as a promising predictive tool for male infertility diagnosis and treatment management – meta-analyses. *Reproductive BioMedicine Online*, *37*(3), 315–326. https://doi.org/10.1016/j.rbmo.2018.06.023

Simon, L., Murphy, K., Shamsi, M., Liu, L., Emery, B., Aston, K., Hotaling, J., & Carrell, D. (2014). Paternal influence of sperm DNA integrity on early embryonic development. *Human Reproduction (Oxford, England)*, *29*(11), 2402–2412. https://doi.org/10.1093/humrep/deu228

Simon, L., Zini, A., Dyachenko, A., Ciampi, A., & Carrell, D. A. (2016). Systematic review and meta-analysis to determine the effect of sperm DNA damage on IVF and ICSI outcome. *Asian Journal of Andrology*, *19* (1), 80–90. https://doi.org/10.4103/1008-682X.182822

Tavalaee, M., Razavi, S., & Nasr-Esfahani, M. H. (2009). Influence of sperm chromatin anomalies on assisted reproductive technology outcome. *Fertility and Sterility*, *91*(4), 1119–1126. https://doi.org/10.1016/j.fertnstert.2008.01.063

Velez de la Calle, J. F., Muller, A., Walschaerts, M., Clavere, J. L., Jimenez, C., Wittemer, C., & Thonneau, P. (2008). Sperm deoxyribonucleic acid fragmentation as assessed by the sperm chromatin dispersion test in assisted reproductive technology programs: results of a large prospective multicenter study. *Fertility and Sterility*, *90*(5), 1792–1799. https://doi.org/10.1016/j.fertnstert.2007.09.021

World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th ed.). WHO Press.

Zhao, J., Zhang, Q., Wang, Y., & Li, Y. (2014). Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. *Fertility and Sterility*, *102*(4), 998.e8–1005.e8. https://doi.org/10.1016/j.fertnstert.2014.06.033

Zheng, W., Song, G., Wang, Q., Liu, S., Zhu, X., Deng, S., Zhong, A., Tan, Y. M., & Tan, Y. (2018). Sperm DNA damage has a negative effect on early embryonic development following in vitro fertilization. *Asian Journal of Andrology*, *20*(1), 75–79. https://doi.org/10.4103/aja.aja_19_17

Zini, A. (2011). Are sperm chromatin and DNA defects relevant in the clinic? *Systems Biology in Reproductive Medicine*, *57*(1–2), 78–85. https://doi.org/10.3109/19396368.2010.515704

Zini, A., Boman, J. M., Belzile, E., & Ciampi, A. (2008). Sperm DNA damage is associated with an increased risk of pregnancy loss alter IVF and ICSI: systematic review and meta-analysys. *Human Reproduction (Oxford, England)*, *23*(12), 2663–2668. https://doi.org/10.1093/humrep/den321

Zini, A., Jamal, W., Cowan, L., & Al-Hathal, N. (2011). Is sperm dna damage associated with IVF embryo quality? A systematic review. *Journal of Assisted Reproduction and Genetics*, *28*(5), 391–397. https://doi.org/10.1007/s10815-011-9544-6

Zini, A., & Sigman, M. (2009). Are tests of sperm DNA damage clinically useful? Pros and cons. *Journal of Andrology*, *30*(3), 219–229. https://doi.org/10.2164/jandrol.108.006908