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ARTICLE

Impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes

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Abstract A prospective study was performed to assess the impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes. The study population included 178 couples (62 cycles of IVF, 116 of intracytoplasmic sperm injection (ICSI)) with own (n = 77) and donor (n = 101) oocytes. DNA fragmentation was evaluated by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling assay. Correlation between DNA damage to oocyte fertilization, embryo quality and clinical pregnancy, implantation and miscarriage rates was evaluated. DNA fragmentation was not related to fertilization rates in either IVF (r = 0.08) or ICSI (r = −0.04) cycles. DNA fragmentation was similar in patients with <50% embryo utilization rate compared with ≥50%, in cancelled and in embryo transfer cycles and in miscarriages and in successful deliveries. Moreover, DNA fragmentation was similar in pregnant and non-pregnant women as well as in IVF with own or donor oocytes. In the multivariable analysis, the odds ratio of DNA after controlling by age was 1.0. Using a 36% sperm fragmentation threshold, results did not vary. It is concluded that DNA damage was not related to outcomes of IVF or ICSI with own or donor oocytes.

KEYWORDS: DNA fragmentation, embryo quality, IVF, miscarriage, oocyte donors

Introduction

Conventional parameters of semen quality, such as sperm concentration, motility and morphology, are generally modest predictors of reproductive outcomes. Approximately 15% of patients with male factor infertility have a normal semen analysis and although fertile men as a group have higher average sperm parameters than infertile men, there is significant overlap between these groups (Agarwal and Allamaneni, 2005). A definite diagnosis of male infertility

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is often unobtainable through routine semen analysis. Over the past decade, there has been a growing body of research focused on the role of sperm nuclear-DNA integrity in male factor infertility (Schulte et al., 1992). The study of sperm DNA damage is especially relevant in an era in which advanced forms of assisted reproductive technologies are frequently used and in which barriers to natural selection are bypassed.

Any form of sperm chromatin abnormality or DNA damage may result in male infertility. Some reports have indicated that when $\geq$30% of the spermatozoa are identified as having DNA damage, the potential for natural fertility is very low (Evenson et al., 2002). It has also been suggested that sperm DNA integrity may be a more objective marker of sperm function than standard semen analysis. The degree of damage to DNA or DNA fragmentation might adversely affect reproductive outcomes, and spermatozoa of infertile men present substantially more DNA damage than the spermatozoa of fertile men (Saleh et al., 2002). However, although there has been a significant amount of research on human-sperm integrity over the last decade (Zini and Libm, 2006; Zini et al., 2005), the understanding of sperm DNA-damage mechanisms and their effects on reproductive outcomes are far from complete.

Studies focused on a number of specific clinical areas also appear to be controversial. While high levels of sperm DNA damage often correlate with poor seminal parameters, such as reduced count and motility or abnormal morphology, DNA damage can also be found in spermatozoa from infertile men with normal standard sperm parameters (Irvine et al., 2000; Lopes et al., 1998; Saleh, 2002). There remains no consistent relationship between sperm DNA integrity and reproductive outcomes after assisted reproduction treatment. High levels of DNA damage have been associated with decreased fertilization rates, embryo quality and pregnancy rates in some studies (Avendaño et al., 2010; Larson-Cook et al., 2003; Li et al., 2006; Lopes et al., 1998; Virro et al., 2004; Zini et al., 2008), while having no influence on reproductive outcome in other studies (Collins et al., 2008; Lin et al., 2008; Zini and Libm, 2006). Moreover, there is little information on the threshold values of sperm DNA fragmentation indicative of a man's fertility potential, and several discriminating values for the different sperm DNA-integrity assays have been proposed (Duran et al., 2002; Sergerie et al., 2005).

DNA repair systems are known to be present in oocytes. The risk of having embryos with paternally transmitted DNA aberrations depends on the efficiency of maternal DNA repair. Experimental evidence in a number of in-vivo and in-vitro systems indicates clearly that the vertebrate oocyte is capable of repairing endogenous and exogenous DNA damage (Ashwood-Smith and Edwards, 1996). Therefore, the oocyte plays a major role in determining the risks for pregnancy loss and frequencies of offspring with genetic defects of paternal origin (Marchetti et al., 2007). Using young healthy egg donors to obtain embryos, for transfer to prepared recipient women, allows for high pregnancy and implantation rates, independent of the recipient's age.

This study examined the relationship between sperm DNA fragmentation and IVF outcome, both with own and donated oocytes.

Materials and methods

Patients

The present prospective study was carried out between March 2010 and June 2010 at the university-affiliated private reproductive medicine and fertility clinic in Barcelona, Spain. The study included 178 couples, divided into 62 cycles of IVF and 116 of intracytoplasmic sperm injection (ICSI). The woman's own oocytes were used in 77 cycles and donor oocytes in 101. Only cycles carried out with fresh, ejaculated spermatozoa were included in the study. Written informed consent was obtained from all participants and the study was approved by the Instituto Valenciano de Infertilidad's ethics committee.

Causes of infertility in the 77 cases of fertilization with own oocytes were as follows: low ovarian response in 19 cases, endometriosis in eight, irregular ovulation in seven, advanced maternal age in seven, tubal factor in seven, poor oocyte quality in five, previous intrauterine insemination failure in four, recurrent miscarriage in one, adnexectomy in one and idiopathic infertility in 18. Indications for egg donation in the 101 cases of fertilization with donor oocytes included advanced maternal age in 52 cases, ovarian failure in 29, endometriosis in nine, genetic disease in four, menopause in four, previous assisted reproduction treatment failure in two and compromised oocyte quality in one case.

Oocyte donation was anonymous and altruistic. The potential gamete donors were recruited from young ($<$35 years old) volunteers. Details of donor selection and work-up studies have been previously reported (Garrido et al., 2002). Professional confidentiality with regard to the identities of gamete donors and recipients was kept according to the dictates of the Spanish Law of Assisted Reproductive Technologies (14/2006).

Ovarian stimulation of patients and donors was achieved by administering recombinant FSH (Puregon; MSD, Madrid, Spain; and Gonal-F; MerckSerono, Geneva, Switzerland) or human menopausal gonadotrophin (Menopur; Ferring Pharmaceuticals, Copenhagen, Denmark) and hypophyseal suppression with either gonadotrophin-releasing hormone (GnRH) agonists (Procrin; Abbot Laboratories, Madrid, Spain) in a long stimulation protocol or GnRH antagonists (Cetrotide; MerkSerono; and Orgalutran; MSD). Ovulation was triggered with human chorionic gonadotrophin (Ovitrelle, 250 μg, Serono, Barcelona, Spain) when three or more follicles had reached a diameter of $\geq$18 mm and oocyte retrieval was scheduled 36 h later. In patients, following egg retrieval, 400 mg of micronized progesterone (Utrogestan; Laboratorio Seid, Barcelona, Spain) was administered vaginally daily until the pregnancy-test results were negative, or during the next 3 months if tested positive following egg retrieval.

Oral or transcutaneous oestradiol valerate (Estradot; Novartis, Barcelona, Spain) was used in a gradually increasing dose pattern for the recipients endometrial preparation. Endometrial thickness and lining was routinely measured before the embryo transfer. Micronized progesterone (Utrogestan, 800 μg; Laboratorio Seid, Barcelona, Spain) was administered vaginally from the day of the oocyte donation.

Sperm collection and preparation

On the day of oocyte retrieval, semen samples were collected by masturbation into non-toxic sterile plastic jars after 3–5 days of sexual abstinence. The 178 samples analysed were allowed to liquefy for 30 min at room temperature (22°C), and were then evaluated according to the WHO criteria (World Health Organization, 1999, 4th edition).

After microscopic evaluation, the samples were prepared for the assisted reproduction procedure, using discontinuous gradients of SpermGrad (Vitrolife; Göteborg Sweden). SpermGrad 100% was diluted with human tubal fluid (HTF) to obtain 45%, 70% and 90% dilutions. Two gradient columns were prepared in Falcon tubes by gently layering 1 ml of each solution, starting from the 90% fraction at the bottom. One millilitre of the semen sample previously washed for 10 min at 350 *g* with HTF was stratified on top of the discontinuous SpermGrad gradient columns and centrifuged for 18 min at 300*g*. After centrifugation, the 90% SpermGrad fraction was collected and washed twice at 350*g* for 5 min. After density-gradient separation, a second evaluation of sperm parameters was carried out and the prepared sample was used for insemination of the oocytes. One aliquot of the processed spermatozoa was fixed with 4% paraformaldehyde and stored at 4°C until analysis.

DNA fragmentation assay

In situ Cell Death Detection Kit (Roche Diagnostics, Barcelona, Spain) based on the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay was used according to the manufacturer's instructions. Samples were washed with phosphate buffered saline (PBS) (Gibco; Invitrogen, Barcelona, Spain). There was an incubation step in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4°C. Samples were incubated for 1 h at 37°C with TUNEL reaction-mixture label solution and terminal transferase solution. Each experimental setup also included a positive control, where the sample was incubated with 3 IU/ml DNase I recombinant in 50 mmol/l Tris–HCl, pH 7.5 (Roche Diagnostics) for 15 min at 25°C to induce DNA fragmentation prior to labelling procedures, and a negative control without the enzyme solution. Samples were washed twice in PBS during 3 s at 7200 *g* and PBS was added for a final volume of 1 ml. Damage quantification was performed on at least 10,000 cells in a FACScan (Becton Dickinson, NJ, USA) cytometer at 488 nm wavelength of fluorescence intensity.

IVF laboratory procedures and cycle outcomes

Recovered oocytes were inseminated using either conventional IVF or ICSI depending on sperm parameters. Injected or inseminated oocytes were placed into droplets of HTF supplemented with 10% maternal serum under equilibrated mineral oil and incubated in a humidified 5.3% CO_2 atmosphere at 37.3°C.

At 16–18 h after insemination or microinjection, the oocytes were assessed for the two pronuclei stage. Fertilization was calculated as the percentage of resulting embryos from the injected or inseminated oocytes. Embryo morphology was evaluated on days 2 and 3 by assessing the number, symmetry and granularity of blastomeres, as well as the type and percentage of fragmentation and the presence of multinucleation. Intrauterine embryo transfer was performed on day 3 and supernumerary embryos were frozen when their morphological state would allow. An embryo suitable to be transferred or frozen on day 3 had between 6–12 cells, less than 20% fragmentation, symmetric blastomeres and no multinucleation. The embryo utilization rate was calculated as the number of embryos suitable for transfer and freezing/total number of embryos cultured to day 3.

A rise in serum β-human chorionic gonadotrophin concentration 14 days after transfer indicated pregnancy. Each pregnancy, where at least one intrauterine sac was revealed by ultrasonography approximately 3 weeks after transfer, was considered as a clinical pregnancy. The implantation rate was calculated as the ratio of gestational sacs determined by ultrasound in relation to the total number of embryos transferred. Miscarriage was defined as a spontaneous pregnancy loss after an intrauterine pregnancy had been detected by ultrasound.

A threshold value of 36% DNA fragmentation was used to see if DNA fragmentation was predictive for the cycle outcome. Some authors (Henkel et al., 2004) have proposed this cut-off value since a significantly lower clinical-pregnancy rate has been found in patients with >36.5% of TUNEL-positive spermatozoa compared with those with less than 35.5% DNA fragmentation.

Statistical analysis

The statistical analysis was carried out with Statistical Package for the Social Sciences version 12.0 software (SPSS, Chicago, IL) and MedCalc Software (Ghent, Belgium). The Kolmogorov–Smirnov test was used to determine whether the data were random samples from a normal distribution, and non-parametric tests were performed due to a non-normal distribution of the data. Comparisons of median values were calculated by Mann–Whitney *U* test. Pearson's correlation of the variable with fertilization rate and implantation rate was calculated. Differences in proportions between groups were tested using the chi-squared test and Yates' correction when low frequencies were found in any cell. Differences in continuous variables were assessed using the Student's *t*-test. Multivariable analysis was performed to compare sperm DNA fragmentation between pregnant and non-pregnant women adjusted by age. The receiver-operating-characteristic (ROC) curve was calculated to determine the diagnostic usefulness of sperm DNA fragmentation using the TUNEL assay. Statistical significance was set at $P < 0.05$.

Results

The characteristics of the study population in relation to male and female age and insemination technique in the groups of own and donated oocytes are shown in **Table 1**. The global median percentage of DNA fragmentation was 15% (95% confidence interval (CI) 6.1–26). The mean ± standard deviation percentage of sperm DNA fragmentation was 19.5% ± 16.6% (**Figure 1**). There were no statistically

Table 1 Female and male age and insemination technique.

Variables	*Total patients* (n = 178)	*Fertilization using*	
		Own oocytes (n = 77)	*Donor oocytes* (n = 101)
Female age (years)	38.7 ± 5.3	35.3 ± 3.5	41.2 ± 5.0
Male age (years)	40.2 ± 6.7	37.2 ± 5.0	42.5 ± 7.0
Conventional IVF	62	20	42
ICSI	116	57	59

Values are mean ± standard deviation or *n*.
ICSI = intracytoplasmic sperm injection.

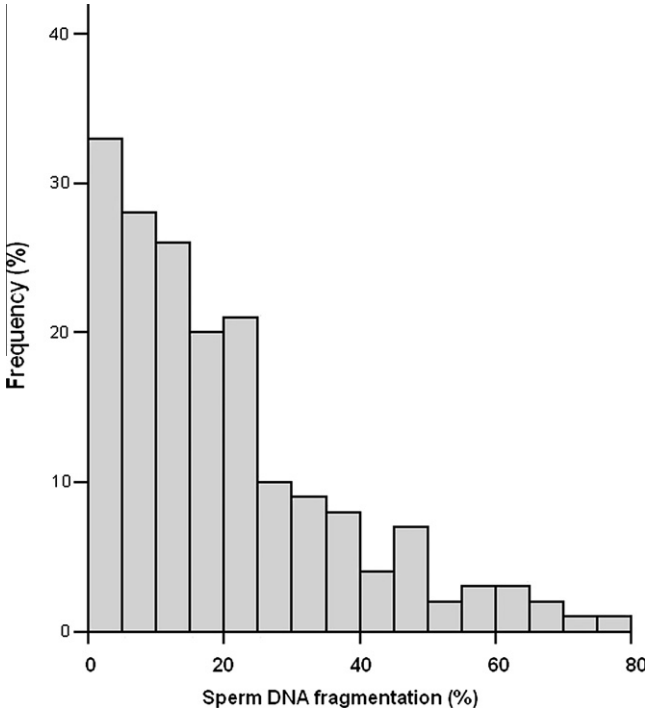

Fig. 1 Percentage of sperm DNA fragmentation distribution in the sperm samples analysed.

significant differences in the median percentage of DNA fragmentation when own oocytes (15%, 95% CI 6.7—28) or donor oocytes (14.4%, 95% CI 6—25) were used.

DNA fragmentation was not related to fertilization rates when all cycles were considered ($r = -0.01$), nor was it considering only the IVF ($r = 0.08$) or the ICSI ($r = -0.04$) cycles. Also, DNA fragmentation was similar in cycles where the embryo utilization rate was <50% (15.5%, 95% CI 9—33) than when the rate was ≥50% (13.7%, 95% CI 5.7—24).

In 161 cycles, embryos were of good quality and ended in embryo transfer, whereas in the remaining 17 cycles, bad-quality embryos were obtained and embryo transfer was cancelled. There was no difference in DNA fragmentation between cancelled embryo-transfer cycles (20%, 95% CI 13.0—29.5) and embryo-transfer cycles (14.8%, 95% CI 16.8—22.0).

The overall clinical-pregnancy rate was 54.0% (87/161). The clinical-pregnancy rate was 46.2% (30/65) for patients using their own oocytes, and 59.4% (57/96) in IVF with donor eggs. As shown in **Table 2**, women who became pregnant were significantly younger than those who did not conceive, both in the groups of own oocytes and donor eggs. Other variables, such as male age, DNA fragmentation and number of transferred embryos were similar. There was no difference in the percentage of DNA fragmentation between patients with miscarriage ($n = 13$) and patients with successful deliveries ($n = 74$) (22%, 95% CI 15—37.0 versus 17%, 95% CI 6.3—25, respectively). DNA fragmentation using a threshold of 36% had no effect on clinical outcomes (**Table 3**).

In the logistic regression analysis, DNA fragmentation was not a predictive variable of clinical pregnancy after controlling by age (odds ratio 1.0, 95% CI 0.98—1.02).

Moreover, the sensitivity and specificity of DNA fragmentation >15% for predicting clinical pregnancy was 56.3% and

Table 2 Differences between pregnant and non-pregnant women.

Study groups	*Female age (years)*	*Male age (years)*	*DNA fragmentation (%)*	*Embryos transferred (n)*
IVF with own oocytes				
Clinical pregnancy ($n = 30$)	33.9 ± 3.1	37.0 ± 4.7	21.2 ± 16.6	2.1 ± 0.3
No pregnancy ($n = 35$)	36.2 ± 3.6	37.4 ± 4.9	18.2 ± 17.5	2.0 ± 0.8
P-value	0.007	NS	NS	NS
IVF with donor oocytes				
Clinical pregnancy ($n = 57$)	40.2 ± 5.2	42.0 ± 6.8	18.7 ± 13.8	2.0 ± 0.3
No pregnancy ($n = 39$)	42.8 ± 4.4	43.1 ± 7.6	19.9 ± 20.0	2.0 ± 0.3
P-value	0.011	NS	NS	NS

Values are mean ± standard deviation.
NS = not statistically significant.

Table 3 Study variables and outcome for a sperm DNA fragmentation threshold of 36%.

Variables	*Recipients of their own oocytes*		*Recipients of donor oocytes*	
	<36% (n = 63)	*≥36% (n = 14)*	*<36% (n = 86)*	*≥36% (n = 15)*
Female age (years)	35.1 ± 3.5	36.2 ± 3.4	41.2 ± 5.2	41.5 ± 3.3
Transferred embryos	2.1 ± 0.6	2.0 ± 0.8	2.0 ± 0.3	2.1 ± 0.5
Embryo transfers	85.7 (54/63)	78.6 (11/14)	94.2 (81/86)	100 (15/15)
Clinical pregnancy rate	48.1 (26/54)	36.4 (4/11)	61.7 (50/81)	46.7 (7/15)
Miscarriage rate	11.5 (3/26)	50.0 (2/4)	10.0 (5/50)	42.9 (3/7)
Implantation rate	30.1 (34/113)	31.8 (7/22)	47.6 (78/164)	32.3 (10/31)

Values are mean ± standard deviation or % (*n*/total).
No statistical significance was found for comparisons of DNA fragmentation within recipient type.

Table 4 Diagnostic accuracy of sperm DNA fragmentation using the TUNEL assay for predicting pregnancy and miscarriage.

Study groups	*DNA fragmentation threshold (%)*	*Sensitivity (%)*	*Specificity (%)*	*Area under the ROC curve*
Pregnancy achievement				
All patients	>15	56.3	64.9	0.552
IVF with own oocytes	>11	76.7	54.3	0.599
IVF with donor oocytes	>15	56.1	64.1	0.528
Miscarriage				
All patients	>35	38.5	91.9	0.666
IVF with own oocytes	>13	100	40.0	0.652
IVF with donor oocytes	>21	62.5	67.3	0.670

ROC = receiver operating characteristic.

64.9%, respectively, with an area under the ROC curve of 0.552. The corresponding figures for miscarriage were 38.5% sensitivity, 91.9% specificity and an area under the ROC curve of 0.666. The diagnostic accuracy of DNA fragmentation with the TUNEL assay in the whole study population as well as in the groups of own and donor oocytes is detailed in **Table 4**. The different thresholds employed for clinical pregnancy and miscarriage were determined as optimal for the data derived from the statistical analysis program, balancing both sensitivity and specificity of the test.

Discussion

The relationship between sperm DNA fragmentation and outcome of assisted reproduction treatment has been admitted, but it remains a subject of controversy. This prospective study was designed to assess the impact of sperm DNA fragmentation, evaluated using the TUNEL assay, on the outcome of IVF/ICSI with own oocytes or with donor eggs. According to the present results, sperm DNA fragmentation has no effect on fertilization rate, clinical pregnancy achievement, miscarriage rate or embryo quality. Donor oocytes were used in a subgroup of participants, which is a novel aspect of the study as compared with previous literature (Avendaño et al., 2010; Bungum et al., 2008; Gandini et al., 2004; Henkel et al., 2004; Lin et al., 2008; Virro et al., 2004).

In the present study, sperm DNA fragmentation was assessed in the same study population scheduled for IVF treatment. Significant intra-individual variability of sperm chromatin-structure assay in a retrospective study of 282 infertile men has been reported (Erenpreiss et al., 2006). Known factors for these variations include drugs, high-fever episodes (Evenson et al., 1991, 2000) and abstinence time (Cohen-Bacrie et al., 2009). It has been suggested that increasing the frequency of ejaculates could reduce the DNA damage presumably by reducing transit time through the epididymis and reducing exposure to reactive oxygen species (Greening, 2007). Although the majority of reports relating sperm DNA damage with assisted reproduction treatment outcome have used unprepared semen values, the present study performed the assessment of DNA fragmentation once the density-gradient selection was done in processed semen samples. Previous studies had found that sperm preparation removes a significant amount of spermatozoa with DNA breaks (Bungum et al., 2008) or at least does not increase the level of DNA damage (Younglai et al., 2001). Regarding this, strengths of the present study include the fact that sperm samples used for assisted reproduction treatment were analysed and that DNA fragmentation was tested in processed samples.

In this study, like in others (Collins et al., 2008), the sperm DNA-integrity assay was not sufficient to discriminate reliably, as a diagnostic test must do, between couples who would or would not conceive. Embryo development and subsequent pregnancy are possible despite high levels of DNA fragmentation in the sperm sample (Bungum, 2004; Gandini et al., 2004; Huang et al., 2005; Payne et al., 2005). No differences were observed in DNA-fragmentation percentages between pregnant and non-pregnant women. In addition, based on the ROC curves and using a 36% threshold, sperm DNA fragmentation and pregnancy achievement remained unrelated. The lack of correlation between sperm DNA fragmentation and pregnancy outcome might be due to embryo selection before transfer. Although there was no difference in the percentage of DNA fragmentation between patients with miscarriage and patients with successful deliveries, when the percentage of DNA fragmentation was $\geq$36%, the miscarriage rate increased fivefold in recipients of their own oocytes and fourfold in those of donor eggs as compared with DNA fragmentation percentages $<$36%. These findings are consistent with data reported in other studies (Bungum, 2004; Carrell et al., 2003; Check et al., 2005; Virro et al., 2004). However, one of the limitations of the present study is the low number of ongoing pregnancies and pregnancy losses that could be analysed.

To date, the clinical value of sperm DNA-damage testing is not fully defined. In a systematic review and meta-analysis of 22 relevant studies to evaluate the predictive value of sperm DNA-integrity tests for pregnancy from IVF treatment (Collins et al., 2008), the evidence is not strong enough to provide a clinical indication for routine use of these tests in infertility evaluation of men. Alternatively, it is difficult to compare data from different laboratories because there is a lack of standardization of the protocol assays. It has been shown that small variations in steps of the TUNEL assay, including concentration of the fixative, time of storage of fixed samples, type of fluorochrome used to label DNA breaks and the method used to analyse flow cytometric data, all greatly affect the measurement of sperm DNA fragmentation (Muratori et al., 2010). It will not be possible to know the real effect of sperm DNA fragmentation on IVF procedures until a large amount of data is published, and results would only be comparable if the semen samples analysed are the same and a step-by-step standardization of the TUNEL assay is agreed upon.

In summary, sperm DNA damage was not related to outcomes of IVF or ICSI with own or donated oocytes. Clinical pregnancy and implantation rates seem to be independent of sperm DNA fragmentation.

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