ORIGINAL ARTICLE

Can DNA fragmentation of neat or swim-up spermatozoa be used to predict pregnancy following ICSI of fertile oocyte donors?

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This study compared the potential of assessing sperm DNA fragmentation (SDF) from neat semen and the subsequent swim-up (SU) procedure to predict pregnancy when conducting ICSI of fertile donor oocytes. Infertile females ($n=81$) were transferred embryos resulting from intracytoplasmic sperm injection (ICSI) of their partner's spermatozoa and proven donor oocytes. This model normalized the impact of female factor in putative sperm DNA repair. Semen was blindly assessed for SDF using Halosperm immediately following ejaculation (NS) and after swim-up at the time of ICSI fertilisation. There was a decrease in SDF values of the ejaculated semen sample following the swim-up protocol ($P=0.000$). Interestingly, pregnancy could be equally predicted from SDF values derived from either neat or swim-up semen samples. Receiver operator curves and the derived Youden's indices determined SDF cutoff values for NS and SU of 24.8% and 17.5%, respectively. Prediction of pregnancy from NS SDF had a sensitivity of 75% and a specificity of 69%, whereas for SU SDF was 78% and 73%, respectively. While increased levels of SDF negatively impact reproductive outcome, we have shown that a reduction in SDF following sperm selection using ICSI with proven donor oocytes is not mandatory for achieving pregnancy. This suggests that a certain level of DNA damage that is not detectable using current technologies could be impacting on the relative success of assisted reproductive technology (ART) procedures. Consequently, we propose a modification of the so called 'iceberg model' as a possible rationale for understanding the role of SDF in reproductive outcome.

INTRODUCTION

Human classical seminal parameters that reliably predict the outcome of human assisted reproduction are controversial,[1–4] but if available, would constitute a valuable tool for the reproductive physician. In this respect, the assessment of sperm DNA fragmentation (SDF) as a potential predictor of fertility is gaining increased acceptance;[5–7] in fact, we would suggest that this might particularly be the case for predictions of pregnancy based on intracytoplasmic sperm injection (ICSI), as this procedure requires no sperm motility and a single 'selected' spermatozoon, so that the effect of other sperm population parameters on reproductive outcome are largely negated or reduced. Under these conditions, it is possible that sperm DNA fragmentation is likely to be a better predictor of male factor infertility, especially that associated with compromised syngamy and early embryonic loss,[8–11] although in other cases, this relationship is still not clear.[12,13] When predictive values for reproductive outcome are compared using classical seminal parameters and sperm DNA damage assessment, the clinical validity of sperm DNA damage in intrauterine insemination (IUI) is higher than that obtained from sperm morphology alone;[14] even so, there is still considerable heterogeneity among the different

clinical contexts as presented in a meta-analysis recently conducted by Castilla *et al.*[15] Reaching a consensus regarding a threshold level for SDF in terms of the 'Take home baby rate' has also been controversial;[16] while a SDF rate ranging from 30% to 40% is considered by some as being predictive of poor fertility,[6,8,17–19] there are some patients that possess a high level of SDF that are still capable of successful pregnancy,[20] particularly when ICSI is used. Recently, Simon *et al.*[21] have reported that sperm DNA fragmentation was lower in couples achieving pregnancies after *in vitro* fertilisation (IVF) but not using ICSI after density gradient centrifugation for sperm selection.

Regarding the predictive role of DNA damage and pregnancy, the scenario is even more complicated when we examine the values obtained from neat semen samples versus selected samples.[22] For example, in a meta-analysis performed by Li *et al.*,[23] there was no explicit reference as to the origin of the semen sample in terms of neat or selected at the time of SDF assessment, so that interpretation of data of this type is problematic. When sperm selection is performed after IVF, the effect of SDF on the pregnancy rate is relatively low but a meta-analysis by Zini[24] has revealed that it was nevertheless apparent. Although this effect is not so clearly observed after sperm selection and

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ICSI,[14,25] there is still evidence showing that embryo quality may be affected.[26,27]

Additionally, the sensitivity (true positive) and specificity (1: false positive) of using SDF as a predictor of pregnancy varies among different laboratories and is likely to be dependent on the type of ART employed and the varying skill of individual clinicians (see, for example, Tables 3 and 4 in Zini and Sigman[28] and Zini[29]). This inconsistency is probably due to the fact that the genesis of SDF in the male is not a consequence of a single factor but of a series of inter-related events including defective spermatogenesis during chromatin remodelling, the interaction of spermatozoa with oxidative stress, exposure to bacterial infections, the presence of chromosomal abnormalities,[30] constitutive genetic conditions,[31,32] genomic modifications, such as telomere-shortening [33] or environmental stress.[34] The predictive value of SDF is also influenced by the quality and competence of the oocyte, and the capacity of the female gamete to repair sperm DNA damage following syngamy.[35] While there is a scarcity of information available regarding the differential capacity of the human oocyte for sperm DNA repair,[36,37] it is nevertheless still the case, that the oocyte contributes to at least 50% of the predictive value of a successful pregnancy. Consequently, the varying quality of the oocyte represents a major potential confounding variable when making fertility predictions based solely on SDF, so that the use of high-quality oocytes from proven donors, is likely to be a useful strategy for controlling female factor contribution; we propose that under such an experimental model, the influence of SDF should at the very least, be more easily detected.

The current study was conducted to determine the effect of SDF on the pregnancy rate of patients undergoing ICSI to fertilize oocytes from donors with proven fertility. Our aim was to investigate the predictive value of SDF from neat semen samples obtained and assessed directly after liquefaction with sperm of the same sample that had been processed for sperm selection using and swim-up and used in parallel for ICSI. The use of oocytes from proven donors was used in order to reduce female infertility as a confounding variable.

MATERIALS AND METHODS

Oocytes and spermatozoa
All patients in this study consented to their participation according to Spanish legislation and confidentiality for egg donation programs and following adherence to ethical standards. Eighty-one couples with severe female factor infertility (24–35 years old; mean±s.d.: 29.0±3.6 years) were incorporated into this study and all were processed in the Tambre Fertility Clinic, Madrid, Spain. In all cases, donor oocytes were from anonymous young women of proven fertility (22–25 years old, while spermatozoa for ICSI were provided by husbands or partners ranging in age from 26 to 52 years old (mean ± s.d.: 31.6±6.6 years). Semen samples were obtained following masturbation on the day of fertilisation, placed at room temperature for liquefaction and assessed for SDF. Prior to use in ICSI, the neat semen samples were processed using swim-up in modified Ham's F-10 basal medium—HEPES (Irvine Scientific, CA, USA). Following incubation for 30 min, the supernatant was recovered for use in ICSI after a classical sperm selection using PVP medium (PolyVinyl Pyrrolidone; Origio, Firenze, Italy). SDF was conducted under two different scenarios using the same ejaculate: (i) neat or freshly ejaculated semen after liquefaction (NS) and (ii) spermatozoa selected using a swim-up procedure (SU). To avoid the impact of iatrogenic sperm damage, the NS was assessed for SDF immediately after ejaculate liquefaction. The processed sample was also assessed for SDF at the time of ICSI fertilization. This is an important aspect of the study, since DNA damage, far from being a static parameter, increases as the samples are incubated *in vitro*. This effect is observed both in fresh[38] and in cryopreserved thawed samples.[39] Consequently, the observed values at the time of ICSI fertilisation can be very different to those assessed before or after the time of fertilisation. Clinical pregnancy was confirmed when a gestational sac with foetal heartbeat was detected by ultrasound at 7 weeks of pregnancy.

Assessment of DNA fragmentation
SDF was assessed using the Halosperm G2 test (Halotech DNA, SL, Madrid, Spain) with minor modifications to the staining procedure. The sperm chromatin dispersion test is based on a two main steps (i) controlled DNA denaturation and (ii) controlled protamine removal; this gives rise to partially deproteinized nucleoids in which the DNA loops expand, forming halos of chromatin dispersion. Fragmented sperm nucleoids do not develop a dispersion halo or possess a halo with minimal dispersion; non-fragmented sperm show a significant halo of DNA dispersal (**Figure 1**). The use of mild acid DNA denaturation enhances protein removal, thus producing massive protamine depletion while leaving other proteins such as those forming the flagellum relatively intact (**Figure 1**). DNA haloes produced after the test were visualized by fluorescence microscopy using GelRed (Biotium, Hayward, CA, USA) to stain DNA. A Leica DMLA model motorized fluorescence microscope controlled with software for automatic scanning and image digitisation (Leica Microsystems, Barcelona, Spain) was used for SDF analysis. The microscope was equipped with a Leica EL6000 metal halide fluorescence light source, a charge coupled device (Leica DFC350 FX; Leica Microsystems) and Fluotar ×40 objectives for routine scanning. The study was performed as a blind clinical trial. Three hundred spermatozoa were scored per sample and the proportion of sperm containing a fragmented DNA molecule calculated.

Statistical analysis
Non-parametric statistics for a non-Gaussian population, including pairwise comparisons using exact one-sided Wilcoxon and Mann–Whitney tests were performed. Correlation analysis was conducted using a Spearman's rank test. A receiver operating characteristic (ROC) curve and Youden's index were calculated to test the predictive value of SDF with respect to pregnancy. ROC analysis provides the
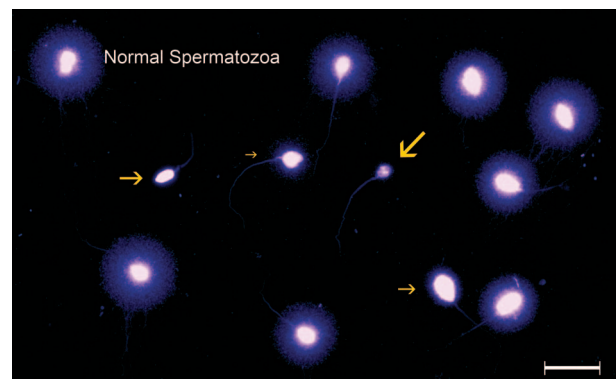

Figure 1 Sperm DNA fragmentation as visualized using Halosperm. Large haloes of chromatin dispersion around a compact core represent sperm showing an orthodox DNA molecule. Sperm presenting damage in the DNA are characterized by small or absent haloes of dispersed chromatin (green arrows). The size of the arrow indicates the extent of the DNA damage. Scale bar=25 µm.

most comprehensive description of diagnostic accuracy available to date because it estimates and reports all of the combinations of sensitivity and specificity that a diagnostic test is able to provide.[40] ROC curves depict a test's ability to correctly identify 'true-positive' and 'true-negative' subjects for various test cutoff points, facilitating a comparison of the overall effectiveness, effectiveness at specified levels of false positives rates and optimal diagnostic ability. The Youden's index, *J*, is a measure of overall diagnostic effectiveness and is a function of sensitivity (probability of a truly diseased individual being diagnosed as such) and specificity (the probability of a truly healthy/non-diseased individual being correctly diagnosed as non-diseased).[41] The index ranges between 0 and 1 with a value of 1 indicating perfect diagnostic effectiveness and 0 indicating an ineffective test. In the majority of cases, there is an inverse relationship between sensitivity and specificity, so moving the 'cut point' increases one while reducing the other; *J* occurs at the 'cut-point' that optimizes the biomarker's differentiating ability when equal weight is given to sensitivity and specificity. Conventionally, *J* is found by evaluating sensitivity and specificity for all possible 'cut points', with the optimal 'cut-point' corresponding to *J*. Statistical analysis was performed using the Statistical Package for the Social Sciences 17 (SPSS Inc., Chicago, IL, USA); $P < 0.05$ was defined as representing a significant difference.

RESULTS

Descriptive statistics

Eighty-one cycles of ICSI using donor oocyte and SU spermatozoa resulted in 49 successful pregnancies, or a pregnancy rate of 60.5%. **Table 1** shows the corresponding mean (±s.d.) SDF values of the NS and SU sperm samples used in the ICSI procedure that subsequently resulted in pregnant and non-pregnant females.

Efficacy of sperm DNA fragmentation via swim-up

Pairwise comparisons using exact one-sided Wilcoxon tests revealed significant differences between the NS and SU SDF values observed within pregnant ($Z=-6.093$; $P=0.000$) and non-pregnant ($Z=-4.938$; $P=0.000$) groups (**Table 1**). To assess whether there was any difference with respect to the efficacy of the post-thaw sperm swim-up procedure on reducing SDF of sperm in either the pregnant and non-pregnant groups, an analysis of covariance of paired samples was performed comparing the change in SDF following swim-up in both groups; pregnancy was used as a cofactor in the model. The results of this analysis are shown in **Figure 2** and revealed that the efficacy for SDF improvement was similar in both groups ($P=0.186$) so that pregnancy was not linked to the efficiency in sperm improvement achieved after swim-up. **Figure 3** shows the relationship between SDF values in NS and SU when data for pregnancy and non-pregnancy were pooled. Using a linear regression model, a significant correlation was found between SDF obtained in NS and SU samples ($R^2=84.8$; ANOVA *F*: 441.9; $P<0.01$; where NS=2.94+1.34×*x*SU).

Table 1 Mean (±s.d.) sperm DNA fragmentation (SDF) of neat (NS) and swim-up (SU) spermatozoa that resulted in pregnancy and non-pregnancy following ICSI of oocytes from proven donors

	Pregnant (n=*49*)	*Non-pregnant* (n=*32*)
Neat semen (NS) SDF	25.3±14.5*	34.9±14.0**
Swim-up (SU) SDF	16.6±9.1*	23.7±10.6**

Significant differences ($P<0.001$) were obtained when NS and SU samples are compared within the pregnant group (*) and non-pregnant-group (**). Data are expressed as mean±s.d.
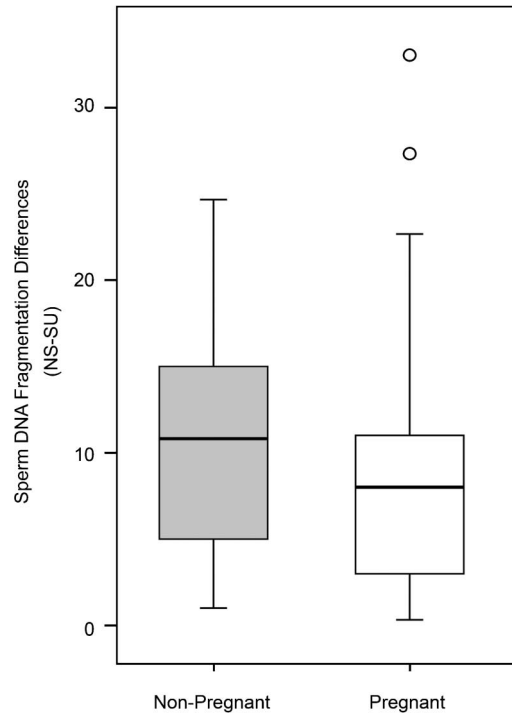

Figure 2 Box and whisker diagram showing the relative improvement in sperm DNA fragmentation following swim-up; sperm samples were grouped into patients where ICSI resulted in pregnancy ($n=49$) or no pregnancy ($n=32$).ICSI, intracytoplasmic sperm injection.

Sperm DNA fragmentation and reproductive outcome

SDF of the NS samples for pregnant and non-pregnant groups were statistically different from each other (**Table 1**; Mann–Whitney *U* test $Z=-3.198$; $P=0.001$), as was the SDF of respective SU samples from the pregnant and non-pregnant groups (**Table 1**; Mann–Whitney test $Z=-3.198$; $P=0.001$). The highest mean SDF value obtained in the experiment was that of the NS of the non-pregnant group, whereas the lowest level was that of the SU of the pregnant group (**Table 1**). It is interesting to highlight that the mean SDF NS value that resulted in pregnancy (25.3 ± 14.5) was not statistically different (Wilcoxon;
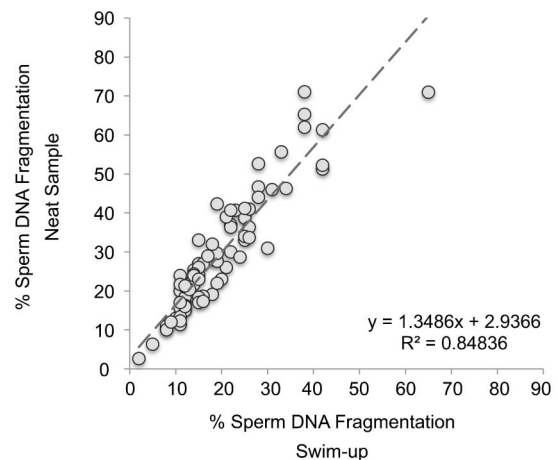

Figure 3 Correlation of % sperm DNA fragmentation for neat semen samples and swim-up spermatozoa. Pooled data from pregnant ($n=49$) and non-pregnant ($n=32$) groups.

$W=785.5$; $P \geqslant 0.05$) to the mean SDF SU value of those patients that failed to produce a pregnancy (23.7 ± 10.6).

Since different values for SDF were obtained using the same ejaculate depending on whether the sample was swim-up or not and given the strong correlation between NS and SU (**Figure 3**), we tested for a possible correlation of SDF values before (NS) and after sperm selection (SU) but also took into account the success of pregnancy. A correlation analysis was performed for both pregnant and non-pregnant groups and a strong correlation was found in both groups (Spearman's rank test: $r=0.79$; $P<0.01$ for pregnant and $r=0.88$; $P<0.01$ for non-pregnant). To further explore this relationship, we tested the performance of our model by calculating the area under the ROC curve for NS and SU (**Figure 4a**). Using this statistical approximation, SDF assessment provided significant areas under the curve (AUC) for predicting pregnancy irrespective of whether the SDF for NS ($AUC=0.711$, $P<0.001$) or SU ($AUC=0.744$, $P<0.01$) was used. Interestingly, not only the AUC but also the shape of the ROC curve depicted under different conditions (NS *vs.* SU) were similar and offered equivalent respective values for sensitivity and specificity (**Figure 4a**). The Youden's *J* statistic, a single statistic that captures the performance of a diagnostic test, was 0.40 for the NS group resulting in a cutoff value for SDF of 26% (**Figure 4b**, 75% sensitivity and 65% of specificity). For the SU group, the value of the Youden's *J* Index was 0.55 with a cutoff value based on SDF of 17.5% (**Figure 4c**,

81% sensitivity and 73% of specificity). In terms of prediction, an NS SDF value of lower than 26% accounted for a pregnancy rate of 80.0% (62.5%–97.5%; 4/20 cases), while an SDF higher than 17.5% shifted this percentage to 54.1% (41.6%–66.6%; 28/61 cases); this gave an odds ratio of 3.39 with an approximate 95% confidence interval of 1.02–11.3. An SU SDF value of lower than 17.5% accounted for a pregnancy rate of 64.2% (52.7%–75.7%; 24/67 cases), while in the group showing an SDF higher than 17.5%, the percentage was 42.9% (17.0%–68.8%; 8/14 cases); this resulted in an odds ratio of 2.39 with an approximate 95% confidence interval (0.74–7.70).

DISCUSSION

This study revealed a significant decrease in the SDF values of the semen following the swim-up protocol; this reduction in SDF occurred in the semen of males irrespective of the reproductive outcome and indicates that a reduction in SDF is, therefore, not necessarily a strong indicator of a successful pregnancy when the sperm samples are used for ICSI. Nevertheless, this study has shown that pregnancy can be reasonably predicted from SDF values derived from either NS or SU semen samples and that in an experimental model that controls for both the quality of the donor oocyte and the SDF assessment at the time of fertilisation, Youden's index SDF 'cutoff' values for NS (26%) and SU (17.5%) produce an assumable predictive capacity for pregnancy. Prediction of pregnancy from NS SDF had sensitivity of 75% and specificity of 69%, whereas the specificity and sensitivity based on SU SDF was 78% and 73%, respectively. Our findings highlight that there is no direct advantage, in terms of predictive value for pregnancy, when using SDF of NS. These results do not in any way imply that neat semen samples should be used for fertilisation or that the reproductive outcome would be similar using directly NS spermatozoa; rather, we propose that SDF assessment of the initial ejaculate has a certain level of predictive value according with the level of confidence obtained from the receiving operation curves and, therefore, could be used as a guideline by the practitioner on initial consultation with the patient. Given that female factor infertility was carefully controlled in this experiment by the use of high quality donor oocytes, our results also reinforce the importance of SDF as a contributing factor of male infertility.

Sperm selection and sperm DNA damage

The efficiency for sperm selection and its association with a decrease in SDF is a topic that has been studied from a range of perspectives[42–45] but deserves further investigation, especially if we take into account possible collateral effects associated with the production of iatrogenic sperm damage via sperm handling.[46] Some studies have reported the benefit of using selected spermatozoa to improve pregnancy[47] and it is logical that the probability of selecting a sperm for ICSI free of DNA damage should increase if density gradient centrifugation or swim-up procedures are implemented. Congruent with this idea are those reports claiming that the predictive value of SDF is low when ICSI is used.[14,15,21] It should be noted that while the incidence of SDF in the selected population may in fact decline after sperm selection, there is still a reasonable chance of accidently selecting one of the remaining underlying sub-population of DNA damaged spermatozoa that was not initially excluded in the selection procedure, i.e., a certain level of damaged spermatozoa still remains in the sample, even under the most stringent and rigorous conditions for sperm selection (see data in Zini *et al.*[42] Gosálvez *et al.*[38] and Enciso *et al.*[43]). By way of an example, if we analyse the data shown by Santiso *et al.*[48] in Figure 1, in some cases and after a swim-up procedure, we are selecting sperm subpopulations
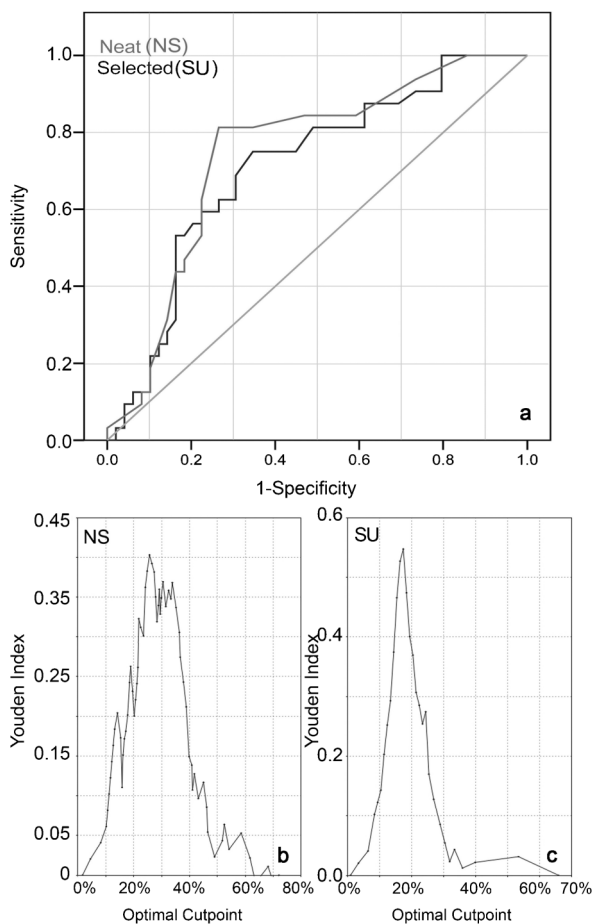

Figure 4 Receiver operating curves for neat (NS) and swim-up (SU) spermatozoa (**a**) and Youden's index plots of optimal cutoff points for NS (**b**) and SU (**c**) sperm DNA fragmentation data.

containing shorter telomeres than those considered as normal. The selection of short telomeres could represent a problem for embryo development, since it produces a large heterozygosity for each chromosome pair with respect to the size of the telomeres. This undesired and undetectable fact may be lethal or at least as deleterious, as it could represent the presence of damaged DNA that was not removed after sperm selection.

We suggest that the swim-up methodology used here, while efficient at removing DNA damaged spermatozoa, did not remove sufficient spermatozoa harbouring apparently sublethal DNA damage in order to improve the capacity of the selected sample to increase reproductive outcome. In more severe male factor infertility, techniques such as PICSI (Physiological ICSI), IMSI (High Magnification Sperm inspection) or MACS (Magnetic Activated Cell Sorting) are potentially able to further refine the efficiency of sperm selection and thereby further increase the probability of a successful pregnancy.[49–51]

Sperm DNA damage and threshold levels

Another interesting finding from this investigation was the specific threshold levels for SDF offered in our predictive ICSI model based on the ROC analysis. As derived from the ROC and the Youden transformation, a neat semen sample with a SDF of 24.8% had the potential to predict pregnancy with sensitivity of 75% and a specificity of 69% (**Figure 4**). If the SDF increased by 10%, then the value for sensitivity would decline to 60% and specificity increase to 73%; a respective decline in SDF by 10% resulted in an increase for sensitivity to 75% and a decline of specificity to 66%. For semen samples recovered after swim-up, and as derived from the ROC and the Youden transformation, the threshold value obtained for SDF was lower (17.5%) than that obtained in neat semen (**Figure 4**); in this case, a sensitivity and specificity of 78% and 73% respectively were obtained. A 10% shift in SDF would result in a decrease of sensitivity to 67% but an increase in specificity to 75%; if SDF declined by 10%, then sensitivity and specificity changed to 78% and 64%, respectively. If we compare these values with previously published data, there appears to be reasonable concordance; for example, it has been proposed that the SCSA technique can predict pregnancy failure when the sperm sample shows an SDF value $\geqslant 25\%$–30%.[18] Spano et al.[8] and Giwercman et al.[19] also showed that a level of sperm DNA damage >20% was associated with decreased fertility and a reduced chance of natural conception. It should be remembered that these predictions have been made on the basis of studying ejaculated neat semen samples, so that these values may change if we use different techniques to determine SDF or if we compare different strategies for ART such as ICSI or IUI (see Table 3 in Zini and Libman[5] and Bungum et al.[14]). Zini and co-workers[28,29] have also shown that for sensitivity and specificity, the confidence interval and the odds ratio will vary when the results from different laboratories are compared. This lack of standardisation in the use of SDF is perhaps not fully appreciated by reproductive clinicians but nevertheless is an extremely important contributing factor when making predictions about pregnancy. The incidence of sperm DNA damage has been shown to be inversely related to fertilisation rate following ICSI.[52,53] Henkel et al.[54] and Benchaib et al.[55] have both reported its negative effect on pregnancy rate if sperm DNA damage was above 36% and 10%, respectively. In IUI cycles, Bungum et al.[14] suggested that SDF can be used as an independent predictor of fertility and proposed that a value of SDF lower than 30% resulted in approximately 19% children per cycle; they also noted that this proportion declined to 1.5%, if SDF increased above 30%. However, in other cases, no specific correlations were found between sperm

DNA damage as observed with the SCD test and pregnancy outcome after IUI.[56] Interestingly in this study, sperm recovered by swim-up did not show a significant improvement in DNA integrity. Using also the sperm chromatin dispersion test and analysing a large cohort of patients attending for IVF or ICSI, Velez de la Calle et al.[57] suggested a threshold sperm DNA fragmentation rate of 18%, above which fragmentation rate was predictive of fertilisation rate. Evidently, the results of the comparison among these ART strategies could be biased since the ART strategies are very different (IUI, FIV-ICSI and egg donation-ICSI). A review of the literature revealed that the predictive value of pregnancy based on SDF appears to be more consistent for IVF/IUI than ICSI.[14,15] The most consistent concept about the impact of sperm DNA damage on pregnancy is that semen prepared by density gradient centrifugation and used for ICSI, has a low power of predictive outcome.[21,22] The situation is quite confusing, and thus, for example, contrasting to those reports where no pregnancy is achieved following assisted reproduction above a sperm DNA fragmentation index of 28%,[58] other authors[14] reported an IUI pregnancy in a man with a DFI of 34%, and also pregnancies following IVF or ICSI with sperm DNA fragmentation indexes above 27%. The negative impact of high levels of SDF on embryo quality as described by Borini et al.[26] or derived from the information supplied by Zini et al.,[27] point to the possible fact that embryo development may be more significant altered in ICSI compared to IVF cycles.

In order to explain these observations, it must be remembered that the oocyte is also playing an important role in the final outcome of the embryo, primarily through its potential ability to repair sperm DNA. In the current experimental design, we assumed a high efficiency of DNA repair and homogeneity as the oocytes we used were all from proven donors; this allowed us to be more confident regarding our predictive SDF values for pregnancy. This is probably the explanation as to why we had reasonably similar predictive SDF values for pregnancy irrespective of whether neat (SDF: 24.8) or swim-up spermatozoa (SDF: 17.5) were used. Where poor quality oocytes from infertile women are involved, the influence of SDF could be even more significant.

The iceberg effect

The unexpected lack of increased predictive power between neat and swim-up SDF reported in this study may be related to the phenomenon previously described by Evenson et al.[59] and Álvarez[60] as the 'iceberg effect' (**Figure 5**). This model proposed that the 'tip of the iceberg' corresponded to sperm DNA damage that is detectable using current available technologies i.e. sperm with massive DNA breakage. These spermatozoa are represented as the first level or the tip of the 'iceberg' in **Figure 5** and correspond to easily detectable highly damaged sperm DNA. Following the swim-up procedure, those cells with detectable DNA damage are removed, but the sperm with undetectable SDF damage still remain hidden or cryptic within the population; these spermatozoa are found within the second level of the iceberg in **Figure 5**. It is possible that this subpopulation has not yet fully expressed itself in terms of SDF at the time of the analysis or selection and thereby represents what might be referred to as spermatozoa with a predisposition to SDF. These sperm are essentially cryptic in terms of SDF detection, waiting 'under the surface', ultimately to be detected, depending on the degree of damage imposed by ex vivo manipulation or iatrogenic damage prior to use in ART. We proposed that dynamic assessment of SDF by incubation of sperm in vitro[38,39] would allow for the detection of this sub-population;[61] this cryptic damage is essentially ignored by single assessments of DNA
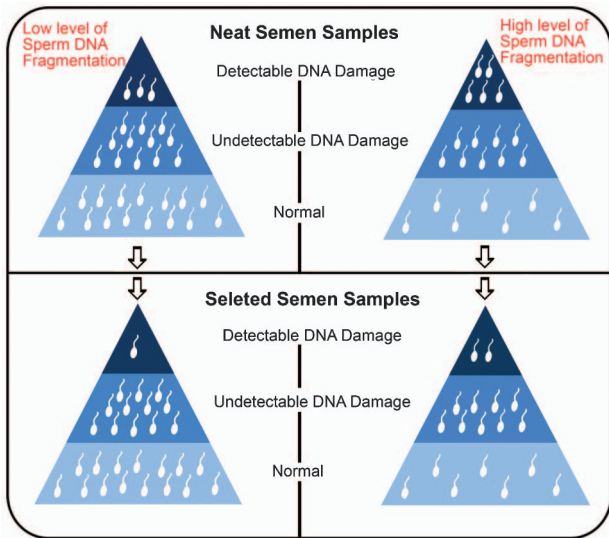
Figure 5 The iceberg effect of cryptic sperm DNA fragmentation adapted from the model originally proposed by Evenson *et al.*[58]

damage. It is possible that this cryptic subpopulation may contain sufficient DNA damage to prevent pregnancy, especially if the oocyte is not capable of DNA repair. The bottom layer of the 'iceberg' model (**Figure 5**) is representative of those spermatozoa that have highly stable DNA, but which under current methodologies cannot yet be physically selected. The primary difference between our model and that originally proposed by Evenson *et al.*[59] is that we are suggesting that there is not always a strong positive correlation between spermatozoa found in the tip of the 'iceberg' and the proportion of spermatozoa in level 2 under the surface. For example, it is possible that a patient may have a low detectable level of SDF but a high underlying undetectable population of sperm with a predisposition for DNA damage. Alternatively, a patient may have a high detectable level of SDF but a low underlying subpopulation of sperm with a predisposition for DNA damage (**Figure 5**). The situation may also exist where a similar detectable level of SDF is present in two individuals, but differences in the underlying undetectable population are present.

Clearly, the amount, quality and distribution of DNA damage may vary among the different spermatozoa in the ejaculate, thus explaining the possibility of successful pregnancies despite a high SDF level.[20] We have already drawn attention to the efficiency of density gradient methodologies and swim-up techniques in eliminating spermatozoa containing double-strand DNA damage and sperm with highly damaged DNA,[43] showing that density gradients are generally more efficient than swim-up at selecting spermatozoa that are free from single-strand DNA damage.[43] Recently, Meseguer *et al.*[35] showed that high-quality donated oocytes can potentially overcome the adverse influence of SDF on pregnancy. It is only when these type of issues and the compounding and interrelated influence of these factors are fully acknowledged that it is possible to make any sense of SDF as a predictor of reproductive success.

In conclusion, our experimental model appears to clarify, within the context of a proven egg donation program, why the sensitivity and specificity offered by the ROC curves are similar, despite different levels of SDF being observed between neat and swim-up spermatozoa. The assessment of SDF, when considered as a discrete value, is of importance to understanding reproductive outcome, but we suggest that it only has predictive value when analysed with respect to the specific ART in which it is used.

AUTHOR CONTRIBUTIONS

JG, PC and RNC were responsible for conception, experimental design and clinical follow up of the different patients. CLF and LO were in charge of the experimental content, data acquisition and data organisation. JLF, SDJ and JG were responsible for drafting the manuscript and intellectual content. JAG participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare they have not any competing financial interests.

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