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Dual use of Diff-Quik-like stains for the simultaneous evaluation of human sperm morphology and chromatin status

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BACKGROUND: Sperm chromatin status and nuclear DNA damage can be detected using well-established assays. However, most techniques are time-consuming and/or involve elaborate protocols and equipment. We have recently developed a simple and fast method to monitor sperm chromatin status in field conditions using the Diff-Quik assay which is employed in fertility clinics to assess sperm morphology with standard bright field microscopy. In the present study, we demonstrate that any Diff-Quik-like stain can easily, reproducibly and routinely monitor human sperm chromatin status as well.

METHODS: Different Diff-Quik-like stains were used to assess sperm morphology and the presence of abnormal dark nuclear staining in human sperm from four ART centres. The TUNEL assay was performed in the same samples, and fertility outcomes were assessed.

RESULTS: A significant correlation was found between TUNEL-positive sperm and dark sperm nuclei. Moreover, associations were also found between the percentage of dark sperm nuclei and seminal parameters, embryo development rate, embryo quality and clinical pregnancy, as well as with cryptorchidism, and there was a tendency towards an association with age. A value of 32% abnormal staining is suggested as a predictive threshold for embryo development and pregnancy.

CONCLUSIONS: Our results show that any Diff-Quik-like stain, already implemented in most laboratories to assess sperm morphology, can be adapted as an indicator for chromatin status in human sperm.

Key words: human sperm / chromatin / nuclear DNA / Diff-Quik staining / sperm morphology

† These authors contributed equally to this work, and should be considered joint first authors.

Introduction

Mounting evidence has shown that standard semen parameters (sperm concentration, morphology and motility; WHO, 1999) do not accurately predict fertility outcomes or the ability of sperm from a given sample to fertilize an oocyte (Agarwal and Allamaneni, 2005), but merely indicate some degree of semen quality and the function of the male reproductive tract.

The role of a spermatozoon as a functional gamete is dependent on many factors, such as the integrity of sperm nuclear DNA, which is required for the correct transmission of paternal genetic information. Several studies have indicated that there is a relationship between sperm DNA integrity and fertility outcomes (reviewed in Agarwal and Allamaneni, 2004; Sharma *et al*., 2004; O'Brien and Zini, 2005; Spano *et al*., 2005; Li *et al*., 2006; Shamsi *et al*., 2008). Although the possible relationship between sperm DNA damage and fertilization rates remains controversial (Sun *et al*., 1997; Lopes *et al*., 1998; Evenson *et al*., 2002; Henkel *et al*., 2004), it seems consensual that a negative correlation between sperm DNA fragmentation and pregnancy rates and/or embryo development does exist (Seli *et al*., 2004; Borini *et al*., 2006; Benchaib *et al*., 2007). Importantly, DNA fragmentation has been pointed out as an important marker of male infertility (Evenson *et al*., 2002; Bungum *et al*., 2004; Erenpreiss *et al*., 2008), and infertile men seem to present significantly higher levels of sperm DNA damage than fertile donors (Chohan *et al*., 2006).

Sperm DNA damage has been detected using a variety of assays (for review, see Evenson *et al*., 2002; Sharma *et al*., 2004; Agarwal and Allamaneni, 2005), such as the sperm chromatin structure assay (SCSA; Evenson *et al*., 1980), the acridine orange test (Tejada *et al*., 1984), the single cell gel electrophoresis assay (COMET; Aravindan *et al*., 1997), the *in situ* nick translation assay (Gorczyca *et al*., 1993) and the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling assay (TUNEL; Sailer *et al*., 1995). Using these assays, attempts have been made towards establishing threshold values for the percentage of sperm with damaged DNA, values above which fertility would be affected. Ultimately, the assessment of sperm DNA status seems to be important for a complete evaluation of sperm quality. However, most of the techniques available are time-consuming and/or involve elaborate protocols, reagents and equipment (e.g. fluorescence microscope and flow cytometer) that do not exist in standard andrology laboratories. Although specific cases may warrant more detailed analysis, it is therefore unlikely that most laboratories can introduce the assessment of sperm DNA status as a routine parameter in semen evaluation (Perreault *et al*., 2003). Possible alternatives to assess chromatin integrity involve colorimetric tests, either directly on sperm with the use of dyes such as Toluidine Blue (Erenpreiss *et al*., 2001) or Aniline Blue (Terquem and Dadoune, 1983) or indirectly on sperm nuclear DNA, such as has been proposed with the sperm chromatin dispersion test (Fernandez *et al*., 2003). However, so far, these simpler methods have not been routinely introduced, probably because these assays require specific stains and other reagents that are not commonly present, or simply because of the difficulty in implementing routine-altering procedures. It is also important to note that the particular compacted nature of sperm chromatin makes it difficult to assess to what degree the defects monitored using the several assays described may also depend on chromatin availability to bind and interact with the several dyes and reagents involved in each case. In fact, an open question remains as to whether what is being monitored using the several assays described above is solely direct DNA damage or changes in chromatin status, notably chromatin packing, which would change DNA availability for reactions in the distinct protocols.

We have recently developed a simple and fast method to monitor sperm DNA status for wild animals under field conditions (Mota and Ramalho-Santos, 2006). This assay uses the Diff-Quik stain and is based on the intensity of nuclear staining: normal sperm heads/nuclei stain lightly, whereas heads/nuclei with fragmented/damaged DNA present a darker stain. Although there are several commercial Diff-Quik-like kits available, with different brand names, the protocol always consists of methanol fixation, followed by sequential exposure to eosin, an anionic/acid dye that stains positively charged/basic proteins red, and a thiazin dye (often Methylene Blue or its oxidation products, such as Azure B; or even a mix of several thiazins) which stains DNA blue. The important issue is that this stain is already widely used clinically to assess sperm morphology with standard bright field microscopy. In the present study, we demonstrate that any Diff-Quik-like stain commonly used in Andrology laboratories to assess sperm morphology can also easily, reproducibly and routinely serve to give an indication on the status, not necessarily of nuclear DNA, but, more broadly, of the chromatin in human sperm.

Materials and Methods

All chemicals were from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise. All patients signed informed consent forms, and all human material was used in accordance with the appropriate ethical and internal review board (IRB) guidelines provided by the participating institutions.

Biological material

Human sperm samples were obtained from the reproduction laboratories of the University Hospitals of Coimbra, from the Pasteur Saint-Esprit clinic in Brest, from CEIE in Oporto and from the Hospital Center of V.N. Gaia, from patients undergoing routine semen analysis or fertility treatments involving either *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). A total of 483 samples were collected and used in this study: 364 for assessment of fertility outcome parameters and 119 for optimization, and for comparisons with the TUNEL assay and between stains. Fresh semen samples were obtained by masturbation after 2–5 days of abstinence and prepared as described earlier (Varum *et al*., 2007). Seminal parameters, namely sperm concentration and sperm motility, were evaluated (WHO, 1999).

Fertility outcome parameters

A total of 364 cycles were included in this analysis. Fertility results from IVF and ICSI cycles were evaluated (Table I). Fertilization rate (number of 2PN oocytes/number of inseminated or injected oocytes) and embryo development rate (number of embryos/number of inseminated or injected oocytes) were calculated. Embryos were classified according to the number, form and symmetry of blastomeres and the presence of blastomere fragmentation, and were graded from I to IV as described (Elder and Dale, 2000). Clinical pregnancy rate was also recorded.

Table I Mean percentages of clinical parameters, including together data from all participating institutions, in a total of 364 cycles*

Parameter	Mean (%)
Fertilization rate	64.7
Embryo development rate	58.7
Transfer rate	83.0
Pregnancy rate	30.5

*Fertilization rate, number of 2PN oocytes/number of inseminated or injected oocytes; embryo development rate, number of embryos/number of inseminated or injected oocytes; transfer rate, number of cases transferred/number of cycles; pregnancy rate, number of clinical pregnancies/number of transfers.

Diff-Quik®, Hemacolor® and Giemsa® assays

Both sperm morphology and staining features were assessed using three different commercial kits: Diff-Quik® (Dade Behring Inc., Newark, NJ, USA), Hemacolor® (Merck, Darmstadt, Germany) and Giemsa® (Merck). Each of these Diff-Quik-like kits is composed of methanol (fixative), eosin (dye that stains basic proteins red) and by a thiazin-like stain (which stains DNA blue). In the classic Giemsa kit, eosin and the thiazin are mixed in the same solution, whereas in the two other kits the application of each dye is sequential. Smears were prepared as previously described (Mota and Ramalho-Santos, 2006). Briefly, 10 µl of a sperm suspension was dragged with a coverslip and allowed to air dry. Slides were then immersed, sequentially and for 10–20 s, in each kit solution, and then rapidly dipped in water to remove excess dye. Slides were allowed to air dry and were observed with a bright field microscope. The staining characteristics were always analysed within each sample, and two staining categories were established: sperm with light heads/nuclei (normal staining) and sperm with dark heads/nuclei (abnormal staining). In order to determine the percentage of dark sperm nuclei, 200 cells were counted per sample in four different fields. Sperm morphology was assessed using strict criteria (Kruger *et al*., 1986). All assays were done blindly and results were reproducible for three different observers and on several optical microscopy setups. Following initial studies, five more observers were trained, with consistent reproducibility.

In addition to commercially available kits, the assay was also performed using in-house prepared solutions. Accordingly, fixation was carried out using 10%, 60% or 100% methanol, or 100% ethanol; protein staining was performed using 0.1, 1, 5 or 10 g/l of eosin and DNA staining was performed using 0.1, 1, 5 or 10 g/l solutions of Methylene Blue, Azure A, Azure B or Toluidine Blue. Furthermore, three sample exposure times (5, 10 or 20 s) to each solution were used. In all cases, the percentage of abnormal dark sperm nuclei was assessed, as previously described, and compared with results obtained with the commercial Diff-Quik kit.

Positive controls for sperm chromatin damage

Sperm samples were exposed to 50 U/ml deoxyribonuclease I (DNase I, Ambion, Austin, TX, USA) for 10 min (to promote DNA fragmentation), to 0.5% hydrogen peroxide (H_2O_2) overnight (to induce apoptosis) or to a temperature of 75°C for 5 min (to induce chromatin decondensation). After each treatment, the Diff-Quik staining was performed as described above, and the percentage of abnormal dark sperm nuclei was assessed.

TUNEL assay

DNA fragmentation was monitored using the APO-BrdU TUNEL assay kit (Molecular Probes, Eugene, OR, USA), as previously described (Varum *et al*., 2007). Briefly, 5×10^6 sperm/ml were fixed with 2% formaldehyde in phosphate-buffered saline (PBS; pH = 7.2) for 35 min, permeabilized in PBS with 1% Triton X-100 for 15 min and washed with the washing buffer provided. Samples were then incubated with a DNA labelling solution (10 µl of reaction buffer, 0.75 µl of TdT enzyme, 8.0 µl of BrdUTP and 31.25 µl of H_2O) for 60 min at 37°C. Cell suspensions were then washed in 200 µl of rinse buffer and cell pellets were incubated with Alexa Fluor 488 anti-BrdU antibody (diluted 1:20 in rinse buffer) for 45 min in the dark at room temperature. Sperm were counterstained with 4,6-diamino-2-phenylindole (DAPI; Molecular Probes) and mounted in VectaShield mounting medium (Vector Labs, Burlingame, CA, USA). Slides were observed using a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band-pass filter, and 200 sperm per sample were counted in four fields, by two independent observers.

Statistical analysis

Statistical analysis was carried out using the SPSS version 13.0 software for Windows (SPSS Inc., Chicago, IL, USA). All values are expressed as means ± standard deviation (SD). The criterion of data normality was evaluated using the Kolmogorov–Smirnov test. Pearson's correlation coefficients (r) and multiple linear regression (r^2) analysis were performed to determine the correlation and relationship of the results obtained with the three different Diff-Quik-like kits, respectively, both for abnormal staining and for morphology. Similar analyses were performed between the percentages of dark sperm nuclei and TUNEL-positive sperm. A Bland–Altman test (Bland and Altman, 1986) was also performed to check agreement between the percentage of abnormal dark sperm nuclei and TUNEL-positive sperm, using the Analyse-it software version 2.11. Comparisons between stains were also carried out by paired samples t-test for the mean percentages of morphologically normal sperm. Correlations between abnormal staining and seminal parameters were also investigated. As data of fertilization rate and embryo development rate do not present a normal distribution, Spearman's non-parametric correlation coefficient test was performed to determine if there were any correlations with abnormal staining. t-Test for equality of means was performed for embryo quality, pregnancy outcome, male patient age and incidence of cryptorchidism. Statistical relevance was considered when $P < 0.05$.

Results

The use of the Diff-Quik®, Hemacolor® and Giemsa® kits was optimized as previously described for cat sperm (Mota and Ramalho-Santos, 2006). Previous observations in this model system have shown that controlled incubation of sperm samples in the Diff-Quik® stain rendered most cell nuclei in the majority of samples lightly stained (thus considered 'normal' staining), whereas some sperm depicted darkly stained nuclei (thus considered 'abnormal' staining). The optimization of each kit is mostly related with the incubation time needed in each staining solution, which, in turn, is dependent of each observer's ability to discriminate sperm structures and may also vary with different Diff-Quik-like kits. Exposure for short periods may not allow enough contrast to properly distinguish sperm structures, whereas long incubation periods (e.g. 1–5 min), especially in the solutions containing thiazins, resulted in an uniform dark blue stain on all sperm heads. Although sperm morphology could still easily be monitored

Table II Correlation coefficients (r) and relationships (r^2) between the three Diff-Quik-like stains in human sperm in terms of normal morphology ($n = 42$) and abnormal staining ($n = 40$)

		Normal morphology		Abnormal staining	
		Hemacolor	Giemsa	Hemacolor	Giemsa
Diff-Quik	r	0.658	0.810	0.833	0.849
	r^2	0.433	0.656	0.694	0.721
Giemsa	r	0.634		0.847	
	r^2	0.402		0.718	

All values are statistically significant ($P < 0.001$).

under these conditions, cells with distinct staining hues could no longer be identified. After establishing the optimal parameters for each kit, the assay was very reproducible.

Sperm morphology was assessed with the three kits, in the same samples, using the strict criteria (Kruger *et al.*, 1986). Significant high correlations (r) and relationships (r^2) were found between the results of different Diff-Quik-like stains (Table II). Furthermore, there were no statistical differences between the mean percentages of morphologically normal sperm detected using each assay (6.27 $\pm$ 4.8, 6.88 $\pm$ 4.9 and 7.52 $\pm$ 5.5, for Diff-Quik, Hemacolor and Giemsa, respectively, $P > 0.05$; Fig. 1).

Whatever the assay used, the presence of lightly and darkly stained sperm heads was observed in every sample (Fig. 2A and B, Table II). Different combinations of staining kits, optical microscopes, lamp intensities and camera settings were employed, resulting in different background illuminations and sperm contrast (Fig. 2A and B). However, the same samples gave similar percentages of abnormal staining using different kits (Table II), and each of these individual settings was always consistent. Following an initial experimental period, the same conditions should be maintained for routine analysis. In addition, sperm morphology values obtained using the dual-purpose version of the stain were indistinguishable to those determined in parallel assays, in which only morphology was evaluated in the same sample by an independent observer (data not shown). In order to explore the possible variations that can exist between different commercially available kits, different concentrations of fixative and of both protein and DNA stains were used. Different compounds of the thiazin family were also tested. Fixation with different concentrations of methanol (10%, 60% and 100%) and ethanol (100%) was performed and the results were compared with the results obtained with the fixation solution included in the commercial Diff-Quik kit (60% methanol). Similarly, protein staining with different eosin solutions (0.1–10 g/l) was performed and compared with the commercial Diff-Quik kit. In both cases, results obtained with the different solutions were indistinguishable from those obtained with the kit (data not shown), suggesting that different concentrations of methanol or the use of ethanol, or different concentrations of eosin, do not modify sperm staining patterns.

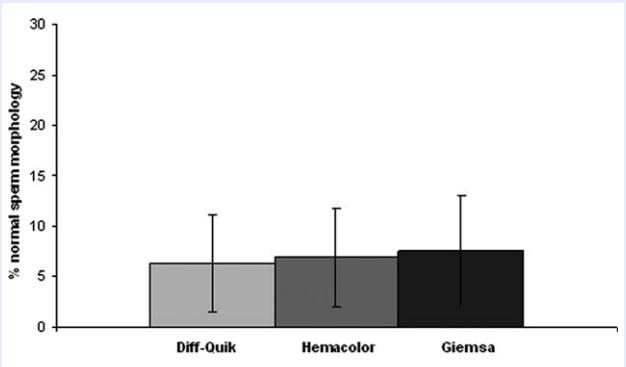

Figure 1 Mean percentage of human sperm with normal morphology assessed by each Diff-Quik-like stain, using strict criteria.

No statistical differences were found ($P > 0.05$) between the three kits. Results represent the mean $\pm$ SD in terms of percentage of cells, from 200 sperm observed for each sample ($n = 42$).

For the theoretically more crucial process of DNA staining, we tested different thiazins, such as Methylene Blue and its oxidation products, Azure A and Azure B, as well as another compound of the thiazin family, Toluidine Blue. For all dyes, different concentrations were used (0.1, 1, 5 and 10 g/l). Results obtained were similar whatever the thiazin solution used (data not shown). However, comparing with the commercial Diff-Quik kit, the results obtained indicate that the use of thiazin concentrations below 1 g/l causes a homogeneously light sperm staining impairing the evaluation of the presence of both normal and abnormal staining sperm. It should be noted that Toluidine Blue was not used as has been described for the Toluidine Blue test (Erenpreiss *et al.*, 2001), but as a thiazin family compound that could be part of a Diff-Quik-like kit, with a simpler protocol.

Using the commercially available Diff-Quik, we found that the percentage of dark sperm heads was highly and significantly correlated with the proportion of sperm with damaged nuclear DNA, as monitored using the TUNEL assay (TUNEL-positive sperm; Fig. 2C) ($r = 0.681$, $r^2 = 0.464$, $P < 0.001$; Fig. 3). Despite the existing correlation between the proposed method and the TUNEL assay, Bland–Altman analysis monitoring agreement between the two methods indicated that they do not measure the same parameter. The mean difference found was +11.5 (meaning the average Diff-Quik measurement is 11.5% higher than a TUNEL measurement in the same sample), but with limits from +31.8 to −8.8, suggesting a wide variation. Given that the two methods are not measuring exactly the same thing, positive controls were performed in order to understand what kind of sperm chromatin impairments may be represented by dark sperm nuclei. When sperm samples were submitted to DNase I, hydrogen peroxide or excess heat, there was a statistically significant ($P =$

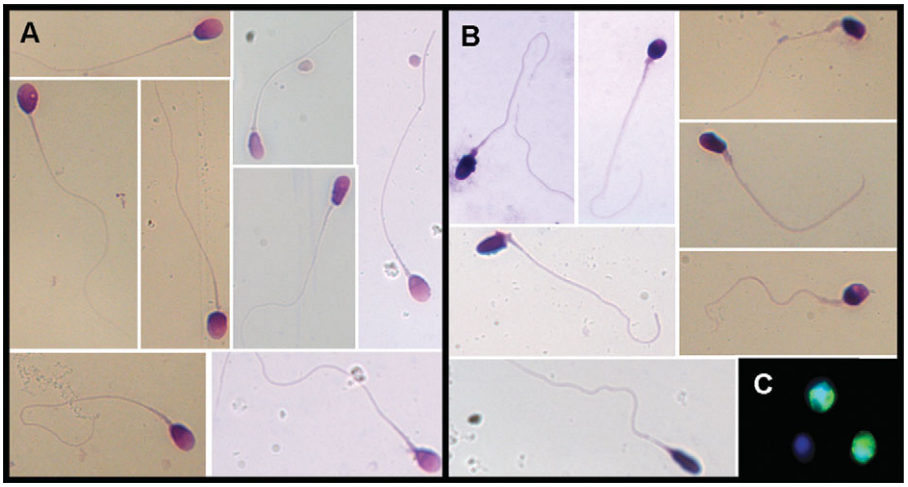

Figure 2 Human spermatozoa assessed for chromatin status.

(**A** and **B**) Diff-Quik-like assay with a standard bright field microscope. Using any of the three assays, spermatozoa with different staining intensities can be observed, which include normal staining patterns (**A**), and sperm with abnormal dark staining heads (**B**). Note that different assays and microscope/camera settings can lead to different backgrounds. (**C**) TUNEL assay, observed by fluorescence microscopy: sperm with DNA damage (TUNEL-positive sperm) present green fluorescence, and sperm with intact DNA (TUNEL-negative sperm) are only labelled with the blue DAPI counterstain.

0.007, $P = 0.045$ and $P < 0.0001$, respectively) increase in the percentage of abnormal dark sperm nuclei (66.9 ± 12.2%, 63.9 ± 13% and 72.9 ± 10%, respectively) compared with the control situation (54.8 ± 11.2%) (Table III), especially for heat, suggesting that chromatin availability to bind dyes may be the limiting factor.

Besides sperm morphology, other routine seminal parameters were also assessed, such as sperm concentration and sperm motility (WHO, 1999). Significant negative correlations were found between the percentages of abnormal dark sperm nuclei and sperm concentration ($r = -0.16$, $P < 0.05$), progressive motility ($r = -0.16$, $P < 0.05$) and normal morphology ($r = -0.30$, $P < 0.0001$).

Regarding the relationship between fertility outcome parameters and sperm staining with Diff-Quik, there was a statistically significant correlation between the percentage of dark sperm nuclei and the embryo development rate (number of embryos/oocytes; $r = -0.109$, $P = 0.045$); however, no correlation with fertilization rate was found. On the other hand, there was a correlation with embryo quality. Embryos were classified from Grades I to IV taking into account the number, form and symmetry of blastomeres and the presence of blastomere fragmentation (Elder and Dale, 2000). Cases where all embryos were classified as Grade I were considered as the 'G1' group, and cases where some or all embryos presented a grade other than Grade I were included in the 'other grade' group (Table IV). When we compared the mean percentage of abnormal dark sperm nuclei from the two embryo quality groups, a significant difference ($P = 0.047$) was found, with the G1 group corresponding to a lower mean percentage of sperm with abnormal dark nuclei (60.18 ± 19.4 and 64.68 ± 19.8, respectively). In addition, clinical pregnancies were also recorded and the mean percentage of abnormal dark sperm nuclei from the pregnant and non-pregnant groups was

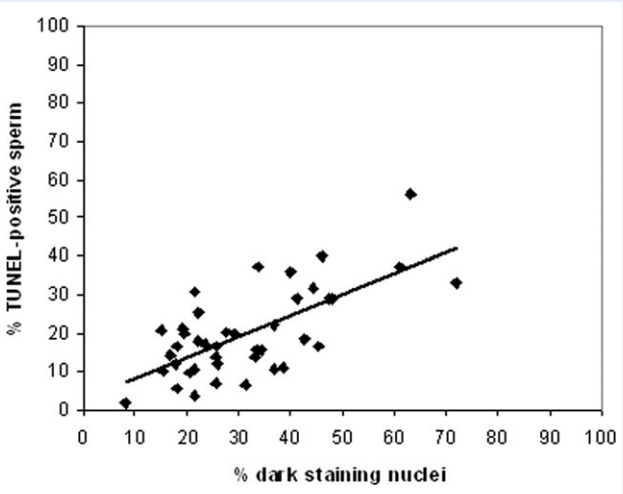

Figure 3 Correlation between the percentage of TUNEL-positive sperm and the proportion of sperm with dark heads/nuclei.

A significant high correlation was found ($r = 0.681$; $P < 0.001$). The data fit the linear regression curve: $y = 0.5514x + 2.5977$; $r^2 = 0.464$. Results represent the percentage of cells from 200 sperm observed for each sample ($n = 42$).

Table III Comparison of mean percentage (±SD) of dark sperm nuclei in samples subjected to induced DNA/chromatin damage

Treatment	Mean ± SD	*P*-value
Control	54.81 ± 11.2	
DNase I	66.88 ± 12.2	0.007
H_2O_2	63.93 ± 13	0.045
Heat	72.88 ± 10	0.0001

Table IV Comparison between the groups according to embryo quality (good quality embryos, 'G1', and poor quality embryos, 'other grade'), clinical pregnancy, male age and cryptorchidism incidence

Parameters	Groups	Mean ± SD	*P*-value
Embryo quality	G1	60.18 ± 19.4	0.047
	Other grade	64.68 ± 19.8	
Clinical pregnancies	Yes	65.87 ± 18.7	0.027
	No	72.08 ± 16.3	
Age	20–34	57.21 ± 21.9	0.074
	≥35	61.10 ± 20	
Cryptorchidism incidence	Cryptorchid	76.04 ± 13.8	0.034
	Non-cryptorchid	68.60 ± 19.1	

Values are mean percentages (±SD) of dark sperm nuclei assessed using a Diff-Quik-like stain.

compared (Table IV). There was a significant difference ($P = 0.027$) between the groups, with a lower mean percentage of sperm with abnormal dark nuclei in the pregnant group (65.87 ± 18.7 and 72.08 ± 16.3, respectively).

Using the linear regression curve, we determined the cut-off values corresponding to the TUNEL thresholds described in the literature (Benchaib *et al*., 2003; Sergerie *et al*., 2005). Thus, the 20% threshold assessed using the TUNEL assay correspond to a cut-off value of 32% for abnormal staining, assessed using a Diff-Quik-like stain. Furthermore, a cut-off value of 32% for abnormal staining was indicative of embryo development and pregnancy: for values above 32% of abnormal dark sperm nuclei, there was a significant decrease in the embryo development rate (71–58%, $P < 0.05$) and a decrease in the pregnancy rate of ~2-fold (67–35%).

To assess a possible effect of age on sperm chromatin damage, we divided our samples in order to obtain two groups with a similar number of cases: a group with age ranging from 20 to 34 years old, and a second group of patients aged 35 or older (Table IV). There was a tendency for an increase in the percentage of sperm with abnormal dark nuclei in the older group than in the younger group (61.10 ± 20 and 57.21 ± 21.9, respectively); however, this increase was not statistically significant ($P = 0.074$).

Finally, at the Brest Centre, men involved in both IVF and ICSI cycles answer a questionnaire that includes a mention of cryptorchidism history, which has a high incidence in the region, possibly due to endocrine disruption (Phillips and Tanphaichitr, 2008). Of the 136 patients from this clinic, 23 patients (16.9%) presented a history of cryptorchidism (Table IV), and that same group of cryptorchid patients showed a significantly higher percentage of abnormal dark sperm nuclei than the non-cryptorchid group (76.04 ± 13.8 and 68.60 ± 19.1, respectively, $P = 0.034$), in accordance with what has been described for this condition (Stronati *et al*., 2006).

Discussion

Routine semen parameters are not able to assess sperm nuclear status and men with abnormal sperm DNA may present normal spermiograms (Alvarez, 2003; Agarwal and Allamaneni, 2004). Thus, an important aspect of sperm status, certainly relevant to monitor the status of male gametogenesis and reproductive tract, and with possible influence on the outcome of ART, is not evaluated (O'Brien and Zini, 2005; Spano *et al*., 2005; Li *et al*., 2006).

Several studies have used tests that purport to monitor DNA integrity (Evenson *et al*., 2002; Fernandez *et al*., 2003; Sharma *et al*., 2004; Agarwal and Allamaneni, 2005), although what is being monitored may be more accurately defined as 'chromatin status' rather than 'DNA status', since sperm chromatin compaction may affect the interactions with DNA. Despite their possible clinical value, these techniques are expensive, time-consuming and/or involve elaborate protocols, reagents and equipment that are not available in most fertility clinics or laboratories. Realistically, at least at an initial stage, recruitment for large studies in several locations will have to rely on simple and time-efficient methodology (Perreault *et al*., 2003). The present data suggest a simple, inexpensive and fast method that assesses chromatin status using, in essence, any Diff-Quik-like staining which is routinely used to monitor sperm morphology.

A highly significant correlation was observed between sperm with dark heads/nuclei, measured using a Diff-Quik-like stain, and sperm with fragmented DNA, as detected using the TUNEL assay. However, given that the percentage of abnormal dark sperm nuclei obtained is always higher than the percentage of TUNEL-positive sperm and that the two methods do not measure exactly the same thing, it is important to understand what is being monitored. Thiazins are DNA-binding dyes (unlike protein-binding dyes such as Aniline Blue) and it is possible that changes in sperm chromatin, and thus nuclear DNA, alter thiazin-DNA interactions, as is the case with the acridine orange interaction with DNA in the SCSA assay. For example, both breaks in DNA or changes in compaction could lead to more dye-binding sites, thus increasing the percentage of darker nuclei. Furthermore, as already noted, interaction depends on dye accessibility to sperm DNA, which can vary greatly (Bungum *et al*., 2007,2008), be donor-dependent (Erenpreiss *et al*., 2006) and even be influenced by semen composition (Richthoff *et al*., 2002). Adding to this accessibility hypothesis, results from positive controls described to cause an increase in DNA fragmentation/chromatin decondensation (Sun *et al*., 1997; Lopes *et al*., 1998; Varum *et al*., 2007), i.e. an increase in DNA accessibility, showed significant increase in the percentage of abnormal dark sperm nuclei. Overall, these data suggest that an increase in the percentage of abnormal staining is primarily indicating an increase in dye access to sperm DNA, and thus nuclear damage in the form of a less compacted state likely including, but not exclusively, DNA fragmentation.

As mentioned, this assay is already implemented, in some form, in most laboratories, and different versions give similar results. Given that in most samples analysed, a high percentage of sperm (>40%) was lightly coloured (i.e. 'normal'), with minimal optimization it would be possible to routinely have at least some evaluation of sperm chromatin status and assess its true importance as a marker of male infertility using a Diff-Quik-like stain. Furthermore, sperm morphology can also be easily and simultaneously monitored.

Interestingly there are several relationships between outcome parameters and chromatin status, as assessed using Diff-Quik-like stains. Our results indicate that there is no correlation between abnormal staining and the fertilization rate. Although this finding is contrary to

some studies (Sun *et al*., 1997; Lopes *et al*., 1998; Benchaib *et al*., 2007; Velez de la Calle *et al*., 2007), it is in accordance with the majority (Host *et al*., 2000; Larson-Cook *et al*., 2003; Henkel *et al*., 2004; Lin *et al*., 2008). Since the paternal genome is only activated 2 days after fertilization (Braude *et al*., 1988), the status of sperm DNA may not dramatically influence fertilization. However, and confirming previous findings from several other groups, there was an association between the percentage of dark sperm nuclei and the embryo development rate (Morris *et al*., 2002; Tesarik *et al*., 2004), suggesting that the presence of higher levels of sperm chromatin damage might impair embryo development. Similarly, when the influence of sperm chromatin status on embryo quality was analysed, we determined that good quality embryos are associated with a lower mean percentage of sperm with damaged DNA, in accordance with other data (Zini *et al*., 2005; Velez de la Calle *et al*., 2007). Finally, the establishment of a clinical pregnancy was associated with a lower mean percentage of sperm with abnormal dark nuclei; these results are in accordance with several studies, where relationships between sperm DNA integrity and pregnancy are described (Tomlinson *et al*., 2001; Benchaib *et al*., 2003; Henkel *et al*., 2003; Larson-Cook *et al*., 2003).

In conjunction with male infertility problems, other factors have been associated with an increase in sperm DNA damage. In our population, there was a tendency for an increase in the percentage of sperm with abnormal dark nuclei in men aged 35 or older. Moreover, as has been recently described (Smith *et al*., 2007; Velez de la Calle *et al*., 2007), levels of sperm chromatin damage are significantly higher in cryptorchid subjects.

The assay described here could be useful in large-scale studies assessing sperm chromatin status, such as those concerning the impact of environmental factors on sperm integrity in human populations (Bian *et al*., 2004; Stronati *et al*., 2006; Long *et al*., 2007). Indeed, it could even be carried out in field conditions, as is currently the case for wild carnivores (Mota and Ramalho-Santos, unpublished data).

Several thresholds for sperm DNA or chromatin integrity have been proposed, in terms of their possible predictive power in ART procedures. For example, a 20% or above threshold for TUNEL-positive sperm in a sample has been proposed to distinguish fertile from infertile men (Sergerie *et al*., 2005), and thus define a value above which pregnancy is unlikely to occur (Benchaib *et al*., 2003). This value mathematically corresponds to a cut-off value of ~32% of abnormally stained sperm. Interestingly, when this value is used as a threshold for our method, there are strong indications in terms of embryo development and pregnancy. This threshold is quite similar to the threshold suggested for SCSA (DFI > 30%; Evenson *et al*., 2002; Bungum *et al*., 2007), possibly due to the fact that, as for SCSA, the Diff-Quik-like kits measure DNA accessibility, which may include both DNA fragmentation and chromatin decondensation.

It is clear that several aspects will need to be validated by further work. An important issue relates to the fact that all optical microscopy observations can have some degree of subjectivity, especially with the pressure to produce timely analyses. Even with assays such as TUNEL, proper training is necessary to distinguish 'bona fide' staining from non-specific speckling. But, once this is established, direct comparisons of TUNEL results using fluorescence microscopy and 'blind' flow cytometry in the same samples show complete consistency (Varum *et al*., 2007).

In summary, the present study shows that by using a Diff-Quik-like stain, it would be possible to routinely evaluate chromatin status in human sperm, whether it is related to direct DNA damage or DNA compaction, and assess its true importance as a marker of male infertility. The assay is already implemented, in some form, in most laboratories to assess sperm morphology.

Author Roles

J.R.-S. established the concept. J.R.-S., A.P.M.S. and R.S.T. designed experiments. A.P.M.S. and R.S.T acquired data, and all authors contributed to analysis and interpretation of data, drafting, revising and approving the article.

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