ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
João Ramalho-Santos, Department of Life Sciences, University of Coimbra, Coimbra 3001-401, Portugal. E-mail: jramalho@ci.uc.pt

[a]These authors contributed equally to this work, and should be considered joint first authors.

Keywords:
ART fertility outcomes, chromatin status, Diff-Quik staining, human spermatozoa, sperm preparation techniques

Received: 11-Apr-2013
Revised: 8-Jul-2013
Accepted: 30-Jul-2013

doi: 10.1111/j.2047-2927.2013.00127.x

Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following in vitro fertilization

[1,2,a]R. S. Tavares, [1,a]A. F. Silva, [1]B. Lourenço, [3,4]T. Almeida-Santos, [1,3]A. P. Sousa and [1,2] J. Ramalho-Santos

[1]Biology of Reproduction and Stem Cell Group, CNC – Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal, [2]Department of Life Sciences, University of Coimbra, Coimbra, Portugal, [3]Human Reproduction Service, University Hospitals of Coimbra, Coimbra, Portugal, and [4]Faculty of Medicine, University of Coimbra, Coimbra, Portugal

SUMMARY

Sperm chromatin/DNA damage can be measured by a variety of assays. However, it has been reported that these tests may lose prognostic value in Assisted Reproductive Technology (ART) cycles when assessed in post-prepared samples, possibly due to the normalizing effect promoted by sperm preparation procedures. We have recently implemented a modified version of the Diff-Quik staining assay that allows for the evaluation of human sperm chromatin status in native samples, together with standard sperm morphology assessment. However, the value of this parameter in terms of predicting in vitro fertilization (IVF) and Intracytoplasmic sperm injection (ICSI) outcomes after sperm selection is unknown. In this study, data from 138 couples undergoing in vitro fertilization (IVF) or Intracytoplasmic sperm injection (ICSI) treatments showed that sperm chromatin integrity was significantly improved after density gradient centrifugation and swim up ($p < 0.001$), but no correlations were found with fertilization or embryo development rates ($p > 0.05$). However, sperm samples presenting lower percentages of damaged chromatin were associated with better quality (Grade I) embryos in both ART procedures ($p < 0.05$) and clinical pregnancy among IVF couples ($p < 0.05$). Furthermore, regression analysis confirmed the clinical value of Diff-Quik staining in predicting IVF (but not ICSI) clinical pregnancy (OR: 0.927, 95% CI: 0.871–0.985, $p = 0.015$), and a threshold value of 34.25% for this parameter was established. The proportion of IVF couples achieving a clinical pregnancy was reduced 1.9-fold when the percentage of abnormal dark staining was ≥34.25% ($p = 0.05$). In conclusion, the Diff-Quik staining assay provides useful information regarding ART success, particularly in IVF cycles, where some degree of 'natural' sperm selection may occur; but not in ICSI, where sperm selection is operator dependent. This quick and low-cost assay is suggested as an alternative method to detect sperm chromatin status in minimal clinical settings, when no other well-established and robust assays (e.g. Sperm chromatin structure assay, terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling) are available.

INTRODUCTION

Male (in)fertility diagnosis usually relies on the microscopic assessment of standard WHO-determined semen parameters – sperm concentration, motility and morphology – in the native, unprocessed, sample. However, emerging reports have shown that sperm DNA integrity, required for the proper delivery of the paternal genome to the offspring, may be a better predictor of male fertilizing potential (Agarwal & Allamaneni, 2004; Bungum *et al.*, 2011). Indeed, substantially higher levels of sperm DNA damage have been reported in both infertile men with normal and abnormal standard semen parameters, when compared with fertile donors (Saleh *et al.*, 2002; Zini *et al.*, 2002; Sergerie *et al.*, 2005). As a consequence, DNA damage has been often related to a wide variety of poor reproductive outcomes, including decreased fertilization rates (Sun *et al.*, 1997; Velez de La Calle *et al.*, 2008; Simon *et al.*, 2011), impaired embryo development (Muriel *et al.*, 2006a; Benchaib *et al.*, 2007), lower embryo quality (Saleh *et al.*, 2003; Velez de La Calle *et al.*, 2008; Simon *et al.*, 2011), reduced chances of pregnancy (Saleh *et al.*, 2003; Henkel *et al.*, 2004; Borini *et al.*, 2006) and increased risk of fathering a child with genetic anomalies (Marchetti & Wyrobek, 2005; Aitken & Koppers, 2011). Regardless, the existing data are conflicting,

especially when it concerns the relationship between DNA damage and fertilization rates (Sun *et al.*, 1997; Gandini *et al.*, 2004; Lin *et al.*, 2008; Velez de La Calle *et al.*, 2008; Simon *et al.*, 2011).

Sperm chromatin structure assay (SCSA; Evenson *et al.*, 1980), terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling assay (TUNEL; Gorczyca *et al.*, 1993), Comet (single-cell gel electrophoresis assay; Hughes *et al.*, 1996) and sperm chromatin dispersion test (commercially available as the Halosperm kit; Fernandez *et al.*, 2003) are well-described tests that monitor sperm chromatin/DNA status, but there is still no consensus on which test should be preferentially used (Zini & Sigman, 2009). As they involve extensive procedures and/or expensive reagents and equipment, sperm DNA analysis has not been routinely established in most standard Andrology laboratories (Perreault *et al.*, 2003).

Recently, we have described a simple, inexpensive and quick method to analyse sperm chromatin status in both feline and human spermatozoa using a simple modification in the Diff-Quik stain, a stain already implemented worldwide to evaluate sperm morphology under a standard bright-field microscope, by detecting spermatozoa with different colour intensities using a very short staining protocol (Mota & Ramalho-Santos, 2006; Sousa *et al.*, 2009). In fact, there was a high correlation between the proportion of spermatozoa with dark stained nuclei and TUNEL-positive cells assayed in the same samples. Moreover, a significant increase in spermatozoa with dark nuclei was detected when male gametes were exposed to DNAse I, hydrogen peroxide and heat, conditions known to promote DNA fragmentation and chromatin decondensation in vitro. Taken together, these data suggest that a modified Diff-Quik stain can also be indicative of abnormal/damaged sperm chromatin (either decondensed or with fragmented DNA). A possible mechanism is that, as is the case with other DNA dyes, changes in chromatin/DNA create more stain-binding sites, thus increasing the percentage of sperm nuclei with a darker colour (Sousa *et al.*, 2009). As observed with other assays, the abnormal chromatin status in native spermatozoa assessed by the Diff-Quik method was negatively correlated with embryo development rate and higher levels were associated with lower quality embryos and negative clinical pregnancies among Assisted Reproductive Technology (ART) couples (Sousa *et al.*, 2009).

Density gradient centrifugation (DGC) and swim-up techniques, either alone or in combination, often allow the selection of motile spermatozoa with normal morphology for ART treatments (Bungum *et al.*, 2008). These procedures have also been shown to improve chromatin/DNA integrity levels as detected by a wide range of assays (Spanò *et al.*, 1999; Tomlinson *et al.*, 2001; Gandini *et al.*, 2004; Marchesi *et al.*, 2010), although unchanged levels were also reported by a small number of studies (Zini *et al.*, 1999; Muriel *et al.*, 2006b). As theoretically only the best spermatozoa are recovered after DGC and/or swim-up selection, it is argued that a certain degree of homogenization occurs (Tomlinson *et al.*, 2001). Consequently, although some DNA integrity tests, such as SCSA or TUNEL, have been shown to predict ART fertilization and pregnancy rates in raw heterogeneous samples, some authors have reported that their prognostic value is lost in both in vitro fertilization (IVF) and Intracytoplasmic sperm injection (ICSI) when using homogeneous populations (Larson *et al.*, 2000; Gandini *et al.*, 2004; Seli *et al.*, 2004; Bungum *et al.*, 2008), possibly because of this 'normalizing' effect promoted by the sperm preparation techniques.

Thus, this work aimed at using the Diff-Quik staining assay to (i) evaluate sperm chromatin status after DGC followed by swim-up sperm preparation procedures; and (ii) assess its relationship with ART fertility outcomes in the motile sperm fraction.

MATERIAL AND METHODS

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Patients

This study was carried out in the Human Reproduction Service at University Hospitals of Coimbra, Portugal, between January 2012 and February 2013. A total of 138 cycles (57 IVF and 81 ICSI) from 138 couples experiencing infertility for at least 1 year, with no viral infections, and whose female partners were <40 years old and presented baseline Follicle-stimulating hormone (FSH)<12 IU/L were included in this study. Couples with normal standard semen parameters according to the World Health Organization criteria (WHO, 2010) were referred to IVF, whereas ICSI was only performed on couples with poor sperm quality (e.g. low concentration and/or motility), or with a previous history of failed IVF fertilization or low fertilization rates.

Sperm samples were used in agreement with the appropriate ethical and Internal Review Board of the Institution, who approved all the experimental work. All individuals signed informed consent forms and samples were obtained by masturbation after 3–5 days of sexual abstinence. Sperm cells were treated according to the WHO guidelines (WHO, 2010).

Sperm preparation for ART

After sample collection and liquefaction, spermatozoa were isolated by sequential DGC (Isolate Sperm Separation Medium; Irvine Scientific, Santa Ana, CA, USA) and swim-up techniques (Amaral *et al.*, 2007; Sousa *et al.*, 2011). Briefly, sperm samples were placed on top of the upper layer (50%) and centrifuged at 528 × ***g*** for 10 min. Spermatozoa previously collected from the 90% lower layer were then washed with Sperm Preparation Medium (Origio Medicult, Jyllinge, Denmark) and centrifuged at 528 × ***g*** for another 10 min. Motile cells were subsequently allowed to swim up for 30 min at 37 °C and 5% CO_2 in the latter medium. The motile sperm fraction was used in ART.

Sperm chromatin status assessment from both unprocessed samples and motile sperm fractions

Sperm chromatin status from both native samples and the respective motile sperm fractions after sperm selection (n = 138) was evaluated by the Diff-Quik (Dade Behring Inc., Newark, NJ, USA) staining as stated elsewhere (Ramalho-Santos *et al.*, 2007; Sousa *et al.*, 2009). This commercially available kit is composed of a fixative (methanol), a dye that stains basic proteins red (eosin) and a thiazin which stains sperm DNA blue. Briefly, 10 μL of the sample was dragged with a cover slip and allowed to air dry. Slides were then sequentially dipped in each kit solution for *no longer* than 10–20 sec each and finally rinsed in water to remove excess dye. This step is crucial to avoid a uniformly dark staining on all spermatozoa, useful to assess morphology, but which does not provide any chromatin status information. Slides

were observed under a bright-field microscope (Nikon Instruments Inc., Melville, NY, USA) and staining features were constantly evaluated within each slide. Both light- and darkly stained sperm heads were visible in each slide, the latter representing abnormal chromatin status, as not only is their proportion strongly correlated with the proportion of TUNEL-positive cells (Mota & Ramalho-Santos, 2006; Sousa *et al.*, 2009) but also significantly increased when exposed to positive control conditions reported to cause DNA fragmentation and chromatin decondensation (Sousa *et al.*, 2009). In general, changes in sperm chromatin (and thus nuclear DNA), whether because of DNA strand breaks or changes in compaction, may alter thiazin–DNA interactions, leading to more dye-binding sites and therefore may raise the percentage of darker sperm nuclei (Sousa *et al.*, 2009). As such, the proportion of spermatozoa with abnormal dark nuclei representing abnormal/damaged chromatin was established after scoring 200 cells in four different fields in each slide. Counts were performed blindly by at least two observers, and intra- and inter-observer variability was negligible. No clinical data were available to the researchers performing this analysis.

Ovarian stimulation

Individualized ovarian stimulation protocols were performed after evaluation of each patient reproductive status (e.g. ovarian reserve and hormone levels). Long and short protocols of pituitary desensitization with GnRH agonists and antagonists, respectively, were performed. Follicular growth was stimulated by recombinant FSH [37.5–325 IU/day GONAL-f (Merck Serono, London, UK) or Puregon (N.V. Organon, Oss, the Netherlands)] or hMG (50–300 IU/day Menopur; Ferring Pharmaceuticals, West Drayton, UK) and when at least one leading follicle reached a 18 mm diameter (monitored by ultrasound), ovulation was induced with hCG (5000 IU Pregnyl; N.V. Organon). Ultrasound-guided vaginal oocyte aspiration was performed 35–36 h post-hCG administration.

IVF and ICSI protocols

Following sperm preparation and oocyte retrieval, IVF and ICSI procedures were performed as previously described (Santos *et al.*, 2006). For ICSI cycles, cumulus cells were removed from the cumulus–oocyte complexes (COCs) by incubation with 50 μL of hyaluronidase (SynVitro Hyadase; Origio Medicult) and intermittent pipetting for a maximum of 30 sec. Sperm suspensions were placed in SpermSlow medium (Origio Medicult) and only free and motile spermatozoa were immobilized and injected into the oocytes. For IVF, each COC insemination was carried out with 100 000 selected spermatozoa. Injected and inseminated oocytes were cultured at 37 °C and 6% CO_2 in IVF medium (Origio Medicult). Fertilization was assessed after 17–20 h.

Fertility outcome parameters

As previously described, fertilization and embryo development rates were scored as the number of 2PN oocytes/number of inseminated or injected oocytes and the number of embryos/number of inseminated or injected oocytes respectively (Sousa *et al.*, 2009). Embryos were graded from I to IV in accordance with the number, form and symmetry of blastomeres and the presence of blastomere fragmentation 48 h post-fertilization (Elder & Dale, 2000). Grade I embryos, that is embryos with regular blastomere shape and symmetry, light cytoplasmic appearance and blastomere fragmentation of up to 10%, from couples only displaying this high embryo quality were included in the 'G1' group, whereas couples having at least one embryo classified differently were included in the 'other grade' group (Sousa *et al.*, 2009). Embryo transfer rate was determined as the number of transfers performed/number of cycles which obtained embryos. Finally, clinical pregnancies were determined by ultrasound detection of the gestational sac(s) 4 weeks after embryo transfer. Clinical pregnancy rate was scored as the number of couples with positive clinical pregnancy detected by ultrasound/number of transfers.

Statistical analysis

Statistical analysis was carried out using the SPSS version 20.0 software for Windows (SPSS Inc., Chicago, IL, USA). Values are expressed as mean ± SEM. All variables were checked for normal distribution by the Kolmogorov–Smirnov test (or the Shapiro–Wilk test for groups with $n < 25$) and the independent *t*-test for normal variables were performed to compare dark sperm staining before and after sperm selection, between the two embryo quality groups and between pregnancy outcome groups in both IVF and ICSI cycles. As fertilization and embryo development rates presented a non-normal distribution, Spearman's non-parametric correlation coefficient test was performed to determine if there were any correlations with dark sperm staining. The sample sizes used in this study provided power values equal or greater than 80% to detect a 10–15% difference in the proportion of dark sperm staining between groups in each condition assessed, using the independent *t*-test, $p < 0.05$ and 95% CIs. Demographic data comparisons between ART treatments and pregnant vs. non-pregnant couples were performed by the independent *t*-test or the related Mann–Whitney test for non-normal variables and the chi-squared test for categorical data. Logistic regression analysis was carried out to assess the predictive value of several factors in ART pregnancy outcomes. Receiver operating characteristic (ROC) curve analysis allowed the determination of a significant threshold for clinical pregnancy following IVF and the chi-squared test was performed to find a possible threshold effect. Statistically significant differences were considered when $p \leq 0.05$.

RESULTS

Characteristics of the study population

Demographic data from 57 IVF and 81 ICSI cycles are displayed in Table 1. Although female factor was the main cause of infertility in both IVF and ICSI couples (64.91 and 51.90% respectively), the percentage of couples experiencing infertility exclusively because of a male factor was significantly higher among couples referred to ICSI cycles (22.78 and 1.75% $p = 0.001$). As one of the criteria used to perform ICSI is poor semen quality (e.g. low concentration and/or motility), the decrease observed in the sperm concentration of these patients was not surprising ($74.21 \pm 6.88 \times 10^6$/mL and $93.40 \pm 6.5 \times 10^6$/mL, $p = 0.018$). However, total motility (progressive motility + in situ) did not differ between treatments ($p > 0.05$). Among IVF couples, the number of inseminated/injected (11.03 ± 0.88 and 4.07 ± 0.32, $p < 0.001$) and fertilized

Table 1 Background information on both IVF and ICSI cycles

	IVF	ICSI	*p*-value
Cycles/couples included (*n*)	57	81	
Female age (years ± SEM)	33.00 ± 0.49	33.14 ± 0.36	>0.05
Male age (years ± SEM)	35.16 ± 0.73	35.37 ± 0.57	>0.05
Diagnosis of infertility (%)			
Unexplained	21.05	12.66	>0.05
Male factor	1.75	22.78	0.001
Female factor	64.91	51.90	>0.05
Male and female factors	12.28	12.66	>0.05
Type of infertility (%)			
Primary	72.73	72.15	>0.05
Secondary	27.27	27.85	>0.05
Duration of infertility (years ± SEM)	5.16 ± 0.45	6.09 ± 0.35	>0.05
No. of inseminated/injected oocytes (mean ± SEM)	11.03 ± 0.88	4.07 ± 0.32	<0.001
No. of 2PN oocytes (mean ± SEM)	6.11 ± 0.62	2.49 ± 0.20	<0.001
Fertilization rate (%)	57.22 ± 3.77	64.33 ± 3.53	>0.05
No. of embryos (mean ± SEM)	4.85 ± 0.50	2.09 ± 0.13	<0.001
Embryo development rate (%)	50.00 ± 3.44	56.70 ± 3.37	>0.05
No of transferred embryos (mean ± SEM)	1.95 ± 0.07	2.05 ± 0.01	>0.05
Embryo transfer rate (%)	86	94.2	>0.05
Clinical pregnancy rate (%)	41.86	27.69	>0.05
Mean sperm concentration (10^6/mL ± SEM)	93.40 ± 6.50	74.21 ± 6.88	0.018
Sperm motility (mean % ± SEM)	60.89 ± 2.52	56.68 ± 2.46	>0.05

2PN: 2 pronuclei. Fertilization rate = number of 2PN oocytes/number of inseminated or injected oocytes; Embryo development rate = number of embryos/number of inseminated or injected oocytes; Embryo transfer rate = number of transfers performed/number of cycles that obtained embryos; Clinical pregnancy rate = number of pregnant couples/number of transfers.

oocytes (6.11 ± 0.62 and 2.49 ± 0.20, $p < 0.001$) as well as the number of embryos retrieved (4.85 ± 0.50 and 2.09 ± 0.13, $p < 0.001$) were significantly higher than the ones obtained by couples undergoing ICSI treatments. No differences were observed regarding male and female age, type and duration of infertility; and fertilization, embryo development, embryo transfer or clinical pregnancy rates ($p > 0.05$, Table 1).

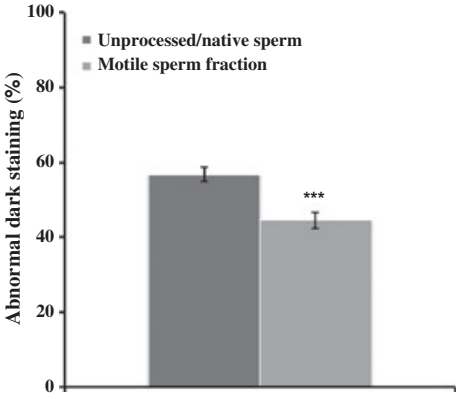
Levels of sperm chromatin damage after DGC and swim-up selection

To potentially maximize ART outcomes, and thus achieve pregnancy, spermatozoa are traditionally processed by DGC and/or swim-up procedures. In our unit, these procedures are routinely carried out sequentially, not as alternatives. Sperm chromatin integrity was significantly improved after this selection, as observed by the decreased proportion of abnormal dark staining in the motile sperm fraction when compared with their unprocessed counterparts (41.13 ± 2.15 and 51.40 ± 1.92, $n = 138$; $p < 0.001$, Fig. 1). It seems therefore that the Diff-Quik staining is able to efficiently detect an enrichment of spermatozoa with chromatin integrity in post-prepared samples, as others have reported using different assays (Spanò *et al.*, 1999; Tomlinson *et al.*, 2001; Gandini *et al.*, 2004; Marchesi *et al.*, 2010).

Chromatin damage, fertilization and embryo development rates

No correlation was detected between the percentage of spermatozoa with dark nuclei and IVF ($\rho = -0.105$, $n = 57$, $p > 0.05$) or ICSI fertilization rates ($\rho = -0.123$, $n = 81$, $p > 0.05$). Moreover, the same lack of association was found between abnormal dark staining and embryo development rates in both IVF and ICSI cycles ($\rho = -0.029$, $n = 51$ and $\rho = -0.067$, $n = 74$, respectively, $p > 0.05$).

Figure 1 Mean proportion of abnormal dark staining spermatozoa in both the native and motile sperm fractions (following sequential application of density gradient and swim up) from the same samples ($n = 138$). ***$p < 0.001$.



Chromatin damage, embryo quality and clinical pregnancy

To further determine if dark sperm nuclei had any relevance in terms of embryo quality, embryos were graded from I to IV according to several embryo features (Elder & Dale, 2000) and couples with only good quality embryos, commonly classified as Grade I, were included in the 'G1' group, whereas couples who had at least one embryo with a different grade were included in the 'other grade' group. For IVF cycles, samples that generated the 'G1' group had a significant lower proportion of spermatozoa with dark staining (32.41 ± 4.03%, $n = 22$, and 47.04 ± 5.77%, $n = 22$, respectively, $p = 0.044$). Similarly, ICSI couples included in the 'G1' group ($n = 38$) presented a significantly lower percentage of dark sperm staining than the ones ($n = 30$) in the 'other grade' group (35.26 ± 3.97 and 47.8 ± 4.69%, respectively, $p = 0.044$).

Several factors may influence the achievement of pregnancy in both IVF and ICSI treatments (Table 2). Pregnant IVF couples presented a higher number of inseminated (11.22 ± 0.95 and 9.00 ± 1.39, $p = 0.023$) and fertilized oocytes (6.78 ± 0.70 and 4.89 ± 0.60, $p = 0.031$) and a decreased proportion of spermatozoa with dark nuclei (36.89 ± 4.52 and 51.75 ± 5.63%, $p = 0.047$) than their non-pregnant counterparts (Table 2). On the other hand, only the number of embryos obtained (2.60 ± 0.19 and 2.09 ± 0.14, $p = 0.05$) and transferred (2.40 ± 0.16 and 1.89 ± 0.12, $p = 0.023$) were significantly increased among couples who became pregnant following an ICSI cycle (Table 2). However, no difference was found between the percentages of dark stained sperm when pregnant and non-pregnant ICSI groups were compared (35.45 ± 4.40% and 44.93 ± 4.15%, $p > 0.05$, Table 2). From all the variables listed in Table 2, only female age (OR: 0.632; 95% CI: 0.431–0.926, $p = 0.018$, Table 3), total sperm motility (OR: 1.092; 95% CI: 1.016–1.174, $p = 0.017$, Table 3) and dark sperm staining (OR: 0.927, 95% CI: 0.871–0.985, $p = 0.015$, Table 3) were found to be predictors of clinical pregnancy among IVF cycles. Although female age seems to be the factor that most contributes to

Table 2 Comparison of several variables among pregnant and non-pregnant couples undergoing IVF or ICSI treatments

	IVF			ICSI		
	Pregnant	Non-pregnant	*p*-value	Pregnant	Non-pregnant	*p*-value
No. of couples/cycles included	18	25		18	47	
Female age (years ± SEM)	31.67 ± 0.79	33.72 ± 0.93	>0.05	34.27 ± 0.62	32.59 ± 0.51	>0.05
Male age (years ± SEM)	33.33 ± 0.67	35.65 ± 1.50	>0.05	35.91 ± 1.22	35.17 ± 0.88	>0.05
No. of inseminated/injected oocytes (mean ± SEM)	11.22 ± 0.95	9.00 ± 1.39	0.023	3.93 ± 0.37	4.61 ± 0.50	>0.05
No. of 2PN oocytes (mean ± SEM)	6.78 ± 0.70	4.89 ± 0.60	0.031	3.13 ± 0.34	2.77 ± 0.28	>0.05
Fertilization rate (mean % ± SEM)	63.87 ± 5.68	60.57 ± 5.29	>0.05	81.00 ± 5.07	68.80 ± 4.06	>0.05
No. of embryos (mean ± SEM)	5.17 ± 0.54	4.44 ± 0.59	>0.05	2.60 ± 0.19	2.09 ± 0.14	0.05
Embryo development rate (mean % ± SEM)	50.70 ± 6.07	53.36 ± 4.75	>0.05	71.33 ± 6.05	58.21 ± 4.13	>0.05
No. of transferred embryos (mean ± SEM)	2.11 ± 0.08	1.89 ± 0.11	>0.05	2.40 ± 0.16	1.89 ± 0.12	0.023
Mean sperm concentration (10^6/mL ± SEM)	112.22 ± 9.55	85.39 ± 12.45	>0.05	89.14 ± 18.26	72.25 ± 8.76	>0.05
Motility (mean % ± SEM)	68.83 ± 3.30	58.67 ± 4.51	>0.05	63.47 ± 3.83	55.55 ± 3.74	>0.05
Abnormal dark staining (mean % ± SEM)	36.89 ± 4.52	51.75 ± 5.63	0.047	35.45 ± 4.40	44.93 ± 4.15	>0.05

2PN: 2 pronuclei. Fertilization rate = number of 2PN oocytes/number of inseminated or injected oocytes; Embryo development rate = number of embryos/number of inseminated or injected oocytes.

Table 3 Odds ratio and 95% CI from several predictors of clinical pregnancy success in both IVF and ICSI cycles

		OR (95% CI)	*p*-value
IVF	Female age	0.632 (0.431–0.926)	0.018
	Motility	1.092 (1.016–1.174)	0.017
	Abnormal dark staining	0.927 (0.871–0.985)	0.015
ICSI	No. of embryos	4.054 (1.308–12.561)	0.015

OR: odds ratio; CI: confidence interval.

pregnancy success in IVF treatments in this particular study, sperm Diff-Quik staining foresees a decrease in pregnancy chances of 7.3% *per* each 1% increase in abnormal dark staining observed. Contrary to this, the number of embryos obtained (OR: 4.054; 95% CI: 1.308–12.561, $p = 0.015$, Table 3), but not sperm staining (OR: 1.01, 95% CI: 0.957–1.106, $p > 0.05$), was predictive of clinical pregnancy for ICSI cycles.

Considering the prognostic value of this modified Diff-Quik assay in IVF pregnancy success a ROC analysis was performed, in an attempt to identify a threshold value for dark sperm staining beyond which clinical pregnancy would be compromised. With an area under the curve of 0.700 cm^2 ($p = 0.046$), a threshold value was set at 34.25% with a sensitivity of 77.8% and a specificity of 52.9%. The proportion of pregnant couples having an abnormal dark staining ≥34.25% was found to be reduced 1.9-fold when compared with the fraction of pregnant couples with sperm dark nuclei below this cut-off ($p = 0.05$).

DISCUSSION

Routine semen analysis does not include the evaluation of sperm DNA damage, despite the multitude of available assays. This lack of assessment has been extensively criticized, as it has become more evident that men with normal standard semen parameters may possess abnormal levels of DNA damage (Agarwal & Allamaneni, 2004), thus potentially contributing to the limited success of ART.

Recently, we modified the Diff-Quik staining method to allow the assessment of sperm morphology and abnormal chromatin status in the same slides, by detecting spermatozoa with dark stained nuclei (Mota & Ramalho-Santos, 2006; Sousa *et al.*, 2009). Despite its clinical value, when used in unprocessed samples, its relationship with ART fertility outcomes after sperm selection was further probed in this study. As many tests may lose their predictive value when assessed in post-prepared samples (Larson *et al.*, 2000; Gandini *et al.*, 2004; Seli *et al.*, 2004; Muriel *et al.*, 2006b; Bungum *et al.*, 2008), it has been suggested that the evaluation of sperm DNA integrity must be carried out in the whole ejaculate when it concerns in vivo conception, and after sperm selection when ART treatments are used (Tomlinson *et al.*, 2001).

Sperm preparation techniques such as DGC and swim up favour the selection of live, highly motile and morphologically normal spermatozoa that will be used in ART cycles (Bungum *et al.*, 2008). However, some conflicting results exist on whether these techniques, alone or in combination, select spermatozoa with lower levels of DNA damage. Nevertheless, as also previously reported by several authors (Spanò *et al.*, 1999; Tomlinson *et al.*, 2001; Gandini *et al.*, 2004; Marchesi *et al.*, 2010), a significant improvement in sperm chromatin integrity following sperm preparation procedures, was found in this study, thus suggesting the use of better quality spermatozoa in ART procedures. Furthermore, these results support the clinical usefulness of this modified Diff-Quik staining assay.

Although many reports have indicated an obvious influence of sperm DNA damage on fertilization rates (Sun *et al.*, 1997; Lopes *et al.*, 1998; Benchaib *et al.*, 2003; Velez de La Calle *et al.*, 2008; Simon *et al.*, 2011), we did not observe any relationship between abnormal chromatin status, as monitored by this assay, and fertilization rates in both IVF and ICSI treatments. These findings are, however, in agreement with several other studies involving both IVF (Tomlinson *et al.*, 2001; Tomsu *et al.*, 2002; Henkel *et al.*, 2003, 2004; Lin *et al.*, 2008) and ICSI cycles (Høst *et al.*, 2000; Henkel *et al.*, 2003; Lin *et al.*, 2008). Given that the activation of the paternal genome is thought to occur only at 4–8-cell stage embryo (Borini *et al.*, 2006), paternal chromatin status might not greatly affect fertilization (Ahmadi & Ng, 1999). However, abnormal levels of DNA damage may influence later stages of development (Virro *et al.*, 2004; Borini *et al.*, 2006; Simon *et al.*, 2010). In this study, we failed to observe any relationship between the percentages of dark sperm nuclear staining and embryo development rates in post-prepared samples in both IVF and ICSI cycles, but embryo quality and, importantly, pregnancy success were adversely affected by abnormal chromatin status monitored by this assay. Abnormal dark staining was found to

have a small, but significant prognostic value in terms of pregnancy achievement when using IVF. Obviously one may not exclude that several other factors besides sperm chromatin/DNA damage (e.g. female age) may also influence these reproductive parameters.

Reports focusing on the relationship between DNA damage assessed after sperm preparation techniques, and embryo and pregnancy outcomes in ART are conflicting probably because the type and degree of DNA injury differs among studies, as do the DNA integrity assays employed. Recently, Simon *et al.* (2010) have also found a decrease in embryo quality and pregnancy rates with increased sperm DNA fragmentation assessed by the Comet assay after DGC among IVF couples, but not between pregnant and non-pregnant groups after ICSI cycles, as we also show. However, contrary to what we describe, they failed to observe any relationship with embryo quality after ICSI treatments. In addition, an inverse correlation between embryo quality and Comet sperm DNA damage among IVF couples was detected in processed samples by Tomsu *et al.*; however, no correlation with pregnancy outcomes was shown (Tomsu *et al.*, 2002). In other studies, positive clinical pregnancies and lower pregnancy loss rates were observed among ICSI couples that presented a lower proportion of sperm with fragmented DNA detected by TUNEL after DGC (Benchaib *et al.*, 2003; Borini *et al.*, 2006). Conversely, others failed to observe any relationship between DNA damage, evaluated in post-prepared spermatozoa by SCSA, TUNEL or in situ nick translation, and embryo quality or clinical pregnancies in IVF and/or ICSI cycles (Sun *et al.*, 1997; Larson *et al.*, 2000; Tomlinson *et al.*, 2001; Benchaib *et al.*, 2003; Gandini *et al.*, 2004; Seli *et al.*, 2004; Borini *et al.*, 2006; Bungum *et al.*, 2008).

Although the percentage of spermatozoa with DNA damage may considerably decrease after sperm preparation techniques, as we demonstrate here, there is still a reasonable likelihood of the technician choosing a spermatozoon with damaged chromatin (i.e. partially decondensed chromatin and/or DNA damage) when performing ICSI, which may explain our findings. This is particularly worrisome given that DNA damage may not be fully repaired by the oocyte machinery, but still allow for embryo development, increasing the risk of conceiving a child with genetic anomalies (Marchetti & Wyrobek, 2005; Aitken & Koppers, 2011). On the other hand, our data suggest that there may be some degree of 'natural' selection in IVF cycles, thus favouring spermatozoa with no or less fragmented DNA to successfully achieve pregnancy.

In this report, clinical pregnancy success was severely reduced in IVF couples having at least 34.25% of (abnormal) dark spermatozoa. Interestingly several other studies showed similar cut-offs for IVF: lower pregnancy rates were reported when the percentage of TUNEL-positive spermatozoa was ≥35 (Frydman *et al.*, 2008) or >36.5 (Henkel *et al.*, 2003, 2004); and a similar value of ≥30% was shown for the SCSA DNA fragmentation index (Virro *et al.*, 2004).

Taken together, the modified Diff-Quik staining provides useful information about ART success in post-prepared samples, particularly in IVF treatments where an operator does not choose the spermatozoa that will fertilize the oocytes. However, despite its low cost and simple methodology, this staining involves the assessment of ±200 cells per slide, displays a certain degree of subjectivity, and exposure to the thiazin dye for longer periods that those described here will produce a uniformly dark staining that will compromise chromatin damage assessment. Proper training, nevertheless, allows the achievement of consistent and reproducible results, with minimal variability. Although SCSA is a very robust assay that analyses 5000–10 000 sperm cells, using objective, machine-defined criteria and with high levels of repeatability (Evenson *et al.*, 1999, 2002), it is not used in most Andrology laboratories, nor are any other chromatin/DNA integrity tests usually employed, at least as a routine procedure. The need of extensive protocols and/or expensive reagents and equipment (e.g. a flow cytometer and fluorescence microscope) are limiting factors when the goal is to routinely implement DNA damage analysis worldwide. Based on our present and previous results (Sousa *et al.*, 2009), we therefore suggest that the modified Diff-Quik staining method may provide an alternative to detect sperm chromatin damage, in the absence of more robust tests.

ACKNOWLEDGEMENTS

The authors would like to thank Paula Mota for many useful discussions and Marta Baptista for valuable assistance in sperm collections. Ana Barbosa, Conceição Dias, Fátima Veríssimo and Raquel Brito, from the University Hospitals of Coimbra, are also acknowledged for technical assistance. This work was carried out, in part, in order to fulfil the requirements for a Masters Degree in Biology (A.F.S.) and a PhD Degree in Cell Biology (R.S.T.), Department of Life Sciences, University of Coimbra, Portugal. R.S.T. is a recipient of a PhD fellowship from FCT, Portugal (SFRH/BD/46002/2008). CNC funding is supported by FCT, Portugal (PEst-C/SAU/LA0001/2011).

AUTHORS' CONTRIBUTION

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

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