Reproductive BioMedicine Online (2011) 22, 583–589

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Relationship between DNA damage and sperm head birefringence

CG Petersen [a,b,c], LD Vagnini [b], AL Mauri [a,b], FC Massaro [a,b], M Cavagna [a], RLR Baruffi [a,b], JBA Oliveira [a,b,c], JG Franco Jr [a,b,c,*]

[a] *Center for Human Reproduction Prof. Franco Junior, Ribeirão Preto, Brazil*; [b] *Paulista Center for Diagnosis, Research and Training, Ribeirao Preto, Brazil*; [c] *Department of Gynecology and Obstetrics, Botucatu Medical School São Paulo State University, Botucatu, Brazil*

[*] Corresponding author. *E-mail address:* crh@crh.com.br (JG Franco)

Claudia G Petersen graduated in 1985 in biology from Federal University of São Carlos (UFSCAR), Brazil. She works at the Human Reproduction Center Prof Franco Jr at Ribeirão Preto, Brazil since 1990, co-ordinating the IVF laboratory team. In 2003, she obtained her Masters degree in clinical embryology at the School of Medicine at the University of Leeds, UK and in 2009 her PhD in science at the Botucatu Medical School, Brazil. Her current research interests have focused on gamete and embryo morphology, implantation and cryobiology.

Abstract Birefringence or double refraction is the decomposition of a ray of light into two rays when it passes through an anisotropic material such as quartz. Sperm cells have been demonstrated to be optically anisotropic. The objective of this study was to evaluate the relationship between the pattern of human sperm head birefringence (SHBF) and DNA damage. A total of 26 patients with normal semen were included. DNA damage (fragmentation and denaturation) was evaluated in the sperm head in the context of birefringence, both total (SHBF-T) and partial (SHBF-P), by terminal deoxyribonucleotidyl transferase (TdT)-mediated dUDP nick-end labelling assay and acridine orange fluorescence, respectively. Positive DNA fragmentation in spermatozoa with SHBF-T (205/1053; 19.5%) was significantly higher ($P < 0.0001$) than in spermatozoa that presented SHBF-P (60/820; 7.3%). However, the percentage of denatured DNA in spermatozoa with SHBF-T (824/1256; 65.6%) was not significantly different from the ones with SHBF-P (666/1009; 66.0%). In conclusion, the data support a positive relationship between spermatozoa with total SHBF in their head and increased DNA fragmentation.

KEYWORDS: DNA denaturation, DNA fragmentation, MSOME, sperm birefringence, sperm DNA damage, sperm selection

Introduction

DNA damage in human spermatozoa is associated with a range of adverse clinical consequences, including infertility, miscarriage and morbidity in offspring. The origins of this damage may involve abortive apoptosis, unresolved strand breaks created during spermiogenesis to relieve the torsional stresses associated with chromatin remodelling and oxidative stress (Aitken and De Iuliis, 2010).

It is clear that, in cases of intracytoplasmic sperm injection (ICSI), although the 'best' spermatozoon is selected, cells with damaged DNA are routinely injected

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2011.03.017

into the oocyte. Successful human reproduction depends in part on the inherent integrity of sperm DNA. There appears to be a DNA damage threshold beyond which embryo development and subsequent pregnancy outcome are impaired. Clinical evidence now shows that sperm DNA damage is detrimental to reproductive outcomes and that spermatozoa of infertile men possess substantially more DNA damage than do the spermatozoa of fertile men. However, an understanding of the causes of sperm damage on reproductive outcome in humans remains undeveloped (Zini and Sigman, 2009).

Baccetti (2004) demonstrated that it is possible to include a polarization apparatus in an inverted light microscope used for ICSI to analyse the human motile sperm head birefringence (SHBF) in order to indicate the structural normality of spermatozoa. Birefringence or double refraction is the decomposition of a ray of light into two rays when it passes through an anisotropic material. Sperm cells of many different species are birefringent or optically anisotropic, as first reported by Valentin (1861) and Engelmann (1875). When the microscope light enters an anisotropic structure, it is refracted into two different refractive rays indicating this double refraction. Gianaroli et al. (2008) were the first to use SHBF to select spermatozoa for ICSI and demonstrated better embryo development and clinical outcome results after injection of spermatozoa with SHBF when compared with controls (spermatozoa without SHBF). Recently, the same group carefully evaluated two types of SHBF on the basis of their acrosome integrity (partial head birefringence/acrosome-reacted and total head birefringence/acrosome-non-reacted spermatozoa) and demonstrated that acrosome-reacted spermatozoa produced better clinical results when used in ICSI (Gianaroli et al., 2010). The data from this study point to the injection of reacted spermatozoa as the best way to achieve pregnancy in couples with male factor infertility. It has also reported that induction of the acrosome reaction in human spermatozoa is associated with improved fertilization and embryo development (Lee et al., 1997; Mansour et al., 2008; Sathananthan et al., 1997). On the other hand, negative correlations between increased DNA damage and acrosome reaction have been identified (Ozmen et al., 2007). With this in mind, the objective of this study was to investigate whether there was any relationship between DNA damage (fragmentation and denaturation) and two types of SHBF: total and partial.

Materials and methods

Patient selection

This prospective study consisted of 26 semen samples from 26 men, mean age (± SD) 39.3 ± 6.1 years, from a group of couples who underwent infertility investigation and treatment at the Centre for Human Reproduction Prof. Franco Jr. Patients presented a mean of 4.0 ± 3.0 years of infertility and mean sperm concentration of $57.9 \pm 28.1 \times 10^6$/ml with mean motile sperm concentration of $37.8 \pm 19.0 \times 10^6$/ml and mean percentage of morphologically normal sperm nuclei of 1.1 ± 1.0% by modified Bartoov classification (Bartoov et al., 2002).

Semen evaluation and processing

Semen samples were collected in sterile containers by masturbation after a period of 2–5 days of sexual abstinence. Semen analysis assessed the sperm concentration, volume and motility quality. Sperm morphology was evaluated by using the motile sperm organelle morphology examination (Bartoov et al., 2002; Oliveira et al., 2009). Morphology was examined at high magnification using an inverted microscope (Eclipse TE 2000 U; Nikon, Japan) equipped with high-power differential interference contrast optics (Nomarski). The total calculated magnification was 8450× (total magnification = objective magnification (100) × magnification selector (1.0) × video coupler magnification (1.0) × calculated video magnification (84.50×))).

A spermatozoon was classified as morphologically normal when it exhibited a normal nucleus, acrosome, post-acrosomal lamina, neck and tail, as well as not presenting cytoplasm around the head. For the nucleus, the morphologically normal state was defined by the shape and content of the chromatin. The criterion for normality of nuclear shape was a smooth, symmetric and oval configuration. Normal means for length and width were estimated as 4.75 ± 2.8 and 3.28 ± 0.20 μm, respectively, with absence of extrusion or invagination of the nuclear chromatin (regional abnormality of nuclear form). The criterion for normality of chromatin content was the absence of vacuoles occupying >4% of the sperm nuclear area. A spermatozoon was classified as morphologically abnormal when it exhibited an abnormal nucleus (form or chromatin content) as well as severe abnormality (such as pin, amorphous, tapered, round or multinucleated head, double tail). The criteria for specifying that a spermatozoon had abnormal nuclear/chromatin content were: (i) small or large oval nuclear forms (length ≤4.19 μm or ≥5.31 μm); (ii) wide or narrow nuclear forms (width >3.7 or <2.9 μm); (iii) an extrusion or invagination of the nuclear mass; (iv) vacuoles occupying >4–50%<!Query id="Q5" desc="Is this '4–50%' or '5–50%'?" /–> of the nuclear area; or (v) vacuoles occupying >50% of the nuclear area (large nuclear vacuoles).

The liquefied fresh semen samples were prepared using the swim-up method, which consisted of permitting the ejaculated sperm sample to migrate in human tubal fluid (HTF; Irvine Scientific, CA, USA) with 10% human serum albumin (HSA), in the proportion of 2:1, deposited on top of the fresh semen sample, for 30 min at 37°C. The portion of motile spermatozoa was resuspended in HTF/10% HSA medium and the concentration was adjusted to 3×10^6 spermatozoa/ml. A total of 1 μl of each semen sample was evaluated according to SHBF and spermatozoa were selected according to the two types of birefringence (total and partial head birefringence).

Birefringence evaluation

The SHBF was assessed using an inverted microscope equipped with Hoffman contrast and polarizing lenses. The total calculated magnification of 2500× was obtained by using a Hoffman objective of 20×, a camera, C-mount and 21-inch monitor (**Figure 1**). SHBF assessment was performed after incubating 1 μl prepared spermatozoa in a 10 μl microdrop of 7% polyvinylpyrrolidone solution (Irvine

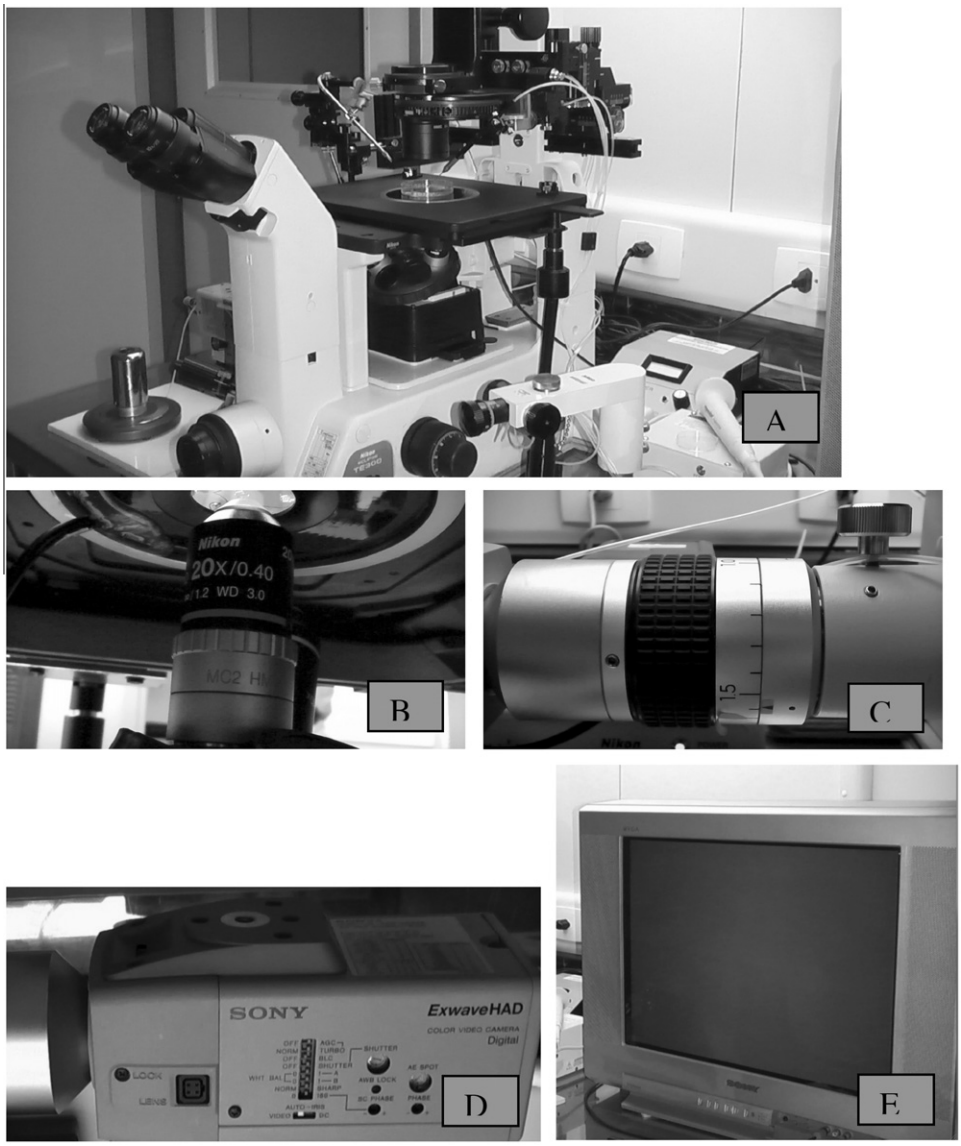

Figure 1 (A) The polarization inverted microscope 1 (TE 300; Nikon, Japan) equipped with Hoffman contrast and polarizing lens; (B) Hoffman objective of 20×; (C) C-mount; (D) camera; and (E) 21-inch monitor.

Scientific), in a plastic Petri dish (430166; Corning) and covered with oil (Vitrolife, Sweden), at room temperature for 30 min. Two types of SHBF were identified: group 1 consisted of motile spermatozoa that presented birefringence throughout the entire head, i.e. total SHBF (SHBF-T) (**Figure 2A**); while group 2 comprised those motile spermatozoa with the presence of birefringence in 50% of each head, with this birefringence being localized in the post-acrosomal region, i.e partial SHBF (SHBF-P) (**Figure 2B**). Cells showing no birefringence were not evaluated since the percentage of spermatozoa without birefringence presented in these semen samples was very low (2.6%).

Experimental design

For evaluation of DNA fragmentation from each sperm sample a mean of ±80 motile spermatozoa (40 with SHBF-T and 40 with SHBF-P) was selected and deposited onto two different slides (**Figure 3**; 1 and 2), previously branded with a circle, using an ICSI pipette (Humagen, USA).

For evaluation of DNA denaturation, from each sperm sample a mean of ±80 motile spermatozoa (40 with SHBF-T and 40 with SHBF-P) was selected and deposited onto two other different slides (**Figure 3**; 3 and 4) for DNA denaturation evaluation.

The same technician, blinded to subject identity, performed all the experiments to rule out inter-technician variability.

Determination of DNA fragmentation

DNA fragmentation in spermatozoa was measured using the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay, which was performed using an in-situ cell death detection kit with tetramethylrhodamine-labelled dUTP (Roche; Monza, Italy) according to the modified description by Tesarik et al.

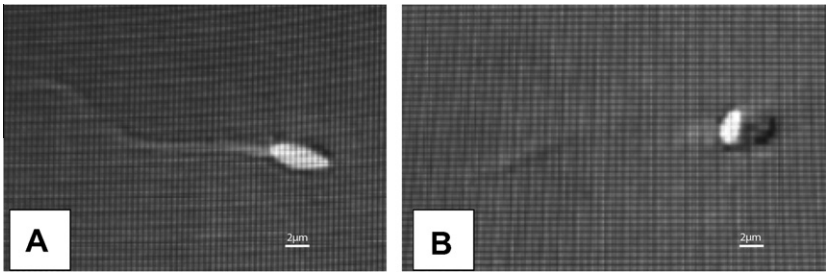

Figure 2 (A) Spermatozoon with total sperm head birefringence (SHBF-T) observed at 2500× magnification; (B) spermatozoon with partial sperm head birefringence (SHBF-P) observed at 2500× magnification.

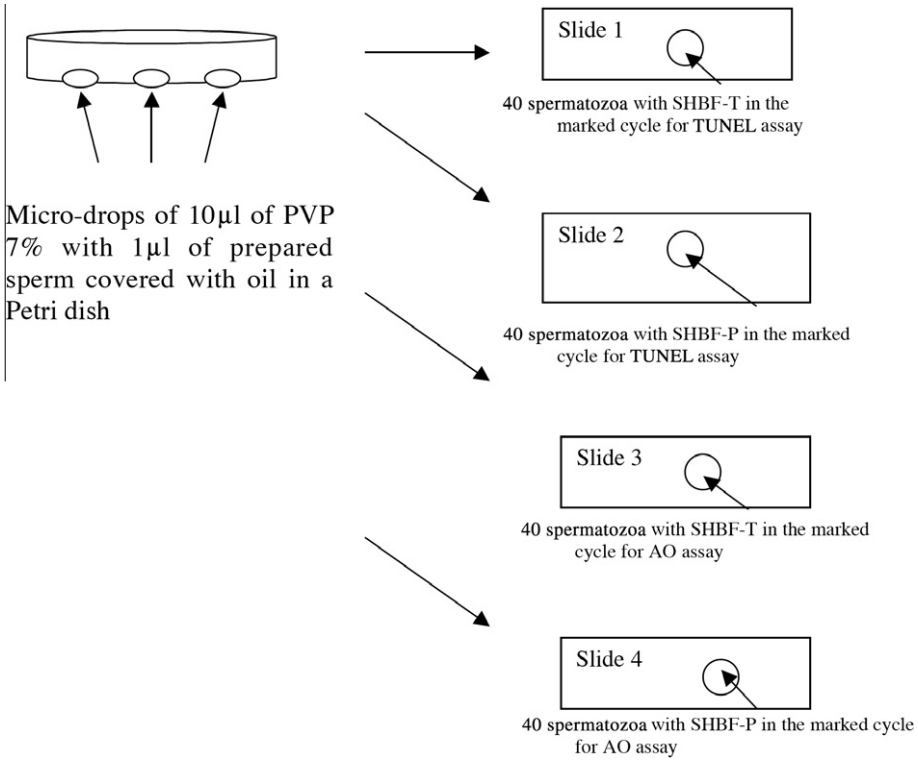

Figure 3 Sperm head birefringence selection and processing for DNA evaluation. AO = acridine orange; PVP = polyvinylpyrrolidone; SHBF-P = partial sperm head birefringence; SHBF-T = total sperm head birefringence; and TUNEL = TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling.

(2004). TUNEL identifies single- and double-stranded DNA breaks by labelling free 3′-OH termini with modified nucleotides in an enzymatic reaction with TdT. Terminal deoxyribonucleotidyl transferase catalyses the polymerization of labelled nucleotides to free 3′-OH DNA ends in a template-independent manner. Slides with different spermatozoa (SHBF-T and SHBF-P) selected specifically for DNA fragmentation were air-dried and then fixed at 4°C in Carnoy's solution (methanol/glacial acetic acid, 3:1), and permeabilized with 0.1% Triton X-100 (VETEC Química Fina; Duque de Caxias, Brazil) in 0.1% sodium citrate at 4°C for 2 min. After washing with phosphate-buffered saline (PBS), the slides were then processed for TUNEL assay. The TdT-labelled nucleotide mix was added to each slide and incubated in the dark in a humidified atmosphere for 2 h at 37°C. After stopping the enzyme reaction, slides were rinsed twice in PBS and then counterstained with Vectashield Mounting Medium with 4,6-diamidino-2-phenylindole 1.5 μg/ml (DAPI; Vector Laboratories, Burlingame, CA, USA). The final evaluation was achieved using a fluorescent microscope in which the percentage of TUNEL-positive spermatozoa was determined. The number of cells per field stained with DAPI (blue) was first counted; in the same field, the number of cells with red fluorescence (TUNEL positive) was expressed as a percentage of DNA fragmentation. Controls were included in every experiment: for negative control, TdT was omitted in the nucleotide mix; positive controls were generated by pre-incubating the fixed and permeabilized sperm cells using DNase I (1 μg/ml; New England Biolabs, Ipswich, MA, USA) for 30 min at 37°C. TUNEL labelling of positive controls varied between 89% and 98% of cells.

Determination of denatured DNA by acridine orange fluorescence

Slides with different spermatozoa (SHBF-T and SHBF-P, respectively) selected specifically for acridine orange fluorescence (Tejada et al., 1984) were air-dried and then fixed overnight at 4°C in Carnoy's solution (methanol/glacial acetic acid, 3:1). After fixation, the slides were air-dried, stained with acridine orange for 5 min and then gently rinsed with distilled water. The acridine orange staining solution was prepared daily as follows: a mixture of 4 ml 0.1 mol/l citric acid and 0.25 ml 0.3 mol/l $Na_2HPO_4.7H_2O$ was added to 1 ml 1% acridine orange stock solution (6014; Sigma-Aldrich Brazil, São Paulo, Brazil) in distilled water. The acridine orange stock solution was stored in the dark at 4°C for 4 weeks. In order to reduce variation in fluorescence intensity, each stained slide was read immediately after washing. Spermatozoa with single (denatured) or double (normal)-stranded DNA were identified under a fluorescence microscope at 400× and 1000× magnification with 450—490 nm excitation. Spermatozoa with double-stranded DNA were fluorescent green and those with denatured DNA were fluorescent red or yellow. To determine intra-technician and intra-assay variabilities for the acridine orange test, before each series of analyses, duplicate slides were made from at least two randomly selected donors to analyse the percentage of spermatozoa with double-stranded DNA and percentage of spermatozoa with denatured DNA. Intra-individual variability for the percentages of green-fluorescing spermatozoa and of red/yellow-fluorescing spermatozoa was ≈5% (for both), which is comparable to those of classical sperm quality parameters (Auger et al., 2000).

Sample size and statistical analysis

Sample size was calculated planning a comparison between two proportions, control and experimental. Usually, DNA fragmentation assay values are abnormal in ≤30% of spermatozoa in the fertile male population. In the same way, usually acridine orange assay values are abnormal in ≤50% of spermatozoa in the fertile male population. Thus a sample size of 400 spermatozoa in each group has 80% power to detect an increase of 10% with a significance level alpha of 0.05 (two-tailed). At least double this number of spermatozoa was observed in each arm (SHBF-P and SHBF-T) of the two analyses (DNA fragmentation and acridine orange fluorescence) to increase the strength of the study. Data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer, Cupertino, CA, USA). The chi-squared test was used. The level of significance was set at $P < 0.05$.

Results

Tables 1 and 2 display the values for DNA fragmentation in a total of 1873 spermatozoa, comprised of SHBF-T ($n = 1053$) and SHBF-P ($n = 820$), and DNA denaturation in a total of 2265 spermatozoa, composed of SHBF-T ($n = 1256$) and SHBF-P ($n = 1009$). The spermatozoa with SHBF-T presented a significantly higher percentage of positive DNA fragmentation (205/1053, 19.5%) than those with SHBF-P (60/820, 7.3%, $P < 0.0001$). However, the percentage of denatured/single-stranded DNA (824/1256, 65.6%) in spermatozoa with SHBF-T was not significantly different (666/1009, 66.0%) from those with SHBF-P.

Table 1 DNA fragmentation in spermatozoa with total and partial sperm head birefringence.

DNA fragmentation	*Spermatozoa (n)*	
	SHBF-T	*SHBF-P*
Positive	205	60
Negative	848	760

$P < 0.0001$.
SHBF-P = partial sperm head birefringence; SHBF-T = total sperm head birefringence.

Table 2 Denatured and double-stranded DNA evaluated by acridine orange fluorescence in spermatozoa with SHBF-T and SHBF-P.

DNA denaturation	*Spermatozoa (n)*	
	SHBF-T	*SHBF-P*
Denatured	824	666
Double-stranded	432	343

There were no statistically significant differences.
SHBF-P = partial sperm head birefringence; SHBF-T = total sperm head birefringence.

Discussion

The polarization microscopy analysis of birefringence in sperm cells has been reported to be an indicator of nuclear structural normality. The presence of SHBF purportedly expresses an organized and very compacted texture that characterizes normal sperm nuclei, acrosomes and motile tails (Baccetti, 2004).

Gianaroli et al. (2008) proposed the injection of a spermatozoon with a birefringent head as a diagnostic tool to improve clinical outcome in treatment of the most severe male factor cases. Along this line of thought, the same group has recently defended the hypothesis that human spermatozoa possess characteristics of birefringence that reflect the state of their inner protoplasmic structures (Gianaroli et al., 2010). The authors argue that abnormalities in the sperm head protoplasmic compartment could be related to anomalies of sperm chromatin packaging and incomplete nuclear remodelling that occur in the final phase of spermatogenesis. However, there are no studies in the literature defining the real correlation between birefringence and protoplasmic structure.

In the literature, only one study analysed the relationship between SHBF and DNA damage (Crippa et al., 2009). According to Crippa et al. (2009), a negative correlation between SHBF and DNA fragmentation was observed, i.e. they showed that the proportion of birefringent spermatozoa was inversely correlated with the incidence

of fragmented DNA. The study evaluated the total semen sample both by the presence of SHBF in sperm heads and the DNA fragmentation, but did not describe the types of SHBF (partial and total).

The present study evaluated two SHBF types (total and partial) and their relationship with DNA damage (fragmentation and denaturation) directly from spermatozoa selected according to their birefringence. The first part of the data shows that DNA fragmentation values are significantly higher in spermatozoa with total SHBF compared with those with partial SHBF. This fact could explain the results obtained by Gianaroli et al. (2010), who reported a higher embryo implantation rate (39% versus 8.6%) when oocytes were injected with acrosome-reacted spermatozoa (SHBF-P) compared with those injected with acrosome-non-reacted (SHBF-T) spermatozoa. But the correlation between acrosome integrity and SHBF, suggested by Gianaroli et al. (2010), is a hypothesis that remains to be proven. However, DNA damage should alter the special cellular functions of human spermatozoa and lead to diminished acrosome reaction with reduced fertilization rates. Moreover, negative correlation was identified between DNA damage and acrosome reaction and/or viability of human spermatozoa (Ozmen et al., 2007).

Acridine orange staining is an established cytochemical method for determining sperm DNA integrity which allows differentiation between normal double-stranded and abnormal denatured/single-stranded DNA by using the metachromatic properties of the stain (Tejada et al., 1984). In samples with high levels of DNA stainability by acridine orange, there is higher acceptability of sperm DNA upon staining, which suggests that the chromatin may be less compact and, consequently, more vulnerable (Evenson et al., 1986; Henkel et al., 2010). However, it does not indicate real DNA damage as found by TUNEL. Although these two assays are used for the same purpose, which is to determine sperm DNA damage, they are based on different principles, i.e. the susceptibility of sperm nuclear DNA to induced DNA denaturation (acridine orange fluorescence) and the detection of actual DNA strand breaks (TUNEL) (Henkel et al., 2010). The second part of the data does not reveal significantly more denaturated DNA in spermatozoa with total SHBF compared with those with partial SHBF, showing that both groups appear to be equally vulnerable. However, the higher incidence of DNA fragmentation in the SHBF-T group suggests that some of the factors proposed for the origin of the DNA damage, i.e. abortive apoptosis, abnormal spermatid maturation (protamination disturbances), chromatin remodelling during spermiogenesis and oxidative stress (reactive oxygen species and other radical molecules) (Erenpreiss et al., 2006; Fernández et al., 2009; Henkel et al., 2004), are more prevalent in these spermatozoa. In this context, the selection of spermatozoa by the presence of SHBF could be inefficient at detecting abnormalities in chromatin packaging. Recently Vagnini et al. (2010) have demonstrated that these specific patterns of SHBF (total and partial) were not efficacious in detecting sperm chromatin packaging abnormalities, at least when observed by chromomycin A3 staining. The percentage of positive chromomycin A3 staining (abnormal chromatin packaging) in spermatozoa with SHBF-T was (40.5%) not significantly different from the percentage (37.3%) with SHBF-P (Vagnini et al., 2010). However, it should be emphasized that the analysis of the birefringence as an indicator of sperm quality is a very recent technique. Additional controlled trials with standardized techniques are necessary before final conclusions about the real meaning of these measurements can be made.

In conclusion, there was significantly more DNA fragmentation in spermatozoa with SHBF-T than in those with SHBF-P. The present results support a positive relationship between spermatozoa with SHBF-T and increase in DNA fragmentation. As far as is known, this is the first study that relates DNA damage with different types of SHBF.

Acknowledgements

The authors wish to thank the Research Support Group, UNESP, for revising the English text.

References

Aitken, R.J., De Iuliis, G.N., 2010. On the possible origins of DNA damage in human spermatozoa. Mol. Hum. Reprod. 16, 3—13.

Auger, J., Eustache, F., Ducot, B., et al., 2000. Intra- and inter-individual variability in human sperm concentration, motility and vitality assessment during a workshop involving ten laboratories. Hum. Reprod. 15, 2360—2368.

Baccetti, B., 2004. Microscopical advances in assisted reproduction. J. Submicrosc. Cytol. Pathol. 36, 333—339.

Bartoov, B., Berkovitz, A., Eltes, F., Kogosowski, A., Menezo, Y., Barak, Y., 2002. Real-time fine morphology of motile human sperm cells is associated with ivf-icsi outcome. J. Androl. 23, 1—8.

Crippa, A., Magli, M.C., Paviglianiti, B., Boudjema, E., Ferraretti, A.P., Gianaroli, L., 2009. DNA fragmentation and characteristics of birefringence in human sperm head. Hum. Reprod. 24, i95.

Engelmann, T.W., 1875. Pfluergers Arch. 11, 432.

Erenpreiss, J., Spano, M., Erenpreisa, J., Bungum, M., Giwercman, A., 2006. Sperm chromatin structure and male fertility: biological and clinical aspects. Asian J. Androl. 8, 11—29.

Evenson, D.P., Darzynkiewicz, Z., Jost, L., Janca, F., Ballachey, B., 1986. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry 7, 45—53.

Fernández, J.L., de la Calle, J.F.V., Tamayo, M., Cajigal, D., Agarwal, A., Gosálvez, J., 2009. Sperm DNA integrity and male infertility: current perspectives. Arch. Med. Sci. 5, S55—S62.

Gianaroli, L., Magli, M.C., Collodel, G., Moretti, E., Ferraretti, A.P., Baccetti, B., 2008. Sperm head's birefringence: a new criterion for sperm selection. Fertil. Steril. 90, 104—112.

Gianaroli, L., Magli, M.C., Ferraretti, A.P., et al., 2010. Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. Fertil. Steril. 93, 807—813.

Henkel, R., Hajimohammad, M., Stalf, T., et al., 2004. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil. Steril. 81, 965—972.

Henkel, R., Hoogendijk, C.F., Bouic, P.J.D., Kruger, T.F., 2010. TUNEL assay and SCSA determine different aspects of sperm DNA damage. Andrologia 42, 305—313.

Lee, D.R., Lee, J.E., Yoon, H.S., Roh, S.I., 1997. Induction of acrosome reaction in human spermatozoa accelerates the time of pronucleus formation of hamster oocytes after intracytoplasmic sperm injection. Fertil. Steril. 67, 315—320.

Mansour, R.T., Serour, M.G., Abbas, A.M., et al., 2008. The impact of spermatozoa preincubation time and spontaneous acrosome

reaction in intracytoplasmic sperm injection: a controlled randomized study. Fertil. Steril. 90, 584—591.
Oliveira, J.B., Massaro, F.C., Mauri, A.L., et al., 2009. Motile sperm organelle morphology examination is stricter than tygerberg criteria. Reprod. Biomed. Online 18, 320—326.
Ozmen, B., Caglar, G.S., Koster, F., Schopper, B., Diedrich, K., Al-Hasani, S., 2007. Relationship between sperm DNA damage, induced acrosome reaction and viability in icsi patients. Reprod. Biomed. Online 15, 208—214.
Sathananthan, A.H., Szell, A., Ng, S.C., Kausche, A., Lacham-Kaplan, O., Trounson, A., 1997. Is the acrosome reaction a prerequisite for sperm incorporation after intra-cytoplasmic sperm injection (icsi)? Reprod. Fertil. Dev. 9, 703—709.
Tejada, R.I., Mitchell, J.C., Norman, A., Marik, J.J., Friedman, S., 1984. A test for the practical evaluation of male fertility by acridine orange (ao) fluorescence. Fertil. Steril. 42, 87—91.
Tesarik, J., Greco, E., Mendonza, C., 2004. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum. Reprod. 19, 611—615.
Vagnini, L.D., Petersen, C.G., Mauri, A.L., et al., 2010. Can sperm-head birefringence indicate sperm chromatin packaging abnormalities? Hum. Reprod. 25, i279.
Valentin, G., 1861. Die Untersuchung der Pflanzenund der Thriegewebe in polarisiertem Engelmann, Leipzig.
Zini, A., Sigman, M., 2009. Are tests of sperm DNA damage clinically useful? Pros and cons. J. Androl. 30, 219—229.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 8 November 2010; refereed 14 February 2011; accepted 14 March 2011.