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ORIGINAL ARTICLE *Andrology*

Development of a novel synthetic oligopeptide for the detection of DNA damage in human spermatozoa

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BACKGROUND: The integrity of DNA in spermatozoa is considered an additional parameter of semen quality and a potential fertility predictor. Significant progress has been made in recent years towards the development of reliable tests for sperm chromatin integrity and DNA damage assessment. However, most of the techniques available are labor intensive, require expensive instrumentation or utilize enzymes whose activity could be compromised by the highly condensed nature of sperm chromatin. In addition, all the methods currently available involve the destruction of the sperm tested; none is able to select intact spermatozoa that could then be used for fertilization. The aim of the present study was to create a peptide ligand-based stain, capable of binding specific DNA structures, thereby revealing the presence of DNA damage, preferably in living cells.

METHODS: The peptide was bioinformatically modelled on the critical region of the p53 protein associated with DNA binding and fluorescently labeled with a terminal rhodamine B dye. The ability of this 21 amino acid synthetic peptide (DW1) to detect DNA damage in intact and fixed human spermatozoa was assessed in detail. Human sperm samples ($n = 20$) were treated with reagents that induce single- and/or double-stranded DNA breaks. The effect of these treatments on peptide-labelling was measured and compared with results obtained using established tests for the evaluation of DNA damage, such as comet assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and sperm chromatin dispersion test.

RESULTS: The peptide had a high affinity for single-stranded DNA, and DNA lesions such as double- and single-stranded breaks. The proportion of spermatozoa with intense staining was found to be closely associated with the percentage of cells possessing DNA damage. The analysis of 10 sperm samples using DW1 staining and TUNEL technique showed a significant correlation between the extent of DNA fragmentation for the two methods ($r = 0.892$, Pearson's correlation, $P < 0.05$).

CONCLUSIONS: We have produced a novel peptide-based stain capable of detecting DNA damage in individual sperm cells. Evaluation of sperm DNA fragmentation using this peptide may be an inexpensive and easier to use alternative to the tests in current use. Additionally, although DW1 currently requires removal of the membrane using a detergent, further research may allow this approach to be applied to the selection of viable spermatozoa with intact DNA for use in ICSI and/or intra-cytoplasmic morphologically selected sperm injection.

Key words: spermatozoa / DNA damage / oligopeptide / DNA fragmentation / p53

Introduction

Traditionally, the diagnosis of male infertility has been based on the assessment and analysis of spermatozoa concentration, motility and morphology, using guidelines from the World Health Organization (WHO). Although much progress has been made in the understanding of sperm physiology and interaction with the oocyte, these measures continue to be the most important and widely used means of evaluating male factor infertility (WHO, 2010). However, it is widely accepted that semen parameters convey only a limited degree of prognostic and diagnostic information (Guzick *et al.*, 2001; Jequier, 2005; Pacey, 2006; Lewis, 2007). In some cases, conventional semen analysis is unable to detect the presence of subtle alterations in spermatozoa that might affect functionality. Therefore, there is a need for new markers allowing improved discrimination of sperm samples of high- and low quality, and capable of providing a more accurate prediction of the likelihood of pregnancy, and perhaps even risk of miscarriage.

Sperm DNA integrity has been proposed as an additional parameter of semen quality and a potential fertility predictor (Zini and Sigman, 2009; Barratt, 2010; Barratt and De Jonge, 2010). It has also been suggested that chromatin and DNA integrity are essential to ensure that the fertilizing spermatozoa can support subsequent embryonic development (Morris *et al*., 2002; Virro *et al*., 2004). Injecting spermatozoa with proven DNA integrity into human oocytes could be a useful therapeutic addition to ICSI treatment. Some studies have indicated that elevated levels of DNA damage can be associated with parameters which indicate poor semen quality (Irvine *et al*., 2000; Acharyya *et al*., 2005). Others, however, have shown that sperm DNA integrity is independent of conventional parameters and suggest that evaluation of DNA integrity could provide valuable additional information (Giwercman *et al*., 2003). Sperm DNA damage has been recognized as a possible explanation for a significant proportion of idiopathic infertility cases (Saleh *et al*., 2002).

In recent years, many studies have been performed in order to assess the significance of sperm DNA fragmentation and its impact on human reproduction. Although it remains a controversial issue, there is growing evidence indicating that sperm DNA damage has a negative impact on both natural conception (Evenson and Wixon, 2008) and assisted reproduction treatment (ART) cycles (Larson *et al*., 2000). In the case of IVF, recent reports confirm that the fertilization rate is negatively correlated with sperm DNA fragmentation (Huang *et al*., 2005) and that DNA fragmentation has a negative impact on pregnancy rates (Borini *et al*., 2006; Benchaib *et al*., 2007). In cycles employing ICSI, reductions in fertilization and embryo implantation rates, which are associated with levels of sperm DNA damage, have also been recorded (Lopes *et al*., 1998, Benchaib *et al*., 2003; Larson-Cook *et al*., 2003; Bungum *et al*., 2004; Gandini *et al*., 2004). Moreover, sperm DNA fragmentation has been also associated with high miscarriage rate (Zini and Sigman, 2009).

Significant progress has been made towards the development of reliable tests for sperm chromatin integrity and DNA damage assessment. Methods include sperm chromatin structure assay (SCSA; Evenson and Jost, 1994); terminal deoxynucleotidyltransferase-mediated dUTP-nick-end labeling (TUNEL) assay (Gorczyca *et al*., 1993); single-cell gel electrophoresis (SCGE) or comet assay (Ostling and Johanson, 1984) and sperm chromatin dispersion test (SCDt; Fernández *et al*., 2003). However, most of the techniques available for the evaluation of sperm DNA integrity are either labor intensive, require expensive instrumentation, necessitate the use of enzymes whose activity and accessibility could be compromised by the highly condensed sperm chromatin, or are difficult to incorporate into the demanding routine of an andrology/IVF laboratory (SCGE/comet assay, SCSA, TUNEL or SCDt, respectively). Most of these procedures may be easier to use in a research context than to apply clinically. In addition to these drawbacks, an important limitation of all the techniques currently available for the assessment of sperm DNA fragmentation is that none is able to select spermatozoa that could subsequently be used for fertilization; all involve the destruction of the sperm tested. The proportion of spermatozoa with fragmented DNA in a sample is revealed but none of the sperm that have been tested are viable following analysis.

It is obvious that for the creation of a healthy embryo, only one viable spermatozoon is needed per oocyte. Therefore, a method allowing sperm to be tested for DNA fragmentation, while maintaining their viability, could allow poor quality samples to be rescued. Motile spermatozoa with intact DNA and normal morphology could be identified and selectively used for fertilization via ICSI, even if they only represent a small minority of the total sperm population. Such an approach could also be desirable in the case of good quality sperm samples, facilitating fertilization by using the best quality spermatozoa. This may be particularly useful for cases in which few oocytes have been produced and fertilization with abnormal spermatozoa must be avoided at all costs.

Short synthetic peptides can be designed to mimic certain protein domains that bind to specific epitopes (Merrifield, 2001). In the present study, we aimed to develop novel peptides that bind to fragmented DNA and could potentially be used to reveal cells containing high levels of DNA damage. In most cases, peptides of this type do not have any impact on cell viability when used *in vivo* to target living cells (Pasqualini and Ruoslahti, 1996; Pieczenik *et al*., 2006). Consequently, it might be possible to select spermatozoa, tested using a peptide-based stain and found to be free of DNA damage, for fertilization using ICSI.

Materials and Methods

Oligopeptide design and preparation

A well-known molecule, with DNA-binding properties, p53, was selected to be the focus for the development of peptides for detection of DNA lesions (Fig. 1). A region towards the C-terminus of the p53 protein is able to bind to a wide variety of DNA structures including single-strand DNA ends (Bakalkin *et al*., 1994), short single DNA strands (Bakalkin *et al*., 1995), irradiated or enzymatically damaged DNA (Reed *et al*., 1995), four-way junctions (Lee *et al*., 1997) and insertion/deletions (Lee *et al*., 1995). A synthetic oligopeptide (DW1) corresponding to 21 amino acids of the human p53 protein was designed and labeled with a terminal rhodamine B dye. This oligopeptide was designed using a variety of bioinformatic approaches based on analysis of 3D protein structure. Specifically, the 21 amino acid sequence corresponds to an exposed epitope in the single-stranded DNA (ssDNA) binding region of the protein, deduced from the crystal structure of p53 binding to DNA.

Semen sample collection

A total of 20 semen samples with normal and abnormal conventional semen parameters, according to the WHO guidelines (WHO, 2010), were collected from couples undergoing ART treatment. All necessary ethical approvals were obtained along with written consent from patients.

Preparation of agarose-embedded sperm slides

Sperm samples were washed twice in phosphate-buffered saline (PBS; Fischer Scientific International, Pittsburgh, PA, USA, by centrifugation at 220*g*, 10 min) and diluted to a concentration of 1×10^7 spermatozoa/ml in PBS. Next, 25 μl of the cell dilution was mixed at 37°C with 50 μl of 1% low-melting point agarose (Sigma, St Louis, MO, USA) previously liquefied at 90°C. An aliquot of 15 μl of the mixture was pipetted onto a glass slide pre-coated with 0.65% standard agarose, covered with a coverslip and transferred to an ice-cold metal plate to promote fast gelling. As soon as the gel solidified, coverslips were smoothly removed and the slides were used for direct DW1 staining, SCDt or comet assay.

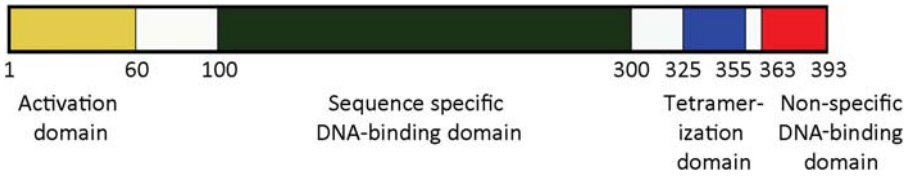

Figure 1 Domain organization of p53 (modified from Ahn and Prives, 2001). p53 is a multidomain protein constituted by an N-terminal transcription-activation domain (TAD) that lies within residues 1–60, a central sequence-specific DNA-binding core domain that lies within residues 100–300 and a multifunctional C-terminal domain that lies within residues 300–393. The p53 C-terminus can be subdivided further into three regions, a flexible linker (residues 300–320) that connects the DNA-binding domain to the tetramerization domain, the tetramerization domain itself (residues 325–355), and, at the extreme carboxyl terminus, a stretch of 30 amino acids that is rich in basic residues (residues 363–393).

SCDt

The SCDt was performed using the Halosperm® kit (Halotech, Madrid, Spain) according to the manufacturer's instructions. After processing, slides were stained with either 4′,6-Diamidino-2-phenylindole (DAPI) combined with Vectashield (125 ng/ml, Abbott Molecular, Inc., Des Plaines, IL, USA), DW1 oligopeptide (described below) or acridine orange (AO described below). The frequency of sperm cells with fragmented DNA, i.e. the sperm DNA fragmentation index (SDF), was established by measuring at least 500 sperm cells per slide, following the criteria established by Fernández *et al.* (2003).

Neutral and alkaline comet assays

For alkaline and neutral comet assays, the 2T comet assay protocol described by Enciso *et al.* (2009) was used with some modifications. For the alkaline comet assay, only the steps involving ssDNA breaks detection were utilized, while for the neutral comet assay the only steps used were those for the detection of double-stranded DNA (dsDNA) breaks (see Supplementary data for a detailed protocol). Slides were stained with either DAPI combined with Vectashield (125 ng/ml, Abbott Molecular Inc., Des Plaines, IL, USA), DW1 oligopeptide (described below) or AO (described below). In the comet assays, results were assessed by visual scoring. The frequency of sperm cells with fragmented DNA, i.e. the SDF index, was established by measuring at least 200 sperm cells per slide. Cells displaying no DNA migration were classified as undamaged. In the case of the alkaline comet assay, cells with evidence of migrated DNA were considered to have ssDNA damage, while in the case of the neutral comet assay cells with characteristic 'comet tails' were classified as having dsDNA damage (Enciso *et al.*, 2009).

TUNEL

The TUNEL technique was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IL, USA) according to the instructions of the manufacturer. Briefly, sperm samples were washed three times by centrifugation in PBS (300*g*, 10 min), diluted to a concentration of 1×10^7 spermatozoa/ml and fixed with 4% (W/V) paraformaldehyde (USB Corporation, OH USA) for 60 min at room temperature. Samples were then washed and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma-Aldrich, St Louis, MO, USA) for 2 min on ice. Samples were then incubated with TUNEL reaction mixture (50 µl per 100 µl of sample) for 1 h at 37°C. Samples were finally washed, counterstained with DAPI or propidium iodide (PI) and analysed using fluorescence microscopy.

Ethanol-fixed spermatozoa

Slides were prepared by smearing 40 µl of a previously PBS-washed (centrifugation at 220 *g*, 10 min) diluted sperm sample (1×10^7 spermatozoa/ml) across the surface followed by air drying and fixation by immersion in ice-cold 95% ethanol (Sigma) for 5 min and air drying a second time.

Triton-X-100 treated spermatozoa

Fresh sperm suspensions were treated with Triton X-100 (Sigma-Aldrich) to permeabilize the plasma membranes (Kasai *et al.*, 1999). An aliquot (400 µl) of fresh, previously PBS-washed, diluted sperm suspension (1×10^7 spermatozoa/ml) was mixed with an equal volume of PBS (Fischer Scientific International, Pittsburgh, PA, USA) containing 0.2% (w/v) Triton X-100 and agitated for 3 min. Then, 400 µl of PBS were added and the sperm cell suspension centrifuged for 10 min at 500*g*. Pelleted spermatozoa were finally resuspended in 400 µl of PBS.

DW1 staining

Fresh spermatozoa in a suspension

Briefly, 15 µl of a PBS-washed diluted sperm suspension (1×10^7 spermatozoa/ml) was exposed to 7.5 µl of DW1 (2.5×10^{-3} mg/ml) and immediately examined on a slide using an epifluorescence microscope (Olympus BX61, Hamburg, Germany). For the optimization of the protocol, a range of DW1 concentrations was tested (2.5 mg/ml–2.5×10^{-4} µg/ml). In some samples, Hoechst 33342 (100 µg/ml, Sigma-Aldrich) was used as a counterstain for DNA.

Ethanol-fixed spermatozoa

Slides were stained with DW1 at a concentration of 2.5×10^{-3} mg/ml and immediately examined on a slide with an epifluorescence microscope. In some samples, Hoechst 33342 (100 µg/ml) was used as a counterstain for DNA.

Triton-X-100-treated spermatozoa

Briefly, 15 µl of sperm suspension was exposed to 7.5 µl of DW1 (1.25×10^{-2} µg/ml) and immediately examined on a slide with an epifluorescence microscope. In certain cases, Hoechst 33342 (100 µg/ml) was used as a counterstain for DNA.

Agarose-embedded spermatozoa

Unprocessed sperm, SCDt-processed and/or comet assay-processed slides were stained with freshly prepared 2.5×10^{-3} mg/ml solution of DW1. A small amount (15 µl) of this peptide was added to a microgel slide containing spermatozoa, covered with a coverslip and immediately evaluated under an epifluorescence microscope. In some cases, DAPI

combined with Vectashield (125 ng/ml, Abbott Molecular, Inc., Des Plaines, IL, USA) was used as a counterstain for DNA.

AO staining

Unprocessed sperm, SCDt-processed and comet assay-processed slides were stained with freshly prepared AO stain (0.5×10^{-4} M, Sigma). Briefly, 15 μl of this solution was placed on a slide, covered with a coverslip and immediately evaluated under an epifluorescence microscope.

Assessment of membrane integrity: Hoechst 33342/PI

To assess sperm membrane integrity, 15 μl of PBS-washed sperm suspension at a concentration of 1×10^7 spermatozoa/ml were exposed to 2.5 μl of Hoechst 33342 (100 μg/ml, Sigma-Aldrich) and 2.5 μl of PI (100 μg/ml, Sigma). Each sample was immediately evaluated under an epifluorescence microscope. Viable sperm were defined as those stained with Hoechst 33324 while resisting the uptake of PI; dead sperm were defined as those showing both PI and Hoechst 33342 fluorescence (Cai *et al*., 2005).

Acrosome integrity: FITC-PSA and Hoechst 33342

Acrosomal status was assessed using the acrosome-specific fluorochrome fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin (FITC-PSA, Sigma). Briefly, 50 μl of PBS-washed sperm suspension (1×10^7 spermatozoa/ml) was exposed to 2.5 μl of FITC-PSA (100 μg/ml) and 2.5 μl Hoechst 33342 (100 μg/ml) for 15 min at room temperature. Spermatozoa were then examined with an epifluorescence microscope. Acrosomal status was assessed according to two staining patterns: completely green acrosome (intact acrosome) and no fluorescence or only a fluorescent band at the equatorial segment of the sperm head (reacted acrosome). In some experiments acrosome integrity and DNA fragmentation analysis by DW1 were combined. In those cases 7.5 μl of DW1 (2.5×10^{-3} mg/ml) were added to the FITC-PSA and Hoechst 33342 mixture.

Immunodetection of ssDNA

Unprocessed sperm, and SCDt-processed and neutral and alkaline comet assay-processed slides were neutralized (two washes in 400 mM Tris–HCl, pH 7.4, Sigma), drained and exposed to 50 μl of a mouse antibody specific to ssDNA, following the procedure described by Wojewodzka *et al.* (2002).

Induction of ssDNA damage: hydrogen peroxide

Ten human sperm samples were washed in PBS, diluted to a concentration of 1×10^7 spermatozoa/ml in PBS (Fischer Scientific International) and divided into three aliquots of 200 μl each, allowing a control group to be compared with the treated cells. To induce ssDNA damage (Yamamoto, 1969), spermatozoa were incubated with 0.03 and 0.3% hydrogen peroxide (H_2O_2, Sigma) for 30 min at room temperature. Next, SCDt, alkaline comet assay and DW1 staining of ethanol-fixed slides were performed.

Induction of dsDNA damage: bleomycin

Ten human sperm samples were washed in PBS, diluted to a concentration of 1×10^7 spermatozoa/ml in PBS and divided into three aliquots of 200 μl each, allowing a control group to be compared with the treated cells. To induce dsDNA damage, spermatozoa were incubated with 2 or 4 mg/ml of bleomycin (Sigma) for 3 h at 37°C (Povirk *et al*., 1989). Next, SCDt, neutral comet assay and DW1 staining of ethanol-fixed slides were performed.

Fluorescence microscopy evaluation and image capture

Slides were analysed using a digital image-analysis platform based on a Olympus BX 61 fluorescence microscope (Olympus BX 61) equipped with a triple-band pass fluorescence filter block (for simultaneous visualization of red, green and blue stains) and two single-band pass fluorescence filter block [red, rhodamine/tetramethylrhodamine (TRITC); blue, DAPI]. Images were captured as tiff files using an Olympus digital camera and processed with Cytovision software (Genetix Ltd., Hampshire, UK).

Digital image analysis of DW1-DAPI stained spermatozoa, SCDt nucleoids and neutral and alkaline comets

The image-processing software Image J (available at http://rsb.info.nih.gov/nih-imageJ; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was used for the quantification of DW1 and DAPI fluorescence (details about the digital image analysis performed can be found in the Supplementary data).

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences v14. (SPSS, Inc., Chicago, IL, USA) and a one-tail *P*-value of 0.05 was considered significant. Details about the statistical tests performed can be found in the Supplementary data. The analyses included the *t*-test, one-way analysis of variance (ANOVA), Dunnett's test, Pearson's correlation coefficient, Mann–Whitney *U*-test and Kruskal–Wallis analysis, as appropriate.

Results

Staining of fresh spermatozoa in a suspension

When fresh intact spermatozoa in suspension were exposed to DW1, it was clear that the peptide was capable of binding to a subpopulation of the spermatozoa (Fig. 2). It was also apparent that the peptide bound to the sperm head where the sperm nucleus is located. No signal corresponding to DW1 could be seen in the tail. Binding of the peptide was extremely rapid at room temperature and persisted indefinitely (up to 24 h was tested).

The combination of rhodamine-labeled DW1 (red) and the DNA-specific fluorochrome DAPI (blue) showed distinct staining patterns among different sperm nuclei. Some nuclei were entirely blue (DW1-) and others red and blue (DW1+), providing further evidence that DW1 staining is specific for DNA and that spermatozoa differ in the degree of peptide binding, perhaps due to differences in levels of DNA damage (Fig. 2D–F).

The DW1 staining process was found to be compatible with continued sperm motility for at least 15 min. However, it was noted that none of the motile spermatozoa exhibited DW1 staining. The spermatozoa showing peptide labeling were restricted to a subset of the immotile sperm. This result suggested that the staining may be dependent not only on DNA damage but also on sperm viability or perhaps membrane integrity. To test this possibility, an experiment to permeabilize the plasma membrane was designed (results shown below). Additionally, we visualized the acrosome to assess whether

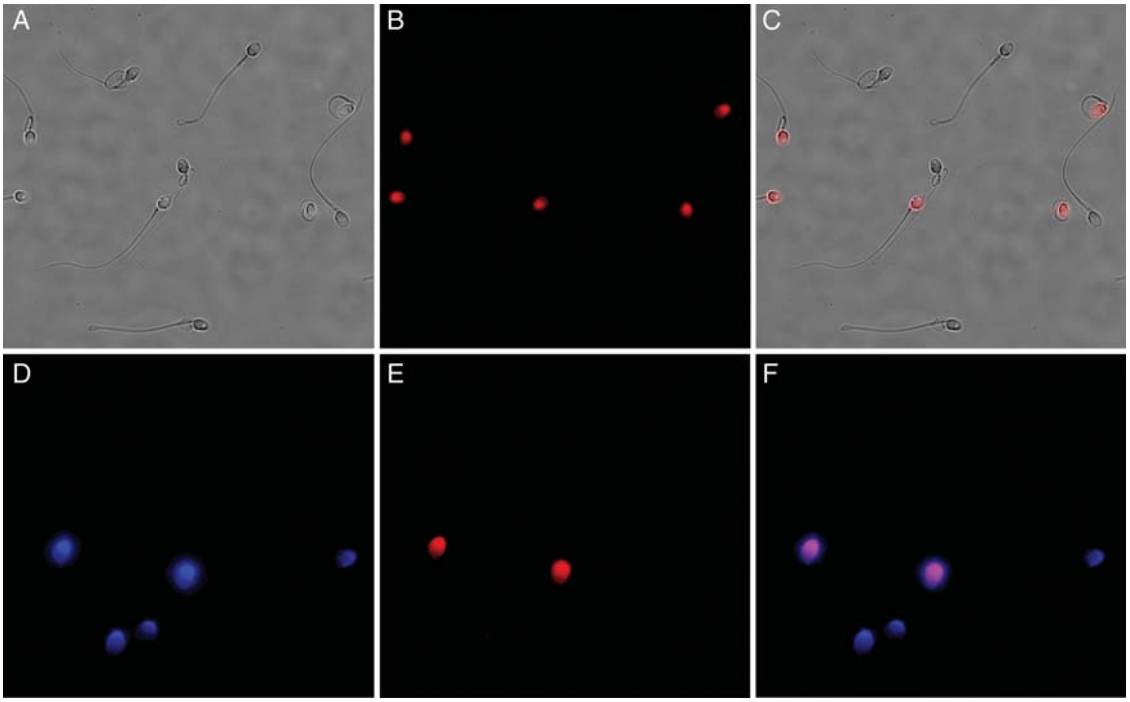

Figure 2 (**A**–**C**) Human spermatozoa stained with DW1 (red). (**A**) Brightfield microscopy. (**B**) Fluorescence microscopy. (**C**) Combined image of brightfield and fluorescence microscopy. (**D**–**F**) Human spermatozoa stained with DW1 (red) and DAPI (blue). (**D**) Blue (DAPI) channel. (**E**) Red (DW1) channel. (**F**) Red (DW1) and blue (DAPI) channels.

the difference in staining could be related to membrane changes associated with the acrosome reaction.

Analysis of peptide staining of acrosome-reacted spermatozoa

When DW1 was used in combination with a nucleus-specific fluorochrome such as Hoechst 33342 and the acrosome-specific stain FITC-PSA, the DNA specificity of the oligopeptide was confirmed again. No DW1 signal could be seen in the acrosome (Fig. 3A–D). Four staining patterns were observed: green acrosome fluorescence and blue nucleus fluorescence (intact acrosome and DNA); green acrosome fluorescence and pink nucleus fluorescence (intact acrosome and damaged DNA); no green acrosome fluorescence and blue nucleus fluorescence (reacted acrosome, intact DNA); no green acrosome fluorescence and pink nucleus fluorescence (reacted acrosome and damaged DNA; Fig. 3E). This suggests that the acrosome reaction does not influence the binding of DW1.

Staining of ethanol-fixed and Triton X-treated spermatozoa to assess DW1 staining in the absence of the plasma membrane

Ethanol fixation was used to remove sperm plasma membranes in order to ensure the access of DW1 to the nucleus in all cells, revealing whether differential membrane integrity could explain the various peptide staining patterns observed in the previous experiments. When ethanol-fixed spermatozoa were stained, binding to the sperm head where the sperm nucleus is located, was seen once again (Fig. 2). The proportion of DW1-stained spermatozoa increased after ethanol fixation as compared with fresh samples (*t*-test, $P < 0.05$; $32.30 \pm 5.12\%$ (mean $\pm$ SEM) versus $23.40 \pm 4.52\%$, respectively). Similar results were obtained when Triton-X was used to remove sperm plasma membranes.

In another analysis, sperm plasma membranes were progressively permeabilized in a suspension containing increasing concentrations of ethanol. In this case, the percentage of DW1-stained spermatozoa significantly increased as ethanol concentration was increased from 10 to 50% (Dunnett's test subsequent to ANOVA, $P < 0.05$). No further increases in the frequency of DW1-labelled sperm cells were seen once a concentration of 50% ethanol had been reached (75 and 95% ethanol were also assessed). The increase in DW1 staining was consistent with a significant decrease (Dunnett's test subsequent to ANOVA, $P < 0.05$) in membrane integrity as assessed by the Hoechst 33342/PI assay ($r = -0.752$, Pearson's correlation, $P < 0.01$; Fig. 4). These experiments suggest that DW1 specifically stains DNA, but cannot readily cross an intact plasma membrane. Similar results were obtained when increasing concentrations of Triton-X were used to disrupt or remove sperm plasma membranes.

What types of DNA does DW1 bind to and can the peptide yield information on the extent of DNA damage?

Another important question concerns the forms of DNA for which the DW1 peptide has affinity. It was important to verify whether

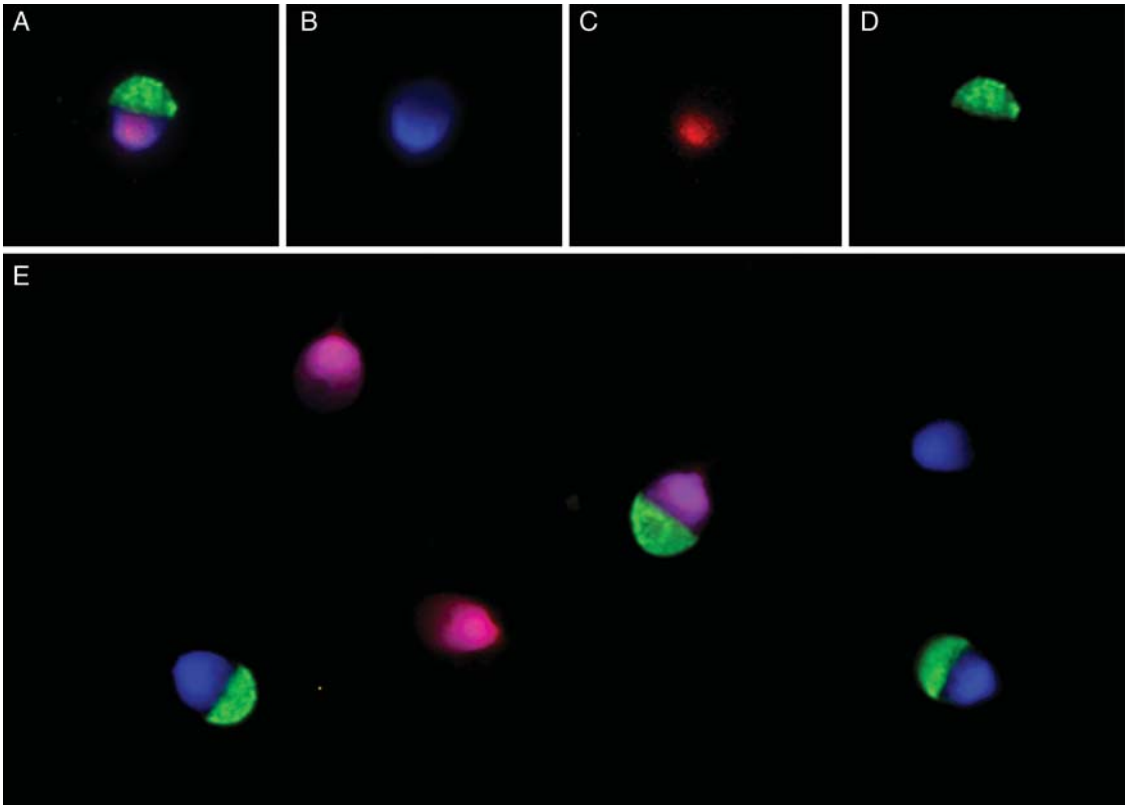

Figure 3 Human spermatozoa stained with DW1, Hoechst 33342 and FITC-PSA. (**A**) Red (DW1), blue (Hoechst 33342) and green (FITC-PSA) channels. (**B**) Blue (Hoechst 33342) channel. (**C**) Red (DW1) channel. (**D**) Green (FITC-PSA) channel. (**E**) Four staining patterns found in a sperm sample.

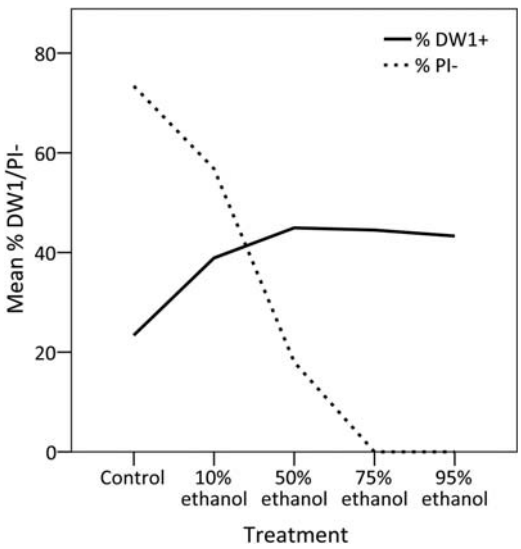

Figure 4 DW1 staining detection (% DW1 positive) and membrane integrity assessment (% PI negative) of five human sperm samples treated with increasing concentrations of ethanol.

the peptide acts as a general DNA stain, or whether it has specificity for specific DNA structures, such as breaks and single strands. For this purpose, several different investigations were undertaken, as detailed below.

Staining of spermatozoa processed with the SCDt

When DW1 was used to stain spermatozoa processed with the SCDt, results demonstrated no binding to the 'halo' of DNA around the core of the nucleoid, believed to represent undamaged loops of dsDNA. However, the peptide did bind to the core of all types of nucleoids generated by the assay: spermatozoa with large, small or no halo of dispersed DNA loops (Fig. 5A–C).

When the staining pattern was studied in detail with digital image analysis, a higher level of DW1 staining was observed in spermatozoa containing high levels of DNA damage, as assessed by the SCDt (i.e. spermatozoa with a small or non-existent halo of dispersed DNA loops) compared with those defined by the SCDt as having intact DNA (i.e. spermatozoa with a large or medium halo of dispersed DNA loops). Some of the parameters measured, in particular, area (red) and integrated density (red) presented significantly higher values in spermatozoa with a small/no halo of dispersed DNA loops as compared with spermatozoa with large/medium haloes of

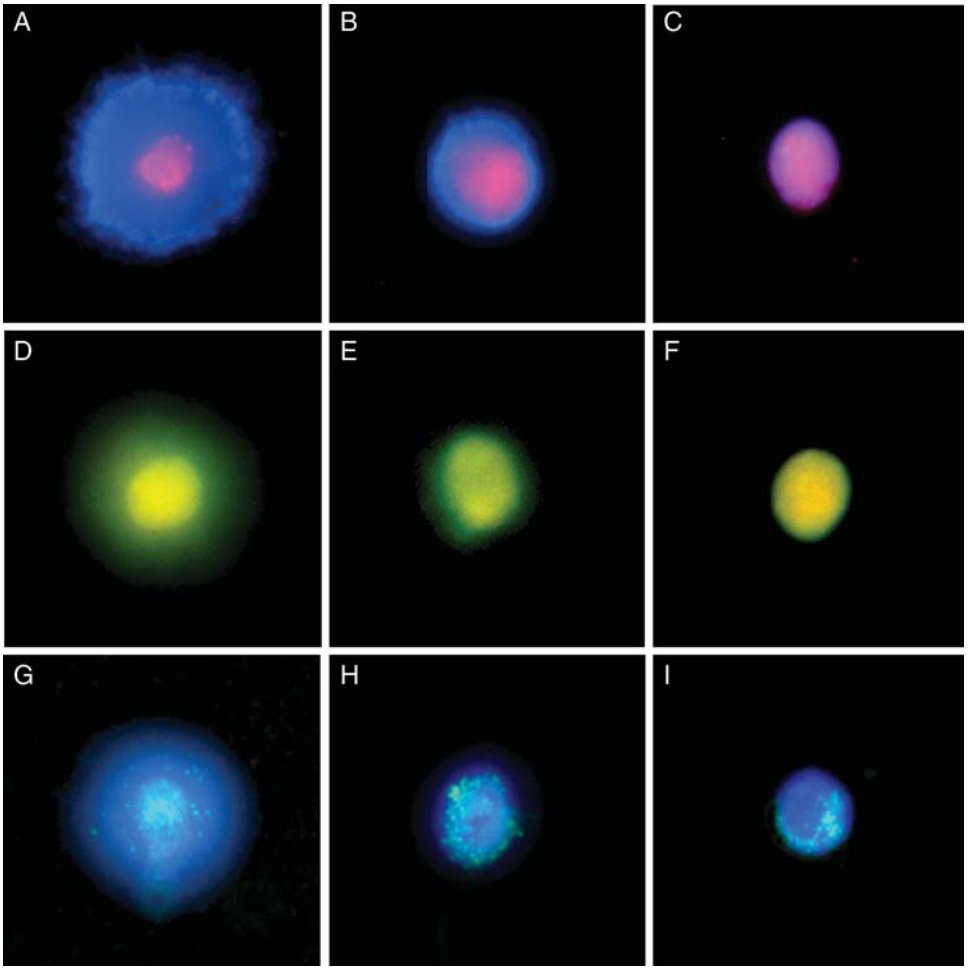

Figure 5 SCDt-processed spermatozoa. (**A**–**C**) Stained with DW1 (red) and DAPI (blue) (**D**–**F**) Stained with AO. AO binds to ssDNA emitting red fluorescence and to dsDNA emitting green fluorescence. (**G** and **H**) ssDNA immunodetection using an FITC-labelled anti-ssDNA antibody (green) and DAPI (blue).

dispersed DNA loops (*t*-test, $P < 0.05$; Supplementary data, Table SI). These results suggest that DW1 staining may indeed provide information about the DNA damage present in spermatozoa.

A similar pattern to that resulting from the staining with DW1 combined with DAPI could be observed in the SCDt nucleoids stained with AO (Fig. 5D–F). AO is a metachromatic dye that binds to ssDNA emitting red fluorescence and to dsDNA emitting green fluorescence. A similar distribution of an anti-ss antibody in SCDt nucleoids was observed as well (Fig. 5G–I). The similar distribution of DW1 fluorescence, AO red fluorescence and the anti-ss antibody observed suggests that DW1 binding sites might correspond to ssDNA.

Staining of spermatozoa processed with the alkaline comet assay

When DW1 was used to stain spermatozoa processed with the alkaline comet assay, the fluorescently labeled oligopeptide bound to both the head and the tail of ssDNA fragments of the alkaline comet. However, when combined with DAPI, it was clear that the peptide had greatest affinity for the DNA fragments present in the comet tail (Fig. 6A).

When this staining pattern was studied in detail with digital image analysis, an inverse correlation ($r = -0.501$, Pearson's correlation, $P < 0.05$) between the amount of blue fluorescence (integrated density DAPI) in the comet head and the amount of red fluorescence (integrated density DW1) in the comet tail of the alkaline comets was found. In addition, a significant positive correlation between the length/density of the tail (area DAPI) and the level of DW1 staining in the alkaline comet (integrated density DW1) was found ($r = 0.768$, Pearson's correlation, $P < 0.01$). Those comets with longer/denser tails (i.e. those nuclei presenting DNA damage) presented significantly higher levels of DW1 staining.

A similar pattern to that resulting from the staining with DW1 combined with DAPI can be observed in the alkaline comets stained with AO (Fig. 6B). A similar distribution of the anti-ss antibody used in the alkaline comets was observed as well (Fig. 6C). As in the case of the SCDt nucleoids, the similar distribution of DW1 fluorescence, AO red fluorescence and anti-ss antibody suggests that DW1 binding sites might correspond to ssDNA.

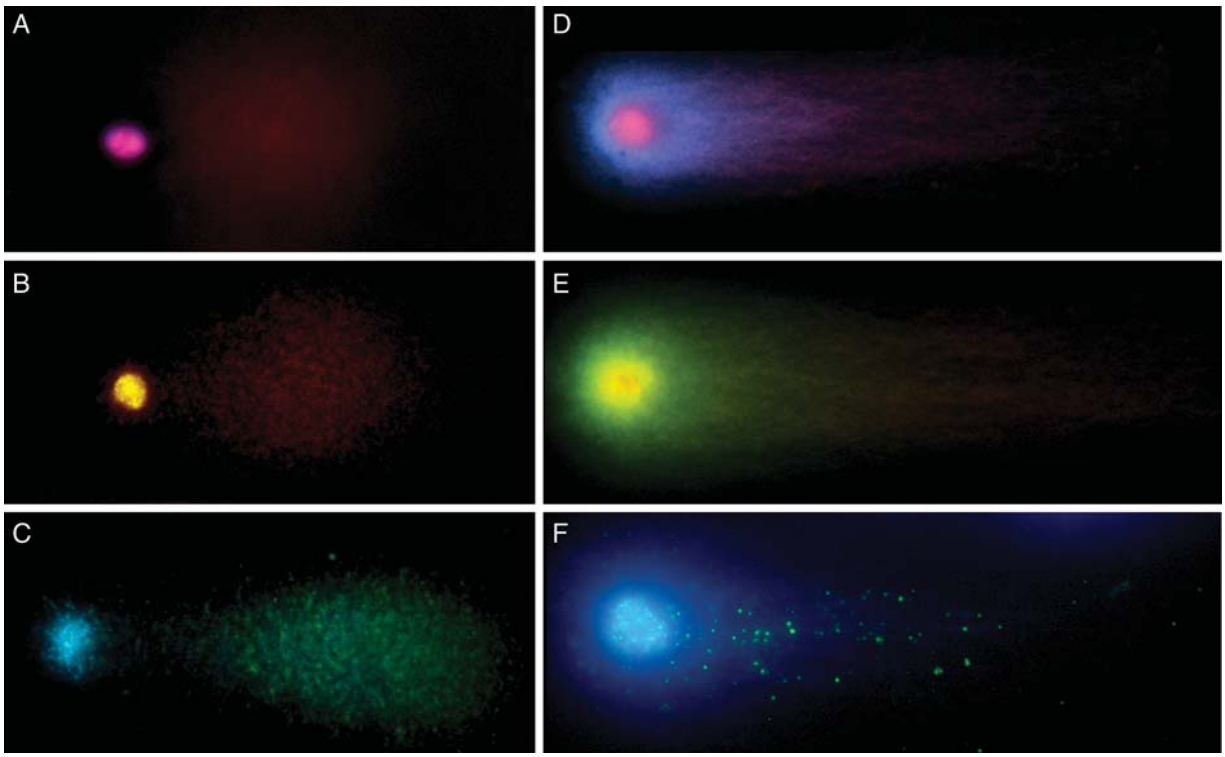

Figure 6 (**A**–**C**) Alkaline comet assay-processed spermatozoa. (**A**) Stained with DW1 (red) and DAPI (blue) (**B**) Stained with AO. AO binds to ssDNA emitting red fluorescence and to dsDNA emitting green fluorescence (**C**) ssDNA immunodetection using an FITC-labelled anti-ssDNA antibody (green) and DAPI (blue). (**D**–**F**) Neutral comet assay-processed spermatozoa. (**D**) Stained with DW1 (red) and DAPI (blue) (**E**) Stained with AO. AO binds to ssDNA emitting red fluorescence and to dsDNA emitting green fluorescence (**F**) ssDNA immunodetection using a FITC-labelled anti-ssDNA antibody (green) and DAPI (blue).

Staining of spermatozoa processed with the neutral comet assay

When DW1 was used to stain spermatozoa processed with the neutral comet assay protocol, it bound to both the head and the tail of the neutral comet; however, when combined with DAPI, it was clear that the peptide had greatest affinity for the terminal region of the comet tail (Fig. 6D).

A significant positive correlation between the length/density of the tail (area DAPI) and the level of DW1 staining in the alkaline comet (integrated density DW1) was found ($r = 0.835$, Pearson's correlation, $P < 0.01$). Those comets with longer/denser tails (i.e. those nuclei presenting DNA damage) presented significantly higher levels of DW1 staining.

A similar pattern to that resulting from the staining with DW1 combined with DAPI can be observed in the neutral comets stained with AO (Fig. 6E). A similar distribution of the anti-ss antibody used in the neutral comets was observed as well (Fig. 6F). As in the case of the SCDt nucleoids and alkaline comets, the distribution of DW1 fluorescence suggests that DW1-binding sites might correspond to ssDNA.

Induction of ssDNA damage: H_2O_2

In samples treated with H_2O_2, the percentage of cells positive for DW1 staining (DW1+) significantly increased with H_2O_2 concentration (Dunnett's test subsequent to ANOVA, $P < 0.05$). A significant increase in the frequency of sperm cells with fragmented DNA detected by the SCDt (i.e. SCDt-SDF) and the alkaline comet assay (i.e. ssSDF), consistent with the increasing concentration of damaging agent, was also found (ANOVA, $P < 0.05$; Fig. 7A).

Not only did the number of DW1+ cells increase after the treatment but also the intensity of DW1 staining in DW1+ sperm cells was significantly higher, especially in the 0.3% H_2O_2-treated samples. Some of the parameters measured such as area (red) and mean grey value (red) presented significantly higher values in the red-type cells found in the H_2O_2-treated samples compared with those of the control (Mann–Whitney *U*-test, $P < 0.05$; Supplementary data, Table SII). These results indicate that DW1 may be detecting DNA damage, in particular the ssDNA breaks generated by H_2O_2.

Induction of dsDNA damage: bleomycin

In samples treated with bleomycin, the frequency of sperm cells with fragmented DNA detected by the SCDt (i.e SCDt-SDF) and the neutral comet assay (i.e. dsSDF) significantly increased with bleomycin concentration (Mann–Whitney *U*-test, $P < 0.05$; Fig. 7B). A significant increase in the percentage of red, DW1 positive, cells (%DW1+), consistent with the increasing concentration of damaging agent, was also found (Mann–Whitney *U*-test, $P < 0.05$; Fig. 7B). Not only did the number of DW1+ cells increase after the treatment but also the intensity of DW1 staining per DW1+ sperm cell was significantly

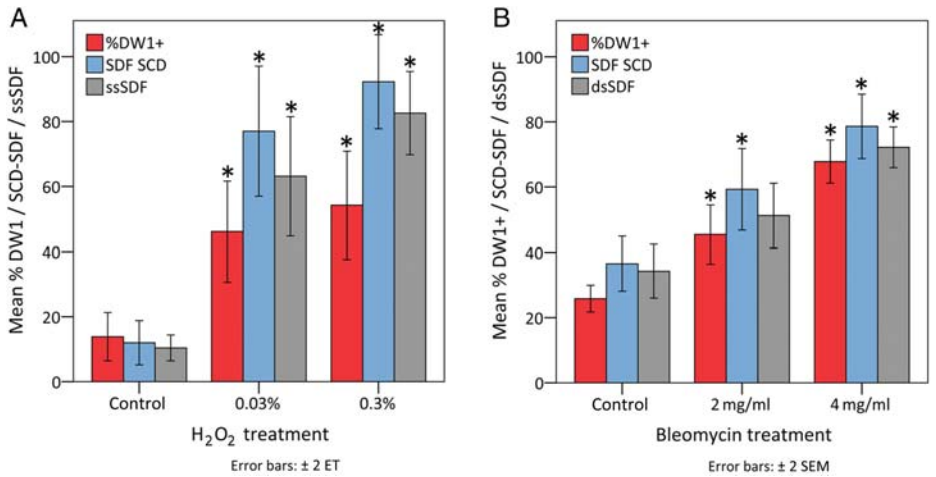

Figure 7 DNA fragmentation detection by DW1 staining, SCDt and comet assay. (**A**) In 10 human sperm samples treated independently with H_2O_2. Groups significantly different from control (Dunnett's test subsequent to ANOVA, $P < 0.05$) are highlighted with an asterisk. (**B**) In 10 human sperm samples treated independently with bleomycin. Groups significantly different from control (Mann–Whitney *U*-test, $P < 0.05$) are highlighted with an asterisk.

higher especially in the 4 mg/ml bleomycin-treated samples. Some of the parameters measured such as area (red) and mean grey value (red) presented significantly higher values in the red-type cells found in the bleomycin-treated samples (Mann–Whitney *U*-test, $P < 0.05$; Supplementary data, Table SII). These results indicate that DW1 may be detecting DNA damage, in particular the dsDNA breaks generated by bleomycin.

Finally, from the analysis of all the samples studied, we found that there is a significant and positive correlation between the percentage of DW1+ sperm cells after DW1-DAPI staining and the SDF calculated by the SCDt ($r = 0.789$, Pearson's correlation, $P < 0.01$). A significant and positive correlation was also found between the percentage of DW1+ sperm cells and the SDF calculated by alkaline and neutral comet assays ($r = 0.902$, Pearson's correlation $P < 0.01$ and $r = 0.933$, Pearson's correlation, $P < 0.01$, respectively). These results confirm that %DW1+ sperm cells gives information about the degree of DNA damage present in a sperm sample. Although the percentage of sperm cells containing fragmented DNA established by DW1 staining seems to be lower than the SDF established by the other two methods (Fig. 7), these differences were not significant (one-factor ANOVA), with the exception of samples treated with 0.3% H_2O_2 where an apparent difference between DW1 and SCDt was recorded (one-factor ANOVA, $P = 0.016$).

Detection of DNA damage: DW1 staining and TUNEL assay

Results from a further experiment involving the analysis of 10 sperm samples using DW1 staining and TUNEL technique showed a significant correlation between the SDFs established by both methods ($r = 0.892$, Pearson's correlation, $P < 0.05$; Supplementary data, Table SIII, Fig. 8). No significant differences were found between the frequencies of DNA damaged sperm cells scored by these methods in the 10 samples analysed.

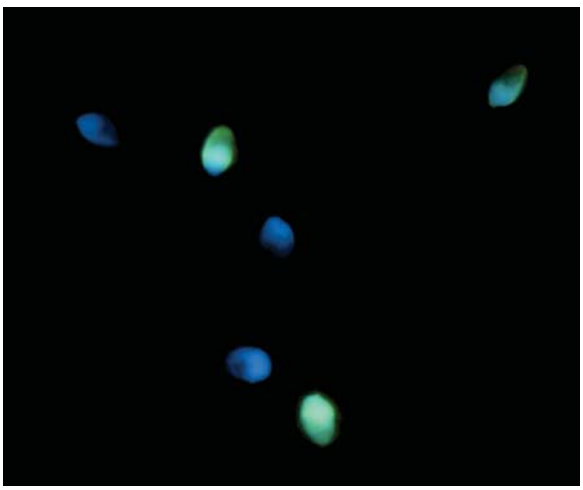

Figure 8 Sperm nuclei processed with TUNEL assay and counterstained with DAPI. Sperm nuclei containing DNA damage are detected by TUNEL and fluorescence green from FITC; sperm nuclei with intact DNA fluorescence blue from the nuclear counterstained DAPI.

Digital image analysis of DW1-DAPI stained spermatozoa

In addition to visual scoring, digital image analysis was performed to produce a prototype for the automatic measurement of DNA damage by means of DW1 staining. Results from digital image analysis of 95% ethanol-fixed slides showed that the two sperm cell types (DW1+ and DW1−) were readily distinguishable. After image capture, Image J software was used to quantify red and blue fluorescence levels for each sperm type (Supplementary data, Table SIV).

Some of the parameters measured, in particular, mean grey value (red), integrated density (red), mean grey value (red/blue) and integrated density (red/blue), presented significantly different values in the two sperm types (Kruskal–Wallis, $P < 0.05$). This allowed the quantitative characterization of DW1+ and DW1− sperm types by digital image analysis.

Discussion

The use of a novel 21 amino acid peptide based on the p53 protein (DW1) to stain spermatozoa in suspension or after fixation on microscope slides showed that the oligopeptide is nucleus specific. The binding of DW1 to its corresponding epitopes was extremely rapid, no incubation was required. Specific labelling was easily achieved by simple adjustment of oligopeptide concentration. The peptide provided highly specific binding to the sperm nucleus at concentrations of 1.25×10^{-3} mg/ml, although a range of concentrations were also found to be suitable (1.25×10^{-2}–1.25×10^{-3} mg/ml). No labelling was observed in either the tail or the acrosome demonstrating that DW1 has a high affinity for DNA. This was also confirmed in experiments in which the peptide was used to stain ssDNA and dsDNA ladders following electrophoresis on agarose gels (Supplementary data, Figures).

Rather than having a generalized affinity for DNA, DW1 was shown to have specificity for particular nucleic acid structures. When DW1 was combined with DAPI, the two dyes did not stain all regions of the nucleus with equal intensity. This indicates that DW1 and DAPI may differ in their preferred binding sites. We propose that DW1 is detecting specific DNA lesions, while DAPI is detecting the whole DNA content of the sperm cell. This rationale is based on the increased DW1 staining seen in sperm samples exposed to genotoxic agents (discussed below) and the fact that previous investigations have shown that the C-terminal domain of p53, the protein upon which DW1 is modelled, specifically binds to irradiated or enzymatically damaged DNA (Reed *et al.*, 1995), insertions/deletions (Lee *et al.*, 1995) or four-way junctions (Lee *et al.*, 1997).

The affinity of DW1 for different DNA structures was explored in experiments performed on spermatozoa processed using established techniques for the detection of DNA damage, such as SCDt, comet assay and TUNEL. Taken together, the results provide strong evidence that DW1 can be used as an indicator of DNA damage in sperm. Regardless of the method of DNA damage assessment used, results using the peptide were always concordant, with staining only seen in cells containing damaged DNA. The use of neutral and alkaline versions of the comet assay provided more detailed data about the specific type of DNA damage being detected by DW1. It is thought that neutral comet tails consist of extended DNA loops still attached to structures within the comet head, loop extension being produced by the presence of dsDNA breaks. In contrast, alkaline comet tails are made up of ssDNA fragments produced by unwinding of the DNA at the break points (Klaude *et al.*, 1996; Collins *et al.*, 1997; Afanasieva *et al.*, 2010).

The DW1 peptide was found to bind to the terminal region of the neutral comet tails, which most likely represent the ends of broken DNA loops, providing a further indication that the peptide has an affinity for DNA ends. A strong DW1 binding was also seen in the head of the comet, likely corresponding to ssDNA breaks present in the comet head, which are unable to migrate in electrophoretic neutral conditions.

In the case of alkaline comets, DW1 binds to both the comet head and tail. In the alkaline protocol two types of DNA fragments are produced. DNA between closely spaced breaks in the same strand will unwind completely and become released as ssDNA fragments, forming a migrating tail during electrophoresis. However, if the breaks are far apart the unwinding will cease before all DNA has become single stranded. Such fragments, only partially single stranded, are anchored in place by their contiguous double-stranded regions and their exit from the comet head is further impeded by the high probability of entangling with other DNA molecules in the lysed nucleus (Klaude *et al.*, 1996). The DW1 signal seen in the alkaline comet tail indicates that the peptide binds ssDNA fragments and DNA ends with high affinity.

Comparison of DW1 staining patterns with AO staining of spermatozoa subjected to SCDt, neutral or alkaline comet assays provided further evidence concerning the affinity of the peptide for specific DNA structures. AO is a selective metachromatic dye that interacts with DNA by intercalation or electrostatic attractions (Mitsuaki *et al.*, 1971). When AO binds to dsDNA motifs, it intercalates in the DNA structure as a monomer, reaching a maximum emission at 525 nm (green). However, when AO binds to ssDNA it forms non-ordered aggregates where the maximum emission shifts to 650 nm (red; Kasten, 1967). DW1 staining demonstrated a similar distribution to AO red fluorescence, providing further evidence of an affinity for ssDNA. This distribution was also seen when ssDNA was detected using immunoassays applied to SCDt nucleoids, as well as neutral and alkaline comets. This characteristic of the DW1 peptide is consistent with the results of Jayaraman and Prives (1995), which suggest that the C-domain of p53 interacts with ssDNA, and with other studies showing that the C-terminus of p53 is capable of binding to ssDNA ends (Bakalkin *et al.*, 1995; Selivanova *et al.*, 1996). Taken together, the data suggest that the DW1 peptide successfully mimics the ssDNA-binding activity of p53. This is an encouraging finding, because this property of the peptide is likely to be useful for the detection of ssDNA breaks in sperm nuclei.

Once the affinity of DW1 for different DNA structures was explored, experiments to test the ability of the peptide to quantify DNA damage in human semen samples (i.e. provide a population overview of damage levels) were undertaken. Results showed that the proportion of DW1-positive sperm cells correlates with the proportion of spermatozoa possessing DNA damage of various kinds, as revealed by SCDt, TUNEL and alkaline and neutral comet assays. These findings show that the peptide not only stains damaged DNA but can also provide a potentially useful measure of the proportion of spermatozoa in a sperm sample that are affected by DNA damage. The SDF for control samples (normozoospermic for conventional WHO semen parameters) ranged from 4.70 to 32.25% for SCDt, 5.00 to 25.31% for comet and from 6.05 to 22.50% with the DW1 staining method. In the experiment comparing TUNEL and DW1 staining results that included not only normozoospermic samples but also samples with abnormal conventional semen parameters, the SDF of samples ranged from 8.00 to 56.73% for TUNEL and from 8.50 to 62.32% for DW1 staining. A normal reference value needs to be established for DW1 in future studies; however, given the high correlation of the results obtained between

DW1 and the currently established methods for evaluating sperm DNA damage, the threshold level of 30% established for sperm DNA fragmentation (Bungum et al., 2007) could be considered as a starting reference value to distinguish between good and poor semen samples.

In most cases the percentages of DNA damaged spermatozoa calculated using DW1 were not significantly different from those obtained using established tests (SCDt, comet and TUNEL). The only exception was the SDF established by DW1 and SCDt in samples treated with 0.3% H_2O_2, where DW1 recorded a lower value. It may be that the harsh conditions (high H_2O_2) leave cells more sensitive to handling and that the processing involved in SCDt leads to artefactual DNA damage. This damage would not be present in DW1-stained spermatozoa because very little manipulation and processing of spermatozoa is involved in this method. Alternatively, it may be that DW1 is not able to identify all the DNA breaks produced by this high concentration of H_2O_2. There have been concerns with all methods of sperm DNA assessment that the highly condensed sperm nucleus might prevent some of the DNA damage present in the nucleus from being detected, potentially leading to an underestimate of the amount of damage (Barratt et al., 2010). The fact that the procedure presented in this study does not involve decondensation of the nucleus, might make access to the nuclear interior even more difficult. This could potentially lead to a loss of resolution once DNA damage exceeds a certain threshold (i.e. once the entire nuclear surface has been damaged and is saturated with peptide). However, the results of the current study are reassuring, clearly indicating a very close association between DNA damage and DW1 staining.

Digital image analysis showed that not only the proportion of sperm positive for DW1 staining but also the intensity of staining in individual cells (integrated density DW1) was associated with the amount of dsDNA and ssDNA damage. The ability to assess the degree of damage in an individual sperm cell might be valuable clinically, particularly as our understanding increases concerning the extent to which sperm DNA damage can be repaired by the oocyte after fertilization. It might be the case that low or even moderate levels of DNA fragmentation are tolerable due to the potent DNA repair capacity of the oocyte. Consequently, a measurement of the extent of DNA damage might be more important than a simple assessment of presence/absence.

No motile spermatozoa presented DW1 staining, suggesting that the peptide is unable to reach the nucleus of viable cells with intact membranes. Accurate detection of sperm DNA damage was only accomplished after the plasma membrane had been permeabilized using ethanol or detergent. Modification of DW1 to include a TAT peptide (YGRKKRRQRRRG), an amino acid sequence which has been shown to facilitate transduction of peptides or proteins into various cells, failed to improve access to the sperm cell interior (data not shown). Unfortunately this means that, at present, the use of DW1 to distinguish spermatozoa with intact membranes and undamaged DNA from those with intact membranes and fragmented DNA is not straightforward.

It is still possible that the peptide could be used to choose spermatozoa for fertilization using ICSI but only if the membrane is first removed using a detergent. Previous studies have shown that sperm treated in this way remain fertilization competent (Kasai et al., 1999). However, more work will need to be done to assess any potential toxicity of this approach. Other modifications to DW1 such as different TAT configurations or the use of an alternative protein transduction domain might allow penetration into the cell interior. Further experiments are needed to determine which of these approaches, if any, will allow the peptide to be used to reveal viable non-permeabilized spermatozoa with intact DNA.

Regardless of whether the peptide can be used for selection of individual spermatozoa, our results show that DW1 is capable of providing an accurate measure of the proportion of cells in a sperm sample which is affected by DNA damage. In membrane-free cells, DW1 staining successfully quantified the presence of DNA fragmentation, indicating that the application of the peptide may be considered as a rapid and inexpensive alternative to the sperm DNA fragmentation detection tests in current use. Furthermore, at higher concentrations DW1 can also be used to assess sperm membrane integrity, thus revealing that potentially viable cells could be used for ICSI in a manner similar to Sybr14/PI testing.

In conclusion, this study shows that the novel synthetic DW1 peptide has affinity for various DNA lesions. DW1 staining displays a close correlation with the results of established tests for sperm DNA and chromatin structure damage (SCDt, comet assay and TUNEL). Although current protocols do not allow the peptide to cross the plasma membrane, preventing it from being used for the selection of sperm with intact DNA for use in ICSI, it can still be used to assess the level of DNA damage present in permeabilized or fixed samples. As such, the peptide could represent a less expensive, faster and easier to use, alternative to existing methods used for the evaluation of DNA damage in sperm samples. Studies assessing samples with a wide variation in semen quality parameters are now required in order to establish appropriate thresholds for DNA damage and to explore the clinical utility of this new approach. Future work will also focus on the development and optimization of peptides which can cross the plasma membrane and target DNA damage, allowing application of this novel technique to viable spermatozoa.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

M.E. participated in the design of the study, collected and analysed the data and drafted the manuscript. G.P. modelled the oligopeptide, participated in the design of the study and manuscript preparation. J.C. participated in the design of the study and contributed to manuscript preparation and D.W. came up with the concept, participated in the design of the study, supervised data analysis and was involved in manuscript preparation.

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Conflict of interest

None declared.

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