Double stranded DNA breaks hidden by the neutral Comet assay suggest a role of the sperm nuclear matrix in DNA integrity maintenance

Running Title: Sperm dsDNA breaks suggesting a role of the nuclear matrix

Ribas-Maynou, J.[1]; Gawecka, J. E.[2]; Benet, J.[1]; Ward, W. S.[2]

[1] *Unitat de Biologia Cellular, Fisiologia i Immunologia. Departament de Biologia Cel·lular, Fisiologia i Immunologia. Facultat de Medicina. Universitat Autònoma de Barcelona. 08193 Bellaterra, Spain.*

[2] *Institute for Biogenesis Research. Department of Anatomy, Biochemistry and Physiology. John A. Burns School of Medicine. University of Hawaii at Manoa. Honolulu, Hawaii, 96822United States of America.*

Corresponding Author:

W. Steven Ward, Ph.D.
Institute for Biogenesis Research
John A. Burns School of Medicine
University of Hawaii at Manoa
1960 East-West Rd.
Honolulu, HI 96822
Tel: 808-956-5189
Fax: 808-956-7316
E-mail: wward@hawaii.edu

Conflict of interest: The authors declare no conflict of interest.

Key Words: Neutral Comet Assay, Sperm Nuclear Matrix, Sperm DNA Breaks

ABSTRACT

We used a mouse model in which sperm DNA damage was induced to understand the relationship of double stranded DNA (dsDNA) breaks to sperm chromatin structure and to the Comet assay. Sperm Chromatin Fragmentation (SCF) produces dsDNA breaks located on the matrix attachment regions, between protamine toroids. In this model, epididymal sperm induced to undergo SCF can religate dsDNA breaks while vas deferens sperm cannot. Here, we demonstrated that the conventional neutral Comet assay underestimates the epididymal SCF breaks because the broken DNA ends remain attached to the nuclear matrix, causing the DNA to remain associated with the dispersion halo, and the Comet tails to be weak. Therefore, we term these hidden dsDNA breaks. When the Comet assay was modified to include an additional incubation with SDS and DTT after the conventional lysis, thereby solubilizing the nuclear matrix, the broken DNA was released from the matrix, which resulted in a reduction of the sperm head halo and an increase in the Comet tail length, exposing the hidden dsDNA breaks. Conversely, SCF-induced vas deferens sperm had small halos and long tails with the conventional neutral Comet assay, suggesting that the broken DNA ends were not tethered to the nuclear matrix. These results suggest that the attachment to the nuclear matrix is crucial for the religation of SCF-induced DNA breaks in sperm. Our data suggest that the neutral Comet assay identifies only dsDNA breaks that are released from the nuclear matrix and that the addition of an SDS treatment can reveal these hidden dsDNA breaks.

INTRODUCTION

Sperm DNA is mostly compacted by protamines rather than histones, that form toroids containing about 25 to 50 kb of DNA (Hud *et al*, 1995). These toroids are linked by matrix attachment regions (MARs), which are nuclease sensitive, and bind them to the nuclear matrix (Aoki and Carrell, 2003; Sotolongo *et al*, 2003; Ward, 2010). MARs are also associated with DNA replication, DNA repair, and gene regulation in somatic cells (Boulikas, 1995; Codrington *et al*, 2007). During the transit through the epididymis, the final DNA compaction of the sperm chromatin occurs through the formation of di-sulfide bonds which stabilize different chromatin interactions (Bjorndahl and Kvist, 2010; Chapman and Michael, 2003; Haidl *et al*, 1994). This renders the mature sperm chromatin more resistant to damage by reactive oxygen species (ROS) (Bennetts and Aitken, 2005; Sakkas and Alvarez, 2010).

Sperm DNA damage can be an obstacle to pregnancy, and it may induce miscarriage or abnormal fetal development (Carrell *et al*, 2003; Evenson *et al*, 1999; Lewis and Simon, 2010; Ribas-Maynou *et al*, 2012b). Several studies have shown that sperm DNA damage is higher in infertile men when compared to fertile donors (Evenson and Jost, 2000; Fernandez *et al*, 2005; Ribas-Maynou *et al.*, 2012b; Sharma *et al*, 2010; Simon *et al*, 2013). In mice, the injection of sperm cells with previously induced DNA damage leads to delayed DNA replication, chromosomal aberrations and, consequentially, the arrest of the embryo at early developmental stages (Gawecka *et al*, 2013). Oxidative stress, which is the main inducer of single stranded DNA breaks, affects all parts of the sperm chromatin – DNA that is protamine bound, within toroids, or histone bound, and between toroids (Agarwal *et al*, 2008; Aitken and De Iuliis, 2010; Ribas-Maynou *et al.*, 2012b). Sperm DNA damage can also be physiologically induced by nucleases in an apoptotic-like process (Aitken and De Iuliis, 2010; Sakkas and Alvarez, 2010), which would affect the toroid linker regions more than the DNA within the protamine toroids.

Mammalian spermatozoa can be induced to cleave their DNA at the MAR regions into 25 to 50 kb fragments by incubation with $MnCl_2$ and $CaCl_2$, in a process termed sperm chromatin fragmentation (SCF) (Yamauchi et al., 2007). These dsDNA SCF-produced breaks in epididymal sperm are most likely produced by inducing topoisomerase 2 (TOP2) to cleave the DNA (Shaman *et al*, 2006). A recent study has confirmed that mature mammalian spermatozoa contain TOP2 (Chauvin *et al*, 2012). TOP2 unwinds DNA by inducing a dsDNA break, then passing another DNA strand through this break, then religating the originally cut strands (Nelson *et al*, 1986) (Suppl. Fig. 1). Both of the broken DNA ends remain covalently attached to a TOP2 monomer during the DNA breakage step, and the enzyme religates the DNA after strand passage. During apoptosis in somatic cells, TOP2 is the initial nuclease that degrades the DNA and in this case the dsDNA breaks are not religated (Champoux, 2001; Li and Liu, 2001). Because TOP2 requires magnesium for activity, treatment with EDTA causes TOP2 to religate the dsDNA breaks, reversing the DNA cleavage step (Li *et al*, 1999).

SCF induced dsDNA breaks in epididymal sperm can be religated by treatment with EDTA (Yamauchi *et al*, 2007a; Yamauchi *et al*, 2007b),

supporting the hypothesis that these breaks are induced by TOP2 or a similar enzyme present in sperm. SCF can only be induced by $MnCl_2$ with or without $CaCl_2$, and not by $MgCl_2$ so it is likely that the divalent cations used to induce SCF do so by activating a pathway that leads to TOP2 or a related enzyme cleaving the DNA, rather than by activating the TOP2 directly. We have suggested that these breaks are part of an apoptotic like mechanism present in sperm that contribute to the eradication of faulty sperm in the male reproductive tract. Injection of epididymal SCF-induced sperm in which the dsDNA breaks were religated by EDTA treatment into oocytes does not lead to normal development to blastocysts, suggesting that the religation step is not a complete DNA repair. When vas deferens sperm is incubated with the same divalent ions, the DNA undergoes further degradation and the DNA can no longer be religated with EDTA (Yamauchi *et al.*, 2007a).

Multiple tests to assess sperm DNA damage have been developed for human and animal sperm. Of these, the Comet assay, is unique in that it can assess differentially single stranded DNA (ssDNA) and double stranded DNA (dsDNA) breaks depending on whether alkaline or neutral pH is used, respectively (Enciso *et al*, 2009; Ribas-Maynou *et al*, 2012a; Singh *et al*, 1988). It relies on the DNA migration in an electrophoresis field after releasing the protamines thereby forming a sperm nuclear halo. The alkaline Comet assay, can distinguish between fertile donors and infertile patients, as well or better than other commonly used assays (Chohan *et al*, 2006; Ribas-Maynou *et al*, 2013). The neutral Comet assay is not capable of detecting infertile patients, but it does have the unique ability to identify men whose sperm are likely to result in pregnancy loss within the first trimester of gestation (Ribas-Maynou *et al.*, 2012b).

The aim of the present study was to understand the mechanism through which the neutral Comet assay recognizes DNA breaks and the type of DNA damage it identifies, in order to enable the correct interpretation of results obtained when this assay is applied with male infertility patients. We used a murine model with induced sperm chromatin fragmentation (SCF) in which we could generate mild and severe DNA damage that had previously been shown to result in dsDNA breaks. Our goal was to understand why there is a relationship between the neutral Comet assay and recurrent pregnancy loss, but not with pregnancy achievement. A secondary objective was to extend the understanding of the mechanisms of this mouse model so that SCF could be subsequently used as a model for different human diseases.

MATERIAL AND METHODS

Animals

B6D2F1 mice were obtained from the National Cancer Institute (Raleigh, NC, USA). Mice were maintained in the University of Hawaii vivarium in accordance with the guidelines of the Laboratory Animal & Veterinary Service and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council. The Institutional Animal Care and Use Committee of the University of Hawaii reviewed the protocols for animal handling and the treatment procedures.

Treatments

Sperm and the corresponding fluid from epididymides and vasa deferentia were collected in TKB buffer (25 mM Tris-HCl, 150 mM KCl, pH 7.5) supplemented with 0.25% Triton X-100. Then, the suspension was mixed gently and different treatments were performed.

SCF and reparability test

To induce SCF, sperm samples were treated with 10 mM $MnCl_2$ and 10 mM $CaCl_2$ for one hour at 37 ºC. To test for the ability of the sperm chromatin to religate dsDNA breaks, samples were incubated with 100 mM EDTA for half an hour at 37 ºC. Controls were incubated for the same time without EDTA (Yamauchi *et al.*, 2007a).

Nuclease treatment

In order to cause a complete digestion of the MAR regions, samples were treated with DNase I (New England Biolabs, Ipswich, MA, USA) at 40 Units/ml in 10 mM $MgCl_2$ for 1 hour at 37ºC.

Hydrogen Peroxide treatment

Hydrogen peroxide causes ssDNA breaks in all the genome regions (Ribas-Maynou *et al.*, 2012a). To produce a high number of DNA breaks, an samples were incubated with 0.128 M H_2O_2 was performed for 30 minutes at 37ºC.

Neutral and alkaline Comet assays

The sperm Comet assay can be performed in neutral or alkaline conditions, which allows this technique to detect double stranded and single stranded DNA breaks, respectively. Sperm samples were diluted to $1 \cdot 10^6$ sperm/ml in TKB, and 25 μl of the suspension was mixed with 50 μl of 1% low melting point agarose (LMP). Next, 15 μl of the sperm-agarose mixture was placed on two 1% LMP agarose-pretreated slides for gel adhesion, covered with coverslips and allowed to solidify for 5 minutes at 4 ºC on a cold metal plate. Afterwards, coverslips were gently removed and slides were incubated in lysis solution 1 (0.8M Tris-HCl, 0.8M DTT, 1% SDS, pH 7.5), then in lysis solution 2 (0.4 M Tris-HCl, 50 mM EDTA, 2 M NaCl, 0.4 M DTT, pH 7.5) for 30 minutes

each at room temperature, and then washed in TBE (0.445 M Tris-HCl, 0.445 M Boric Acid, 10 mM EDTA) for 10 minutes.

At this point, the slide designated for the neutral Comet assay was placed on the electrophoresis canister in TBE buffer and electrophoresis was performed at 1 V/cm for 4 or 12.5 minutes. Afterwards, the neutral Comet assay slide was washed in 0.9% NaCl. In parallel, the alkaline Comet slide was submerged in the alkaline solution (0.03 M NaOH, 1 M NaCl) for 2.5 minutes at 4ºC and then electrophoresed at 1 V/cm in alkaline buffer (0.03M NaOH) for 4 or 12.5 minutes. Finally, both slides were washed in neutralization solution (0.4M Tris-HCl, pH 7.5) for 5 minutes, then in an ethanol series (70%, 90% and 100%) for 2 minutes each, and allowed to dry horizontally.

SDS-neutral Comet assay

To test whether some double stranded DNA breaks remain associated with the nuclear matrix preventing the DNA from migrating in the Comet tail, slides that were prepared for the neutral Comet assay were incubated for an additional 30 minutes with a solution containing 0.4 M Tris-HCl, 1% SDS and 0.8 mM DTT, pH 7.5, after the two conventional lysis solutions incubation.

Comet Assay analysis

Comet slides were stained with DAPI SlowFade® Gold antifade (Invitrogen, Eugene, OR, USA) and images of about 100 sperm for each sample were captured at 10x magnification under an epifluorescence microscope (Olympus IX81, Olympus Optical Co., Hamburg, Germany). Total Comet Tail length and Head Halo mean diameter were analyzed using the CometScore 1.0 software (TriTek, Sumerduck, VA, USA). Moreover, the Length-Halo ratios (LH ratio) defined as the ratio between Comet Tail length and Head Halo diameter (Comet Length/Head halo diameter) were calculated.

RESULTS

Analysis of SCF with the Neutral Comet Assay

We first analyzed mouse SCF with the neutral Comet assay to test whether previous experiments demonstrating that SCF-induced double stranded breaks could be repeated with this test. Examples of four different treatments performed with epididymal and vas deferens sperm are shown in Figure 1, and larger fields showing several Comets are shown in Suppl. Figs. 2 and 3., The Comet length histograms of the population for each treatment are shown in Figure 2. Our standard neutral Comet assay in sperm uses 12.5 min of electrophoresis time while our standard alkaline Comet assay uses only 4 min. Because we were comparing the two techniques, and attempting to understand the relationship between sperm nuclear structure and the Comet assay, we used both electrophoresis times for our studies in each assay. For the neutral Comet assay, an increase in the Comet tails was seen when increasing the electrophoresis time from 4 min to 12.5 min. However, the Comet tail length did not appear at 12.5 min in samples that had no Comet tails at 4 min indicating that the increased time of electrophoresis did not introduce artifactual Comet tails (Figs. 1 and 2). Untreated samples showed no Comet tails and large head halos (Fig. 1 and Fig. 2a,f,k,p, Suppl. Figs. 2 and 3). Nuclease treatment, our positive control for double stranded DNA breaks, caused an intense damage in all the sperm cells, showing a small or inexistent head halo, and an increase in Comet tail length ranging between 120 and 200 μm for 4 minutes of electrophoresis (Fig. 2d,n) and between 180 and 280 μm, for 12.5 minutes of electrophoresis (Fig. 2i,s) for both epididymal and vas deferens sperm. Incubations with hydrogen peroxide, which induces only single stranded DNA breaks, caused no Comet tail in the neutral Comet assay, as expected, with head halo sizes similar to untreated samples (Fig. 2e,j,o,t).

We next examined SCF. Previous reports using pulsed-field gel electrophoresis demonstrated a degradation of epididymal sperm DNA to 25 kb, which could be religated by EDTA treatment after the dsDNA breaks were induced by divalent cations. Vas deferens sperm DNA was digested further to smaller fragments, which could not be religated with subsequent EDTA incubation (Yamauchi *et al.*, 2007a). Neutral Comet assays for epididymal sperm SCF resulted in weak but measureable Comet tails and large head halos. These Comet tails disappeared when the samples were treated with EDTA after the divalent cation treatment (Fig. 1A and Fig 2b,c,g,h, Suppl. Fig. 2). Vas deferens SCF sperm showed longer Comet tails than epididymal sperm but significantly smaller head halos (Fig. 1B, Suppl. Fig. 2). Incubations of these spermatozoa with EDTA showed a bimodal distribution in 4 min electrophoresis, and a broad distribution of Comet tail lengths similar to that observed in vas deferens SCF with 12.5 min electrophoresis. However, the head halo was not recovered after EDTA treatment (Fig. 1 and Fig. 2l,m,q,r).

Analysis of SCF with the Alkaline Comet assay

We next analyzed the SCF-induced sperm with the alkaline Comet assay to test whether single stranded DNA breaks were induced as well as double stranded breaks. Examples of alkaline sperm Comet assays for each treatment

performed with epididymal and vas deferens sperm are shown in Figure 3, and the Comet length histograms of the population for each treatment are shown in Figure 4. As with the neutral Comet assay, Comet tails were longer in all untreated and treated samples when a 12.5 min electrophoresis was performed rather than 4 min. (Figs. 3 and 4). Untreated samples showed a short comet tail and a large head halo (Figs. 3 and 4a,f,k,p). Nuclease treatment showed Comet tail lengths ranging between 120 and 180 μm for 4 minutes of electrophoresis (Fig. 4d,n) and between 230 and 350 μm, for 12.5 minutes of electrophoresis (Fig. 4i,s) for epididymal and vas deferens sperm. Nuclease treatment resulted in the removal of most of the DNA from the head halo in both epididymal and vas deferens sperm, with slightly more DNA remaining associated with the halo in nuclease treated vas deferens sperm (Fig. 3B). Hydrogen peroxide treatment caused a complete removal of head halo and a longer Comet tail compared with untreated samples (Fig. 3). However, the same hydrogen peroxide treatment caused longer Comet tails in epididymal sperm than vas deferens sperm (Fig. 4e,j,o,t). SCF treatment showed a reduction of the head halo diameter and an increase of Comet tail lengths compared to untreated samples, and this effect was slightly greater for epididymal sperm. Finally, treatments with EDTA showed no DNA religation, as compared to SCF (Fig. 3 and Fig. 4 b,c,l,m,g,h,q,r). These data suggested that SCF induced a significant level of single stranded DNA breaks in addition to the documented double stranded breaks.

Comet Length-Halo ratio (LH ratio)

The experiments above clearly demonstrated that Comet tail length, alone, was insufficient to quantitate the differences in DNA damage between the epididymal and vas deferens sperm. Epididymal SCF-induced sperm had large halos and weak tails in the neutral Comet assay (Fig. 1A) while vas deferens SCF-induced sperm had much more intensely stained tails and very small halos (Fig. 1B) However, the actual Comet tail lengths were not very different. Therefore, we calculated the ratios (LH) between the comet lengths and the head halo mean diameters,(Table IA). As expected, there was only a small difference between LH values of the epididymal and vas deferens sperm in the alkaline Comet assay. However, there was a much larger difference in LH between the two sperm types in the neutral Comet assay which became even greater when the samples were electrophoresed for 12.5 min as compared with 4 min.

SDS-Neutral Comet

The data above suggested the possibility that most of the double stranded DNA breaks remained bound to the nuclear matrix in epididymal SCF-induced sperm but not in vas deferens SCF-induced sperm. This, in turn, implied that the sperm nuclear matrix remained relatively intact in the conventional neutral Comet assay. This is probable since during sperm preparation for the neutral Comet assay, the SDS treatment occurs before the protamines are extracted with high salt, which is one protocol for preparing sperm nuclear matrices (Ward, 2013). To test this, we introduced an additional incubation with SDS and DTT after the treatments that take place during the sperm preparation for the neutral Comet assay. Examples of sperm Comet

images after the treatments are shown in Figure 5, and Suppl. Fig. 4, the accompanying histogram analysis is shown in Figure 6, and the results of LH ratio in the population are shown in Table IIB.

Compared to the traditional neutral Comet assay, the additional SDS treatment caused an increase of the LH ratio in epididymal sperm by reducing the head halo diameter and increasing the Comet tails, but no changes were found on vas deferens sperm. Moreover, after this treatment, no differences were found in the LH ratio between epidydimal SCF and vas deferens SCF (Table IIB). Finally, EDTA incubation after SCF caused a complete reduction of epididymal sperm LH ratio to that of untreated samples, while vas deferens sperm LH ratios did not change with EDTA treatment.

DISCUSSION

Sperm DNA damage is a leading cause of several different problems in fertility and embryo development (Carrell *et al.*, 2003; Evenson and Jost, 2000; Lewis and Simon, 2010). As discussed below, our data support three major conclusions about dsDNA breaks in mature spermatozoa, their relationship to chromatin structure, and how to analyze them. We have also defined a mouse model that has similarities to two human infertility disorders.

The Neutral Comet Assay Does Not Disrupt DNA Attachment Sites to the Sperm Nuclear matrix

The first major finding is that the conventional neutral Comet assay preserves at least one component of the sperm chromatin structure, the nuclear matrix. This was reported previously in somatic cells (Afanasieva *et al*, 2010; Anderson and Laubenthal, 2013), but it was not clear that the same would be true for the sperm Comet assay. This was seen most clearly in the epididymal sperm SCF treatments. They showed an increase in the neutral Comet tail lengths compared to untreated samples but were shorter than nuclease-treated sperm (Fig. 1 and 2b,g). Moreover, most of the DNA was contained in the large dispersion halos in the nuclear core, which were similar to those in untreated samples. These results did not agree with previously published results using PFGE that demonstrated a degradation of all the DNA to loop-size fragments (Yamauchi *et al.*, 2007a). When the neutral Comet assay was modified to include additional SDS and DTT treatments after salt extraction, the dispersion halos became much smaller, and the tail lengths and the LH ratio values increased to values that were similar to conventional neutral Comet assay results with nuclease treated sperm (compare Figs. 3B and 5A).

These data support a model, shown in Fig. 7, in which the dsDNA breaks in SCF-induced epididymal sperm remain attached to the sperm nuclear matrix. The fact that the sperm nuclear matrix can survive the extractions of the conventional neutral Comet assay is not surprising because they mirror the isolation procedures for sperm nuclear matrices (Choudhary *et al*, 1995; Ward *et al*, 1989). The sperm chromatin condensed by disulfide-linked protamines protects the sperm nuclear matrix until after protamine extraction by high salt and DTT. However, when salt extracted sperm nuclei are then treated with SDS, the nuclear matrix is no longer stable (Fig. 7C).

Two Different Types of dsDNA Breaks Exist in Sperm

The second major finding is that we have defined two different types of sperm dsDNA breaks. SCF-induced epididymal sperm had dsDNA breaks but the DNA remained associated with the nuclear matrix, and most of these breaks were therefore not revealed in the conventional neutral Comet assay. These dsDNA breaks were only identified in SDS-neutral Comet assays (Table 1B). In contrast, SCF-induced vas deferens sperm examined in the conventional neutral Comet assay had long Comet tails and small dispersion halos, similar to nuclease treated sperm, suggesting that the dsDNA breaks in this case were

not associated with the nuclear matrix (Fig. 7D). There was some evidence for dsDNA break religation with EDTA treatment in vas deferens SCF-induced sperm in that the neutral Comet assay showed a bimodal distribution when short electrophoresis is performed (compare Figs. 2l and m). However, with the longer electrophoresis time, there was no difference in the neutral Comet assay with or without EDTA (compare Figs. 2q and r). We propose that the two types of sperm dsDNA breaks are related in that the matrix associated breaks progress to matrix unassociated breaks as SCF progresses from the early, TOP2 induced reversible dsDNA breaks to the irreversible DNA degradation that is associated with other nucleases (Suppl. Fig. 1). In the vas deferens, this progression is more advanced, while in the epididymal sperm the degradation is largely arrested at the first step.

Because the conventional and SDS-neutral Comet assays result in the release of loop-sized fragments from SCF-induced epididymal sperm, it is possible to assign a rough fragment length of the Comet tails. We propose that that loop-size fragments, which measure between 25 and 50 kb, are located between 120 and 200 μm in the neutral Comet assay with an electrophoresis lasting 4 minutes, and between 180 and 280 μm in neutral Comet with an electrophoresis of 12.5 minutes.

The Nuclear Matrix Plays a Critical Role in DNA Repair

This led to our third major finding, that only the matrix associated breaks could be religated by EDTA treatment (Fig. 1). Previous work had shown that epididymal SCF-induced sperm dsDNA breaks could be religated with EDTA treatment, but those from the vas deferens could not (Boaz *et al*, 2008; Yamauchi *et al.*, 2007a), but the data reported here are the first to demonstrate that the dsDNA breaks remained associated with the nuclear matrix in epididymal sperm. In somatic cells, TOP2 induced breaks on the nuclear matrix are the first step of DNA degradation during apoptosis and it has been speculated that because it is reversible, it serves as a type of checkpoint for DNA degradation (Li *et al.*, 1999). The fact that dsDNA breaks can only be religated when they are still attached to the sperm nuclear matrix raises the possibility that the matrix may play a role in DNA repair even in the zygote by maintaining the two ends of the dsDNA break in close proximity to each other. This is similar to a process recently reported in somatic cells in which persistent double stranded breaks that are not rapidly repaired are recognized by different proteins, such as Mps3, that hold them close to the nuclear envelope, allowing the DNA repair processes to take place with a greater efficiency (Gartenberg, 2009; Oza and Peterson, 2010). This may have implications for the repair of some types of damaged sperm DNA after fertilization in the zygote, just before zygotic S-phase (Derijck *et al*, 2008; Menezo *et al*, 2010). It has been proposed that the paternal pronucleus inherits the DNA organization on the nuclear matrix from the sperm (Sotolongo and Ward, 2000). If so, the paternal pronucleus may also inherit this template for religation of matrix associated dsDNA breaks in the sperm that may serve as a template for the DNA repair mechanisms in the oocyte.

SCF Also Produces Single Stranded DNA Breaks

Several reports have shown that SCF in both epididymal and vas deferens spermatozoa result in dsDNA breaks (Shaman *et al.*, 2006; Shaman and Ward, 2006; Yamauchi *et al.*, 2007a; Yamauchi *et al.*, 2007b), but the presence of ssDNA breaks were never tested. The alkaline Comet assay measures both single and double stranded DNA breaks, and positive tests have been associated with human male infertility (Ribas-Maynou *et al.*, 2013; Simon *et al.*, 2013). In this case, it is clear that the nuclear matrix is not preserved, because the alkaline treatment also denatures proteins (Afanasieva *et al.*, 2010). The main cause of ssDNA breaks identified by the alkaline Comet assay is likely to be oxidative stress (Aitken and De Iuliis, 2010; Enciso *et al.*, 2009; Ribas-Maynou *et al.*, 2012a). We found that SCF-induced epididymal and vas deferens spermatozoa had long Comet tails and small dispersion halos. These DNA breaks could not be religated in the epididymal sperm after EDTA treatment, as they could in the neutral Comet assay (Figs. 3A and 4b,c,g,h). This suggests that SCF also produces single stranded DNA breaks through an oxidative stress mechanism, which would result in an extensive DNA damage all along the genome that is not reversible by *in vitro* EDTA incubation. This additional damage would also explain why proper development to the blastocyst stage was not possible even after EDTA treatment of epidydimal SCF-induced sperm (Yamauchi *et al.*, 2007a). Single-stranded DNA breaks detected by the alkaline Comet assay prevent pregnancy in humans (Ribas-Maynou *et al.*, 2013; Simon *et al*, 2011), and a high level of these ssDNA breaks produced by SCF would be difficult to repair *in vivo* by the known mechanisms available in the embryo. This may lead to several DNA aberrations (Gawecka *et al.*, 2013) that would arrest the embryo development at early stages.

Epididymal Sperm is More Sensitive to Nuclease Treatment than Vas Deferens Sperm

We used nuclease treatment as a control for dsDNA breaks that were detected by both the alkaline and neutral Comet assays. In both assays control vas deferens sperm treated with nuclease had much less detectable damage than nuclease treated control sperm from the epididymis. The data suggest that the chromatin compaction near the MAR regions, which are most susceptible to exogenous nuclease degradation, is greater in vas deferens sperm. This is in contrast to SCF induction, which clearly results in more dsDNA damage in vas deferens sperm. This suggests that the endogenous mechanisms for SCF may be internal to the sperm chromatin structure and are different from those that render the chromatin sensitive to external nucleases.

Relationship of the SCF Mouse Model for Sperm DNA Damage to Clinical Conditions

Our results suggest that SCF in epididymal and vas deferens sperm can be related to two specific clinical conditions. The Comet assay results for epididymal SCF are similar to previous results obtained with males from couples undergoing treatment for recurrent pregnancy loss without female factor (Ribas-Maynou *et al.*, 2012b). An increase in the percentage of sperm showing long tails in the neutral Comet assay but low alkaline Comet assay

positive sperm was found in one third of fertile donors analyzed and in most men from couples with recurrent pregnancy loss without female factor. If the mechanisms of epididymal sperm SCF and those underlying recurrent pregnancy loss are similar, it would have important implications for the oocyte regarding dsDNA repair. Thus, the use of epididymal sperm SCF in mice could be a very suitable model for the study of male-factor dependent recurrent miscarriage.

On the other hand, two different processes seem to take place in vas deferens sperm, the reversible dsDNA breaks followed by irreversible DNA digestion that releases the DNA from the matrix. This combination resulted in a great reduction of the dispersion halo and an increase in Comet tail length after SCF, in both alkaline and neutral Comet assays. Sperm from varicocele patients behaved the same way (Ribas-Maynou *et al.*, 2012a), suggesting a similar mechanism of DNA damage. This was corroborated with the SCD test which demonstrated a sperm subpopulation from varicocele patients with a strongly damaged nuclear core (Gosalvez *et al*, 2013). Therefore, it also seems suitable to use vas deferens SCF-treated as a model for the DNA damage in varicocele patients.

Modifications to the Comet Assay

We have made two modifications to the Comet assay in this work that may have useful implications for future clinical analyses. First, we found that differences in the nuclear core halo could also be indicative of DNA damage in neutral Comet assay and defined a new parameter, the LH ratio. The LH ratio showed much clearer differences between epididymal and vas deferens SCF than Comet tail length or head halos diameter, alone (Table 1A). Moreover, the LH ratio represented the actual Comet images more accurately. Second, we added a simple treatment, incubation in SDS and DTT after the final extraction to distinguish between dsDNA breaks that are attached to the matrix and those that are not (Fig. 7).

Conclusions

The main conclusion of this study is that the conventional neutral Comet assay underestimates the level of repairable DNA breaks in the MAR regions that remain attached to the sperm nuclear matrix. We also provide evidence that the sperm nuclear matrix may play an essential role in DNA repair after fertilization by holding together the dsDNA breaks. Finally, we demonstrated that murine epididymal and vas deferens SCF are useful models for investigations of DNA damage in human recurrent pregnancy loss and varicocele patients, respectively.

FUNDING

This work has been supported by NIH Grant 1R01HD060722 (WSW), Instituto de Salud Carlos III (Madrid), project PI11/0630 (JB) and Generalitat de Catalunya (2009 SGR 1107) (JB) and Generalitat de Catalunya (JR).

AUTHOR'S ROLES

Jordi Ribas-Maynou contributed in experimental design, experimental procedures, image analysis, data collection, graphics and table elaboration and manuscript writing.
Joanna Gawecka contributed in experimental design, experimental procedures and manuscript writing.
Jordi Benet and Steve Ward contributed in experimental design, manuscript writing and revising, and direction and coordination of the work.

FIGURES AND TABLE

Figure 1. Examples of the most common neutral Comet assay results for two different electrophoresis times after different treatments on A) epididymal sperm, and B) vas deferens sperm. See text for explanations of the different treatments.

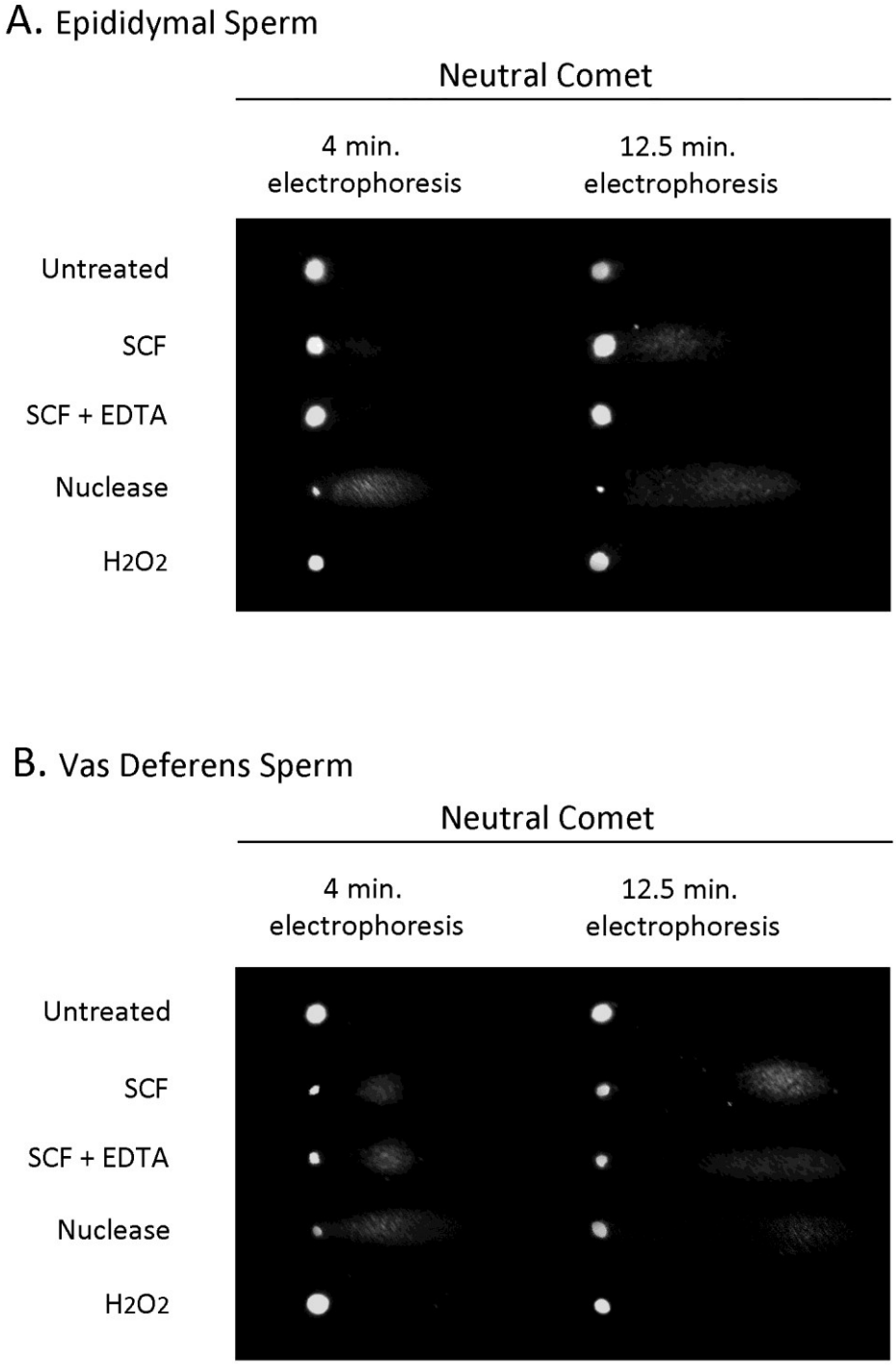

Figure 2. Histograms showing the distribution of total Comet lengths for the neutral Comet assay with different electrophoresis times after different treatments on A) epididymal sperm, and B) vas deferens sperm. See text for explanations of the different treatments.

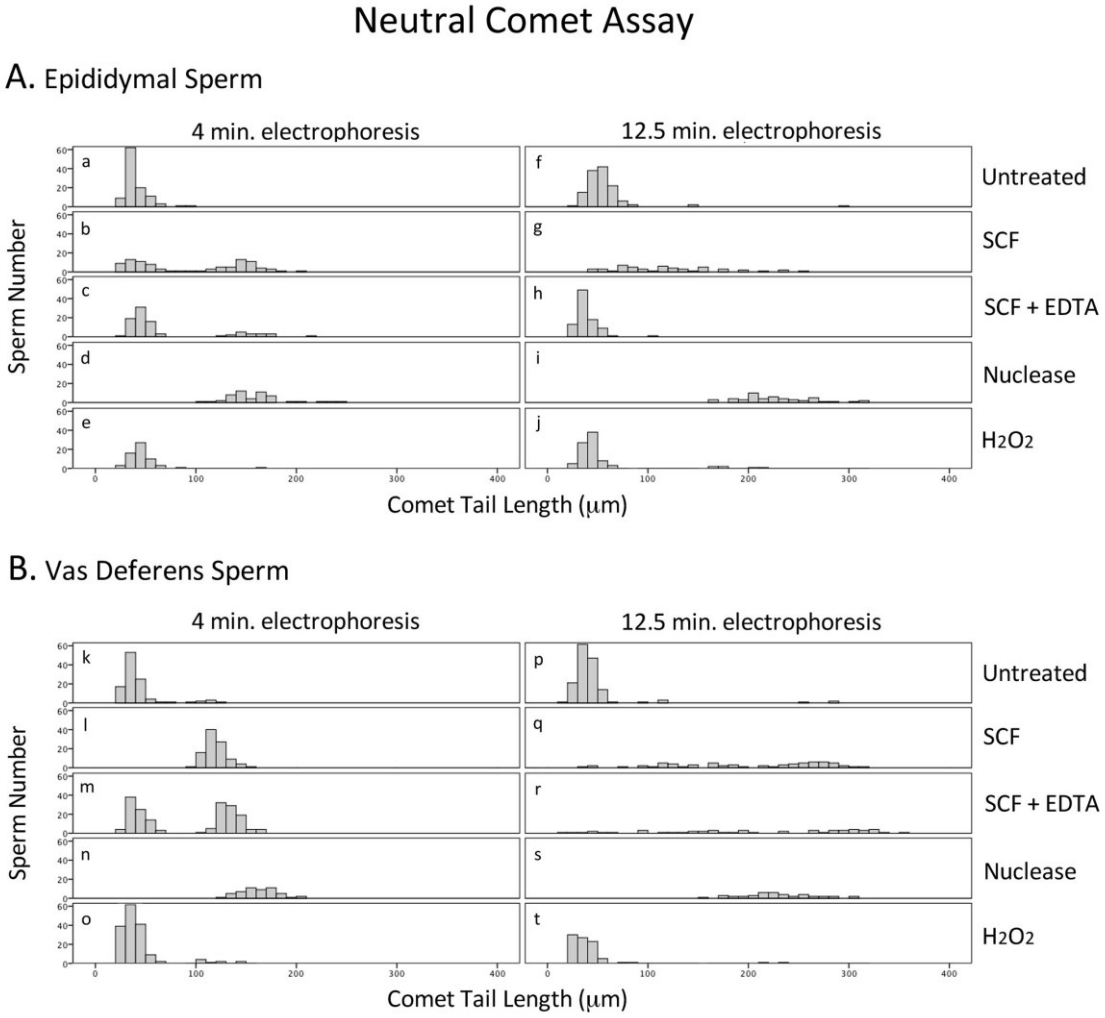

Figure 3. Examples of the most common alkaline Comet assay results for two different different electrophoresis times after different treatments on A) epididymal sperm, and B) vas deferens sperm. See text for explanations of the different treatments.

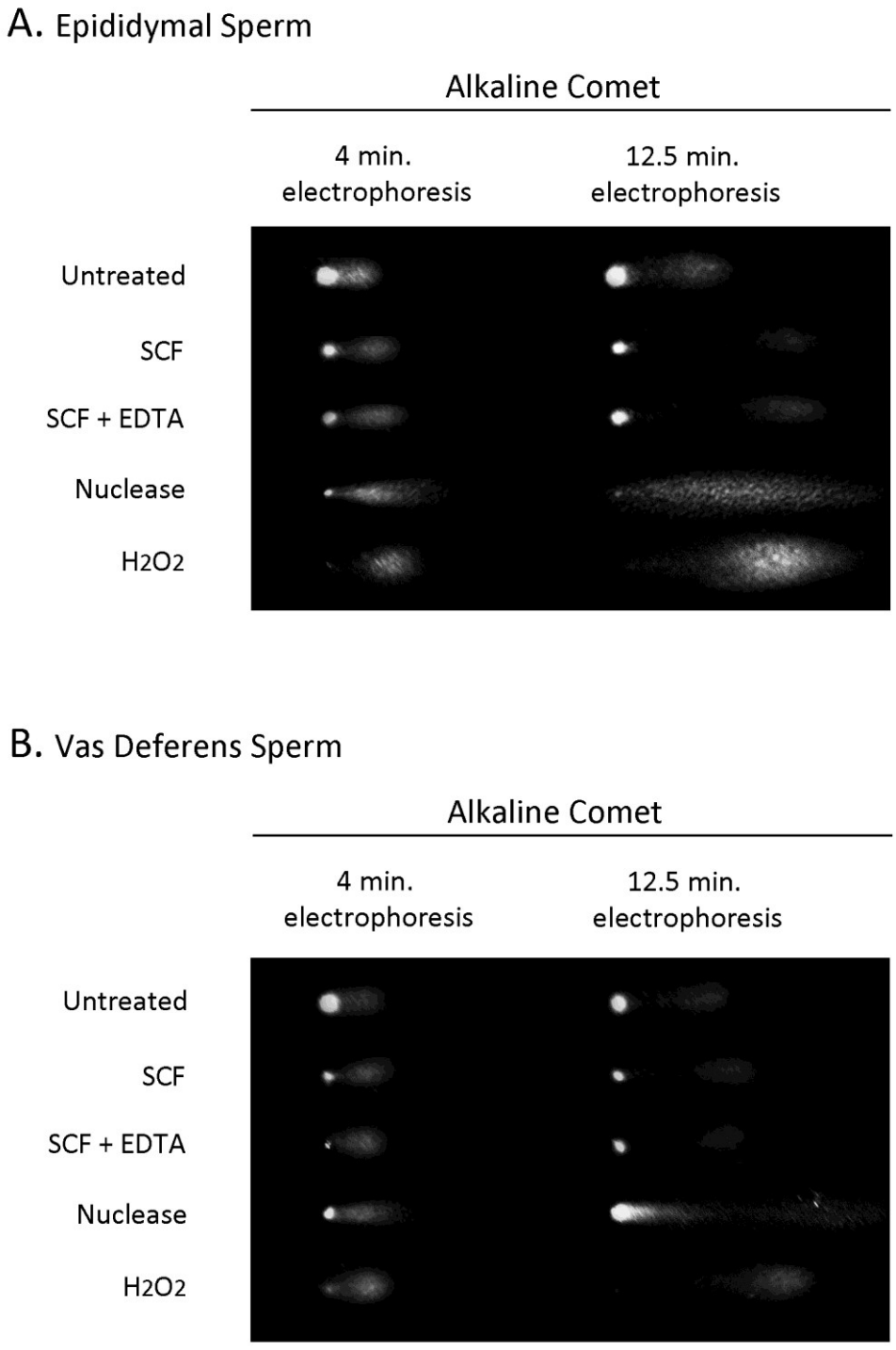

Figure 4. Histograms showing the distribution of total Comet lengths for the alkaline Comet assay with different electrophoresis times after different treatments on A) epididymal sperm, and B) vas deferens sperm. See text for explanations of the different treatments.

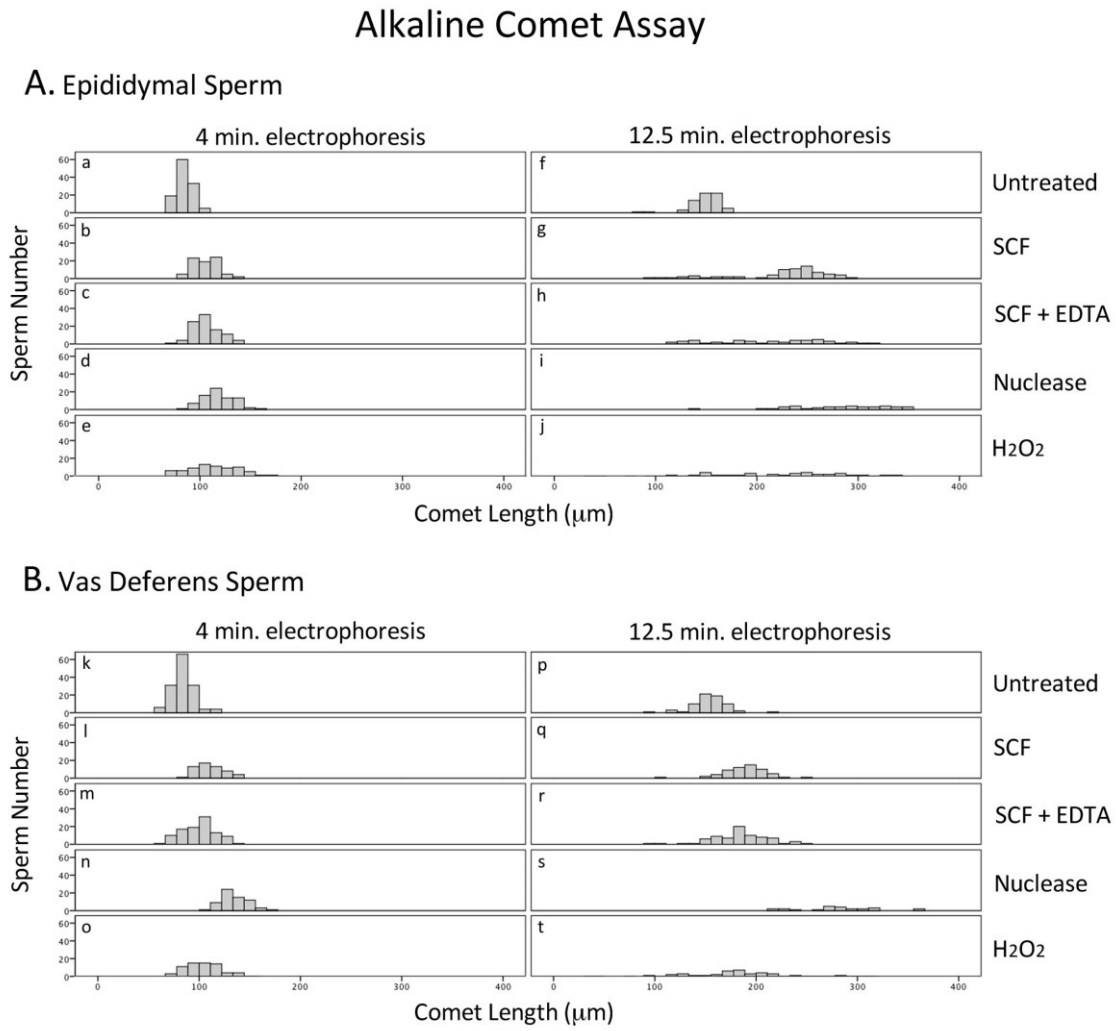

Figure 5. Examples of the most common SDS-neutral Comet assay in different electrophoresis times after different treatments on A) epididymal sperm, and B) vas deferens sperm. See text for explanations of the different treatments.

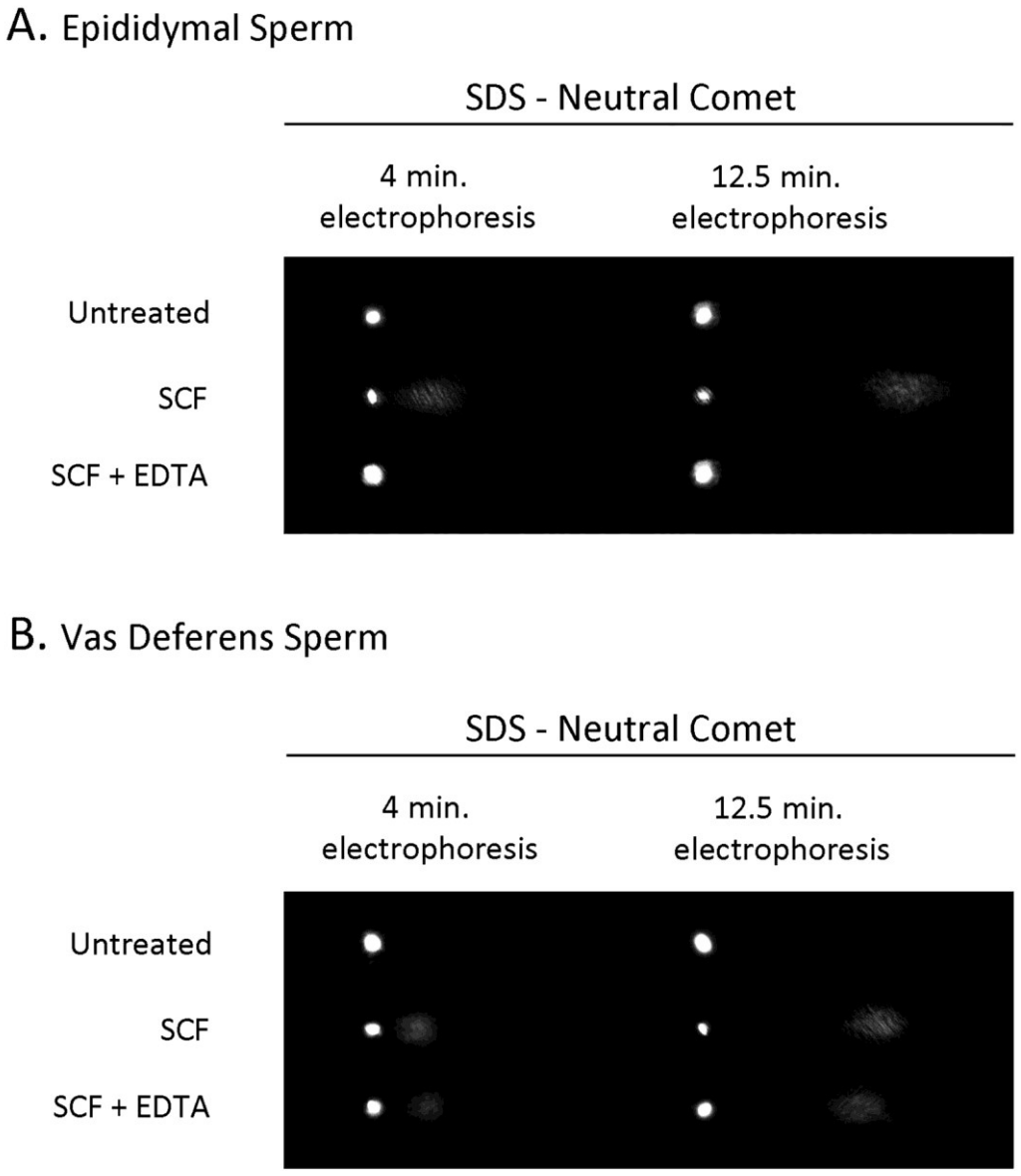

Figure 6. Histograms showing the distribution of total Comet lengths for the SDS-neutral Comet assay after different treatments on A) epididymal sperm, and B) vas deferens sperm. See text for explanations of the different treatments.

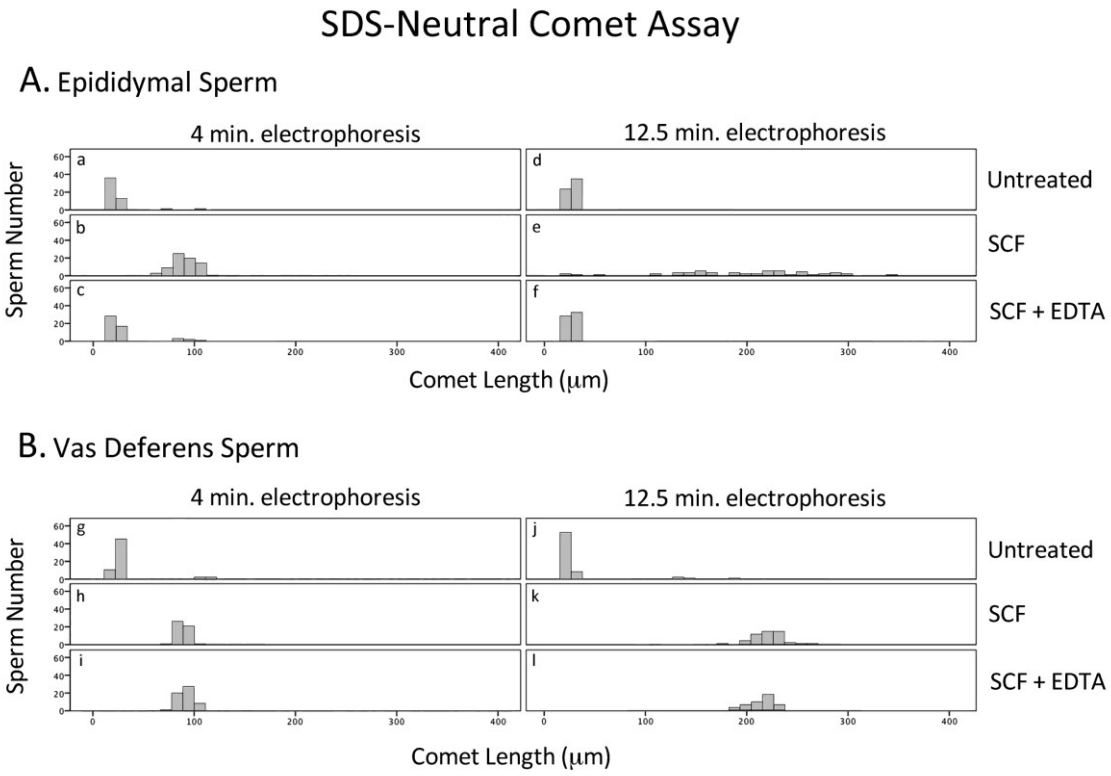

Figure 7. Model for the relationship between the nuclear matrix and the neutral Comet assay. The left column depicts our model for the effect of various treatments on the toroid linker regions, or MARs, in situ. The right column depicts the nuclear matrix (green) and exposed DNA loops after the Comet lysis treatments, and what happens to the DNA during electrophoresis. A) In untreated samples, both SDS treated and conventional neutral Comet assays result in a dispersion halo around the core, and no Comet tail. The DNA is held in the dispersion halo by virtue of its large size. B) In nuclease-treated samples, the matrix attachment regions are digested, while toroid bound DNA remains intact because the protamines protect the DNA from digestion. The DNA loops (each protamine toroid represents one DNA loop domain) are released from the nuclear matrix, and both the conventional and SDS-neutral Comet assays result in small or nonexistent dispersion halos, and long Comet tails, corresponding to loop size fragments. C) When epididymal sperm are induced to undergo SCF, a break is produced at or near the matrix attachment site, and the free ends remain attached to the nuclear matrix. Therefore, the conventional neutral Comet assay results in a dispersion halo around the core and a small Comet tail because the nuclear matrix remains intact. However, the SDS-neutral Comet assay results in a small or nonexistent dispersion halo and large tail corresponding to loop size fragments, because the nuclear matrix is disrupted by the second SDS treatment releasing the DNA loop domains. These breaks are repairable with EDTA (Fig. 5, and Table 1). D) In vas deferens SCF-induced sperm, two processes occur. The first is the repairable, double stranded DNA break in which the broken strands remain attached to the nuclear matrix, just as in epididymal SCF-induced sperm. Most of the vas deferens SCF-induced sperm undergo a second, irreversible digestion that releases the DNA loops from the matrix, similar to nuclease digestion. Most of the DNA is therefore released into the Comet tail in both the conventional and SDS-neutral Comet assays, but all the DNA is released in the SDS-neutral Comet assay.

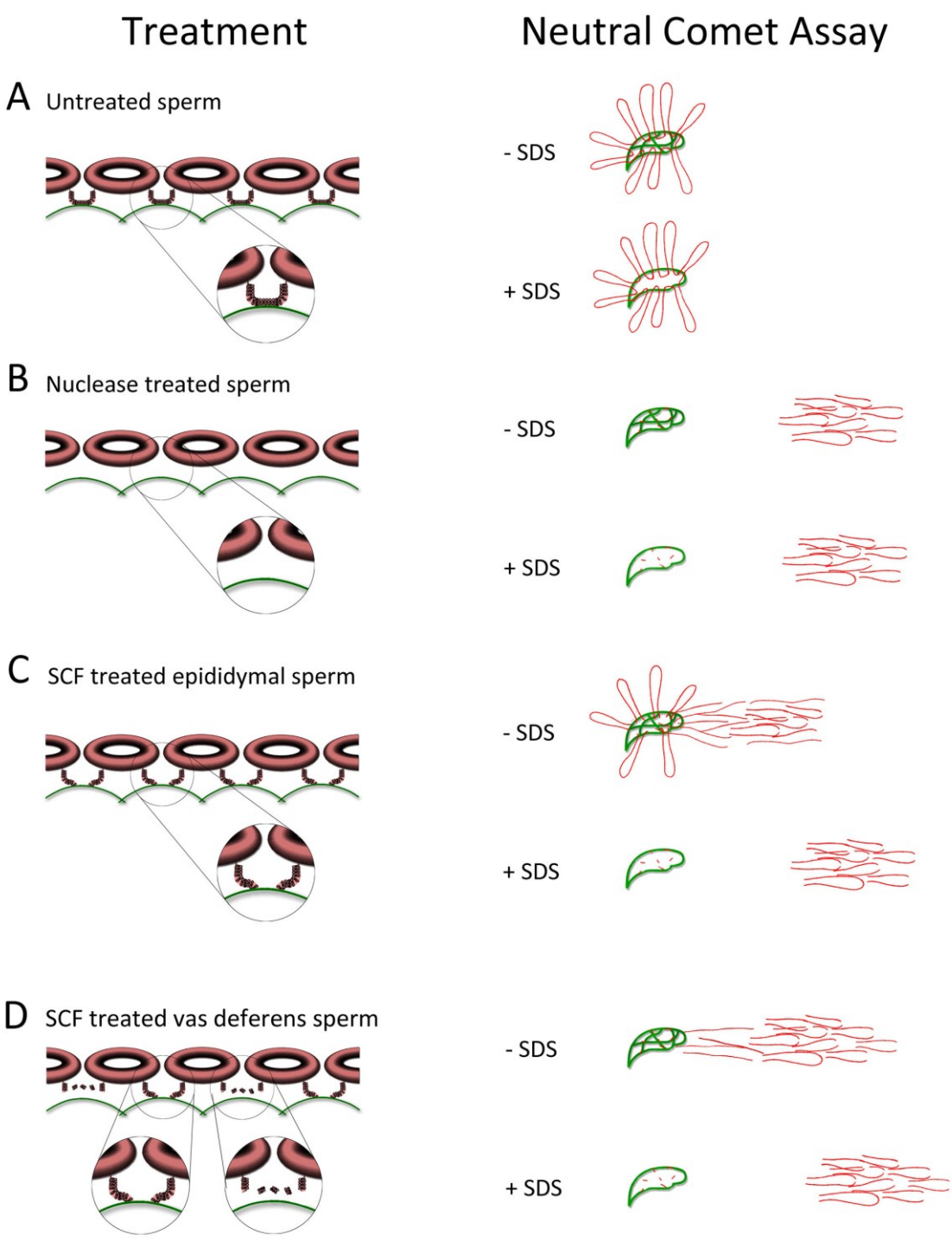
Treatment
Neutral Comet Assay
A Untreated sperm
- SDS
+ SDS
B Nuclease treated sperm
- SDS
+ SDS
C SCF treated epididymal sperm
- SDS
+ SDS
D SCF treated vas deferens sperm
- SDS
+ SDS

Table 1. Data (mean ± standard deviation) for Length-Halo ratios (LH ratio) defined as Comet Length/Head Halo diameter in conventional Comet assays A) without the SDS and DTT treatment after the two Comet lysis solutions, and B) with SDS-Neutal Comet Assays in which and additional SDS and DTT treatment was performed after the two Comet lysis solutions.

A. Conventional Comet Assays

		Neutral Comet Assay		Alkaline Comet Assay	
		4 min	12.5 min	4 min	12.5 min
Epididymal sperm	Untreated	1.1 ± 0.1	1.2 ± 0.2	2.8 ± 0.4	4.7 ± 0.7
	SCF	3.3 ± 1.3	3.7 ± 1.4	4.8 ± 0.8	11.3 ± 1.9
	SCF + EDTA	1.9 ± 2.1	1.1 ± 0.1	4.2 ± 0.5	9.9 ± 2.3
Vas deferens sperm	Untreated	1.2 ± 0.8	1.1 ± 0.1	2.5 ± 0.3	6.0 ± 1.0
	SCF	6.6 ± 1.2	14.6 ± 2.3	5.2 ± 1.1	11.4 ± 2.3
	SCF + EDTA	7.2 ± 1.2	15.2 ± 3.3	6.0 ± 1.1	8.1 ± 1.4

B. SDS-Neutral Comet Assay

		SDS - Neutral Comet SDS	
		4 min	12.5 min
Epididymal sperm	Untreated	1.2 ± 1.0	1.1 ± 0.1
	SCF	6.4 ± 1.5	12.6 ± 4.1
	SCF + EDTA	1.5 ± 1.1	1.3 ± 1.7
Vas deferens sperm	Untreated	1.4 ± 1.1	1.6 ± 2.1
	SCF	5.2 ± 0.9	12.0 ± 1.9
	SCF + EDTA	6.1 ± 1.2	12.2 ± 2.4

REFERENCES

Afanasieva K, Zazhytska M, Sivolob A (2010) Kinetics of comet formation in single-cell gel electrophoresis: loops and fragments. *Electrophoresis* **31**, 512-9.

Agarwal A, Makker K, Sharma R (2008) Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* **59**, 2-11.

Aitken RJ, De Iuliis GN (2010) On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* **16**, 3-13.

Anderson D, Laubenthal J (2013) Analysis of DNA Damage via Single-Cell Electrophoresis. *Methods Mol Biol* **1054**, 209-18.

Aoki VW, Carrell DT (2003) Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility. *Asian J Androl* **5**, 315-24.

Bennetts LE, Aitken RJ (2005) A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol Reprod Dev* **71**, 77-87.

Bjorndahl L, Kvist U (2010) Human sperm chromatin stabilization: a proposed model including zinc bridges. *Mol Hum Reprod* **16**, 23-9.

Boaz SM, Dominguez KM, Shaman JA, Ward WS (2008) Mouse Spermatozoa Contain a Nuclease that Is Activated by Pretreatment with EGTA and Subsequent Calcium Incubation. *J Cell Biochem* **103**, 1636-1645.

Boulikas T (1995) How enhancers work - juxtapositioning of DNA control elements by synergistic interaction of Mars - (review-hypothesis). *Int J Oncol* **6**, 1313-8.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B (2003) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* **49**, 49-55.

Champoux JJ (2001) DNA topoisomerases: structure, function, and mechanism. *Annu Rev Biochem* **70**, 369-413.

Chapman JC, Michael SD (2003) Proposed mechanism for sperm chromatin condensation/decondensation in the male rat. *Reprod Biol Endocrinol* **1**, 20.

Chauvin T, Xie F, Liu T, Nicora CD, Yang F, Camp DG, 2nd, Smith RD, Roberts KP (2012) A systematic analysis of a deep mouse epididymal sperm proteome. *Biol Reprod* **87**, 141.

Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT (2006) Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* **27**, 53-9.

Choudhary SK, Wykes SM, Kramer JA, Mohamed AN, Koppitch F, Nelson JE, Krawetz SA (1995) A haploid expressed gene cluster exists as a single chromatin domain in human sperm. *J Biol Chem* **270**, 8755-62.

Codrington AM, Hales BF, Robaire B (2007) Chronic cyclophosphamide exposure alters the profile of rat sperm nuclear matrix proteins. *Biol Reprod* **77**, 303-11.

Derijck A, van der Heijden G, Giele M, Philippens M, de Boer P (2008) DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. *Hum Mol Genet* **17**, 1922-37.

Enciso M, Sarasa J, Agarwal A, Fernandez JL, Gosalvez J (2009) A two-tailed Comet assay for assessing DNA damage in spermatozoa. *Reprod Biomed Online* **18**, 609-16.

Evenson D, Jost L (2000) Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci* **22**, 169-89.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* **14**, 1039-49.

Fernandez JL, Muriel L, Goyanes V, Segrelles E, Gosalvez J, Enciso M, LaFromboise M, De Jonge C (2005) Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril* **84**, 833-42.

Gartenberg MR (2009) Life on the edge: telomeres and persistent DNA breaks converge at the nuclear periphery. *Genes Dev* **23**, 1027-31.

Gawecka JE, Marh J, Ortega M, Yamauchi Y, Ward MA, Ward WS (2013) Mouse zygotes respond to severe sperm DNA damage by delaying paternal DNA replication and embryonic development. *PLoS One* **8**, e56385.

Gosalvez J, Rodriguez-Predreira M, Mosquera A, Lopez-Fernandez C, Esteves SC, Agarwal A, Fernandez JL (2013) Characterisation of a subpopulation of sperm with massive nuclear damage, as recognised with the sperm chromatin dispersion test. *Andrologia,* May 26. doi: 10.1111/and.12118.

Haidl G, Badura B, Schill WB (1994) Function of human epididymal spermatozoa. *J Androl* **15 Suppl**, 23S-27S.

Hud NV, Downing KH, Balhorn R (1995) A constant radius of curvature model for the organization of DNA in toroidal condensates. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3581-5.

Lewis SE, Simon L (2010) Clinical implications of sperm DNA damage. *Hum Fertil (Camb)* **13**, 201-7.

Li TK, Chen AY, Yu C, Mao Y, Wang H, Liu LF (1999) Activation of topoisomerase II-mediated excision of chromosomal DNA loops during oxidative stress. *Genes Dev* **13**, 1553-60.

Li TK, Liu LF (2001) Tumor cell death induced by topoisomerase-targeting drugs. *Annu Rev Pharmacol Toxicol* **41**, 53-77.

Menezo Y, Dale B, Cohen M (2010) DNA damage and repair in human oocytes and embryos: a review. *Zygote* **18**, 357-65.

Nelson WG, Liu LF, Coffey DS (1986) Newly replicated DNA is associated with DNA topoisomerase II in cultured rat prostatic adenocarcinoma cells. *Nature* **322**, 187-9.

Oza P, Peterson CL (2010) Opening the DNA repair toolbox: localization of DNA double strand breaks to the nuclear periphery. *Cell cycle (Georgetown, Tex* **9**, 43-9.

Ribas-Maynou J, Garcia-Peiro A, Abad C, Amengual MJ, Navarro J, Benet J (2012a) Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Hum Reprod* **27**, 652-8.

Ribas-Maynou J, Garcia-Peiro A, Fernandez-Encinas A, Abad C, Amengual MJ, Prada E, Navarro J, Benet J (2013) Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology*.

Ribas-Maynou J, Garcia-Peiro A, Fernandez-Encinas A, Amengual MJ, Prada E, Cortes P, Navarro J, Benet J (2012b) Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. *PLoS One* **7**, e44679.

Sakkas D, Alvarez JG (2010) Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* **93**, 1027-36.

Shaman JA, Prisztoka R, Ward WS (2006) Topoisomerase IIB and an Extracellular Nuclease Interact to Digest Sperm DNA in an Apoptotic-Like Manner. *Biol Reprod* ***75***, 741-748.

Shaman JA, Ward WS. Sperm chromatin stability and susceptibility to damage in relation to its structure Cambridge: Cambridge University Press; 2006.

Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A, Agarwal A (2010) TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology* **76**, 1380-6.

Simon L, Lutton D, McManus J, Lewis SE (2011) Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil Steril* **95**, 652-7.

Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D, Lewis SE (2013) Sperm DNA damage has a negative association with live-birth rates after IVF. *Reprod Biomed Online* **26**, 68-78.

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175**, 184-91.

Sotolongo B, Lino E, Ward WS (2003) Ability of Hamster Spermatozoa to Digest Their Own DNA. *Biol Reprod* **69**, 2029-2035.

Sotolongo B, Ward WS (2000) DNA Loop Domain Organization: The Three Dimensional Genomic Code. *Journal of Cellular Biochemistry* **35**, 23-26.

Ward WS (2010) Function of sperm chromatin structural elements in fertilization and development. *Mol Hum Reprod* **16**, 30-6.

Ward WS (2013) Isolation of sperm nuclei and nuclear matrices from the mouse, and other rodents. *Methods Mol Biol* **927**, 437-44.

Ward WS, Partin AW, Coffey DS (1989) DNA loop domains in mammalian spermatozoa. *Chromosoma* **98**, 153-9.

Yamauchi Y, Shaman JA, Boaz SM, Ward WS (2007a) Paternal Pronuclear DNA Degradation Is Functionally Linked to DNA Replication in Mouse Oocytes. *Biol Reprod* **77**, 407-415.

Yamauchi Y, Shaman JA, Ward WS (2007b) Topoisomerase II Mediated Breaks in Spermatozoa Cause the Specific Degradation of Paternal DNA in Fertilized Oocytes. *Biol Reprod* **76**, 666-672.