EFECTE DE LA FRAGMENTACIÓ DE CADENA SENZILLA i DOBLE DEL DNA ESPERMÀTIC EN LA FERTILITAT MASCULINA

Memòria presentada per **Jordi Ribas Maynou** per aspirar al grau de Doctor per la Universitat Autònoma de Barcelona en el programa de Doctorat en Biologia Cel·lular.

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CERTIFIQUEN

Que Jordi Ribas Maynou ha realitzat, sota la seva direcció, a la Unitat de Biologia Cel·lular i Genètica Mèdica de la Facultat de Medicina de la UAB, el treball per optar al títol de Doctor per la Universitat Autònoma de Barcelona en el programa de Doctorat en Biologia Cel·lular, amb el títol:

EFECTE DE LA FRAGMENTACIÓ DE CADENA SENZILLA i DOBLE DEL DNA ESPERMÀTIC EN LA FERTILITAT MASCULINA

Jordi Ribas Maynou Jordi Benet Català Joaquima Navarro Ferreté

Bellaterra, Abril de 2014

A en Pau, la Paquita i en Josep,

perquè gràcies a vosaltres

he arribat fins aquí.

Moltíssimes Gràcies.

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ÍNDEX

Abreviatures **9**

Resum **11**

1. Introducció **13**

1.1 L'espermatogènesi: meiosi i espermiogènesi 15

1.2 El DNA espermàtic 18

1.2.1 Estructura de la cromatina nuclear de l'espermatozoide 18

1.2.2 Estat de compactació de la cromatina: model Ward 20

1.2.3 Arquitectura del nucli espermàtic: model Zalensky 22

1.2.4 DNA mitocondrial 24

1.3 Infertilitat 24

1.4 Marcadors d'infertilitat masculina 26

1.4.1 Seminograma 27

1.4.2 Varicocele 29

1.5 Fragmentació del DNA espermàtic 30

1.5.1 Etiologia de fragmentació 32

1.5.1.1 Estres oxidatiu 33

1.5.1.2 Apoptosi 35

1.5.1.3 Activitat nucleasa 36

1.5.1.4 Alteracions de l'empaquetament del DNA 37

1.5.1.5 Infeccions i inflamacions 39

1.5.1.6 Quimioteràpia I radiacions 40

1.5.1.7 Exposició a tòxics, radiació electromagnètica i estil de vida 40

1.5.2 Tècniques d'anàlisi de la fragmentació del DNA espermàtic 42

1.5.2.1 Terminal transferase dUTP Nick-End Labelling (TUNEL) 42

1.5.2.2 Sperm Chromatin Structure Assay (SCSA) 44

1.5.2.3 Sperm Chromatin Disperson test (SCD) 46

1.5.2.4 Assaig Comet 47

1.5.2.5 Gels Camp polsant 50

1.6 L'anàlisi del DNA espermàtic per grups clínics 51

2. Objectius 53

2.1 Hipòtesi 55

2.2 Objectiu 1: Estudi de les metodologies de fragmentació del DNA 57

2.2.1 Efecte de la criopreservació 57

2.2.2. Sensibilitat de les tècniques 57

2.3 Objectiu 2: Pacients infèrtils i fragmentació del DNA 58

2.3.1 Grups de pacients infèrtils amb alteracions conegudes 58

2.3.2 Avortaments de repetició 58

2.4 Objectiu 3: Tipus de trencaments al DNA espermàtic 59

2.4.1 Model *Sperm Chromatin Fragmentation* en ratolí 59

2.5 Objectiu 4: Relació de la compactació del DNA i la fragmentació del DNA 60

2.5.1 Etiologia dels espermatozoides amb alta degradació nuclear 60

2.5.2 Relació de la compactació i la fragmentació del DNA en grups de pacients 60

3. Material i Mètodes 63

3.1 Mostres de semen humanes 65

3.1.1 Procedència 65

3.1.2 Consentiment informat i comitè d'ètica 65

3.1.3 Grups de pacients i controls 65

3.1.4 Obtenció de les mostres de semen humà 67

3.2 Mostres espermàtiques de ratolí 67

3.2.1 Procedència i comitè d'ètica 67

3.2.2 Obtenció de mostres de fluid epididimal i de vas deferent de ratolí 68

3.3 Criopreservació de les mostres de semen 69

3.3.1 Medi de criopreservació 69

3.3.2 Criopreservació en Test-Yolk buffer 70

3.3.3 Descongelació i rentat de la mostra de semen 70

3.4 Tècniques d'anàlisi del semen 71

3.4.1 Seminograma 71

3.5 Inducció del dany al DNA *in vitro* 72

3.5.1 Dany per estrès oxidatiu 72

3.5.2 Dany per activitat nucleasa 72

3.5.3 Dany a les regions MAR (SCF) 72

3.6 Tècniques d'anàlisi de la fragmentació del DNA espermàtic 73

3.6.1 Assaig Comet 73

3.6.1.1 Solucions necessàries 73

3.6.1.2 Protocol 74

3.6.1.3 Anàlisi per microscòpia de fluorescència 76

3.6.2 TUNEL (Terminal transferase dUTP Nick-End Labelling) 77

3.6.2.1 Solucions necessàries 77

3.6.2.2 Protocol 78

3.6.2.3 Anàlisi amb citometria de flux 79

3.6.2.4 Anàlisi amb microscopi de fluorescència 80

3.6.3 SCSA (Sperm Chromatin Structure Assay) 81

3.6.3.1 Solucions necessàries 81

3.6.3.2 Protocol 81

3.6.3.3 Anàlisi 82

3.6.4 SCD (Sperm Chromatin Dispersion test) 83

3.6.4.1 Solucions necessàries 83

3.6.4.2 Protocol 84

3.6.4.3 Anàlisi 84

3.6.5 PFGE (Pulsed-Field Gel Electrophoresis) 85

3.6.5.1 Solucions necessàries 85

3.6.5.2 Protocol 86

3.6.5.3 Anàlisi 87

3.7 Anàlisi de la compactació del DNA espermàtic 88

3.7.1 Determinació de la ràtio entre protamina 1 i protamina 2 88

3.7.1.1 Solucions necessàries 88

3.7.1.2 Protocol 89

3.7.1.3 Anàlisi 90

3.7.2 Test de la Cromomicina A3 (CMA3) 91

3.7.2.1 Solucions necessàries 91

3.7.2.2 Protocol 91

3.7.2.3 Anàlisi 92

3.8 Estadística 93

4. Resultats 95

4.1 Resultats referents a l'objectiu 1.1: Article 1 97

4.2 Resultats referents a l'objectiu 1.2: Article 2 105

4.3 Resultats referents a l'objectiu 2.1: Article 3 117

4.4 Resultats referents a l'objectiu 2.2: Article 4 127

4.4.1 Millora en la predicció del risc d'avortaments en el reanàlisi de les dades amb una mostra ampliada de la inclosa en l'article 4 138

4.5 Resultats referents a l'objectiu 3: Article 5 141

4.6 Resultats referents a l'objectiu 4.1: Article 6 159

4.7 Resultats referents a l'objectiu 4.2: Article 7 189

5. Discussió 215

5.1 Detecció diferencial dels trencaments de cadena senzilla i doble del DNA espermàtic 217

5.1.1 Comet Alcalí: trencaments de cadena senzilla del DNA 217

5.1.2 Comet Neutre: trencaments de cadena doble del DNA 218

5.2 La criopreservació de mostres de semen causa únicament trencaments de cadena senzilla del DNA 220

5.3 La incidència de fragmentació de cadena senzilla del DNA constitueix una bona aproximació per a la predicció de la capacitat fèrtil 222

5.4 Els trencaments de cadena doble del DNA detectats per Comet neutre no estan relacionats amb la capacitat de generar un embaràs clínic 224

5.5 L'anàlisi de la fragmentació de cadena senzilla i doble permet una millor caracterització de les diferents tipologies clíniques de pacients infèrtils. 226

5.6 Els trencaments de cadena senzilla del DNA es corresponen amb un dany extensiu 229

5.7 Els trencaments de cadena doble del DNA estan relacionats amb un risc d'avortament associat a factor masculí 230

5.8 Els trencaments de cadena doble del DNA corresponen a un dany a les regions MAR 232

5.9 Els trencaments de cadena doble del DNA espermàtic en un model de ratolí: implicació de la matriu nuclear espermàtica en la relligació del DNA 234

5.9.1 La lisi cel·lular que s'utilitza en l'aplicació de l'assaig Comet no desestabilitza la matriu nuclear espermàtica 235

5.9.2 Existeixen almenys dos tipus de trencaments de cadena doble del DNA........ 237

5.9.3 La matriu nuclear té un paper crític en la reparació dels trencaments de cadena doble del DNA 239

5.9.4 La inducció de dany de cadena doble mitjançant MnCa i la seva posterior relligació causa trencaments de cadena senzilla del DNA 240

5.10 La relació de la compactació i la fragmentació del DNA espermàtic 241

5.10.1 La compactació del DNA en l'espermatozoide està relacionada amb els trencaments de cadena senzilla del DNA i no amb els trencaments de cadena doble 241

5.10.2 La compactació del DNA com a predicció de la infertilitat 242

5.11 Espermatozoides amb una alta degradació nuclear: efecte d'activitat nucleasa sobre espermatozoides amb una pobre compactació del DNA 243

6. Conclusions 247

7. Bibliografia 253

ABREVIATURES

8-OHdG	8-hidroxi-2'-deoxiguanosina
DNA	Àcid desoxiribonucleic
ATP	Adenosina trifosfat
ATZ	Astenoteratozoospèrmia
BSA	Albúmina de Sèrum Bovina
CMA3	Chromomycin A3
dsSDF	Fragmentació de cadena doble del DNA
DTT	Dithiothreitol
FIV	Fecundació *in vitro*
HDS	High DNA Stainability (SCSA)
ICSI	Intra Cytoplasmatic Sperm Injection
IIU	Inseminació intra-uterina
Kb	kilobases
MAR	Matrix Attachment Region
OATZ	Oligoastenoteratozoospèrmia
ROS	Espècies reactives d'oxigen
SCD	Sperm Chromatin Dispersion
SCF	Sperm Chromatin Fragmentation
SCSA	Sperm Chromatin Structure Assay
SDF	Sperm DNA Fragmentation
SDS	Sodium Dodecyl Sulfate
ssSDF	Fragmentació de cadena senzilla del DNA
TdT	Transferasa terminal
TUNEL	Terminal transferase dUTP Nick-End-Labeling
TYB	Test Yolk buffer
WHO (OMS)	Organització Mundial de la Salut

RESUM

Al llarg dels darrers anys, s’ha demostrat que la fragmentació del DNA espermàtic té un paper important sobre la fertilitat masculina. A més, sembla lògic que trencaments en el DNA espermàtic poden comprometre la viabilitat del zigot, afectant al desenvolupament embrionari.

Tenint en compte la hipòtesi que els trencaments de cadena senzilla i doble del DNA poden tenir efectes diferenciats en la clínica del pacient, s’han desenvolupat diferents objectius: i) en quan a metodologies, ii) efectes clínics en diferents grups de pacients, iii) aspectes bàsics dels trencaments de doble cadena, i iv) la relació amb la compactació del DNA.

Com a aspectes rellevants, s’han establert valors llindar per la detecció d’infertilitat mitjançant les diferents metodologies d’anàlisi de la fragmentació del DNA, s’ha descrit l’efecte clínic de la fragmentació de doble cadena del DNA espermàtic en humans i s’ha proposat un mecanisme bàsic, utilitzant un model de ratolí. Els diferents resultats han conclòs que aquest tipus de trencaments són responsables d’un increment en el risc d’avortament associat al factor masculí.

Aquests descobriments han permès desenvolupar noves eines diagnòstiques que permeten un millor consell reproductiu, així com la possibilitat de recerca de possibles tractaments per pal·liar aquest efecte.

1. INTRODUCCIÓ

1.1 L'espermatogènesi: meiosi i espermiogènesi

L'espermatozoide és la cèl·lula especialitzada en la reproducció masculina encarregada principalment de transmetre la informació genètica masculina al futur embrió (Yanagimachi, 2005). Per a transmetre aquesta informació és necessari un procés de diferenciació cel·lular, conegut com a espermatogènesi, que té com a objectiu la producció de milions d'espermatozoides. Aquest procés té lloc a la gònada masculina, el testicle, s'inicia amb la pubertat i és continu al llarg de tota la vida fèrtil de l'individu. Consta de tres fases principals: una primera etapa de proliferació de les espermatogònies, una segona etapa que té com a objectiu la reducció de la dotació cromosòmica a la meitat, anomenada meiosi, i una tercera etapa de diferenciació cel·lular, anomenada espermiogènesi.

En la primera etapa, les cèl·lules mare de la gònada, anomenades espermatogònies (diploide; 2n), pateixen successives divisions per mitosi donant lloc a dues cèl·lules mare filles, amb la mateixa dotació cromosòmica (diploide; 2n), de forma que una d'elles es prepara per a entrar a la fase de meiosi, i l'altra resta a l'epiteli seminífer per a seguir amb les divisions mitòtiques que permeten la generació de noves espermatogònies, donant lloc així a un procés de renovació continu al llarg de tota la vida fèrtil de l'individu (Figura 1).

En la segona etapa, l'espermatogònia entra en el procés de la meiosi, amb l'objectiu de reduir la dotació cromosòmica a la meitat i així donar lloc a quatre cèl·lules filles, les espermàtides, amb la meitat de la dotació genètica. Així, mentre el genoma de les espermatogònies o qualsevol altra cèl·lula somàtica presenten una dotació cromosòmica diploide (2n), a l'espècie humana 46 cromosomes (23, XY o 23, XX), després de la meiosi, les espermàtides presentaran una dotació cromosòmica haploide

(n), amb un sol cromosoma de cada parella (1-22, X o 1-22, Y), amb l'objectiu de que, en fecundar un oòcit també haploide, donar lloc a un zigot diploide (2n=46). D'aquesta manera s'aconsegueix mantenir estable el nombre de cromosomes que defineix l'espècie (Figura 1). A més a més, la meiosi també aporta una característica crucial per a l'evolució de l'espècie, la recombinació homòloga. Aquest procés es dóna a la profase I de la meiosi I, i consisteix en l'intercanvi d'informació genètica entre cromosomes homòlegs, donant lloc a variabilitat genètica. Assegura una correcta segregació dels cromosomes homòlegs, sent el principal mecanisme per mantenir l'euploïdia. D'aquesta manera, al final de la meiosi, cada espermàtida contindrà una informació genètica que varia segons la recombinació homòloga, de forma que la probabilitat de trobar finalment dos espermatozoides amb la mateixa informació genètica és pròxima a 0 (Figura 1).

Finalitzada la meiosi, cada espermàtida entra en un procés de diferenciació cel·lular anomenat espermiogènesi. Durant aquest procés, es dóna principalment una reducció important del citoplasma, l'aparició de l'acrosoma que permetrà la penetració a l'oòcit, l'adquisició d'un flagel que permetrà el desplaçament cel·lular, i una remodelació de la cromatina, que passarà d'estar compactada en histones a estar compactada amb protamines, fet que permet una molt elevada compactació del DNA (Figura 2). Aquestes característiques finals són les que permetran a l'espermatozoide independitzar-se de l'epiteli seminífer, nedar per el tracte genital femení i fecundar l'oòcit. Un cop fecundat l'oòcit, el material genètic de l'espermatozoide donarà lloc al pronucli masculí del zigot. D'aquesta manera, s'obtindrà un embrió amb una dotació cromosòmica 2n. En aquest estadi, l'oòcit aporta a l'embrió una capacitat de reparació de trencaments que pot actuar també sobre el pronucli masculí, a més d'iniciar-se la

transcripció de gens necessaris per el procés de desenvolupament preimplantacional, bàsicament guiat per un cicle cel·lular en el que s'alternen la fase S i la fase M, sense passar per un estadi d'interfase.

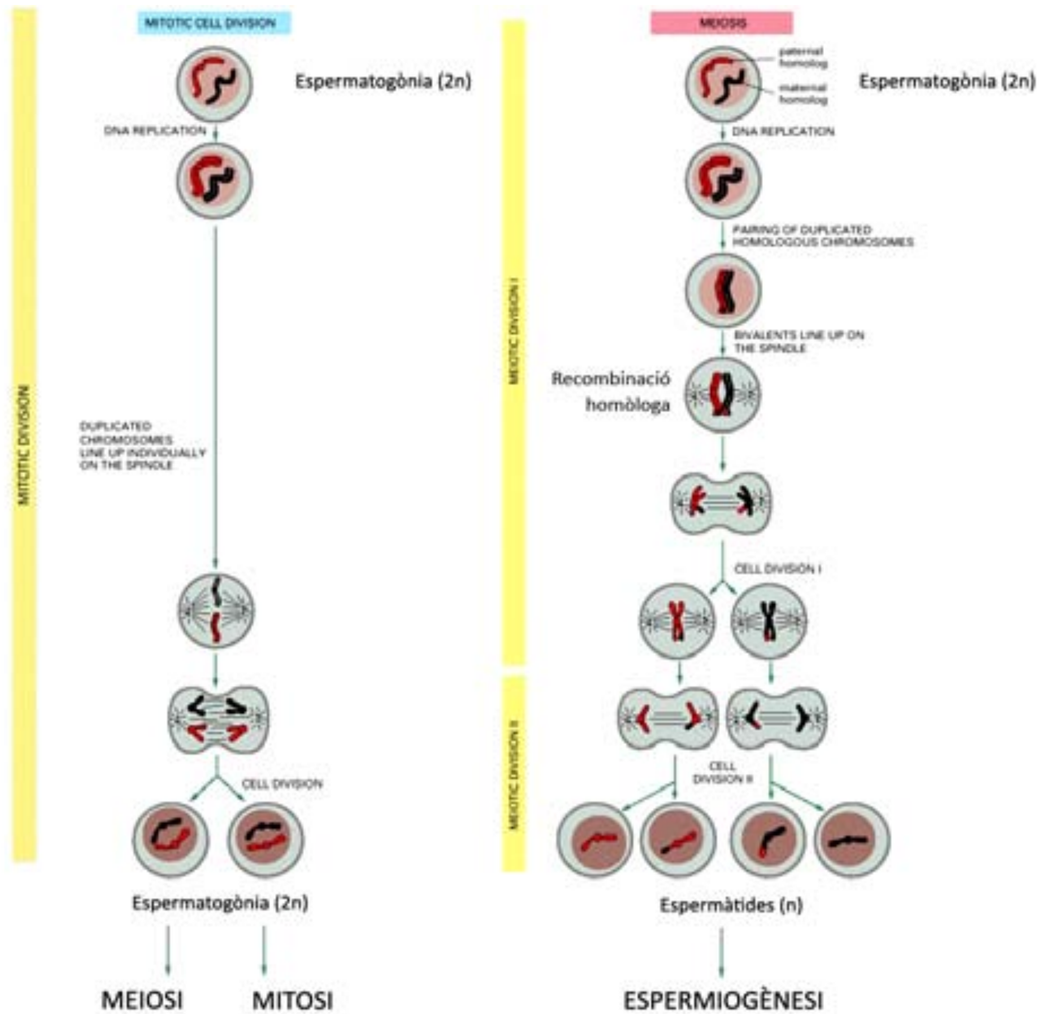


Figura 1. Processos de la mitosi i la meiosi. Mitjançant la mitosi una cèl·lula mare o espermatogònia (2n) produeix dues espermatogònies filles idèntiques (2n). Una d'elles entrarà dins el procés de la meiosi mentre que l'altra residirà al epiteli seminífer per mantenir la continuïtat de la línia germinal. Mitjançant la meiosi una espermatogònia (2n) dóna lloc a quatre espermàtides amb la meitat de material genètic (n) que entraran en el procés d'espermiogènesi per donar lloc a espermatozoides (Adaptada de: Alberts, Molecular Biology of the Cell).

1.2 El DNA espermàtic

Com qualsevol cèl·lula eucariota, l'espermatozoide presenta dos tipus de DNA: el DNA nuclear i el DNA mitocondrial. Mentre el primer sembla cabdal en la seva implicació en el zigot (Alvarez, 2003; Aitken i De Iuliis, 2010; Sakkas i Alvarez, 2010), el segon ha estat molt menys estudiat des del punt de vista de la infertilitat (St John i col., 2005). Així, diversos problemes de fertilitat i fallades de diferents tractaments de reproducció assistida han estat relacionats amb una mala integritat del DNA nuclear espermàtic (Lewis i Simon, 2010; Gosálvez i col., 2013; Lewis i col., 2013).

1.2.1 Estructura de la cromatina nuclear de l'espermatozoide

La cromatina nuclear espermàtica presenta diferències úniques respecte la cromatina de les cèl·lules somàtiques (Ward i Coffey, 1991). Aquestes diferències estan directament relacionades amb la funció principal que l'espermatozoide desenvolupa: la transmissió del DNA patern a l'embrió. Així doncs, mentre la cromatina nuclear de les cèl·lules somàtiques està empaquetada mitjançant unes proteïnes bàsiques anomenades histones, la cromatina nuclear espermàtica està empaquetada principalment per protamines. Les protamines són també proteïnes bàsiques però molt més petites que les histones (Oliva, 2006). Durant l'espermiogènesi, les histones pateixen una sèrie de canvis (principalment acetilacions), que provoquen que aquestes siguin intercanviades per unes proteïnes de transició, i finalment, per protamines, que s'uneixen al solc major de la doble hèlix, al llarg de tot el DNA espermàtic (Figura 2) (Oliva i Castillo, 2011). Així doncs, l'espermatozoide madur humà està compactat en un 85% per protamines, que li confereixen un superior grau de compactació, que s'estima que és de l'ordre de 6 vegades més que els cromosomes metafàsics (Fuentes-Mascorro

i col., 2000). Un cop l'espermatozoide ha fecundat l'oòcit, el procés de recanvi d'histones per protamines que ha tingut lloc durant l'espermiogènesi, es realitza a la inversa, amb l'objectiu d'aconseguir la descompactació del pronucli masculí i obtenir un altre cop un genoma compactat amb histones a punt per començar a transcriure's, iniciant el desenvolupament embrionari (Figura 2) (McLay i Clarke, 2003).

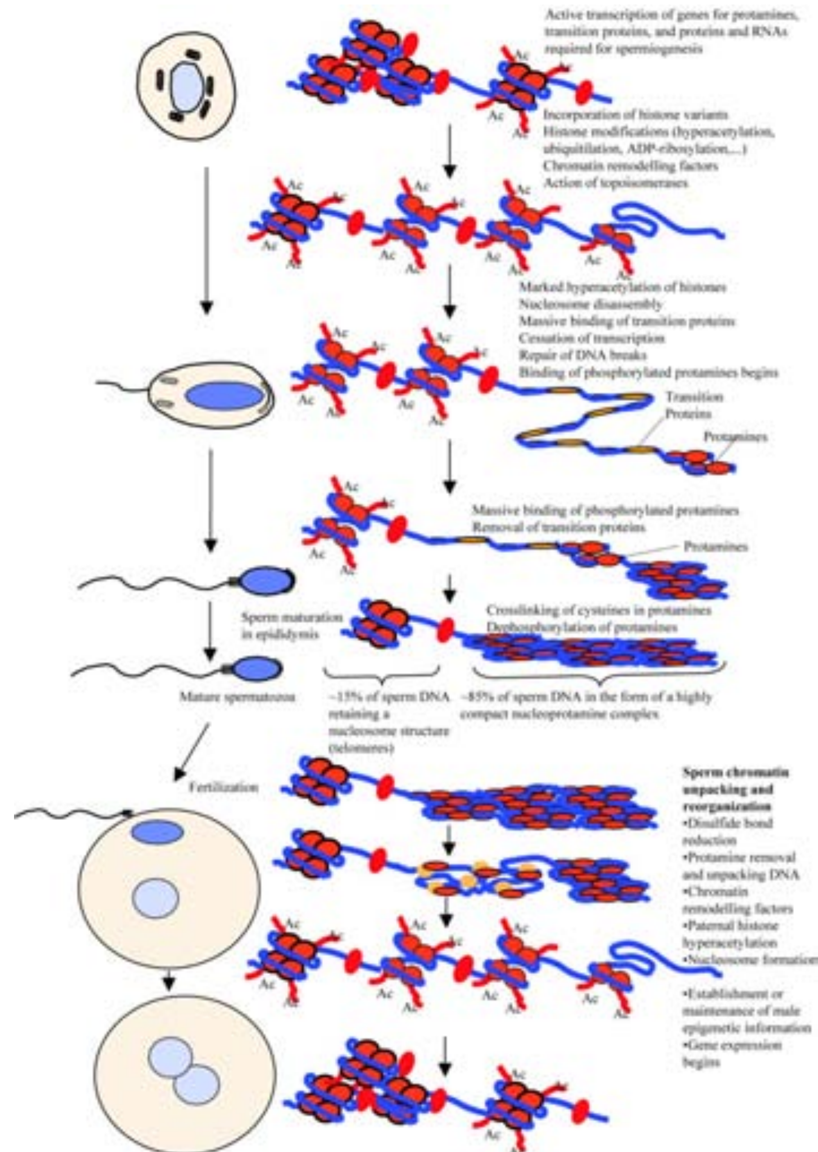


Figura 2. Procés de recanvi d'histones per protamines durant l'espermiogènesi. Aquest procés confereix una gran compactació al DNA espermàtic, aportant una protecció en front a agents externs que puguin comprometre la seva integritat (Oliva, 2006).

1.2.2 Estat de compactació de la cromatina: model Ward

La estructura del DNA espermàtic humà, compactat en protamines, és clau per a poder entendre els mecanismes que causen una reducció de la integritat del DNA, causant problemes diversos de fertilitat (Björndahl i Kvist, 2011). Inicialment, es creia que el DNA compactat amb protamines era inert en front de qualsevol agent físic o químic, i que la cromatina espermàtica formava una estructura cristal·lina (Balhorn, 1982) que era fins i tot resistent a tractaments amb detergent, que provoca una descompactació nuclear en cèl·lules somàtiques però no en espermatozoides (Perreault i Zirkin, 1982; Kvist i col., 1987). Uns anys més tard, diferents estudis de descompactació nuclear van permetre establir el que es coneix com a model del dònut de la cromatina espermàtica, que estableix que el DNA espermàtic compactat amb protamines forma unes estructures toroïdals, en les que els ponts di-sulfur entre protamines estabilitzen l'estructura (Ohsumi i col., 1988; Ward i col., 1989; Ward i Coffey, 1991; Ward, 2010). Tot i que encara no es coneix amb exactitud el procés de formació de les estructures toroïdals, s'atribueix a un fenomen d'auto-organització passiva. Aquest seria depenent de les diferents forces fisico-químiques intrínseques de les protamines, hipòtesi recolzada per diferents estudis que demostren la possibilitat de formació espontània de toroides *in vitro* per el sol fet d'estar en contacte protamines i DNA (Brewer i col., 1999, 2003).

La Figura 3 mostra el model de compactació del DNA amb toroides. Cada toroide permet compactar de 25 a 50 kilobases de DNA, corresponent a la mida d'un llaç (*loop)* de DNA (Hud i col., 1993), i està unit al següent toroide i a la vegada a la matriu nuclear mitjançant regions anomenades Matrix Attachment Regions (MAR) (Aoki i Carrell, 2003; Sotolongo i col., 2003; Wykes i Krawetz, 2003; Ward, 2010). Aquestes regions

MAR mantenen tota l'estructura del DNA unida d'una forma estreta a la matriu nuclear espermàtica, possiblement a través d'altres proteïnes. A més, les regions MAR estan compactades amb histones i, per tant, són susceptibles al dany per nucleases, a diferència de les regions toroïdals (Figura 3) (Ward, 2010).

En cèl·lules somàtiques, les regions MAR semblen estar associades a replicació del DNA, reparació del DNA i regulació gènica (Boulikas, 1995; Codrington i col., 2007a, 2007b). Així, diverses hipòtesis han suggerit les regions del DNA empaquetades amb histones (entre el 5% i el 15% segons l'espècie) podrien tenir una importància en etapes molt primerenques del desenvolupament embrionari, per exemple, sent regions amb una transcripció molt primerenca.

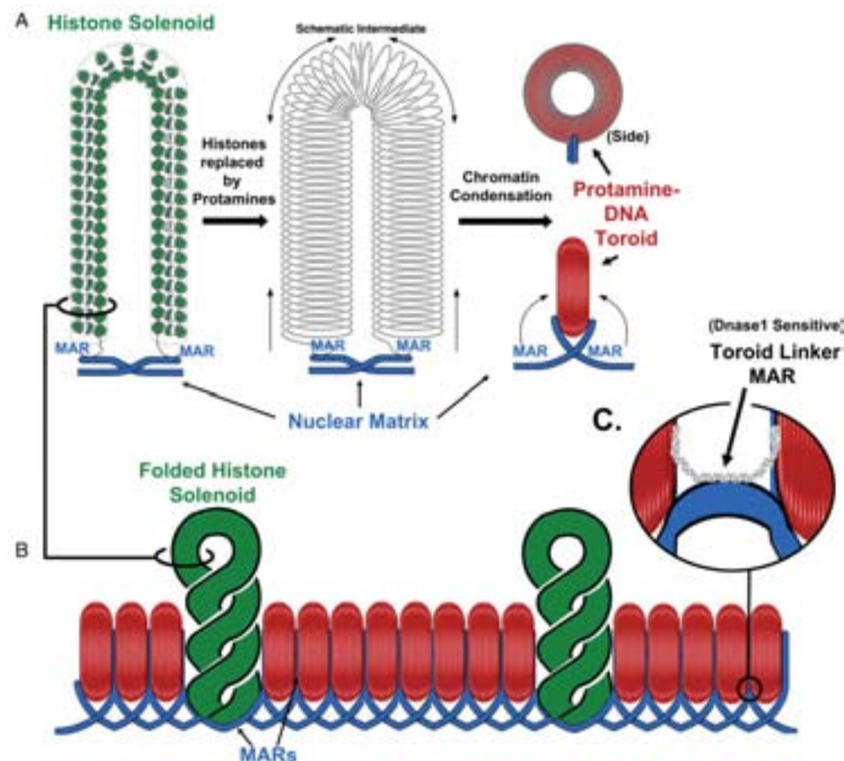


Figura 3. Model que exposa la disposició del DNA compactat amb protamines: aquestes protamines formen unes estructures toroïdals que compacten d'entre 23 a 50 kb de DNA, i es mantenen units entre ells i a la matriu nuclear espermàtica per les regions MAR (Ward, 2010).

1.2.3 Arquitectura del nucli espermàtic: model Zalensky

Mentre el model anterior resol l'estructura fonamental del DNA espermàtic, empaquetat amb protamines, un altre aspecte d'interès és com organitza el nucli espermàtic els diferents cromosomes. Així, els models proposats per Zalensky suggereixen que l'arquitectura cromosòmica en l'espermatozoide no és trivial, sinó tot el contrari: cada cromosoma ocuparia un territori del nucli espermàtic (Zalensky i col., 1995), disposant els telòmers cap a l'exterior del cap de l'espermatozoide, units a la membrana nuclear formant dímers o tetràmers, mentre que els centròmers quedarien disposats cap al centre del nucli espermàtic (Solov'eva i col., 2004; Zalenskaya i Zalensky, 2004; Mudrak i col., 2005) (Figura 4).

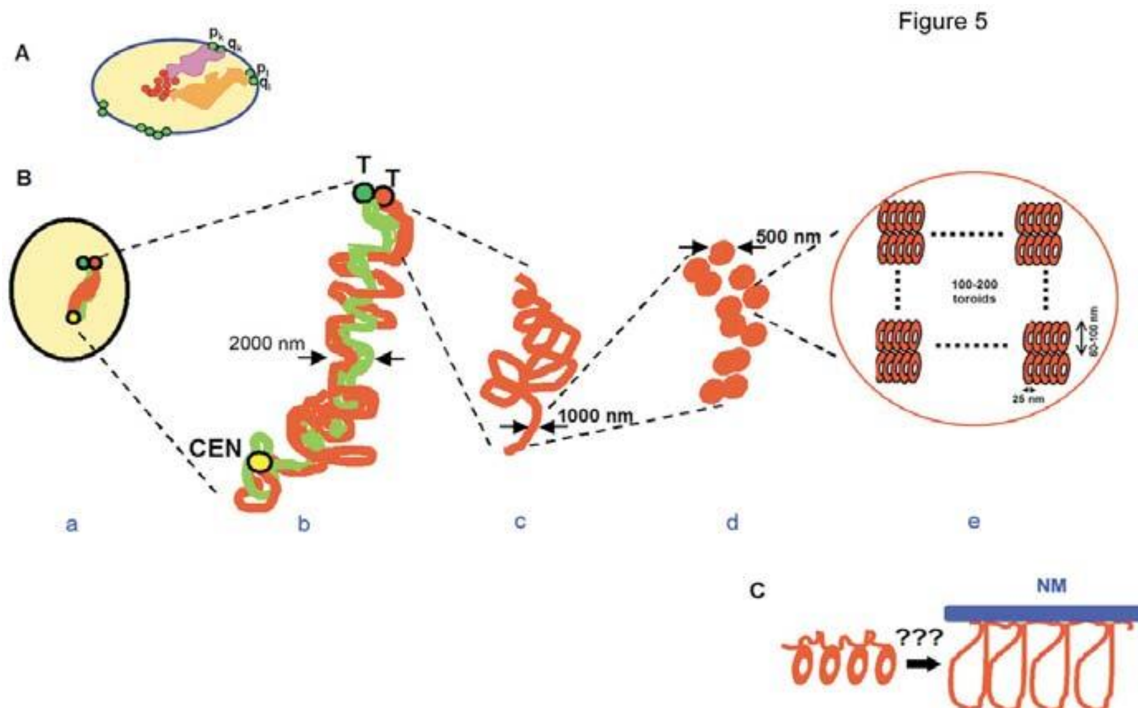


Figura 4. Model d'arquitectura del nucli espermàtic: els cromosomes espermàtics situen els centròmers al centre del nucli, i els telòmers disposats per parelles i units a la membrana nuclear (Mudrak i col., 2005)

A més, treballs més recents del mateix grup demostren que regions més riques en gens quedarien també situades a l'interior del nucli espermàtic, i per tant amb una major protecció i empaquetament, mentre que regions més pobres en gens quedarien situades cap a l'exterior. Finalment, també s'ha descrit que l'organització dels diferents cromosomes en el nucli espermàtic podria dependre de l'organització dels centròmers o pericentròmers a la zona interna del nucli (Figura 5) (Mudrak i col., 2012).

Així doncs, s'espera que aquesta organització no aleatòria dels cromosomes espermàtics tingui implicacions en la formació del pronucli masculí i el conseqüent procés de recanvi de protamines per histones d'aquest en el zigot, per a tot seguit donar lloc a una activació de l'embrió (Mudrak i col., 2009).

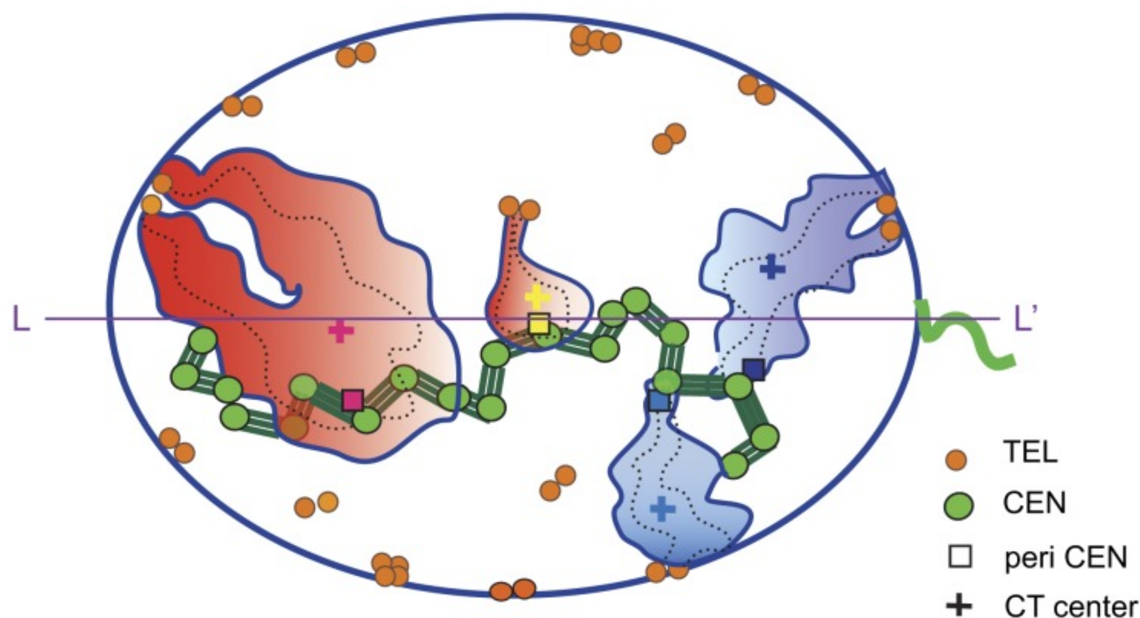


Figura 5. Model d'organització dels cromosomes espermàtics: Els cromosomes reben un ordre d'acord amb l'ordre que presenten els centròmers o pericentròmers al centre del nucli espermàtic, en verd. En blau, les zones pobres en gens quedaran exposades a l'exterior del nucli, mentre que les regions riques en gens, en vermell, quedaran situades més a l'interior d'aquest nucli, amb una major protecció (Mudrak i col., 2012).

1.2.4 DNA mitocondrial

En l'espermatozoide, els mitocondris estan situats a la peça intermèdia i, de la mateixa manera que en les cèl·lules somàtiques, la seva principal funció és la producció d'energia en forma d'ATP que, en aquest cas, serà utilitzada bàsicament per al moviment del flagel. Com a subproducte d'aquest procés, del 1% al 5% de l'oxigen consumit es converteix en espècies reactives d'oxigen (Chance i col., 1979), susceptibles d'atacar al DNA mitocondrial i també el DNA nuclear espermàtic, a part de causar danys a les membranes plasmàtiques (Agarwal i col., 2006; Koppers i col., 2008; Aitken i De Iuliis, 2010).

A diferència del DNA nuclear espermàtic, el DNA mitocondrial no presenta una compactació amb protamines ni histones, presentant una susceptibilitat molt més elevada al dany (Sawyer i col., 2001). Tot i que aquest DNA mitocondrial codifica per alguns polipèptids necessaris en la producció d'energia i que el dany en aquest DNA podria alterar aquest procés, la implicació d'aquest en la fertilitat és limitada, afectant bàsicament a la mobilitat espermàtica (Amaral i col., 2013). Tot i això, diversos autors han senyalat la importància dels mitocondris espermàtics en la fertilitat masculina (Wang i col., 2003; Sousa i col., 2011; Amaral i col., 2013).

1.3 Infertilitat

La infertilitat és considerada una malaltia per l'Organització Mundial de la Salut, i afecta a aproximadament al 15% de les parelles en edat reproductiva en els països desenvolupats (WHO, 2010; Louis i col., 2013), podent arribar a ser de l'ordre del 25-30% en països subdesenvolupats (Petraglia i col., 2013). Considerant que la incidència

de malalties considerades comunes com per exemple la diabetis és al voltant del 5%, es pot considerar la infertilitat com a una malaltia comuna.

Per definició, es considera una parella infèrtil quan aquesta ha mantingut relacions sexuals amb finalitats reproductives sense èxit durant 12 mesos, considerant tant a parelles que no aconsegueixen un embaràs com a parelles amb avortaments (WHO, 2010; American Society for Reproductive Medicine, 2013). Aquesta definició es basa en estudis que proven que durant el primer any de relacions sexuals sense anticonceptius, prop del 90% de les parelles aconsegueixen un embaràs (Figura 6).

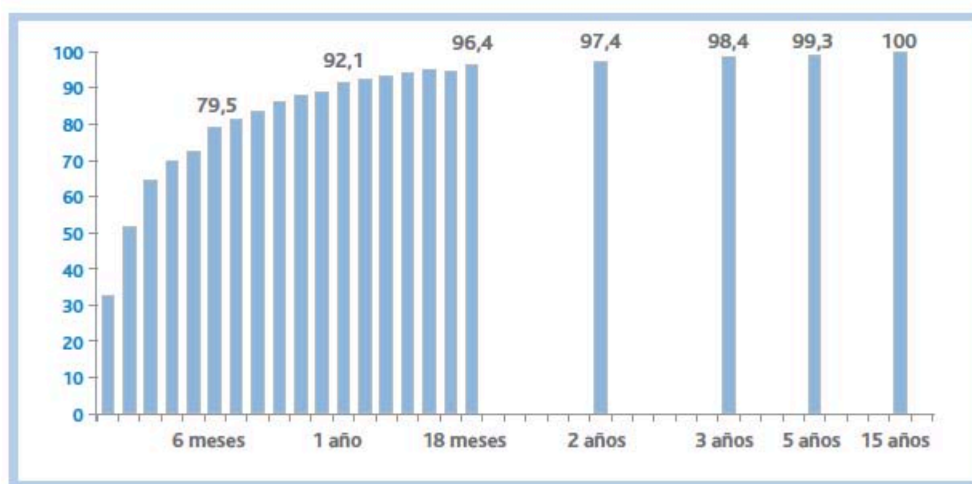
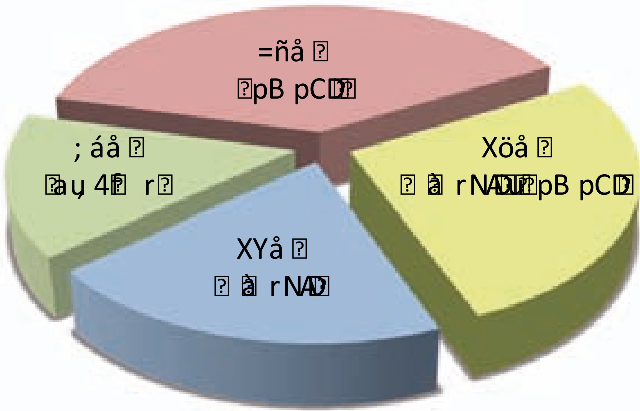
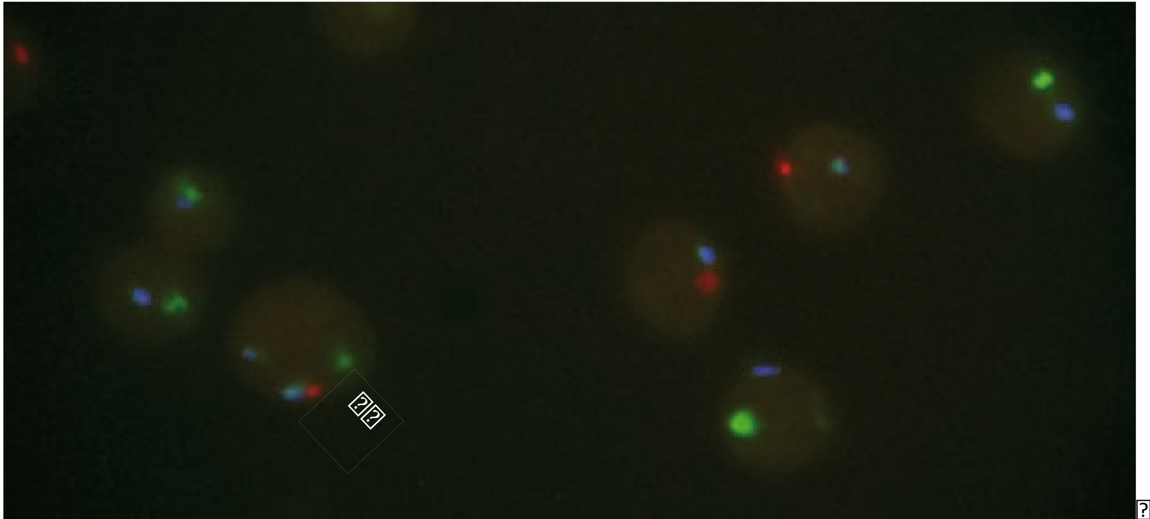


Figura 6. Distribució en el temps de la taxa d'embaràs en cicles d'exposició coital sense anticoncepció (Sociedad Española de Fertilidad).

Així doncs cal diferenciar el concepte d'infertilitat amb el d'esterilitat, que es refereix a una parella que no podrà tenir descendència al llarg de tota la seva vida fèrtil (WHO, 2010).

Tot i que durant molt temps s'ha considerat que la infertilitat és un problema bàsicament d'origen femení, diversos estudis han provat que l'etiologia d'aquesta està repartida a parts iguals entre els factors femení i masculí. Tenint en compte aquest fet,





espermàtica (Rolland i col., 2013) fet que es pot atribuir a diferents etiologies, bàsicament efectes mediambientals i socials, entre altres (Jurewicz i col., 2013a; Martenies i Perry, 2013; Miranda-Contreras i col., 2013).

Tot i que l'anàlisi del seminograma ha estat millorat gràcies a programes informàtics específics (Figura 9), aquest tipus d'estudi no sempre és conclusiu per a determinar l'origen de la infertilitat (Lewis, 2007), i són necessaris altres estudis complementaris per al diagnòstic de la infertilitat.

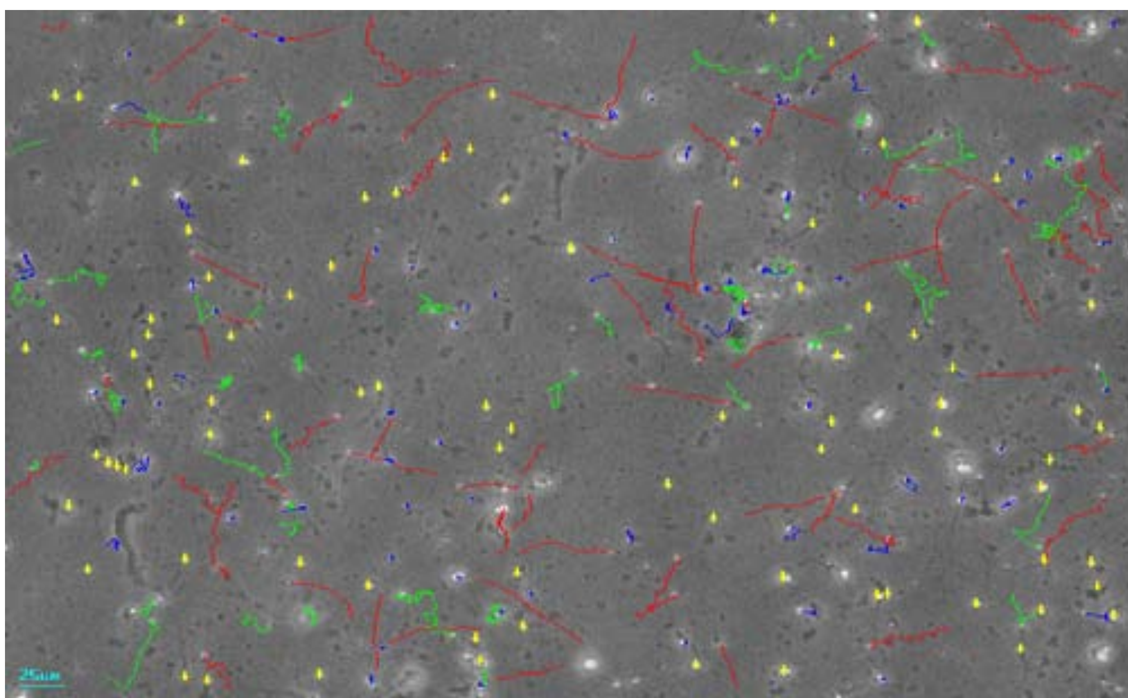


Figura 9. Anàlisi de la mobilitat espermàtica assistit per programa informàtic específic (Sperm Class Analyzer, SCA®, Microptic, Barcelona, Espanya). En vermell estan representats els espermatozoides amb mobilitat de tipus progressiu, en verd els que presenten una mobilitat no progressiva, en blau els que presenten una mobilitat reduïda, i en groc els espermatozoides immòbils.

1.4.2 Varicocele

Entre les causes d'infertilitat més comunes es troba el varicocele, la prevalença del qual es calcula que és al voltant del 15% dels homes, essent aproximadament un 35% dels casos d'infertilitat primària, i fins un 75-80% dels casos d'infertilitat secundària (Chan i Goldstein, 2002).

El varicocele és una dilatació de la vena del plexe pampiniforme que és visible a l'escrot i pot ésser diagnosticat mitjançant exploració física. Es classifica en tres graus depenent de la seva gravetat: en el grau I, les venes afectades són petites i es palpen en situacions d'esforç mitjançant la maniobra de Valsalva; en el grau II les venes són palpables sense aquesta maniobra; i en el grau III, el més greu, és visible sense necessitat de palpació.

La presència d'un varicocele produeix un reflux de la sang venosa, que causa principalment un augment de temperatura testicular i que a la vegada provoca un augment de les espècies reactives d'oxigen i una disminució de la capacitat antioxidant del testicle (Agarwal i col., 2006). Aquest augment de la temperatura i de l'estrès oxidatiu poden provocar canvis hormonals al testicle causant infertilitat masculina. Tan es així que nombrosos treballs han descrit un augment de la fragmentació del DNA espermàtic en aquests pacients (Agarwal i col., 2008; García-Peiró i col., 2011c; Zini i Dohle, 2011). Tot i que el varicocele és de les poques afectacions de la infertilitat masculina corregibles quirúrgicament, la millora d'aquests tractaments quirúrgics han estat motiu de controvèrsia, ja que depèn en gran part del grau d'afectació i de l'edat del pacient (Grasso i col., 2000; Cayan i col., 2009; Baazeem i col., 2011; Will i col., 2011). Tot i això, en l'actualitat s'estan desenvolupant noves tècniques quirúrgiques

que permeten un millor tractament del varicocele provocant substancials millores en la qualitat espermàtica (Mehta i Goldstein, 2013; Parekattil i Gudeloglu, 2013).

1.5 Fragmentació del DNA espermàtic

Diferents grups de recerca han centrat el seu interès en l'anàlisi de la integritat del DNA espermàtic per la necessitat d'incorporar tests més eficaços en el diagnòstic de la infertilitat masculina (Evenson, 2013; Gawecka i col., 2013; Gosálvez i col., 2013; Lewis i col., 2013; Sharma i col., 2013). Molts són els treballs que han relacionat la fragmentació del DNA espermàtic amb la impossibilitat de concebre de forma natural o mitjançant tractaments de reproducció assistida (Esbert i col., 2011; Simon i col., 2011; Nuñez-Calonge i col., 2012; Robinson i col., 2012; Alkhayal i col., 2013).

Per a fragmentació del DNA espermàtic s'entenen tot el conjunt d'alteracions que poden ser presents a la doble hèlix del DNA, com trencaments de cadena senzilla del DNA, trencaments de cadena doble del DNA, modificacions de bases nitrogenades, llocs abàsics, oxidació de bases nitrogenades, o fenòmens d'entrecreuament (cross-link) entre DNA i proteïnes o DNA i DNA que poden donar lloc, posteriorment, a nous trencaments a la doble hèlix (Barratt i col., 2010) (Figura 10). Tots aquests fenòmens no són incompatibles amb la fecundació, ja que espermatozoides que presenten una pobre integritat de la cromatina són capaços de fecundar a l'oòcit, donant lloc a un embrió anòmal des del punt de vista del DNA masculí, comprometent així la viabilitat d'aquest. A més, donada la dificultat d'analitzar la fragmentació del DNA d'un espermatozoide de forma que no l'invalidi per a ser utilitzat per un tractament de reproducció, aquestes tècniques poden utilitzar espermatozoides amb trencaments al DNA, la qual cosa pot comportar riscos pels futurs nadons nascuts. Així, s'ha vist que la

fecundació amb espermatozoides que presenten una pobre integritat de la cromatina poden donar lloc a embrions amb un augment d'anomalies cromosòmiques, un augment de les taxes d'avortaments, o fins i tot un risc més elevat de càncer infantil (Cooke i col., 2003; Aitken i col., 2009).

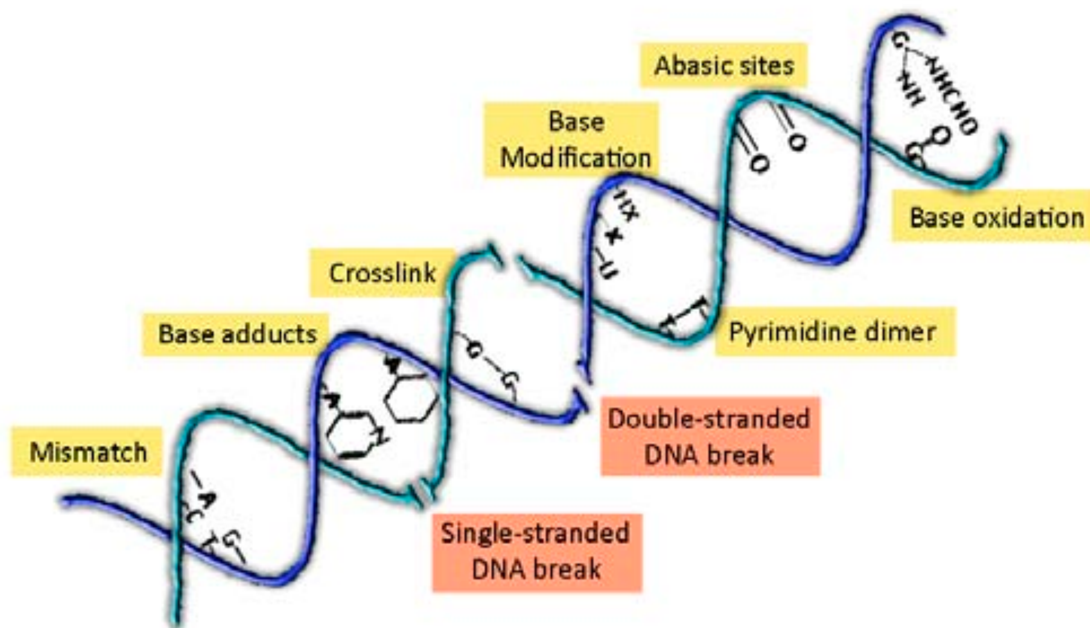


Figura 10. Possibles alteracions amb un potencial de donar lloc a trencaments al DNA espermàtic.

Tot i això, no es pot obviar la potencialitat de reparació del DNA de la que disposa el zigot, sent capaç de reparar algunes de les afectacions al DNA espermàtic mencionades anteriorment (Ménézo i col., 2010).

Així doncs, l'anàlisi de la fragmentació del DNA espermàtic s'ha consolidat com a una tècnica complementària de diagnòstic a les actualment disponibles, amb el fi de realitzar un millor anàlisi de l'estat del genoma patern. En aquest aspecte, diversos estudis han ajudat a estandarditzar les tècniques d'anàlisi, així com a establir valors

llindar aplicables al diagnòstic i consell reproductiu (Lewis i Simon, 2010; Sharma i col., 2010). Tot i això, cal deixar clar que la fragmentació del DNA espermàtic no té un poder predictiu total sobre la reproducció de la parella, donat que es tracta d'una anàlisi únicament del factor masculí. Llavors, en molts casos, és necessari fer un estudi exhaustiu de la parella per a poder establir l'origen concret de la incapacitat de reproducció.

1.5.1 Etiologia de fragmentació

L'origen de la fragmentació del DNA espermàtic pot ser degut a motius diversos, i diferents estudis han provat diferents vies mitjançant les quals el DNA espermàtic pot sofrir trencaments, comprometent així la integritat d'aquest i tots els efectes comentats anteriorment. Aquestes vies que provoquen trencaments al DNA poden actuar de forma aïllada, però el dany al DNA pot esdevenir com a resultat d'una combinació d'aquests factors (Aitken i De Iuliis, 2010). Com a factors intrínsecs del dany al DNA espermàtic destaquen l'efecte de l'estrès oxidatiu, l'apoptosi (més recentment entesa com a un procés similar a l'apoptosi, o *apoptosi-like*), el dany per nucleases i l'alteració en l'empaquetament de la cromatina. Tanmateix, l'exposició a tòxics ambientals, inflamacions, infeccions, l'efecte de la quimioteràpia o les radiacions electromagnètiques poden actuar sobre el DNA espermàtic com a factors extrínsecs (Aitken i De Iuliis, 2010; Sakkas i Alvarez, 2010). Tots aquests efectes poden estar presents durant l'espermatogènesi a testicle, o bé també després de la producció dels espermatozoides, a l'epidídim, al vas deferent, i posteriorment, a l'ejaculat (Figura 11).

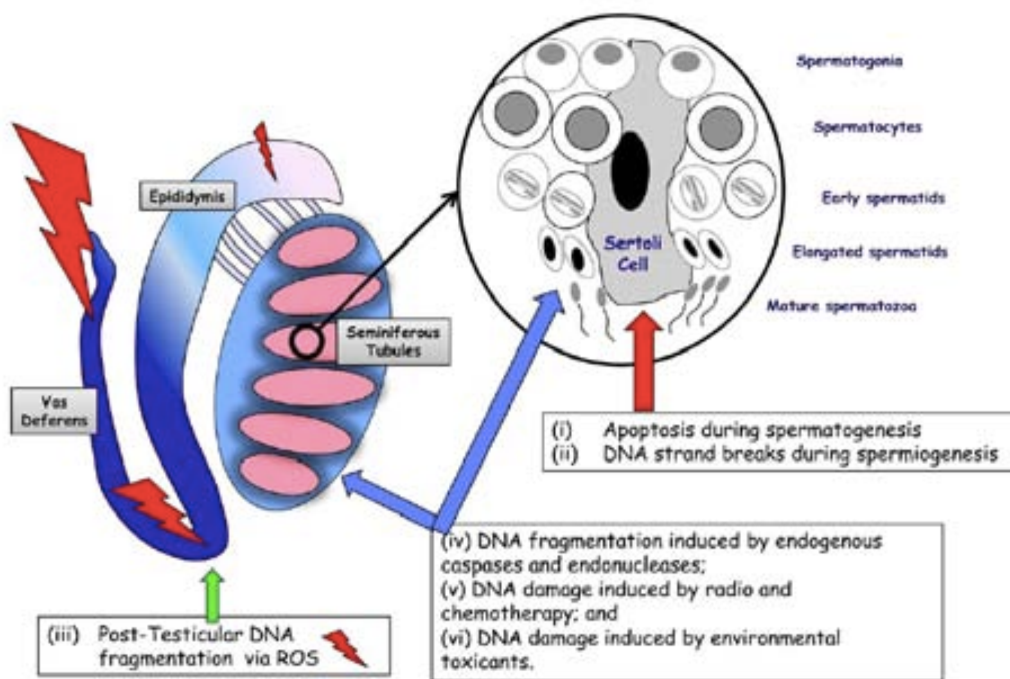
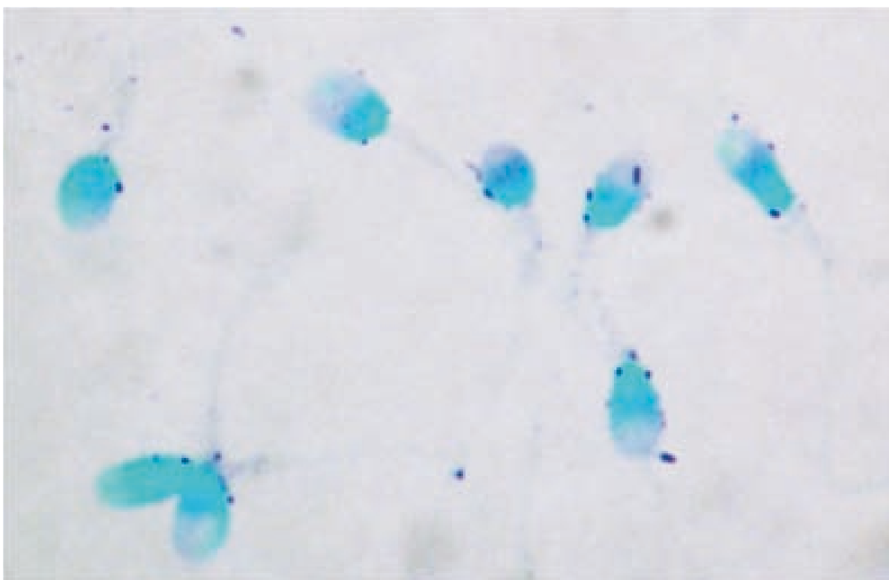


Figura 11. Etiologia del dany al DNA espermàtic esquematitzada a tres nivells: a nivell testicular, a nivell d'epidídim, i a nivell de vas deferent (Sakkas i Alvarez, 2010)

1.5.1.1 Estres oxidatiu

La producció i la presència d'espècies reactives d'oxigen (ROS) és una de les principals causes del dany al DNA espermàtic (Bennetts i Aitken, 2005) i s'ha descrit que una gran part dels homes infèrtils presenten nivells elevats de ROS (Iwasaki i Gagnon, 1992; de Lamirande i Gagnon, 1995; Agarwal i col., 2008; De Iuliis i col., 2009; García-Peiró i col., 2011c). Les espècies reactives d'oxigen són produïdes principalment pels processos de respiració cel·lular dels mitocondris de la peça intermèdia en generar ATP que serà utilitzat per el moviment del flagel. Tot i això, nivells basals de ROS podrien realitzar funcions fonamentals en processos com ara la capacitació i la reacció acrosòmica (Aitken i col., 2009; Marzec-Wróblewska i col., 2012). Aquests processos estan regulats mitjançant diferents enzims presents al espermatozoide i al fluid seminal, com ara el



Aquest efecte de l'estrès oxidatiu està, a més, condicionat per diferents factors com per exemple la capacitat antioxidant del espermatozoide i del fluid seminal. Aquesta protecció s'ha demostrat que és més elevada al epidídim (Bennetts i Aitken, 2005), on l'espermatozoide roman uns dies després de la seva producció al testicle, i manté uns nivells basals, tot i que menors, en el fluid seminal. Aquesta capacitat antioxidant del fluid seminal, bàsicament associada amb la vitamina C (Song i col., 2006), carnitines (De Rosa i col., 2005), o coenzim Q10 (Mancini i col., 2005), ha estat correlacionada amb l'estat de fertilitat de l'individu (Pasqualotto i col., 2008) i es veu disminuïda, per exemple, en individus fumadors (Fraga i col., 1996; Saleh i col., 2002).

L'efecte antioxidant ha estat motiu de diversos d'estudis i complements alimentaris que disminueixen l'estrès oxidatiu al semen i milloren la qualitat del DNA espermàtic i altres paràmetres seminals com ara la mobilitat (Hamada i col., 2012; Wirleitner i col., 2012; Abad i col., 2013), generant millors expectatives en la capacitat reproductiva de l'individu.

1.5.1.2 Apoptosi

Una gran part de les cèl·lules somàtiques del cos humà, i també de les cèl·lules germinals moren diàriament degut a processos d'apoptosi. Aquest fet també és aplicable en l'espermatogènesi, on les cèl·lules de Sertoli mantenen l'equilibri normal al testicle. D'aquesta manera s'eliminen les cèl·lules meiòtiques o post-meiòtiques alterades (Braun, 1998), afavorint el control de qualitat que requereix la producció d'espermatozoides a testicle.

En aquest procés d'eliminació, hi pren un paper important el sistema Fas/FasL. Les cèl·lules que han de ser eliminades expressen el marcador Fas a la seva membrana,

mentre que les cèl·lules de Sertoli expressaran el lligand (FasL). La unió del lligand amb el marcador causa una fagocitosi de les cèl·lules apoptòtiques per les cèl·lules de Sertoli (Lee i col., 1997; Eguchi i col., 2002). En cas de que aquest mecanisme no funcioni correctament, possiblement degut a una saturació de les cèl·lules de Sertoli (Sakkas i col., 1999), es podran observar espermatozoides apoptòtics amb el marcador Fas a la seva membrana, hipòtesi que es coneix com a apoptosi abortiva. Tot i això, aquests espermatozoides Fas positius no són capaços d'iniciar processos d'apoptosi (Lachaud i col., 2004). No obstant, tot i que l'expressió de Fas no s'ha relacionat amb la fragmentació del DNA (McVicar i col., 2004), l'apoptosi abortiva es relacionaria amb una via intrínseca, per la qual l'estrès oxidatiu mitocondrial activaria caspases, i aquestes activarien nucleases internes de l'espermatozoide que digeririen les regions accessibles del DNA.

1.5.1.3 Activitat nucleasa

Diferents autors han demostrat la presència de nucleases a l'espermatozoide de diferents animals (Sotolongo i col., 2005). Existeix la possibilitat de que nucleases equivalents puguin afectar també a espermatozoides humans. Donat que aquests presenten un menor grau de compactació degut al major contingut en histones (Bianchi i col., 1993) el seu efecte podria ser encara més sever. Les nucleases, a més, poden ser endògenes de l'espermatozoide, o també exògenes, presents al fluid seminal (Nadano i col., 1993; Maione i col., 1997).

Les nucleases presents al semen, doncs, podrien danyar el DNA espermàtic en les zones sensibles com ara les regions MAR (Ward, 2010), provocant una reducció de la capacitat fèrtil. En aquest aspecte, fins i tot alguns autors han demostrat que

l'espermatozoide és capaç d'internalitzar fragments de DNA exogen, que seria integrat al seu genoma mitjançant l'activació de nucleases endògenes (Lavitrano i col., 1992; Maione i col., 1997; Spadafora, 1998).

1.5.1.4 Alteracions de l'empaquetament del DNA

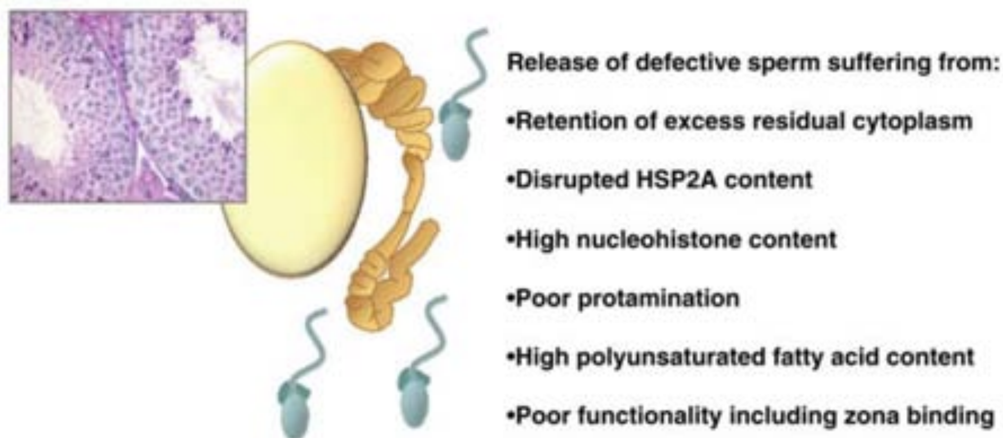
Durant el procés de l'espermiogènesi, cada una de les espermàtides pateix una sèrie de canvis bioquímics i morfològics que donaran lloc a espermatozoides madurs. D'entre aquests canvis, cal destacar una notable condensació de la cromatina, duta a terme en dues fases, i que, mitjançant els processos anteriorment explicats (Figura 2) portaran aquest DNA fins a un estat de compactació extremadament elevat (Fuentes-Mascorro i col., 2000). En la fase testicular s'intercanvien les histones per protamines que, segons els models actuals, s'organitzaran en forma de toroide, i posteriorment, al epidídim i vas deferent, es formaran els ponts disulfur necessaris per a estabilitzar aquestes estructures (Manfredi Romanini i col., 1986; Seligman i Shalgi, 1991; Björndahl i Kvist, 2010).

Aquest elevat estat de compactació de la cromatina representa un punt clau per a entendre la gran protecció del DNA espermàtic en vers a diferents agents susceptibles de produir-li trencaments. Així, qualsevol estat alterat de la cromatina o del DNA espermàtic pot donar lloc a alteracions en aquest procés d'empaquetament i, com a conseqüència, donar lloc a un nucli pobrament compactat i susceptible de patir trencaments per altres mecanismes presents al espermatozoide, com l'estrès oxidatiu (Figura 13) (Aitken i De Iuliis, 2010). D'entre aquestes alteracions, hi prenen part les reorganitzacions cromosòmiques, que causaran una alteració en l'arquitectura normal del nucli espermàtic, ja sigui per translocacions robertsonianes o recíproques,

inversions, duplicacions o delecions. En aquest sentit, diversos treballs demostren una menor integritat del DNA espermàtic i una menor compactació nuclear en aquests tipus de pacients (García-Peiró i col., 2011a, 2011b, 2012).

D'altra banda, possibles alteracions en la relació entre protamina 1 i protamina 2 (P1/P2) podrien donar lloc també a un erroni empaquetament, fet que es traduirà en el mateix efecte. De fet, alguns treballs de la literatura demostren una alteració d'aquesta relació P1/P2 en pacients portadors d'alteracions cromosòmiques (García-Peiró i col., 2011a), amb una menor capacitat d'embaràs en cicles de FIV o ICSI (Torregrosa i col., 2006).

Tanmateix, un altre factor amb una potencial capacitat d'alteració de la compactació del DNA espermàtic és el varicocele. L'increment de temperatura provocat per el reflux de sang en la o les venes testiculars afectades pot causar alteracions en l'espermiogènesi, donant lloc al final a un elevat percentatge d'espermatozoides immadurs des del punt de vista de la cromatina (García-Peiró i col., 2012), susceptibles a degradació per diferents agents exposats en aquesta introducció, entre ells, activitat nucleasa (Sotolongo i col., 2003, 2005).



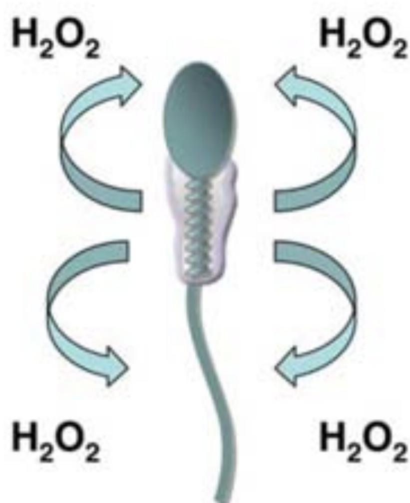


Figura 13. Hipòtesi del doble pas: segons aquesta hipòtesi, les diferents causes de fragmentació del DNA no actuen de forma aïllada, sinó que de forma cooperativa. En una primera etapa, algunes etiologies causen una situació compromesa en la cromatina espermàtica, mentre que en un segon terme, aquesta cromatina pot ésser danyada per altres agents com ara l'estrès oxidatiu (Aitken i de Iuliis, 2010).

1.5.1.5 Infeccions i inflamacions

Les infeccions són la principal causa d'infertilitat corregible. Generalment, les infeccions al tracte genital masculí poden anar acompanyades d'inflamacions i, com a conseqüència, un transvasament de leucòcits al tracte seminal (Al-Moushaly, 2013; Alshahrani i col., 2013; La Vignera i col., 2013; Weidner i col., 2013). Aquest increment de leucòcits activats per a combatre la infecció dóna lloc a un increment substancial de l'estrès oxidatiu, amb tots els efectes descrits prèviament, com ara l'atac a les membranes plasmàtiques, membranes mitocondrials, o al DNA espermàtic, dificultant l'embaràs o les tècniques de reproducció assistida (Henkel i col., 2003; Yilmaz i col., 2005; Seshadri i col., 2012; Aitken i Baker, 2013). A més, cal afegir el dany que poden

causar els patògens sobre el tracte genital, l'espermatozoide, o l'efecte que poden tenir sobre l'espermatogènesi.

Aquestes afectacions poden ser detectades amb una observació microscòpica del semen o bé mitjançant anàlisis microbiològics, presentant un increment de leucòcits (o leucocitospermia) i/o bacteris, i són corregibles mitjançant l'administració d'antibiòtics al pacient per tal de combatre la infecció i el conseqüent increment d'estrès oxidatiu produït pels leucòcits presents al semen.

1.5.1.6 Quimioteràpia i radiacions

Diversos tractaments de quimioteràpia i radioteràpia han estat relacionats directament amb una afectació de la fertilitat, ja sigui actuant a nivell d'espermatogònies o bé també a nivell d'espermatogènesi, provocant diversos efectes, entre ells azoospèrmia temporal o permanent (Meistrich, 2013) o també fragmentació del DNA espermàtic (Ståhl i col., 2006; Sakkas i Alvarez, 2010). Aquests efectes descrits per la quimioteràpia o radioteràpia poden variar depenent del tipus de procediment, de la dosi, o de l'edat del pacient, entre altres. D'altra banda, aquests tractaments també provoquen una més elevada taxa de mutacions a les cèl·lules germinals, amb la potencialitat de produir espermatozoides alterats genèticament i, en conseqüència, descendència amb susceptibilitat a carcinogènesi (Hoyes i col., 2001).

1.5.1.7 Exposició a tòxics, a radiació electromagnètica i estil de vida

Existeixen evidències de l'efecte de determinats productes tòxics sobre la fertilitat, causant efectes sobre paràmetres com la motilitat, concentració, o fragmentació del DNA espermàtic. Productes com per exemple dissolvents, resines fenòliques, metalls pesants, gasolina, pintura o altres substàncies provinents de la industria química,

electrònica o del motor han estat objecte d'estudi, demostrant el seu efecte carcinogen transgeneracional (Wilkins i Hundley, 1990). A més, contaminants producte del trànsit com monòxid de carboni o òxids de sofre, presents diàriament en zones urbanes, incideixen negativament sobre la mobilitat espermàtica (De Rosa i col., 2003). Finalment, s'ha demostrat que productes com l'estirè, utilitzat àmpliament per la indústria per a produir plàstics, i diversos insecticides o pesticides, provoquen un efecte directe sobre el DNA espermàtic, comprometent la seva integritat (Migliore i col., 2002; Bian i col., 2004; Sánchez-Peña i col., 2004).

La majoria d'aquestes substàncies afecten principalment a l'equilibri d'oxidació-reducció i per tant, capaces d'afectar a les cèl·lules germinals, que presenten una alta sensibilitat al dany (Fabricant i col., 1983).

Val la pena destacar, a més, que diferents hàbits no saludables tenen també el seu efecte sobre la fertilitat masculina: el consum de tabac (Taha i col., 2012; Yu i col., 2013), cànnabis i altres drogues (Vine, 1996; Ricci i col., 2007; Bari i col., 2011), alcohol i cafeïna (Anderson i col., 2010; Joo i col., 2012; Jurewicz i col., 2013b).

També poden contribuir a una menor capacitat fèrtil l'estrès psicològic (Zorn i col., 2008), l'obesitat (Vagnini i col., 2007; Paasch i col., 2010), la dieta, i les radiacions electromagnètiques produïdes per aparells electrònics, especialment si estan situats a prop de la zona testicular com ara en el cas de telèfons mòbils (Jurewicz i col., 2013).

1.5.2 Tècniques d'anàlisi de la fragmentació del DNA espermàtic

1.5.2.1 Terminal transferase dUTP Nick-End Labelling (TUNEL)

La tècnica TUNEL és un mètode àmpliament utilitzat per al marcatge de cèl·lules apoptòtiques i que, per tant, presenten un dany al DNA (Gorczyca i col., 1993). Aquesta tècnica es basa en la incorporació d'oligonucleòtids (dUTP) conjugats amb un fluorocrom, que s'incorporen mitjançant una transferasa terminal (TdT) als extrems 3'OH lliures del DNA conseqüència dels trencaments en aquest (Figura 14). Així doncs, donat que només és necessari un extrem 3'OH lliure al DNA sobre el qual s'addicionen nucleòtids, aquesta tècnica hauria de ser capaç de detectar indistintament trencaments de cadena senzilla i trencaments de cadena doble del DNA (Sergerie i col., 2005). En aquest cas, la intensitat de fluorescència està directament relacionada amb la quantitat de trencaments presents al DNA i es pot observar tant al microscopi de fluorescència en espermatozoides fixats, com quantificar-la amb un citòmetre de flux, fet que augmenta la seva sensibilitat (Domínguez-Fandos i col., 2007). Tot i això, sembla necessari estandarditzar la tècnica, ja que petits canvis en la metodologia i en l'anàlisi donen lloc a variacions del resultat de fragmentació (Muratori i col., 2008; Mitchell i col., 2011). Així mateix, aquesta tècnica també es pot aplicar a talls de biòpsia testicular, permetent observar *in situ* possibles apoptosi en el procés d'espermatogènesi.

Finalment, en mostres de semen, s'obté el percentatge d'espermatozoides amb fragmentació del DNA respecte el total (%SDF). Aquest percentatge d'espermatozoides amb fragmentació s'ha relacionat amb la infertilitat masculina, havent-hi diverses publicacions al respecte (Chohan i col., 2006; Lewis, 2007; Mehdi i col., 2009; Sharma i col., 2010, 2013; García-Peiró i col., 2012; Perrin i col., 2013), i algunes d'elles establint

valors llindar al voltant del 20%-25% per a la distinció fèrtil-infèrtil (Sergerie i col., 2005; Sharma i col., 2010).

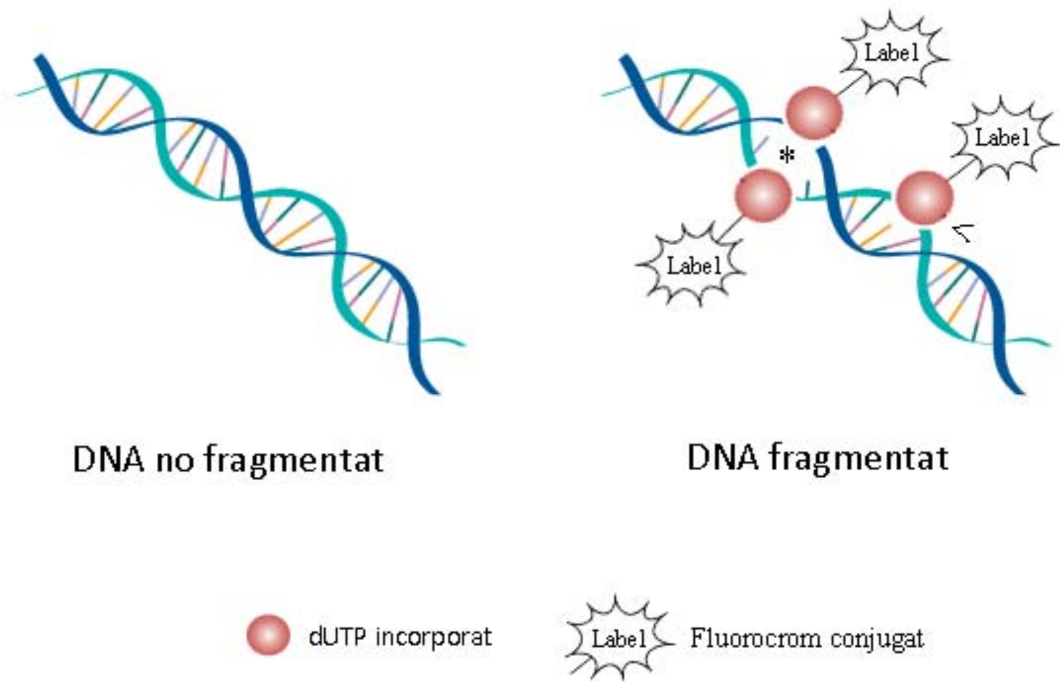


Figura 14. Esquema de funcionament a nivell molecular de la tècnica TUNEL: en els extrems 3’ del DNA que presenta un trencament s’incorpora un nucleòtid (dUTP) marcat amb un fluorocrom. D’aquesta manera, es poden detectar indistintament trencaments de cadena doble del DNA (*), i trencaments de cadena senzilla del DNA (<).

1.5.2.2 Sperm Chromatin Structure Assay (SCSA)

La tècnica SCSA determina la susceptibilitat del DNA a la desnaturalització després d'un tractament a pH àcid. Llavors, com més nombre de trencaments presents al DNA i més alteracions a la cromatina existeixin, més sensible serà el DNA a la desnaturalització. Per a la mesura d'aquesta susceptibilitat a desnaturalització s'aprofita la propietat metacromàtica del colorant taronja d'acridina (Evenson i col., 1980; Evenson, 2013). Aquest colorant s'intercala en el DNA de cadena doble en forma de monòmer, emetent en una longitud d'ona verda (510 - 515 nm) o bé s'agrega en el DNA de cadena senzilla emetent en una longitud d'ona vermella (630 nm). Així doncs, espermatozoides amb una major emissió de color vermell indicaran un major nombre d'alteracions a la cromatina i, indirectament, un major nombre de trencaments al seu DNA (Figura 15). Un paràmetre addicional que ofereix aquesta tècnica és el percentatge d'espermatozoides immadurs, que presenten una pobre compactació del DNA. Aquests espermatozoides mostren un major grau de fluorescència en el verd donat que el taronja d'acridina pot accedir a parts més internes del DNA espermàtic (Evenson i Wixon, 2006).

Aquests percentatges de fluorescència en el verd i el vermell es quantifiquen mitjançant un citòmetre de flux, permetent extrapolar el percentatge d'espermatozoides amb fragmentació del DNA (%SDF) i el percentatge d'immadurs (%HDS). Diversos treballs han estudiat la capacitat de predicció d'infertilitat d'aquesta tècnica, situant el valor llindar al voltant del 30% de fragmentació del DNA (Evenson i Wixon, 2006). Tot i això, diferents treballs mostren diferents valors llindar amb una variabilitat entre el 20% i el 40% (Evenson i Jost, 2000; Evenson i col., 2002; Larson-Cook i col., 2003; Bungum i col., 2004; Payne i col., 2005; Boe-Hansen i col., 2006;

Venkatesh i col., 2011; Evenson, 2013). A més, s'ha trobat relació d'alts percentatges d'espermatozoides immadurs amb pacients afectats de varicocele (García-Peiró i col., 2012).

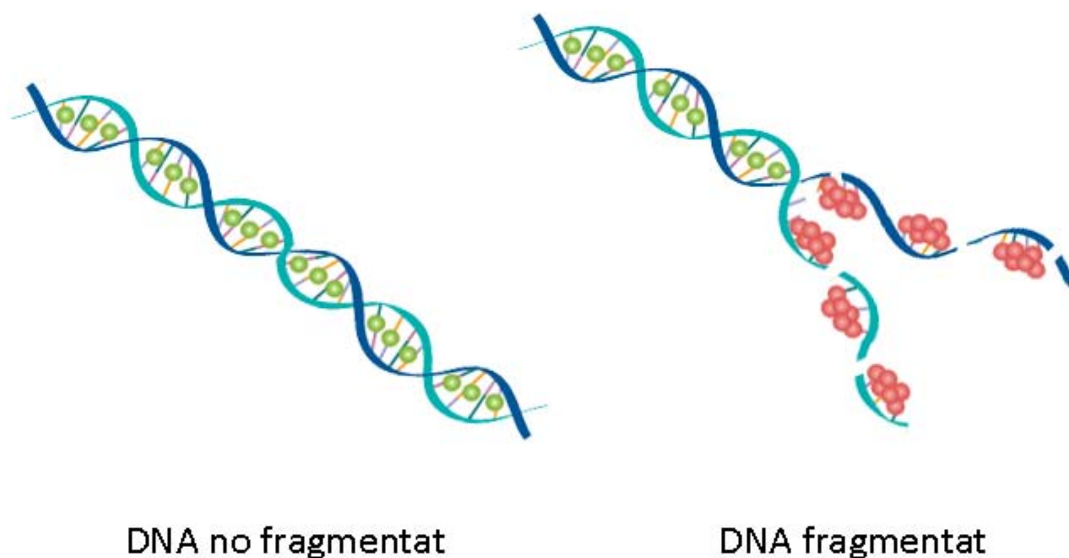


Figura 15. Esquema de funcionament a nivell molecular de la tècnica SCSA: quan el DNA no presenta alteracions, no es desnaturalitza en tractament àcid, el taronja d'acridina s'uneix en monòmers, donant una emissiófluorescent en verd. Alternativament, quan el DNA presenta trencaments és més susceptible a la desnaturalització àcida. Llavors, el taronja d'acridina s'uneix en forma d'agregats, emetent en vermell.

1.5.2.3 Sperm Chromatin Disperson test (SCD)

El test de la dispersió de la cromatina o SCD test es basa en una desnaturalització àcida seguida d'una descompactació del DNA espermàtic mitjançant una extracció de les protamines i les histones, de manera que la cromatina forma un halo de dispersió al voltant de la matriu proteica del cap de l'espermatozoide, que està inclòs dins una matriu d'agarosa. En el cas de presentar trencaments, el DNA es dispersa formant un halo molt més gran, que no pot ser detectat amb la resolució del microscopi òptic, així doncs, en espermatozoides amb fragmentació del DNA, s'observa el nucli espermàtic sense halo (Figura 16) (Fernández i col*.*, 2005).

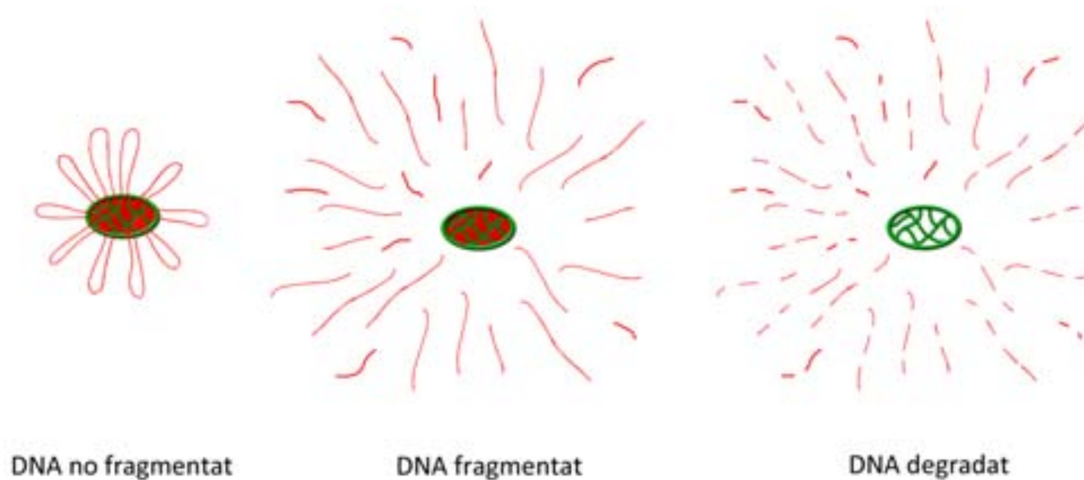


Figura 16. Esquema de funcionament a nivell molecular de la tècnica SCD: els espermatozoides no fragmentats presenten un halo de dispersió al voltant del nucli espermàtic. Els espermatozoides amb fragmentació del DNA presenten un halo molt extens, no sent visible per microscòpia òptica, així, s'observa un nucli espermàtic fortament tenyit. Els espermatozoides amb una alta degradació nuclear són similars als fragmentats, però presenten una tinció no homogènia del nucli de l'espermatozoide.

Un paràmetre addicional que ofereix aquesta tècnica és la capacitat de distingir espermatozoides amb una alta degradació del DNA nuclear, els quals no presenten halo i, a més, presenten un nucli amb una tinció pobre i no homogènia, evidenciant una extensiva degradació del DNA (Enciso i col., 2006; García-Peiró i col., 2012; Gosálvez i col., 2013).

Aquesta tècnica d'anàlisi s'ha utilitzat per a la detecció de dany al DNA espermàtic humà (Fernández i col., 2005; Gallegos i col., 2008; Abad i col., 2013) i també de diversos animals (Johnston i col., 2007; Cortés-Gutiérrez i col., 2009; López-Fernández i col., 2009, 2010; Portas i col., 2009). Així doncs, s'han establert valors llindar de fragmentació del DNA al voltant del 25-30% per a infertilitat masculina (Fernández i col., 2005; Velez de la Calle i col., 2008; Nuñez-Calonge i col., 2012).

1.5.2.4 Assaig Comet

L'assaig Comet és una tècnica molt emprada tradicionalment en cèl·lules somàtiques per a estudis toxicològics (Shaposhnikov i col., 2008), i que va ser adaptada a espermatozoides per Singh i col., 1988. Tot i així, es tracta d'una tècnica no estandarditzada, ja que existeixen multitud de protocols que difereixen entre laboratoris, bàsicament en la lisi dels espermatozoides i en els temps d'electroforesi (Singh i col., 1988; Enciso i col., 2009; Villani i col., 2010; Simon i col., 2011; Kumar i col., 2013). L'assaig Comet es basa en una lisi de les cèl·lules espermàtiques incloses en una matriu d'agarosa, la qual allibera el DNA de les seves proteïnes annexes i permet que pugui migrar en un camp electroforètic. Així, les regions on el DNA presenti trencaments, aquest migrarà formant una cua amb aspecte de cometa, la grandària de la qual estarà en funció del grau de fragmentació del DNA. Aquesta electroforesi es pot

realitzar en condicions neutres o en condicions de desnaturalització alcalina. En la primera variant, el DNA roman en forma de cadena doble i, per tant, la tècnica reflectirà trencaments de cadena doble del DNA, tot i que alguns autors han apuntat a la possibilitat de que el DNA de la cua del cometa correspongui a fibres de cromatina amb un sol trencament en un extrem (Shaposhnikov i col., 2008; Kaneko i col., 2012). En el cas de realitzar una desnaturalització alcalina, el DNA restarà en forma de cadena senzilla durant l'electroforesi i la tècnica reflectirà trencaments de cadena senzilla del DNA (Figura 17) (Simon i Carrell, 2013).

La valoració de la fragmentació del DNA es pot expressar com a percentatge d'espermatozoides amb fragmentació de cadena senzilla del DNA (%ssSDF) i com percentatge d'espermatozoides amb fragmentació de cadena doble del DNA (%dsSDF). Tot i que el percentatge de fragmentació permet la comparació amb altres mètodes, una anàlisi de l'assaig Comet a través de programes informàtics especialitzats pot oferir diferents mesures com per exemple llargada de la cua del cometa, percentatge de DNA a la cua, relació entre llargada de cua i diàmetre del cap, etc. Tant el percentatge d'espermatozoides com alguns d'aquests paràmetres proporcionats per l'anàlisi computeritzat del Comet han estat estudiats per diferents autors, mostrant una aplicabilitat del Comet alcalí en el diagnòstic clínic de la infertilitat (Hussein i col., 2008; Dobrzynska i col., 2010; Lewis i Simon, 2010; Simon i col., 2010, 2011, 2013; Kumar i col., 2011). El Comet neutre, en canvi, ha estat menys estudiat, i no existeixen resultats en relació a la fertilitat masculina.

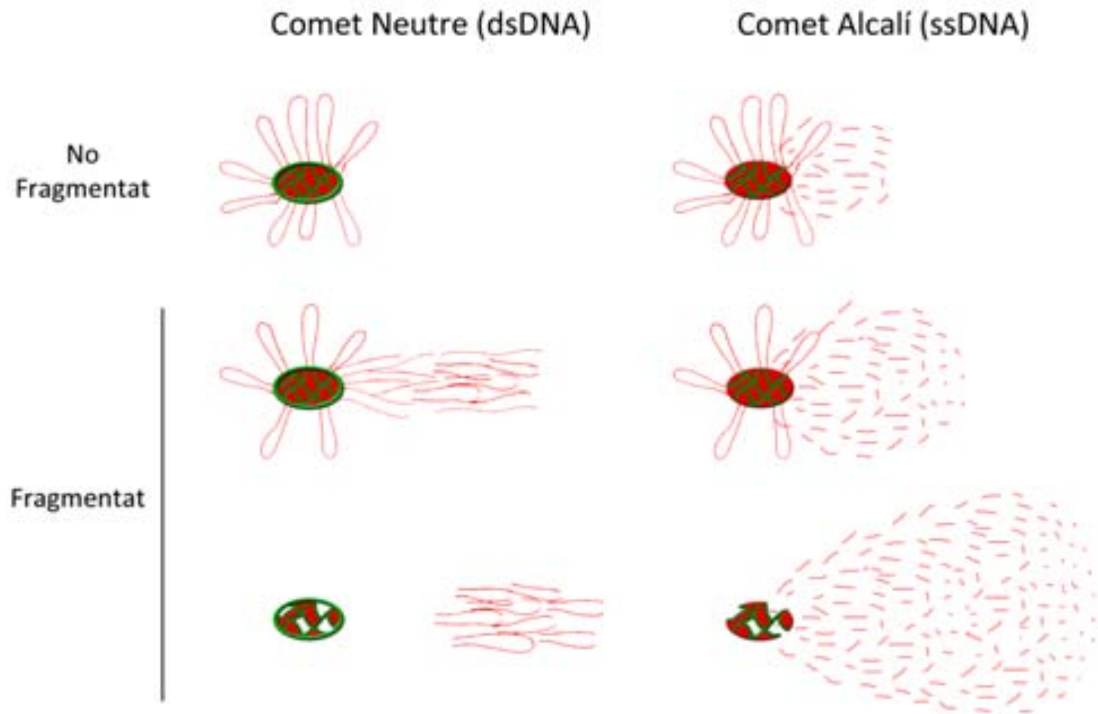


Figura 17. Esquema de funcionament a nivell molecular del Comet assay: quan al Comet neutre, en espermatozoides no fragmentats s'observa un halo de dispersió. Aquest halo de dispersió disminueix a favor d'un increment del DNA a la cua del cometa a mesura que hi ha un increment en els trencaments al DNA espermàtic. En el Comet alcalí, en canvi, espermatozoides sense fragmentació presenten una cua de cometa estructural corresponent al trencament de les regions alcalí-làbils en la desnaturalització del DNA. Aquesta cua s'incrementa en cas de ser-hi presents trencaments en el DNA espermàtic.

1.5.2.5 Gels Camp polsant

L'electroforesi en gels d'agarosa es basa en el moviment del DNA mitjançant un camp elèctric a través d'una matriu d'agarosa. D'aquesta manera, el DNA es separa en funció de la seva mida. L'electroforesi en gels d'agarosa convencional pot resoldre correctament fragments de 10-15 kb o menys. Així doncs, per a separar fragments més grans de DNA són necessàries variacions de la direcció del camp elèctric. Els gels d'agarosa en camp polsant es basen en variar la direcció del camp elèctric periòdicament, generalment en angles de 120º (Figura 18), per a aconseguir un augment de resolució. Així, la tècnica d'electroforesi en camp polsant és més adequada separar molècules grans de DNA.

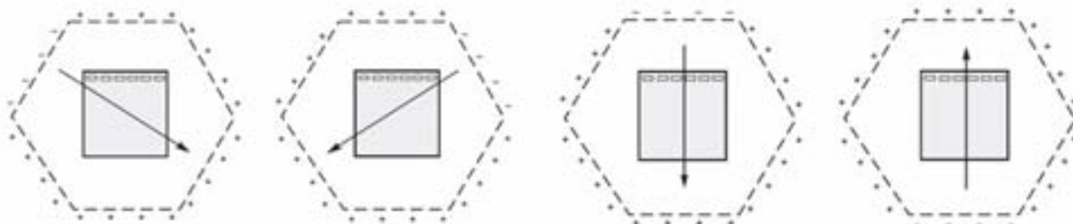


Figura 18. Esquema de la variació de la direcció del camp elèctric en l'electroforesi de camp polsant (Adaptada de: AES, Electrophoresis Society).

Aquesta tècnica s'ha utilitzat poc dins l'àmbit de l'anàlisi de la fragmentació del DNA, tot i això, alguns treballs han demostrat que en presència de manganès i calci es produeixen trencaments a les regions d'unió a la matriu nuclear d'espermatozoide de ratolí, que podrien estar relacionats amb alteracions citogenètiques en les primeres etapes embrionàries (Yamauchi i col., 2007a, 2007b; Gawecka i col., 2013).

1.6 L'anàlisi del DNA espermàtic per grups clínics

Dins aquest marc d'afectació del DNA espermàtic, l'estudi de diferents grups de pacients, tenint en compte les seves característiques clíniques, presenta un gran interès de cara al desenvolupament de noves eines diagnòstiques, ja sigui per al diagnòstic de la infertilitat masculina, o per a la investigació de l'etiologia d'aquesta infertilitat. En aquest sentit, un gran nombre de treballs han demostrat l'associació entre la fragmentació del DNA espermàtic no només amb la infertilitat, sinó també amb avortaments de repetició i fallades de les tècniques de reproducció assistida (Evenson i Wixon, 2006; Bungum i col., 2007; Lewis i Simon, 2010; Brahem i col., 2011; Venkatesh i col., 2011; Zini, 2011). A més, diferents estudis també han demostrat una associació amb la presència d'alteracions com ara afectació en els paràmetres del seminograma (Saleh i col., 2002b), varicocele (Baker i col., 2013; Smit i col., 2013) o reorganitzacions cromosòmiques (Perrin i col., 2009, 2011; Brugnon i col., 2010). Tot i això, el grau de fragmentació del DNA difereix lleugerament en funció de la tècnica utilitzada, i per això és necessària una comparativa entre les tècniques més tradicionals, com ara el TUNEL i l'SCSA i les més innovadores com és el Comet. En aquest sentit, una exhaustiva caracterització de la fragmentació del DNA en pacients segons la seva condició clínica pot ajudar a entendre millor els diferents mecanismes que tenen lloc en la fragmentació del DNA i, a més, millorar el diagnòstic, pronòstic i tractament de les parelles en qüestió.

2. HIPÒTESI i OBJECTIUS

2.1 Hipòtesi

La relació entre la fragmentació del DNA i la infertilitat masculina, demostrada en una tesi prèvia del grup, evidencia que l'avaluació de la integritat del DNA espermàtic és una eina d'utilitat en el diagnòstic de la infertilitat masculina.

El present treball pretén donar resposta a la següent hipòtesi: diferents tipus de fragmentació del DNA espermàtic, de cadena senzilla o doble, poden donar lloc a diferents efectes en la infertilitat masculina.

2.2 Objectiu 1: Estudi de les metodologies de fragmentació del DNA

El primer objectiu de la present tesi doctoral es centra en caracteritzar les diferents metodologies utilitzades per a la determinació de la fragmentació del DNA espermàtic per a determinar quina ha de ser la tècnica d'anàlisi d'elecció, i determinar l'efecte de la criopreservació sobre la fragmentació del DNA espermàtic.

2.2.1 Efecte de la criopreservació

Caracterització de l'efecte general de la criopreservació sobre la fragmentació de cadena senzilla del DNA, utilitzant el Comet alcalí, i sobre la fragmentació de cadena doble del DNA, utilitzant el Comet neutre.

Comparació de l'efecte específic en controls de fertilitat provada, en avortaments de repetició sense factor femení i en pacients infèrtils amb seminograma alterat.

2.2.2 Sensibilitat de les tècniques

Avaluació del potencial de les tècniques TUNEL, SCSA, SCD, Comet alcalí i Comet neutre, en relació a la predicció d'infertilitat. Determinació dels seus valors llindar, sensibilitat i especificitat en una mateixa subpoblació de pacients i controls fèrtils analitzats amb totes les tècniques.

2.3 Objectiu 2: Pacients infèrtils i fragmentació del DNA

Caracterització de la fragmentació de cadena senzilla i de cadena doble del DNA en diferents grups de pacients infèrtils, així com la comparació d'aquests amb controls de fertilitat provada, per a poder determinar les possibles etiologies del dany al DNA que poden donar-se en cada grup concret.

2.3.1 Grups de pacients infèrtils amb alteracions conegudes

Comparació de la fragmentació de cadena senzilla i de cadena doble en diferents grups de pacients infèrtils amb diferents alteracions conegudes: a) pacients infèrtils que presenten un seminograma d'astenoteratozoospèrmia; b) pacients infèrtils que presenten un seminograma d'astenoteratozoospèrmia i a més el diagnòstic d'un varicocele clínic; c) pacients infèrtils que presenten un seminograma d'oligoastenoteratozoospèrmia; d) individus portadors de reorganitzacions cromosòmiques; i comparació d'aquests grups amb controls fèrtils amb descendència.

2.3.2 Avortaments de repetició

Caracterització de la fragmentació del DNA de mostres d'avortaments de repetició idiopàtics sense factor femení. Caracterització de l'origen del dany present al DNA. Establiment de valors llindar, sensibilitat i especificitat per a la predicció d'avortament i comparació amb els valors llindar obtinguts per infertilitat.

2.4 Objectiu 3: Tipus de trencaments al DNA espermàtic

Estudi bàsic dels diferents tipus de fragmentació del DNA, principalment aquells que afecten a les regions d'unió a la matriu nuclear espermàtica.

2.4.1 Model *Sperm Chromatin Fragmentation* en ratolí

Determinació mitjançant l'assaig Comet del dany al DNA de regions d'unió a la matriu nuclear induït en espermatozoide de ratolí. Comparació amb perfils de fragmentació de cadena senzilla i doble del DNA espermàtic en diferents patologies humanes.

2.5 Objectiu 4: Relació de la compactació del DNA i la fragmentació del DNA

Estudi de la relació entre el grau de compactació del DNA espermàtic i la integritat del DNA.

2.5.1 Etiologia dels espermatozoides amb alta degradació nuclear

Determinació de l'etiologia de la subpoblació d'espermatozoides amb una elevada degradació del DNA detectats amb la tècnica SCD.

2.5.2 Relació de la compactació i la fragmentació del DNA en grups de pacients

Caracterització de la compactació del DNA en diferents grups de pacients mitjançant la tècnica de la cromomicina. Correlació del grau de compactació amb la fragmentació del DNA present a la mostra de semen.

3. MATERIAL i MÈTODES

3.1 Mostres de semen humanes

3.1.1 Procedència

Per a la consecució dels objectius plantejats ha estat necessària l'obtenció de mostres de semen humanes. Per això, s'han establert diverses col·laboracions amb hospitals públics de l'àmbit sanitari Català. D'una banda, l'Hospital Universitari Parc Taulí de Sabadell, mitjançant el departament d'Urologia del mateix hospital i el centre de diagnòstic UDIAT (Parc Taulí), d'on s'han obtingut la major part d'ejaculats de pacients infèrtils. D'altra banda, s'ha establert una col·laboració amb el departament de Ginecologia de l'Hospital Universitari Mutua de Terrassa, d'on s'han obtingut les mostres d'ejaculat de parelles amb avortaments de repetició sense factor femení conegut. Les mostres d'ejaculat d'individus control de fertilitat provada s'han obtingut a través del banc de semen CEFER i de donants recollits a la pròpia UAB.

3.1.2 Consentiment informat i comitè d'ètica

Els pacients participants en els diferents estudis han signat prèviament un consentiment informat en el qual accedeixen a donar part de la seva mostra per a recerca, que en cap cas ha interferit en els procediments mèdics o diagnòstics de rutina. A més, els procediments duts a terme amb aquestes mostres han estat prèviament aprovats per el comitè d'ètica de la Corporació Sanitària Parc Taulí de Sabadell.

3.1.3 Grups de pacients i controls

Aquesta tesi empra mostres d'ejaculat de pacients de diferents orígens i etiologies. Els diferents pacients han estat caracteritzats atenent al seu seminograma, utilitzant com a referència el manual de l'OMS de l'any 2010 (Taula I). Les mostres d'ejaculat dels diferents individus infèrtils s'han classificat en:

- Seminograma normal: Pacients que presenten una concentració, mobilitat i morfologia espermàtica dins els valors de la normalitat.
- Seminograma Astenozoospèrmic: Pacients que presenten una alteració en la mobilitat espermàtica, però no en la concentració ni en la morfologia.
- Seminograma Teratozoospèrmic: Pacients que presenten una alteració en la morfologia espermàtica, però no en la concentració ni en la mobilitat.
- Seminograma Astenoteratozoospèrmic: Pacients que presenten alteracions de mobilitat i morfologia espermàtica, però no en la concentració d'espermatozoides.
- Seminograma Oligoastenoteratozoospèrmic: Pacients que presenten alteracions en els paràmetres de concentració, mobilitat i morfologia.

A més a més, s'ha inclòs un grup de pacients astenoteratozoospèrmics amb presència de varicocele clínic, prèviament diagnosticat en consulta d'urologia.

Els individus portadors de reorganitzacions cromosòmiques inclosos en els estudis presenten algun tipus de reorganització identificable al analitzar el seu cariotip per bandes G. D'entre aquestes reorganitzacions hi ha 9 individus portadors de translocacions recíproques equilibrades, 1 portador d'una translocació robertsoniana equilibrada, 2 portadors d'una doble translocació equilibrada i 1 portador d'una inversió cromosòmica equilibrada. Tots aquests pacients s'han identificat en acudir a consulta d'andrologia/urologia per infertilitat.

Finalment, també s'ha inclòs un grup de pacients de parelles amb avortaments de repetició. L'historia clínica d'aquestes parelles mostra més de dos avortaments de primer trimestre i, a més, han estat descartats tots els factors de risc d'origen femení potencialment responsables d'aquesta història d'avortaments de repetició.

Els resultats procedents dels estudis de tots aquests grups de pacients s'han comparat sempre amb els d'un grup d'individus amb fertilitat provada, procedents de donants voluntaris i amb descendència confirmada.

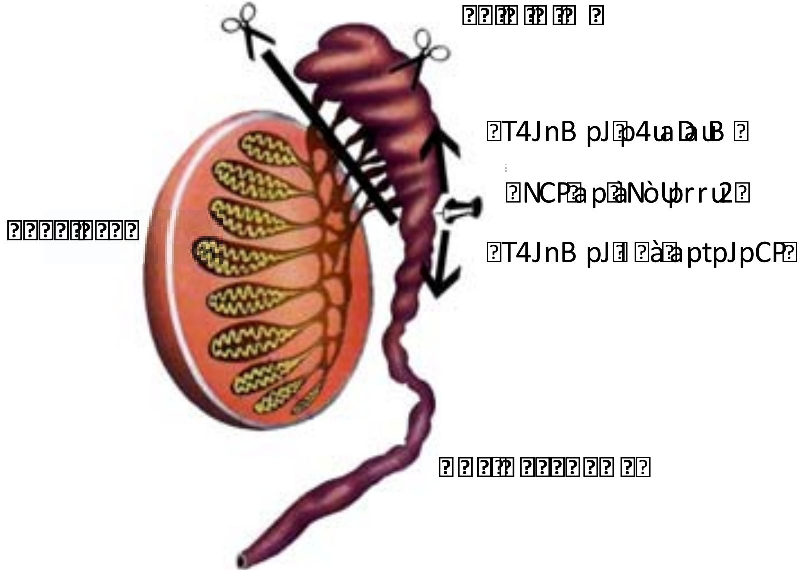
3.1.4 Obtenció de les mostres de semen humà

Les mostres de semen, tant de pacients com de controls fèrtils, s'han obtingut per masturbació i s'han recollit en flascons estèrils després d'un període d'abstinència sexual d'entre tres i set dies. La recollida de les mostres s'ha realitzat als diferents hospitals i centres col·laboradors, o bé al laboratori de la Unitat de Biologia Cel·lular i Genètica Mèdica de la UAB. Totes les mostres s'han estudiat d'acord amb els criteris bàsics del seminograma establerts per l'Organització Mundial de la Salut (WHO, 2010). Posteriorment s'ha procedit a la seva criopreservació per al seu posterior estudi.

3.2 Mostres espermàtiques de ratolí

3.2.1 Procedència i Comitè d'ètica

Els estudis amb animals han estat realitzats a l'Institute for Biogenesis Research (IBR) de la University of Hawaii at Manoa. Ratolins de la soca B6D2F1 s'han obtingut del *National Cancer Institute (Raleigh, NC, USA)* i s'han mantingut al estabulari de l'IBR d'acord amb les normatives del *Laboratory Animal Service i del Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council*. El *Animal Care and Use Committee* de la Universitat de Hawaii ha revisat els protocols de maneig i tractament dels animals en qüestió.



3.3 Criopreservació de les mostres de semen

En cas de no indicar-se el contrari, tots els reactius utilitzats en aquest apartat i els següents provenen de *Sigma Aldrich; St Louis, MO, USA*.

3.3.1 Medi de criopreservació

El medi de criopreservació utilitzat per a les mostres de semen s'ha adaptat d'altres autors (Chernos i Martin, 1989). Aquest medi de criopreservació conté:

- 30 ml de rovell d'ou
- 1,98 g de glucosa
- 1,72 g de citrat sòdic
- 56 ml d'aigua mil·liQ
- 14 ml de glicerol
- 2 g de glicina

Per a preparar aquest medi, se separen les clares del rovell d'ou i s'obtenen 30 ml de rovell d'ou sense el tel que l'envolta. Posteriorment s'homogeneïtzen en un vas de precipitats i s'afegeixen la glucosa, el citrat sòdic, l'aigua mil·liQ i el glicerol. Aquest últim actua com a criopreservant, impedint que durant la congelació es formin petits cristalls d'aigua que trenquin les membranes plasmàtiques de les cèl·lules. Un cop la mescla anterior és homogènia, aquesta s'incuba en un bany a 56ºC durant 30 minuts per inactivar les proteïnes que conté el rovell d'ou. Passat aquest temps, es deixa refredar a temperatura ambient, s'afegeix la glicina i s'ajusta el pH entre 7,2 i 7,4. El medi obtingut es guarda a -20ºC en alíquotes per tal de descongelar-ne la part necessària en el moment de la congelació de la mostra. És aconsellable no utilitzar el mateix medi passats 6 mesos de la seva preparació.

3.3.2 Criopreservació en Test-Yolk buffer

Un cop obtinguda la mostra de semen, aquesta es deixa liquar durant 30 minuts. Passat aquest temps, es mesura la quantitat de semen a congelar amb una pipeta, es transfereix a un tub estèril i s'afegeix la mateixa quantitat del medi de criopreservació al mateix tub. D'aquesta forma s'obté una proporció 1:1 de mostra i medi de criopreservació. Finalment, s'homogeneïtza la mescla i es transfereix en alíquotes de 0,5 ml en criotubs resistents al fred. Aquests tubs es congelen a -80ºC en un bany d'isopropanol per assegurar una rampa de refredament de -1ºC/min durant 6 – 12 hores. Passat aquest temps, es submergeixen els criotubs en un tanc de nitrogen líquid (< -195ºC).

3.3.3 Descongelació i rentat de la mostra de semen

Just abans de realitzar l'anàlisi mitjançant qualsevol de les tècniques emprades, la mostra criopreservada es descongela a temperatura ambient fins que estigui liquada. Immediatament després, es recull el contingut del criotub amb una pipeta, i es mescla en un tub eppendorf amb PBS sense Ca^{2+} ni Mg^{2+} en una proporció 1:3. A continuació, es centrifuga a 400g durant 4 minuts. Es decanta el sobrenedant i es repeteix aquesta operació dues vegades més. Finalment, es resuspèn el botó cel·lular en la solució necessària per cada tècnica, ajustant la concentració d'espermatozoides al valor desitjat.

3.4 Tècniques d'anàlisi del semen

3.4.1 Seminograma

Les mostres de semen humanes s'han caracteritzat tradicionalment mitjançant el seminograma. Aquest estudi permet la seva classificació a través de diferents propietats macroscòpiques i microscòpiques. D'una banda, les propietats macroscòpiques són caracteritzades per l'investigador o tècnic a ull nu. D'entre aquestes, cal destacar el volum de la mostra, la liqüefacció (completa o incompleta), l'aspecte (opac, translúcid o transparent), el color (blanc, blanc-groc) i el pH. D'altra banda, les propietats microscòpiques s'han analitzat mitjançant el programa *Sperm Class Analyzer* (SCA) (Microptic; Barcelona, Espanya) que proporciona dades sobre la concentració d'espermatozoides, la mobilitat distribuïda en quatre categories, i la morfologia, seguint el criteri estricte de Kruger (Kruger i col., 1987).

L'OMS estableix uns valors llindar de normalitat per a totes aquestes característiques del semen, resumits a la Taula I, extreta del *Manual for Semen Analysis* (WHO, 2010):

Taula I. Valors llindar de normalitat segons l'Organització Mundial de la Salut (WHO, 2010)

Paràmetre	*Valor de referència*
Volum (ml)	1,5
pH	≥ 7,2
Concentració d'espermatozoides	15 x 106 espermatozoides/ml
Nombre total d'espermatozoides	39 x 106 espermatozoides
Mobilitat total (a+b)%	32%
Morfologia (% Formes normals)	4%
Vitalitat	58%

3.5 Inducció del dany al DNA *in vitro*

En estudis *in vitro*, la fragmentació del DNA s'ha induït específicament en cada cas segons el seu origen:

3.5.1 Dany per estrès oxidatiu

El dany al DNA per estrès oxidatiu s'ha induït realitzant una incubació amb peròxid d'hidrogen (H_2O_2) a diferents concentracions, bàsicament entre 0,01% a 0,3%.El peròxid d'hidrogen genera trencaments de cadena senzilla del DNA de forma extensiva a l'espermatozoide, tant a les regions MAR com en regions intra-toroïdals.

3.5.2 Dany per activitat nucleasa

El dany al DNA per activitat nucleasa pot actuar únicament a regions del DNA no protegides per protamines, per tant, actua digerint les regions d'unió a la matriu nuclear, trencant així les unions del DNA i la matriu nuclear espermàtica. Al laboratori, aquest s'indueix realitzant incubacions a 37ºC en 0.5 mg/ml de ribonucleasa I de pàncrees boví. Això assegura una completa digestió de les regions inter-toroïdals, mantenint intacte el DNA toroïdal.

3.5.3 Dany a les regions MAR (SCF)

El dany del DNA causat per una incubació amb ions Mn^{2+} i Ca^{2+} produeix un trencament de cadena doble del DNA a la regió d'unió a la matriu nuclear de l'espermatozoide. Aquest model d'inducció, desenvolupat per el grup del Dr. Ward, s'ha aplicat a espermatozoides de ratolí incubant-los en una solució 10 mM $MnCl_2$ i 10 mM $CaCl_2$ durant una hora a 37ºC. Aquest tipus de trencaments reverteixen en incubar la mostra en 100 mM EDTA 30 minuts a 37ºC (Yamauchi i col., 2007b).

3.6 Tècniques d'anàlisi de la fragmentació del DNA espermàtic

Per a l'anàlisi de la fragmentació del DNA espermàtic s'han utilitzat diferents tècniques: l'assaig Comet, TUNEL, SCSA i SCD, a més d'electroforesis en gels de camp polsant (PFGE).

3.6.1 Assaig Comet

L'assaig Comet es basa en una descondensació completa de la cromatina i una electroforesi en condicions neutres o alcalines. Utilitzant condicions neutres, les cues de Comet mostraran espermatozoides amb trencaments de cadena doble del DNA, mentre que en condicions alcalines es podran avaluar trencaments de cadena senzilla del DNA (Figura 20).

3.6.1.1 Solucions necessàries

- Portaobjectes tractats amb una capa d'agarosa de baix punt de fusió: es submergeixen portaobjectes en una solució d'agarosa de baix punt de fusió al 1% en aigua mil·liQ, s'eixuguen amb un paper suau i es deixen assecar a l'aire sobre un paper de filtre.
- Solució d'agarosa de baix punt de fusió al 1% en aigua mil·liQ.
- Solució PBS 1x: A partir de la solució de PBS 10x preparada al laboratori es realitza una dilució 1:10 en aigua mil·liQ. La solució de PBS 10x conté 1,37M NaCl, 27mM KCl, 100mM $Na_2HPO_4 \cdot 2H_2O$, 20mM KH_2PO_4 ajustada a pH 7,4.
- Solució TBE 1x: Es realitza una dilució 1:10 en aigua mil·liQ a partir de la solució de TBE 10x (Bio-Rad; Hercules, CA, USA).
- Solució de Lisi 1: Es prepara una solució en aigua mil·liQ contenint 0,4M Tris-HCl, 0,8M DTT, 1% SDS ajustada a pH 7,5.

- Solució de Lisi 2: Es prepara una solució en aigua mil·liQ contenint 0,4M Tris-HCl, 0,4M DTT, 2M NaCl, 50mM EDTA ajustada a pH 7,5.
- Solució NaCl 0,9%: Es prepara una solució de NaCl al 0,9% en aigua mil·liQ pesant 4,5 grams de NaCl per 500 ml d'aigua.
- Solució de neutralització: Es prepara una solució en aigua mil·liQ contenint 0,4M Tris-HCl ajustant el pH a 7,5.
- Solució alcalina freda: Es prepara una solució en aigua mil·liQ contenint 30mM NaOH i 1M NaCl. El pH d'aquesta solució queda ajustat al barrejar exactament aquestes quantitats. Aquesta solució es guarda a 4ºC ja que s'ha d'utilitzar en fred.
- Solució d'electroforesi alcalina: Per a preparar una solució 10x es fa una dissolució 300mM NaOH en aigua mil·liQ. Per a obtenir la solució 1x es realitza una dilució 1:10 en aigua mil·liQ.
- Etanols al 70%, 90% i 100%.

3.6.1.2 Protocol

Un detall rellevant és que totes les incubacions que es realitzen en el protocol del assaig Comet es duen a terme amb el portaobjectes en posició horitzontal.

Preparació de la mostra i lisi dels espermatozoides

Primer de tot, s'ajusta la concentració de la mostra al voltant de 2 milions d'espermatozoides per ml amb una cambra de Neubauer o bé observant uns 4 o 5 espermatozoides per camp microscòpic a 400 augments. Un cop ajustada aquesta concentració es liqua una alíquota d'agarosa de baix punt de fusió i es barregen 25 μl de la mostra amb 50 μl d'agarosa de baix punt de fusió, temperada a 37ºC. Ràpidament, es posen entre 10 μl i 15 μl d'aquesta barreja sobre dos portaobjectes

tractats amb agarosa i es cobreixen amb dos cobreobjectes. Un d'aquests portaobjectes serà destinat al Comet neutre i l'altre al Comet alcalí.

Es deixa gelificar l'agarosa sobre una placa metàl·lica a 4ºC durant 5 minuts i posteriorment es retiren els cobreobjectes suaument. Immediatament després i evitant que l'agarosa s'assequi, es submergeixen els portaobjectes en les solucions de lisi 1 i 2 durant 30 minuts cada una. La solució de lisi 1 provoca una digestió de les membranes plasmàtiques de l'espermatozoide mitjançant el SDS i una reducció dels ponts disulfur presents a les protamines que empaqueten el DNA. El NaCl present a la solució de lisi 2 neutralitza la unió de les protamines amb el DNA, de manera que aquestes queden en solució, deixant el DNA nu i formant un halo de dispersió al voltant del nucli o *core*.

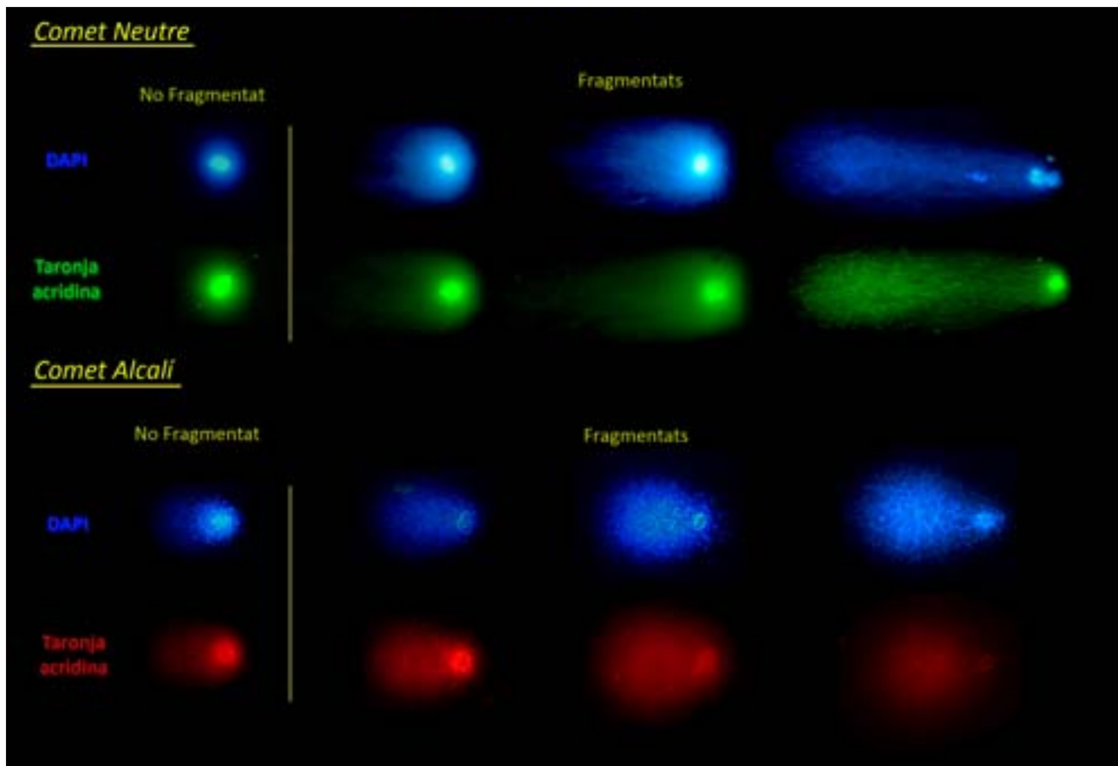
Posteriorment a la incubació en les dues solucions de lisi, es realitza un rentat amb TBE durant 10 minuts.

Electroforesi

L'etapa d'electroforesi difereix segons si es realitza en condicions neutres o bé amb una desnaturalització alcalina. En aquest punt, el portaobjectes destinat a cada tipus de Comet rep un tractament diferenciat:

Per al Comet neutre, es realitza una electroforesi en tampó salí TBE a 1V/cm durant 12,5 minuts en una cubeta Horizon (GE Healthcare; Uppsala, Sweden). Posteriorment es submergeix el portaobjectes en NaCl 0,9% durant 2 minuts.

Per al Comet alcalí, es realitza una incubació en la solució alcalina freda durant 2,5 minuts a 4ºC per a desnaturalitzar el DNA, i posteriorment es realitza l'electroforesi utilitzant com a tampó salí la solució d'electroforesi alcalina, a 1V/cm durant 4 minuts.



Comet Neutre
No Fragmentat
Fragmentats
DAPI
Taronja acridina
Comet Alcalí
No Fragmentat
Fragmentats
DAPI
Taronja acridina

criteris establerts a la Figura 20 (Ribas-Maynou i col., 2012a). D'aquí s'obté el percentatge d'espermatozoides amb fragmentació del DNA. Donades les possibilitats d'anàlisi del assaig Comet, per a l'objectiu 3, s'ha analitzat la llargada de les cues i el diàmetre mitjà del halo, utilitzant el programa informàtic *CometScore 1.0 (TriTek; Sumerduck, VA, USA*), establint així una relació entre aquests paràmetres.

3.6.2 TUNEL (Terminal transferase dUTP Nick-End Labelling)

La tècnica TUNEL utilitzada en aquest estudi ha estat mitjançant el kit *In Situ Cell Death Detection*, comercialitzat per Roche (Roche Diagnostic Gmbh, Penzberg, Alemanya).

Aquesta tècnica detecta de manera directa els trencaments en el DNA a través de nucleòtids marcats, que s'uneixen al DNA fragmentat mitjançant un enzim transferasa. A causa de que la intensitat de la fluorescència que es dóna en un sol espermatozoide depèn directament de la quantitat de trencaments que existeixen al DNA d'aquella cèl·lula, l'anàlisi de la fragmentació del DNA en aquesta tècnica es pot realitzar tant amb un microscopi de fluorescència com amb un citòmetre de flux, obtenint en ambdós casos un percentatge d'espermatozoides amb fragmentació respecte el total d'espermatozoides de la mostra.

3.6.2.1 Solucions necessàries

- Solució PBS 1x: A partir de la solució de PBS 10x preparada al laboratori es realitza una dilució 1:10 en aigua mil·liQ. La solució de PBS 10x conté 1,37M NaCl, 27mM KCl, 100mM $Na_2HPO_4 \cdot 2H_2O$, 20mM KH_2PO_4 ajustada a pH 7,4.
- Solució de PBS suplementada amb 1% d'albúmina de sèrum bovina (BSA).
- Solució de Fixació: Es prepara una solució al 4% de para-formaldehid en PBS ajustant el pH a 7,4.

- Solució de permeabilització: Es prepara una solució al 0,25% de Triton-X100 en aigua mil·liQ.
- Solució de marcatge proporcionada per el kit de Roche.
- Enzim transferasa terminal proporcionada per el kit de Roche.

3.6.2.2 Protocol

Preparació de la mostra

Després de la descongelació de la mostra, es realitzen dos rentats addicionals amb PBS suplementat amb 1% de BSA centrifugant a 400g durant 4 minuts. L'albúmina de sèrum bovina redueix la quantitat d'espermatozoides que es perden en cada centrifugació. Aquests rentats es realitzen en fred per a prevenir la generació de nous trencaments al DNA, ja que la mostra encara no està fixada.

Fixació

Posteriorment, es resuspèn el botó de la mostra en para-formaldehid al 4% i s'incuba durant una hora a temperatura ambient per a fixar les cèl·lules. Després d'aquesta fixació es realitzen dos rentats en PBS/1%BSA a 400g durant 4 minuts per a eliminar les restes de para-formaldehid.

Permeabilització

Es procedeix a la permeabilització dels espermatozoides incubant el botó de la mostra en 100 μl de solució de permeabilització durant 2 minuts a temperatura ambient. A continuació, es realitzen dos rentats amb PBS/1%BSA per a eliminar el Tritón-X100

Reacció TUNEL, controls positiu i negatiu

En l'últim rentat, es divideix la mostra en dos tubs Eppendorf, un destinat a ser control negatiu, sense enzim, i l'altre amb la mostra problema, amb l'enzim. Així doncs, es

resuspenen els dos botons obtinguts en 45 ml de la solució de marcatge. Aquesta solució és altament tòxica, per tant, cal seguir totes les instruccions del fabricant tant per a la manipulació com per a la gestió dels residus.

El tub destinat a ser la mostra problema serà en el que es donarà la reacció amb la transferasa terminal, així doncs, s'afegeixen 5 μl d'aquest enzim i s'incuba durant 1 hora a 37ºC.

En el tub destinat a control negatiu, s'omet aquest darrer pas i no s'inclou enzim, per així poder establir el llindar de soroll de fons en l'anàlisi.

Rentats

Finalment, es realitzen tres rentats amb PBS/1%BSA centrifugant a 400g durant 4 minuts, per a reduir el soroll de fons donat per unions inespecífiques del marcatge a les membranes de l'espermatozoide i a altres estructures.

3.6.2.3 Anàlisi amb citometria de flux

La mostra es dilueix fins a 500 μl en PBS, i es transfereix a un tub de citòmetre, mantenint-se en la foscor fins al moment de l'anàlisi. Per a l'anàlisi s'utilitza un citòmetre de flux (FACSCalibur; Becton Dickinson; Franklin Lakes, NJ, USA), i el programa d'anàlisi CELLQUEST (Becton Dickinson; Franklin Lakes, NJ, USA). En cada mostra es comptabilitzen 10.000 espermatozoides analitzant la seva fluorescència (FITC) amb el filtre de 530nm a una velocitat d'entre 200 i 300 espermatozoides per segon.

Per a l'anàlisi, s'estableix el valor de fluorescència de fons utilitzant el control negatiu sense enzim, i a partir d'aquest valor llindar, s'obté el percentatge d'espermatozoides que són positius per la reacció TUNEL en la mostra problema, tal i com mostra la Figura 21.

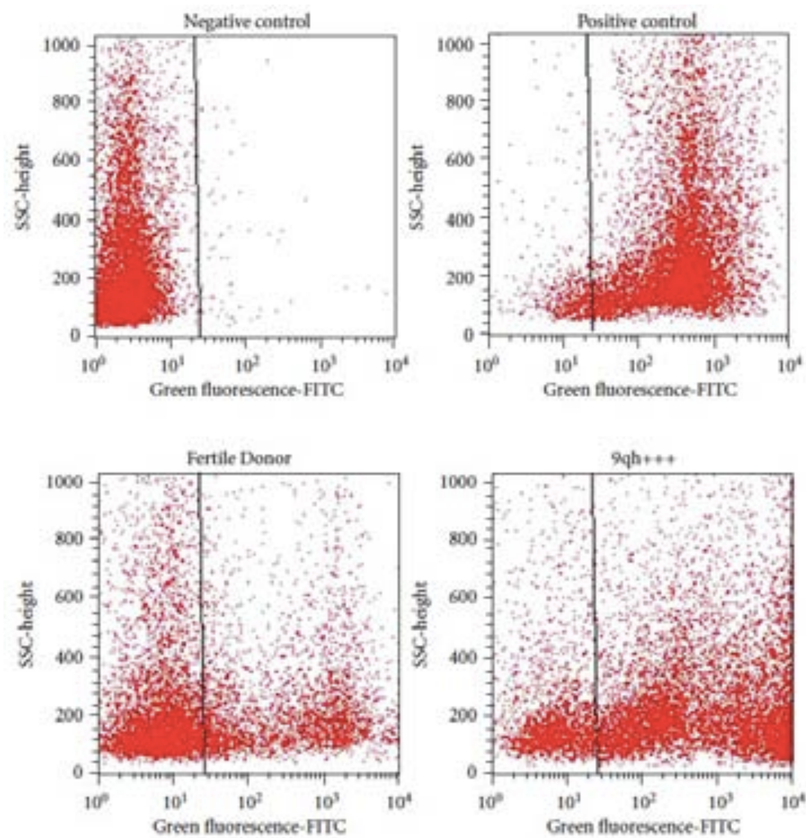


Figura 21. Citogrames obtinguts al analitzar per TUNEL un control negatiu, control positiu, un donant fèrtil, i un portador d'un polimorfisme 9qh+++ (García-Peiró i col., 2011).

3.6.2.4 Anàlisi amb microscopi de fluorescència

La mostra es dilueix amb 100 µl de PBS i es realitza una extensió sobre un portaobjectes, que es deixa assecar a la campana d'extracció de gasos. Posteriorment, s'afegeix un marcatge de contrast com DAPI SlowFade Gold antifade (Invitrogen; Eugene, OR, USA) i s'observa al microscopi de fluorescència. Utilitzant els filtres verd i blau es podran observar les cèl·lules TUNEL positives, marcades en verd i en blau, i les cèl·lules TUNEL negatives, marcades únicament en blau.

3.6.3 SCSA (Sperm Chromatin Structure Assay)

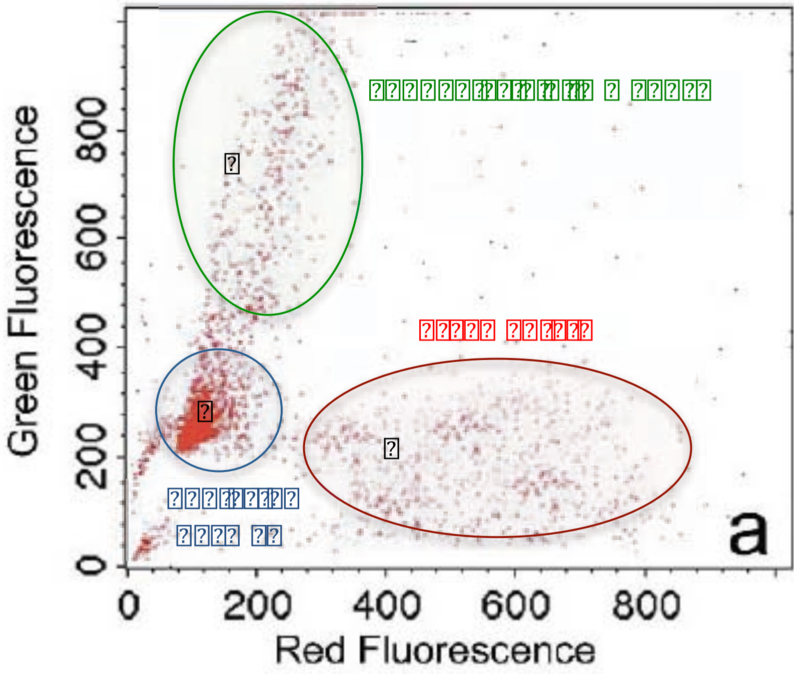
La tècnica SCSA mesura les diferents emissions en el verd i el vermell que provoca la tinció amb taronja d'acridina, un colorant metacromàtic que emet diferencialment si s'intercala al DNA de cadena doble (verd) o si s'agrega al DNA de cadena senzilla (vermell). La capacitat de l'espermatozoide a desnaturalitzar-se es relaciona amb el grau de fragmentació del DNA quantificant aquestes emissions al citòmetre de flux comptabilitzant 5.000 cèl·lules.

3.6.3.1 Solucions necessàries

- Solució tampó/salina TNE: Es prepara una solució contenint 10mM Tris-HCl, 150mM NaCl, 1mM EDTA en aigua mil·liQ, ajustant el pH final a 7,4.
- Solució de tinció: Es prepara una solució contenint 37mM d'àcid cítric, 125mM de Na_2HPO_4, 150mM NaCl i 1mM EDTA en aigua mil·liQ, ajustant el pH a 6.
- Solució de tinció de taronja d'acridina: A partir d'una solució de taronja d'acridina a 1mg/ml, es fa una dilució en proporcions 6:1 d'aquesta en solució de tinció.
- Solució àcid-detergent: Es prepara una solució contenint 80mM HCl, 150mM NaCl, 0.1% Triton X-100 en aigua mil·liQ, ajustant el pH a 1,2.
- Solució de tinció: En el moment de realitzar la tècnica s'han d'afegir 6 ml de la solució de tinció per ml de TNE.

3.6.3.2 Protocol

Es renta la mostra amb el procediment habitual de descongelació i es fan dos rentats addicionals en solució tampó TNE. Un cop rentada la mostra, s'ajusta la concentració d'espermatozoides sobre els 5 milions per mil·lilitre. Llavors, s'afegeixen 400 μl de la



Green Fluorescence
0
200
400
600
800
Red Fluorescence
0
200
400
600
800
a

3.6.4 SCD (Sperm Chromatin Dispersion test)

El test de la dispersió de la cromatina espermàtica es basa en la formació d'un halo de dispersió, la mida del qual depèn de si existeixen més o menys trencaments al DNA. En cas que existeixin molts trencaments, l'halo format és tan gran que no s'aprecia material nuclear tenyit al microscopi. En cas que el DNA no estigui fragmentat, es pot observar un halo de dispersió corresponent a DNA nuclear al voltant del cap de l'espermatozoide. El test SCD, que va ser descrit per Fernández i col., 2005, es realitza utilitzant un kit comercialitzat per l'empresa Halotech (Madrid, Espanya).

3.6.4.1 Solucions necessàries

- Portaobjectes tractats amb una capa d'agarosa de baix punt de fusió: Es submergeixen portaobjectes en una solució d'agarosa de baix punt de fusió al 1% en aigua, s'eixuguen amb un paper suau i es deixen assecar a l'aire sobre un paper de filtre.
- Solució d'agarosa de baix punt de fusió al 1% en aigua mil·liQ.
- Solució àcida: Àcid clorhídric concentrat. Al moment d'utilització cal diluir 80μl en 10 ml d'aigua mil·liQ.
- Solució de lisi: Solució que conté DTT i NaCl en una concentració que no descriu el fabricant.

A més, és necessari disposar de:

- Solució PBS 1x: A partir de la solució de PBS 10x preparada al laboratori es realitza una dilució 1:10 en aigua mil·liQ. La solució de PBS 10x conté 1,37M NaCl, 27mM KCl, 100mM $Na_2HPO_4 \cdot 2H_2O$, 20m M KH_2PO_4 ajustada a pH 7,4.
- Etanol al 70%, 90% i 100%.

3.6.4.2 Protocol

Després de realitzar el rentat de la mostra, s'ajusta la concentració d'aquesta en PBS al voltant de 6 milions d'espermatozoides per mil·lilitre. Posteriorment, es barregen 25 μl de la mostra amb 50 μl d'agarosa de baix punt de fusió prèviament liquada. D'aquesta mescla, es col·loquen 15 μl en un portaobjectes tractat amb agarosa i es deixa gelificar 5 minuts a 4ºC cobert amb un cobreobjectes. Un cop l'agarosa està gelificada, es retira suaument el cobreobjectes i s'incuba la mostra 7 minuts amb la solució àcida amb el fi de desnaturalitzar el DNA. Passat aquest temps la mostra s'incuba en la solució de lisi durant 25 minuts, procés en el qual s'extrauran les histones i les protamines que compacten el DNA espermàtic. Finalment, la mostra es renta 5 minuts amb aigua destil·lada i es deshidrata amb series d'etanol 70%, 90% i 100% durant dos minuts cada un.

3.6.4.3 Anàlisi

La tinció dels portaobjectes es realitza utilitzant un agent intercalant del DNA, en aquest cas s'utilitza *DAPI SlowFade Gold antifade* (Invitrogen; Eugene, OR, USA), que conté un agent protector de la fluorescència.

S'analitzen 400 espermatozoides classificant-los en no fragmentats, que presenten un halo de dispersió; fragmentats, que no presenten un halo de dispersió i el nucli està uniformement tenyit; i degradats, que no presenten halo de dispersió i el nucli presenta una tinció no uniforme (Figura 23).

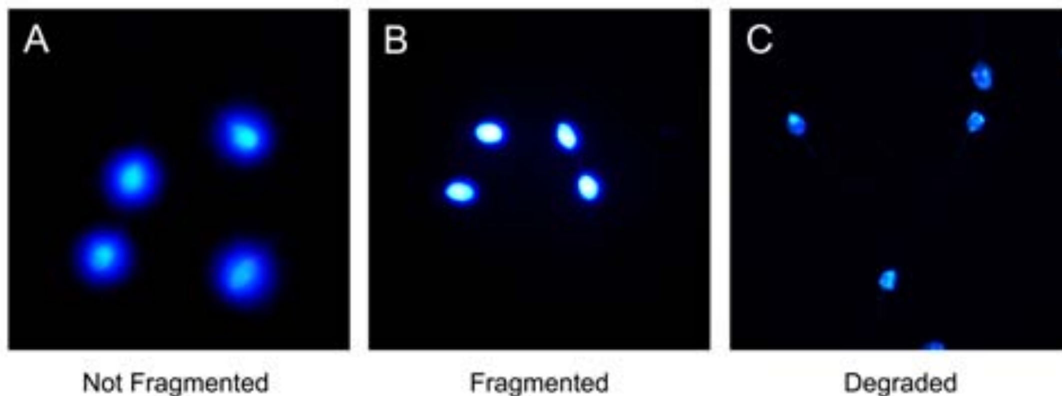


Figura 23. Criteris de classificació dels espermatozoides en la tècnica *Sperm Chromatin Dispersion* segons: A) no fragmentats; B) fragmentats i C) degradats.

3.6.5 PFGE (Pulsed-Field Gel Electrophoresis)

La tècnica d'electroforesi en camp polsant es basa en una extracció del DNA espermàtic que avança en el camp elèctric. Com qualsevol tècnica d'electroforesi, els fragments més petits avançen més ràpidament i, en aquest cas, l'electroforesi de camp polsant s'utilitza per a una millor resolució en separar fragments de mides més grans de 10 kb.

3.6.5.1 Solucions necessàries

- Solució d'agarosa de baix punt de fusió al 2% en TBE.
- Solució TBE 1x: Es realitza una dilució 1:10 en aigua mil·liQ a partir de la solució de TBE 10x (Bio-Rad; Hercules, CA, USA).
- Solució de digestió: Es prepara una solució contenint 10 mM Tris, 50 mM DTT, 100 mM NaCl, 5 mM EDTA i 0.5% SDS en aigua mil·liQ, i s'ajusta el pH a 7,5.
- *CHEF pulsed field plugs mold.* Motlles per a la realització de *plugs* per a camp polsant (Bio-Rad; Hercules, CA, USA).

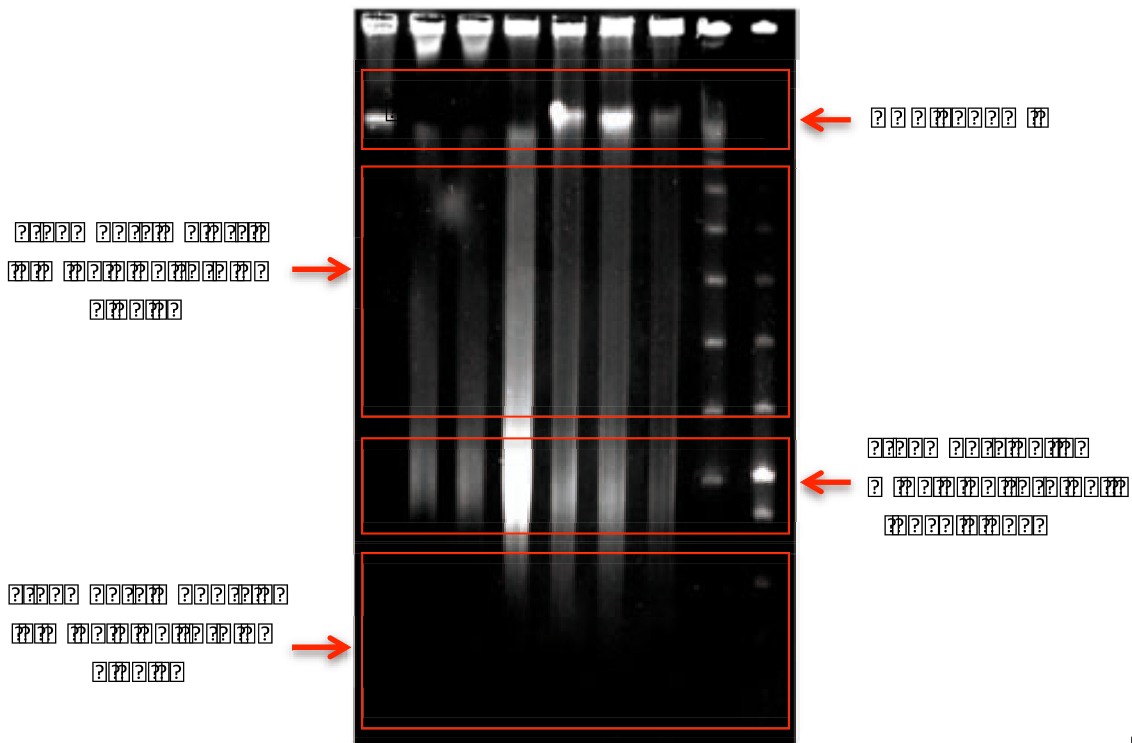
- *SYBR green*
- Agarosa certificada per electroforesi de camp polsant: Es dilueix la quantitat necessària d'agarosa per a fer un gel al 0,8% en TBE, s'afegeix un agent intercalant del DNA (SYBR green) (Invitrogen; Eugene, OR, USA) i es deixa gelificar a temperatura ambient.
- Marcadors de pes molecular: *Lambda Hind III* (Invitrogen; Eugene, OR, USA); i concatàmers de *Lambda* (New England Biolabs; Ipswich, MA, USA).

3.6.5.2 Protocol

S'obté una mostra espermàtica en fresc o bé es realitza el protocol de descongelació i es dilueix a una concentració de 10-20 milions d'espermatozoides per mil·lilitre. Es liqua una alíquota d'agarosa de baix punt de fusió al 2% i es barregen a parts iguals 50μl de mostra amb 50μl d'agarosa de baix punt de fusió al 2%. Aquests 100 μl de la barreja es col·loquen en el motlle per a realitzar els *plugs* i es deixen gelificar 15 minuts a 4ºC. Mentrestant, es prepara un gel d'agarosa de camp polsant al 0.8% en TBE, afegint la tinció per a DNA (SYBR green) i es deixa gelificar a temperatura ambient amb una pinta per a generar els pouets on es carregarà la mostra.

Un cop els *plugs* s'han solidificat, aquests es submergeixen en solució de digestió i s'incuben una hora a 51-53ºC amb el fi de digerir la membrana espermàtica, a més d'extreure les histones i protamines, deixant nu el DNA espermàtic. Un cop realitzada aquesta incubació, els *plugs* es carreguen als pouets del gel de camp polsant amb l'ajuda de dues espàtules.

La electroforesi de camp polsant es realitza en una cubeta de camp polsant (CHEF DRIII System, Bio-Rad; Hercules, CA, USA) en les següents condicions:



3.7 Anàlisi de la compactació del DNA espermàtic

3.7.1 Determinació de la ràtio entre protamina 1 i protamina 2 (P1/P2)

Es tracta d'una tècnica en la que es determina la proporció de la protamina 1 i la protamina 2 mitjançant una extracció de proteïnes espermàtiques i electroforesi en gels d'acrilamida. La determinació d'aquesta ràtio entre els dos tipus de protamines pot indicar possibles alteracions en el recanvi d'histones per protamines que es dóna durant l'espermatogènesis.

3.7.1.1 Solucions necessàries

- Solució de permeabilització: Es prepara una solució contenint 0,5% de Tritón X-100, 1M Tris-HCl 1M y 1M de $MgCl_2$ en aigua mil·liQ, i s'ajusta el pH a 8.
- Solució amb 1mM de PMSF en aigua mil·liQ.
- Solució amb 20mM EDTA, 1mM PMSF i 100mM Tris-HCl en aigua mil·liQ, ajustant el pH a 8.
- Solució GuHCl/DTT: Es prepara una solució contenint 575mM DTT, 6M Hidroclorur de guanidina (GuHCL).
- Solució 1% de β-mercaptoetanol en acetona.
- Tampó de mostra: Es prepara una solució contenint 5,5M Urea, 20% β-mercaptoetanol, 5% de àcid acètic.
- Solució per a tenyir els gels: *Comassie Brilliant Blue* G-250 (Bio-rad; Hercules, CA, USA).

3.7.1.2 Protocol

Extracció àcida de les proteïnes

Primer de tot, s'elimina el fluid seminal o el medi de criopreservació realitzant tres centrifugacions a 400g durant 5 minuts, ajustant la concentració d'espermatozoides a 15 milions per mil·lilitre a l'últim rentat. A continuació, s'afegeix 1 ml de medi Ham F10 1x (Gibco BRL, Life Technologies Ltd; Paisley, UK) i es torna a centrifugar la mostra a 400g durant 5 minuts. Es retira el sobrenedant i s'afegeixen 300 μl de medi Ham F10 i es torna a repetir aquest pas. A continuació, per a desestabilitzar les membranes cel·lulars espermàtiques, es realitza una incubació amb la solució de permeabilització i es centrifuga la mostra 5 minuts a 400g. Un cop retirat el sobrenedant, es realitzen dos passos de rentat utilitzant 200 μl de PMSF 1mM, el qual llisa les cèl·lules mitjançant un xoc hiposmòtic. Es resuspèn el sediment en 50 μl d'una solució contenint EDTA, PMSF i Tris-HCl i s'afegeixen 50 μl d'una solució amb hidroclorur de guanidina (GuHCl) i DTT. S'afegeixen 100 μl d'aigua mil·liQ i s'incuba la mescla durant 30 minuts a temperatura ambient i protegida de la llum. Aquest tractament redueix els ponts disulfur de les protamines, produint una descompactació del nucli espermàtic. Passat aquest temps, s'afegeixen 500 μl d'etanol fred i s'incuba durant 20-30 minuts a -20ºC. Passat aquest punt, es centrifuga novament la mostra a 700g durant 5 minuts i s'elimina el màxim de sobrenedant possible. S'afegeixen 500 μl d'una solució 0,5M HCl i s'incuba durant 5 minuts a 37ºC. Es centrifuga a 800g la mostra i es guarda el sobrenedant en un tub amb 20% de TCA. En aquest sobrenedant s'hi troben les proteïnes extretes de les cèl·lules. Aquest tub s'incuba 10 minuts a 4ºC per a que precipitin les proteïnes, i es centrifuga per eliminar el sobrenedant. Es renta amb 500 μl d'una solució que conté 1% de β-mercaptoetanol en acetona i es centrifuga a 800g durant 5 minuts, repetint

dos vegades aquest pas. Finalment, es deixa assecar el botó cel·lular a l'aire, i es guarda a -20ºC fins el moment de carregar el gel.

Pre-electroforesi

Es col·loca un gel d'acrilamida (5,5M urea, 5% d'àcid acètic, 15,1% acrilamida/bisacrilamida) a la cubeta d'electroforesi utilitzant una solució que contingui 0,9N d'àcid acètic. Es col·loquen els pols d'electroforesi invertits a la font d'alimentació i s'ajusta el voltatge a 150V i s'espera a que l'amperatge sigui constant. Aquest pas és molt important per a eliminar tots els ions que pugui contenir el gel, ja que les proteïnes no es separen per pes molecular sinó per càrrega.

Electroforesi

Abans de realitzar l'electroforesi cal renovar la solució d'electroforesi que s'ha utilitzat prèviament. Llavors, es carreguen les proteïnes extretes que s'han redissolt prèviament en 20 μl de tampó de mostra. Es carreguen 2,5 μl per cada mostra i s'afegeixen 3 μl de tampó de mostra amb verd de metilè (10mg/100μl).

S'efectua l'electroforesi durant una hora a 150V.

3.7.1.3 Anàlisi

Es realitza una tinció dels gels per a proteïnes amb *Comassie Brilliant Blue* G-250 (Bio-rad; Hercules, CA, USA), s'escaneja el gel i es quantifiquen les bandes corresponents a les protamines 1 i 2 utilitzant un programa especialitzat: *Quantity One* (Bio-Rad; Hercules, CA, USA).

3.7.2 Test de la Cromomicina A3 (CMA3)

La tècnica d'anàlisi de la compactació del DNA mitjançant cromomicina es basa en que aquesta competeix amb les protamines per la unió al solc menor del DNA, així doncs, espermatozoides amb una protaminització anòmala quedaran ocupats per CMA3, generant fluorescència verda. Aquesta mesura permet extrapolar el grau de compactació dels espermatozoides de la mostra en general.

3.7.2.1 Solucions necessàries

- Solució PBS 1x: A partir de la solució de PBS 10x preparada al laboratori es realitza una dilució 1:10 en aigua mil·liQ. La solució de PBS 10x conté 1,37M NaCl, 27mM KCl, 100mM $Na_2HPO_4 \cdot 2H_2O$, 20mM KH_2PO_4 ajustada a pH 7,4.
- Solució McIlvine: Per a preparar 60 ml de la solució cal barrejar 18 ml d'una solució 0,1M d'àcid Cítric i 42 ml d'una solució 0,2M de Na_2HPO_4. Posteriorment, cal ajustar el pH a 7. A l'hora d'utilitzar aquesta solució cal suplementar-la amb 10mM de $MgCl_2$.
- Cromomicina A3: Cal preparar la cromomicina comercial a una concentració de 5mg/ml en etanol 100%.
- Solució de tinció: Per a realitzar la tinció, caldrà diluir 10 ml de cromomicina 5mg/ml en 190 ml de solució McIlvine suplementat amb $MgCl_2$.
- Solució de fixació: Es prepara una solució al 4% de para-formaldehid en PBS
- Solució de permeabilització: Es prepara una solució al 0,5% de Triton-X100.

3.7.2.2 Protocol

Es renta la mostra seguint el protocol de descongelació i posteriorment es realitza una incubació en solució de fixació durant 20 minuts. Posteriorment es renta la mostra en PBS i es realitza una incubació en la solució de permeabilització durant 15 minuts a

temperatura ambient. Es realitza un rentat en PBS centrifugant a 400g durant 5 minuts. Es resuspèn el botó en PBS fins a una concentració d'uns 10 milions per mil·lilitre i es col·loquen unes gotes d'aquesta suspensió sobre un portaobjectes deixant assecar-ho a l'aire. Quan l'extensió està completament seca, es realitza la tinció amb CMA3 en solució McIlvine afegint 50 μl de la solució de tinció, cobrint-ho amb parafilm, durant 20 minuts a temperatura ambient i protegit de la llum.

3.7.2.3 Anàlisi

La mostra es contra-tenyeix utilitzant D*API SlowFade Gold antifade* (Invitrogen; Eugene, OR, USA), i s'analitzen 400 espermatozoides al microscopi de fluorescència. Aquells espermatozoides amb fluorescència blava i verda seran positius per a la CMA3, mentre que aquells que només presentin fluorescència blava seran negatius. S'expressa el resultat com a percentatge d'espermatozoides positius (Figura 25).

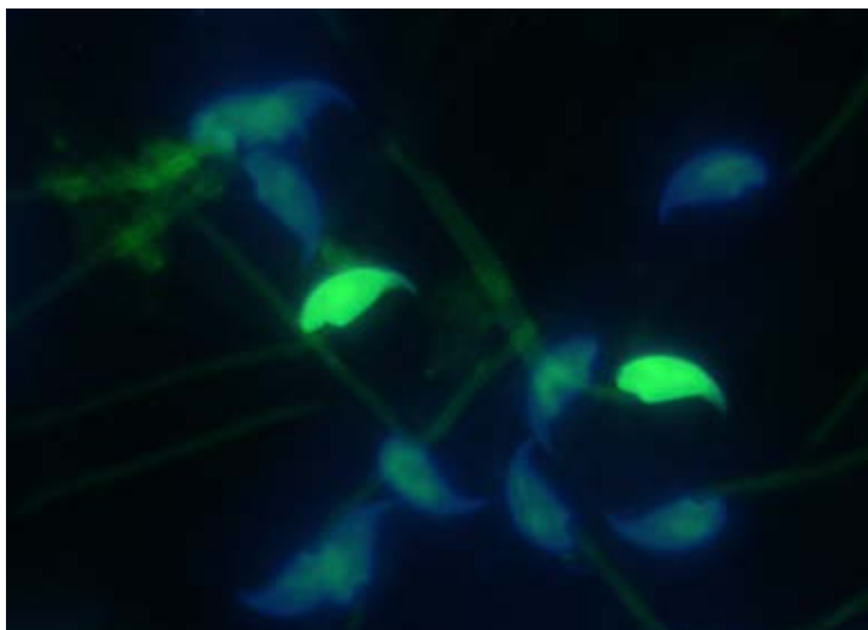


Figura 25. Anàlisi d'espermatozoides de ratolí amb la tècnica CMA3. Els espermatozoides marcats en verd són positius per a la cromomicina.

3.8 Estadística

L'anàlisi estadístic d'aquest treball ha estat realitzat amb el programa estadístic SPSS v20 (Statistic Package for Social Sciences, SPSS Inc.; Chicago, IL, USA). Així, s'han utilitzat diferents tests en funció de les característiques de cada estudi. Per a comparacions entre diferents grups independents de dades s'han utilitzat proves no paramètriques per a mostres independents, com la U de Mann-Whitney. Per a estudis comparant grups dependents de mostres s'han utilitzat tests no paramètrics per a mostres relacionades, com el test de Wilcoxon. Els estudis de correlacions s'han realitzat mitjançant la prova de Spearman. Finalment, per a estudis de sensibilitat i especificitat de les diferents tècniques s'ha utilitzat l'anàlisi ROC (Receiver Operating Characteristic), que permet maximitzar la sensibilitat, especificitat i punt de tall d'una tècnica respecte un resultat, en el nostre cas una variable dicotòmica, embaràs o no embaràs.

4. RESULTATS

4.1 Resultats referents a l'objectiu 1.1

Els resultats aconseguits en el desenvolupament de l'objectiu 1.1 han donat lloc al següent treball, publicat en una revista de l'àrea de l'Andrologia indexada en el JCR en 1er quartil (1/6).

Article 1

Títol: Human semen cryopreservation: a sperm DNA fragmentation study with alkaline and neutral Comet assay.

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Human semen cryopreservation: a sperm DNA fragmentation study with alkaline and neutral Comet assay

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SUMMARY

Sperm cryopreservation is widely used for both research and reproduction purposes, but its effect on sperm DNA damage remains controversial. Sperm DNA fragmentation (SDF) has become an important biomarker to assess male infertility. In particular, the differentiation between single- and double-stranded DNA fragmentation (ssSDF and dsSDF) has clinical implications for male infertility where ssSDF is associated with reduced fertility, whereas dsSDF is associated with increased risk of miscarriage. In this study, semen samples from 30 human males have been analysed in both fresh and cryopreserved using the alkaline and neutral Comet assays. Results show an increase of about 10% of ssSDF, assessed by the alkaline Comet assay, regardless of the male fertility status. Neutral Comet analysis of dsSDF does not show any statistical increase when comparing fresh and cryopreserved samples in any of the patient groups. Results support previous reports that oxidative stress is the major effector in DNA damage during sample cryopreservation, as, on one hand, ssSDF has previously been related to oxidative damage and, on the other hand, we have not found any effect on dsSDF. Therefore, there might be a slight risk of decreased fertility after using a freezed sample, but no evidence for increased miscarriage risk from cryopreserved spermatozoa should be expected.

INTRODUCTION

The sperm DNA damage analysis has become a complementary biomarker in determining male infertility, which is mainly diagnosed through macroscopic and microscopic semen parameters, determination of chromosomal aneuploidies, meiotic studies, hormonal analysis and karyotype (Egozcue *et al.*, 1997; Benet *et al.*, 2005; Martin, 2006; Carrell, 2008; Templado *et al.*, 2011). Sperm DNA fragmentation (SDF) has been developed as a marker of sperm DNA quality, and many studies have shown an increase in SDF in infertile patients compared with fertile donors, and have established clinical threshold values for infertility using different techniques (Sergerie *et al.*, 2005; Evenson & Wixon, 2008; Velez de la Calle *et al.*, 2008; Sharma *et al.*, 2010; Simon *et al.*, 2011; Ribas-Maynou *et al.*, 2012b). Moreover, a distinction of different groups of infertile patients such as varicocoele patients, recurrent miscarriage patients or chromosomal rearrangement carriers can be performed by using methods with higher sensitivity for SDF analysis such as the Comet assay (Ribas-Maynou *et al.*, 2013). The aetiology of SDF has also been widely discussed, locating the DNA damage at different levels (Aitken & De Iuliis, 2010; Sakkas & Alvarez, 2010): (i) at the testicular level, where there can occur apoptosis during spermatogenesis, DNA breaks during spermiogenesis as a result of nuclease activity, radiotherapy and chemotherapy or environmental toxicants (Maione *et al.*, 1997; Sailer *et al.*, 1997; Sotolongo *et al.*, 2005; Rubes *et al.*, 2007; O'Flaherty *et al.*, 2008); (ii) at the epididymis level, where the DNA damage would be mainly caused by oxidative stress and (iii) at vas deferens level, where the oxidative stress is increasing with respect to the epididymis (Agarwal *et al.*, 2008; Makker *et al.*, 2009; Aitken & Koppers, 2011).

The effect of the sperm DNA damage on the embryo has been less studied owing to a lack of physiological studies. However, some authors report that fertilization with a DNA-damaged spermatozoon might lead to DNA errors at different levels of embryogenesis (Aitken & De Iuliis, 2007; Lewis & Simon, 2010) or a slower embryo development (Gawecka *et al.*, 2013). Moreover, if the DNA breaks carried by the sperm cell are not repaired, the embryo might be miscarried (Ribas-Maynou *et al.*,

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2012b) or the child affected by different childhood diseases (Cooke *et al.*, 2003; Aitken *et al.*, 2009).

Gamete cryopreservation is widely used for a variety of purposes, such as fertility preservation previous to chemotherapy treatment, donor or conjugal sperm cryopreservation or research (Sanger *et al.*, 1992; Anger *et al.*, 2003; Jensen *et al.*, 2011; Di Santo *et al.*, 2012). Because of that, it is important to understand the effects of cryopreservation to preserve the better quality of the thawed sample. It has been shown that cryopreservation reduces sperm motility and sperm vitality (Thomson *et al.*, 2010; Lee *et al.*, 2012; Satirapod *et al.*, 2012). Recent studies have been focused on the effect of cryopreservation on sperm DNA damage, showing that the main effector of DNA damage during the process of freezing and thawing a semen sample are the reactive oxygen species (Lasso *et al.*, 1994; Thomson *et al.*, 2009; Said *et al.*, 2010). However, the effect of cryopreservation on sperm DNA integrity remains controversial with some reports showing an effect (Spano *et al.*, 1999; Donnelly *et al.*, 2001; de Paula *et al.*, 2006; Thomson *et al.*, 2009; Zribi *et al.*, 2010), whereas others report none (Host *et al.*, 1999; Duru *et al.*, 2001; Isachenko *et al.*, 2004). These controversial data may be resolved by controlling for additional factors that affect sperm DNA integrity during freeze/thawing, such as the previous state of the sample (Donnelly *et al.*, 2001; Kalthur *et al.*, 2008; Ahmad *et al.*, 2010), the technique used for cryopreservation or the cryoprotectant applied (Di Santo *et al.*, 2012).

Different techniques have been used to assess sperm DNA damage in cryopreservation, such as TUNEL (Duru *et al.*, 2001; de Paula *et al.*, 2006; Thomson *et al.*, 2009; Zribi *et al.*, 2010), SCSA (Spano *et al.*, 1999; Gandini *et al.*, 2006), SCD (Gosálvez *et al.*, 2010) and the Comet assay (Donnelly *et al.*, 2001; Kalthur *et al.*, 2008; Ahmad *et al.*, 2010). However, to our knowledge there have been no cryopreservation studies differentiating single-stranded sperm DNA fragmentation (ssSDF) and double-stranded sperm DNA fragmentation (dsSDF) on the same semen sample, using both fertile and subfertile patients. This differentiation could be helpful to understand the mechanisms through which DNA fragmentation is produced in cryopreservation. In this sense, it has been proposed that ssSDF can be related to oxidative stress DNA damage and would be extensively distributed throughout the genome, whereas dsSDF is associated with some kind of enzymatic activity having acting in a non-extensive manner (Sotolongo *et al.*, 2005; Ribas-Maynou *et al.*, 2012a,b). The sperm Comet assay allows researchers to distinguish between these two types of DNA damage, depending on whether it is performed with a previous alkaline denaturation or with neutral conditions respectively (Enciso *et al.*, 2009; Ribas-Maynou *et al.*, 2012a). The Comet assay has a higher sensitivity than the SCD test because of the electrophoresis component of the former (Ribas-Maynou *et al.*, 2013). The SCD test has a similar sensitivity as two other common SDF assays, the TUNEL assay and SCSA (Chohan *et al.*, 2006; Garcia-Peiró *et al.*, 2011).

The main aim of the present work is to evaluate the effect of cryopreservation on semen samples attending single-stranded or double-stranded sperm DNA fragmentation using the Comet assay methodology. A secondary objective of this work was to analyse the effect of cryopreservation in different groups, taking into account their clinical status.

MATERIALS AND METHODS

Sample collection

Semen samples from 44 human males were obtained by masturbation after an abstinence period of 3–7 days. Samples were divided into three clinical groups: fertile donors (n = 10), recurrent miscarriage patients without a female factor (RPL) (n = 8) and a group of subfertile patients (n = 26) which includes altered semenogram samples from subfertile couples (7 asthenozoospermic, 4 teratozoospermic, 9 asthenoteratozoospermic, 1 oligoasthenoteratozoospermic and 5 asthenoteratozoospermic with varicocoele) who had unprotected intercourse without a pregnancy during 12 months. The age of all donors ranged from 18 to 38 years and there were no differences among different groups. Informed consent was obtained for all donors and the appropriate ethics committee approved the study.

Semen parameters

After allowing the sample to liquefy for 30 minutes, semen parameters according to WHO 2010 guidelines were analysed by using SCA software (Sperm Class Analyzer; Microptic, Barcelona, Spain). Sperm count (10^6 spermatozoa/mL), motility (% A+B) and morphology (% normal forms) for the samples were (mean ± standard deviation): 124.35 ± 58.42, 50.95 ± 9.63 and 8.11 ± 2.89, respectively, for fertile donors; 122.14 ± 128.02, 46.5 ± 17.46 and 4.14 ± 2.12, respectively, for recurrent miscarriage patients and 61.93 ± 63.33, 24.54 ± 13.00 and 2.86 ± 3.35, respectively, for subfertile patients.

Cryopreservation

The cryopreservation technique used in this work has previously been published in Ribas-Maynou *et al.* (2012a). The total semen sample was mixed in equal proportions with test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose and 1.72% sodium citrate, pH 7.5) and, after homogenizing, each sample was divided into cryotubes and frozen in isopropanol at −80 °C overnight, which allows a cooling ramp of −1 °C/min. The following day, samples were transferred to liquid nitrogen until they were thawed to start the analysis of DNA fragmentation.

Thawing and sample preparation

Samples were thawed at room temperature. Then, three washes were performed using PBS without Ca^{2+} or Mg^{2+}, centrifuging at 600 g for 5 min. Finally, the sperm concentration was adjusted at 1×10^6 spermatozoa/mL to assess sperm DNA damage.

SDF analysis

The SDF analysis was performed twice using the alkaline and neutral Comet assays: once starting within the first hour after obtaining the fresh sample and again starting immediately after thawing a cryopreserved fraction of the same sample.

Comet assay

The Comet assay was performed in alkaline or neutral conditions to analyse single-stranded DNA fragmentation and double-stranded DNA fragmentation, respectively, as previously described (Ribas-Maynou *et al.*, 2012a). Briefly, 15 μL of semen sample was mixed with 25 μL of LMP agarose, and allowed to solidify with a coverslip on two slides. After coverslips were

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removed, slides were incubated in two lysis solutions for half an hour each. Then slide designated for alkaline Comet was denatured in an alkaline buffer for 2.5 min and electrophoresed for 4 min, and slide designated for neutral Comet was electrophoresed for 12.5 min and washed in a NaCl buffer. Finally, both slides were washed in neutralization buffer and ethanol series. After allowing to dry horizontally, staining was performed using DAPI SlowFade® Gold antifade (Invitrogen, Eugene, OR, USA) and 400 spermatozoa were classified as fragmented or non-fragmented following the criteria reported before (Ribas-Maynou *et al.*, 2012a) using a fluorescence microscope (Olympus AX70, Hamburg, Germany). Results were expressed as a percentage of the fragmented spermatozoa (%SDF).

Statistical analysis

Statistical analysis was performed with SPSS v20 software (Statistics Package for the Social Sciences software, Inc., Chicago, IL, USA). As the two fresh and cryopreserved groups are related samples, the comparisons between them were performed using the Wilcoxon test for paired samples. The significance level was established at 95% of the confidence interval to be considered statistically significant.

RESULTS

Cryopreservation and SDF

The data were classified attending the clinical status of the donors into three groups: fertile donors, recurrent miscarriage without female factor patients and general subfertile patients including altered semenogram patients as described in Material and Methods. The SDF analysed with alkaline and neutral Comet assay regarding these three groups before and after cryopreservation is shown in Table 1.

The alkaline Comet results showed statistical differences between fresh and cryopreserved spermatozoa in all three groups (Table 1). These differences were greater for the fertile donors ($p = 0.005$) and subfertile males group (0.000) than for males from couples with recurrent pregnancy loss ($p = 0.045$). Overall, there was approximately a 10% increase in ssDNA damage in cryopreserved spermatozoa as measured by the alkaline Comet assay ($p < 0.001$). Interestingly, the neutral Comet assay did not show any statistical difference between fresh and cryopreserved samples in any of the groups ($p > 0.05$).

DISCUSSION

SDF and cryopreservation

Semen cryopreservation has become widely used technique in reproduction, applied to both assisted reproduction techniques and research. The human sperm cryopreservation has been studied in many publications, with different results between them. Some studies have been focused on the effect of cryopreservation to seminal parameters such as sperm motility, vitality and morphology, showing a decrease on these parameters (Thomson *et al.*, 2010; Di Santo *et al.*, 2012; Lee *et al.*, 2012; Satirapod *et al.*, 2012). However, the growing interest on SDF assessment requires studies to approach the actual DNA damage on the cryopreserved spermatozoa. In this sense, opposite results have been described on literature, some showing DNA damage after cryopreservation (Spano *et al.*, 1999; Donnelly *et al.*, 2001; de Paula *et al.*, 2006; Thomson *et al.*, 2009; Zribi *et al.*, 2010), and some showing no effect of cryopreservation (Host *et al.*, 1999; Duru *et al.*, 2001; Isachenko *et al.*, 2004). Nevertheless, cryopreservation studies have been performed with different techniques and, because of the controversy on this topic (Garcia-Peiró *et al.*, 2011), it might be necessary to perform the analysis at the same time with different techniques, or using the most sensitive ones, such as Comet assay (Ribas-Maynou *et al.*, 2013). For that, in this work we performed the analysis through the alkaline and neutral Comet assays. Comet results showed a statistical increase on SDF (Table 1), agreeing with some previous studies using this technique (Donnelly *et al.*, 2001; de Paula *et al.*, 2006; Thomson *et al.*, 2009). In this sense, a remarkable result obtained is that the percentage of spermatozoa with single-stranded DNA fragmentation is increased by a 10% after cryopreservation (Table 1). This would mean that a semen sample would have roughly 10% more fragmented spermatozoa, which would have worse DNA integrity, and therefore, they would be less likely to end up with a pregnancy. Regarding neutral Comet, no differences have been observed between before and after cryopreservation (Table 1), showing no effect on double-stranded DNA integrity. To our knowledge there have not been results using this technique related to cryopreservation, but taking into account that ssSDF has recently been related to oxidative damage (Enciso *et al.*, 2009; Ribas-Maynou *et al.*, 2012a), these results would fit to the consideration that oxidative stress would be the main effector of DNA damage during cryopreservation (Mazzilli *et al.*, 1995; Thomson *et al.*, 2009).

Table 1 Percentage of spermatozoa showing DNA fragmentation assessed by the Comet assay before and after cryopreservation (mean ± standard deviation)

	Fresh Alkaline Comet		Cryopreserved Alkaline Comet	Fresh Neutral Comet		Cryopreserved Neutral Comet
Total samples (*n* = 44)	40.13 ± 17.67		49.80 ± 17.64	67.36 ± 19.57		68.88 ± 17.83
p value		0.000**			0.086	
Fertile donors (*n* = 10)	21.05 ± 10.63		33.63 ± 12.34	63.70 ± 28.52		65.57 ± 24.80
p value		0.005**			0.169	
RPL patients (*n* = 8)	34.97 ± 18.51		38.55 ± 17.23	84.60 ± 16.11		84.88 ± 14.74
p value		0.049*			0.889	
Subfertile patients (*n* = 26)	49.05 ± 12.79		59.48 ± 12.43	63.46 ± 13.29		65.23 ± 12.81
p value		0.000*			0.073	

*Statistical differences between fresh and cryopreserved spermatozoa, Wilcoxon paired samples test ($p < 0.05$).
**Statistical differences between fresh and cryopreserved spermatozoa, Wilcoxon paired samples test ($p < 0.01$).

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Moreover, this increase only on ssSDF might have a clinical effect on pregnancy achievement, but the lack of increase on dsSDF would not produce an increase on the miscarriage risk (Ribas-Maynou *et al.*, 2012b). In relation to that, when different clinical statuses were analysed, all fertile donors, recurrent miscarriage patients and subfertile patients showed a statistical increase on alkaline Comet after cryopreservation, but none of them showed an increase on neutral Comet (Table 1). Therefore, as different cryopreservation protocols can have different effects on DNA integrity, a comparative study of the cryopreservation techniques and a standardization of the best one would be necessary to solve the different effects found in the literature (Donnelly *et al.*, 2001; Kalthur *et al.*, 2008; Ahmad *et al.*, 2010).

Conclusion

The effect of cryopreservation on alkaline Comet assay showed an increase of 10% of ssSDF, whereas the neutral Comet assay showed no effect after thawing. Therefore, these results show that a mean of 10% of the cryopreserved sperm cells present worse single-stranded DNA integrity than before cryopreservation. This suggests that cryopreservation may affect the pregnancy capacity of the sperm cell without increasing the associated miscarriage risk.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J.R.-M. contributed in experimental procedures, statistical analysis, table elaboration and document writing. A.F.-E. contributed in experimental procedures. A.G.-P. contributed in experimental design, results discussion and statistical analysis. E.P., C.A. and M.J.A. contributed in recruitment of patients, samples collection, storage and semen parameters analysis. J.N. and J.B. contributed in experimental design and direction and coordination of the work.

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4.2 Resultats referents a l'objectiu 1.2

Els resultats aconseguits en el desenvolupament de l'objectiu 1.2 han donat lloc al següent treball, publicat en una revista de l'àrea de l'Andrologia indexada en el JCR en 1er quartil (1/6).

Article 2

***Títol**:* Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay.

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Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay

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SUMMARY

Sperm DNA fragmentation (SDF) is becoming an important test to assess male infertility. Several different tests are available, but no consensus has yet been reached as to which tests are most predictive of infertility. Few publications have reported a comprehensive analysis comparing these methods within the same population. The objective of this study was to analyze the differences between the five most common methodologies, to study their correlations and to establish their cut-off values, sensitivity and specificity in predicting male infertility. We found differences in SDF between fertile donors and infertile patients in TUNEL, SCSA, SCD and alkaline Comet assays, but none with the neutral Comet assay. The alkaline COMET assay was the best in predicting male infertility followed by TUNEL, SCD and SCSA, whereas the neutral COMET assay had no predictive power. For our patient population, threshold values for infertility were 20.05% for TUNEL assay, 18.90% for SCSA, 22.75% for the SCD test, 45.37% for alkaline Comet and 34.37% for neutral Comet. This work establishes in a comprehensive study that the all techniques except neutral Comet are useful to distinguish fertile and infertile men.

INTRODUCTION

In recent years, the sperm DNA fragmentation (SDF) has become a biomarker for male infertility because it has been shown that fertilization of a spermatozoa with fragmented DNA could cause defects in embryo development, giving rise to the risk of undergoing a pregnancy loss at early pregnancy stages, or problems with foetal development (Evenson *et al.*, 1999; Carrell *et al.*, 2003; Lewis & Simon, 2010). Moreover, high SDF has been associated with recurrent miscarriage, higher difficulty in achieving a pregnancy and different childhood diseases (Cooke *et al.*, 2003; Aitken *et al.*, 2009; Brahem *et al.*, 2011; Zini, 2011; Absalan *et al.*, 2012). Etiological studies have concluded that oxidative stress is one of the most common factors associated with sperm DNA damage (Agarwal *et al.*, 2008; Makker *et al.*, 2009; Aitken & De Iuliis, 2010). Other factors involved in sperm nuclear DNA fragmentation include incorrect chromatin remodelling, nuclease activity or different external factors such as radiation (Maione *et al.*, 1997; Sailer *et al.*, 1997; Sotolongo *et al.*, 2005; Aitken & De Iuliis, 2010; Sakkas & Alvarez, 2010).

Several methodologies have been developed to assess SDF, and most of them have been applied for clinical purposes by establishing their cut-off values for predicting pregnancy, and monitoring their sensitivity and specificity (Evenson *et al.*, 2002; Sergerie *et al.*, 2005; Velez de la Calle *et al.*, 2008; Nijs *et al.*, 2009; Sharma *et al.*, 2010; Simon *et al.*, 2011; Venkatesh *et al.*, 2011). First, the TUNEL assay (Gorczyca *et al.*, 1993) uses a terminal TdT transferase to label the 3′ free ends of DNA, resulting in a higher labelling on spermatozoa with fragmented DNA. For this methodology, different cut-off values have been reported to assess the fertility status of the male (Sergerie *et al.*, 2005; Sharma *et al.*, 2010). It has been demonstrated that sensitivity and specificity can be increased by analyzing the results with a cytometer instead of an epifluorescence microscope (Dominguez-Fandos *et al.*, 2007), by

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decompaction of the DNA with DTT (Mitchell *et al.*, 2011) or not including the apoptotic bodies on the final result (Marchiani *et al.*, 2007). Second, the Comet assay (Singh *et al.*, 1988) has the unique feature that it can distinguish between single and double stranded DNA breaks (ssSDF and dsSDF respectively) when it is performed under alkaline or neutral conditions. It is based on nuclear decompaction followed by electrophoresis and visualization of individual spermatozoa. Clinical cut-off values for male infertilty, assessing Comet tail DNA and percentage of fragmented spermatozoa have been published using the alkaline Comet assay for both total semen sample (Simon *et al.*, 2011; Ribas-Maynou *et al.*, 2012a) and also differentiating swim-up sperm cells (Simon *et al.*, 2011). Moreover, our group demonstrated a clinical association of dsSDF assessed by neutral Comet with recurrent miscarriage risk in couples without female factor (Ribas-Maynou *et al.*, 2012b), showing that differences in the DNA break type, ssSDF or dsSDF, has different implications for human reproduction.

Other methods such as Sperm Chromatin Structure Assay (SCSA) (Evenson *et al.*, 1980) and the Sperm Crhomatin Dispersion (SCD) test (Fernandez *et al.*, 2005) base their detection of SDF on the denaturing capacity of the sperm chromatin. The SCSA uses acridine orange staining to label the double stranded DNA with green and the single stranded DNA with red. The proportion of these two emissions, with a previous acid-denaturing step, has widely been demonstrated to determine the percentage of DNA fragmentation, and several reports for clinical usage have been published (Evenson & Jost, 2000; Evenson *et al.*, 2002; Bungum *et al.*, 2004; Virro *et al.*, 2004; Nijs *et al.*, 2009; Venkatesh *et al.*, 2011). Moreover, SCSA provides also an additional parameter named high DNA stainability (HDS). This parameter is a measure of the percentage of immature spermatozoa within the semen sample, which can also be taken into account on the male infertility assessment (Evenson *et al.*, 1999).

Finally, the SCD test assesses the capacity of the sperm chromatin to form dispersion halos, and allows differentiating the non-fragmented spermatozoa (with halo) from the fragmented spermatozoa (without halo). Like the other methods, studies showing the infertility cut-off value for the SCD test have been performed (Fernandez *et al.*, 2005; Velez de la Calle *et al.*, 2008; Nunez-Calonge *et al.*, 2012; Ribas-Maynou *et al.*, 2012b).

Although many studies reported different clinical values using these techniques, only a few studies have proved the correlation between TUNEL, SCSA and SCD (Chohan *et al.*, 2006; Garcia-Peiró *et al.*, 2011); however, these studies have not reported the sensitivity and specificity values for each technique.

On the other hand, although a study found a relationship between SDF and embryo quality using the SCSA (Niu *et al.*, 2011), some studies failed in finding a relationship between the SDF predictive value and assisted reproduction techniques such as in vitro fertilization (IVF) or intra-cytoplasmatic sperm injection (ICSI) (Esbert *et al.*, 2011; Bungum *et al.*, 2012; Simon *et al.*, 2013). This lack of the predictive quality of SCSA could be because of the presence of a female factor, such as differences between oocytes on their efficiency of DNA repair after fertilization (Payne *et al.*, 2005; Evenson & Wixon, 2006).

We have previously shown that extensive sperm ssSDF may prevent pregnancy, but that spermatozoa with dsSDF can fertilize ooctyes achieving pregnancy, but compromise the foetus viability within the first trimester (Ribas-Maynou *et al.*, 2012b). Moreover, the lack of relationship of most SDF assays with IVF or ICSI found by other authors might also be related to the method used to assess the sperm DNA damage, or to differences in sensitivity and specificity in detecting the total SDF in the semen sample between methods may be because of a lack of method standardization. However, these two facts have not been exhaustively studied among methods, although it seems to be important because there is still a limitation in the knowledge about the effects that DNA fragmentation could have on the embryo and the embryonic development.

The objectives of this study were to compare the five most commonly used techniques to assess DNA damage, to establish the correlations between them, and finally, to compare their sensitivity, specificity and threshold values attending male infertility.

MATERIALS AND METHODS

Sample collection

Semen samples from 240 human males were collected in collaboration with reproduction centres and hospitals from the Barcelona area. Samples from couples showing female factors have been excluded from the study. An informed consent was obtained from all donors and the appropriate ethics committee approved the study.

Samples were divided into fertile donors, who achieved a clinical pregnancy, and infertile patients, obtaining a group size of 50 and 190 respectively. Semen samples were obtained with a minimum of 3 days and maximum of 7 days of sexual abstinence, and were cryopreserved in test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose, 1.72% sodium citrate) until the SDF analysis. The total sample sizes that were analyzed for the different methods were 183 for alkaline Comet, 183 for neutral Comet, 123 for SCD test, 93 for TUNEL assay and 98 for SCSA.

TUNEL assay

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostic Gmbh, Penzberg, Germany) following the protocol previously described (Barroso *et al.*, 2000). The analysis of SDF was performed by flow cytometer analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA), and a total of 10 000 spermatozoa were analyzed at a flow rate of 200–300 spermatozoa/sec taking into account a negative control without the TdT enzyme. Data were processed using CellQuest analysis software (Becton Dickinson) after gating out cell debris.

SCSA

The SCSA methodology has been described elsewhere by Evenson *et al.* (1999). Briefly, each semen sample was diluted to reach a concentration of 2×10^6 spermatozoa/mL in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) in a total volume of 200 μL. Then, the sample was treated with an acid solution (150 mM NaCl, 0.1% Triton X-100, pH 1.2) and after 30 sec a staining was performed using acridine orange 6 μg/mL for 3 min. Finally, a total of 5000 sperm cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson). The percentage of spermatozoa with DNA fragmentation shows increased red fluorescence, unlike the non-fragmented population that shows a normal level of red fluorescence. The percentage of HDS spermatozoa had not been included in this SDF comparative study.

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SCD test

The SCD test was performed using the Halosperm kit (Halotech DNA; Madrid, Spain) following the manufacturer's instructions. Samples were stained with propidium iodide and 250 spermatozoa were assessed and classified as fragmented or non-fragmented spermatozoa using a fluorescence microscope (Olympus AX70, Olympus Optical Co., Hamburg, Germany).

Comet assay

The alkaline and neutral Comet assay was performed simultaneously in two different slides to assess single and double stranded DNA fragmentation respectively. The assay has been performed following the protocol reported before (Ribas-Maynou *et al.*, 2012a,b). Briefly, samples were washed and sperm concentration was adjusted to 10×10^6 spermatozoa/mL. Then incubations with two lysis solutions were performed and the samples were electrophoresed, using alkaline or neutral buffer depending on the assay, with a previous denaturation on alkaline Comet slide. Finally, both the slides were submerged on a neutralization solution and were dehydrated in ethanol series of 70, 90 and 100%. Samples were stained with DAPI SlowFade Gold antifade (Invitrogen, Eugene, OR, USA), and 400 spermatozoa were classified according fragmented or non-fragmented following the criteria reported before (Fig. 1 at Ribas-Maynou *et al.*, 2012a).

Statistical analysis

Statistical analysis was performed using the Statistics Package for the Social Sciences software, version 20 (SPSS Inc., Chicago, IL, USA). Comparisons of SDF between different groups were assessed using the Mann–Whitney *U*-test. Correlations between techniques were assessed using the Spearman test and the Receiver Operating Characteristic (ROC) analysis was performed to obtain the sensitivity, specificity and the cut-off value for each test. All statistical tests were performed taking into account the 95% of the confidence interval.

RESULTS

SDF regarding male infertility

For each assay, the percentage of spermatozoa in the sample that was positive for the test was calculated. The average percentage of SDF for fertile and infertile patients using the five different techniques is shown in Table 1, and a histogram for the same results is displayed in Fig. 1 to show their distribution.

Statistical differences were found between fertile and infertile patients through TUNEL assay, SCSA, SCD test and alkaline Comet assay ($p < 0.001$); however, no differences were found when comparing fertile donors and infertile patients through neutral Comet assay ($p = 0.862$).

Correlation between techniques

Correlation between all techniques was assessed using the Spearman test. High correlations were found between the SCD test and SCSA ($r = 0.71$; $p < 0.001$), between SCD test and TUNEL assay ($r = 0.70$; $p < 0.001$) and between SCSA and the TUNEL assay ($r = 0.79$; $p < 0.001$), the latter being the highest correlation.

Moderate correlations were found between the alkaline Comet assay and the SCD test ($r = 0.61$; $p < 0.001$), between the alkaline Comet and SCSA ($r = 0.59$; $p < 0.001$) and between the alkaline Comet and TUNEL assay ($r = 0.72$; $p < 0.001$).

Finally, no correlation was found between the neutral Comet assay and the other four methodologies.

ROC analysis, sensitivity, specificity, cut-off values

The sensitivity, specificity, the cut-off values for male factor infertility and the area below the curve obtained by the ROC analysis are shown in Table 2, and a graphical representation of ROC curves for all techniques is shown on Fig. 2. The alkaline Comet showed the highest area below the curve (0.937 cm^2), and a cut-off value of 45.37% of SDF with a sensitivity and specificity of 0.850 and 0.920 respectively. TUNEL assay showed an area below the curve of 0.903 cm^2, and a cut-off value of 20.05% of SDF with a sensitivity and specificity of 0.764 and 0.952 respectively. The SCD test showed an area below the curve of 0.869 cm^2, and a cut-off value of 22.75% of SDF with a sensitivity and specificity of 0.730 and 0.918 respectively. The SCSA showed lower association with male infertility, with an area below the curve of 0.792 cm^2, and a cut-off value of 18.90% of SDF with a sensitivity of 0.595 and a specificity of 0.875. Finally, the neutral Comet assay showed no association with male infertility, with the lowest area below the curve (0.516 cm^2), a cut-off value of 34.37% of SDF with a sensitivity and specificity of 0.970 and 0.320, respectively.

DISCUSSION

Although the use of different methodologies to assess sperm DNA damage has been widely discussed, a few reports have compared the clinical utility and the correlation between the most common methods in a comprehensive manner (Erenpreiss *et al.*, 2004; Chohan *et al.*, 2006; Garcia-Peiró *et al.*, 2011). Therefore, we performed this comparative analysis to test their correlation and to determine the different clinical cut-off values among the most used techniques.

The analysis of SDF showed statistical differences between fertile and infertile patients in the TUNEL assay, SCSA, the SCD test and the alkaline Comet assay as different reports have previously found (Gandini *et al.*, 2000; Irvine *et al.*, 2000; Zini *et al.*, 2001; Saleh *et al.*, 2002; Chohan *et al.*, 2006; Garcia-Peiró *et al.*, 2012; Ribas-Maynou *et al.*, 2012a). However, no differences were found between fertile donors and infertile patients with the neutral Comet assay. This was also found in a previous study from our group demonstrating that neutral Comet assay is related to the miscarriage risk and it is not involved in the fertility status. Moreover, a bimodal distribution has also been found in fertile donors, showing the presence of two subgroups of fertile donors, as it has previously been described (Ribas-Maynou *et al.*, 2012b). On the other hand, the neutral Comet assay showed a normal distribution on infertile samples, presenting mostly high values of dsSDF (Fig. 1 and Table 1). In fact, the distribution of infertile patients in the neutral Comet assay mirrored that of the alkaline Comet assay, suggesting that for infertile patients, at least, these two assays identify similar populations of patients.

When comparing the SDF and the SDF ranges among different methodologies, differences were found in fertile donors between the alkaline Comet assay and the SCD test, SCSA or TUNEL assay. These differences between the alkaline Comet assay and the other techniques might bebecause of the electrophoresis

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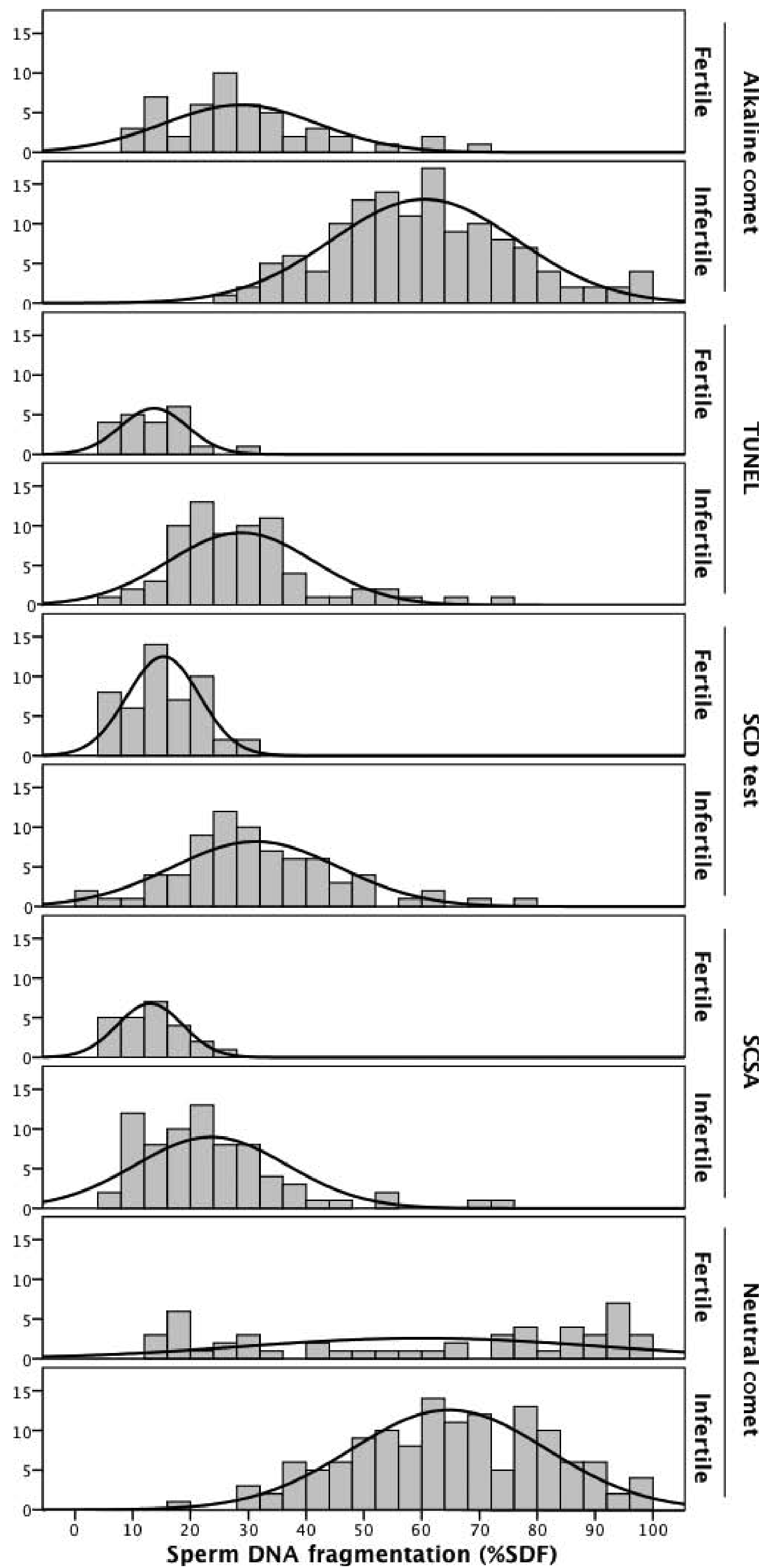

Figure 1 Fertile and infertile sperm DNA fragmentation distribution in the five different techniques. Curves show the approximation to a normal distribution.

step, which could be increasing the sensitivity of the detection of the DNA breaks with respect to other methodologies. Regarding infertile patients, values of SDF obtained by the alkaline Comet assay were statistically higher than SCD test, SCSA and TUNEL methodologies, showing that Comet assay seems to have higher sensitivity on detecting the sperm DNA breaks as Comet assay show values up to 100% of Spermatozoa with DNA fragmentation in some infertile patients, and the other methodologies do

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Table 1 Sperm DNA fragmentation (%SDF) values for fertile donors and infertile patients in each assay

Technique	*n*	Fertile donors	Range	*n*	Infertile patients	Range
TUNEL assay	21	13.67 ± 5.79	(6.6–29.3)	72	28.75 ± 12.56*	(7.1–74.1)
SCSA	24	13.01 ± 5.64	(5.0–27.3)	74	23.58 ± 13.17*	(7.7–74.5)
SCD test	49	15.32 ± 6.25	(4.1–31.5)	74	31.26 ± 14.41*	(6.5–78.0)
Alkaline Comet	50	28.64 ± 13.40	(9.3–70.0)	133	60.48 ± 16.03*	(17.4–99.0)
Neutral Comet	50	60.09 ± 30.57	(12.2–99.0)	133	64.74 ± 16.90	(26.8–100.0)

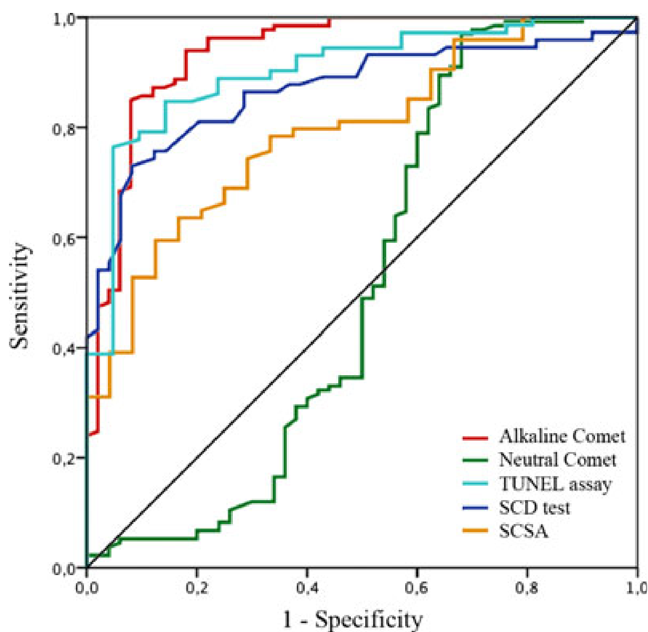
*Statistical differences with fertile donors ($p < 0.001$).

Table 2 Cut-off values with sensitivity and specificity obtained for each technique

Technique	*n*	Area*	Cut-off value (%)	Sensitivity	Specificity
Alkaline Comet	183	0.937	45.37	0.850	0.920
Neutral Comet	183	0.516	34.37	0.970	0.320
SCD test	123	0.869	22.75	0.730	0.918
SCSA	98	0.792	18.90	0.595	0.875
TUNEL	93	0.903	20.05	0.764	0.952

*Area below the ROC curve.

Figure 2 ROC curve comparing the five SDF techniques to assess male infertility.



not reach this value (Fig. 1). SCSA showed statistically lower values than SCD and TUNEL assay, which do not show statistical differences between their values. These data suggest that different methodologies might be detecting different aspects of the SDF, as SCD and SCSA might be detecting some aspects related to chromatin fragmentation, and Comet and TUNEL assays could be detecting DNA breaks directly (The Practice Committee of the American Society for Reproductive Medicine, 2008; Henkel *et al.*, 2010).

Regarding the correlations between the methods, the best correlation was found between the cytometric assays (TUNEL and SCSA), as has been previously reported (Chohan *et al.*, 2006; Villani *et al.*, 2010; Garcia-Peiró *et al.*, 2011). This is interesting given that the two assays are thought to be measuring different aspects of SDF (Henkel *et al.*, 2010). It also seems to be necessary to standardize the TUNEL methodology as it is known that it shows variations in SDF detected depending on minor variations in the procedure (Dominguez-Fandos *et al.*, 2007; Muratori *et al.*, 2008; Mitchell *et al.*, 2011) or in its analysis (Marchiani *et al.*, 2007). Nevertheless, despite the differences between the TUNEL assay and SCSA and the need for standardization of the former, both assays had very similar values for SDF. Moreover, they also present a good correlation with the SCD test, which is based on the capacity of the chromatin to form different dispersion halos depending on its SDF (Fernandez *et al.*, 2005). The correlation between SCD and the two cytometric assays has been tested before, with similar results to the present work (Chohan *et al.*, 2006; Villani *et al.*, 2010; Garcia-Peiró *et al.*, 2011).

Similarly, the alkaline Comet assay showed a moderate correlation with the SCD test, the TUNEL assay and SCSA, as has been described before by different laboratories (Donnelly *et al.*, 2000; Villani *et al.*, 2010). This correlation was not as strong as the correlations found among the latter three techniques, which might be because of a possible higher sensitivity of the alkaline Comet assay with respect to the other methodologies.

In contrast, the neutral Comet assay does not show any correlation with the other four methodologies to assess SDF. As has been proposed before, the neutral Comet assay is related to the risk of having a miscarriage, as the dsDNA breaks could be a non-extensive type of DNA damage located only in a few points along the genome (Kaneko *et al.*, 2012), preferently in the matrix attachment regions between toroids (Ribas-Maynou *et al.*, 2012b) and might be occuring by an acute or fractionated exposition to radiation, as it has been demonstrated in tumour cells (Jayakumar *et al.*, 2012). Although it is known that techniques such as the TUNEL assay and SCSA are detecting both single and double stranded DNA damage (The Practice Committee of the American Society for Reproductive Medicine, 2008; Villani *et al.*, 2010), our data show a correlation between both TUNEL or SCSA and the alkaline Comet assay, which would be detecting mainly ssSDF. However, they do not show a correlation with the neutral Comet assay, which has been demonstrated to assess mostly dsDNA breaks (Van Kooij *et al.*, 2004; Ribas-Maynou *et al.*, 2012a). Moreover, the neutral and alkaline Comet assays showed a tendency to a moderate correlation in infertile patients, a fact that could be related to the possibility that the presence of many single stranded DNA breaks could lead to double stranded DNA breaks.

To test the clinical utility of the different DNA damage tests on predicting male infertility, an analysis using ROC curves was performed. The higher area below the curve has been shown by alkaline Comet assay, followed by the TUNEL assay, the SCD test, SCSA and the neutral Comet assay (Table 2 and Fig. 2).

First, the alkaline Comet assay showed a threshold value in predicting infertility of 45.37% of DNA fragmentation with an

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area below the curve of 0.937. This cut-off value shows a very high sensitivity and specificity, and is consistent with previous results from our group (Ribas-Maynou *et al.*, 2012b). However, it is not comparable with previous studies, where the percentage of damaged DNA and not the percentage of fragmented sperm cells have been assessed (Simon *et al.*, 2011).

The TUNEL assay showed a threshold value for male infertility of 20.05% of SDF, with very high values of area below the curve and specificity (0.903 and 0.952 respectively); however, a lower value of sensitivity with respect to alkaline Comet was obtained (0.764). These results were comparable to those obtained by Sharma *et al.* (2010), who obtained a cut-off value of 19.25%, with an area below the curve, sensitivity and a specificity of 0.890, 0.649 and 1.000 respectively. However, sensitivity found in this work slightly differs from those obtained by Sergerie *et al.* (2005), who obtained a higher value of 0.896.

The cut-off, sensitivity and specificity results obtained by the SCD test in this study (Table 2) do not differ from previously published works (Fernandez *et al.*, 2005; Velez de la Calle *et al.*, 2008; Nunez-Calonge *et al.*, 2012; Ribas-Maynou *et al.*, 2012b), showing a good capacity of this technique to assess male infertility.

Reported values for SCSA threshold vary from 20 to 30% (Evenson & Jost, 2000; Evenson *et al.*, 2002; Larson-Cook *et al.*, 2003; Bungum *et al.*, 2004; Payne *et al.*, 2005; Boe-Hansen *et al.*, 2006; Venkatesh *et al.*, 2011; Evenson, 2013). Our results show a threshold value of 18.9% of SDF, which is at the low end of the published range. Despite being the lowest, it does not differ from studies that find threshold values about 20%. Moreover, it is very well-known that SCSA is the most standardized technique between different laboratories (Evenson, 2013).

Finally, the neutral Comet assay showed a very weak association with male infertility, as fertile donors can show low or high values of dsDNA fragmentation analyzed with this method. However, infertile patients always show high values. Because of that, the threshold value established was 34.37% of SDF with a high sensitivity, but a very low specificity, as a bimodal distribution in fertile donors overlaps the infertile values, as it has also been shown before. This would mean that male infertility could be predictable, but always taking into account that high values are associated with the risk of suffering a miscarriage because of a male factor (Ribas-Maynou *et al.*, 2012b).

For further assessment, as different techniques may measure different aspects of chromatin integrity, a double analysis using more than one SDF technique would allow to confirm the diagnosis.

CONCLUSION

This work provides data from the five most used methodologies to assess the SDF on the same patient population. With this data, it can be concluded that the alkaline Comet assay, the SCD test, SCSA and the TUNEL assay are useful to distinguish fertile and infertile patients, with the alkaline Comet assay being the best predictor of male infertility. However, the neutral Comet shows no capacity on differentiating fertile donors and infertile patients. Moreover, threshold values have been compared in a comprehensive work to assess infertility. Finally, this work provides a comprehensive comparison in fertile donors and infertile patients, which could be useful to technique standardization.

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AUTHOR'S CONTRIBUTION

J.R-M. contributed to experimental procedures, statistical analysis, graphics and table elaboration and document writing. A.G-P. contributed to experimental design, results discussion, statistical analysis and document writing and revising. A.F-E contributed to experimental procedures. M.J.A. and C.A contributed to recruitment of patients, sample collection, storage and semen parameter analysis. J.N. and J.B. contributed to experimental design and direction and coordination of the work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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4.3 Resultats referents a l'objectiu 2.1

Els resultats aconseguits en el desenvolupament de l'objectiu 2.1 han donat lloc al següent treball, publicat en una revista de l'àrea de la Biologia de la Reproducció, indexada en el JCR en 1er quartil (2/28).

Article 3

***Títol**:* Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups.

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ORIGINAL ARTICLE *Andrology*

Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups

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BACKGROUND: The analysis of sperm DNA fragmentation has become a new marker to predict male infertility, and many techniques have been developed. The sperm Comet assay offers the possibility of differentiating single- and double-stranded DNA (ssDNA and dsDNA) breaks, which could have different effects on fertility. The objective of this study was to perform a descriptive characterization of different groups of patients, such as those with asthenoteratozoospermic (ATZ) with or without varicocele, oligoasthenoteratozoospermic (OATZ) or balanced chromosome rearrangements, as compared with fertile donors. The Comet assay was used to investigate sperm samples for ssDNA and dsDNA breaks.

METHODS AND RESULTS: The analysis of alkaline and neutral Comet assays in different groups of patients showed different sperm DNA damage profiles. Most fertile donors presented low values for ssDNA and dsDNA fragmentation (low-equivalent Comet profile), which would be the best prognosis for achieving a pregnancy. OATZ, ATZ and ATZ with varicocele presented high percentages of ssDNA and dsDNA fragmentation (high-equivalent Comet assay profile), ATZ with varicocele being associated with the worst prognosis, due to higher levels of DNA fragmentation. Rearranged chromosome carriers display a very high variability and, interestingly, two different profiles were seen: a high-equivalent Comet assay profile, which could be compatible with a bad prognosis, and a non-equivalent Comet assay profile, which has also been found in three fertile donors.

CONCLUSIONS: Comet assay profiles, applied to different clinical groups, may be useful for determining prognosis in cases of male infertility.

Key words: sperm / DNA fragmentation / Comet assay / chromosomal rearrangement / varicocele

Introduction

Infertility is a health problem affecting 15% of all couples of reproductive age. The male factor is present in ~50% of all infertility cases; moreover, an exclusive male factor accounts for ~20% of cases (de Kretser, 1997). Consequently, the study of implicated causes of male factor infertility is a subject of increasing interest. Traditional methods to assess male infertility diagnosis have been mainly based on seminogram parameters. Although this information is necessary, results obtained are not conclusive in accurately determining the fertility status of many patients (Lewis, 2007). More recently, the genomic status of the sperm cell has been investigated in meiotic studies to determine synapsis alterations and recombination (Egozcue *et al.*, 1997; Carrell, 2008; Templado *et al.*, 2011). At a single sperm level, determination of chromosomal aneuploidy using fluorescent *in situ* hybridization methods have also significantly improved the field of male infertility diagnosis (Benet *et al.*, 2005; Martin, 2006). However, prediction of infertility in a reliable manner is still not possible (Collins *et al.*, 2008b). In spite of the progress made, the diagnosis of sperm quality remains controversial (Practice Committee of American Society for Reproductive Medicine, 2008; Zini and Sigman, 2009; Lewis and Simon, 2010; Zini, 2011).

In recent years, the analysis of sperm DNA fragmentation (SDF) has become another marker of genome quality, and for this reason, many tests have been developed for both research and clinical applications (Evenson *et al.* 1980, 2002; Gorczyca *et al.*, 1993; Evenson and Jost 2000; Fernandez *et al.*, 2003; Sharma *et al.*, 2010; Mitchell *et al.*

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2011). Characterization of mechanisms and causes of DNA fragmentation are not easy, because there are many intrinsic and extrinsic factors involved. Different factors causing SDF have been proposed (Aitken and De Iuliis 2010; Sakkas and Alvarez 2010). Principally, oxidative stress (Agarwal *et al.*, 2008; Makker *et al.*, 2009; Aitken and Koppers, 2011), endogenous endonuclease and caspase activation (Maione *et al.*, 1997; Sailer *et al.*, 1997), alterations to chromatin remodeling during spermiogenesis (Marcon and Boissonneault, 2004; Carrell *et al.*, 2007) and apoptosis of germ cells at the beginning of meiosis (Pentikainen *et al.*, 1999; Sakkas *et al.*, 1999, 2004) have been identified as intrinsic factors. External factors causing DNA damage have also been described, such as radiotherapy, chemotherapy and environmental toxicants (Morris, 2002; Rubes *et al.*, 2007; O'Flaherty *et al.*, 2008). All of these mechanisms can affect DNA strands in a various manners, producing, in the end, single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) breaks.

Although conventional DNA damage methodologies have established a threshold value based on the percentage of sperm with fragmented DNA (Sergerie *et al.*, 2005; Evenson and Wixon, 2008; Sharma *et al.*, 2010), the methods previously mentioned are not capable of distinguishing between ssDNA and dsDNA breaks in a separate form. Characterization of the type of DNA break could be interesting from the clinical point of view because it can give guidance regarding which mechanisms may be relevant in producing the DNA damage. Single-cell gel electrophoresis (Comet assay) allows the distinction between ssDNA and dsDNA breaks, depending on whether alkaline denaturing or neutral conditions are performed (Singh *et al.*, 1988; Morris *et al.*, 2002; Van Kooij *et al.*, 2004; Enciso *et al.*, 2009). This information from the Comet assay could provide DNA strand break profiles in patient subgroups classified according to their clinical features.

Therefore, this research was conducted to characterize the ssDNA and dsDNA fragmentation profiles, assessed by alkaline and neutral Comet assays, in fertile donors and different groups of patients. The patients were selected according to anomalies in sperm count, motility and morphology, such as oligoasthenoteratozoospermic (OATZ) and asthenoteratozoospermic (ATZ), or due to having pathologies with a high incidence of infertility such as varicocele or balanced chromosomal rearrangements.

Materials and Methods

Semen samples and cryopreservation

Semen samples from 73 men were divided into 5 groups: 15 fertile donors with proven fertility, 15 ATZ with clinical varicocele, 15 ATZ without varicocele, 15 OATZ and 13 patients with structural chromosome rearrangements that include: 9 reciprocal translocations, t(1;13), t(2;13), t(3;8), t(3;19), t(4;8), t(9;17), t(10;14), t(11;17), t(12;16); 1 Robertsonian translocation, t(14;21); 2 double translocations, both t(2;17), t(14;21) and 1 inversion, inv7. Sperm counts (spermatozoa/ml), motility (A B %) and morphology (normal forms %), respectively, were: 83 ± 48 sperm/ml, 37 ± 23% and 8 ± 3% for fertile donors; 140 ± 122 sperm/ml, 17 ± 10 and 5 ± 2% for ATZ with clinical varicocele; 94 ± 51 sperm/ml, 14 ± 7 and 5 ± 5% for ATZ without varicocele and 11 ± 4 sperm/ml, 16 ± 7 and 5 ± 2% for OATZ. Details of seminograms and meiotic chromosome segregation of 9 reciprocal translocation patients and of the inversion patient have been reported elsewhere (Perrin *et al.*, 2009).

Samples were obtained by masturbation after a minimum of 3 days of abstinence. Seminograms were performed according to the World Health Organization 2010 criteria (WHO, 2010), and samples were cryopreserved in the Test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose and 1.72% sodium citrate; Garcia-Peiro *et al.*, 2011a,b). Informed consent was obtained from all patients and the study was approved by the appropriate ethics committee.

Neutral and alkaline Comet assay

The Comet assay protocol was performed on all semen samples according to the Enciso *et al.* (2009) method, with slight modifications. Neutral and alkaline Comet assays were carried out simultaneously in two different slides. First, an aliquot of the total semen was thawed and washed three times in phosphate-buffered saline. Then, sperm cells were diluted to a concentration of 10×10^6 spermatozoa/ml, and 25 µl was mixed with 50 µl of 1% low-melting point agarose (Sigma Aldrich, St Louis, MO, USA) in distilled water. Quickly, 15 µl of the mixture was placed on two different pre-treated slides for gel adhesion (1% low-melting point agarose), covered with coverslips and allowed to gel on a cold plate at 4°C for 5 min. Next, coverslips were carefully removed and slides were submerged for 30 min in two lysing solutions (Comet lysis solutions, Halotech, Madrid, Spain) and washed for 10 min in tris borate EDTA (TBE) (0.445 M Tris–HCl, 0.445 M Boric acid and 0.01 M EDTA). For the neutral Comet assay, electrophoresis was performed in the TBE buffer at 20 V (1 V/cm) for 12 min and 30 s, with a subsequent wash in 0.9% NaCl for 2 min. For the alkaline Comet assay, the slide was incubated in a denaturing solution (0.03 M NaOH and 1 M NaCl) for 2 min and 30 s at 4°C, and electrophoresis was then performed in 0.03 M NaOH buffer at 20 V (1 V/cm) for 4 min. Both neutral and alkaline slides were then incubated in the neutralizing solution (0.4 M Tris–HCl, pH 7.5) for 5 min, in TBE for 2 min and finally dehydrated in an ethanol series of 70, 90 and 100% for 2 min each.

Induction of ssDNA breaks: H_2O_2 treatment

In order to induce ssDNA breaks, incubations of 30 min at room temperature with hydrogen peroxide (Sigma Aldrich) at 0, 0.03, 0.15 and 0.30% were performed on five samples from fertile donors with a known low percentage of neutral and alkaline Comet SDF. After hydrogen peroxide treatment, samples were diluted at 10×10^6 spermatozoa/ml and the Comet assay protocol was performed as described above.

Induction of dsDNA breaks: Alu1 restriction enzyme treatment

Induction of dsDNA breaks was performed on the same five samples from fertile donors with a known low DNA fragmentation rate as mentioned above.

After two lysis treatments, slides were rinsed with 50 µl of reaction buffer and treated with the Alu1 restriction enzyme (Sigma Aldrich) for different times: 15 IU for 15 min, 15 IU for 25 min and 0 IU as a control. Afterwards, slides were washed in TBE for 5 min and the protocol was continued at the electrophoresis step, depending on neutral or alkaline Comet assays as described above.

Staining and evaluation of samples

All Comet assay samples were stained with DAPI SlowFade® Gold antifade (Invitrogen, Eugene, OR, USA) and were evaluated using a fluorescence microscope (Olympus AX70), counting at least 400 spermatozoa per sample. Sperm cells were classified according to fragmented and non-fragmented sperm. Different levels of DNA damage are shown in Fig. 1.

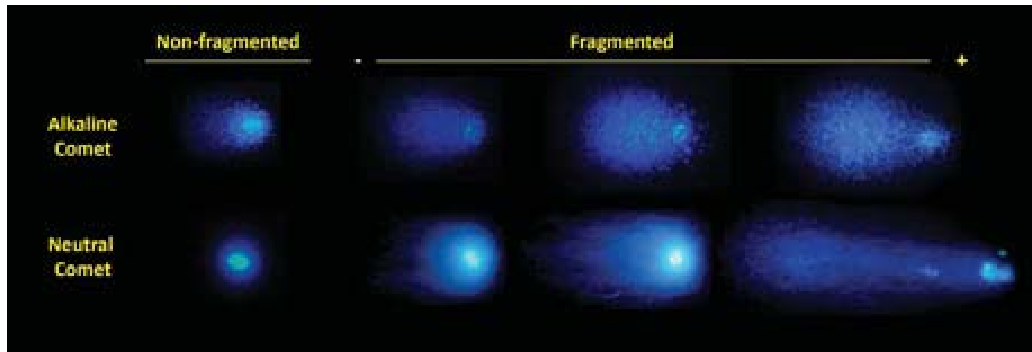

Figure 1 Non-fragmented and fragmented spermatozoa in alkaline and neutral Comet assays. Different levels of sperm DNA fragmentation (SDF) are shown for fragmented spermatozoa (DAPI staining).

Statistical analysis

Statistical analyses of data were performed using the Statistics Package for the Social Sciences software, version 17 (SPSS, Inc., Chicago, IL, USA). Values were compared using a non-parametric, Mann–Whitney *U*-test. A 95% confidence interval was set as being statistically significant.

Results

Oxidative and enzymatic DNA damage induction

Figure 2 shows data pertaining to SDF induction in samples from five donors with proven fertility and a known low alkaline and neutral SDF (<25%; Simon *et al.*, 2011). The effect of incubation of these samples with increasing concentrations of hydrogen peroxide, on both the alkaline and neutral Comet assay SDF, is shown in Fig. 2A. The effect of oxidative stress treatment in controls significantly increased SDF in both alkaline and neutral Comet assays ($P = 0.008$; $P = 0.032$), respectively. This effect was about three times higher in the alkaline Comet assay, with respect to the neutral Comet assay, even at low concentrations of H_2O_2. Contrasting results were obtained when restriction enzyme incubations were performed on samples from the same five fertile donors (Fig. 2B). AluI incubation statistically increased SDF ($P = 0.009$; $P = 0.009$) shown by both alkaline and neutral Comet assay, respectively, but produced more than two times more SDF in the neutral Comet assay, with respect to the alkaline Comet assay, after 15 min of incubation.

SDF assessment in different groups of patients

SDF values from both alkaline and neutral Comet assays for different clinical patient groups are shown in Table 1 and Fig. 3. Statistically significant differences were observed in both alkaline and neutral Comet assays between fertile controls and the entire group of infertile patients ($P < 0.01$). Attending to their clinical classification, statistical differences were also found between fertile controls and each of the infertility subgroups ($P < 0.01$).

Higher values of SDF were observed in the ATZ with varicocele subgroup, being statistically different for both Comet assay methods

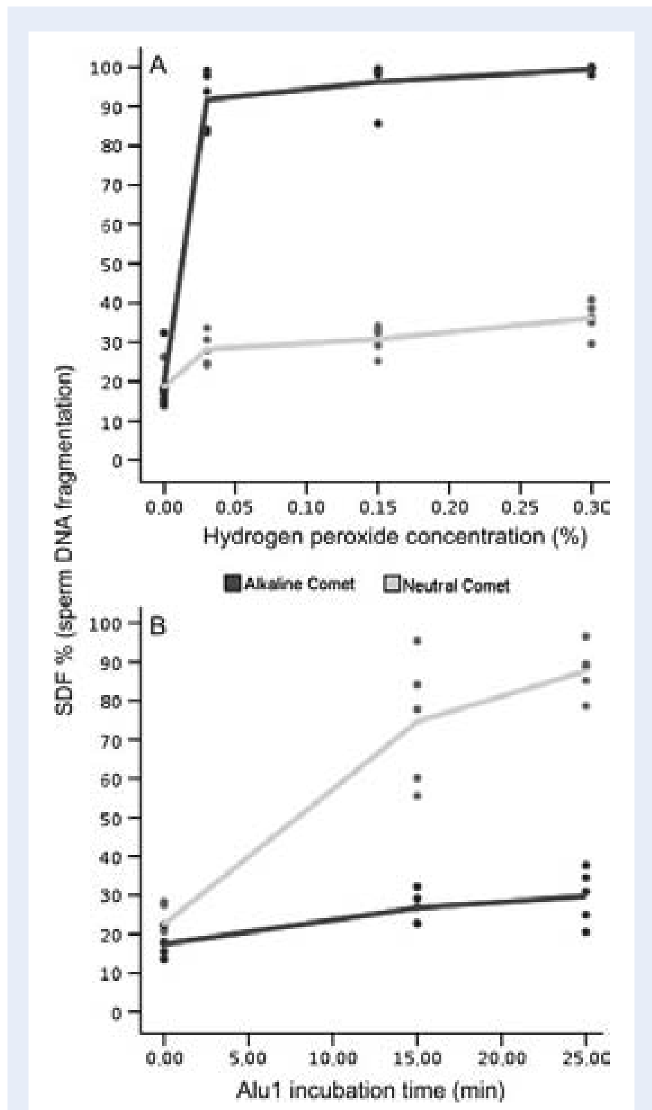

Figure 2 Alkaline and neutral Comet assays evaluating ssDNA and dsDNA breaks, respectively, in incubations with (**A**) increasing concentrations of H_2O_2 and (**B**) AluI restriction enzyme for different times.

Table I Sperm DNA fragmentation (SDF) values (mean ± SD) in different groups of patients.

	% SDF	
	Alkaline Comet	Neutral Comet
Fertile controls ($n = 15$)	21.1 ± 5.91	31.59 ± 26.85
Infertile patients		
ATZ without varicocele ($n = 15$)	60.52 ± 11.05[a,b]	65.38 ± 11.18[a,b]
OATZ ($n = 15$)	60.81 ± 11.08[a,b]	61.86 ± 16.48[a,b]
ATZ with varicocele ($n = 15$)	78.98 ± 8.49[a]	78.80 ± 13.66[a]
Rearranged chromosome carriers ($n = 13$)	73.24 ± 27.63[a]	66.61 ± 27.99[a]
Total infertile ($n = 58$)	68.22 ± 17.46[a]	68.22 ± 18.74[a]

[a]Significant differences, with respect to fertile controls ($P < 0.01$).
[b]Significant differences, with respect to ATZ with varicocele ($P < 0.01$).

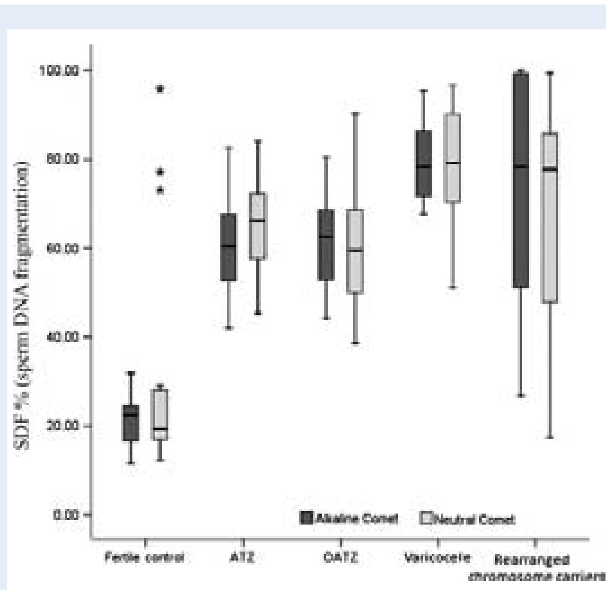

Figure 3 Alkaline and neutral Comet assay SDF in fertile controls and four groups of patients.

when compared with ATZ without varicocele and OATZ subgroups ($P < 0.01$).

No significant differences was observed between the ATZ without varicocele, OATZ and rearranged chromosome carrier subgroups, although a high variability of SDF was observed in both alkaline and neutral Comet assays for the carriers of balanced chromosomal rearrangements (Table I and Fig. 3).

Discussion

Interest in SDF has mainly been focused on predicting male infertility. Although different threshold values for the different methodologies have been proposed (Evenson *et al.*, 1999; Evenson and Wixon, 2008; Velez de la Calle *et al.*, 2008; Sharma *et al.*, 2010; Simon *et al.*, 2011), no differentiation about the relative presence of ssDNA or dsDNA breaks has been reported in infertile or subfertile patients. This distinction may have significant consequences for fertility because sperm DNA damage can occur through different mechanisms (Aitken and De Iuliis, 2010; Sakkas and Alvarez, 2010), and the resulting DNA damage profile could be linked with yet unknown pathophysiological aspects of the patient. Regarding this assumption, to our knowledge, only one report has demonstrated an association between ssDNA breaks and oxidative stress, and dsDNA breaks and enzymatic nuclease activity in human sperm cells using the 2D-Comet assay methodology (Enciso *et al.*, 2009). In the present work, similar results were found using the same experimental conditions but applying alkaline and neutral Comet assays separately. Although there were different levels of fragmentation in each assay, our results did show a statistical increase in both alkaline and neutral DNA strand breaks for both H_2O_2 and Alu I treatments, suggesting that the two types of DNA damage may be linked in some way. Recently, it has been proposed that oxidative stress can activate caspases and endonucleases in sperm (Sakkas and Alvarez, 2010). Results reported here are in agreement with this proposal. Therefore, oxidative and enzymatic DNA damage are probably related in infertile patients. Despite this, the alkaline Comet assay was much more sensitive in detecting ssDNA breaks, while the neutral Comet assay was more sensitive in detecting dsDNA breaks (Fig. 2).

Once the sensitivity of alkaline and neutral Comet assay for ssDNA and dsDNA breaks was confirmed, the analysis of different groups of patients was performed in order to characterize their DNA damage profile.

Fertile control group

Low percentages of SDF were observed in the fertile control group for both alkaline and neutral Comet assays (Fig. 3). Similar low values for both alkaline and neutral Comet assay will be referred to as a low-equivalent Comet assay profile. Alkaline Comet assay DNA fragmentation in all controls was lower than the fertility threshold value recently proposed for native semen using ART (52%; Simon *et al.*, 2011) and the majority showed lower DNA fragmentation than the 25% threshold value for natural conception (Simon *et al.*, 2011). Mostly low levels for the neutral Comet assay were shown, although three fertile donors presented high values (Fig. 3). Profiles showing low levels of alkaline SDF (< 52%) and high levels of neutral SDF are referred to as a non-equivalent Comet assay profile. There are no data in the literature about the amount of sperm DNA damage from the neutral Comet assay for fertile males. However, it has been suggested that, in somatic cells, the neutral Comet assay may be more related to the chromatin structure rather than to DNA breaks (Collins *et al.*, 2008a), although our results point out that there is a relationship between neutral Comet assay results and double-stranded breaks caused by nuclease activity. In the three fertile men, there appears to be a DNA damage mechanism that is not related to oxidative stress and has unknown consequences on fertility. In this regard, activation of nucleases has been proposed (Sotolongo *et al.*, 2005). Since the cleavage of dsDNA breaks is one of the origins of chromosomal rearrangements, dsDNA damage may contribute to an increased

risk of having embryos with chromosomal instability (Voet et al., 2011). Consistent with this, sperm DNA damage has been related to an increased risk of recurrent miscarriage (Carrell et al., 2003; Lewis and Simon, 2010).

ATZ without varicocele and OATZ patients

Oligoasthenoteratozoospermic patients are known to have the worst prognosis for becoming fertile, due to their low number of spermatozoa. This low number may lead one to think that a complex etiology could affect them (Burrello et al., 2004). High levels of DNA fragmentation for both alkaline and neutral Comet assays were found in both OATZ and ATZ samples (Fig. 3). Profiles showing high values of alkaline and neutral Comet SDF are referred to as high-equivalent Comet assay profiles. This reinforces the idea that oxidative and enzymatic DNA damage are related, at least in these groups of patients. Moreover, our results suggest that a low sperm number is not related to DNA fragmentation. According to this, in IVF/ICSI treatments, OATZ patients would have the same fertilization potential as would ATZ patients.

ATZ with varicocele

Varicocele patients have an altered spermatogenesis due to different factors (Naughton et al., 2001). High levels of oxidative stress are known to be one of the major contributors to damaging sperm function and DNA (Hendin et al., 1999; Aitken and Krausz, 2001; Hauser et al., 2001). Therefore, the results expected in varicocele patients would be higher in the alkaline Comet than in the neutral Comet assay. However, the high-equivalent Comet assay profile found in varicocele shows higher values of SDF than that in ATZ without varicocele for both alkaline and neutral Comet assays, suggesting that varicocele oxidative stress conditions intensify the two types of DNA damage. These results reinforce the fact that there is a relation between oxidative DNA fragmentation assessed by the alkaline Comet assay and enzymatic DNA damage assessed by the neutral Comet assay. Due to their oxidative damage etiology, varicocele patients could be a group likely to be successfully treated with antioxidants. Nevertheless, there are several antioxidant treatments, and they have a different effect depending on the antioxidant and on the patient (Greco et al., 2005; Agarwal and Sekhon, 2011; Gharagozloo and Aitken, 2011; Zini and Al-Hathal, 2011). Assuming that an antioxidant treatment would work on varicocele patients, we would expect a decrease in DNA fragmentation not only for the alkaline, but also for the neutral Comet assay.

Chromosomal rearrangement carriers

Chromosomal rearrangements have been traditionally associated with an increased risk of miscarriage and infertility (De Braekeleer and Dao, 1991; Benet et al., 2005). Some papers have reported that there are abnormally increased values of SDF in patients carrying Robertsonian translocations (Brugnon et al., 2010), reciprocal translocations and inversions (Perrin et al., 2009, 2011). However, a high variability of SDF has also been observed using TUNEL, SCSA and SCD, suggesting that susceptibility to DNA damage could depend on each specific type of chromosomal reorganization (Garcia-Peiro et al., 2011a,b). In order to gain information about the origin of DNA fragmentation, alkaline and neutral Comet assays were performed, and a high variability was observed for both techniques in these patients (Fig. 3).

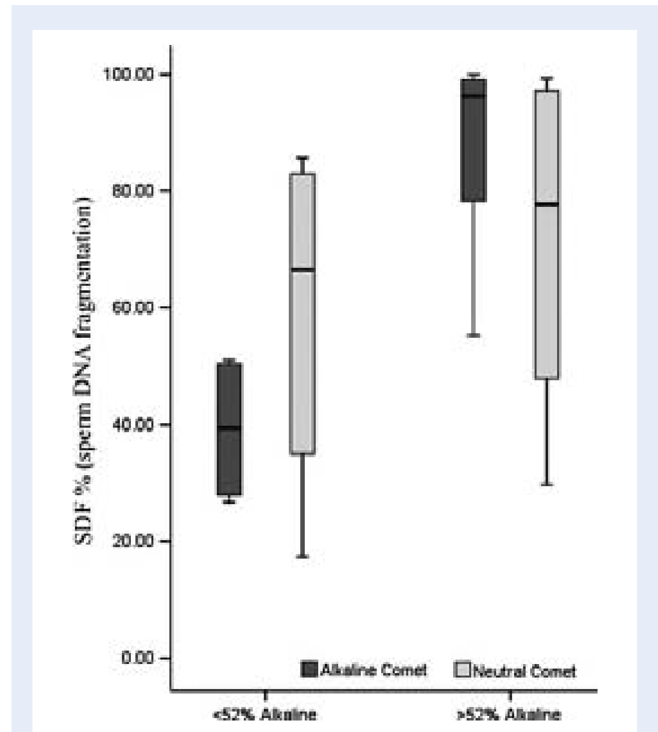

Figure 4 Alkaline and neutral Comet assay SDF in rearranged chromosome carriers, classified according to the 52% alkaline Comet threshold value. Mean $\pm$ standard deviations for the <52% alkaline Comet group were 39.18 $\pm$ 12.96% for alkaline Comet and 59.03 $\pm$ 31.28% for neutral Comet. For the >52% alkaline Comet group, the values were 88.37 $\pm$ 15.65% for alkaline Comet and 69.97 $\pm$ 27.70% for neutral Comet.

Interestingly, two DNA fragmentation profiles were found when samples were classified according to the 52% alkaline Comet assay fertility threshold proposed (Simon et al., 2011). First, a high-equivalent Comet assay profile was found when the alkaline Comet assay was >52% and, second, a non-equivalent Comet assay profile was found in patients with an alkaline Comet assay <52% (Fig. 4). The high-equivalent Comet assay profile in carriers was similar to that found in ATZ, OATZ and varicocele, and the levels of DNA fragmentation were more similar to varicocele patients than to the other groups of patients, although differences are not significant. This may lead one to think that oxidative stress could be one of the main origins of DNA fragmentation in chromosomal reorganization carriers with a high-equivalent Comet assay profile. This oxidative stress could increase neutral Comet assay DNA fragmentation by activating caspases or endonucleases (Sakkas and Alvarez, 2010). Chromosomal reorganization carriers with a non-equivalent Comet assay profile should have a better prognosis for achieving a pregnancy, considering that they had <52% alkaline Comet assay (Simon et al., 2011), and their profile was similar to the three fertile donors analyzed who also had a non-equivalent Comet assay profile, although there are not enough cases to compare them statistically.

Moreover, in our set of patients carrying chromosomal reorganizations, the analysis of the alkaline–neutral DNA profile in two brothers carrying a double translocation 45,XY,t(2;17)(q14.2;q23); t(14;21)(q10;q10) was performed and the data obtained revealed that they had different Comet assay profiles. In particular, one had a non-equivalent Comet assay profile and a baby born naturally, while the other had a high-equivalent Comet assay profile and a baby born after two cycles of PGD (Rius *et al*., 2011). This may suggest that a non-equivalent Comet assay profile may have a better prognosis than a high-equivalent Comet assay profile, while low-equivalent Comet assay profile would correspond with the most fertile donors. In this regard, the 52% alkaline Comet assay threshold may predict infertility (Simon *et al*., 2011), but high values for neutral Comet assay could be indicative of another unknown alteration. In this regard, further studies are needed.

Conclusion

In summary, the combination of alkaline and neutral Comet assays allows researchers to establish relationships between oxidative stress and enzymatic DNA damage, providing a very high sensitivity. DNA fragmentation profiles showed no difference between OATZ and ATZ, while the worst DNA integrity was found in varicocele patients, probably caused by oxidative stress. Different Comet assay profiles can be distinguished in carriers of balanced chromosomal rearrangements, such as the high-equivalent Comet assay profile and the non-equivalent Comet assay profile. Our results suggest that the former would have the worst prognosis, while the latter may have a better chance of achieving a pregnancy.

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Authors' roles

J.R.-M. contributed to the experimental procedure, statistical analysis, graphics and table elaboration and document writing. A.G.-P. contributed to the experimental design, results, discussion and document writing. C.A. and M.J.A. contributed to recruitment of patients, sample collection and storage. J.N. and J.B. contributed to the experimental design and direction and coordination of the work.

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Conflict of interest

None declared.

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4.4 Resultats referents a l'objectiu 2.2

Els resultats aconseguits en el desenvolupament de l'objectiu 2.2 han donat lloc al següent treball, publicat en una revista de l'àrea de la Ciències Multidisciplinàries, indexada en el JCR en 1er quartil (7/56).

Article 4

***Títol**:* Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor.

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PLOS ONE

Double Stranded Sperm DNA Breaks, Measured by Comet Assay, Are Associated with Unexplained Recurrent Miscarriage in Couples without a Female Factor

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Abstract

It is known that sperm samples from recurrent pregnancy loss (RPL) couples have an increase in their sperm DNA fragmentation (SDF), but no studies have been performed in order to identify differences between single stranded SDF (ssSDF) and double stranded SDF (dsSDF) in these patients. This could be relevant because the type of DNA damage could have different effects. Semen samples were classified attending their clinical status: 25 fertile donors and 20 RPL patients with at least two unexplained first trimester miscarriages. SDF was analysed using alkaline and neutral Comet assay, SCD test and pulsed-field gel electrophoresis (PFGE), and ROC analysis including data from 105 more infertile patients (n = 150) was performed to establish predictive threshold values. SDF for alkaline and neutral Comet, and the SCD test was analysed in these categories of individuals. Data revealed the presence of two subgroups within fertile donors. The values obtained were 21.10±9.13, 23.35±10.45 and 12.31±4.31, respectively, for fertile donors with low values for both ssSDF and dsSDF; 27.86±12.64, 80.69±12.67 and 12.43±5.22, for fertile donors with low ssSDF and high dsSDF; and 33.61±15.50, 84.64±11.28 and 19.28±6.05, for unexplained RPL patients, also showing a low ssSDF and high dsSDF profile. This latter profile was seen in 85% of unexplained RPL and 33% of fertile donors, suggesting that it may be associated to a male risk factor for undergoing RPL. ROC analysis regarding recurrent miscarriage set the cut-off value at 77.50% of dsDNA SDF. PFGE for low ssSDF and high dsSDF profile samples and positive controls treated with DNase, to induce dsDNA breaks, showed a more intense band of about 48 kb, which fits the toroid model of DNA compaction in sperm, pointing out that some nuclease activity may be affecting their sperm DNA in RPL patients. This work identifies a very specific SDF profile related to the paternal risk of having RPL.

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Introduction

Recurrent pregnancy loss (RPL) is defined as having at least two consecutive embryo miscarriages within the first or early second trimester of pregnancy [1]. Due to the complex aetiology involved in miscarriages, up to 40%–50% of RPLs remain unexplained [1,2]. Taking into account that sperm cells and oocytes provide half of the nuclear embryo DNA, it may be assumed that both males and females could be involved in either infertility or RPL [3,4].

Regarding female factors that may be involved in RPL, they can be classified as genetic or chromosomal causes, advanced maternal age, antiphospholipid syndrome, hormonal abnormalities, uterine abnormalities or metabolic disorders [1,2,5–8]. The male factor has been less studied for many years, mainly basing the infertility diagnosis on semen parameters and, although this information is necessary, it is not always conclusive [9]. It has been described that the male factor may be involved in RPL when poor semen parameters, Y chromosome microdeletions, or a higher percentage of sperm aneuploidies detected by FISH are found [10–18]. However, normal sperm parameters are shown in many reported cases of RPL [17]. As a consequence, the paternal effect in these cases is being underestimated, and only a few recent reports provide data suggesting the possible relation of the sperm DNA status in the aetiology of RPL [12,19]. Sperm DNA fragmentation has now become a new biomarker for male infertility diagnosis and different methods have been developed [20–26]. In fact, some studies have shown that sperm DNA fragmentation (SDF) is increased in semen samples from RPL couples by using Sperm

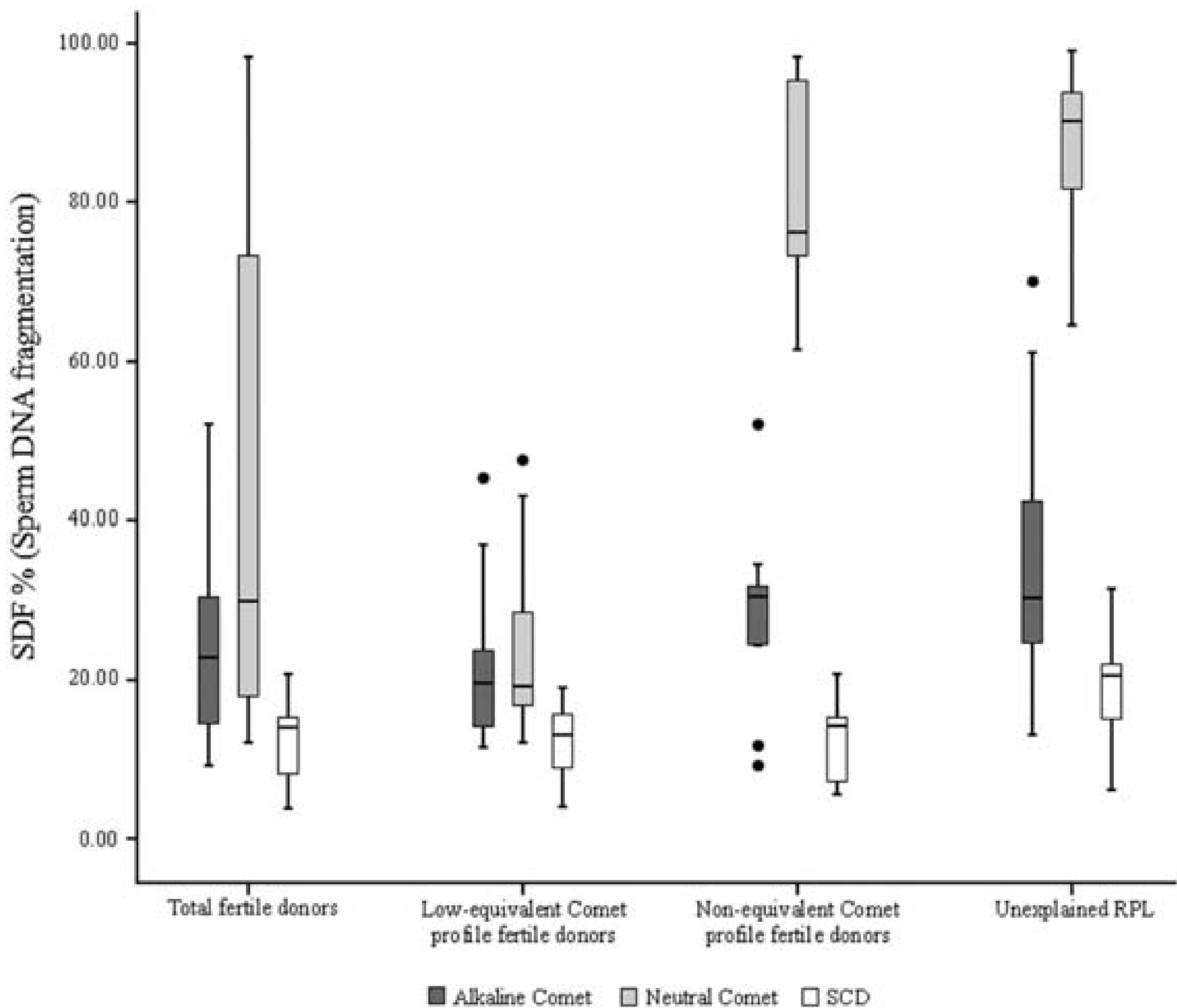

Figure 1. Alkaline Comet, neutral Comet and Sperm Chromatin Dispersion for total fertile donors, for low dsSDF and high dsSDF fertile donor subgroups and for the unexplained RPL group.
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Chromatin Dispersion test (SCD) [12,15], Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) [11,19] or Sperm Chromatin Structure Assay (SCSA) [10] methodologies. However, no studies have been performed analysing both single and double stranded DNA fragmentation in RPL patients. It has been recently reviewed that fertilisation with damaged spermatozoon may result in an increase of DNA damage in the embryo genome, which could result in DNA errors at different levels of embryogenesis [4], and it could end up as a miscarriage or different childhood diseases [27,28].

The higher sperm DNA fragmentation found in previous works studying RPL patients may have its origin in poor DNA packaging, at chromatin remodelling during spermiogenesis, which could leave DNA more vulnerable basically in front of oxidative stress [29–32] and DNA nucleases [33,34]. Some papers have described the sperm chromatin compaction showing the toroids as the basic structural elements separated by a linker DNA attached to the nuclear matrix, known as matrix attachment region (MAR), which would be more susceptible to being cut by nucleases [35–37]. Each toroid compacts about 48 kb of DNA, which represents a unique degree of DNA packaging in sperm [37]. Moreover, other authors showed the importance of chromosome organisation in the sperm nucleus, pointing out that centromeres might be grouped in internal regions of the sperm and telomeres would be associated in pairs at more outer layers [38–40].

In a previous study, alkaline Comet assay, identifying mostly single stranded DNA fragmentation (ssSDF), and neutral Comet assay, identifying mostly double stranded DNA fragmentation (dsSDF), were compared in controls and in different groups of patients [41,42]. Different DNA damage profiles were found due to different aetiologies of DNA fragmentation in different infertile patients and chromosome reorganisation carriers [41]. Then, different single and double stranded DNA damage profiles were established: a) a profile with low percentages of sperm with both ssSDF and dsSDF, which has been seen in most fertile donors; b) a profile with low percentages of sperm with ssSDF and high percentages of dsSDF, which was seen in chromosome reorganisa-

Table 1. Sperm DNA fragmentation (mean ± SD) in fertile donors and unexplained RPL samples.

	% SDF (Sperm DNA fragmentation)		
	Alkaline Comet	**Neutral Comet**	**SCD**
Total fertile donors (n = 25)	23.53±10.79	44.00±30.18	12.35±4.55
Low dsSDF fertile donors (n = 16)	21.10±9.13	23.35±10.45[c]	12.31±4.31
High dsSDF fertile donors (n = 9)	27.86±12.64	80.69±12.67[a, b]	12.43±5.22
Unexplained RPL (n = 20)	33.61±15.50[a, b]	84.64±11.28[a, b]	19.28±6.05[a, b, c]

[a]Statistical differences with total fertile donors ($p<0.01$).
[b]Statistical differences with low dsSDF fertile donors ($p<0.01$).
[c]Statistical differences with high dsSDF fertile donors ($p<0.01$).
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tion carriers and three fertile donors [41] and with still unknown consequences on fertility and c) a profile with both high percentages of ssSDF and dsSDF, which has been shown in varicocele patients [41] and linked to the worst prognosis for fertility.

The aim of the present work is to describe the single and double stranded DNA fragmentation, by using alkaline and neutral Comet and SCD test, in semen samples from RPL couples without female factors. Then, to establish different threshold values for both pregnancy and recurrent miscarriage, and additionally, to improve the knowledge of the causes and the possible localisation of these dsDNA breaks by using pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Semen Samples

Semen samples from 45 human males were obtained in collaboration with reproduction centres of the Barcelona area and were divided into two groups: 25 donors with proven fertility and without experiencing any previous miscarriage (15 previously reported, [41]) and 20 donors from couples with at least two

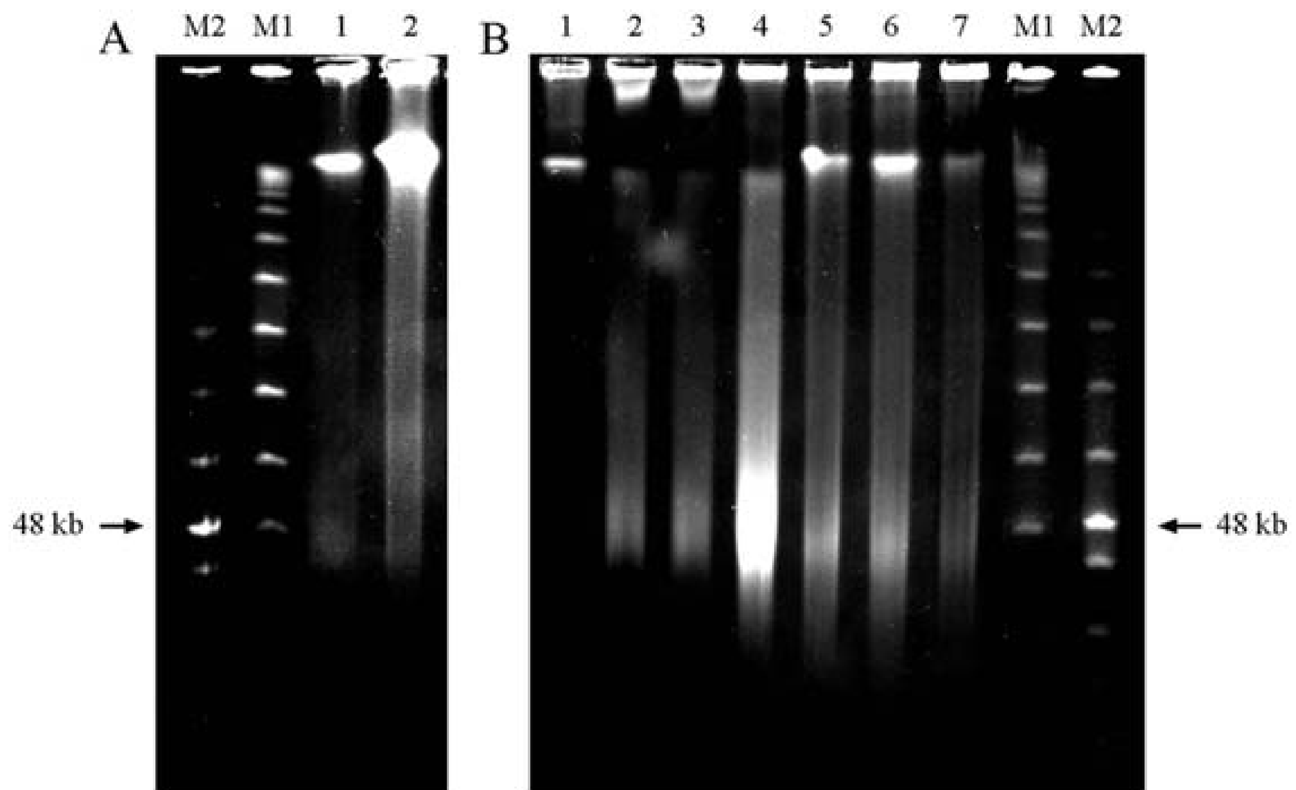

Figure 2. Pulsed-field gel electrophoresis of semen samples DNA from fertile donors (A, lanes 1 and 2; B, lane 1), negative control (B, lane 1), positive controls with DNAse 0.5 mg/ml, 30 minutes (B, lanes 2, 3 and 4) and RPL samples (B, lanes 5, 6 and 7). DNA molecular weight markers consisting of Low Range PFG Marker (M1) and Lambda ladder PFG marker (M2) are detailed. Negative controls in B, lane 1 show a thin compression zone. Positive controls in B, lanes 2, 3 and 4 show DNA digestion into sizes of around the 48 Kb. Sperm DNA fragmentation of the specific samples of this figure is shown in Table 2.
doi:10.1371/journal.pone.0044679.g002

Table 2. Relation of samples shown in Figure 2 with their sperm DNA fragmentation.

% SDF		A		B						
	Lane	**1**	**2**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Alkaline Comet (ssSDF)		11.75	24.5	14.6	–	–	–	30.4	24.2	21.25
Neutral Comet (dsSDF)		98.25	95.75	18.0	–	–	–	96.6	90.0	94.75

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consecutive miscarriages within the first or early second trimester of pregnancy. In the RPL samples, abnormal female factors for advanced maternal age, karyotype, antiphospholipid antibodies, uterine abnormalities and thrombophilias were discarded.

Samples were obtained by masturbation after a minimum of three days of abstinence. A semenogram was performed according to WHO 2010 and samples were cryopreserved in test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose, 1.72% sodium citrate) [41,43]. Sperm count (spermatozoa/mL), motility (A+B %) and morphology (Kruger strict criteria, normal forms %) were (mean ± standard deviation): 109.88±114.54, 37.20±23.02 and 7.20±1.87, respectively, for the fertile donor group and 116.65±115.83, 39.18±19.44 and 5.00±2.45, respectively, for the RPL group.

Informed consent was obtained from all donors and the present study was approved by the appropiate ethics committee.

Neutral and Alkaline Comet Assay

Alkaline and neutral Comet assay procedures, staining and classification of fragmented or non-fragmented sperm were performed on all semen samples according to the protocol reported before [41]. Intra-individual differences were measured in five samples and the variability mean was less than 5% of SDF for both alkaline and neutral Comet. These results have been previously published [41].

Alkaline and neutral Comet assays were perfomed simultaneously in two different slides. First, an aliquot of the total semen was thawed and washed three times in PBS. Then, sperm cells were diluted to a concentration of 10×10^6 spermatozoa/ml, and 25 µl were mixed with 50 µl of low melting point agarose 1% (Sigma Aldrich; St Louis, MO, USA) in distilled water. Rapidly, 15 µl of the mixture were placed on two different pre-treated slides for gel adhesion (1% low melting point agarose), covered with coverslips and allowed to jellify on a cold plate at 4°C for 5 minutes. Next, coverslips were carefully removed and slides were

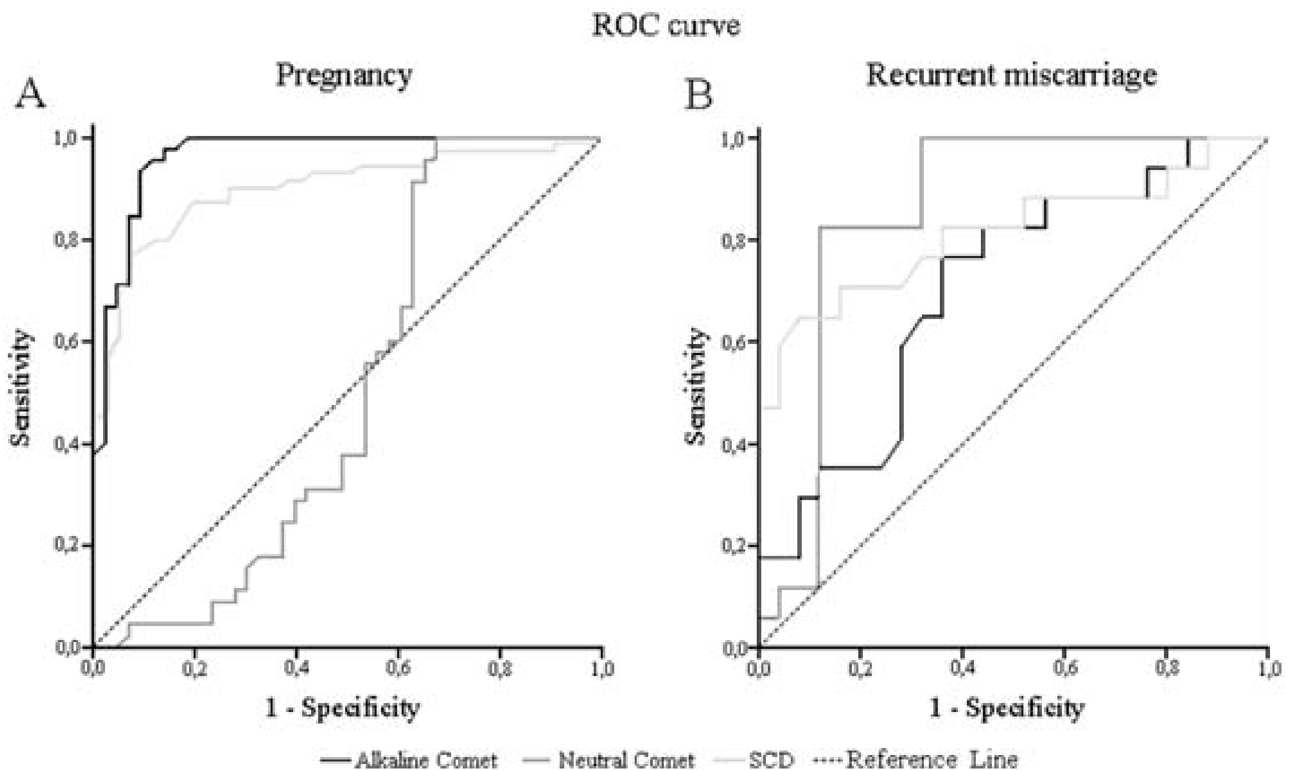

Figure 3. ROC curves analysis for alkaline Comet, neutral Comet and SCD attending: Pregnancy without miscarriage (A), and recurrent miscarriage (B).
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submerged for 30 minutes in two lysing solutions (Comet lysis solutions, Halotech; Madrid, Spain) and washed for 10 minutes in TBE (0.445 M Tris-HCl, 0.445 M Boric acid, 0.01 M EDTA). For the neutral Comet assay, electrophoresis was performed in TBE buffer at 20 V (1 V/cm) for 12 minutes and 30 seconds, and then washed in 0.9% NaCl for 2 minutes. For the alkaline Comet assay, the slide was incubated in denaturing solution (0.03 M NaOH, 1 M NaCl) for 2 minutes and 30 seconds at 4°C, and afterwards, electrophoresis was then performed in 0.03 M NaOH buffer at 20 V (1 V/cm) for 4 minutes. After that, both neutral and alkaline slides were incubated in the neutralizing solution (0.4 M Tris-HCl, pH 7.5) for 5 minutes, in TBE for 2 minutes and finally dehydrated in an ethanol series of 70%, 90% and 100% for 2 minutes each.

Sperm Chromatin Dispersion Test (SCD)

Sperm DNA damage using the SCD test was performed using the Halosperm kit (Halotech DNA; Madrid, Spain) following the manufacturer's instructions. Samples were stained with DAPI SlowFade® Gold antifade (Invitrogen; Eugene, OR, USA) and 400 spermatozoa were assessed and classified as fragmented or non-fragmented sperm, according to the manufacturer's criteria, using a fluorescence microscope (Olympus AX70).

Pulsed-field Gel Electrophoresis (PFGE) Method

23 of 45 samples from all the groups were analysed through PFGE in order to find size patterns of the DNA fragments.

Negative and positive controls. Sperm samples with a known profile of both low values of ssSDF and dsSDF were considered to be negative controls. Positive controls were induced using the same Comet assay profile samples, but with the following procedure: after thawing on ice and being washed twice in PBS for 2 minutes, sperm cells were centrifuged at 700 g to achieve a concentration between $15 \cdot 10^6$ and $30 \cdot 10^6$ spermatozoa per 100 µl. Then, sperm cells were permeabilised with 0.25% Triton X100 for 2 minutes on ice and two more washings in TBE 0.5X were performed. After that, in order to produce dsDNA breaks, a treatment with 0.5 µg/mL ribonuclease I from bovine pancreas (Sigma; St Louis, MO, USA) was performed for 30 minutes at 37°C and nuclease action was stopped with 50 mM EDTA. PFGE protocol was continued making the PFGE plugs as following described immediately below.

PFGE analysis. The pulsed-field gel electrophoresis protocol applied was similar to the protocol reported before [36]. Sperm cells were concentrated at 15–30 million spermatozoa in 100 µl and mixed with 1% 100 µl pulsed-field certified agarose (BioRad; Hercules, CA, USA), poured into insert moulds and allowed to solidify. For lysis, the resulting plugs were placed in 2 ml of lysis buffer (10 mM Tris HCl, 10 mM EDTA, 100 mM NaCl, 20 mM DTT, 2% SDS and 20 µg/mL proteinase K, pH 8.0) and incubated for 24 h at 53°C. The plugs were washed three times in TE+Glycine (10 mM Tris-HCl, 0.1 mM EDTA, pH 8 and 1 M Glycine) for 10 minutes, and then twice more in TE buffer for 10 minutes.

A quarter slice of each plug was cut off and placed on 1% gel and resolved by electrophoresis on a contour-clamped homogeneous electric field apparatus (Bio-Rad CHEF DRIII system) in TBE 0.5 X (Tris-borate 50 mM, EDTA 0.1 mM) at a 120° angle, 14°C, 4 V/cm and with the following pulses: 6.7 tp 33.7 seconds for 27.1 hours.

DNA molecular weight markers consisting of Lambda ladder PFG marker and Low Range PFG Marker (New England Biolabs; Ipswich, MA, USA) were included in each electrophoretic run.

Gels were stained with ethidium bromide and visualised and photographed under ultraviolet light using the GelDoc System (BioRad; Hercules, CA, USA).

Statistical Analysis

Statistical analysis of SDF data was performed using the Statistics Package for the Social Sciences software, version 17 (SPSS Inc.; Chicago, IL). The Mann-Whitney U test was used to compare samples, setting the confidence interval at 95%, and ROC analysis was performed, including previous data of 105 infertile patients from our group ($n = 150$) ([41] and Garcia-Peiró unpublished data) in order to obtain the sensitivity, specificity and the cut-off value for each test.

Results

Sperm DNA Fragmentation: Alkaline and Neutral Comet Assay and SCD Test

Of all 25 collected semen samples from fertile donors, 16 samples (64%) presented a profile with low values of both ssSDF and dsSDF and nine samples (36%) presented a profile with low values of ssSDF and high values of dsSDF. Regarding RPL study samples, 17 out of 20 (85%) showed low values of ssSDF and high values of dsSDF.

Results and statistical comparisons of data obtained by using both alkaline and neutral Comet assays and the SCD test are shown in Table 1 and Figure 1. No statistical differences were obtained for either alkaline or neutral Comet assays with the increase of 10 more samples in the fertile donor group, with respect to the previously published control group [41] ($p > 0.05$). However, this enlargement of the previously reported control group allowed for the observation of a bimodal distribution is dsSDF, suggesting the presence of two subgroups within it. These two fertile donor subgroups, one with a low ssSDF and low dsSDF profile and the other with a low ssSDF and high dsSDF profile, showed statistical differences in neutral Comet ($p < 0.01$), nevertheless, no statistical differences were found between them regarding alkaline Comet ($p > 0.05$) or the SCD test ($p > 0.05$) (Table 1).

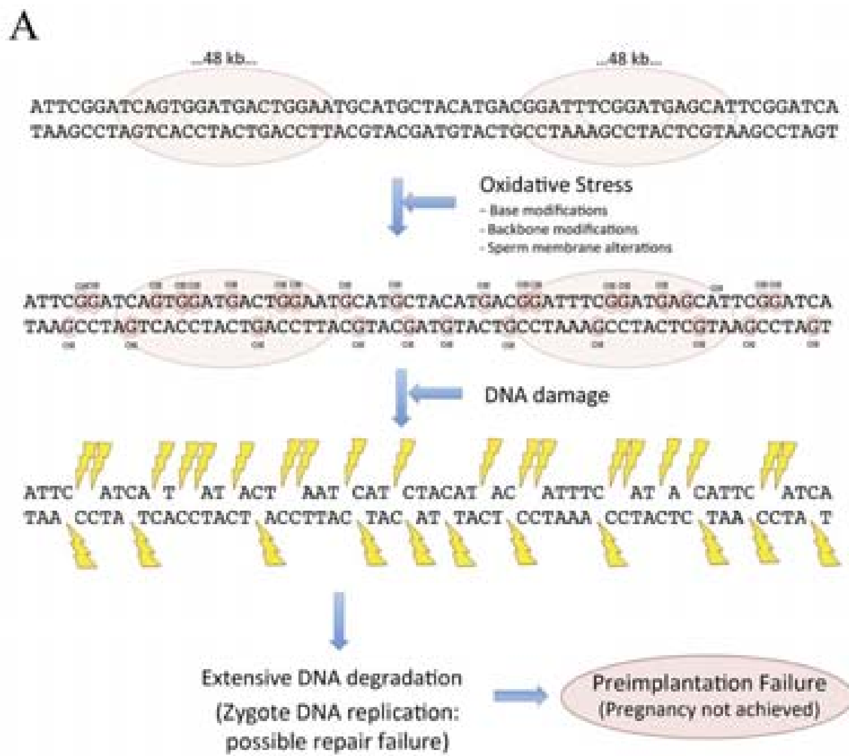
On the other hand, statistical differences were found in all three techniques when comparing unexplained RPL group SDF with total fertile donors SDF ($p < 0.01$). Regarding the RPL group and low dsSDF fertile donor group, differences were found between them for all three techniques analysed ($p < 0.01$). No differences were found for ssSDF or dsSDF between RPL and high dsSDF fertile donor group ($p > 0.05$), however, statistical differences were found by using the SCD test ($p < 0.01$).

Pulsed-field Gel Electrophoresis (PFGE)

PFGE showed good reproducibility regarding the bands shown within the sample groups analysed. A PFGE analysis on sperm DNA is shown in Figure 2 as an example, and the relationship with the SDF of the samples analysed in that gel is shown in Table 2.

Negative and positive controls. The negative control obtained from a sample with a known low SDF for both ssDNA and dsDNA is shown in Figure 2B, lane 1, and shows a thin compression zone. Positive controls made using the same sample with incubations of DNAse to induce dsDNA breaks (Fig. 2B lanes 2, 3 and 4) showed DNA digestion into sizes of around 48 Kb.

Samples from fertile donors and RPL patients. Samples from three fertile donors are shown in: Figure 2A lanes 1 and 2, for samples with a low ssSDF and high dsSDF profile; and in Figure 2B, lane 1, for a sample with both low ssSDF and dsSDF.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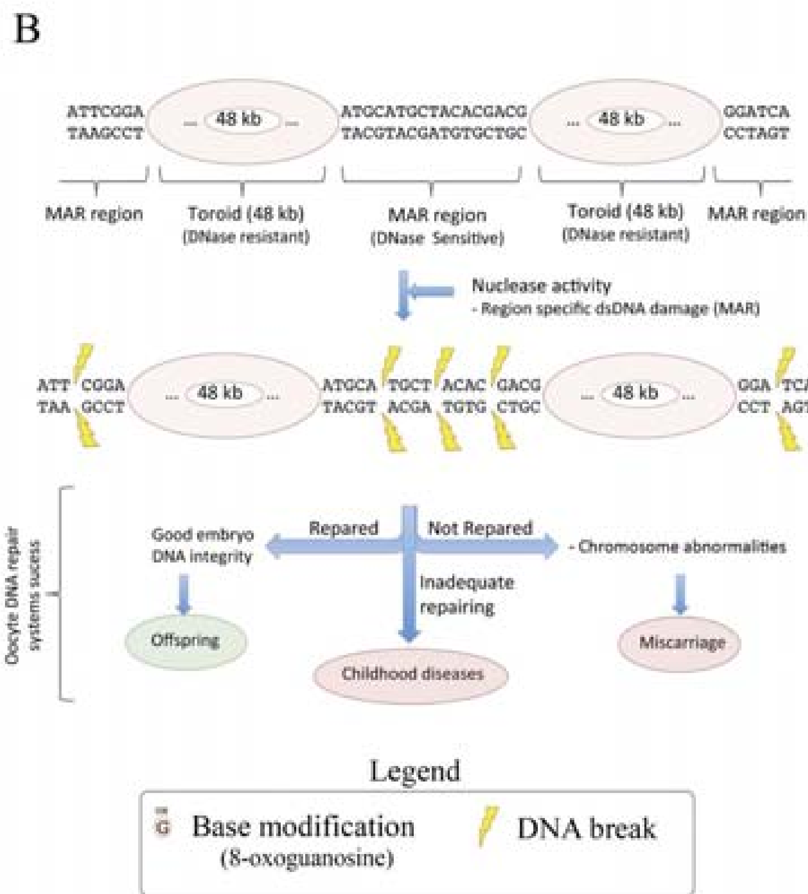
Oxidative Stress
- Base modifications
- Backbone modifications
- Sperm membrane alterations
DNA damage
Extensive DNA degradation
(Zygote DNA replication:
possible repair failure)
Preimplantation Failure
(Pregnancy not achieved)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Nuclease activity
- Region specific dsDNA damage (MAR)
Oocyte DNA repair systems sucess
Good embryo DNA integrity
Repared
Not Repared
- Chromosome abnormalities
Inadequate repairing
Offspring
Childhood diseases
Miscarriage
Legend
Base modification
(8-oxoguanosine)
DNA break

Figure 4. Model for ssDNA and dsDNA breaks mechanisms and clinical outcomes. ssSDF model (A) dsSDF model (B).
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Samples with a low ssSDF and high dsSDF profile showed a slight compression band and also a band at about 48 kb, similar to positive controls with nuclease (Figure 2B; lanes 2, 3 and 4). The fertile donor shown in Figure 2B, lane 1, with both a low ssSDF and dsSDF, presented a compression band with good DNA integrity, and no 48 kb band was seen.

Results from RPL samples are shown in Figure 2B, lanes 5, 6 and 7. Both the thin compression band and the 48 kb sized fragments were present in these patients.

ROC Analysis

ROC analysis results are shown in Figure 3, for either achieving a pregnancy without taking into account a possible subsequent miscarriage, and for undergoing a recurrent miscarriage associated with a male factor and without the female factors mentioned previously. Regarding the achievement of a pregnancy in all three techniques, ROC analysis set the cut-off value at 45.62% of alkaline Comet SDF, with a sensitivity and specificity of 0.933 and 0.907, respectively, and an area below the curve of 0.965 cm^2. SCD data showed a cut-off value of 22.5% of SDF with a sensitivity and specificity of 0.768 and 0.929, respectively, and an area below the curve of 0.899 cm^2. Neutral Comet showed lower combined sensitivity and specificity and less area below the curve in predicting pregnancy: 0.911, 0.349, 0.503 cm^2, respectively (Figure 3A). Otherwise, regarding male-factor associated recurrent miscarriage, neutral Comet assay set the threshold value at 77.5% of SDF, with a sensitivity and specificity of 0.833 and 0.880, respectively, and an area below the curve of 0.858 cm^2. SCD established the cut-off value at 18.5% of SDF, with a sensitivity and specificity of 0.647 and 0.920, respectively, and an area below the curve of 0.814 cm^2. Alkaline Comet showed lower combined sensitivity and specificity and less area below the curve in predicting recurrent miscarriage: 0.944, 0.057, 0.303, respectively (Figure 3B).

Discussion

Measurement of sperm DNA fragmentation is an area of growing interest due to its capacity of predicting male infertility [4,44–46]. In a previous paper a descriptive study was performed on different groups of patients discussing the relationship about the different profiles of alkaline and neutral Comet assay regarding the aetiology of DNA breaks [41]. In the present work, ssSDF and dsSDF have been analysed in fertile donors group and RPL patients by using alkaline and neutral Comet assay, the SCD test and PFGE. Regarding fertile donors, a bimodal distribution has been observed in neutral Comet assay SDF, corresponding to dsDNA breaks, suggesting that two different subgroups could be identified within them: fertile donors with low ssSDF and low dsSDF, and fertile donors with low ssSDF and high dsSDF (Figure 1 and Table 1). These results point out that dsDNA breaks would not have implications on the achievement of a pregnancy. Low values of alkaline Comet assay SDF ($<$52%) are shown in both subgroups of fertile donors, showing its importance in achieving a pregnancy, as has been proposed recently for native semen using ART [47], and most of them showed a lower SDF than the 25% threshold value for natural conception [47].

About 85% of unexplained RPL patients included in the study showed low values of ssSDF and high values of dsSDF, and no differences were found when comparing them with the high dsSDF fertile donors group (Figure 1 and Table 1). Otherwise, statistical differences were found for both alkaline and neutral Comet assays upon comparing them with the low dsSDF fertile donors. However, alkaline Comet assay always showed values below the 52% threshold value established for the achievement of a pregnancy [47]. These Comet assay profiles applied to fertile donors and unexplained RPLs are consistent with previous reports because they were compatible with pregnancy by having a low percentage of single stranded DNA damage [47] and also with the fact that dsSDF might be a quality biomarker in sperm, that could be indicative about the progressive embryonic development. About that, some unknown parameter related to the oocyte capacity of repairing these double stranded DNA damage presented by the fertilising sperm could be important for appropriate **embryonic development (Figure** 1) [48]. In this sense, while a profile with low values for both ssSDF and dsSDF would mean a good prognosis of pregnancy and offspring, the profile with low ssSDF and high dsSDF would indicate a good prognosis for pregnancy, but with a risk of undergoing a male-factor associated miscarriage. It has also been described that fertilisation with damaged sperm could lead to errors in DNA replication, transcription and translation [4] because of the differential repair of single or double stranded breaks. For that, the distinction of ssDNA and dsDNA breaks seems to have an interest in the male factor diagnosis area, and the knowledge of the DNA breaks aetiology could provide new clues to understanding part of idiopathic RPL. Moreover, the sperm DNA damage assessment could be especially interesting in those patients with normal semen parameters, who are classified as idiopathic infertility.

The analysis of SDF by the SCD test showed no statistical differences between the two different fertile donor groups and, in consequence, the SCD test would not have the ability of distinguishing the high percentage of double stranded DNA breaks presented by the fertile group with high dsSDF found by the neutral Comet assay. The unexplained RPL group presented higher levels of SDF, when compared with the two fertile donor groups, in agreement with previous studies [11,12,15]. However, RPL samples presented a SDF at about the threshold value required for this method (20%–30%) [24] for achieving a pregnancy.

Data obtained with both Comet assay and the SCD test allowed for the establishment of different threshold values for each SDF technique for fertilisation success and miscarriage prognosis. Results displayed a threshold value of 45.62% SDF for the alkaline Comet assay related to pregnancy achievement. This result is in agreement with the 52% threshold for ART fertility proposed by Simon et al. [47], taking into account that this study did not differentiate natural conception and fertilisation after ART treatment, and that the Comet assay protocol used was slightly different [41,42]. Regarding the SCD test, different threshold values have been proposed for achieving pregnancy [23,49], and our analysis obtained a similar threshold value of 22.5% of SDF. Although both techniques can distinguish between fertile and infertile donors, alkaline Comet assay demonstrated higher sensitivity and specificity than the SCD test, in relation to fertility. Otherwise, neutral Comet assay, evaluating dsDNA breaks incidence, had no association with pregnancy achievement (Figure 3A). This lack of association with pregnancy might be due to the different oocyte repair mechanisms (Figure 4). Single stranded DNA damage is produced mainly due to oxidative stress, which induces base modifications, DNA backbone modifications

and membrane alterations [50]. This DNA damage is extensive, being produced both in the MAR regions and within the DNA compacted by toroids, and could even be stronger if a bad DNA compaction is present. This extensive DNA damage finally produces a high number of DNA breaks and, because the ssDNA breaks are being repaired during the first embryo DNA replication [51], the presence of such extensive damage would make it difficult to be all repaired in the first embryo cleavage. This lack of repair due to this extensive damage would cause, in the end, a failed pregnancy. On the other hand, double stranded DNA damage is produced mainly due to nuclease activity, which directly produces DNA breaks in unprotected regions (MAR regions that are not compacted by protamines) [37] (Figure 2B). In consequence, this dsDNA damage is not as much extensive as ssDNA damage, and must be repaired before the replacement of protamines by histones in the embryo. There are three possible scenarios about the final outcome: a) If the dsDNA damage is not repaired by the embryo, it would cause chromosome abnormalities that would end up as a miscarriage; b) If the dsDNA breaks are repaired, then DNA integrity is recovered and the pregnancy and posterior birth can be carried out, and c) If dsDNA breaks have an inadequate repair, then there would be a few DNA alterations that could lead to childhood diseases (Figure 4). In this sense, our results show that the neutral Comet assay (dsSDF) had a good association with the male-factor associated miscarriage risk, induced by sperm DNA damage, with a threshold value of 77.5% of SDF and an acceptable sensitivity (0.833) and specificity (0.880) to be used as a diagnostic tool. For predicting the male-factor associated miscarriage risk, the SCD test established a threshold of 18.5%, but with lower sensitivity than neutral Comet assay. Otherwise, the alkaline Comet assay did not have any association with recurrent miscarriage, being the worst of the three techniques in RPL prognosis (Figure 3B). As the effect of ssSDF and dsSDF could have different implications in reproduction, our data suggest that semen samples need to be analysed with both alkaline and neutral Comet assay in order to obtain an accurate diagnosis. First, the alkaline Comet assay threshold of 52% would indicate the fertilisation capacity of the sample. Then, if neutral Comet is higher than 77.5%, the low ssSDF and high dsSDF profile shown would indicate the possibility of suffering a miscarriage, depending on the oocyte capacity of repairing the double stranded sperm DNA breaks. In fact, it has been demonstrated that better outcomes are obtained when oocytes from donors are used, compared with standard IVF cycles [52]. The combination of the two Comet techniques could also improve the global sensitivity and specificity of predicting a pregnancy, which could result in miscarriage.

Finally, regarding the possible origin of the dsDNA breaks shown by neutral Comet assay, it has been previously described the existence of some nuclease activity in sperm cells [36] whose activation should be linked to oxidative stress [53]. Both fertile donors with high dsSDF and unexplained RPL showed low values of oxidative damage, which is detected by alkaline Comet assay (ssSDF) [41] and high values of nuclease damage, which is detected by neutral Comet assay (dsSDF) [41], a reason that leads us to think that nuclease activity independent of oxidative stress should also exist. To confirm this approach, PFGE was performed on the different sample groups (Figure 2 and Table 2). Fertile donors with both low ssSDF and dsSDF showed a thin compression band (Figure 2B, lane 1), and incubations of this same sample with DNase, to induce dsDNA breaks, resulted in fragment sizes of around 48 kb (Figure 2B, lanes 2, 3 and 4). These results fit the toroid model of DNA compaction. Toroids of 48 kb are compacted on the sperm head, leaving a region of about two kilobases, the matrix attachment regions, which would be linked to the nuclear matrix, packaged by histones and, because of that, sensitive to nuclease activity [37]. DNases would not be able to cut toroid DNA compacted by protamines, but MAR regions, which are linked to histones, would be exposed to their nuclease activity [35,36]. Samples from fertile donors with high dsSDF and from unexplained RPL patients showed both bands, one compression band at a high number of kb and the other at 48 kb, similar to the band that appeared with DNase treatment, which would agree with the approach that some nuclease activity, independent of oxidative stress, affects the DNA of these donors and RPL patients. The compression band would have a relation with a low level of ssSDF, and the 48 kb band would be related to a high percentage of dsSDF (Table 2 and Figure 2).

Conclusion

The results support the fact that single stranded DNA damage allows to predict the fertilisation potential, and suggest that double stranded DNA damage is related to the risk of undergoing a male-factor associated miscarriage, possibly due to a possible lack of repair of sperm dsDNA breaks by the oocyte, as we have proposed in a model. For that, it would be essential to have good quality oocytes on couples where the male show this low ssSDF and high dsSDF profile. The establishment of the 77.5% SDF threshold for neutral Comet assay offers an opportunity for idiopathic RPL without female factor patients to be diagnosed. Finally, PFGE treatments with DNase in sperm showed 48 kb bands, suggesting that the dsDNA breaks are being produced in MAR regions, which are known to be DNase sensitive. Finally, the analysis of high dsSDF fertile donors and RPL samples pointed out that non-oxidative dependent enzymatic activity could be producing the double stranded breaks detected by neutral Comet assay in these donors and patients. In this sense, the research on different strategies of sperm selection to reduce the dsSDF could improve the miscarriage rates in these patients.

Author Contributions

Conceived and designed the experiments: JRM AGP JN JB. Performed the experiments: JRM AFE MJA EP PC. Analyzed the data: JRM AGP PC JN JB. Wrote the paper: JRM AGP JN JB.

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4.4.1 Millora en la predicció del risc d'avortaments en el reanàlisi de les dades amb una mostra ampliada respecte la inclosa en l'article Ribas-Maynou i col., 2012b

L'anàlisi estadístic ampliat de les dades de Comet neutre i alcalí de 28 pacients de parelles que presenten avortaments de repetició sense factor femení, 25 controls de fertilitat provada, i 131 pacients infèrtils ha permès realitzar una corba ROC més acurada per al diagnòstic del risc d'avortament de repetició associat a factor masculí.

Tal i com ja s'ha discutit anteriorment (Ribas-Maynou i col*.,* 2012b), el Comet alcalí no presenta poder predictiu per a aquest diagnòstic. El reanàlisi del Comet neutre presenta una sensibilitat, especificitat i àrea sota la corba de de 0,517; 0,890 i 0,708; respectivament, amb un valor llindar del 84% de dsSDF. Donat que el diagnòstic d'avortaments està associat a una baixa ssSDF i alta dsSDF, la realització d'una ràtio dsSDF/ssSDF pren sentit, ja que pacients amb risc d'avortaments presentaran valors elevats d'aquesta ràtio, mentre que tant pacients infèrtils com pacients fèrtils presentaran valors similars a 1. Així doncs, els valors predictius de sensibilitat, especificitat i àrea sota la corba de la ràtio dsSDF/ssSDF són 0,724; 0,955 i 0,875; respectivament, amb un valor llindar de 2. Aquesta ràtio representa una millora en la predicció del risc d'avortaments respecte l'anàlisi únicament per Comet neutre (Figura 26), obtenint una millora diagnòstica substancial.

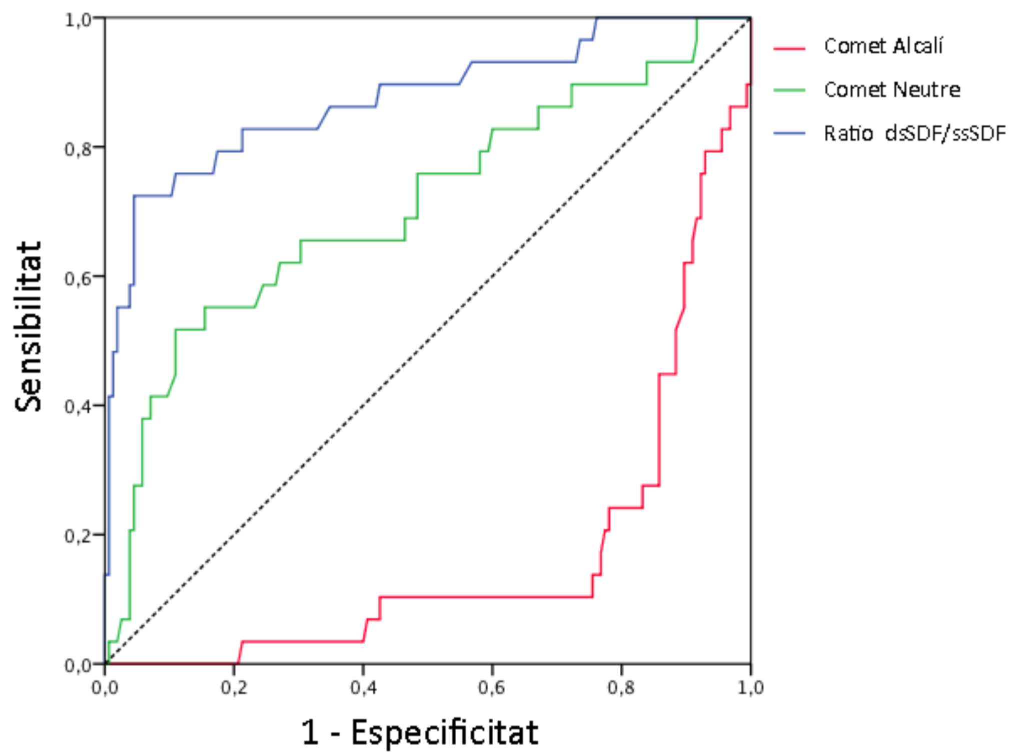

Figura 26. Anàlisi ROC comparatiu per al diagnòstic d'avortament amb Comet alcalí, Comet neutre, i la ràtio dsSDF/ssSDF.

4.5 Resultats referents a l'objectiu 3

Els resultats aconseguits en el desenvolupament de l'objectiu 3 han donat lloc al següent treball, publicat en una revista de l'àrea de la Biologia de la Reproducció, indexada en el JCR en 1er quartil (3/28).

Article 5

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

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MHR ORIGINAL RESEARCH

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

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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 sodium dodecyl sulfate (SDS) and dithiothreitol (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.

Key words: neutral Comet assay / sperm nuclear matrix / sperm DNA 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 (Haidl *et al.*, 1994; Chapman and Michael, 2003; Bjorndahl and Kvist, 2010). This renders the mature sperm chromatin more resistant to damage by reactive oxygen species (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 (Evenson *et al.*, 1999; Carrell *et al.*, 2003; Lewis and Simon, 2010; Ribas-Maynou *et al.*, 2012b). Several studies have shown that sperm DNA damage is higher in infertile men when compared with fertile donors (Evenson and Jost, 2000; Fernandez *et al.*, 2005; Sharma *et al.*, 2010; Ribas-Maynou *et al.*, 2012b; 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 (ssDNA) 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

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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., 2007a,b). 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 has the ability to unwind and untangle knotted pieces of DNA through transient double-stranded DNA (dsDNA) breaks. TOP2 binds to one part of the knotted DNA and induces a dsDNA break. Another part of the knotted or twisted DNA is then passed between the two broken ends of the first DNA cut. TOP2 then religates the originally cut strands (Nelson et al., 1986) (Supplementary data, Fig. S1). 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, b), 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 ssDNA and dsDNA breaks depending on whether alkaline or neutral pH is used, respectively (Singh et al., 1988; Enciso et al., 2009; Ribas-Maynou et al., 2012a). 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 as 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 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.

Materials 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 1 h at 37°C. To test for the ability of the sperm chromatin to religate dsDNA breaks, samples were incubated with 100 mM ethylenediaminetetraacetic acid (EDTA) for half an hour at 37°C. 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 h at 37°C.

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, samples were incubated with 0.128 M H_2O_2 for 30 min at 37°C.

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 1×10^6 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 min at 4°C 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 min each at room temperature, and then washed in TBE (0.445 M Tris–HCl, 0.445 M boric acid, 10 mM EDTA) for 10 min.

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 min. 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 min at 4°C and then electrophoresed at 1 V/cm in alkaline buffer (0.03M NaOH) for 4 or 12.5 min. Finally, both slides were washed in neutralization solution (0.4 M Tris–HCl, pH 7.5) for 5 min, then in an ethanol series (70, 90 and 100%) for 2 min each, and allowed to dry horizontally.

SDS-neutral Comet assay

To test whether some dsDNA 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 min 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 × 10 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 Fig. 1, and larger fields showing several Comets are shown in Supplementary data, Figs S2 and S3. The Comet length histograms of the population for each treatment are shown in Fig. 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 tails 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 (Figs 1 and 2a,f,k and p, Supplementary data, Figs S2 and S3). Nuclease treatment, our positive control for dsDNA 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 min of electrophoresis (Fig. 2d and n) and between 180 and 280 μm, for 12.5 min of electrophoresis (Fig. 2i and s) for both epididymal and vas deferens sperm. Incubations with hydrogen peroxide, which induces only ssDNA breaks, caused no Comet tail in the neutral Comet assay, as expected, with head halo sizes similar to untreated samples (Fig. 2e,j,o and t).

We next analyzed SCF with the neutral Comet assay. Previous reports using pulsed-field gel electrophoresis (PFGE) demonstrated a degradation of epididymal sperm DNA to 25 kb, which could be religated by

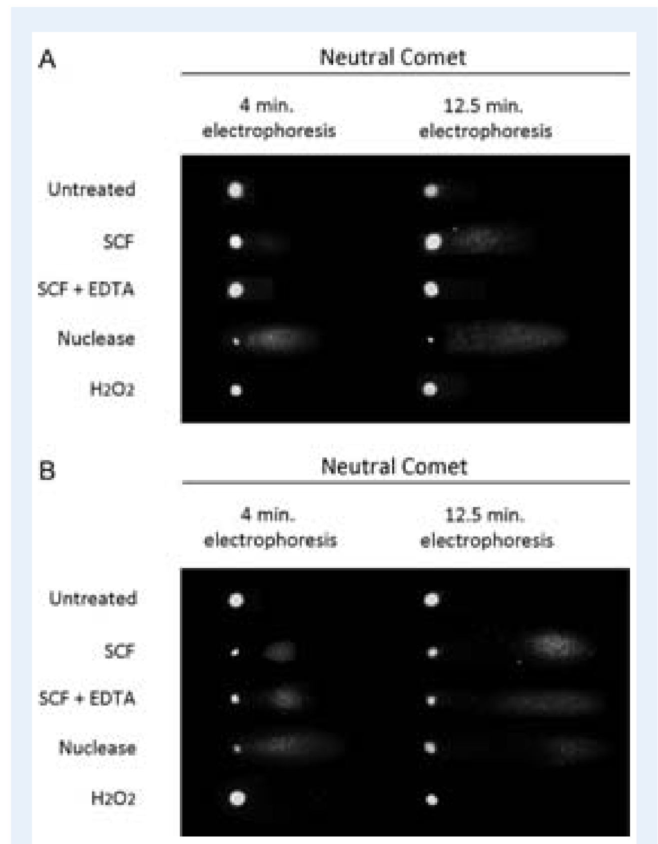

Figure 1 Examples of the most common neutral Comet assay results for two different electrophoresis times after different treatments on (**A**) epididymal sperm and (**B**) vas deferens sperm. See text for explanations of the different treatments.

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 (Figs 1A and 2b,c,g and h, Supplementary data, Fig. S2). Vas deferens SCF sperm showed longer Comet tails than epididymal sperm but significantly smaller head halos (Fig. 1B, Supplementary data, Fig. S2). 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 (Figs 1 and 2l,m,q and r).

Analysis of SCF with the alkaline Comet assay

We next analyzed the SCF-induced sperm with the alkaline Comet assay to test whether ssDNA 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 Fig. 3, and the Comet length histograms of the population for each

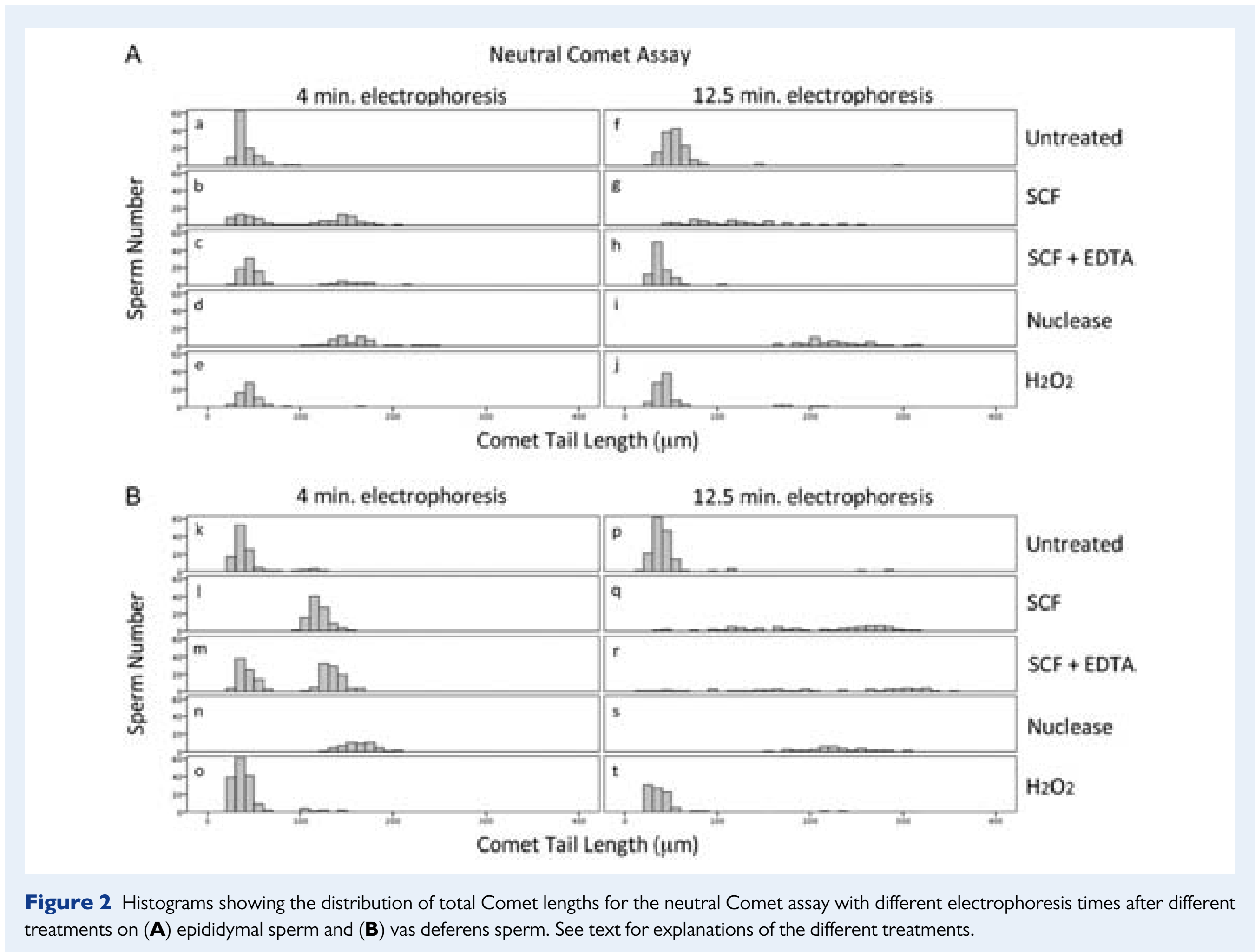

Figure 2 Histograms showing the distribution of total Comet lengths for the neutral Comet assay with different electrophoresis times after different treatments on (**A**) epididymal sperm and (**B**) vas deferens sperm. See text for explanations of the different treatments.

treatment are shown in Fig. 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 and p). Nuclease treatment showed Comet tail lengths ranging between 120 and 180 μm for 4 min of electrophoresis (Fig. 4d and n) and between 230 and 350 μm, for 12.5 min of electrophoresis (Fig. 4i and 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 and t). SCF treatment showed a reduction of the head halo diameter and an increase of Comet tail lengths compared with untreated samples, and this effect was slightly greater for epididymal sperm. Finally, treatments with EDTA showed no DNA religation, when compared with SCF (Figs 3 and 4b,c,l,m,g,h,q and r). These data suggested that SCF induced a significant level of ssDNA breaks in addition to the documented double-stranded breaks.

Comet length-halo ratio

The experiments above clearly demonstrated that Comet tail length, alone, was insufficient to quantify 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 1A). 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 when compared with 4 min.

SDS-neutral Comet

The data above suggested the possibility that most of the dsDNA 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

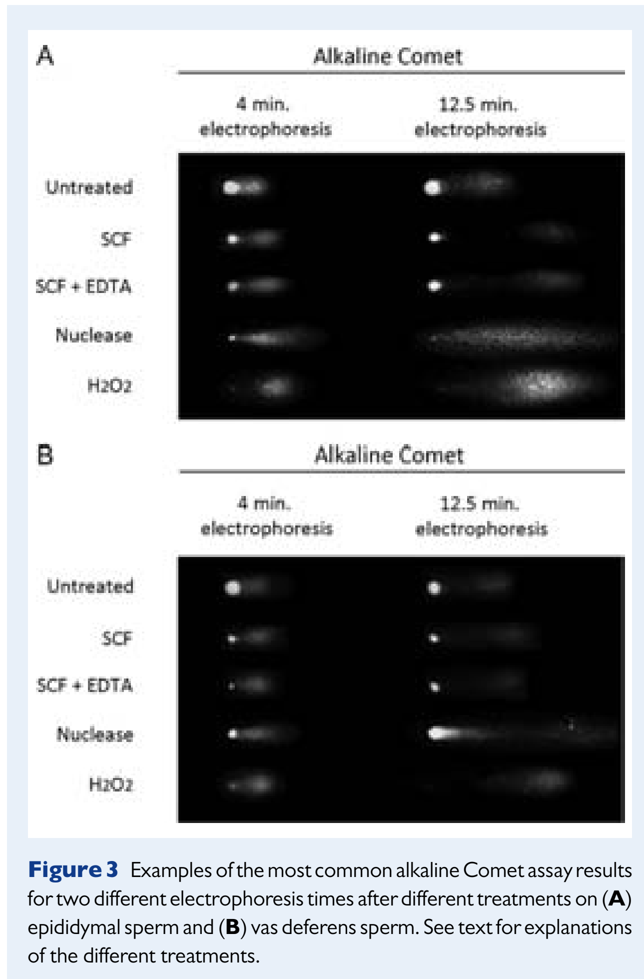

Figure 3 Examples of the most common alkaline Comet assay results for two different electrophoresis times after different treatments on (**A**) epididymal sperm and (**B**) vas deferens sperm. See text for explanations of the different treatments.

neutral Comet assay. This is probable since during sperm preparation for the neutral Comet assay, the sodium dodecyl sulfate (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 dithiothreitol (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 Fig. 5, and Supplementary data, Fig. S4, the accompanying histogram analysis is shown in Fig. 6, and the results of LH ratio in the population are shown in Table IB.

Compared with 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 epididymal SCF and vas deferens SCF (Table I). 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 (Evenson and Jost, 2000; Carrell *et al.*, 2003; 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 with untreated samples but were shorter than nuclease-treated sperm (Figs 1 and 2b and 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 (Ward *et al.*, 1989; Choudhary *et al.*, 1995). 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 IB). 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 Fig. 2l and m). However, with the longer electrophoresis time, there was no difference in the neutral Comet assay with or without EDTA (compare Fig. 2q and r). We propose that the two types of sperm dsDNA

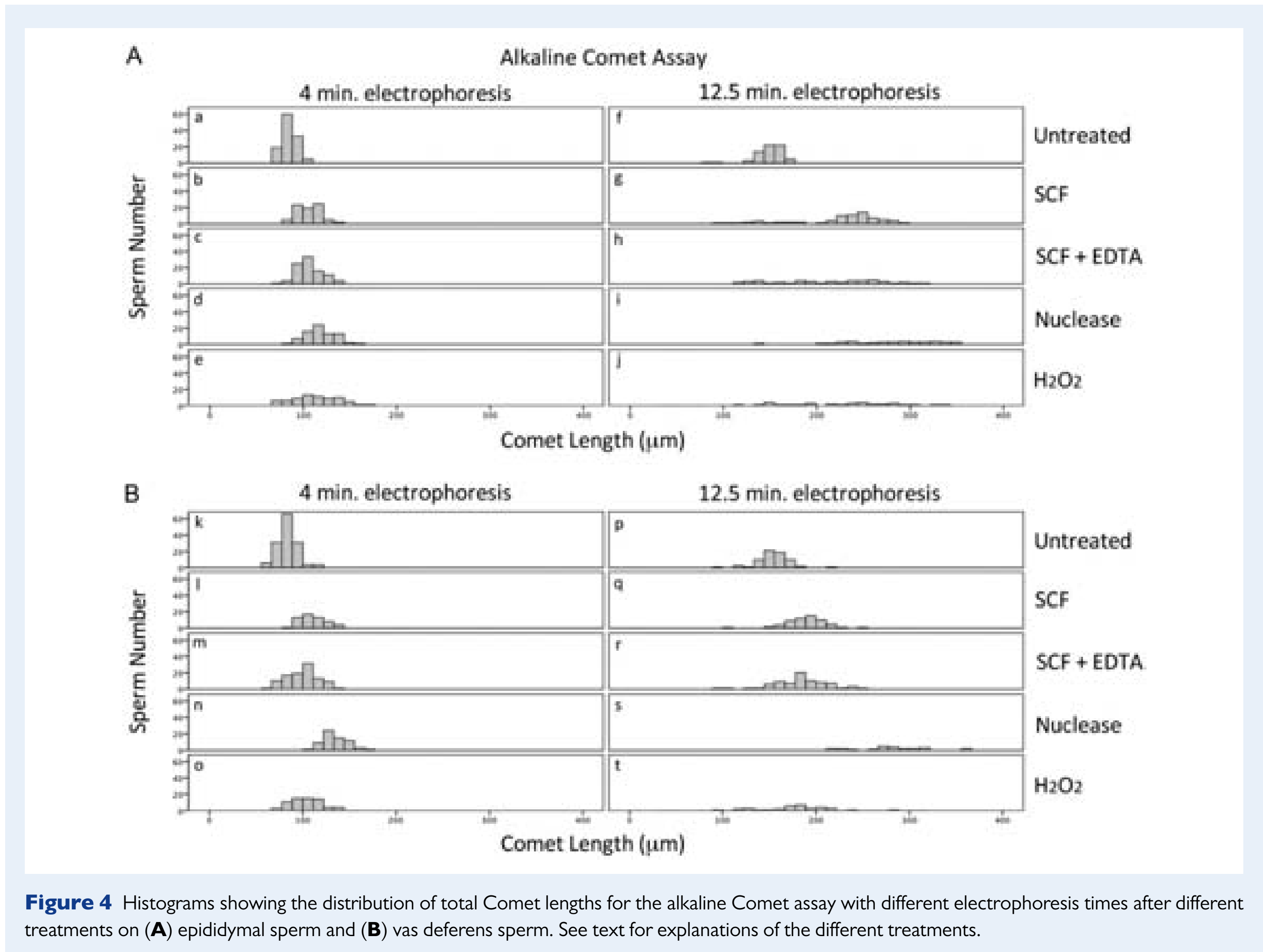

Figure 4 Histograms showing the distribution of total Comet lengths for the alkaline Comet assay with different electrophoresis times after different treatments on (**A**) epididymal sperm and (**B**) vas deferens sperm. See text for explanations of the different treatments.

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 (Supplementary data, Fig. S1). 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 min, and between 180 and 280 µm in neutral Comet with an electrophoresis of 12.5 min.

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 (Yamauchi *et al.*, 2007a; Boaz *et al.*, 2008), 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.

Table I **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-neutral Comet assays in which an 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

SCF also produces ssDNA breaks

Several reports have shown that SCF in both epididymal and vas deferens spermatozoa results in dsDNA breaks (Shaman *et al.*, 2006; Shaman and Ward, 2006; Yamauchi *et al.*, 2007a,b), 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 (Enciso *et al.*, 2009; Aitken and De Iuliis, 2010; 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 and h). This suggests that SCF also produces ssDNA 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 epididymal SCF-induced sperm (Yamauchi *et al.*, 2007a). ssDNA breaks detected by the alkaline Comet assay prevent pregnancy in humans (Simon *et al.*, 2011; Ribas-Maynou *et al.*, 2013), 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, 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 mouse sperm as a model for the DNA damage in varicocele patients.

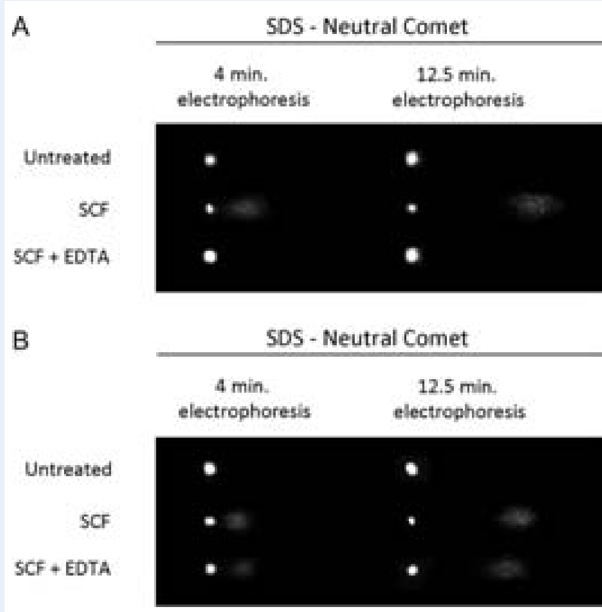

Figure 5 Examples of the most common SDS-neutral Comet assay in different electrophoresis times after different treatments on (**A**) epididymal sperm and (**B**) vas deferens sperm. See text for explanations of the different treatments.

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 IA). 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

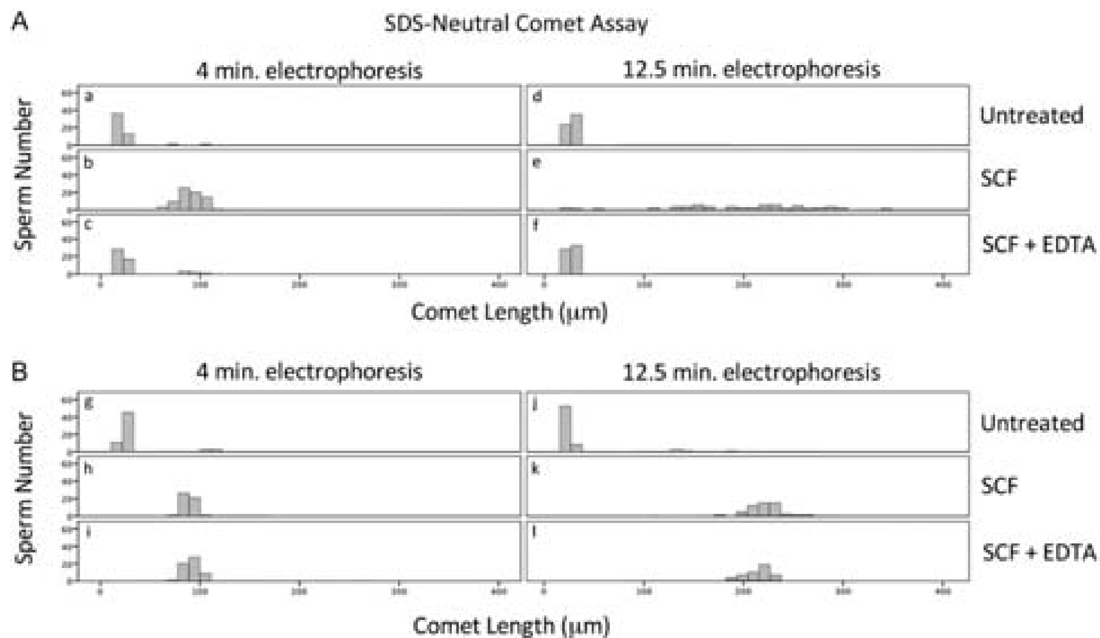

Figure 6 Histograms showing the distribution of total Comet lengths for the SDS-neutral Comet assay after different treatments on (**A**) epididymal sperm and (**B**) vas deferens sperm. See text for explanations of the different treatments.

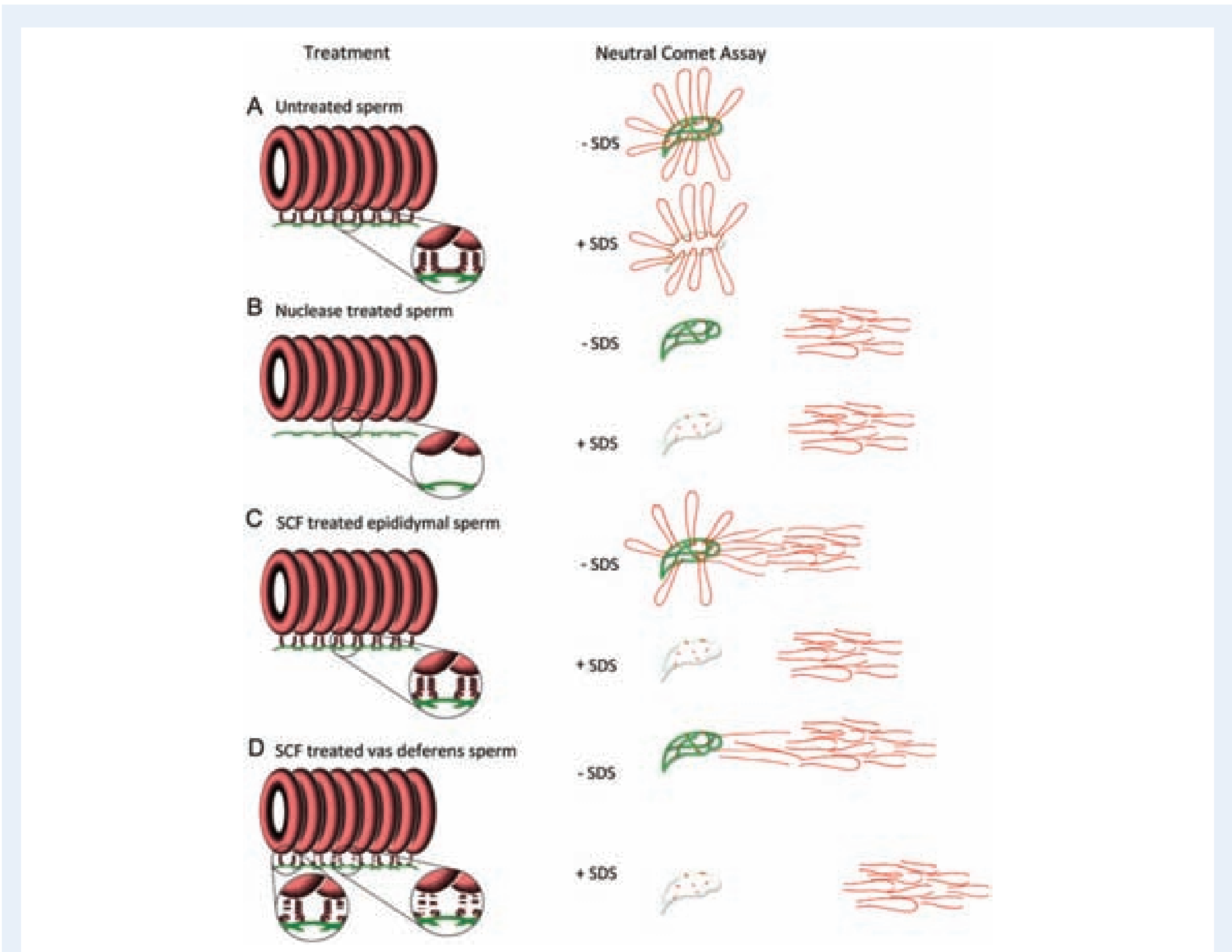

Figure 7 Model for the relationship between the nuclear matrix and the neutral Comet assay. The left column depicts our model for the effect of various treatments on the toroid linker regions, or MARs, *in situ*. The right column depicts the nuclear matrix (green) and exposed DNA loops after the Comet 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 I). (**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.

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.

Supplementary data

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

Authors' roles

J.R.-M. contributed in experimental design, experimental procedures, image analysis, data collection, graphics and table elaboration and manuscript writing. J.E.G. contributed in experimental design, experimental procedures and manuscript writing. J.B. and W.S.W. contributed in experimental design, manuscript writing and revising, and direction and coordination of the work.

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Conflict of interest

None declared.

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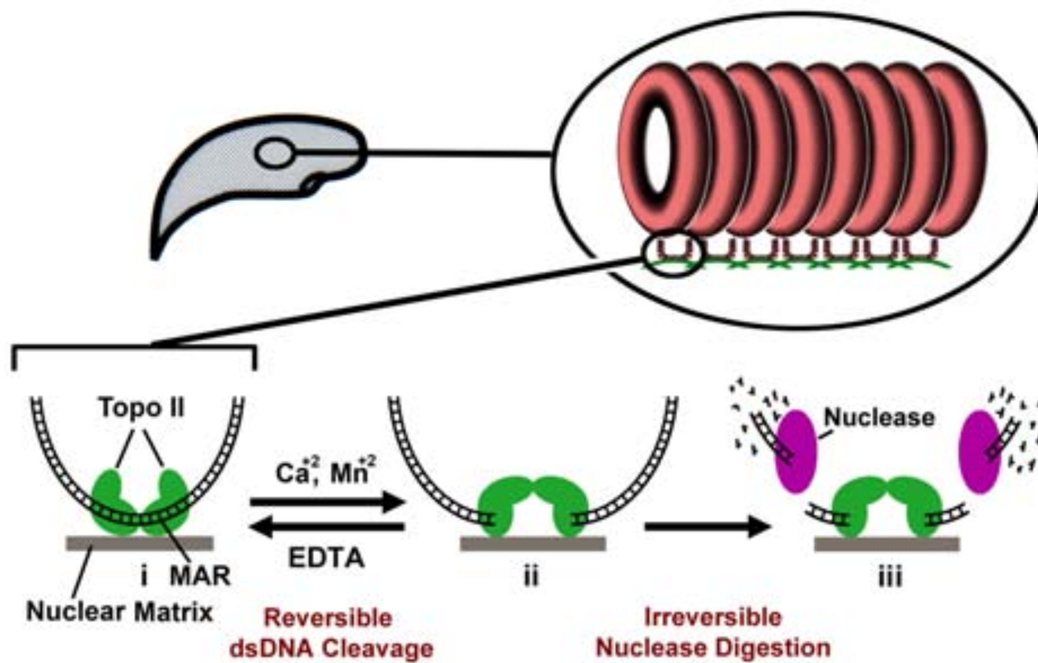
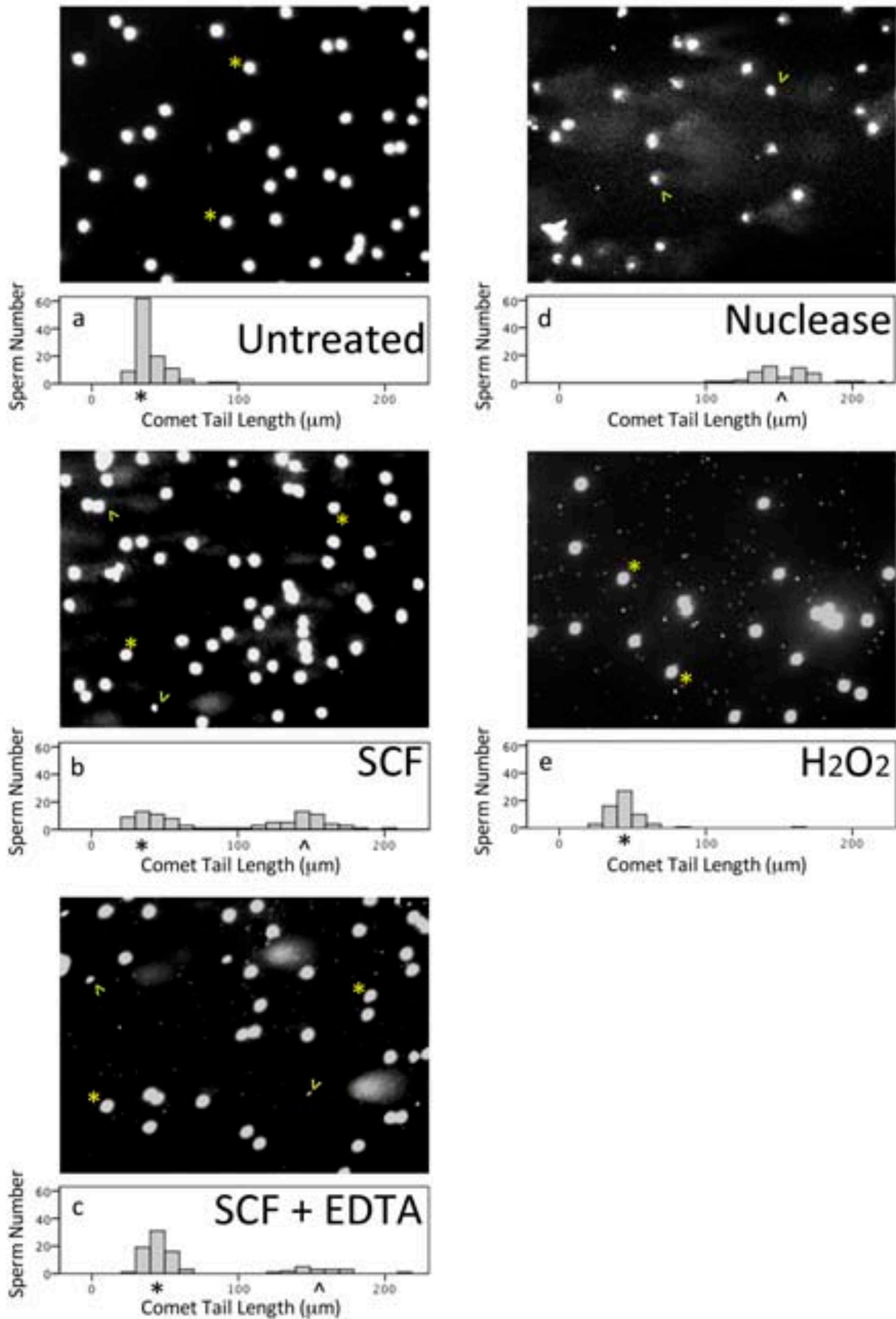
Supplementary Figures

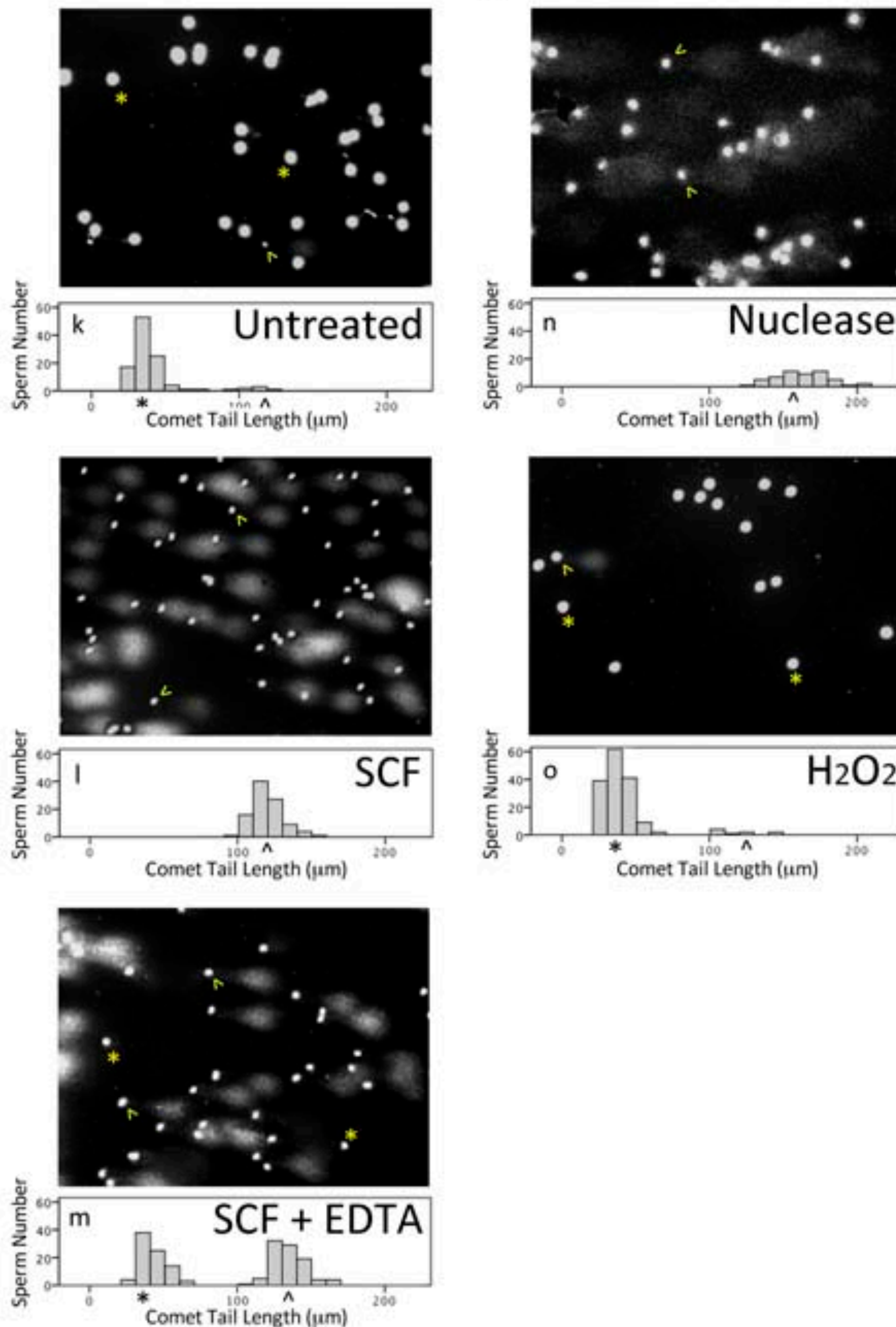
Supplemental Figure 1. A model for Sperm Chromatin Fragmentation. Mammalian sperm DNA is packaged by protamines into toroids which are linked at their bases to the sperm nuclear matrix. As discussed in the text, several publications have demonstrated that divalent cations induce dsDNA breaks in epdidymal sperm, and that subsequent treatment with EDTA causes these breaks to be religated. This is a characteristic mechanism of TOP2, and our current working model for SCF is that either TOP2 or a TOP2 like enzyme present in mature spermatozoa is responsible for the first, reversible DNA cleavage in SCF. Because TOP2 is associated with the nuclear matrix, and it is covalently attached to the broken DNA strands, the broken DNA remains associated with the nuclear matrix (ii). Vas deferens sperm, however, progress to the next step in which a nuclease further digests the DNA, releasing it from the nuclear matrix.



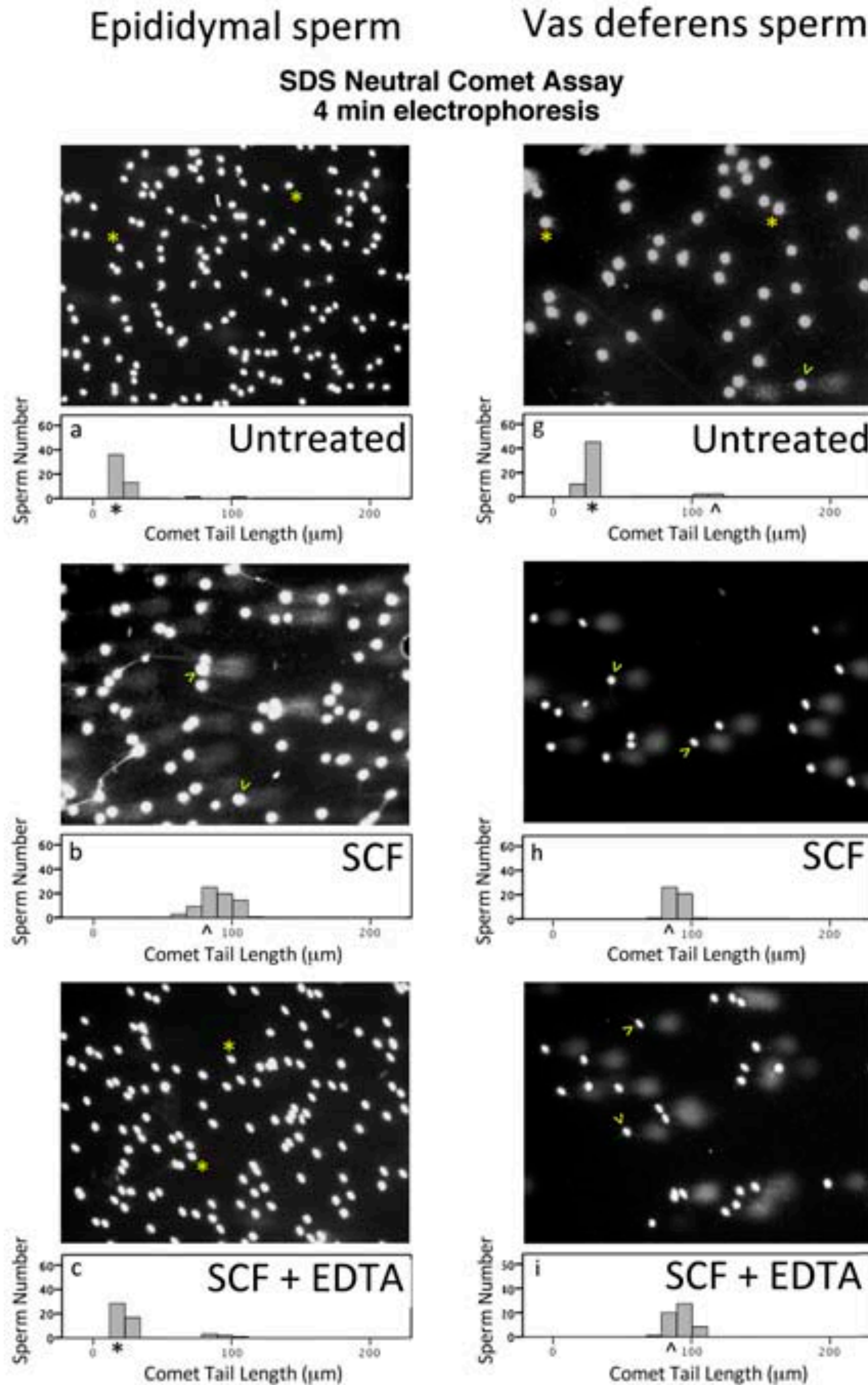
Supplemental Figure 2. Representative low magnification images of Comet assay results for the neutral Comet assays for epididymal sperm shown in Figure 1. For each representative image in Fig. 1, a low magnification image showing several comets is shown. The histogram analysis for each image in Fig. 1, shown in Fig. 2, is reproduced below each image in Suppl. Fig. 2 for comparison.



Supplemental Figure 3. Representative low magnification images of Comet assay results for the SDS neutral Comet assays for vas deferens sperm shown in Figure 1. For each representative image in Fig. 1, a low magnification image showing several comets is shown. The histogram analysis for each image in Fig. 1, shown in Fig. 2, is reproduced below each image for comparison.



Supplemental Figure 4. Representative low magnification images of Comet assay results for the SDS neutral Comet assays shown in Figure 5. For each representative image in Fig. 5, a low magnification image showing several comets is shown. The histogram analysis for each image in Fig. 5, shown in Fig. 6, is reproduced below each image in Suppl. Fig. 3 for comparison.



4.6 Resultats referents a l'objectiu 4.1

Els resultats aconseguits en el desenvolupament de l'objectiu 4.1 han donat lloc al següent treball, publicat en una revista de l'àrea de l'Andrologia, indexada en el JCR en 4rt quartil (5/6).

Article 6

***Títol**:* Nuclear degraded sperm subpopulation is affected by poor chromatin compaction and nuclease activity

Autors: J. Ribas-Maynou, A. García-Peiró, J. Martínez-Heredia, A. Fernández-Encinas, C. Abad, MJ. Amengual, J. Navarro i J. Benet

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Nuclear degraded sperm subpopulation is affected by poor chromatin compaction and nuclease activity

Running Title: Nuclear degraded sperm etiology

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

ABSTRACT

There is an interest in the nuclear degraded sperm subpopulation because, although it is present in a low percentage in all semen samples, patient groups such as varicocele and rearranged genome carriers show high levels of these degraded spermatozoa. This study is designed with two objectives in mind. First, incubations of H_2O_2 and nuclease on DTT-treated and untreated samples to show the etiology of this subpopulation and, second, assessment of the correlation between the protamine ratio and nuclear degraded sperm. A very high increase of the nuclear degraded subpopulation has been found with nuclease incubation, and it is even higher when it has been merged with nuclear decompaction using DTT. Alternatively, incubation with H_2O_2 with and without DTT did not show such a significant increase in nuclear degraded sperm. The protamine ratio correlated with this subpopulation, showing, in patients, that poor nuclear compaction would turn the sperm susceptible to degradation. Then, the assessment of nuclear degraded sperm might not be only a measure of DNA degradation but also an indicator of chromatin compaction in the sperm. Different patient groups would fit this model for sperm nuclear degradation, such as varicocele patients, who show a high percentage of immature sperm and nuclear degraded sperm, and reorganized genome carriers, where reorganization might also cause poor chromatin compaction on the sperm nucleus.

Key words: nuclear degraded sperm, nuclease activity, oxidative stress, sperm, sperm DNA fragmentation, sperm subpopulation, rearranged genome carriers, varicocele.

INTRODUCTION

Recent studies have focused on sperm DNA fragmentation (SDF), demonstrating that sperm DNA integrity is a limiting factor for the correct transmission of paternal genetic information (Lewis et al., 2008). Alterations at the DNA level could cause errors in fertilization and embryo development, and have been associated with several childhood diseases (Aitken et al., 2009; Lewis and Simon, 2010). Main endogenous mechanisms producing SDF are oxidative stress, nuclease activation in an apoptosis-like process (Aitken and De Iuliis, 2010) or topoisomerase deregulation in spermatogenesis (Ward, 2011). As external factors, different papers show that alcohol, smoking, pesticide exposure and environmental toxins, obesity, diabetes and an increase in testicle temperature could negatively affect correct sperm development, exposing sperm DNA to be degraded by oxidative stress and DNA nucleases (Aitken and De Iuliis, 2010).

Many methods have been developed to characterize SDF, the main ones being Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL), Sperm Chromatin Structure Assay (SCSA), Sperm Chromatin Dispersion (SCD) and Comet assay (Evenson et al., 1980 and 2002; Gorczyca et al., 1993; Evenson and Jost, 2000; Fernandez et al., 2005; Enciso et al., 2009; Sharma et al., 2010; Mitchell et al., 2011). Moreover, some techniques can provide extra information about sperm quality, such as immature sperm, defined as high DNA stainability (HDS) subpopulation, and detected by the SCSA method (Zini et al., 2009), which, according to the authors, has a poorly compacted nucleus, meaning some morphological abnormalities (Zini et al., 2009). These HDS spermatozoa are especially augmented in varicocele patients and reorganized chromosome carriers (García-Peiró et al., 2012).

Another parameter, which is determined by the SCD test, is the percentage of nuclear degraded sperm, a subpopulation of sperm with a massively fragmented DNA in both single and double stranded DNA (Gosálvez et al., 2013) and protein depletion (Enciso et al., 2006; García-Peiró et al., 2012). Interestingly, although this sperm subpopulation is present in all semen samples in a low percentage, it also shows a significant increase in varicocele patients and rearranged genome carriers (Enciso et al., 2006; García-Peiró et al., 2012). In this sense, it has recently been described that a degradation index (%nuclear degraded sperm/%SDF) by the SCD test higher than 0.3 is associated with a varicocele affectation (Rodriguez et al., 2012; Gosálvez et al., 2013).

Although both HDS and nuclear degraded sperm subpopulations can coexist in the same semen sample, no correlation has been found between them, suggesting that they are not related (García-Peiró et al., 2012). In a previous work, Zini et al. (2009), using controlled DTT incubations to induce nuclear sperm decompaction, showed that the HDS subpopulation is associated with an immature sperm chromatin status. Recently, some works have determined the presence of the degraded subpopulation in different infertile patients using the SCD test (Enciso et al., 2006; García-Peiró et al., 2012; Rodriguez et al., 2012; Gosálvez et al., 2013). Nevertheless, little is known about their etiology in semen samples.

In addition to these parameters, the analysis of the P1/P2 ratio measures the relative amount of each protamine present in the sperm nucleus through a protein gel. Altered values of this ratio can also be the origin of an abnormal sperm nuclear compaction and associated with high sperm DNA fragmentation. In this regard, its relation with male infertility has been proven (Oliva, 2006; Nanassy et al., 2011; García-Peiró et al., 2011).

Two groups of infertile patients that show interest due to their increase in both HDS and degraded sperm subpopulations are varicocele patients and the rearranged genome carriers. First, varicocele patients commonly show an increase in scrotal temperature caused by a dilated vein, which causes abnormal chromatin compaction, abnormal P1/P2 ratio, oxidative stress, and an increase in DNA fragmentation (Love and Kenney, 1999; Bacchetti et al., 2002; Paul et al., 2008; Barratt et al., 2010; Björndahl and Kvist, 2010). On the other hand, reorganized chromosome carriers show a genetic reorganization which can also cause an alteration in the sperm nuclear organization, which can cause an immature state of the sperm chromatin, poor DNA compaction and, usually, DNA fragmentation (Baccetti et al., 2002 and 2003; Perrin et al., 2009; García-Peiró et al., 2011; Ribas-Maynou et al., 2012).

The objective of the present study is to show evidence regarding the origin of the nuclear degraded subpopulation in human sperm. For that reason, this study has been divided into two objectives: first, to assess the ability of oxidative stress and nuclease activity on generating degraded sperm cells in different nuclear compaction states, induced by DTT, and second, to test the correlation between the nuclear degraded sperm assessed by the SCD test and the protamine P1/P2 ratio in control and patient samples.

MATERIAL AND METHODS

Sample collection

To assess the first objective, a semen sample of six donors with normal semen parameters, following the World Health Organization 2010 criteria (WHO, 2010), were obtained by masturbation after an abstinence of three days. For the first objective, six donors showing low values for SDF and nuclear degraded sperm were used, in order to show the increase produced by the treatments performed.

For the second objective, 32 semen samples were used: six samples from donors with proven fertility, seven samples from patients who are carriers of a rearranged genome, and 19 samples from clinical varicocele patients.

Informed consent was obtained for all donors, and the corresponding ethics committee approved the study.

Sample cryopreservation and thawing

The total semen sample was mixed 1:1 with test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose, and 1.72% sodium citrate) (García-Peiró et al., 2011; Ribas-Maynou et al., 2012), and each sample was divided into cryotubes and frozen in isopropanol at -80ºC overnight. The next day, samples were submerged in liquid nitrogen until the realization of the experiment.

Samples were thawed at 37ºC for 30 seconds. Then, three washes were performed using PBS without Ca^{2+} or Mg^{2+}, centrifuguing them at 600g for five minutes. Finally, sperm concentration was adjusted to 1 x 10^6 spermatozoa/mL.

H_2O_2, nuclease and DTT treatments

For the first objective, each sample was divided into six aliquots and subjected to different treatments with H_2O_2, nuclease incubation (Ribas-Maynou et al., 2012) and

DTT (Zini et al., 2009). Untreated samples with PBS were also included as a control. The treatments consisted of: 0.5 μg/μl DNase I (New England Biolabs; Ipswich, MA, USA) incubation for one hour at 37ºC, and a 0.3% H_2O_2 (Sigma-Aldrich; St. Louis, MO, USA) incubation for 30 minutes at room temperature. Previous results regarding these treatments produced very high DNA fragmentation (Ribas-Maynou et al., 2012), and were chosen with the aim of inducing sperm nuclear degradation. Then, the same H_2O_2 and nuclease treatments were applied, but including a previous incubation of 5mM DTT for 30 minutes at room temperature, with the aim of inducing a controlled nucleus decompaction simulating *in vitro*, chromatin immature sperm, also called high DNA stainability (HDS) (Zini et al., 2009).

SCD test

Treated samples for the first objective and control and patient samples for the second objective were analyzed through the SCD test to obtain the values of degraded sperm, and sperm DNA fragmentation, which includes both degraded sperm and fragmented sperm, since degraded sperm is a subpopulation of fragmented sperm.

Semen samples were washed twice in PBS and the SCD test was performed using the halosperm kit (Halotech DNA, S.L.; Madrid, Spain) following the manufacturer's instructions. Samples were stained with DAPI gold antifade (Invitrogen; Eugene, OR, USA) and 400 spermatozoa were classified for each treatment as non-fragmented, fragmented, and degraded, allowing to obtain the percentage of sperm with DNA fragmentation, which includes nuclear degraded sperm, and the % of nuclear degraded sperm alone, according to the manufacturer's criteria. A sample of each type of spermatozoa is shown in Figure 1.

Another parameter calculated has been the degradation index, which is definded as % nuclear degraded sperm / %SDF (Rodriguez et al., 2012; Gosálvez et al., 2013).

Protamine ratio

Protamine ratio data concerning samples used for the second objective had been previously published by our group, therefore, the protocol used was exactly the same as previously performed (García-Peiró et al., 2011). Briefly, sperm cells were washed with Ham F10 media (Gibco; Grand Island, NY, USA), resuspended with phenylmethylsulphonyl fluoride (Sigma-Aldrich; St. Louis, MO, USA) and processed as previously described (Torregrosa et al., 2006). Finally, the sample was resuspended in a buffer containing urea, β-mercaptoethanol and acetic acid, and acid-urea polyacrylamide gel electrophoresis was performed at 150V for 1 hour in acetic acid buffer. The gel was stained with Coomassie blue (Bio-Rad; Hercules, CA, USA) and protamine bands were quantified with Quantity One software (Bio-Rad).

Protamine ratio data were tested for correlation with the % of nuclear degraded sperm obtained for the same samples.

Statistical analysis

Statistical analysis was performed with SPSS v17 software (Statistics Package for the Social Sciences software, Inc.; Chicago, IL). Comparisons between values with or without DTT were performed through the Mann-Whitney U test, and correlation between nuclear degraded sperm and the P1/P2 ratio was performed using the Pearson test. A significance level of 95% of the confidence interval has been applied to the tests to be considered statistically significant.

RESULTS

Oxidative stress and nuclease treatment effects

Figure 1 shows the effect of treatments on sperm DNA integrity (SDF and nuclear degraded sperm) using the SCD test. Table I displays a summary of the results for both variables (mean ± standard deviation) (n=6).

Treatments effects on SDF

Values of %SDF in untreated samples with and without DTT were 19.17±9.26 and 17.37±7.96 respectively, and no statistical differences were found between them (p=0.671). Treatments with 0.3% H_2O_2 caused a high increase of SDF, when compared with the untreated group (p<0.001) for DTT (+) (99.96±0.10) and DTT (–) (99.58±0.80). However, no differences were found between the treated DTT and untreated DTT samples (p=0.405). In the same way, treatment with 0.5 µg/µl DNase produced a high increase, with respect to the control group (p<0.001), for DTT (+) (99.87±0.21) and DTT (–) (88.87±24.18), also without statistical differences between them (p=0.325) (Table I).

Treatments effects on the nuclear degraded sperm subpopulation

Nuclear degraded sperm percentages (mean ± standard deviation) are also shown in Table I. Control group values with DTT and without DTT were 3.10±1.30 and 3.23±2.19, respectively, and no statistical differences were found between them (p=0.887).

H_2O_2 treatment caused a statistical increase on the nuclear degraded subpopulation, with respect to the control group (p<0.05). However, in H_2O_2 treated samples, no differences were found between DTT (+) (11.54±6.44) and DTT (-) (13.25±12.73) (p=0.873). On the other hand, DNase treatment also caused a statistical increase, with respect to the control group and H_2O_2 treatment (p<0.05), and statistical differences

were found between the untreated DTT (48.54±26.99) and treated DTT (79.12±12.61) samples ($p<0.05$) (Table I).

Effect on degradation index

Data regarding the degradation index (DI) is shown in Table I. The untreated samples showed normal values of the degradation index parameter, being 0.19 and 0.16 for untreated DTT and treated DTT samples, respectively. When treatments with H_2O_2 were performed, no significant increase with the untreated group was found in the degradation index, for either DTT (-) or DTT (+) ($p>0.05$). However, when samples were treated with DNase, a statistical increase on the degradation index was seen ($p>0.05$), being higher in DTT treated samples: 0.55 for DTT (-) and 0.79 for DTT (+).

Nuclear degraded sperm and the P1/P2 ratio correlation

Protamine ratio results had previously been published by our group (García-Peiró et al., 2011), and an example of a protamine gel is shown in Figure 2. Data concerning the P1/P2 ratio and degraded sperm were (mean ± standard deviation): 1.23±0.48 and 2.17±1.17, respectively, for fertile donors; 1.70±0.46 and 8.86±4.41, respectively, for rearranged genome carriers, and 1.91±0.75 and 16.63±5.13, respectively, for varicocele patients. A scatter-plot regarding the degraded sperm subpopulation of 32 semen samples analyzed by the SCD test, the protamine ratio and their correlation is shown in Figure 3.

The protamine ratio and the nuclear degraded subpopulation showed a statistical ($p<0.001$) correlation with a correlation coefficient of 0.586, showing that an abnormal P1/P2 ratio corresponds to an increase in nuclear degraded sperm (Figure 3).

DISCUSSION

The aim of the study is to add evidence concerning the origin of the nuclear degraded sperm subpopulation and to find clues about the mechanisms involved in DNA degradation. About that, it has been demonstrated that this sperm subpopulation is increased in such different etiologies such as varicocele and chromosome reorganization carriers. To assess the origin of this DNA degradation, strong treatments with hydrogen peroxide and DNase (simulating the main DNA fragmentation effectors) have been performed, imitating oxidative stress and enzymatic DNA damage, respectively (Enciso et al., 2009; Ribas-Maynou et al., 2012), in different nucleus compaction stages originated by DTT treatments, taking into account that DTT treatment induces the formation of immature-like sperm (HDS), as demonstrated by Zini et al. (2009), using SCSA. Afterwards, nuclear degraded sperm and the protamine 1/protamine 2 ratio were assessed in different sperm samples from donors and patients to analyze the correlation between these two variables.

Regarding the effect of the treatments on DNA fragmentation and degradation, no statistical increase in SDF has been found between DTT (+) and DTT (-) samples, measured by the SCD test (Table I). In this sense, our results agree with the previous observations assessed by SCSA (Zini et al., 2009). However, this type of comparison cannot be performed between the two treatments because the use of such an intense treatment, which produces almost 100% of SDF, would not be the most appropriate to assess differences in SDF.

Although 0.3% hydrogen peroxide and 0.5 μg/μl DNase I are very strong treatments, which are not at physiological concentrations, a less aggressive treatment would have fragmented fewer spermatozoa, and this would have produced less nuclear degraded

sperm, not helping the main goal of this study. The reason for using these strong treatments and DTT incubation was mainly to uncover the etiology of the nuclear degraded sperm subpopulation in normal semen samples, and to assess the effect of DTT on the nuclear degraded sperm subpopulation, not to find differences in fragmented DNA between compacted and decompacted sperm, as it is well known that these treatments produce a high DNA fragmentation in both single and double stranded DNA fragmentation (Enciso et al., 2009; Ribas-Maynou et al., 2012).

For both hydrogen peroxide and DNase treatments, a significant increase in degraded sperm has been shown in DTT treated samples, as compared with untreated samples (Table I). However, the increase has been much higher when dealing with nuclease treatment than with hydrogen peroxide.

Within the treatments, DTT incubation has only produced differences in DNase treatment. Therefore, as it has been demonstrated that DTT increases the immature subpopulation assessed with SCSA (Zini et al., 2009), our results suggest that poor nuclear compaction is a condition that would be more susceptible to highly degrading its DNA mainly mediated by nuclease activity, which, in patients, could be endogenous (Sotolongo et al., 2005) or activated through oxidative stress or an increase of testicular temperature (Sakkas and Alvarez, 2010). Indeed, the immature sperm subpopulation is increased in varicocele patients and chromosome reorganization carriers, and both also present a high percentage of nuclear degraded sperm (García-Peiró et al., 2012; Gosalvez et al., 2013).

Therefore, results show that DTT treated sperm, simulating sperm with poor DNA compaction, undergo nuclear DNA degradation when nuclease activity is present.

Regarding the second objective of this study, samples from fertile donors, varicocele patients and rearranged genome carriers were analyzed through the SCD test to obtain the percentages of nuclear degraded sperm cells. These data were analyzed with the P1/P2 ratio obtained before for the same samples (García-Peiró et al., 2011), and a correlation between these two parameters has been found. This fact reinforces the idea that patients with poor chromatin compaction, such as those with an abnormal protamine ratio, would show a higher percentage of nuclear degraded sperm by making the sperm DNA more accessible to nuclease activity, which would be the main final effector.

Different patient groups fit the model proposed for nuclear degraded sperm generation (Figure 4): On one hand, varicocele patients present high oxidative stress, altered protamine ratio (Figure 3), and high nuclear degraded sperm (Enciso et al., 2006; Zini and Dohle, 2011; García-Peiró et al., 2012). In this patients, the elevated scrotal temperature produced by the dilated vein may affect the di-sulfide bridges or destabilize the zinc content. This fact might result in an abnormal DNA compaction, abnormal protamine ratio, and a high percentage of immaturity in the sperm nucleus (Love and Kenney, 1999; Bacchetti et al., 2002; Paul et al., 2008; Barratt et al., 2010; Björndahl and Kvist, 2010). Then, an explanation might be that oxidative stress could be activating nucleases (Sakkas and Alvarez, 2010) that might be the final effectors of DNA damage, producing, in the end, the high percentage of nuclear degraded sperm. Because of that, oxidative stress would not be the last effector, but rather it would be an intermediary to activate the nucleases that would induce DNA degradation (Figure 4B). In fact, in a previous study, single stranded DNA fragmentation (ssSDF) has been associated with oxidative stress, and double stranded DNA fragmentation (dsSDF) has

been associated with nuclease activity (Ribas-Maynou et al., 2012). Moreover, varicocele patients showed high values of ssSDF and high values of dsSDF, suggesting that these two mechanisms are related (Ribas-Maynou et al., 2012).

On the other hand, in rearranged genome carriers, the chromosomes involved in the reorganization can produce a high percentage of immaturity (Baccetti et al., 2002 and 2003), sperm with both poor DNA compaction and an altered protamine ratio (Figures 2, 3 and 4). Then, the poorly compacted nucleus is susceptible to being degraded by active nucleases or DNases that might be present in the sperm (Sotolongo et al., 2005), or oxidative stress dependent nucleases that could be activated (Sakkas and Alvarez, 2010). In any case, the percentage of nuclear degraded sperm in rearranged genome carriers is less than in varicocele patients, which would mean that varicocele patients could have, in general, worse DNA compaction than chromosome reorganization carriers, which is usually a group with very high heterogeneity due to the different reorganizations (García-Peiró et al., 2011; Ribas-Maynou et al., 2012).

Another group of patients that could have increased oxidative stress are patients with leukocytospermia (Saleh et al., 2002), but it has been described that they do not show an increase in the nuclear degraded subpopulation (Rodriguez et al., 2012; Gosálvez et al., 2013). This fact could be explained in the sense that they might not have testicular affectation, as with varicocele, and because of that, they would present good sperm nuclear DNA compaction. This would not allow any nuclease activity to degrade the sperm DNA and, thus, produce nuclear degraded sperm (Figure 4A).

Other causes that might explain the etiology of the nuclear degraded subpopulation are those that especially affect chromatin compaction, such as possible alterations in topoisomerases, which are responsible for histone replacement by protamines in

spermiogenesis. Alterations at this level might result in poor nuclear compaction and, as a result, higher DNA fragmentation (Simon et al., 2011) and nuclear degraded sperm cells (Figure 4B).

In a recent paper, a relation has been established between varicocele affectation and the degradation index using the SCD test. Degradation index values above 0.3 have been proposed as a marker for varicocele affectation (Rodriguez et al., 2012; Gosálvez et al., 2013). Degradation index data showed lower values than 0.3 in untreated samples and in hydrogen peroxide treatments with and without DTT. However, when dealing with DNase treatment, and especially when sperm DNA is decompacted with DTT, the values for the degradation index overcome this proposed threshold. This would reinforce the idea that the final effector of nuclear degraded sperm would be some kind of nuclease activity, activated or not by oxidative stress (Sotolongo et al., 2005; Sakkas and Alvarez, 2010), depending on the patient's infertility etiology. Moreover, this fact could be inferred in varicocele patients, who would have poor nuclear DNA compaction that would lead to nuclease activity activated through oxidative stress or an increase in testicular temperature, this would degrade sperm DNA producing nuclear degraded sperm *in vivo* and, because of that, increase the degradation index, as we have seen in our study *in vitro.*

It has been seen that other patient groups such as asthenoteratozoospermic (ATZ) patients without varicocele show low values of nuclear degraded sperm, without any statistical difference to fertile donors, always much lower than varicocele patients (Rodriguez et al., 2012; Gosálvez et al., 2013), although it is known that they present higher DNA fragmentation (Ribas-Maynou et al., 2012). As these ATZ patients do not present varicocele, there is not any testicular affectation that could affect chromatin

compaction. This would agree with the fact that better nuclear compaction would result in a very low percentage of the nuclear degraded sperm subpopulation and, in consequence, a low associated degradation index. In this sense, it could be interesting to identify the different factors that can cause a poor compaction in sperm DNA, especially in those patients who present a high percentage of sperm nuclear degradation, such as varicocele patients. The identification of the causes could be important to improve the chromatin compaction status in the different patients, with the objective of reducing the nuclear degraded sperm cells and, in consequence, reducing SDF.

The observed relation between DTT treatments and nucleases suggests that poor nuclear DNA compaction in sperm is a factor that predisposes it to degradation. In this sense, nuclease activity, and not oxidative stress, would be the final effector mechanism that generates these nuclear degraded sperm. Since no specific nucleases, but only the presence of nuclease activity have been described in sperm cells, more studies are needed to determine what the specific effector of this activity is. Moreover, this nuclease activity could also be activated by oxidative stress or an increase in testicular temperature in some patient groups such as varicocele patients. In this sense, the correlation between nuclear degraded sperm and the protamine ratio supports the belief that an abnormal chromatin condensation would lead this nuclease activity to completely degrade the sperm DNA. Because of that, the presence of nuclear degraded sperm in a semen sample could also be an indicator not only for low DNA integrity, but also for poor nuclear compaction.

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AUTHOR'S ROLES

Jordi Ribas-Maynou contributed in experimental design, experimental procedures, statistical analysis, graphics and table elaboration and document writing.

Agustín García-Peiró contributed in experimental design, experimental procedures results discussion, statistical analysis and document writing and revising.

Juan Martínez-Heredia contributed in protamine ratio experimental procedures.

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Table 1. Values of sperm DNA fragmentation (%SDF), nuclear degraded sperm (%), and degradation index (DI) (%Nuclear degraded sperm/%SDF) for different treatments applied to sperm samples from donors.

	Untreated Samples		H_2O_2 0.3%		DNase 0.5 mg/ml	
	DTT (-)	DTT (+)	DTT (-)	DTT (+)	DTT (-)	DTT (+)
% SDF	17.37 ± 7.96	19.17 ± 9.26	99.58 ± 0.80 [a]	99.96 ± 0.10 [a]	88.87 ±24.18 [a]	99.87 ± 0.21[a]
% Nuclear degraded sperm	3.23± 2.19	3.10 ± 1.30	13.25 ± 12.73 [a]	11.54 ± 6.44 [a]	48.54 ± 26.99 [a, b]	79.12 ± 12.61 [a, b, c]
DI	0.19	0.16	0.13	0.12	0.55 [a, b]	0.79 [a, b, c]

[a] Statistical differences with the respective untreated group ($p<0.05$).
[b] Statistical differences with the respective H_2O_2 0.3% treatment ($p<0.05$).
[c] Statistical differences with the respective DTT (-), DNase 0.5 μg/μl ($p<0.05$).

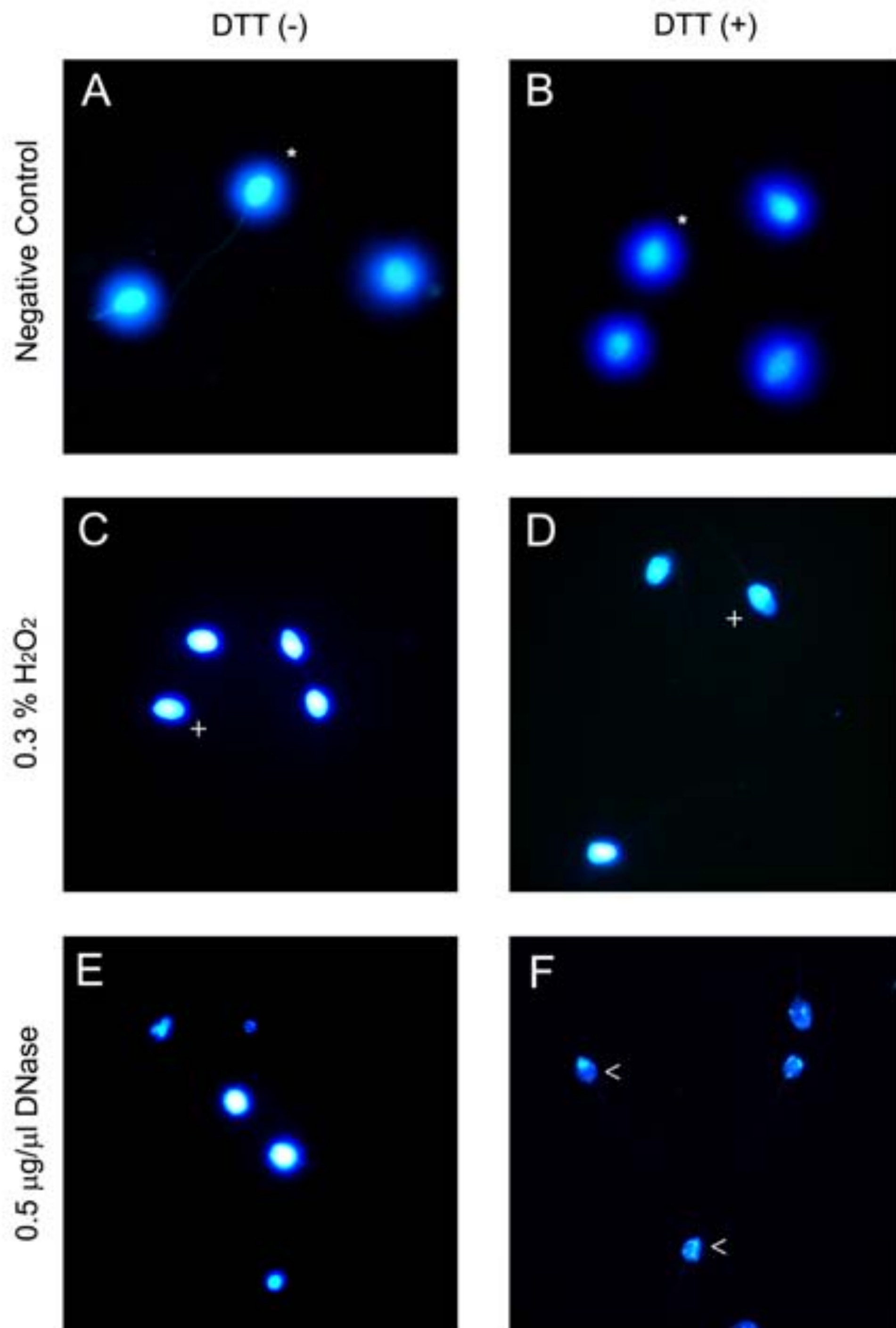

Figure 1. Sperm Chromatin Dispersion test in DTT (-) treatment (A, C, E) and DTT (+) treatment (B, D and F). Untreated samples are shown in A and B. H_2O_2 treatments are shown in C and D. DNase treatments are shown in E and F. Stars (*) represent non-fragmented sperm, crosses (+) represent fragmented sperm, arrows (>) represent nuclear degraded sperm.

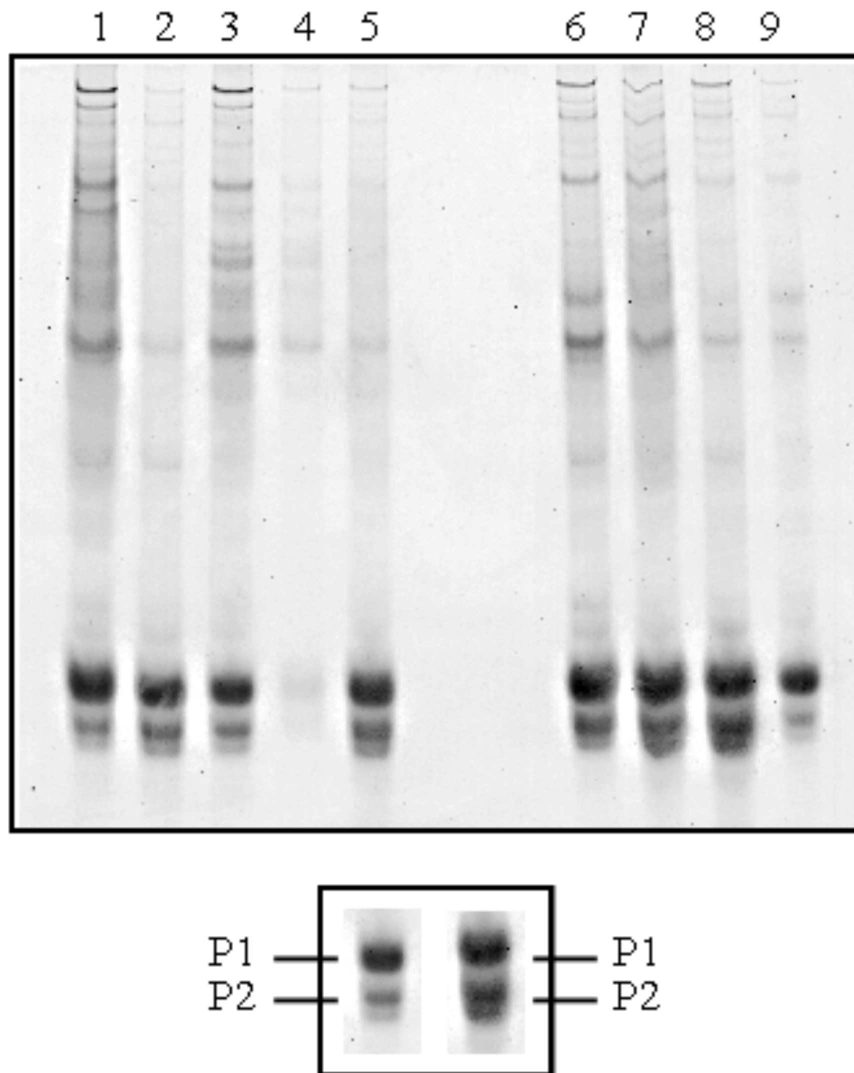

Figure 2. Analysis of the protamine 1 (P1) and protamine 2 (P2) ratio. (**A**) Sperm proteins extracted from spermatozoa, separated on a polyacrylamide acetic-urea gel (Coomassie blue staining). Lanes 1–5 and 6–9 correspond to different sperm samples from fertile donors and chromosome reorganization carriers, respectively. (**B**) Example from a fertile donor sample with a normal P1/P2 ratio (left) and a chromosome reorganization patient with a highly altered P1/P2 ratio (right).

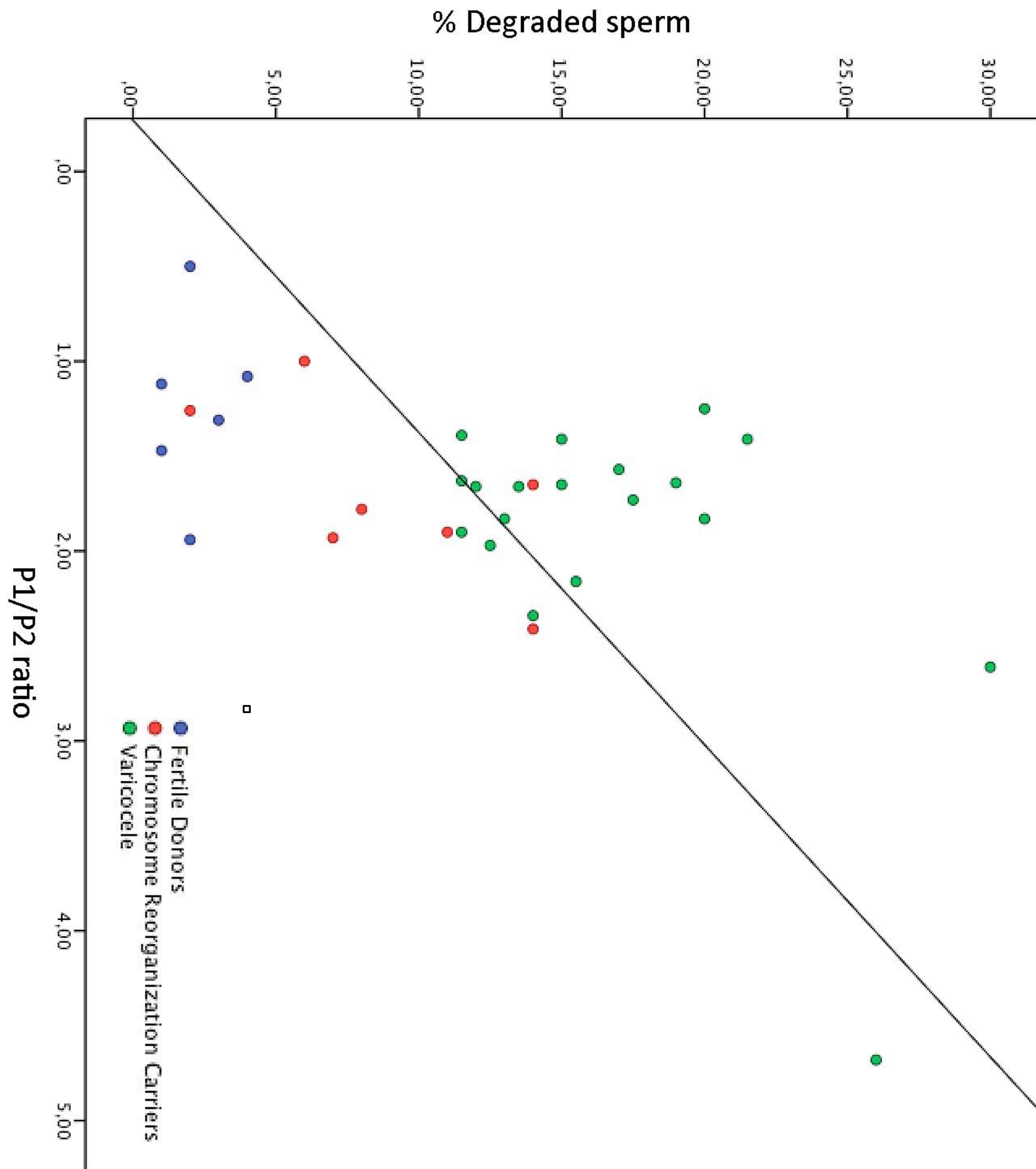

Figure 3. Scatter diagram showing the relation between nuclear degraded sperm and the protamine 1 and protamine 2 ratio calculated with protamine gels. This plot shows the correlation between these two variables.

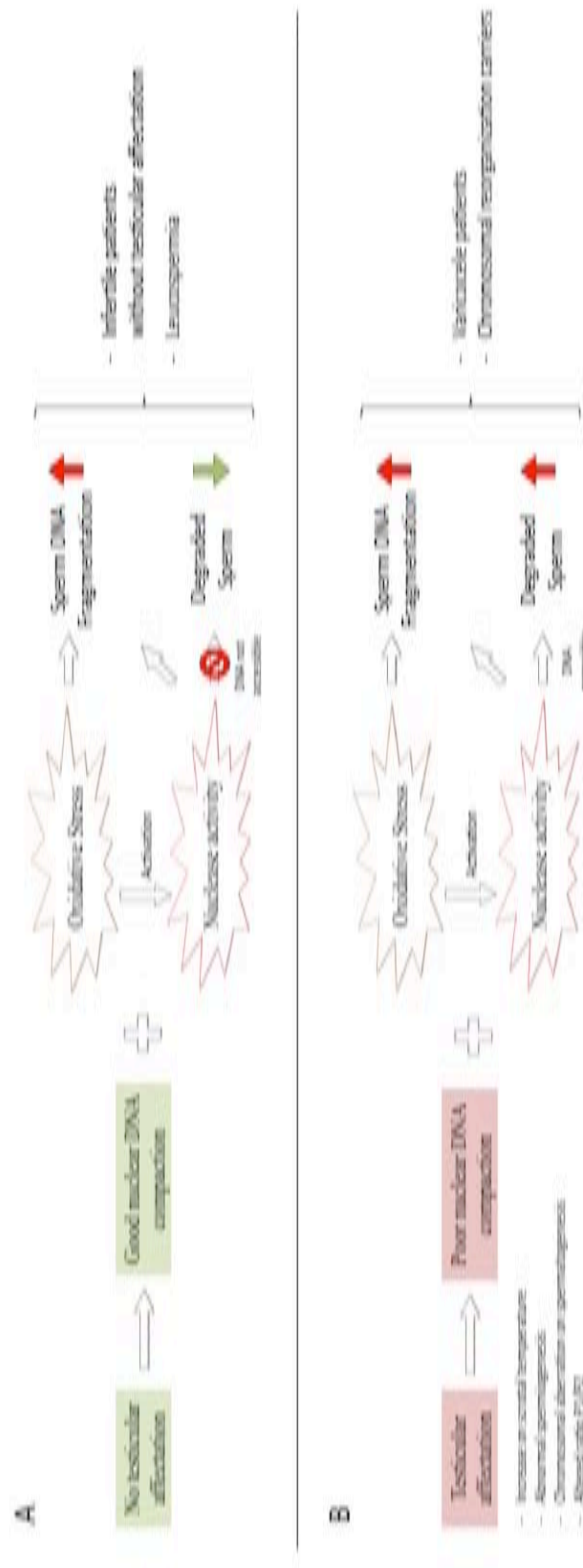

Figure 4. Model for SDF and sperm nuclear degradation through oxidative stress and nuclease activity in a scenario of: A) no testicular affectation and B) testicular affectation.

4.7 Resultats referents a l'objectiu 4.2

Els resultats aconseguits en el desenvolupament de l'objectiu 4.2 han donat lloc al següent treball, pendent de publicació a una revista de l'àrea de Biologia de la Reproducció indexada al JCR

Article 7

***Títol**:* Clinical analysis of sperm DNA damage and DNA compaction: Comet assay and chromomycin A3

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Clinical analysis of sperm DNA damage and DNA compaction: Comet assay and chromomycin A3.

Running Title: DNA damage and DNA compaction in clinical groups of patients

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ABSTRACT

Study question: Is DNA compaction related to single or double stranded DNA damage?

Summary answer: DNA compaction is related to single stranded DNA damage, and has detrimental implications on pregnancy achievement.

What is known already: Sperm DNA compaction is one of the origins through DNA damage can occur. A poor DNA compaction turns the sperm nucleus accessible to different mechanisms causing DNA breaks. Different studies have shown its relation to male infertility, but no studies about the relation to single or double stranded DNA damage have been performed.

Study design, size, duration: This cohort study includes 90 semen samples from males with different clinical features: 23 patients with recurrent miscarriage without female factor (RPL), 47 infertile patients divided according their semen parameters, and 23 fertile donors with proven fertility.

Participants/materials, setting, methods: Sperm DNA compaction through Chromomycin A3 was performed to all samples, and compared to DNA fragmentation assessed through alkaline and neutral Comet assay.

Main results and the role of chance: About DNA compaction, fertile donors showed differences with all other groups ($p<0.001$), RPL patients showed differences with infertile patients ($p<0.05$), and among infertile patients, varicocele patients showed the highest percentage of CMA3 positive cells. The comparison to sperm DNA fragmentation shows that as CMA3 increases, single stranded DNA fragmentation also increases ($r=0.456$; $p<0.001$). However, double stranded DNA fragmentation did not show relation to CMA3 ($r=0.172$ and $p=0.104$). The predictive power of CMA3 test on

infertility showed a sensitivity and specificity of 0.936 and 0.512, respectively, with a cut-off value of 40% of positive cells.

Limitations, reasons for caution: The CMA3 analysis can be done using flow cytometer, which could increase the assessed spermatozoa per sample. However, the analysis through microscopy turns the technique easier to perform in different laboratories.

Wider implications of the findings: The present study agrees the fact that DNA compaction and DNA fragmentation have a relation. It is demonstrated that DNA compaction is related to single stranded but not double stranded DNA fragmentation, having implications on fertility.

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INTRODUCTION

Infertility is defined as the impossibility to achieve a pregnancy after one year of regular sexual relations without contraception (WHO, 2010). It has clearly been demonstrated that infertility is affecting equally males and females, however, the origin of infertility in some infertile couples remain unexplained (de Kretser, 1997). In this sense, recent studies show that sperm cell would not only be providing male genome complement, but also could be higher involved in the early embryo development. Thus, the nuclear structure of the sperm could be essential in the first embryo cleavage (Ward, 2010; Gawecka *et al.,* 2013) and the sperm cell could be bringing epigenetic signals to the embryo (Dada *et al.,* 2012; Boissonnas *et al.,* 2013) or being a source of important proteins or mRNAs used in the first stages of embryogenesis (Hamatani, 2012; Li and Zhou, 2012).

In contrast to somatic cells, the sperm cell presents a unique degree of DNA compaction, by having exchanged the most part of histones by protamines during spermatogenesis and having lost the most part of cytoplasm during spermiogenesis. Then, the mature sperm shows its DNA compacted into protamine toroids of about 23 to 48 kb length, linked between them by a DNA linker regions, the matrix attachment regions or MARs, which are attached to the nuclear matrix and are sensitive to being cut by nucleases (Ward, 2010). Moreover, it has been shown that the paternal chromosomes are organized on different territories in the sperm cell, pointing their centromeres in the more internal regions and their telomeres grouped by pairs and liked to the inner cell membrane (Solov'eva *et al.,* 2004; Zalenskaya and Zalensky, 2004; Mudrak *et al.,* 2005, 2012).

Then, the result is a very small cell with a highly compacted DNA, which should be able to fertilize the oocyte and then decompact its DNA by replacing the protamines by histones at the first stage of the embryo development to allow subsequent activation of some important genes at this stage (Oliva, 2006).

Defects on the sperm protamination and, in consequence, to its DNA compaction, have been associated to male infertility and DNA fragmentation, as it has been shown by a good positive correlation between Chromomycin A3 (CMA3) test and DNA fragmentation (De Iuliis *et al.,* 2009). Moreover, values above or below the normal protamine1/protamine2 ratio (P1/P2) (ranged between 0.8 and 1.2 in human fertile males) would lead into poor-compacted spermatozoa and, in consequence, sperm head morphology defects, sperm DNA fragmentation and infertility (Oliva and Castillo, 2011). In this sense, patient groups such as varicocele patients or balanced chromosome reorganization carriers have been shown to have abnormal protamine ratio (García-Peiró *et al.,* 2011a), however, it seems that they would have different etiologies (Ribas-Maynou *et al.,* 2012a).

As a consequence of a poor DNA compaction the DNA would be more accessible to any nuclease activity and oxidative stress, then, sperm DNA fragmentation would appear (Yamauchi *et al.,* 2007a, 2007b; Aitken and De Iuliis, 2010). In the recent years, the assessment of SDF has become a new biomarker for male fertility and there are many studies that show its implication in the couple's infertility (Evenson, 2013; Gosálvez *et al.,* 2013a; Lewis *et al.,* 2013; Sharma *et al.,* 2013). It seems coherent that a damaged DNA would block the embryo development unless it could be repaired by a yet unknown mechanism carried by the oocyte (Ribas-Maynou *et al.,* 2013b). In this sense, exhaustive analysis in patients an controls males have been performed using different

DNA integrity assays, such as deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) (Gorczyca *et al.,* 1993), Sperm Chromatin Structure Assay (SCSA) (Evenson, 2013), Sperm Chromatin Dispersion test (SCDt) (Fernández *et al.,* 2005) and Comet assay (Singh *et al.,* 1988; Enciso *et al.,* 2009; Ribas-Maynou *et al.,* 2012a). Different threshold values for fertility have been proposed for each assay (Fernández *et al.,* 2005; Sergerie *et al.,* 2005; Evenson and Wixon, 2006; Simon *et al.,* 2011b). Moreover, it has been demonstrated that it could be important to distinguish the single and double stranded DNA fragmentation (ssSDF and dsSDF), by using the alkaline and neutral Comet assay respectively, as they could have different implications on the outcome (Ribas-Maynou *et al.,* 2012a, 2012b). Attending pregnancy prediction, it has been demonstrated that alkaline Comet assay (which detects ssSDF) shows the best sensitivity and specificity compared to all other techniques (Ribas-Maynou *et al.,* 2013a). Recent works demonstrate that a high ssSDF could be an extensive DNA damage difficult to be repaired by the oocyte and, consequently, would not allow biochemical pregnancy, by blocking the embryo development at the first stages (Ribas-Maynou *et al.,* 2012b). On the other hand, dsSDF could be a specific DNA damage that may allow the embryo development but could induce a subsequent miscarriage within the first trimester (Ribas-Maynou *et al.,* 2012b) or a delayed embryo development (Gawecka *et al.,* 2013). These recurrent miscarriage patients with low ssSDF and high dsSDF are of high importance, since they are the only clinical group showing these features, and no distinction from one subgroup of fertile donors with the same DNA fragmentation profile can be found (Ribas-Maynou et al., 2012b). In a recent study on mice, two different types of double stranded DNA damage with different implications upon fertility have been found, one of them linked to the nuclear matrix, defined as

Sperm Chromatin Fragmentation (SCF), and the other that implies a release from the nuclear matrix, named Sperm DNA Degradation (SDD) (Yamauchi *et al.,* 2007a, 2007b; Ribas-Maynou *et al.,* 2013b).

Since both issues, DNA compaction and DNA fragmentation, seem to show a clinical interest the aim of the present work is to analyze the relation of sperm DNA compaction in relation to the different profiles of sperm DNA fragmentation: low ssSDF and low dsSDF, found in some fertile donors; low ssSDF and high dsSDF, found in some fertile donors and RPL patients; and high ssSDF and high dsSDF, found in infertile patients. This would allow identifying differences not only between fertile and infertile patients but also within infertile patients, as well as to establish a new threshold value for fertility attending sperm DNA compaction

MATERIAL AND METHODS

Sample Collection

Semen samples from 90 human males were obtained by masturbation after having completed between three and seven days of sexual abstinence. Samples were collected in collaboration with hospitals from Barcelona area, an informed consent was obtained from all donors, and the appropriate ethics committee approved the study.

First, macroscopical and microscopical semen analysis was performed according the WHO guidelines (WHO, 2010), and then, samples were divided into two aliquots, cryopreserved in Test Yolk Buffer (14% glycerol, 30% egg yolk, 1.98% glucose, 1.72% sodium citrate) (Ribas-Maynou *et al.,* 2013a) and stored in liquid nitrogen until the analysis. Samples and the subsequent results were classified according their clinical features, being the size of the groups: 20 for fertile donors, 23 for recurrent pregnancy loss patients (RPL) without female factor, and 47 for infertile patients. Within infertile patients, 9 samples showed normal semen parameters, 30 showed altered semen parameters (7 asthenozoospermic, 5 teratozoospermic, 10 asthenoteratozoospermic and 8 oligoasthenoteratozoospermic), and 8 males were diagnosed with clinical varicocele and showed an asthenoteratozoospermic semenogram.

Since the aim of the present work is to assess the relation of DNA compaction to DNA fragmentation. Data from DNA fragmentation assessed through Comet assay in these patients and controls has previously been published in different papers from our group (Ribas-Maynou et al., 2012a, 2012b; Fernandez-Encinas et al., 2014).

Chromomycin A3 test

The CMA3 test was performed to all semen samples after washing them in PBS. First, samples were treated with 4% paraformaldehyde for 30 minutes and with 0.25% Triton X-100 for 15 minutes. Then, fixed sperm cells were extended on a slide, and air-dried until no liquid was present. McIlvaine buffer solution (18mL citric acid 0.1M and 41 mL Na_2HPO_4 0.2M) was prepared and mixed freshly with $MgCl_2$ to a final concentration of 10mM. Then, the final staining solution 190 μl of this fresh solution was mixed with 10 μl of CMA3 (5 mg/ml), obtaining a final concentration of 250 μg/mL.

Finally, the slide was incubated with 50 μl of the final staining solution covered with coverslip and incubated at room temperature and darkness for 20 minutes. After that, coverslip was gently removed and sperm cells were counterstained with DAPI SlowFade® Gold antifade (Invitrogen; Eugene, OR, USA). A total of 400 spermatozoa were analyzed as positive (blue and green) or negative (only blue) at the epifluorescence microscope (Olympus AX70) to obtain the percentage of chromomycin positive cells.

Statistical analysis

Data was analyzed using SPSS v20 software (Statistics Package for the Social Sciences software, Inc., Chicago, IL). To assess differences between clinical groups, non-parametric tests (Mann-Whitney U test) were used. Correlations were analyzed using the Spearman test. To determine the sensitivity and specificity of the test in predicting male infertility, Receiver Operating Characteristic (ROC) curves were used. The significance level was established at 95% of the confidence interval to be considered significant in all tests.

RESULTS

CMA3 test in comparison to sperm DNA fragmentation

Mean and standard deviation of sperm DNA fragmentation and chromomycin A3 test is displayed in Figure 1 and Table I. Attending CMA3 in clinical groups, fertile donors showed the lowest value of positive cells, showing differences to all other groups ($p<0.001$). Recurrent miscarriage patients showed similar values to infertile patients with or without altered semenogram ($p>0.05$), but differences were found when compared to varicocele patients ($p=0.012$). The infertile patients showed higher values of CMA3 positive cells, being the highest values found in varicocele patients. No differences were found among the three groups of infertile patients ($p>0.05$), however, a tendency to signification was found between normal semenogram infertile patients and varicocele patients ($p=0.059$). Interestingly, our results show that as alkaline Comet DNA fragmentation becomes higher, CMA3 positive cells also increase (Figure 1 and Table I).

Correlation between Comet assay and CMA3

The spearman test showed a positive but weak correlation between alkaline Comet (detecting ssSDF) and chromomycin A3 test with a correlation coefficient of 0.456 and $p<0.001$. Alternatively, no correlation has been found ($r=0.172$ and $p=0.104$) between neutral Comet (detecting dsSDF) and CMA3 test.

Male infertility predictive power of CMA3 test

After knowing the fertility outcome of each subject, the ROC analysis was used to assess the ability of CMA3 in predicting male infertility. An important issue is that the RPL patients were considered as fertile because although they experienced miscarriages, they achieved a clinical pregnancy. The specific ROC curve for this test is

displayed in Figure 2. The cut-off value for infertility was 40% of positive CMA3 test, with a sensitivity of 0.936, specificity of 0.512 and an area below the ROC curve of 0.783.

DISCUSSION

The aim of the present work is to analyze the relation between DNA compaction and DNA damage taking into account different clinical statuses. For that, we have analyzed a total of 90 semen samples from individuals grouped into different groups: fertile donors, recurrent pregnancy loss patients without female factor, and three groups of infertile patients: normal semenogram, altered semenogram, and varicocele patients.

Relation of sperm DNA compaction with DNA damage

The DNA compaction and defects of protamination can be evaluated using the chromomycin A3 test (De Iuliis *et al.,* 2009). The chromomycin A3 competes with protamines in DNA binding, and therefore, it binds to zones where a poor protamination exists, giving fluorescence that can be analyzed with an epifluorescence microscope. Defects on protamination are common among infertile patients, being one of the origins of DNA fragmentation (De Iuliis *et al.,* 2009; Aitken and De Iuliis, 2010; Sakkas and Alvarez, 2010; Sivanarayana *et al.,* 2012). Different authors have shown an altered ratio between protamine 1 and protamine 2 in infertile patients, such as varicocele and chromosome reorganization carriers, who show also high values of SDF (Torregrosa *et al.,* 2006; Castillo *et al.,* 2011; García-Peiró *et al.,* 2011a). In this work, we show that an increase of the positive CMA3 sperm cells can be seen in all infertile patients (Figure 1 and Table I) compared to fertile donors. Moreover, this percentage of sperm cells with poor DNA compaction increases as single stranded DNA fragmentation does, indicating some relation between alterations in DNA compaction and DNA damage (De Iuliis *et al.,* 2009; Nijs *et al.,* 2009; Aitken & de Iuliis, 2010; Castillo *et al.,* 2011; Simon *et al.,* 2011a; Sivanarayana *et al.,* 2012; Salehi *et al.,* 2013). Attending DNA compaction, recurrent pregnancy loss patients show statistical

differences compared to fertile donors, but similar values to infertile patients are found (Table I and Figure 1). Interestingly, these RPL patients show a DNA fragmentation profile with low ssSDF and high dsSDF, which is similar to one subgroup of fertile donors but different to infertile patients, who show high ssSDF and high dsSDF (Ribas-Maynou et al., 2012a & 2012b). This fact may indicate that DNA compaction analysis might be helpful in the distinction between fertile donors with low ssSDF and high dsSDF profile and RPL patients, who show the exact DNA fragmentation profile.

The study of the correlation showed a weak but significant correlation between alkaline Comet and CMA3, but no correlation can be found between neutral and CMA3. This result is of interest, since neutral Comet neither did show any correlation with all commonly used tests to assess DNA fragmentation such as TUNEL, SCSA and SCD test (Ribas-Maynou *et al.,* 2013a). An explanation would be that neutral Comet is detecting a very specific dsDNA damage that is located at matrix attachment regions (MAR). The detection of these double stranded DNA breaks using neutral comet depends on their binding to the sperm nuclear matrix (Ribas-Maynou *et al.,* 2012b, 2013b). These dsDNA breaks on the MAR regions can be induced in mice with manganese and calcium and religated with EDTA. To explain this fact, a model where a TOPO-II like enzyme might be performing this activity has been proposed (Yamauchi *et al.,* 2007a, 2007b; Gawecka *et al.,* 2013; Ribas-Maynou *et al.,* 2013b). Therefore, since these dsDNA breaks detected by neutral Comet are located mainly on MAR regions, and these regions are compacted by histones rather than protamines, no relation with CMA3 would be expected.

Different facts should happen when assessing the correlation between CMA3 and ssDNA breaks: these breaks are proposed to be present in an extensive form along all the genome, both inside the toroids and the MAR regions (Ribas-Maynou *et al.,* 2012b). As they have implication in toroids, where protamines are present, a relation with CMA3 should be expected. Moreover, the relation between DNA compaction and DNA fragmentation found in the present work is in agreement to the correlation found in other works (De Iuliis *et al.,* 2009; Nijs *et al.,* 2009; Sivanarayana *et al.,* 2012; Salehi *et al.,* 2013), and the fact that only alkaline Comet shows correlation with CMA3 is in agreement that oxidative stress could be the main factor related to this poor DNA compaction (De Iuliis *et al.,* 2009).

Prediction of male infertility using CMA3 test

To test the usefulness of the technique in predicting male infertility, a ROC analysis was performed and a cut-off value for male infertility of 40% of CMA3 positive sperm cells was obtained, with a high sensitivity of 0.936, and low specificity of 0.512 (Figure 2). Until our knowledge, there is not any CMA3 predictive value in the literature related to male infertility, however, its relation to assisted reproduction has been previously reported (Nasr-Esfahani *et al.,* 2005; Tarozzi *et al.,* 2009).

The CMA3 test would have a good predictive power for male infertility due to its high sensitivity, but it would not be as good to predict fertility due to a low value of specificity (Figure 2). However, compared to other tests to predict male infertility, CMA3 would be an easy and cheap test to detect defects on the sperm chromatin packaging, which would probably lead to DNA fragmentation and, therefore, prevent or add difficulty in achieving a pregnancy.

Conclusion

The chromomycin A3 test is a measure of the sperm DNA compaction status, which is positively correlated to single stranded DNA damage but not to double stranded DNA damage. Moreover, its simplicity and its capacity to distinguish infertile patients results in a good test for the reproductive counseling.

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AUTHOR'S ROLES

Jordi Ribas-Maynou contributed in experimental procedures, statistical analysis, graphics and table elaboration and document writing.

Agustín García-Peiró contributed in experimental design, results discussion and statistical analysis.

Alba Fernandez-Encinas contributed in experimental procedures.

María José Amengual, Carlos Abad and Elena Prada contributed in recruitment of patients, samples collection, storage and semen parameters analysis.

Jordi Benet and Joaquima Navarro contributed in experimental design and direction, coordination of the work and document writing.

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Table 1. Alkaline and Neutral Comet sperm DNA fragmentation and chromomycin A3 positive cells percentages attending different clinical status.

	Alkaline Comet	**Neutral Comet**	**CMA3**
Fertile donors	27.85 ± 17.68	48.67 ± 29.70	27.19 ± 12.01
Recurrent Pregnancy Loss	38.08 ± 17.48	73.75 ± 24.02	52.63 ± 13.85
Total infertile patients	57.78 ± 13.18	64.05 ± 15.18	61.18 ± 17.17
Infertile patients			
- Normal semenogram	43.21 ± 9.03	54.39 ± 14.77	54.48 ± 9.42
- Altered Semenogram	60.71 ± 10.18	65.41 ± 14.32	60.28 ± 17.57
- Varicocele	63.20 ± 16.59	69.79 ± 15.82	72.11 ± 18.98

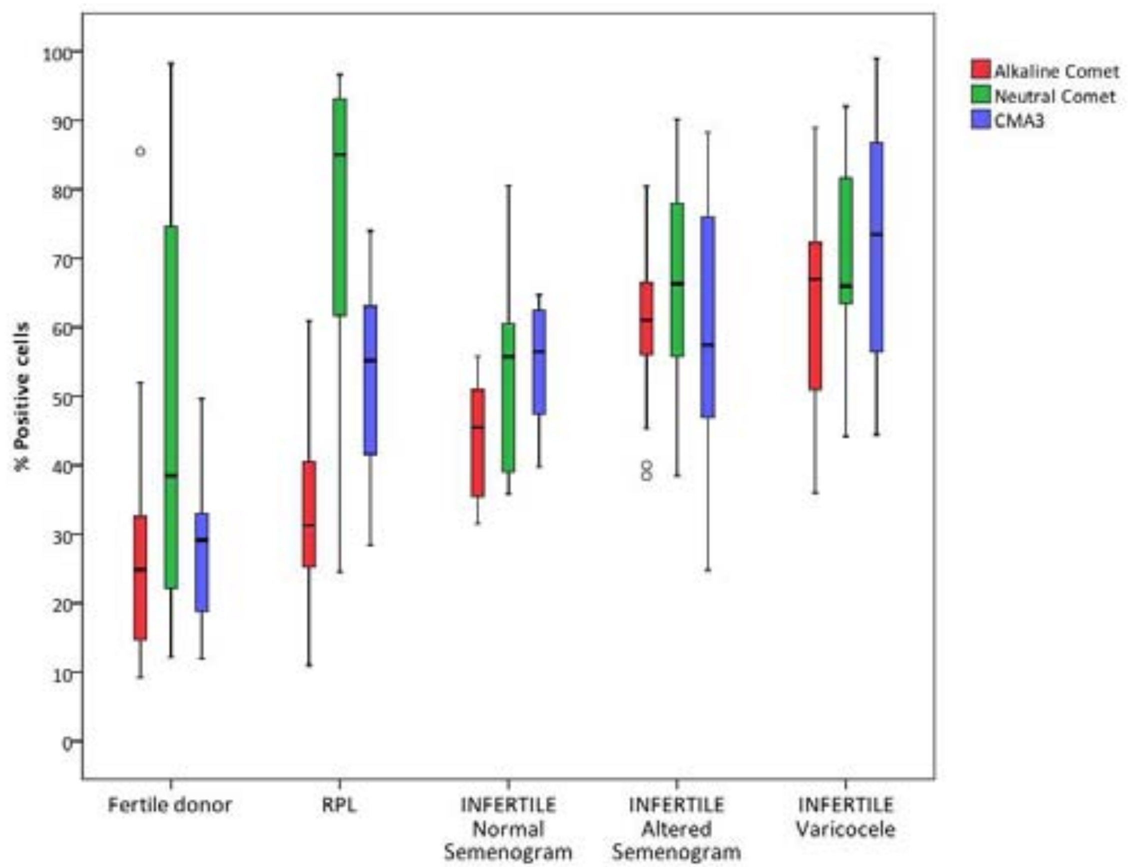


Figure 1. Percentage of single stranded DNA fragmentation assessed by alkaline Comet (red), double stranded DNA fragmentation assessed by neutral Comet (green), and positive chromomycin A3 sperm cells (blue).

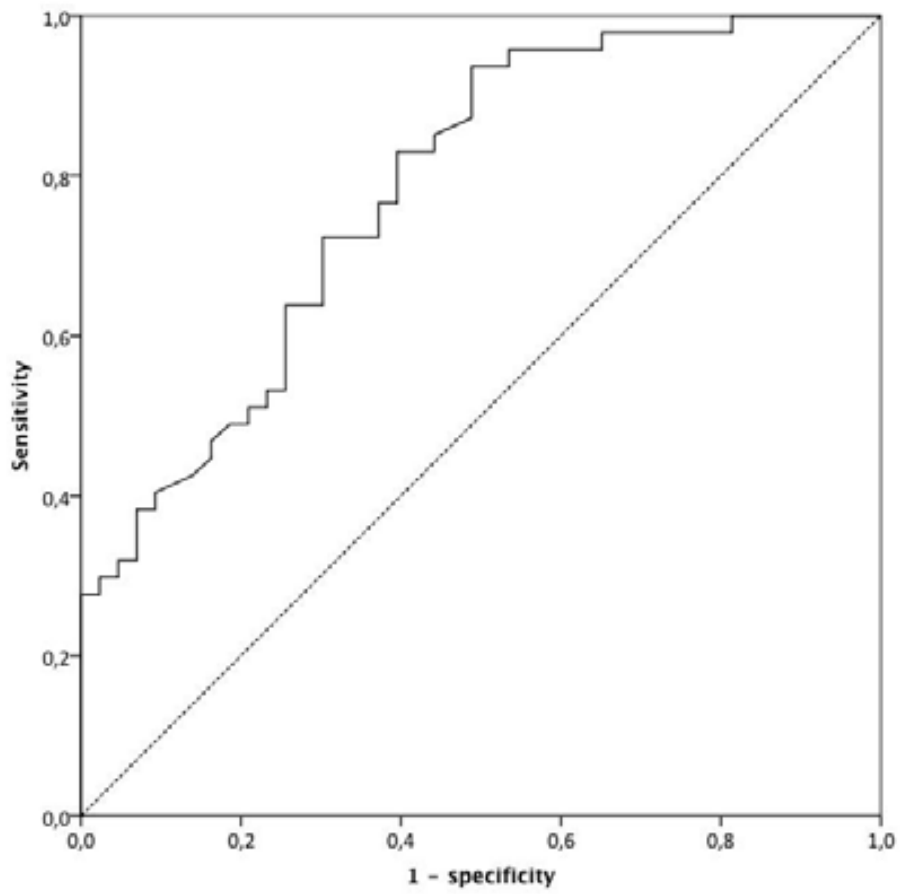


Figure 2. Receiver Operating Characteristic curve to assess the ability of the CMA3 test in predicting male infertility.

5. DISCUSSIÓ

En el marc del diagnòstic de la infertilitat masculina, s'ha establert l'anàlisi de la fragmentació del DNA espermàtic com a un biomarcador sensible i complementari a l'anàlisi tradicional de les mostres de semen. En aquest sentit, aquesta tesi doctoral presenta una sèrie de treballs en els que es discuteixen els aspectes més rellevants de la fragmentació del DNA, en quan a metodologies, en quan a diagnòstic clínic dels pacients infèrtils, en quan a l'efecte diferenciat dels trencaments de cadena senzilla i doble del DNA, i finalment, en quan a la relació de la compactació del DNA espermàtic. Tots aquests aspectes són importants en el si d'una anàlisi global del factor masculí, en el que la fragmentació del DNA pot tenir un paper clau en la decisió diagnòstica final. A continuació, es discuteixen de forma transversal aquells aspectes derivats de l'anàlisi dels treballs presentats.

5.1 Detecció diferencial dels trencaments de cadena senzilla i doble del DNA espermàtic

Les diferents incubacions realitzades utilitzant el peròxid d'hidrogen, que causa trencaments de cadena senzilla del DNA i un enzim de restricció, que causa trencaments de cadena doble del DNA han aconseguit amb èxit la caracterització dels diferents tipus de trencaments de DNA espermàtic detectables amb el Comet alcalí i amb el Comet neutre (Ribas-Maynou i col., 2012a).

5.1.1 Comet Alcalí: trencaments de cadena senzilla del DNA

Les incubacions a diferents concentracions de peròxid d'hidrogen evidencien un augment progressiu de la fragmentació detectada per el Comet alcalí, mentre que l'increment és mínim per el Comet neutre (Figura 27). Així doncs, s'estableix que el Comet alcalí detecta amb èxit preferencialment trencaments de cadena senzilla del DNA, mentre que el Comet neutre no presenta sensibilitat per a la seva detecció. Els

resultats obtinguts en aquesta posada a punt del mètode Comet alcalí són concordants amb els obtinguts en l'estudi publicat per Enciso i col., 2009, utilitzant Comet 2D.

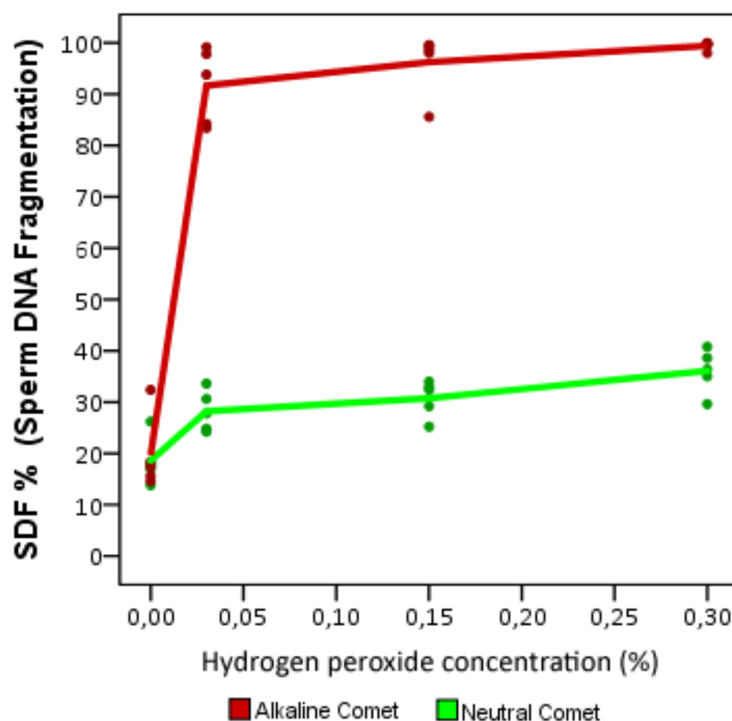


Figura 27. Percentatge de fragmentació del DNA detectada amb el Comet alcalí i el Comet neutre en incubacions amb diferents concentracions de peròxid d'hidrogen, que causa trencaments de cadena senzilla del DNA (Adaptada de: Ribas-Maynou i col., 2012a).

5.1.2 Comet Neutre: trencaments de cadena doble del DNA

El mateix tipus d'estudi d'integritat del DNA espermàtic s'ha realitzat utilitzant tractaments amb un enzim de restricció (Alu1) que provoca trencaments de cadena doble del DNA en la seqüència 5'AGCT 3'.

Els resultats obtinguts en incubacions d'espermatozoides descompactats amb l'enzim de restricció evidencien un elevat increment de fragmentació amb Comet neutre, mentre que el Comet alcalí, tot i augmentar significativament, presenta percentatges

prop d'una tercera part del Comet neutre. Així doncs, s'infereix que el Comet neutre detecta preferencialment els trencaments de cadena doble del DNA, mentre que el Comet alcalí no presenta prou sensibilitat per detectar aquest tipus de trencaments (Figura 28). Tot i que alguns autors postulen que el Comet alcalí seria capaç de detectar tant trencaments de cadena senzilla com de cadena doble del DNA (Simon i Carrell, 2013), el present anàlisi concorda amb resultats publicats utilitzant Comet 2D (Enciso i col., 2009), demostrant que el Comet alcalí no té una bona sensibilitat en la detecció dels trencaments de cadena doble del DNA.

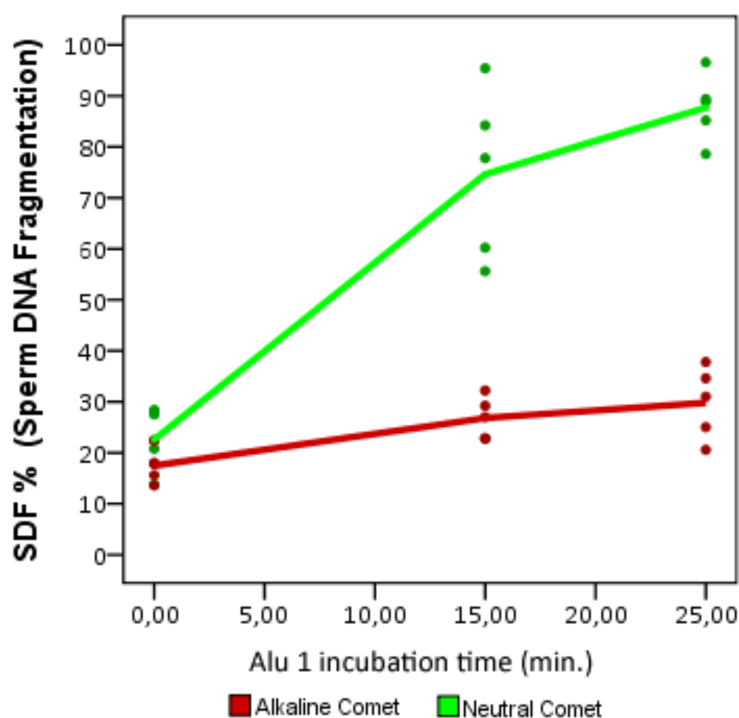


Figura 28. Percentatge de fragmentació del DNA detectada per Comet alcalí i Comet neutre en incubacions a diferents temps amb un enzim de restricció (Alu1), que causa trencaments de cadena doble del DNA (Adaptada de: Ribas-Maynou i col., 2012a).

5.2 La criopreservació de mostres de semen causa únicament trencaments ssDNA

La criopreservació de mostres de semen ha estat tradicionalment una tècnica molt utilitzada per multitud d'aplicacions, tant a nivell de reproducció assistida, com en la preservació de la fertilitat, així com per recerca (Sanger i col., 1992; Anger i col., 2003; Jensen i col., 2011; Di Santo i col., 2012). La criopreservació d'espermatozoides humans ha estat, al llarg dels últims anys, un procediment àmpliament estudiat, amb diversos treballs centrats en l'efecte que produeix sobre els paràmetres seminals com la mobilitat, la morfologia i la vitalitat espermàtica, els quals, després de la criopreservació, mostren una clara davallada (Thomson i col., 2010; Di Santo i col., 2012; Lee i col., 2012; Satirapod i col., 2012). Actualment hi ha un enorme interès en la fragmentació del DNA com a marcador de qualitat espermàtica i, en aquest camp, els diversos estudis de la literatura que avaluen els efectes de la congelació són encara discordants (Høst i col., 1999; Spanò i col., 1999; Duru i col., 2001; Isachenko i col., 2004; de Paula i col., 2006; Thomson i col., 2009; Zribi i col., 2010). Analitzant aquests estudis, s'observa que han estat realitzats utilitzant diferents tècniques d'anàlisi de la fragmentació del DNA, que poden presentar diferències de sensibilitat en quan a la detecció del dany. Per aquest motiu, per al present estudi s'ha utilitzat la tècnica més sensible: l'assaig Comet (Ribas-Maynou i col., 2013b). A més, la determinació de la fragmentació de cadena senzilla i doble del DNA permet la diferenciació entre el dany produït mitjançant mecanismes d'estrès oxidatiu i mecanismes enzimàtics, respectivament (Ribas-Maynou i col., 2012a).

Els resultats obtinguts en el present estudi mostren un increment significatiu del 10% d'espermatozoides amb trencaments de cadena senzilla del DNA després de la criopreservació, mentre que no s'ha observat un increment significatiu en els

trencaments de cadena doble del DNA després de la criopreservació. Fins al moment no hi ha dades a la literatura sobre l'efecte de la criopreservació sobre la fragmentació de cadena doble del DNA. Els resultats utilitzant Comet alcalí són concordants amb els d'estudis previs (Donnelly i col*.,* 2001; de Paula i col*.,* 2006; Thomson i col*.,* 2009), quantificant, per primera vegada, l'increment d'aquest efecte (10% de ssSDF).

Els resultats d'aquest objectiu confirmen la hipòtesi tradicional, segons la que el dany provocat per la criopreservació al DNA espermàtic correspon a un mecanisme d'estrès oxidatiu (Mazzilli i col*.,* 1995; Thomson i col*.,* 2009). A més, donat que els trencaments de cadena senzilla del DNA han estat associats a una menor capacitat d'embaràs i els trencaments de cadena doble del DNA a un major risc d'avortament (Ribas-Maynou i col*.,* 2012b), en la utilització en reproducció assistida de mostres de semen criopreservades no s'hauria d'esperar un augment del risc d'avortament, però si una reducció del 10% en la capacitat de produir un embaràs evolutiu. Aquesta premissa seria vàlida en la major part dels pacients, ja que tots els grups clínics analitzats en aquest estudi presenten un similar increment en la ssSDF i no presenten increment en la dsSDF (Ribas-Maynou i col., 2014a).

Finalment, cal tenir presents aquests efectes de la criopreservació en els posteriors estudis d'aquesta tesi, ja que totes les mostres utilitzades han estat prèviament criopreservades sota el mateix protocol. Per aquest motiu cal contemplar la possibilitat d'un cert biaix en la incidència de trencaments de cadena senzilla del DNA. En qualsevol cas, aquest biaix seria el mateix per a totes les mostres de diferents grups clínics i controls fèrtils, fet que no impediria la comparació entre els diferents resultats que es discuteixen a continuació.

5.3 La incidència de fragmentació de cadena senzilla del DNA constitueix una bona aproximació per a la predicció de la capacitat fèrtil

Fins al moment, només tres estudis han analitzat la utilitat clínica de les tècniques d'anàlisi de fragmentació del DNA comparant de forma exhaustiva la majoria les metodologies existents (Erenpreiss i col*.,* 2004; Chohan i col*.,* 2006; García-Peiró i col*.,* 2011c). Per tant, es fa molt necessari estudiar la fragmentació del DNA espermàtic atenent a la pròpia sensibilitat de cada una de les metodologies disponibles per poder determinar quina d'elles hauria de ser la tècnica d'elecció a l'hora de caracteritzar la infertilitat masculina.

Diferents estudis mostren una correcta diferenciació entre individus fèrtils i infèrtils mitjançant les tècniques TUNEL, SCSA, SCD i Comet alcalí (Gandini i col*.,* 2000; Irvine i col*.,* 2000; Zini i col*.,* 2001; Saleh i col*.,* 2002a; Chohan i col*.,* 2006; García-Peiró i col*.,* 2012; Ribas-Maynou i col*.,* 2012a). En aquest sentit, els resultats que aporta el present treball són concordants (Ribas-Maynou i col*.,* 2013b). A més, les quatre metodologies esmentades presenten una elevada correlació entre elles, sent les tècniques citomètriques les que presenten una millor correlació (Chohan i col*.,* 2006; Villani i col*.,* 2010a; García-Peiró i col*.,* 2011c). Aquest fet és de gran interès donat que els procediments de TUNEL i de SCSA analitzen diferents aspectes del dany al DNA espermàtic: mentre que el TUNEL detecta de forma directa els trencaments mitjançant una terminal transferasa, l'SCSA avalua la susceptibilitat a desnaturalització del DNA per valorar indirectament els trencaments. Addicionalment, la tècnica SCD també presenta una bona correlació amb les anteriors tècniques mencionades, i estudis previs en els que s'analitza aquesta correlació confirmen també aquest resultat (Donnelly i col*.,* 2000; Villani i col*.,* 2010a). D'altra banda, la correlació que existeix

entre el Comet alcalí i les altres tres tècniques presenta un coeficient menor, i la comparació dels rangs de fragmentació del DNA observats amb les diferents tècniques pot aportar una explicació a aquest fet. Mentre que els sistemes TUNEL, SCSA i SCD presenten rangs que van del 0% al 50% de fragmentació del DNA, el Comet alcalí presenta valors pràcticament de 0% a 100% de fragmentació del DNA, indicant que aquest posseeix una major sensibilitat a detectar el dany real de la cèl·lula en qüestió, fet possiblement atribuïble a l'aplicació d'electroforesi, que permet evidenciar millor els trencaments en el DNA. Aquest increment de la sensibilitat del Comet alcalí per a detectar el dany al DNA també es fa palès a l'hora de detectar la capacitat potencial de fertilitat. Per a aquesta predicció, mentre que el Comet alcalí presenta una àrea sota la corba ROC de 0,937 amb un punt de tall del 45% de fragmentació de cadena senzilla del DNA, les tècniques TUNEL, SCSA i SCD presenten àrees sota la corba menors, indicant una menor sensibilitat i especificitat en el diagnòstic de pacients infèrtils (Figura 29). Tenint en compte aquest menor poder de predicció, els diferents valors llindar per al diagnòstic d'infertilitat obtinguts (20% per TUNEL, 19% per SCSA i 22,5% per SCD) són concordants als prèviament publicats per diversos grups (Evenson i col*.,* 2002; Fernández i col*.,* 2005; Payne i col*.,* 2005; Velez de la Calle i col*.,* 2008; Sharma i col*.,* 2010; Venkatesh i col*.,* 2011; Nuñez-Calonge i col*.,* 2012).

Aquests resultats posen de manifest que el Comet alcalí presenta la major sensibilitat i especificitat per a la diferenciació dels individus infèrtils respecte dels fèrtils i, a més, que els trencaments de cadena senzilla del DNA són els més implicats en el potencial d'embaràs de l'individu.

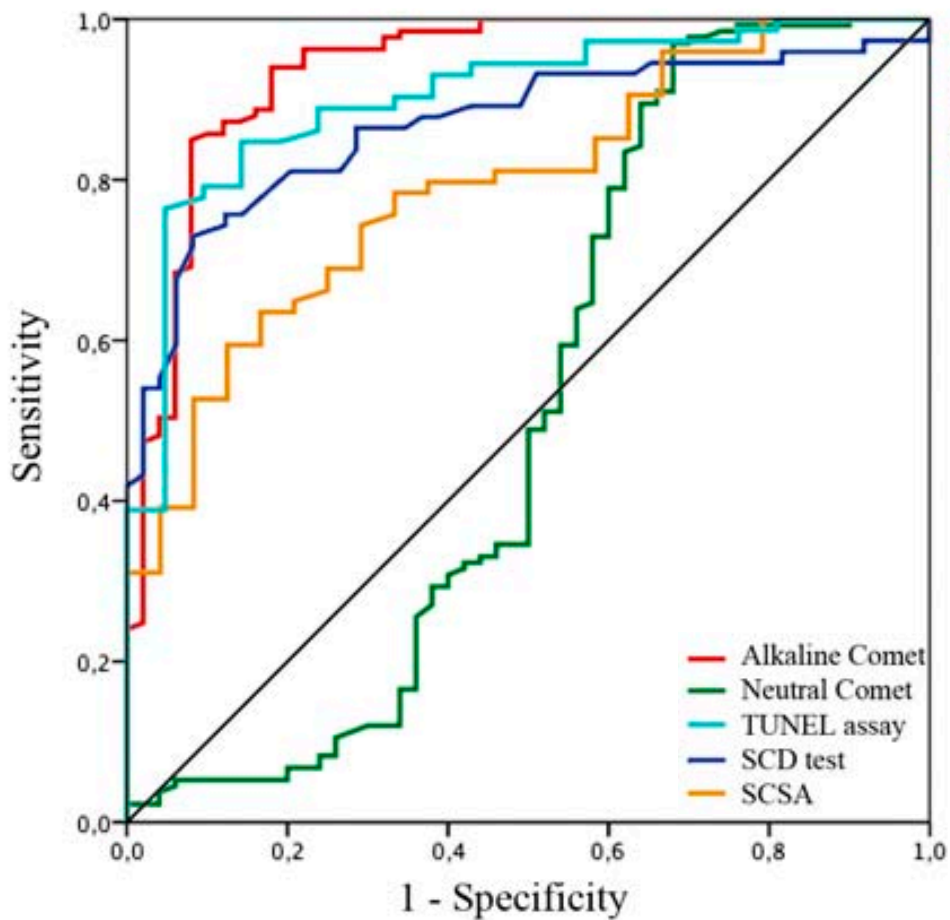


Figura 29. Anàlisi ROC per determinar la sensibilitat i especificitat de cada tècnica (Ribas-Maynou i col., 2013a)

5.4 Els trencaments de cadena doble del DNA detectats per Comet neutre no estan relacionats amb la capacitat de generar un embaràs clínic

A diferència de l'esmentat per als trencaments de cadena senzilla del DNA, els trencaments de cadena doble del DNA d'espermatozoides d'individus infèrtils no presenten diferències en relació a la dels individus fèrtils. En pacients fèrtils, la fragmentació de cadena doble del DNA presenta una distribució bimodal que es discutirà en el següent apartat (Apartat 5.5, Figura 30).

En pacients infèrtils, els resultats de fragmentació de cadena doble del DNA presenten una distribució normal, similar a la distribució dels resultats de fragmentació de cadena senzilla de DNA en pacients infèrtils (Ribas-Maynou i col*.,* 2012a, 2013b). Al contrari que les altres quatre tècniques discutides anteriorment, els resultats de Comet neutre no presenten correlació amb cap de les tècniques d'anàlisi de la fragmentació del DNA, fet que indica que aquesta tècnica detecta un tipus de dany del DNA diferent, majoritàriament relacionat amb trencaments de localització molt específica (Kaneko i col*.,* 2012), situats a les regions MAR, i en una quantitat limitada (Ribas-Maynou i col*.,* 2012b, 2013c). La falta de correlació d'aquests resultats amb els obtinguts amb tècniques SCSA i TUNEL, que teòricament són capaces de detectar trencaments de cadena senzilla i doble del DNA (Van Kooij i col*.,* 2004) es deuria a que aquestes tècniques no presenten suficient sensibilitat per detectar aquests trencaments detectats per el Comet neutre.

Per tots aquests motius, la predicció de la capacitat fèrtil mitjançant l'anàlisi de la fragmentació del DNA amb el mètode del Comet neutre presenta valors molt baixos de sensibilitat i especificitat, sent el seu valor predictiu gairebé nul (Figura 29). Així doncs, per obtenir un diagnòstic acurat de la infertilitat masculina, sembla necessari l'anàlisi diferencial de la incidència de fragmentació de cadena senzilla del DNA, relacionat amb la capacitat fèrtil, i la de cadena doble del DNA, relacionat amb el risc d'avortament associat a factor masculí (Ribas-Maynou i col., 2012b). L'assaig Comet en les seves dues variants, és capaç d'informar de la incidència d'aquests dos tipus de fragmentació del DNA, presentant-se com el sistema que posseeix major capacitat de predicció.

5.5 L'anàlisi de la fragmentació de cadena senzilla i doble permet una millor caracterització de les diferents tipologies clíniques de pacients infèrtils.

Diversos treballs diferencien pacients infèrtils d'individus fèrtils en base als resultats d'estudis de la fragmentació del DNA espermàtic amb diferents tècniques (Gandini i col*.,* 2000; Irvine i col*.,* 2000; Zini i col*.,* 2001; Saleh i col*.,* 2002; Chohan i col*.,* 2006; García-Peiró i col*.,* 2012). Com ja s'ha discutit, l'anàlisi de la fragmentació del DNA amb el mètode del Comet alcalí és capaç de diferenciar aquests dos grups amb una major sensibilitat (Ribas-Maynou i col*.,* 2013b). Els resultats obtinguts en mostres de semen d'homes fèrtils mostren un comú denominador en un baix percentatge de fragmentació de cadena senzilla del DNA, mentre que hi ha una gran dispersió dels valors de fragmentació de cadena doble del DNA (Ribas-Maynou i col*.,* 2012a, 2012b). Dins aquesta dispersió, s'observa una distribució bimodal entre baix i alt percentatge de dsSDF (Figura 30). Això permet l'establiment de dos subgrups de controls fèrtils, uns amb un perfil de baixa ssSDF i baixa dsSDF, i els altres amb un perfil de baixa ssSDF i alta dsSDF.

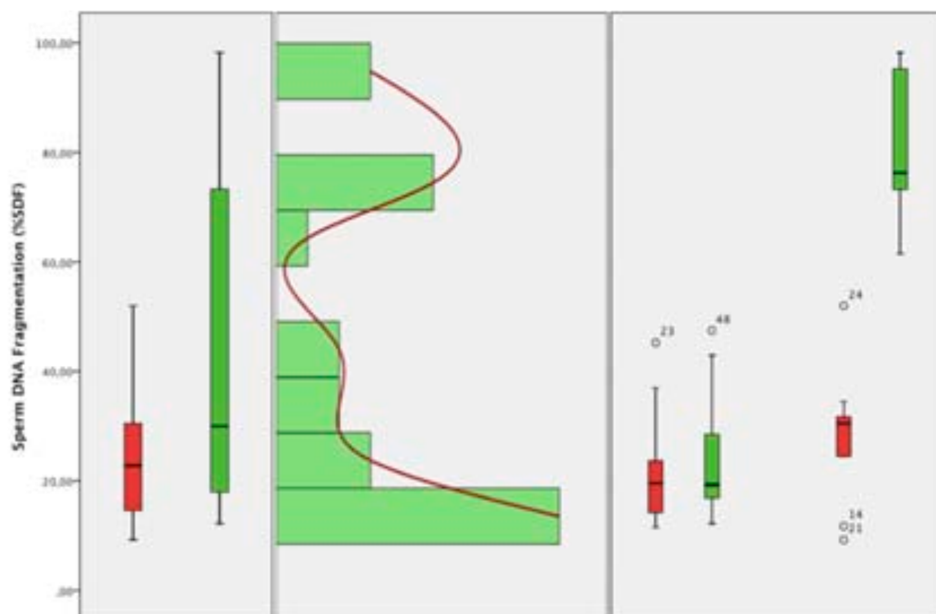


Figura 30. Anàlisi de la dispersió de la dsSDF en mostres espermàtiques d'homes fèrtils

De la mateixa manera, l'anàlisi diferencial de la fragmentació del DNA amb el Comet neutre i el Comet alcalí de pacients infèrtils subdividits segons característiques clíniques específiques, mostra un altre perfil amb elevats percentatges de ssSDF i de dsSDF (Ribas-Maynou i col*.,* 2012a). Tant en pacients ATZ com OATZ, els percentatges de fragmentació de cadena senzilla i de cadena doble del DNA estan al voltant del 60% (Ribas-Maynou i col*.,* 2012a). D'acord amb això, la concentració d'espermatozoides de la mostra no està relacionada amb la fragmentació del DNA espermàtic. Tenint en compte aquest resultat, s'esperaria que aquests dos grups de pacients tinguessin, en circumstàncies d'un tractament de reproducció assistida, la mateixa probabilitat d'èxit reproductiu. Alternativament, pacients ATZ amb varicocele presenten una incidència de fragmentació més elevada, al voltant del 80% de ssSDF i dsSDF, sent aquest grup de pacients el de pitjor pronòstic (Ribas-Maynou i col*.,* 2012a). Aquest fet es podria explicar per l'etiologia del varicocele que causa una més elevada temperatura testicular provocant un estrès oxidatiu addicional respecte el present en ATZ sense varicocele (Aitken i Krausz, 2001). Aquest estrès oxidatiu seria responsable d'una intensificació del dany al DNA espermàtic, especialment de cadena senzilla. A més, l'increment dels trencaments de cadena doble en aquest grup de pacients apunta la possibilitat de que els dos tipus de trencaments del DNA estiguin d'alguna forma relacionats.

Els pacients portadors de reorganitzacions cromosòmiques presenten, generalment, valors de trencaments del DNA força elevats, observant-se una elevada dispersió tant per Comet alcalí com per Comet neutre. A diferència dels resultats de dsSDF en controls fèrtils, aquests no mostren una distribució que permeti distingir-hi diversos subgrups, de forma que la variabilitat observada estaria força condicionada per la

heterogeneïtat de les reorganitzacions incloses en aquest grup de pacients (Perrin i col., 2009; García-Peiró i col*.,* 2011a, 2011b).

En resum, mitjançant el mètode Comet alcalí i neutre, s'evidencien tres perfils: dos d'ells, un amb baixa ssSDF i baixa dsSDF, i un amb baixa ssSDF i alta dsSDF, compatibles amb l'embaràs, i un d'ells, amb elevada ssSDF i elevada dsSDF relacionat amb la infertilitat (Figura 31).

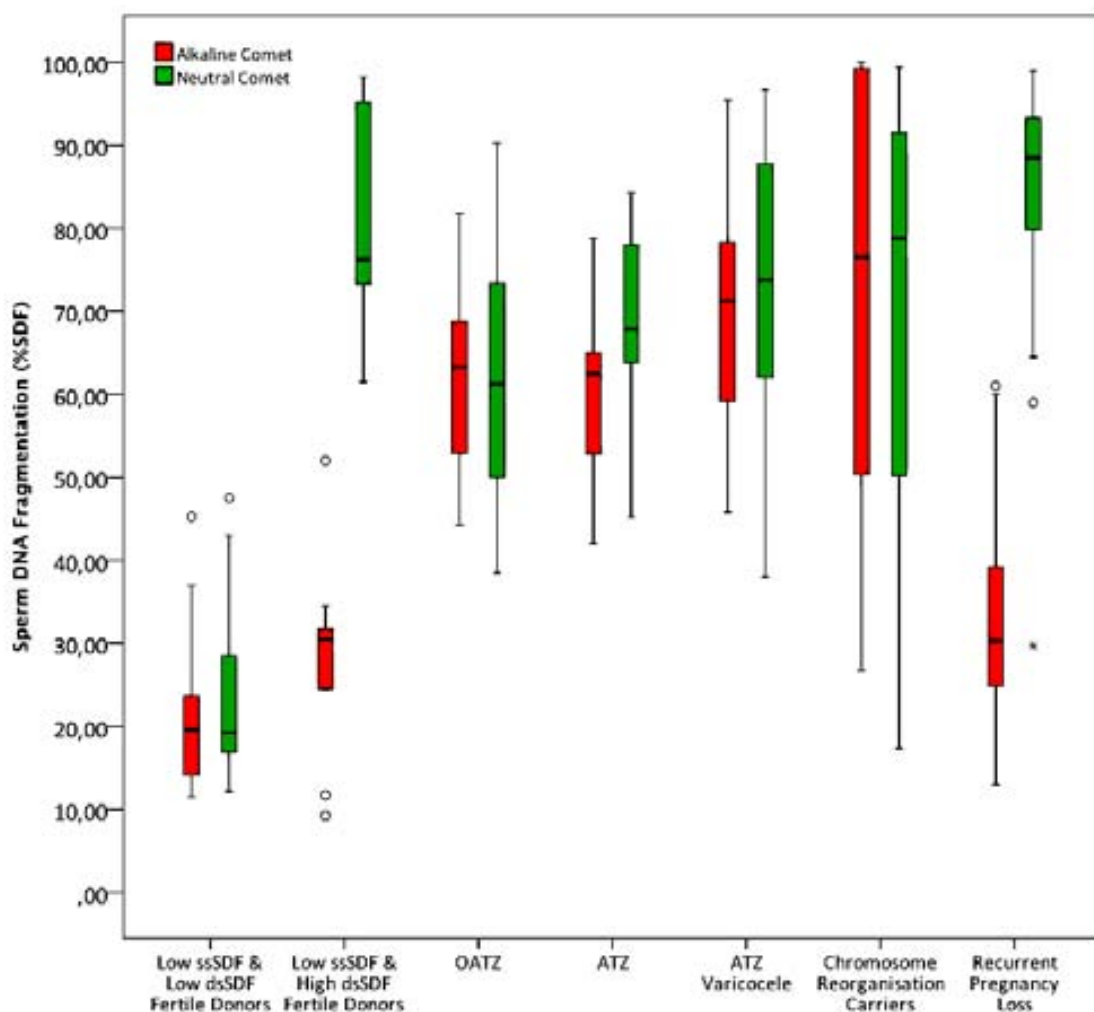


Figura 31. Resultats de fragmentació de cadena senzilla del DNA (vermell) i cadena doble del DNA (verd) en diferents grups de pacients.

5.6 Els trencaments de cadena senzilla del DNA es corresponen amb un dany extensiu

La relació entre l'elevada incidència dels trencaments de cadena senzilla del DNA i la fallada en aconseguir l'embaràs suggereix el següent model (Figura 32), segons el que es postula que els trencaments de cadena senzilla del DNA, produïts bàsicament mitjançant un dany oxidatiu (Aitken i De Iuliis, 2010; Sakkas i Alvarez, 2010), estarien presents en el DNA espermàtic en nombre exhaustiu i distribuïts de forma indistinta entre regions intra-toroïdals i inter-toroïdals (MAR), i sent major encara en cas d'una pobre compactació del DNA espermàtic.

Figura 32. Model de la localització i l'efecte dels trencaments de cadena senzilla del DNA (Adaptada de: Ribas-Maynou i col., 2012b).

Després de la fecundació, es conegut que l'oòcit aporta una capacitat de reparació del DNA al zigot (Derijck i col., 2008; Fulka i col., 2009; Ménézo i col., 2010; Pellestor i col., 2014). Tot i això, donada l'elevada extensió dels trencaments, pot semblar obvia la saturació d'aquests sistemes de reparació, que no aconseguirien reparar completament el DNA espermàtic al pronucli masculí, fet que comportaria errors de replicació i un bloqueig durant l'etapa preimplantacional, impossibilitant l'arribada a l'etapa d'implantació.

5.7 Els trencaments de cadena doble del DNA estan relacionats amb el risc d'avortament associat a factor masculí

L'anàlisi dels trencaments de DNA de mostres de pacients amb avortaments de repetició de primer trimestre sense factor femení conegut presenta un perfil amb baixa incidència de fragmentació de cadena senzilla i alta incidència de fragmentació de cadena doble del DNA. És molt interessant ressaltar que el perfil d'aquest grup de pacients no presenta diferències amb un dels subgrups de controls fèrtils descrit anteriorment i no és present en cap altre grup de pacients analitzats (Figura 31) (Ribas-Maynou i col*.,* 2012b). Així, mentre que l'embaràs està associat a una baixa incidència de ssSDF, una elevada incidència de dsSDF està associada a un elevat risc d'avortament, només supeditat a la capacitat de reparació dels trencaments de cadena doble per el zigot, abans del recanvi de protamines per histones al pronucli masculí. En aquest sentit, una variabilitat en la qualitat oocitària i embrionària ha sigut observada (Baird i col., 2005; Braga i col., 2013; Pellestor i col., 2014). Llavors, una explicació seria que oòcits amb una major qualitat podrien donar lloc a embrions competents per a la reparació aquest dany i, per tant, produir un embaràs evolutiu, com seria el cas dels

controls de fertilitat provada. Per contra, oòcits de pitjor qualitat, incapaços de donar lloc a embrions que puguin reparar els trencaments de cadena doble, donarien lloc a un embaràs no evolutiu. En aquest darrer cas, l'avortament dins el primer trimestre podria relacionar-se amb la producció d'alteracions al DNA. En aquest sentit, s'ha demostrat que la inducció de fragmentació del DNA en un model murí causa múltiples aberracions cromosòmiques (Gawecka i col., 2013; Pellestor i col., 2014). En ser necessària alguna d'aquestes regions per al desenvolupament de l'organisme, aquest podria sofrir un bloqueig, donant lloc a un avortament de primer trimestre.

El fet de que un elevat percentatge d'espermatozoides amb trencaments de cadena doble del DNA estigui relacionat amb un risc d'avortaments permet realitzar estudis estadístics per determinar la sensibilitat i especificitat d'aquesta tècnica en predir aquest risc d'avortament. El valor llindar de Comet neutre per al risc d'avortaments ha estat del 77,5%, però aquest valor llindar està condicionat a que la mateixa mostra espermàtica no presenti valors de ssSDF incompatibles amb l'embaràs (>45% ssSDF).

L'ús d'una ràtio dsSDF/ssSDF presenta un major poder de predicció que l'anàlisi del dsSDF respecte el risc d'avortament associat a factor masculí, amb una sensibilitat i especificitat de 0,724 i 0,955, respectivament, amb un valor llindar de 2 (Figura 26).

5.8 Els trencaments de cadena doble del DNA corresponen a un dany a les regions MAR

L'aprofundiment en l'estudi basic dels trencaments de cadena doble del DNA, detectats mitjançant el Comet neutre, permet entendre millor el seu potencial efecte sobre la fertilitat masculina. Els gels d'electroforesi en camp polsant separen fragments grans de DNA (>10 kb) amb una millor resolució que els gels convencionals gràcies a l'aplicació de variacions de la direcció del camp elèctric. L'ús d'aquesta metodologia de detecció de trencaments de cadena doble de DNA en mostres de pacients d'avortaments, evidencia l'existència d'una banda de DNA de grandària al voltant de les 48kb, similar a la que apareix davant un tractament amb nucleasa com a control positiu (Yamauchi i col*.,* 2007b; Ribas-Maynou i col*.,* 2012b). Aquesta banda correspon a la mida mitjana del DNA que es compacta a cada toroide, i aquest resultat permet extrapolar que, de forma majoritària, els trencaments de cadena doble del DNA es situen a les regions d'unió a la matriu (MAR), que contenen histones i serveixen de nexe entre les diferents regions toroïdals (Ribas-Maynou i col*.,* 2012b). Aquestes regions MAR compactades amb histones tenen la capacitat d'integrar DNA exogen, per la qual cosa haurien de ser regions més sensibles als trencaments de cadena doble de DNA (Spadafora, 1998; Pittoggi i col*.,* 2000).

Aquestes conclusions suggereixen el següent model (Figura 33) en el que es postula que els trencaments de cadena doble del DNA, relacionats amb el risc d'avortaments, han d'estar localitzats a les regions d'unió a la matriu nuclear espermàtica, i en una quantitat limitada, ja que l'embrió hauria de ser capaç de poder reparar aquests trencaments per donar lloc a un embaràs evolutiu.

Considerant una errònia reparació d'aquests trencaments, caldria contemplar la possibilitat de que pugui néixer un nadó amb malalties postnatals com leucèmia i càncer, el risc dels quals s'ha postulat que pot estar relacionat amb la fragmentació del DNA espermàtic (Cooke i col*.,* 2003; Aitken i col*.,* 2009).

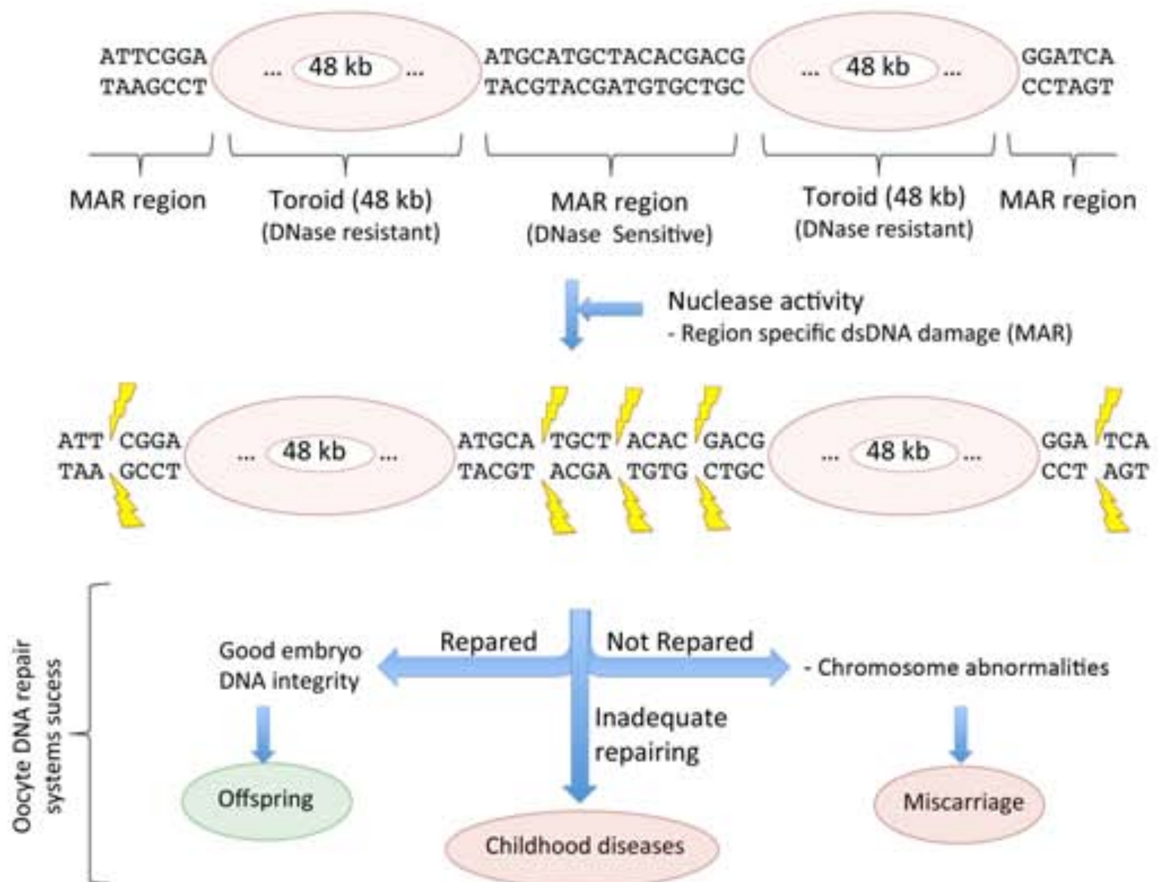
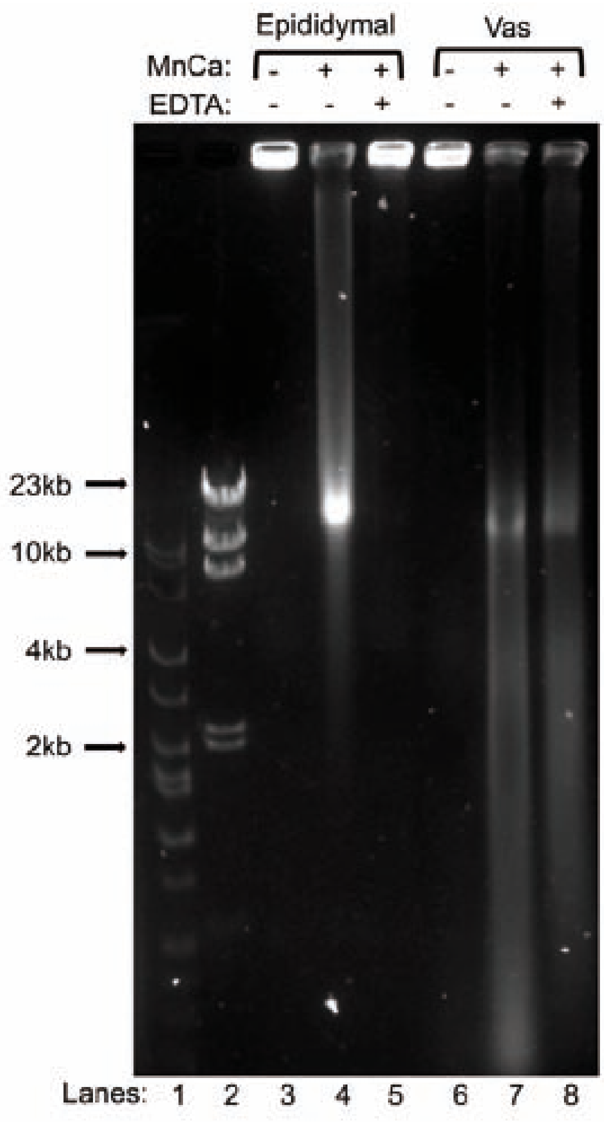


Figura 33. Model de la localització i l'efecte dels trencaments de cadena doble del DNA (Adaptada de: Ribas-Maynou i col., 2012b).



Epididymal
Vas
MnCa: - + + - + +
EDTA: - - + - - +
23kb
10kb
4kb
2kb
Lanes: 1 2 3 4 5 6 7 8

5.9.1 La lisi cel·lular que s'utilitza en l'aplicació de l'assaig Comet no desestabilitza la matriu nuclear espermàtica

En espermatozoides d'epidídim de ratolí l'efecte Sperm Chromatin Fragmentation (SCF) genera cues de Comet neutre més llargues que en el control negatiu, però menys que els tractaments amb nucleasa. A més, la major part dels fragments de DNA semblen estar units a l'halo nuclear del Comet, mostrant la seva interacció amb algun component del nucli espermàtic, probablement la matriu nuclear, de forma que s'estenen fibres de cromatina amb un únic trencament a l'extrem (Figura 35).

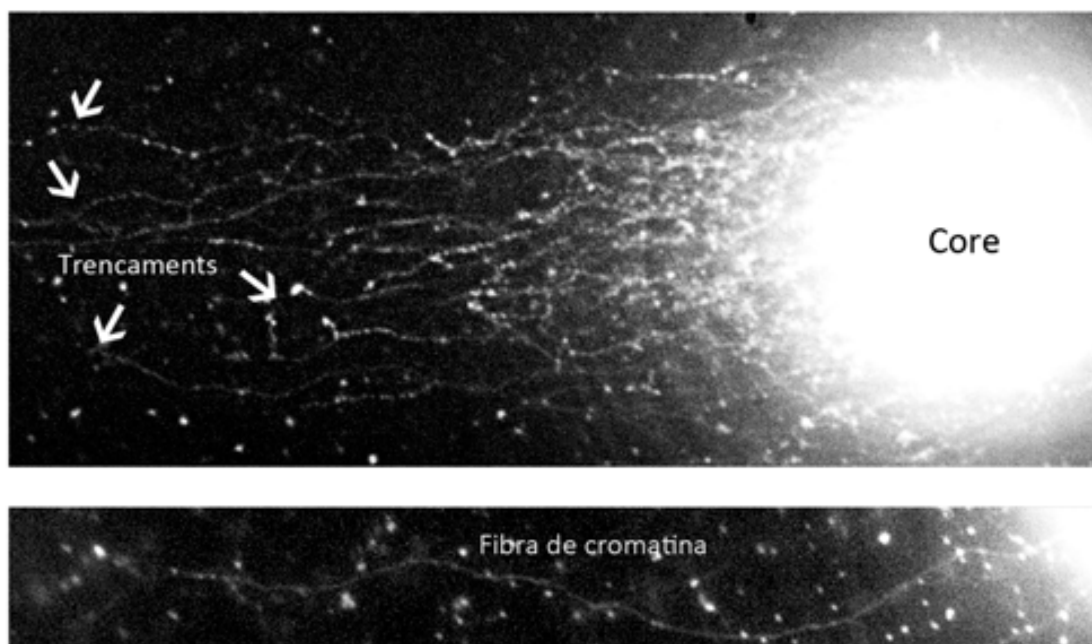


Figura 35. Fotografia d'un Comet neutre mostrant les fibres de cromatina unides al nucli o *core*. La imatge ha estat digitalment tractada d'imatge amb elevat contrast.

Aquests resultats no es corresponen amb els obtinguts en gels de camp polsant (Figura 34), on la lisi cel·lular causa la disrupció de la matriu nuclear espermàtica i, per tant, tant en tractaments amb nucleasa com amb SCF dóna lloc al mateix perfil de fragmentació (Yamauchi i col*.,* 2007b; Gawecka i col*.,* 2013). Aquesta discrepància

entre els diferents mètodes permet inferir que, tot i que tant l'SCF com els tractaments amb DNAsa causen trencaments a les regions MAR, els trencaments de cadena doble causats per l'SCF es mantenen units a la matriu nuclear espermàtica (Suppl.1, Ribas-Maynou i col*.,* 2013b).

La incubació del Comet neutre amb una tercera solució de lisi contenint SDS i DTT causa, en tractaments SCF, una reducció del halo nuclear i un increment en les cues visibles amb el Comet neutre, sent impossible la distinció entre trencaments SCF i tractaments amb nucleasa (Ribas-Maynou i col., 2013b). Aquest fet, doncs, evidencia la no disrupció de la matriu nuclear espermàtica amb les dues solucions de lisi convencionals del Comet, fet ja descrit anteriorment en cèl·lules somàtiques (Afanasieva i col*.,* 2010; Anderson i Laubenthal, 2013). Aquests experiments evidencien que el Comet neutre no és capaç de detectar la totalitat dels trencaments de cadena doble en regions MAR presents al DNA espermàtic. Així doncs, amb la modificació (Comet SDS-neutre) aportada per la tercera solució de lisi podria augmentar la sensibilitat del Comet neutre en la detecció de trencaments de cadena doble del DNA.

5.9.2 Existeixen almenys dos tipus de trencaments de cadena doble del DNA

En espermatozoides d'epidídim i de vas deferent de ratolí, en induir els trencaments de cadena doble amb manganès i calci (SCF) i posteriorment incubar-los amb EDTA, es produeixen dos efectes diferenciats (Figura 34 i Ribas-Maynou i col., 2013b). En espermatozoides d'epidídim es produeixen trencaments a les regions MAR, totalment reversibles amb EDTA, fet que indica que aquests es mantenen units a la matriu nuclear espermàtica. En el vas deferent, el mateix tractament SCF produeix dos tipus de trencaments: uns similars als de l'epidídim, i per tant, reversibles, i altres, de mida inferior a la mida toroïdal, no units a la matriu nuclear i, per tant, irreversibles (Yamauchi i col*.,* 2007b; Ribas-Maynou i col*.,* 2013c). Aquests efectes són observats per el Comet SDS-neutre en la mateixa mesura que en els gels de camp polsant, de forma que són resultats concordants amb estudis previs (Yamauchi i col., 2007b; Gawecka i col., 2013). Els resultats han estat recopilats en un model proposat a la Figura 36, esquematitzant els dos tipus de trencaments de cadena doble del DNA, que es diferencien per la seva capacitat d'unió a la matriu nuclear, directament relacionada amb la seva reparació (Suppl.1, Ribas-Maynou i col*.,* 2013b).

La comparació de les observacions esmentades en el model ratolí amb les observades en pacients amb avortaments de repetició suggereix que aquests podrien presentar trencaments del DNA similars als SCF identificats a espermatozoides d'epidídim de ratolí, ja que mostren el mateix perfil tant en Comet neutre com en gels de camp polsant (Ribas-Maynou i col*.,* 2012b). D'altra banda, la fragmentació del DNA evidenciada en mostres de pacients amb varicocele mostra que la degradació podria ser similar a la que es dóna en tractaments amb manganès i calci al vas deferent de ratolí.

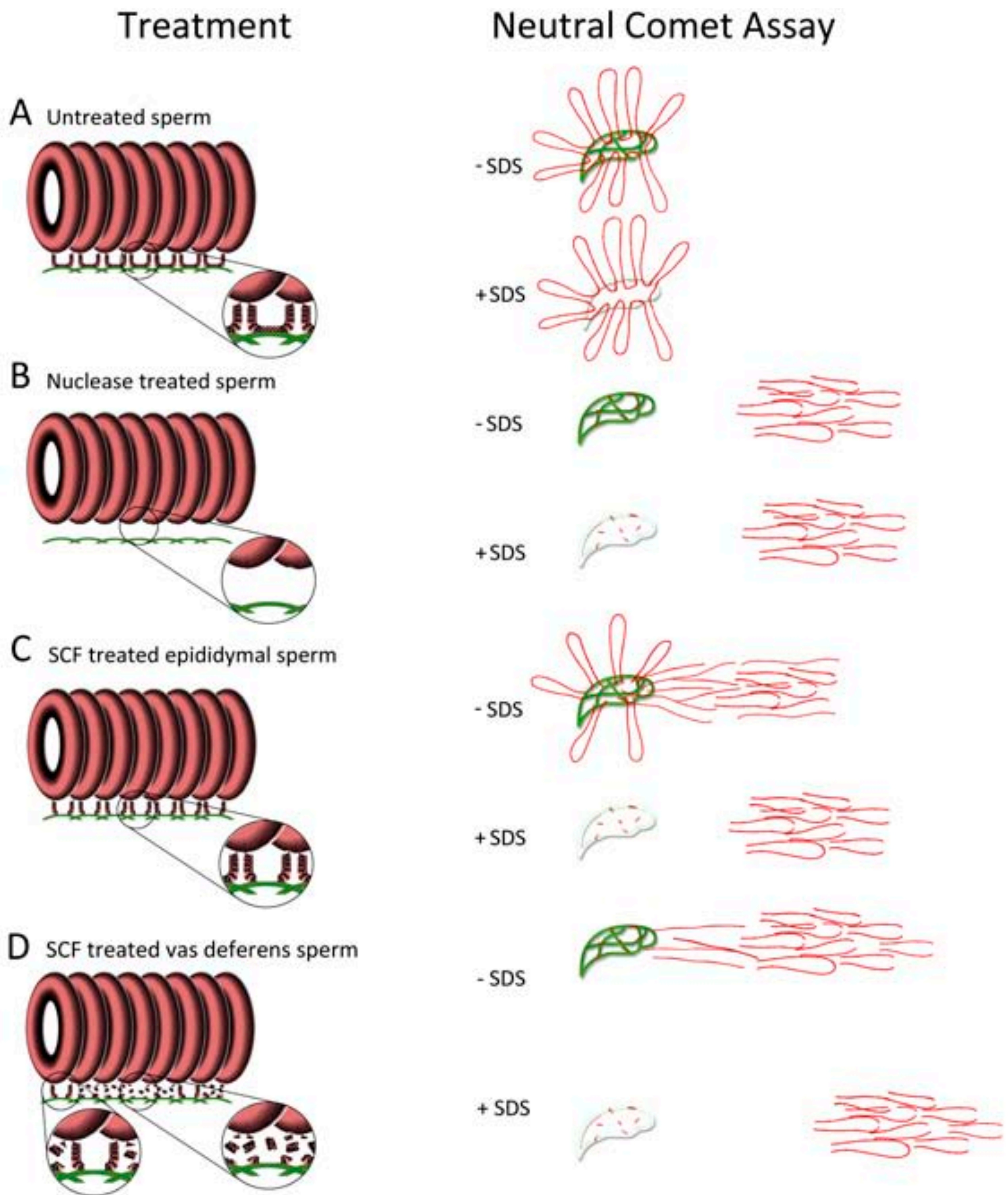


Figura 36. Efecte dels diferents tractaments d'SCF en epidídim i vas deferent sobre el DNA espermàtic i el Comet neutre en presència o absència de SDS (Ribas-Maynou i col., 2013b).

5.9.3 La matriu nuclear pot tenir un paper crític en la reparació dels trencaments de cadena doble del DNA

El fet que només els trencaments de cadena doble del DNA que resten units a la matriu tenen la capacitat de ser relligats *in vitro* permet hipotetitzar que aquests probablement seran reparats per el zigot, necessàriament abans de la primera fase S. En aquest sentit, se sap que la matriu nuclear espermàtica és heretada per el pronucli masculí (Sotolongo i Ward, 2000), i per tant, podria tenir una funció de motlle, mantenint en una estreta proximitat els dos punts del trencament del DNA, possibilitant una correcta reparació. Aquest mecanisme, a més, és similar a un mecanisme descrit recentment en cèl·lules somàtiques, segons el que els trencaments de cadena doble del DNA que no són ràpidament reparats queden segrestats per diferents proteïnes com la Msp3, que els mantenen units a l'embolcall nuclear, permetent una reparació eficient (Gartenberg, 2009; Oza i Peterson, 2010). D'aquesta manera, diferents proteïnes amb una activitat similar a la de la topoisomerasa II/lligasa o bé similar a la recombinació homòloga, com per exemple la SPO11 o la HOP2, funcionals durant l'espermatogènesi, podrien, en l'espermatozoide madur o en el zigot, ser candidats a realitzar aquesta funció (Bates i col*.,* 2011; Borde i de Massy, 2013; Lilienthal i col*.,* 2013; Pezza i col*.,* 2013; Zhao i col*.,* 2013). A més, un estudi recent mostra una colocalització de la topoisomerasa IIβ amb els trencaments al DNA espermàtic (Noblanc i col*.,* 2013) reforçant el model proposat prèviament (Yamauchi i col*.,* 2007b; Ribas-Maynou i col*.,* 2013b).

5.9.4 La inducció de dany de cadena doble mitjançant MnCa i la seva posterior relligació causa trencaments de cadena senzilla del DNA

Mitjançant la ICSI d'espermatozoides sotmesos a la inducció de SCF, s'evidencia una degradació del pronucli masculí i una davallada de la taxa d'implantació (Yamauchi i col*.,* 2007b). Així mateix, la injecció d'espermatozoides amb dany del DNA de cadena doble induït per SCF i posteriorment revertit amb EDTA evidencia el mateix efecte (Yamauchi i col*.,* 2007b). Tot i que s'ha vist que l'EDTA repara els trencaments de cadena doble del DNA, els trencaments de cadena senzilla no varen ser analitzats en aquests estudis. L'anàlisi mitjançant el Comet alcalí d'aquests tractaments de cadena senzilla, posa de manifest un increment de fragmentació de cadena senzilla després de la inducció i la reparació. Un increment d'aquest tipus de dany ha estat prèviament relacionat amb la fallada de l'embaràs en humans (Ribas-Maynou i col*.,* 2013b) fet que aportaria una explicació a la fallada de la ICSI en aquest experiment amb ratolins. Un treball més recent del mateix grup analitza el complement masculí de zigots generats amb espermatozoides tractats amb SCF. Aquest DNA masculí mostra un elevat nombre d'alteracions, probablement causades pels trencaments de cadena senzilla, que impossibiliten la correcta reparació del DNA del zigot, i conseqüentment, la progressió de l'embrió (Gawecka i col*.,* 2013).

5.10 La relació de la compactació i la fragmentació del DNA espermàtic.

5.10.1 La compactació del DNA en l'espermatozoide està relacionada amb els trencaments de cadena senzilla del DNA i no amb els trencaments de cadena doble.

La cromomicina competeix per la unió al DNA amb les protamines, de forma que zones amb una pobre protaminació queden senyalitzades amb fluorescència verda. Aquesta tècnica permet una anàlisi ràpida i efectiva de l'estat de compactació del DNA espermàtic (De Iuliis i col*.,* 2009). Diversos treballs mostren que defectes en la compactació del DNA estan relacionats amb la presència de fragmentació del DNA i amb la infertilitat masculina (Torregrosa i col*.,* 2006; De Iuliis i col*.,* 2009; Aitken i De Iuliis, 2010; Sakkas i Alvarez, 2010; Castillo i col*.,* 2011; García-Peiró i col*.,* 2011a; Sivanarayana i col*.,* 2012).

Els resultats referents a l'objectiu 4 d'aquesta tesi també evidencien un increment significatiu en la incidència d'espermatozoides CMA3 positius en pacients infèrtils respecte de la seva incidència en espermatozoides d'individus fèrtils. A més, s'observa com aquest percentatge s'incrementa amb la presència valors alterats de fragmentació de cadena senzilla del DNA , indicant una relació entre la compactació i la fragmentació del DNA (De Iuliis i col*.,* 2009; Nijs i col*.,* 2009; Castillo i col*.,* 2011; Simon i col*.,* 2011a; Sivanarayana i col*.,* 2012; Salehi i col*.,* 2013). En aquest sentit, s'ha pogut realitzar una caracterització de diferents grups de pacients infèrtils atenent el seu seminograma, observant un augment del percentatge d'espermatozoides CMA3 positius a mesura que s'incrementen les alteracions de seminograma (Article 7, Figura 1).

L'anàlisi estadístic d'aquestes variables mostra una correlació entre la CMA3 i ssSDF, tot i això, no existeix correlació amb dsSDF. L'interès d'aquest resultat rau en el fet que

prèviament s'ha vist que els trencaments de cadena doble del DNA detectats per Comet neutre no correlacionen amb cap altra tècnica d'anàlisi de fragmentació del DNA (Ribas-Maynou i col., 2013b). L'explicació d'aquest fet és que el Comet neutre detecta, com ja s'ha discutit, un dany molt específic situat majoritàriament a les regions MAR, i que a més pot dependre de la unió del DNA amb la matriu nuclear espermàtica (Ribas-Maynou i col., 2012b, 2013c). Donat que les regions MAR, en les que està situat en gran part el dany de cadena doble resten compactades amb histones, és coherent pensar que aquestes regions no estiguin implicades amb la unió de CMA3, ja que aquesta competeix amb els punts d'unió de les protamines al DNA i no d'histones. D'altra banda, el dany de cadena senzilla del DNA es dóna de forma extensiva tant a les regions MAR com en les regions toroïdals (Ribas-Maynou i col., 2012b), així, un increment d'aquest dany pot estar directament relacionat amb una pobre protaminació. En aquest sentit, aquests resultats són coherents amb els obtinguts amb altres tècniques de fragmentació del DNA (De Iuliis i col., 2009; Nijs i col., 2009; Sivanarayana i col., 2012; Salehi i col., 2013).

5.10.2 La compactació del DNA com a predicció de la infertilitat

L'anàlisi ROC tenint en compte la condició clínica de fertilitat o infertilitat de les mostres de semen analitzades resulta en un punt de tall del 44% d'espermatozoides CMA3 positius per predir infertilitat amb una sensibilitat de 0,830 i especificitat de 0,545. Tot i que diversos autors tenen clara la seva relació amb la infertilitat (Nasr-Esfahani i col., 2005; Tarozzi i col., 2009), fins al moment no s'ha descrit un valor predictiu d'infertilitat. Encara que aquest valor predictiu presenta sensibilitat i especificitat inferiors al Comet alcalí, es podria utilitzar el resultat del test de la CMA3, com a mesura orientativa del potencial d'infertilitat.

5.11 Espermatozoides amb una alta degradació nuclear: efecte d'activitat nucleasa sobre espermatozoides amb una pobre compactació del DNA

Diversos estudis demostren l'associació d'espermatozoides amb alta degradació del DNA nuclear amb pacients portadors de reorganitzacions cromosòmiques i amb pacients amb varicocele (Enciso i col*.,* 2006; García-Peiró i col*.,* 2012; Gosálvez i col*.,* 2013b). Els tractaments intensos amb peròxid d'hidrogen i nucleasa, ja prèviament descrits (Enciso i col*.,* 2009; Ribas-Maynou i col*.,* 2012a), produeixen gairebé el 100% de fragmentació del DNA, i la producció d'espermatozoides amb el DNA altament degradat. Tractaments menys agressius no haurien ajudat al objectiu d'aquest estudi, ja que s'hauria produït menys fragmentació al DNA espermàtic, i com a conseqüència, menys espermatozoides degradats.

Un increment significatiu d'espermatozoides degradats en mostres no tractades amb DTT ha estat observat tant amb el tractament amb peròxid d'hidrogen com amb el de nucleasa, tot i això, l'increment major es va obtenir amb incubacions amb nucleasa. A més, afegint prèviament DTT a les mostres, les incubacions amb nucleasa van evidenciar un increment dels espermatozoides degradats respecte dels percentatges detectats en mostres no tractades amb DTT. En canvi, en les mateixes condicions, el tractament amb peròxid d'hidrogen va produir percentatges similars d'espermatozoides degradats. Així, els resultats suggereixen que una compactació nuclear pobre és la condició que causa major susceptibilitat dels espermatozoides a degradar el DNA mitjançant una activitat nucleasa, ja sigui aquesta endògena (Sotolongo i col*.,* 2005) o bé activada per estrès oxidatiu o increment de la temperatura testicular (Sakkas i Alvarez, 2010). De fet, espermatozoides amb pobre compactació del DNA, com són els espermatozoides immadurs (HDS), estan

incrementats en grups de pacients que presenten elevats percentatges d'espermatozoides degradats (Enciso i col*.,* 2006; García-Peiró i col*.,* 2012; Gosálvez i col*.,* 2013b). A més a més, s'ha evidenciat que, en aquest tipus de pacients, existeix una correlació significativa entre una ràtio de protamines P1/P2 alterada i la incidència d'espermatozoides degradats, fet que reforça aquesta hipòtesi.

La relació de les dades de compactació i fragmentació del DNA amb les característiques clíniques de diferents grups de pacients suggereixen el model proposat a la Figura 37. D'una banda, els pacients amb varicocele presenten un increment en la temperatura testicular, fet que causa un increment d'estrès oxidatiu i una pobre compactació del DNA (Love i Kenney, 1999; Baccetti i col*.,* 2002; Paul i col*.,* 2008; Barratt i col*.,* 2010; Björndahl i Kvist, 2010). Llavors, l'estrès oxidatiu podria causar l'activació de nucleases (Sakkas i Alvarez, 2010) que es troben en el nucli pobrament compactat, ocasionant una elevada degradació del DNA.

En pacients portadors de reorganitzacions cromosòmiques, la pròpia reorganització seria causa d'una desestabilització de l'arquitectura del nucli espermàtic, ocasionant una pobre compactació del DNA i alt percentatge d'immaduresa espermàtica (Baccetti i col*.,* 2002, 2003), això possibilitaria l'alta degradació del DNA espermàtic per part de nucleases intrínseques o bé activades per l'estrès oxidatiu generat en les funcions fisiològiques espermàtiques (Sotolongo i col*.,* 2005; Sakkas i Alvarez, 2010). En qualsevol cas, en comparació amb pacients de varicocele, els pacients portadors de reorganitzacions cromosòmiques, presenten en general menys espermatozoides amb DNA degradat ja que la pobra compactació del DNA dependria directament de la presència i tipus de la pròpia reorganització (García-Peiró i col., 2012; Ribas-Maynou i col., 2012a).

Finalment, pacients amb leucocitospèrmia no presenten percentatges incrementats d'espermatozoides degradats tot i presentar un elevat estrès oxidatiu. Aquest fet s'explicaria ja que aquests pacients no solen presentar afectació testicular, i per tant, presenten una bona compactació del DNA, que el manté protegit de l'activitat de les nucleases.

En conclusió, aquells pacients que presentin afectacions testiculars presentaran un DNA espermàtic pobrament compactat, que donarà lloc a una alta degradació del DNA mitjançant una activitat nucleasa (Figura 37).

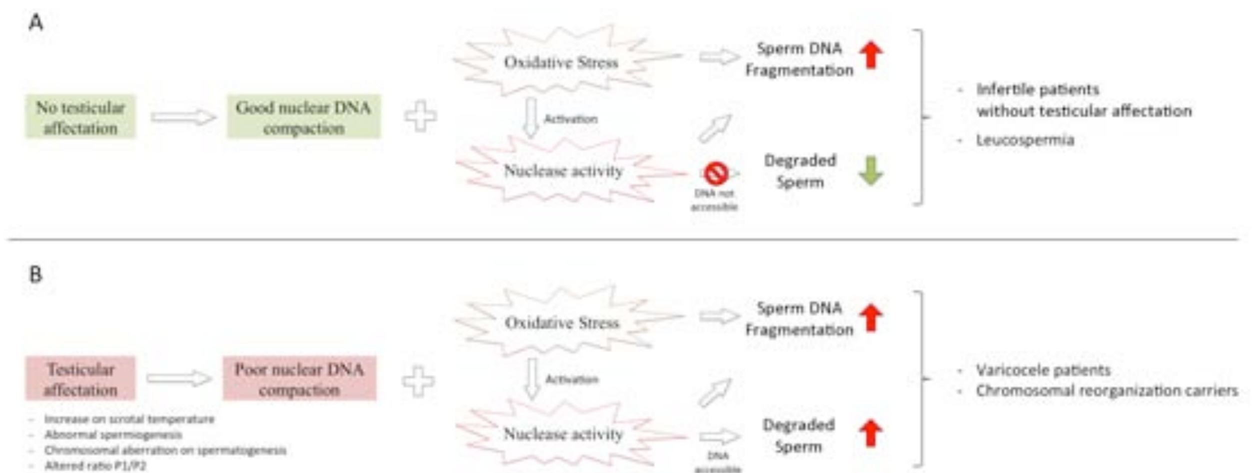


Figura 37. Model que relaciona l'afectació testicular amb la degradació del DNA nuclear espermàtic (Ribas-Maynou i col., 2014, in press).

6. CONCLUSIONS

1. L'assaig Comet alcalí detecta preferentment trencaments de cadena senzilla del DNA i l'assaig Comet neutre detecta preferentment trencaments de cadena doble del DNA.

2. La criopreservació de les mostres de semen mitjançant Test Yolk causa un increment del 10% d'espermatozoides amb fragmentació de cadena senzilla del DNA, i no té efectes en la fragmentació de cadena doble del DNA. Aquest increment descrit ha estat similar per a tots els grups analitzats.

3. Les tècniques SCSA, TUNEL, SCD i Comet alcalí són tècniques vàlides per a la distinció de pacients infèrtils respecte individus fèrtils. A més, les quatre tècniques presenten una bona correlació entre elles. D'altra banda, la tècnica Comet neutre no presenta correlació amb cap de les anteriors metodologies.

4. La tècnica d'anàlisi de la fragmentació del DNA espermàtic que presenta una sensibilitat i especificitat més elevada per predir la capacitat fèrtil és el Comet alcalí. Els trencaments de cadena senzilla estan relacionats amb l'estat de fertilitat masculina.

5. La fragmentació de cadena senzilla del DNA s'incrementa en relació a alteracions dels paràmetres seminals. A més, la presència de varicocele causa una intensificació d'aquest dany. El grup de portadors de reorganitzacions cromosòmiques presenta una gran heterogeneïtat.

6. Els pacients amb avortaments de repetició sense factor femení presenten incrementada la fragmentació de cadena doble del DNA. Aquests trencaments no presenten associació amb la infertilitat, sinó que estan relacionats amb el risc d'avortament associat a factor masculí.

7. Segons la fragmentació de cadena doble del DNA, existeix un subgrup de controls fèrtils amb un elevat risc d'avortament associat al factor masculí.

8. La fragmentació de cadena senzilla del DNA espermàtic correspon a un dany extensiu tant en regions inter-toroïdals com en regions intra-toroïdals. Els trencaments de cadena doble del DNA espermàtic mostren una localització específica a les regions MAR i són presents en una quantitat limitada.

9. Com a mínim, existeixen dos tipus de trencaments de cadena doble del DNA espermàtic, uns amb capacitat de reparació per el fet de mantenir-se units a la matriu nuclear espermàtica i els altres sense aquesta capacitat, degut a que no estan units a la matriu nuclear. La matriu nuclear espermàtica ha de tenir un paper clau en la reparació del DNA espermàtic.

10. Els tractaments d'inducció i reparació del dany a la doble cadena del DNA espermàtic generen trencaments de cadena senzilla del DNA, fet que explicaria la manca d'embaràs evolutiu després d'aquests tractaments en el model murí.

11. La compactació del DNA espermàtic en mostres de semen està relacionada amb la fragmentació de cadena senzilla i no amb la fragmentació de cadena doble del DNA. El test de la cromomicina pot ser una metodologia simple per a la detecció d'infertilitat, tot i que presenta una sensibilitat menor a la tècnica Comet.

12. L'aparició d'espermatozoides amb una alta degradació del DNA nuclear, bàsicament presents en pacients de varicocele i portadors de reorganitzacions, està relacionada amb l'efecte d'una pobra compactació i d'una nucleasa.

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