ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Jordi Benet and Jordi Ribas-Maynou, Departament de Biologia Cellular, Fisiologia i Immunologia. Facultad de Medicina, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain. E-mails: jordi.benet@uab.cat, jordi.ribas@uab.cat

Keywords:
Comet assay, cryopreservation, miscarriage, oxidative stress, sperm DNA fragmentation

Received: 20-Aug-2013
Revised: 8-Oct-2013
Accepted: 15-Oct-2013

doi: 10.1111/j.2047-2927.2013.00158.x

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

[1]J. Ribas-Maynou, [1]A. Fernández-Encinas, [1,2]A. García-Peiró, [3]E. Prada, [4]C. Abad, [5]M. J. Amengual, [1]J. Navarro and [1]J. Benet

[1]Departament de Biologia Cel·lular, Fisiologia i Immunologia, Universitat Autònoma de Barcelona, Bellaterra, Catalunya, Spain, [2]Centro de Infertilidad Masculina y Análisis de Barcelona (CIMAB), Edifici Eureka, PBM5, Parc de Recerca de la UAB (PRUAB), Bellaterra, Spain, [3]Servei de Ginecologia, Hospital Universitari Mútua de Terrassa, Terrassa, Spain, [4]Servei d'Urologia, Corporació Sanitària Parc Taulí, Institut Universitari Parc Taulí – UAB, Sabadell, Spain, and [5]UDIAT, Centre Diagnòstic, Corporació Sanitària Parc Taulí, Institut Universitari Parc Taulí – UAB, Sabadell, Spain

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.*,

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 $\pm$ standard deviation): 124.35 $\pm$ 58.42, 50.95 $\pm$ 9.63 and 8.11 $\pm$ 2.89, respectively, for fertile donors; 122.14 $\pm$ 128.02, 46.5 $\pm$ 17.46 and 4.14 $\pm$ 2.12, respectively, for recurrent miscarriage patients and 61.93 $\pm$ 63.33, 24.54 $\pm$ 13.00 and 2.86 $\pm$ 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

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 ($p < 0.01$).

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.

ACKNOWLEDGEMENTS

This work has been supported by Fondo de Investigación Sanitária, Instituto de Salud Carlos III (PI11/00630), and Generalitat de Catalunya (2009 SGR 1107), and J. Ribas-Maynou has a grant from Generalitat de Catalunya (FI-DGR 2011). We would like to thank Dr. Steve Ward for his exhaustive revision and useful comments on the final manuscript.

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.

REFERENCES

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

Ahmad L, Jalali S, Shami SA, Akram Z, Batool S & Kalsoom O. (2010) Effects of cryopreservation on sperm DNA integrity in normospermic and four categories of infertile males. *Syst Biol Reprod Med* 56, 74–83.

Aitken RJ & De Iuliis GN. (2007) Origins and consequences of DNA damage in male germ cells. *Reprod Biomed Online* 14, 727–733.

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

Aitken RJ & Koppers AJ. (2011) Apoptosis and DNA damage in human spermatozoa. *Asian J Androl* 13, 36–42.

Aitken RJ, De Iuliis GN & McLachlan RI. (2009) Biological and clinical significance of DNA damage in the male germ line. *Int J Androl* 32, 46–56.

Anger JT, Gilbert BR & Goldstein M. (2003) Cryopreservation of sperm: indications, methods and results. *J Urol* 170, 1079–1084.

Benet J, Oliver-Bonet M, Cifuentes P, Templado C & Navarro J. (2005) Segregation of chromosomes in sperm of reciprocal translocation carriers: a review. *Cytogenet Genome Res* 111, 281–290.

Carrell DT. (2008) The clinical implementation of sperm chromosome aneuploidy testing: pitfalls and promises. *J Androl* 29, 124–133.

Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ & Carrell DT. (2006) Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 27, 53–59.

Cooke MS, Evans MD, Dizdaroglu M & Lunec J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* 17, 1195–1214.

Di Santo M, Tarozzi N, Nadalini M & Borini A. (2012) Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. *Adv Urol*, 854837.doi:10.1155/2012/854837.

Donnelly ET, Steele EK, McClure N & Lewis SE. (2001) Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum Reprod* 16, 1191–1199.

Duru NK, Morshedi MS, Schuffner A & Oehninger S. (2001) Cryopreservation-Thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. *J Androl* 22, 646–651.

Egozcue J, Blanco J & Vidal F. (1997) Chromosome studies in human sperm nuclei using fluorescence in-situ hybridization (FISH). *Hum Reprod Update* 3, 441–452.

Enciso M, Sarasa J, Agarwal A, Fernandez JL & Gosálvez J. (2009) A two-tailed Comet assay for assessing DNA damage in spermatozoa. *Reprod Biomed Online* 18, 609–616.

Evenson DP & Wixon R. (2008) Data analysis of two in vivo fertility studies using Sperm Chromatin Structure Assay-derived DNA fragmentation index vs. pregnancy outcome. *Fertil Steril* 90, 1229–1231.

Gandini L, Lombardo F, Lenzi A, Spano M & Dondero F. (2006) Cryopreservation and sperm DNA integrity. *Cell Tissue Bank* 7, 91–98.

Garcia-Peiró A, Oliver-Bonet M, Navarro J, Abad C, Guitart M, Amengual MJ *et al.* (2011) Dynamics of sperm DNA fragmentation in patients carrying structurally rearranged chromosomes. *Int J Androl* 34, 546–553.

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

Gosálvez J, de la Torre J, Lopez-Fernandez C, Perez-Gutierrez L, Ortega L, Caballero P *et al.* (2010) DNA fragmentation dynamics in fresh versus frozen thawed plus gradient-isolated human spermatozoa. *Syst Biol Reprod Med* 56, 27–36.

Host E, Lindenberg S, Kahn JA & Christensen F. (1999) DNA strand breaks in human sperm cells: a comparison between men with normal and oligozoospermic sperm samples. *Acta Obstet Gynecol Scand* 78, 336–339.

Isachenko E, Isachenko V, Katkov II, Rahimi G, Schondorf T, Mallmann P *et al.* (2004) DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Hum Reprod* 19, 932–939.

Jensen JR, Morbeck DE & Coddington CC. (2011) Fertility preservation. *Mayo Clin Proc* 86, 45–49.

Kalthur G, Adiga SK, Upadhya D, Rao S & Kumar P. (2008) Effect of cryopreservation on sperm DNA integrity in patients with teratospermia. *Fertil Steril* 89, 1723–1727.

Lasso JL, Noiles EE, Alvarez JG & Storey BT. (1994) Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation. *J Androl* 15, 255–265.

Lee CY, Lee CT, Wu CH, Hsu CS & Hsu MI. (2012) Kruger strict morphology and post-thaw progressive motility in cryopreserved human spermatozoa. *Andrologia* 44(Suppl. 1), 81–86.

Lewis SE & Simon L. (2010) Clinical implications of sperm DNA damage. *Hum Fertil (Camb)* 13, 201–207.

Maione B, Pittoggi C, Achene L, Lorenzini R & Spadafora C. (1997) Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA. *DNA Cell Biol* 16, 1087–1097.

Makker K, Agarwal A & Sharma R. (2009) Oxidative stress & male infertility. *Indian J Med Res* 129, 357–367.

Martin RH. (2006) Meiotic chromosome abnormalities in human spermatogenesis. *Reprod Toxicol* 22, 142–147.

Mazzilli F, Rossi T, Sabatini L, Pulcinelli FM, Rapone S, Dondero F *et al.* (1995) Human sperm cryopreservation and reactive oxygen species (ROS) production. *Acta Eur Fertil* 26, 145–8.

O'Flaherty C, Vaisheva F, Hales BF, Chan P & Robaire B. (2008) Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. *Hum Reprod* 23, 1044–1052.

de Paula TS, Bertolla RP, Spaine DM, Cunha MA, Schor N & Cedenho AP. (2006) Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia. *Fertil Steril* 86, 597–600.

Ribas-Maynou J, Garcia-Peiro A, Abad C, Amengual MJ, Navarro J & Benet J. (2012a) Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Hum Reprod* 27, 652–658.

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

Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, Abad C, Amengual MJ, Prada E *et al.* (2013) Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology* 5, 715–722.

Rubes J, Selevan SG, Sram RJ, Evenson DP & Perreault SD. (2007) GSTM1 genotype influences the susceptibility of men to sperm DNA damage associated with exposure to air pollution. *Mutat Res* 625, 20–28.

Said TM, Gaglani A & Agarwal A. (2010) Implication of apoptosis in sperm cryoinjury. *Reprod Biomed Online* 21, 456–462.

Sailer BL, Sarkar LJ, Bjordahl JA, Jost LK & Evenson DP. (1997) Effects of heat stress on mouse testicular cells and sperm chromatin structure. *J Androl* 18, 294–301.

Sakkas D & Alvarez JG. (2010) Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 93, 1027–1036.

Sanger WG, Olson JH & Sherman JK. (1992) Semen cryobanking for men with cancer–criteria change. *Fertil Steril* 58, 1024–1027.

Satirapod C, Treetampinich C, Weerakiet S, Wongkularb A, Rattanasiri S & Choktanasiri W. (2012) Comparison of cryopreserved human sperm from solid surface vitrification and standard vapor freezing method: on motility, morphology, vitality and DNA integrity. *Andrologia* 44 (Suppl 1), 786–790.

Sergerie M, Laforest G, Bujan L, Bissonnette F & Bleau G. (2005) Sperm DNA fragmentation: threshold value in male fertility. *Hum Reprod* 20, 3446–3451.

Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A & Agarwal A. (2010) TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology* 76, 1380–1386.

Simon L, Lutton D, McManus J & Lewis SE. (2011) Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil Steril* 95, 652–657.

Sotolongo B, Huang TT, Isenberger E & Ward WS. (2005) An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J Androl* 26, 272–280.

Spano M, Cordelli E, Leter G, Lombardo F, Lenzi A & Gandini L. (1999) Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. *Mol Hum Reprod* 5, 29–37.

Templado C, Vidal F & Estop A. (2011) Aneuploidy in human spermatozoa. *Cytogenet Genome Res* 133, 91–99.

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA & Clark AM. (2009) Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* 24, 2061–2070.

Thomson LK, Fleming SD, Barone K, Zieschang JA & Clark AM. (2010) The effect of repeated freezing and thawing on human sperm DNA fragmentation. *Fertil Steril* 93, 1147–1156.

Velez de la Calle JF, Muller A, Walschaerts M, Clavere JL, Jimenez C, Wittemer C *et al.* (2008) Sperm deoxyribonucleic acid fragmentation as assessed by the sperm chromatin dispersion test in assisted reproductive technology programs: results of a large prospective multicenter study. *Fertil Steril* 90, 1792–1799.

Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A & Ammar Keskes L. (2010) Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. *Fertil Steril* 93, 159–166.

