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

Running Title: Semen sample cryopreservation with Comet assay

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ABSTRACT

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 in that ssDNA is associated with reduced fertility while dsDNA is associated with increased miscarriage. In this study, semen samples from 32 human males have been analyzed in both fresh and cryopreserved using the alkaline and neutral Comet assays and the SCD test. 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 comparing fresh and cryopreserved samples in any of the patient groups. The SCD test demonstrated an increase of 1% of SDF, which is only statistically significant for infertile patients. Our results support previous reports that oxidative stress is the major effector in DNA damage during sample cryopreservation, since we found no effect on dsSDF. Therefore, there would be a slight risk of decreased fertility after thawing, but no evidence for increased miscarriage risk from cryopreserved sperm has been found.

Key words: Cryopreservation, sperm DNA fragmentation, Comet assay, oxidative stress, miscarriage.

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 J 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 to 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 varicocele patients, recurrent miscarriage patients or chromosomal rearrangement carriers can be performed by using methods with higher sensitivity for sperm DNA fragmentation analysis such as the Comet assay (Ribas-Maynou 2013). The etiology of SDF has also been widely discussed, locating the DNA damage at different levels (Aitken & De Iuliis, 2010; Sakkas & Alvarez, 2010): a) at the testicular level, where there can occur apoptosis during spermatogenesis, DNA breaks during spermiogenesis due to 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); b) at the epididymis level, where the DNA damage would be mainly due to oxidative stress; and c) 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 due 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 in order 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 (ROS) (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 (Thomson et al., 2009, Spano et al., 1999; Donnelly et al., 2001; de Paula et al., 2006; Zribi et al., 2010) while 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 (Gosalvez 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 infertile 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 is associated with oxidative stress DNA damage and would be extensively distributed throughout the genome, while dsSDF is associated with an enzymatic activity having acting in a non-extensive manner (Ribas-Maynou et al., 2012b). 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 (Ribas-Maynou et al., 2012a; Enciso et al., 2009). 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 and the SCD test. A secondary objective of this work was to analyze the effect of cryopreservation in different patient groups, taking into account their clinical status.

MATERIAL AND METHODS

Sample collection

Semen samples from 32 human males were obtained by masturbation after an abstinence period of three to seven days. Samples were divided into three clinical groups: Fertile donors (n=12), recurrent miscarriage patients without female factor (RPL) (n=8) and infertile patients (n=12). The total amount of samples successfully analyzed by alkaline and neutral Comet assays was 30, and 30 for SCD test and DDS (Tables I and II). 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 analyzed by using SCA software (Sperm Class Analyzer, Microptic; Spain). Sperm count ($x10^6$ spermatozoa/ml), motility (% A+B) and morphology (% normal forms) for the samples were 124.35 ± 58.42, 50.95 ± 9.63 and 8.11 ± 2.89 (mean ± standard deviation), respectively for fertile donors (n=12), 122.14 ± 128.02, 46.5 ± 17.46 and 4.14 ± 2.12, respectively for recurrent miscarriage patients (n=8), and 38.52 ± 18.79, 33.98 ± 18.14 and 3.83 ± 5.04, respectively for infertile patients (n=12).

Cryopreservation

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) and, after homogenizing, each sample was divided into cryotubes and frozen in isopropanol at -

80ºC overnight. The following day, samples were submerged in liquid nitrogen until they were analyzed.

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 600g for five minutes. Finally, the sperm concentration was adjusted at 1 x 10^6 spermatozoa/mL to assess sperm DNA damage.

Sperm DNA fragmentation analysis

The sperm DNA fragmentation analysis was performed twice: once before cryopreservation and again after thawing, using the alkaline and neutral Comet assays, and the SCD test.

Comet assay

The Comet assay was performed in alkaline or neutral conditions to analyze single stranded DNA fragmentation and double stranded DNA fragmentation, respectively, as previously described (Ribas-Maynou et al., 2012b). Staining was performed using DAPI SlowFade® Gold antifade (Invitrogen; Eugene, OR, USA) and 400 spermatozoa were classified as fragmented or non-fragmented according the criteria reported before (Ribas-Maynou et al., 2012a) using a fluorescence microscope (Olympus AX70). Results were expressed as a percentage of the fragmented spermatozoa (%SDF).

SCD test

The Sperm chromatin dispersion test was performed using the Halosperm kit (Halotech DNA; Madrid, Spain) following the manufacturer's instructions. Samples were stained with propidium iodide, and 400 spermatozoa were classified according to the manufacturer's criteria, using an epifluorescence microscope (Olympus AX70).

Spermatozoa with halos are classified as non-fragmented, spermatozoa with no halos, but with strong staining in the nucleus were classified as fragmented (SDF) and spermatozoa with scarce DNA staining on the core were classified as degraded (DDS). SDF and DDS were expressed as a percentage of the whole (%SDF and %DDS).

Statistical analysis

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

RESULTS

Cryopreservation and sperm DNA fragmentation

The data were classified attending the clinical status of the donors into three groups: fertile donors, recurrent miscarriage without female factor patients, and general infertile patients. The SDF analyzed with SCD test and Comet assay regarding these three groups before and after cryopreservation is shown in Tables I and II.

The results demonstrated that there were slight, but statistically significant differences between fresh and cryopreserved spermatozoa in all groups (Table I). Interestingly, this difference was greater for the fertile donors than for either the infertile males or the males from couples with recurrent pregnancy loss. Overall, there was approximately a 10% increase in ssDNA damage in cryopreserved sperm as measured by the alkaline comet assay. The neutral comet assay did not detect any differences between fresh and cryopreserved samples.

For the SCD test, the only group that had a statistically significant difference was the infertile males for SDF, with roughly the same level of significance as the alkaline comet assay (Table II). Overall, there was a slight, 1%, increase in DNA damage that approached statistical significance ($p= 0.081$) for SCF in the SCD test. For DDS in the SCD test, there were no statistically significant differences. However, the infertile males did approach statistical significance ($p= 0.75$), suggesting that with larger numbers there might be a measureable effect of cryopreservation on infertile males.

DISCUSSION

Sperm DNA fragmentation 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 sperm DNA fragmentation assessment requires studies to approach the actual DNA damage on the cryopreserved sperm. 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 due to the controversy on this topic (Garcia-Peiro 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, and to assess the etiology of the DNA damage (oxidative or enzymatic damage), in this work we performed the analysis through the alkaline and neutral Comet assays, and the SCD test. Results in alkaline Comet showed a statistical increase on sperm DNA fragmentation (Table I and Figure 1), agreeing 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 sperm with single stranded DNA fragmentation is increased on a 10% after cryopreservation (Table I). This would mean that a semen sample would have roughly 10% more spermatozoa that would not be able to result in a pregnancy. Regarding neutral Comet, no differences have been observed between before and after cryopreservation (Table I and Figure 1), showing no effect on double stranded DNA integrity. Until our knowledge, there have not been results using this technique related to cryopreservation, but taking into account that ssSDF has recently been associated to oxidative damage and dsSDF has been associated to nuclease damage (Ribas-Maynou et al., 2013), 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). 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 status were analyzed, all fertile donors, recurrent miscarriage patients and infertile patients showed a statistical increase on alkaline Comet after cryopreservation, but did not show an increase on neutral Comet (Table I). Therefore, it would be necessary to standardize the cryopreservation technique and the comet assay to solve the different results found in the literature (Donnelly et al., 2001; Kalthur et al., 2008; Ahmad et al., 2010).

Regarding SCD test, the increase on sperm DNA fragmentation shows a tendency to signification in the overall semen samples. However, when different clinical groups were analyzed separately, fertile donors and RPL patients did not show a statistical increase after cryopreservation, agreeing with a similar analysis on human fertile donors (Gosalvez et al., 2010). In contrast, infertile patients show a statistical increase in their DNA fragmentation (Table II), being on the order of 1% of SDF (Table II and Figure I), what would not have an implication in clinical assessment. These results do not agree with alkaline Comet assay results, that show an increase in all three groups, however, the higher sensitivity of Comet assay in respect to SCD test on distinguishing the DNA damage would explain these differences (Ribas-Maynou et al., 2012b).

Attending to the DDS analyzed by the SCD test, no statistical increase has been shown in the total group of samples, however, when clinical groups are analyzed, infertile patients showed an increase after cryopreservation with tendency to signification (Table II and Figure 1). This might be explained by the fact that the most samples showed low values of DDS, but a few infertile patients showed high values of DDS, which has been recently associated to a varicocele affectation (Gosalvez et al., 2013). Then, infertile samples that show a higher percentage of DDS might have more susceptible to be damaged after cryopreservation. However, the increase on infertile patients would only be about 0.65% of DDS, which would not have substantial implications on clinical practice, as these DDS might probably be immotile or dead sperm.

Conclusion

In conclusion, the effect of cryopreservation on alkaline Comet assay showed an increase of 10% of ssSDF, while the neutral Comet assay showed no effect after thawing. SCD test of freeze-thawed samples showed an increase of 1% of SDF.

No differential effect has been found attending the clinical status of the sample using Comet assay, however, SCD test showed statistical differences between fertile donors and infertile patients.

Attending previous published works, these results suggest that cryopreservation may affect the pregnancy capacity of the sperm cell, but no affectation should be seen on the miscarriage risk.

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AUTHOR'S ROLES

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

Alba Fernandez-Encinas contributed in experimental procedures.

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

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

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

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FIGURE LEGEND

Figure 1. Sperm DNA fragmentation before and after cryopreservation on alkaline and neutral Comet, SCD test and DDS.

Table I. Percentage of sperm 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=30)	33.75 ± 16.92	43.45 ± 17.06	68.79 ± 21.91	70.64 ± 19.54
p value	0.000 **		0.156	
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	
Infertile patients (n=12)	43.43 ± 13.94	54.89 ± 14.35	62.50 ± 13.64	65.37 ± 12.80
p value	0.010 *		0.272	

* Statistical differences between fresh and cryopreserved sperm, Wilcoxon paired samples test (p< 0.05)

** Statistical differences between fresh and cryopreserved sperm, Wilcoxon paired samples test (p< 0.005)

Table II. Percentage of sperm showing DNA fragmentation through SCD test before and after cryopreservation (mean ± standard deviation).

	Fresh SCD (SDF)	Cryopreserved SCD (SDF)	Fresh DDS	Cryopreserved DDS
Total Samples (n=30)	17.64 ± 9.17	18.46 ± 9.42	3.41 ± 3.25	3.67 ± 3.62
p value	0.081 [a]		0.349	
Fertile Donors (n=12)	11.63 ± 5.85	12.75 ± 5.00	1.47 ± 1.17	1.45 ± 1.15
p value	0.169		0.875	
RPL patients (n=7)	18.06 ± 10.06	17.14 ± 10.53	3.05 ± 2.79	3.00 ± 3.07
p value	0.612		0.865	
Infertile patients (n=11)	23.93 ± 7.65	25.54 ± 8.23	5.75 ± 3.74	6.51 ± 3.97
p value	0.041 *		0.075 [a]	

[a] Tendency to statistical differences between fresh and cryopreserved sperm using Wilcoxon paired samples test ($p < 0.1$)

* Statistical differences between fresh and cryopreserved sperm using Wilcoxon paired samples test ($p < 0.05$)