ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Peter Chan, Department of Urology, McGill University Health Centre, Royal Victoria Hospital, 1001 Boulevard Décarie, Montréal, QC H4A 3J1, Canada.
E-mail: mcgillsperminator@yahoo.com

Keywords:
cryopreservation, DNA breaks, male infertility, sperm chromatin, sperm quality

Received: 25-Jan-2018
Revised: 12-Jun-2018
Accepted: 9-Jul-2018

doi: 10.1111/andr.12529

Effects of different cryopreservation methods on DNA integrity and sperm chromatin quality in men

[1]M. F. Lusignan, [1]X. Li, [2]B. Herrero, [3]G. Delbes and [1,2]P. T. K. Chan

[1]Department of Urology and [2]MUHC Reproductive Centre, McGill University Health Centre, Montreal, QC, Canada, [3]INRS – Institut Armand Frappier, Laval, QC, Canada

ABSTRACT

Background: Cryopreserved human sperm are used in assisted reproductive technology. However, the effect of cryopreservation on sperm DNA integrity is unclear.

Objectives: The objectives of this study were to: (i) determine the impact of semen cryopreservation on human sperm DNA integrity and chromatin structure; (ii) test if parameters obtained from TUNEL and SCSA® correlate; and (iii) verify correlation between sperm motility, morphology and viability with TUNEL and SCSA® parameters.

Materials and methods: Men attending a fertility clinic were recruited and grouped according to their sperm parameters (n = 9/group): normozoospermia, oligoasthenoteratozoospermia and teratozoospermia. Each semen sample was processed as follow: (i) directly frozen at −80 °C; (ii) diluted in Sperm Maintenance Medium, cooled for 30 min at 4 °C and frozen at −80 °C; (iii) diluted in Sperm Maintenance Medium; or (iv) in SpermFreeze. Each mixture from method (iii) and (iv) was then suspended for 30 min in liquid nitrogen vapor and plunged into liquid nitrogen. After at least two months of storage, samples were thawed at room temperature and analyzed for motility and viability, TUNEL and SCSA® assays.

Results: Progressive motility and viability decreased after freeze-thawing. TUNEL scores increased significantly in all samples after freezing-thawing while no significant change in the DNA fragmentation index (DFI) from SCSA® was observed. No change in the percentage high DNA stainability (HDS) was observed in normozoospermic samples; however it was significantly increased in all the methods in oligoasthenoteratozoospermic and in the methods (ii)–(iv) in teratozoospermic samples. The DFI and TUNEL scores correlated significantly with each other and inversely with sperm motility, viability and morphology.

Discussion and conclusion: Cryopreservation seems to be deleterious for the integrity of human sperm DNA and compaction. However, the sperm DFI was not affected during cryopreservation under the various methods of storage tested. Clinicians and investigators should take this information into consideration when using cryopreserved sperm for assisted reproduction.

INTRODUCTION

Sperm cryopreservation is currently the only clinically available technique for preservation of male gametes for subsequent use in assisted reproduction technologies (ART). For example, men and boys at mature ages undergoing cancer therapy or surgical treatments that could impair their fertility status may cryopreserve their spermatozoa before treatment for future use. Despite the advantage of sperm banking, it is well documented that freeze-thawing is harmful for the spermatozoa by causing an important reduction of motility (Smith & Steinberger, 1973) and viability (review in (Nijs & Ombelet, 2001)) and structural damages to the mitochondria and cell membranes, leading to the adverse effects on sperm function (review in (Paoli *et al.*, 2014)).

During freezing, the formation of ice in the aqueous medium in which the spermatozoa are suspended increases the concentration of the solutes in the extracellular environment, which will lead to the dehydration of cell (review in (Paoli *et al.*, 2014)). This process is necessary to protect the cells from the formation of intracellular ice. However, if the extent of dehydration is too severe, and the cell volume reaches approximately 40% of its original size, it could lead to irreversible sperm damage. Then, during thawing, the melting of extracellular ice leads to lower extracellular solutes concentration than the intracellular milieu in spermatozoa. Water then enters back inside the spermatozoa and restores its volume. However, there is a risk of formation of intracellular ice crystals during this phase, which could lead to

irreversible cell damage (review in (Paoli *et al.*, 2014)). Minimizing the formation of intracellular ice crystals is highly important for the survival of spermatozoa after cryopreservation.

Extenders are used to preserve the survival of spermatozoa after freeze-thawing. The composition of these media is variable, but they usually contain a buffer to preserve extracellular pH and osmolarity; sugars as a source of energy for the spermatozoa; antibiotics; and most importantly, a cryoprotectant such as glycerol. Glycerol can pass through sperm membrane and decrease the freezing point of water, thus decreasing the risk of intracellular ice formation during freezing (Hammerstedt *et al.*, 1990; Holt, 2000).

Evaluation of semen quality has gone beyond conventional semen analysis focusing on sperm counts, motility, and morphology. Integrity of sperm DNA is crucial for normal embryo development (Ahmadi & Ng, 1999). Sperm DNA damage is more often observed in infertile men (reviewed in (Barratt *et al.*, 2010)) and may be related to adverse reproductive outcomes such as miscarriage (Robinson *et al.*, 2012). Indeed, it was shown that DNA-damaged spermatozoa can fertilize an oocyte, but the embryonic development is related to the degree of DNA damages (Ahmadi & Ng, 1999). Moreover, sperm DNA breaks are associated with spontaneous recurrent miscarriage (Brahem *et al.*, 2011; ASRM Practice Committee, 2015), a decrease in the pregnancy rate in ART (Benchaib *et al.*, 2003), and blastocyst development following IVF (Seli *et al.*, 2004).

With the growing interest in reproductive medicine on the impact of sperm DNA integrity on reproductive outcomes, especially with assisted reproduction, it is important to evaluate whether cryopreservation could affect sperm DNA integrity. Different assays are used to assess the integrity of sperm chromatin and sperm DNA. Each assay measures different parameters such as sperm DNA strand breaks (TUNEL and COMET assays) (Donnelly *et al.*, 2001a; Ribas-Maynou *et al.*, 2014; Rahiminia *et al.*, 2017), sperm chromatin structure (SCSA® assay), or sperm DNA compaction (CMA3 assay) (Delbes *et al.*, 2010; Rahiminia *et al.*, 2017). The only assay that has a clinical standard protocol is the SCSA®, and this well-known technique gives reliable and repeatable results (Evenson, 2016). The absence of standard protocol in the other techniques and the different parameters measured could lead to the disparity of the results between the studies (Donnelly *et al.*, 2001b; Duru *et al.*, 2001; Paasch *et al.*, 2004; de Paula *et al.*, 2006). Hence, there are values in performing more than one methodology to evaluate the potential correlation of sperm DNA integrity obtained in different tests.

While it was shown that cryopreservation does not affect sperm chromatin measured by SCSA® (Evenson & Jost, 2000), it is unclear whether the cryopreservation has a negative impact on the integrity of the sperm DNA as contradictory results were obtained. For example, it was reported that there was no significant increase of sperm DNA damages, as determined by the TUNEL assay, after freeze-thawing, as compared to fresh samples in fertile and infertile patients (Paasch *et al.*, 2004) or in a pool of spermatozoa from healthy donors (Duru *et al.*, 2001). However, other studies using the TUNEL assay (de Paula *et al.*, 2006) or the COMET assay (Donnelly *et al.*, 2001b) observed that the cryopreservation process induced increased DNA damages after freeze-thawing. It is possible that the different freeze-thawing protocols, methods of storage, and sperm extenders commonly used in laboratories led to such contradictory results. Moreover, the design of the study and the fertility status of the subjects providing the samples (fertile donors or different category of infertile patients) could have an impact on the results.

The objective of this study was to determine the impact of different freezing methods on human sperm DNA and chromatin structure assessed by two complementary assays: the SCSA® and TUNEL. We tested these methods on semen samples from the three most common, well-defined categories of men namely normozoospermic (N), oligoasthenoteratozoospermic (OAT), and teratozoospermic (T).

MATERIALS AND METHODS

Subject recruitment and sperm sample

Semen samples were obtained from a cohort of adult men (22–50 years) recruited at a university-based reproductive center (MUHC RC) based on their semen parameters according to the WHO (2010) criteria (World Health Organization, 2010). This study was approved by the institutional review board (protocol number 15-344-MUHC), and informed consent was obtained from all subjects. As per standard clinical evaluation for male fertility, a semen sample with a standardized 3 days of abstinence was obtained. We recruited nine normozoospermic (27–45 years old), nine oligoasthenoteratozoospermic (33–50 years old), and nine teratozoospermic (22–44 years old) patients (Table 1).

Semen sample analysis

Semen analyses were performed in the crude semen. Sperm concentration and motility were analyzed by computerized semen analysis (SpermVision; Sperm Processor Pvt. Ltd, Garheda, India). The viability of 100 spermatozoa per sample was analyzed using the VitalScreen kit (FertiPro N.V., Beernem, Belgium). The morphology of 100 spermatozoa per sample was evaluated as previously described (World Health Organization, 2010).

Freezing methods

Each sample was divided into four aliquots of 500 μl and processed as follows:

Method 1 (M1), directly frozen in a 1.5 ml plastic microcentrifuge tube with snap cap (VWR International, Ville Mont-Royal, QC, Canada) at −80 °C.

Table 1 Patient's cohort description. Patient's age, sperm parameters, and DNA quality and integrity are described in fresh semen samples for each category of patients. Data presented are means ± SEM

	N	OAT	T
Age (year)	35.2 ± 1.9	38.8 ± 1.7	33.2 ± 2.2
Sperm concentration (million/ml)	97.2 ± 16.5	9.5 ± 1.1**$^{\#\#}$	78.4 ± 10.7
Progressive motility (%)	50.9 ± 4.9	11.1 ± 2.6**$^{\#\#}$	37.4 ± 5.0
Morphology (% NF)	5.56 ± 0.67	1.00 ± 0.24**	2.11 ± 0.26**
Viability (%)	69.9 ± 2.3	43.1 ± 2.8**$^{\#\#}$	69.0 ± 4.1
TUNEL-positive cells (%)	12.5 ± 1.7	40.1 ± 6.2**$^{\#}$	16.6 ± 2.8
DFI (%)	12.3 ± 1.6	40.3 ± 5.7**$^{\#\#}$	16.5 ± 2.4
HDS (%)	9.0 ± 1.4	18.0 ± 2.7*$^{\#}$	8.8 ± 0.7

*$p < 0.01$ or **$p < 0.001$ using one-way ANOVA followed by Tukey's test compared to normozoospermic samples; $^{\#}p < 0.01$ or $^{\#\#}p < 0.001$ using one-way ANOVA followed by Tukey's test compared to teratozoospermic samples.

Method 2 (M2), diluted in Sperm Maintenance Medium (SMM; Irvine Scientific, Santa Ana, CA, USA) with a semen:extender ratio of 3 : 1, cooled for 30 min at 4 °C, and frozen in a 1.5 ml plastic microcentrifuge tube with snap cap at −80 °C.
Method 3 (M3), diluted in SMM at a semen:extender ratio of 3:1, transferred to a 0.5 ml straw (Cryo Bio System-IMV Technologies, L'Aigle, France) that was suspended for 30 min in liquid nitrogen (LN_2) vapor, and then plunged into LN_2.
Method 4 (M4), SpermFreeze TM (FertiPro N.V., Beernem, Belgium) at a semen:extender ratio of 1:0.7, transferred to a 0.5 ml straw that was suspended for 30 min in liquid nitrogen (LN_2) vapor, and then plunged into LN_2.

After at least 2 months of storage, samples were placed on the laboratory bench for thawing at room temperature (at 22 °C) for 10 min and analyzed for sperm motility and viability, and SCSA® and TUNEL assays.

Preparation of spermatozoa for SCSA® and TUNEL assays

Fresh or freeze-thawed spermatozoa were washed in PBS by centrifugation at 500 × *g* for 5 min and resuspended in PBS at 2×10^6 cells/ml.

Sperm chromatin structure assay (SCSA®)

To measure the susceptibility of the sperm nuclear DNA to low pH induced denaturation *in situ*, the SCSA® method was used as previously described (Evenson *et al.*, 2002; Delbes *et al.*, 2013). Sperm sample was analyzed using a MACSQuant Analyser (Miltenyi Biotec, Auburn, CA, USA). WinList software version 5.0 (Verify Software House, Topsham, ME, USA) was used to process raw data. The DNA fragmentation index (DFI; mean red fluorescence/total of red and green fluorescence) was analyzed according to the previously described percentage of cells outside the main population (%DFI) (Evenson & Wixon, 2006). Percentage of high DNA stainability (%HDS), an indication of sperm DNA compaction, was also analyzed as previously described (O'Flaherty *et al.*, 2010). A minimum of 5000 events were analyzed for each sample.

TUNEL assay

The TUNEL assay was used to quantify DNA free 3′-OH ends, as previously described (Delbes *et al.*, 2013). Briefly, 30 µl of PBS washed fresh or thawed spermatozoa was deposited for 45 min at RT on 21-well slides (Teflon Printed Slide 21-well 4 mm diameter, Electron Microscopy Sciences, Hatfield, PA, USA) that were previously coated with Poly-Lysine (Sigma, Oakville, ON, Canada) for 5 min and dried. Sperm samples were fixed for 2 min with cold methanol and were washed in PBS for 5 min. Then, samples were incubated for 45 min at 37 °C with the TUNEL mix (*In Situ* Cell Death Detection Kit, Fluorescein; Roche, Mannheim, Germany) and washed five times in PBS for 3 min, and the slides were mounted using the Vectashield H1000 mounting medium with DAPI (Vector Laboratories, Burlington, ON, Canada). Positive controls were obtained by pre-incubating sperm cell on slide with 210 U/ml deoxyribonuclease I (Sigma) for 20 min at 37 °C; negative controls consisted of sperm cells incubated in the TUNEL mix lacking the terminal deoxynucleotidyl transferase (TdT) enzyme. FITC staining was analyzed under a fluorescent microscope (Axiophot; Zeiss, Toronto, ON, Canada). A minimum of 200 cells were counted for every sample, in duplicate.

Data analysis

Statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad software, La Jolla, CA, USA). Data were tested for normal distribution with the one-sample Kolmogorov–Smirnov test. All data were normally distributed, except where indicated. To adjust for the deviation from normality for data in percentages, a arcsine square root transformation was performed (Bartlett, 1936; Anscombe, 1948). Statistical significance in fresh raw samples (Table 1) has been calculated using one-way ANOVA followed by the Tukey's post hoc test. Impact of the freezing method in each parameter (Figs 1 and 2) was determined using a repeated-measures ANOVA followed by the Tukey's post hoc test. The calculations of correlation coefficients between parameters (Table 2) were tested using the nonparametric Spearman's rank correlation test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of cryopreservation on motility and viability

Three categories of patients, normozoospermic (N), oligoasthenoteratozoospermic (OAT), and teratozoospermic (T), were recruited according to their fresh sperm parameters. Sperm concentration, progressive motility, and morphology of fresh samples are displayed in Table 1. As expected, concentration, progressive motility, and morphology were significantly decreased in the OAT group compared to N samples, and only morphology was affected in the T group compared to N samples. In fresh samples, viability of sperm varied from ~45 to 70% among cohorts and was significantly lower in OAT compared to N and T samples (Table 1).

After freeze-thawing, there was a significant decrease in progressive motility and viability in each of the three categories of semen samples (Fig. 1A and B). M1, that used no extender, seemed to induce the worse outcomes, especially on sperm viability for which it was significantly lower than any other freezing methods, independently of semen category (Fig. 1B).

Effect of cryopreservation on sperm chromatin quality and DNA integrity

Sperm chromatin quality and DNA integrity were assessed in fresh and freeze-thawed sperm samples using SCSA® and TUNEL assays, respectively. Fresh sperm samples in N samples presented with low DFI and TUNEL values (%DFI ≤ 25–27%; % TUNEL-positive ≤ 36%) (ASRM Practice Committee, 2015). Interestingly, whereas fresh T sperm samples showed similar DFI and TUNEL values than N ones, fresh OAT samples presented higher percentages of DFI and TUNEL-positive cells compared to N and T sperm samples (Table 1).

When comparing fresh and frozen sperm samples for each category of patients, no significant difference was observed in the %DFI (Fig. 2A) across all three categories of patients and the four freezing methods. However, when evaluating %HDS, a significant increase was observed in OAT sperm samples after all freeze-thawing methods and in T sperm samples when using freezing methods 2–4 (Fig. 2B). Moreover, in the T sperm samples, the %HDS in M3 and M4 was significantly increased as compared to M1, suggesting these methods induced more effects in sperm compaction. The %TUNEL-positive cells

Figure 1 Effect of different methods of freezing and storage on sperm parameters from N, OAT, and T patients. (A) Percent of sperm progressive motility; (B) Percent of sperm viability. Results represent means ± SEM. Motility data were transformed using the arcsine square root to obtain a normal distribution before analysis. Statistical significance has been calculated using a repeated-measures ANOVA followed by Tukey's test. ***$p < 0.001$ when compared with fresh sample from its own patient category; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ when compared with M1 sample from its own patient category; $+p < 0.05$ when compared with M2 sample from its own patient category.

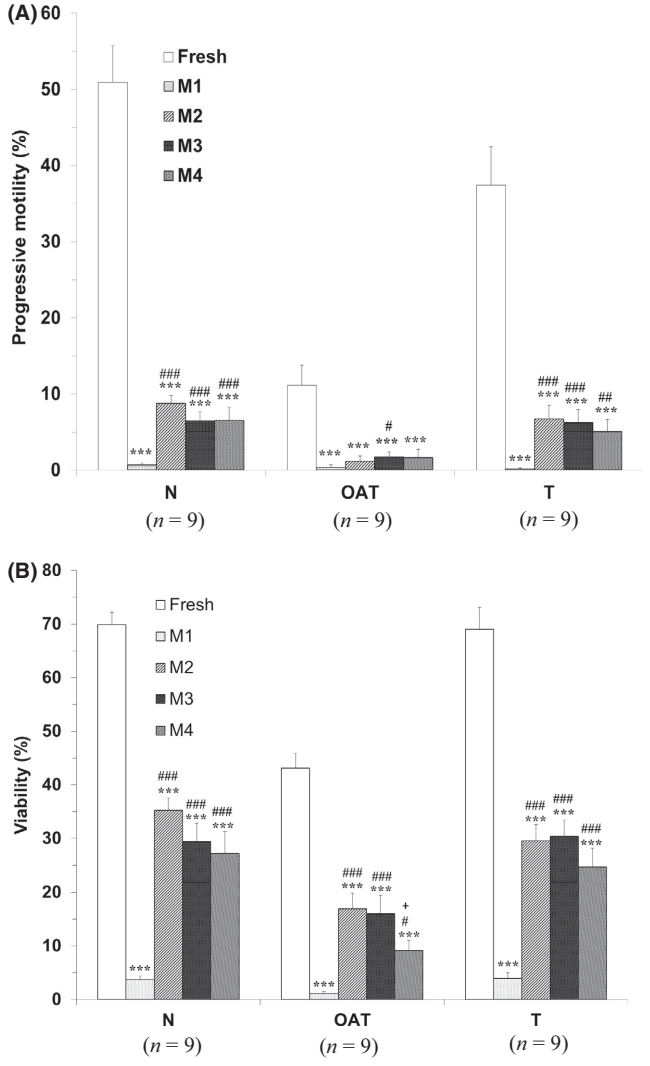
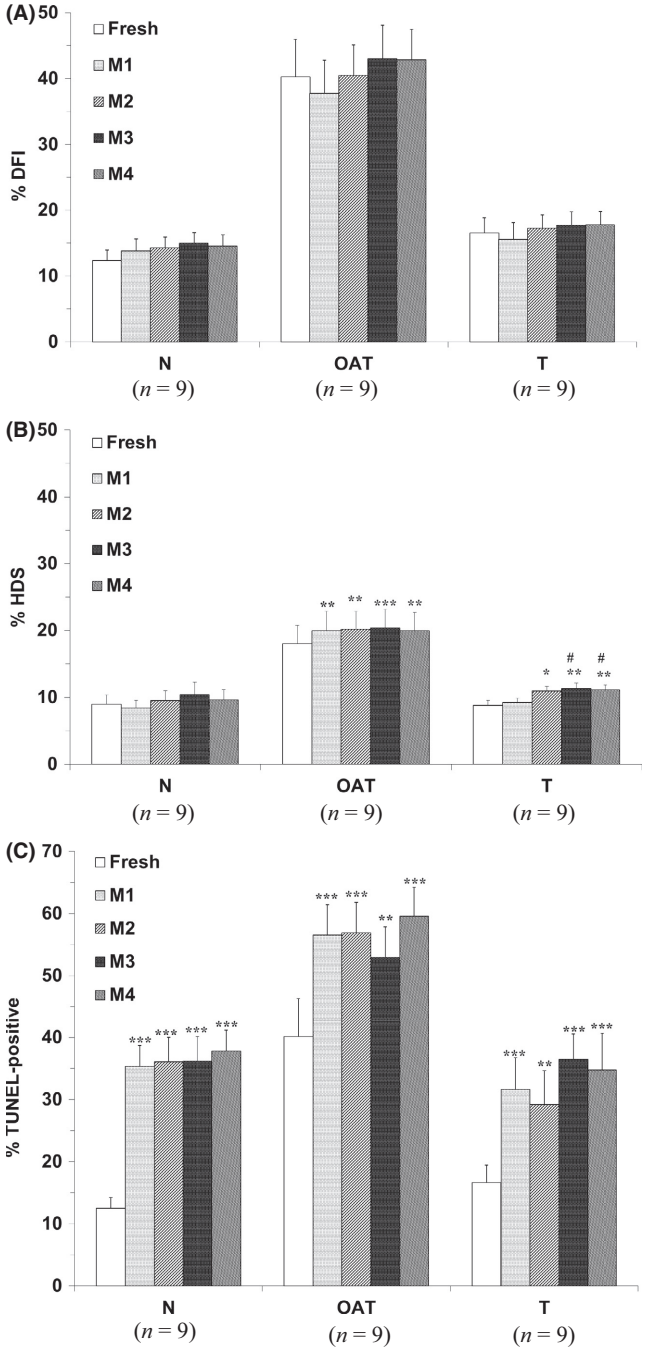


Figure 2 Effect of different methods of freezing and storage on the susceptibility of sperm DNA to acid-induced denaturation (SCSA®) and the DNA strand breaks TUNEL in raw sperm samples from N, OAT, and T patients. (A) Percent of DFI; (B) Percent of HDS; (C) Percent of TUNEL-positive. Results represent means ± SEM. DFI data were transformed using the arcsine square root to obtain a normal distribution before analysis. Statistical significance has been calculated using a repeated-measures ANOVA followed by Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with fresh sample from its own patient category; $^{\#}p < 0.05$ when compared with M1 sample from its own patient category.



increased significantly after freeze-thawing as compared to fresh samples in all three categories of semen samples for all the four freezing methods tested (Fig. 2C). Moreover, there was no significant difference in the freezing methods for each category of patients, suggesting that they all induce DNA damage.

Correlations between sperm parameters and SCSA® and TUNEL assays

All three groups of fresh semen samples were analyzed as one group to evaluate correlation between SCSA parameters, TUNEL assay, and patient's age, sperm motility, morphology, and vitality. No correlation was observed for patient's age (Table 2). However, there was a significant negative correlation in the case of progressive motility, morphology, and viability with % TUNEL-positive or %DFI. As well in our cohort, the %TUNEL-positive spermatozoa positively correlated with %DFI (Table 2).

DISCUSSION

In this study, we evaluated the effect of freezing on the integrity of human sperm chromatin and DNA in men with different semen parameters. The freezing protocols used in this study represent the main principles of various sperm freezing

Table 2 Spearman's correlation coefficients (*r*) of %TUNEL-positive sperm and SCSA® %DFI with age, morphology, progressive motility, and viability in fresh samples for N, OAT, and T samples grouped together ($n = 27$)

	r (*p* value)
%TUNEL-positive	
Age	0.26 ($p = 0.20$)
Motility (%progressive)	−0.79 ($p < 0.0001$)
Morphology (%normal form)	−0.59 ($p = 0.001$)
Viability	−0.81 ($p < 0.0001$)
SCSA® %DFI	0.84 ($p < 0.0001$)
SCSA® %DFI	
Age	0.35 ($p = 0.07$)
Motility (%progressive)	−0.75 ($p < 0.0001$)
Morphology (%normal form)	−0.64 ($p = 0.0003$)
Viability	−0.72 ($p < 0.0001$)

methodologies commonly used clinically and in scientific investigations. Method 1 used no cryoprotectant, while methods 2–4 used several common or commercially available extenders as well as two types of containers. We selected three well-defined and commonly encountered categories of semen parameters: normozoospermic, oligoasthenoteratozoospermic, and teratozoospermic. In addition to conventional semen parameters, we applied two complimentary assays to evaluate the sperm chromatin and DNA quality. SCSA® was used to evaluate sperm chromatin integrity and TUNEL for the DNA integrity. Our results demonstrate that none of the freezing and storage methods used in this study was superior in preserving both the chromatin and DNA quality of the human spermatozoa. In the absence of cryoprotectant (M1), the impact of cryopreservation was the most severe, with motility and viability of the sperm almost completely lost after freezing.

In our study, we compared SMM and SpermFreeze™, two commercial extenders used for human semen cryopreservation. Both media are HEPES-based buffer containing glycerol. It is well established in the literature (review in (Paoli *et al.*, 2014)) that cryopreservation induces an inevitable decrease of sperm motility and viability in spite of the use of extender, consistent with our findings in the current study. Glycerol is a cryoprotectant that passes through sperm membrane and decreases the freezing point of water, thus decreasing the risk of intracellular ice formation during freezing (Hammerstedt *et al.*, 1990; Holt, 2000). Ice crystals are harmful to the spermatozoa by causing membrane break and impairment of organelle function, leading to a decrease of sperm survival (Di Santo *et al.*, 2012). As shown in our results, the absence of an extender (M1) had more deleterious effect on the cell survival for every category of semen samples tested, than when extenders were used, demonstrating the importance of an extender in the maintenance of sperm basic parameters. However, SMM and SpermFreeze™ were equivalent regarding the preservation of motility and viability.

Our results were in agreement with the previous studies in which the freezing and thawing did not have a significant impact on sperm chromatin quality. According to Evenson & Jost (2000), SCSA® data do not significantly change after one time freeze-thawing as compared to the one from fresh samples. Here, the presence or absence of an extender and the type of storage (freezer or liquid nitrogen) did not affect the %DFI in the three categories of sperm samples. However, cryopreservation induced a lower DNA compaction in the OAT and T patients as measure with the %HDS. As men with subnormal semen parameters such as OAT or T may undergo assisted reproduction which may require the use of cryopreserved spermatozoa, clinicians should thus be aware of the potential negative impacts of cryopreservation, as we reported in this study, on the chromatin integrity of sperm.

Interestingly, DNA breaks were significantly increased after freeze-thawing in all categories of patients, as demonstrated by the TUNEL assay. Carrell *et al.* (2003) obtained similar results where they analyzed TUNEL reactive spermatozoa from eight fertile donors before and after cryopreservation in liquid nitrogen, the percentage of TUNEL-positive cells being significantly increased as compared to fresh sample in each donor. Zribi *et al.* (2010) also observed an increase in DNA damage, as shown by TUNEL assay, in both normal and abnormal semen samples after cryopreservation. This suggests that freeze-thawing has a negative impact on DNA quality, independently of the methods of freezing, and TUNEL assay is sensitive enough to detect such damage on sperm DNA quality. The difference in results obtained after freeze-thawing with SCSA® and TUNEL could be due to the fact that these two assays measure different parameters of sperm DNA damage (Delbes *et al.*, 2010). SCSA® requires a step of denaturation before detecting potential DNA damage, following the principle that abnormal DNA is more sensitive to fragmentation by acid denaturation than normal DNA. In TUNEL, the TdT enzyme incorporates biotinylated deoxyuridine triphosphate (dUTP) at the 3′-OH ends found at single- or double-strand DNA breaks within the sperm DNA, making it a more direct assay than SCSA® (Bach & Schlegel, 2016). We have further considered if the differences we observed between SCSA® and TUNEL could be due to the fact that according to the protocol of TUNEL assay, samples are to be incubated for 45 min and that this period of incubation created artifactual changes on sperm chromatin integrity. For this concern, we have conducted a series of experiments using multiple semen samples ($n = 9$, sperm concentration range 18–109.2 M/ml, motility range 3.66–61.57%) that were fixed (i) using the same protocol as described in this paper or (ii) cold methanol was directly added inside the tube containing spermatozoa (no incubation time before fixation) and kept in cold for 10 min before being removed by centrifugation. Sperm pellet was diluted in PBS before being put on the TUNEL slide. We observed that there was no significant difference between the %TUNEL-positive cells obtained whether the same samples were fixed in the tube immediately after thawing or with fixation after 45 min at RT on the slide ($32.3 \pm 5.1\%$ and $37.3 \pm 5.4\%$, respectively, $p = 0.5$). We believe that the observed difference between %DFI and %TUNEL-positive after freezing is real and not caused by the 45-min incubation in the TUNEL protocol (results not shown).

The type of storage is important to preserve sperm quality. It is recommended for long-term storage that samples must stay at a stable temperature; otherwise, a frequent rise and fall of temperature observed in, for example, a refrigerator-freezer that have an automatic defroster could induce damage to the chromatin structure and result in artifacts in the SCSA® results (Evenson & Jost, 2000). We did not observe significant differences in the percentage of damaged sperm in each category of infertile men between the two types of storage used in our study, namely liquid nitrogen or with a −80 °C freezer. We arbitrarily chose a freezing duration of 2 months in this study. To our

knowledge, there are no data to indicate impact on the quality of spermatozoa with longer duration of storage on cryopreserved sperm sample.

Despite the fact that SCSA® and TUNEL measure different aspects of sperm quality, some investigators reported a positive correlation between the results from the two assays, with the coefficient of correlation varies from moderate (r = 0.31) (Stahl *et al.*, 2015) to high correlation (r = 0.87) (Gorczyca *et al.*, 1993) and (r = 0.90) (Chohan *et al.*, 2006) in studies comparing data from fresh vs. frozen samples together or just among frozen samples. In the present study, we obtained a high Spearman's correlation coefficient between SCSA® and TUNEL when we compared results from fresh samples. The variation of the correlation coefficient between the studies may result from the design of the study (the type and number of patients) and the use of different protocols for each assay, especially in the case of the TUNEL assay where no standard clinical protocol exists. Moreover, it was shown that the values obtained when measuring TUNEL-positive cells manually by fluorescence microscopy were lower than the one obtained using flow cytometry analysis (reviewed in (Muratori *et al.*, 2010)).

We also observed a negative correlation between sperm motility, morphology, and viability and the %DFI and %TUNEL-positive in fresh samples. It was previously demonstrated that abnormal morphology, motility, and viability are associated with altered chromatin structure and damaged DNA (Sills *et al.*, 2004; Cohen-Bacrie *et al.*, 2009). However, the mechanisms by which sperm basic parameters are linked to DNA damage and chromatin structure are still unknown.

Paternal aging has been shown to correlate with sperm DNA breaks (Belloc *et al.*, 2014; Carlini *et al.*, 2017). In our study, we did not find any correlation between sperm chromatin structure and DNA damage with age. However, our patients were not selected based on their age in this study and we have a relatively narrow range of age (22–50 years) reflecting the reproductive age of subjects seeking fertility assessment during the study period.

In summary, we demonstrated that sperm cryopreservation negatively affects sperm DNA integrity and HDS, but not the DFI, independently of the type of sperm cryopreservation method evaluated. Moreover, SCSA® and TUNEL data positively correlate in fresh samples. Further, sperm integrity has a negative correlation to sperm basic parameters (motility, morphology, and viability) of the patients. Clinicians and investigators using these assays to evaluate the sperm quality should be aware of the potential impact of cryopreservation and the choice of assays on their results.

ACKNOWLEDGMENTS

This study was funded by grant MOP86636 from the Canadian Institutes for Health Research. We thank Lorraine Lavigne (RN, McGill University Health Center) and Josée Lefebvre (BSc, McGill University Health Center) for their help in this study.

DISCLOSURES

All authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors have made a substantial contribution to the research design, information, or material submitted for this publication. MFL and XL performed the experimental work. All authors contributed to the analysis of data. MFL drafted the paper, and all authors revised it critically and approved the final version.

REFERENCES

Ahmadi A & Ng SC. (1999) Fertilizing ability of DNA-damaged spermatozoa. *J Exp Zool* 284, 696–704.

Anscombe FJ. (1948) The transformation of Poisson, binomial, and negative binomial data. *Biometrika* 35, 195–199.

ASRM Practice Committee. (2015) Diagnostic evaluation of the infertile male: a committee opinion. *Fertil Steril* 103, e18–e25.

Bach PV & Schlegel PN. (2016) Sperm DNA damage and its role in IVF and ICSI. *Basic Clin Androl* 26, 15.

Barratt CL, Aitken RJ, Bjorndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L, Robaire B, Ward S & Zini A. (2010) Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications–a position report. *Hum Reprod* 25, 824–838.

Bartlett MS. (1936) The square root transformation in analysis of variance. *J Royal Statis Soc Suppl* 3, 68–78.

Belloc S, Benkhalifa M, Cohen-Bacrie M, Dalleac A, Amar E & Zini A. (2014) Sperm deoxyribonucleic acid damage in normozoospermic men is related to age and sperm progressive motility. *Fertil Steril* 101, 1588–1593.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H & Guerin JF. (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod* 18, 1023–1028.

Brahem S, Mehdi M, Landolsi H, Mougou S, Elghezal H & Saad A. (2011) Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. *Urology* 78, 792–796.

Carlini T, Paoli D, Pelloni M, Faja F, Dal Lago A, Lombardo F, Lenzi A & Gandini L. (2017) Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss. *Reprod Biomed Online* 34, 58–65.

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

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.

Cohen-Bacrie P, Belloc S, Menezo YJ, Clement P, Hamidi J & Benkhalifa M. (2009) Correlation between DNA damage and sperm parameters: a prospective study of 1,633 patients. *Fertil Steril* 91, 1801–1805.

Delbes G, Hales BF & Robaire B. (2010) Toxicants and human sperm chromatin integrity. *Mol Hum Reprod* 16, 14–22.

Delbes G, Herrero MB, Troeung ET & Chan PT. (2013) The use of complimentary assays to evaluate the enrichment of human sperm quality in asthenoteratozoospermic and teratozoospermic samples processed with Annexin-V magnetic activated cell sorting. *Andrology* 1, 698–706.

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* 2012, 854837.

Donnelly ET, McClure N & Lewis SE. (2001a) Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. *Fertil Steril* 76, 892–900.

Donnelly ET, Steele EK, McClure N & Lewis SE. (2001b) 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.

Evenson DP. (2016) The Sperm Chromatin Structure Assay (SCSA((R))) and other sperm DNA fragmentation tests for evaluation of sperm

nuclear DNA integrity as related to fertility. *Anim Reprod Sci* 169, 56–75.
Evenson D & Jost L. (2000) Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci* 22, 169–189.
Evenson DP & Wixon R. (2006) Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* 65, 979–991.
Evenson DP, Larson KL & Jost LK. (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 23, 25–43.
Gorczyca W, Traganos F, Jesionowska H & Darzynkiewicz Z. (1993) Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Exp Cell Res* 207, 202–205.
Hammerstedt RH, Graham JK & Nolan JP. (1990) Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl* 11, 73–88.
Holt WV. (2000) Basic aspects of frozen storage of semen. *Anim Reprod Sci* 62, 3–22.
Muratori M, Tamburrino L, Marchiani S, Guido C, Forti G & Baldi E. (2010) Critical aspects of detection of sperm DNA fragmentation by TUNEL/flow cytometry. *Sys Biol Reprod Med* 56, 277–285.
Nijs M & Ombelet W. (2001) Cryopreservation of human sperm. *Hum Fert (Camb)* 4, 158–163.
O'Flaherty C, Hales BF, Chan P & Robaire B. (2010) Impact of chemotherapeutics and advanced testicular cancer or Hodgkin lymphoma on sperm deoxyribonucleic acid integrity. *Fertil Steril* 94, 1374–1379.
Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ Jr, Glander HJ & Agarwal A. (2004) Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* 71, 1828–1837.
Paoli D, Lombardo F, Lenzi A & Gandini L. (2014) Sperm cryopreservation: effects on chromatin structure. In: *Genetic Damage in Human Spermatozoa* (eds. E Baldi & M Muratori), pp. 137–150. Springer, New York.
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.
Rahiminia T, Hosseini A, Anvari M, Ghasemi-Esmailabad S & Talebi AR. (2017) Modern human sperm freezing: effect on DNA, chromatin and acrosome integrity. *Taiwan J Obstet Gynecol* 56, 472–476.
Ribas-Maynou J, Fernandez-Encinas A, Garcia-Peiro A, Prada E, Abad C, Amengual MJ, Navarro J & Benet J. (2014) Human semen cryopreservation: a sperm DNA fragmentation study with alkaline and neutral Comet assay. *Andrology* 2, 83–87.
Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J & Coomarasamy A. (2012) The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod* 27, 2908–2917.
Seli E, Gardner DK, Schoolcraft WB, Moffatt O & Sakkas D. (2004) Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* 82, 378–383.
Sills ES, Fryman JT, Perloe M, Michels KB & Tucker MJ. (2004) Chromatin fluorescence characteristics and standard semen analysis parameters: correlations observed in andrology testing among 136 males referred for infertility evaluation. *J Obstet Gynaecol* 24, 74–77.
Smith KD & Steinberger E. (1973) Survival of spermatozoa in a human sperm bank. Effects of long-term storage in liquid nitrogen. *JAMA* 223, 774–777.
Stahl PJ, Cogan C, Mehta A, Bolyakov A, Paduch DA & Goldstein M. (2015) Concordance among sperm deoxyribonucleic acid integrity assays and semen parameters. *Fertil Steril* 104, 56–61. e51
World Health Organization. (2010) *WHO Laboratory Manual for the Examination and Processing of Human Semen*. Department of Reproductive Health and Research, Geneva, Switzerland.
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.