The effect of two cryopreservation methods on human sperm DNA damage

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Abstract

Several methods are currently available for selection when conducting sperm cryopreservation, however, these methods might cause different degrees of damage on sperm DNA. The aim of the this study is to compare the effects of storage at -80 °C (in ultra-low temperature refrigerator) and

at -196 °C (in liquid nitrogen) on sperm DNA damage, thus to provide a reference for choosing the right method according to different aims. We randomly collected 28 semen samples from college students of Chongqing city. The samples stored at -80 °C were neat semen samples and the samples stored at -196 °C were mixed with additional cryoprotectants. Each sample was subjected to two freezing-thawing cycles, and the sperm DNA damage levels of fresh and thawed samples were measured by single cell gel electrophoresis (SCGE) and sperm chromatin structure assay(SCSA). Both SCGE and SCSA assays showed cryopreservation induced significant damage to sperm DNA. However, storage at -196 °C lead to more severe damage to sperm DNA than storage at -80 °C measured by SCSA. Sperm DNA damage increased simultaneously with the higher frequency of freezing-thawing cycles. We concluded that storage of neat semen samples at -80 °C had milder damage to sperm DNA than storage at -196 °C mixed with cryoprotectants. To avoid additional sperm DNA damage, repeated freezing and thawing should be prevented.

Keywords

DNA damage; cryopreservation; SCSA; SCGE; sperm

Abbreviations:

ART: assisted reproduction technique

ROS: reactive oxygen species

VCL: curvilinear velocity

VSL: straight-line velocity

VAP: average path velocity

45 BCF: beat cross-frequency
46 ALH: amplitude of lateral head displacement
47 LIN: linearity
48 WOB: wobble of the curvilinear trajectory
49 STR: straightness
50 PBS: phosphate buffer saline
51 SCGE: single-cell gel electrophoresis assay
52 DFI: DNA fragmentation index
53 %Tail DNA: percent tail DNA
54 SCSA: sperm chromatin structure assay

55

56 **Introduction**

57 Sperm cryopreservation is necessary in Andrology laboratory, either for assisted reproduction
58 technique (ART) or for other assessments. Traditional sperm cryopreservation for neat sample
59 without cryoprotectant is generally used for molecular biology studies such as DNA, RNA, and
60 protein assays but it is harmful to sperm motility and viability. To keep sperm viability and motility
61 for ART purpose, storage of human spermatozoa by cryopreservation has been introduced since
62 1960[1, 2]. Nowadays, many cryopreservation methods and cryoprotectant have been established
63 and played important roles in ART area. However, cryoprotectants were also found to increase
64 sperm ROS, caue extra DNA damage[3, 4], and might cause ultra gene-loads for offspring.

65 When conducting large-scale epidemiological study, as the limitation of time and experimental
66 conditions, many assessments, including sperm DNA damage assay, can not be done immediately

for fresh semen samples. Therefore, it is necessary to cryopreserve the samples and perform the assessment lately. However, many cryopreservation protocols might cause ultra damage to sperm compared with fresh sample[5]. The objective of this study is to investigate the adverse effects of cryopreserve methods and subsequent freezing-thawing procedure on DNA damage of semen samples. We evaluated the effect of two storage methods, neat sample stored in -80 °C and semen mixed with cryoprotectant stored in -196 °C, and the effect of repeated freezing-thawing on sperm DNA damage. We hope our research may provide some practical suggestions on semen sample storage when conducting large-scale epidemiological investigation.

Materials and Methods

Sample collection and semen analysis

The study proposal was reviewed and approved by the Ethical Committee of the Third Military Medical University. 28 semen samples were selected randomly from participants in an epidemiological study(MARHCS) (Yang, 2015), with written consent provided. The semen samples were obtained by masturbating after abstinence for 2-7 days, collected into disposable sterile, preweighed, wide-mouth plastic container, and allowed to liquefy at 37 °C before the routine semen analysis.

5 µl of the liquefied semen sample was pipeted to conducted routine analysis. Semen parameters including appearance, viscosity, liquefaction time, pH value, semen volume, sperm concentration and percent motility, were measured according to WHO 2010 guidelines(5th edition, WHO, Switzerland). Sperm motility parameters including curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), beat cross-frequency (BCF), amplitude of lateral head

displacement (ALH), linearity ($\text{LIN} = \text{VSL}/\text{VCL} \times 100$), wobble of the curvilinear trajectory ($\text{WOB} = \text{VAP}/\text{VCL} \times 100$) and straightness ($\text{STR} = \text{VSL}/\text{VCL}$) were recorded or calculated by computer-aided sperm analysis software (SCA CASA System; Microptic S.L., Barcelona, Spain). Smears of the semen were prepared, air-dried and were stained with a Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for sperm morphology assessment.

Cryopreservation

600 μl of liquefied semen sample was divided equally into three aliquots, one aliquot was used to perform sperm DNA damage assessment immediately, the other two aliquots were stored at -80°C or -196°C respectively.

-80°C (Ultra-low temperature refrigerator) storage: One aliquot containing 200 μl semen sample was transferred to 1 ml cryovials, and stored at -20°C for 8h, then transferred to -80°C for storage. After two weeks of storage, the cryovials were thawed at 37°C water bath for 1 minute, then, 100 μl semen was used for SCGE and SCSA assays, the remaining semen sample was stored in -80°C refrigerator again. Two weeks later, the second thawing and analysis procedure was conducted according to the same protocol as the first cycle.

-196°C (Liquid nitrogen) storage: 200 μl semen was transferred to 1 ml cryovials and mixed with 100 μl (2:1 v/v) test-yolk buffer (14% glycerol, 30% egg yolk, 1.98% glucose and 1.72% sodium citrate, pH7.5). After homogenizing at room temperature for 10 minutes, the cryovials were transferred to -20°C for 8h, then transferred to -80°C for another 8h, and finally transferred to Liquid Nitrogen. Two weeks later, the cryovials were thawed at 37°C water bath for 1 minute, then, 100 μl semen was used for SCGE and SCSA assays, the remaining semen sample was stored again

in -20 °C for 8h, and then -80 °C for 8 h, then was transferred to liquid nitrogen. Another two weeks later, the second thawing and analysis procedure was conducted according to the same protocol as the first cycle.

Sperm DNA damage assessment

SCGE assay: The sperm DNA damage was evaluated using a modified alkaline single-cell gel electrophoresis assay(SCGE)[6-9]. 20 µl of fresh or thawed semen sample was diluted with phosphate buffer saline (PBS) to final concentration of $4 \times 10^6 \text{ ml}^{-1}$, 40 µl of suspension was mixed with 200 µl 0.6% low melting point agarose gel (Sigma-Aldrich Co., MO, USA), and then the mixture was spreaded on the 1% normal melting point agarose gel layer which previously laid on the slide, coverslipped cooling at 4°C for 15 minutes. Slides were submersed in 4°C lysing solution ($2.5 \text{ mol l}^{-1} \text{ NaCl}$, $100 \text{ mmol l}^{-1} \text{ EDTA}$ disodium salt, $10 \text{ mmol l}^{-1} \text{ Tris-base}$ with pH adjusted to 10; 10% Dimethyl sulfoxide and 1% Triton-X100) for 1 hour after removed the coverslips. This step mainly allowed for dissolution of the cell membrane so that the sperm chromatin could be accessed by the next steps. The slides were then transferred for enzyme treatment ($2.5 \text{ mol l}^{-1} \text{ NaCl}$, $100 \text{ mmol l}^{-1} \text{ EDTA}$ disodium salt with pH adjusted to 7.4; 10% Dimethyl sulfoxide and 1% DNase-free proteinase K with final concentration was $100 \mu\text{g ml}^{-1}$) for 16 h at 37°C. This step was crucial to decondense of sperm chromatin and allow migration of DNA fragmentation from the nucleus. Slides were then immersed in alkaline electrophoresis solution($300 \text{ mol l}^{-1} \text{ NaCl}$, $1 \text{ mmol l}^{-1} \text{ EDTA}$ disodium salt with pH adjusted to 13) to unwinding for 20mins before being electrophoresed at 4 °C for 8min, 22V (0.714v/cm) and 160mA. The slides were then transferred in neutralize solution($400 \text{ mmol l}^{-1} \text{ Tris}$ with pH adjusted to 7.5) for 10 mins and fixed with ethanol for 10mins.

The resulting slides were air-dried and subsequently stained with ethidium bromide($20\text{ }\mu\text{g ml}^{-1}$), then examined with an inverted fluorescence microscope (ECLIPSE TE2000-S, Nikon Corporation, Japan) at 200x magnification. Parameters of DNA damage were obtained in Casp software[10](<http://casplab.com/>, version1.2.2).

SCSA: We used the SCSA protocol described by Evenson[11] , with minor modification. This protocol is one of the most repeatable and standardized protocols for sperm DNA analysis. $20\text{ }\mu\text{L}$ of fresh or thawed semen samples were diluted with TNE buffer ($0.15\text{ mol l}^{-1}\text{ NaCl}$, $0.01\text{ mol l}^{-1}\text{ Tris-HCl}$, $1\text{ mmol l}^{-1}\text{ EDTA}$, pH7.4) at a final sperm concentration of $2\times 10^6\text{ ml}^{-1}$. $50\text{ }\mu\text{l}$ sperm suspension was mixed with $100\text{ }\mu\text{l}$ of acid detergent solution(0.1% Triton X-100, $0.15\text{ mol l}^{-1}\text{ NaCl}$, 0.08N HCl , pH1.2), vortexed for 30s, the cells were then stained with $300\text{ }\mu\text{l}$ 6 mg l^{-1} chromatographically-purified AO solution(0.1 mol l^{-1} citric acid, $0.2\text{ mol l}^{-1}\text{ Na}_2\text{HPO}_4$, $1\text{ mmol l}^{-1}\text{ EDTA}$, $0.15\text{ mol l}^{-1}\text{ NaCl}$, pH6.0). Cells were analyzed by flow cytometer FC500 (Beckman Coulter Inc, Brea, USA) at a flow rate of $\sim 200\text{ cell s}^{-1}$. AO intercalated to double-stranded DNA fluoresced green($530\pm 30\text{ nm}$) and associated with single-stranded DNA fluoresced red ($>630\text{ nm}$). About 10000 cells were measured for each sample and all measurements were finished within 5mins after AO staining. The extent of DNA denature ability was expressed as the DNA fragmentation index (DFI), Which was the ratio of the fluorescent intensity of red to the sum of red and green.

Statistical analysis

Statistical analysis was performed with SPSS v20 software(IBM Inc., Chicago, USA). Data were presented as mean $\pm$ SD or median and interquartile range, a normality test was performed using the Shapiro-Wilk test. The comparisons between related samples were performed using

paired t test, Wilcoxon Signed-rank test, or Friedman nonparametric repeated-measure tests as appropriate. Bivariate correlation analysis were performed using spearman correlation or pearson correlation analysis. *P* value of < 0.05 was considered statistically significant.

Results

Basic characteristics and semen parameters

The mean age of the 28 subjects was 22.90 ± 1.08 , the mean duration of sexual abstinence was 4.25 ± 1.62 days. The semen parameters, including volume, concentration, total count, normal morphology, motility, and motility parameters, such as VAP, VSL, VCL , BCF etc., were shown in **Table 1**.

Comparison of the effects of two methods of cryopreservation on sperm DNA damage:

Like the other studies, we used percent tail DNA (%Tail DNA) as the standard to evaluate the DNA damage levels in SCGE, and used DNA fragmentation index (DFI) as the standard to evaluate the DNA damage in SCSA. There was no significant difference in mean of %Tail DNA between two storage methods after two weeks storage($P=0.061$), but median of DFI was significantly higher in -196°C storage than in -80°C storage after two weeks storage($P<0.05$) (**Table 2**).

Effect of repeated freezing-thawing on sperm DNA damage

Repeated freezing and thawing have a significant impact on sperm DNA. %Tail DNA was significantly higher following the repeated freezing-thawing both in -80°C storage ($P=0.000^{*}$) (**Figure 1a**) and in -196°C storage($P=0.000^{*}$)(**Figure 1b**). DFI was also significantly increased

following the repeated freezing-thawing both in -80°C storage ($P=0.000^*$) (**Figure 1c**) and in -196°C storage($P=0.000^*$) (**Figure 1d**).

The correlation between sperm motility, morphology and DNA damage

Motility was negatively correlated with DFI in both -80°C storage following first freezing-thawing cycle($r=-0.413$, $P<0.05$) and in -196°C storage following first cycle($r=-0.429$, $P<0.05$) and second cycle($r=-0.407$, $P<0.05$). Progressive motile was negatively corrected with DFI in -80°C storage following first cycle and second cycle ($r=-0.497$, $P<0.01$ and $r=-0.444$, $P<0.05$ respectively) and in -196°C storage following first cycle and second cycle ($r=-0.521$, $P<0.005$ and $r=-0.488$, $P<0.01$ respectively)(**Table 3**).

Head defect was positive correlated with DFI in -196°C storage following first freezing-thawing cycle($r=0.425$, $P<0.05$), midpiece defect was positive correlated with %Tail DNA in fresh samples($r=0.514$, $P<0.01$), and tail defect was positive correlated with %Tail DNA in -80°C storage following first freezing-thawing cycle($r=0.564$, $P<0.005$) (**Table 3**).

The correlation between %Tail DNA and DFI

Highly positive correlated was found between %Tail DNA and DFI in -196°C storage following first cycle ($r=0.604$, $P<0.005$) and second cycle($r=0.555$, $P<0.005$) (**Table 4**).

Discussion

Multiple sperm cryopreservation methods have been well established either for ART or other assessments. However, the controversy about whether cryopreservation affects sperm chromatin

integrity still exists[12-15]. In present study, our results showed that cryopreservation could induce sperm damage in both two tested protocols.

As the existence of intracellular water, a rapid cooling rate causes the formation of intracellular crystals[16], which would destroy the integrity of cellular membrane, increase the incidence of relaxed chromatin following physical damage of the tertiary structure of the DNA–protamine complex[17], then, induce DNA damage. The extracellular osmotic pressure rises following the solutes excluded from ice formation, which pushes extracellular solution into cell and intercellular water out of the cell, results in excessive dehydration of the cell, increased concentration of intracellular solutes, and produces hyperosmotic stress. Hyperosmotic stress may cause DNA double-strand breaks resulting from cell shrinkage, formation of free radicals, and changes of chromatin compactness and accessibility[18]. For DNA damage assessment, cryopreserved sample usually have higher damage values than fresh samples.

However, when conducting large-scale epidemiological study, hundreds of samples might be collected in one day. Because of the limitation of examination equipment, time and experimenter, samples were usually cryopreserved and transferred to laboratory for further assessment, i.e. DNA fragmentation assay. -80°C neat sample storage and -196°C storage with cryoprotectants were most frequently used protocol for semen preservation. We found that -80°C storage without cryoprotectants have a lower DNA damage than -196°C storage with cryoprotectants. Freezing sperm in seminal plasma can better resist freezing damage, improves postthaw DNA integrity[19]. Sperm mitochondria produce excessive reactive oxygen species (ROS) during freezing-thawing, seminal plasma contains an abundance of antioxidant enzymes including superoxide dismutase and catalase, which remove ROS to protect sperm from ice crystals and oxidative stress induced

damage[13, 20]. Cryoprotective agent (CPA) such as glycerol (a kind of permeating CPAs), can stabilize cell plasma membrane proteins and reduce electrolytes concentration[21] to maintain cell vitality, while it can also affects cytoskeleton, mitochondrial membrane potential and lipid peroxidation when used alone[22], thus may induce sperm DNA damage. Our results suggested that although cryoprotectants were useful to keep sperm viability in cryopreservation, when conducting a DNA damage assessment, -80°C storage with seminal plasma might be a better choice, because cryoprotectants might be detrimental to DNA stability.

Our results showed either for -80°C storage or -196°C storage, repeated freezing-thawing procedure could induce more sperm DNA damage, which was consistent with other studies[23]. DNA damage might be induced in every steps of cryopreservation, including freezing rate, pre- or post-freezing process, cryoprotectants species, thawing temperature, and thawing time etc.[24-28] Cryopreserved sperm samples should avoid multiple freezing-thawing before testing.

Our results also showed a significant correlations between sperm DNA damage and sperm motility as well as morphology defects. Sperm motility was negatively correlated with sperm DNA damage, it seemed that the better motility of fresh sperm the lower DNA damage could be detected after cryopreservation. Sperm morphology defects were positively correlated with sperm DNA damage, which means the lower morphology defects of fresh sperm the lower DNA damage could be detected after cryopreservation. These results suggested that for ART purpose, higher motility and normal morphology percentage samples should be chosen for cryopreservation.

A number of test systems for sperm DNA damage assay at different levels and different molecular sites have been developed, the most frequently used systems include the sperm chromatin structure assay(SCSA)[29], terminal deoxynucleotidyl transferase-mediated dUTP nick

end-labelling assay(TUNEL)[30], neutral/alkaline single-cell gel electrophoresis(SCGE)[31] and sperm chromatin diffusion test(SCD)[32]. In present study, we used SCGE and SCSA to detect sperm DNA damage. These two assays were based on different principles, SCGE was used to detect actual DNA strand breaks while SCSA to detect the susceptibility of sperm nuclear DNA to induce DNA denaturation. Our results showed there were significant correlations between %TailDNA and DFI. Previous studies have showed SCGE had higher sensitivity and specificity[9, 33] than SCSA, and was better in predicting male infertility, but its value depends on alkali-labile sites and was more subjective during the procedure and was difficult to standardize[34, 35]. SCSA values was an indirect index to reflect DNA damage, but as “gold standard” and the most standardized technique between different laboratories, it could be detected by high-throughput assessments, i.e. flow cytometry [36, 37]. Our results suggested that SCSA was more suitable for DNA damage detection in large scale epidemiological study, for the higher throughput and better correlation with semen parameters.

In this study, the 28 selected subjects had a very good homogeneity in age, education level, living environment, and life style. All semen samples were collected, stored and analyzed according to the same standards. Two assessment methods were applied to ensure the accuracy of DNA damage evaluation. However, in this study only short-term preserved samples were evaluated, the situations for long-term preserved samples should be tested subsequently. In conclusion, this study might provide researchers and clinical workers with constructive references when choosing the appropriate sperm cryopreservation methods for DNA damage assay. Storage of neat semen samples at -80 °C had milder damage to sperm DNA than storage at -196 °C mixed with cryoprotectants. To avoid additional sperm DNA damage, repeated freezing and thawing should be

prevented.

Author contributions

ZHC initiated the study and designed the protocol. All authors participate in the subjects recruitment and samples collection. The freezing-thawing operations, SCGE, and SCSA was performed by TXL. ZHC, TXL, JFG,XZ and JC wrote the paper. All authors read and approved the final manuscript.

Competing interests

All authors declare no competing interests.

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References

- [1] J.K. Sherman, Synopsis of the use of frozen human semen since 1964: state of the art of human semen banking, *Fertility and sterility*, 24 (1973) 397-412.
- [2] J.K. Sherman, Practical Applications and Technical Problems of Preserving Spermatozoa by Freezing, *Federation proceedings*, 24 (1965) S288-296.
- [3] J.L. Lasso, E.E. Noiles, J.G. Alvarez, B.T. Storey, Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation, *Journal of andrology*, 15 (1994) 255-265.
- [4] X. Wang, R.K. Sharma, S.C. Sikka, A.J. Thomas, Jr., T. Falcone, A. Agarwal, Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor

infertility, *Fertility and sterility*, 80 (2003) 531-535.

[5] M.S. Aydin, G.E. Senturk, F. Ercan, Cryopreservation increases DNA fragmentation in spermatozoa of smokers, *Acta histochemica*, 115 (2013) 394-400.

[6] P. Villani, M. Spano, F. Pacchierotti, M. Weimer, E. Cordelli, Evaluation of a modified comet assay to detect DNA damage in mammalian sperm exposed in vitro to different mutagenic compounds, *Reproductive toxicology*, 30 (2010) 44-49.

[7] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, *Experimental cell research*, 175 (1988) 184-191.

[8] R. Klaver, A. Bleiziffer, K. Redmann, C. Mallidis, S. Kliesch, J. Gromoll, Routine cryopreservation of spermatozoa is safe--evidence from the DNA methylation pattern of nine spermatozoa genes, *Journal of assisted reproduction and genetics*, 29 (2012) 943-950.

[9] S.M. Duty, N.P. Singh, L. Ryan, Z. Chen, C. Lewis, T. Huang, R. Hauser, Reliability of the comet assay in cryopreserved human sperm, *Human reproduction (Oxford, England)*, 17 (2002) 1274-1280.

[10] K. Konca, A. Lankoff, A. Banasik, H. Lisowska, T. Kuszewski, S. Gozdz, Z. Koza, A. Wojcik, A cross-platform public domain PC image-analysis program for the comet assay, *Mutat Res-Gen Tox En*, 534 (2003) 15-20.

[11] D.P. Evenson, Sperm chromatin structure assay (SCSA(R)), *Methods in molecular biology (Clifton, N.J.)*, 927 (2013) 147-164.

[12] N. Zribi, N. Feki Chakroun, H. El Euch, J. Gargouri, A. Bahloul, L. Ammar Keskes, Effects of cryopreservation on human sperm deoxyribonucleic acid integrity, *Fertility and sterility*, 93 (2010) 159-166.

[13] L.K. Thomson, S.D. Fleming, R.J. Aitken, G.N. De Iuliis, J.A. Zieschang, A.M. Clark, Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis, *Human reproduction*, 24 (2009) 2061-2070.

[14] E. Isachenko, V. Isachenko, Katkov, II, G. Rahimi, T. Schondorf, P. Mallmann, S. Dessole, F. Nawroth, DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification, *Human reproduction (Oxford, England)*, 19 (2004) 932-939.

[15] U. Paasch, R.K. Sharma, A.K. Gupta, S. Grunewald, E.J. Mascha, A.J. Thomas, Jr., H.J. Glander, A. Agarwal, Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa, *Biology of reproduction*, 71 (2004) 1828-1837.

[16] T.M. Said, A. Gaglani, A. Agarwal, Implication of apoptosis in sperm cryoinjury, *Reproductive biomedicine online*, 21 (2010) 456-462.

[17] S.D. Johnston, N. Satake, Y. Zee, C. Lopez-Fernandez, W.V. Holt, J. Gosalvez, Osmotic stress and cryoinjury of koala sperm: an integrative study of the plasma membrane, chromatin stability and mitochondrial function, *Reproduction*, 143 (2012) 787-797.

[18] D. Kultz, D. Chakravarty, Maintenance of genomic integrity in mammalian kidney cells exposed to hyperosmotic stress, *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology*, 130 (2001) 421-428.

[19] E.T. Donnelly, N. McClure, S.E. Lewis, Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity, *Fertility and sterility*, 76 (2001) 892-900.

[20] J.G. Alvarez, B.T. Storey, Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation, *Journal of andrology*, 13 (1992) 232-241.

[21] G.M. Fahy, The relevance of cryoprotectant "toxicity" to cryobiology, *Cryobiology*, 23 (1986) 1-13.

- [22] B. Macias Garcia, C. Ortega Ferrusola, I.M. Aparicio, A. Miro-Moran, A. Morillo Rodriguez, J.M. Gallardo Bolanos, L. Gonzalez Fernandez, C.M. Balao da Silva, H. Rodriguez Martinez, J.A. Tapia, F.J. Pena, Toxicity of glycerol for the stallion spermatozoa: effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential, *Theriogenology*, 77 (2012) 1280-1289.
- [23] L.K. Thomson, S.D. Fleming, K. Barone, J.A. Zieschang, A.M. Clark, The effect of repeated freezing and thawing on human sperm DNA fragmentation, *Fertility and sterility*, 93 (2010) 1147-1156.
- [24] S. Petyim, R. Choavaratana, Cryodamage on sperm chromatin according to different freezing methods, assessed by AO test, *Journal of the Medical Association of Thailand = Chotmaihet thangphaet*, 89 (2006) 306-313.
- [25] T. Vutyavanich, W. Piromlertamorn, S. Nunta, Rapid freezing versus slow programmable freezing of human spermatozoa, *Fertility and sterility*, 93 (2010) 1921-1928.
- [26] J. Gosalvez, R. Nunez, J.L. Fernandez, C. Lopez-Fernandez, P. Caballero, Dynamics of sperm DNA damage in fresh versus frozen-thawed and gradient processed ejaculates in human donors, *Andrologia*, 43 (2011) 373-377.
- [27] L.H. Dalzell, C.M. McVicar, N. McClure, D. Lutton, S.E. Lewis, Effects of short and long incubations on DNA fragmentation of testicular sperm, *Fertility and sterility*, 82 (2004) 1443-1445.
- [28] J.C. Calamera, M.G. Buffone, G.F. Doncel, S. Brugo-Olmedo, S. de Vincentiis, M.M. Calamera, B.T. Storey, J.G. Alvarez, Effect of thawing temperature on the motility recovery of cryopreserved human spermatozoa, *Fertility and sterility*, 93 (2010) 789-794.
- [29] D.P. Evenson, K.L. Larson, L.K. Jost, Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques, *Journal of andrology*, 23 (2002) 25-43.
- [30] W. Gorczyca, F. Traganos, H. Jesionowska, Z. Darzynkiewicz, 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, *Experimental cell research*, 207 (1993) 202-205.
- [31] J. Ribas-Maynou, A. Garcia-Peiro, C. Abad, M.J. Amengual, J. Navarro, J. Benet, Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups, *Human reproduction*, 27 (2012) 652-658.
- [32] M. Meseguer, R. Santiso, N. Garrido, M. Gil-Salom, J. Remohi, J.L. Fernandez, Sperm DNA fragmentation levels in testicular sperm samples from azoospermic males as assessed by the sperm chromatin dispersion (SCD) test, *Fertility and sterility*, 92 (2009) 1638-1645.
- [33] J. Ribas-Maynou, A. Garcia-Peiro, A. Fernandez-Encinas, C. Abad, M.J. Amengual, E. Prada, J. Navarro, J. Benet, Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay, *Andrology*, 1 (2013) 715-722.
- [34] A.R. Collins, Measuring oxidative damage to DNA and its repair with the comet assay, *Biochimica et biophysica acta*, 1840 (2014) 794-800.
- [35] A.R. Collins, A.A. Oscoz, G. Brunborg, I. Gaivao, L. Giovannelli, M. Kruszewski, C.C. Smith, R. Stetina, The comet assay: topical issues, *Mutagenesis*, 23 (2008) 143-151.
- [36] L. Gandini, F. Lombardo, A. Lenzi, M. Spano, F. Dondero, Cryopreservation and sperm DNA integrity, *Cell and tissue banking*, 7 (2006) 91-98.
- [37] M. Spano, E. Cordelli, G. Leter, F. Lombardo, A. Lenzi, L. Gandini, Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay, *Molecular human reproduction*, 5 (1999) 29-37.

380 **Table 1.** Basic characteristics and Semen parameters (n=28)

Variables	Values	
	mean $\pm$ SD	Median(interquartile range)
Age(year)	22.90 $\pm$ 1.08	22.79(22.35-23.25)
Abstinence duration(day)	4.25 $\pm$ 1.62	4.00(3.00-5.00)
Volume(ml)	4.14 $\pm$ 1.33	3.95(3.30-4.76)
Concentration(10^6 ml $^{-1}$)	76.34 $\pm$ 52.92	62.60(38.23-91.15)
Total sperm count(10^6)	292.83 $\pm$ 181.71	256.16(165.62-415.54)
Normal morphology(%)	10.38 $\pm$ 5.16	10.00(6.67-13.00)
Head defect(%)	65.59 $\pm$ 6.80	68.23(61.01-70.00)
Midpiece defect(%)	27.77 $\pm$ 5.85	28.56(25.52-32.36)
Tail defect(%)	20.55 $\pm$ 4.76	21.34(17.29-24.44)
Total motility(%)	83.36 $\pm$ 15.30	84.10(79.58-89.90)
Progressive motile(%)	60.80 $\pm$ 15.30	62.15(51.15-70.95)
Motility parameters		
VCL(μ m s $^{-1}$)	67.30 $\pm$ 20.05	67.50(56.00-79.75)
VSL(μ m s $^{-1}$)	28.18 $\pm$ 9.21	27.50(22.00-32.75)
VAP(μ m s $^{-1}$)	40.46 $\pm$ 11.29	40.00(35.25-46.00)
BCF(Hz)	13.64 $\pm$ 2.86	14.00(13.00-15.75)
ALH(μ m)	2.09 $\pm$ 0.68	2.00(2.00-2.00)
LIN(%)	41.89 $\pm$ 5.69	42.00(38.25-45.00)

STR(%)	69.18±6.07	68.00(64.25-74.00)
WOB(%)	60.36±4.48	60.50(58.00-64.00)

Abbreviations: VCL=curvilinear velocity; VSL=straight line velocity; VAP=average path velocity;
BCF =beat cross frequency; ALH =amplitude of lateral head displacement;10⁶ LIN= linearity
(VSL/VCL); STR=straightness (VSL/VAP); WOB=wobble of the curvilinear trajectory
(VAP/VCL).

Table 2. Comparison of the effects of two methods of cryopreservation on sperm DNA damage.

	-80 °C storage		-196°C storage		<i>P</i>
	Mean ± SD	Median(interquartile range)	Mean ± SD	Median(interquartile range)	
%Tail DNA	20.77±5.30	21.39 (18.15-24.27)	24.02±7.76	22.96 (20.15-29.02)	0.061 ^a
DFI	12.33±7.07	9.77 (7.49-17.25)	13.67±8.96	9.91 (7.57-20.38)	0.023 ^{b, *}

^apaired t test.

^bWilcoxon Signed-rank test.

**P*<0.05.

Figure 1. Effect of repeated freezing-thawing on sperm DNA damage.

(a) Effect of repeated freezing-thawing on sperm DNA damage in -80°C storage measured by
SCGE(*P*=0.000**). (b) Effect of repeated freezingthawing on sperm DNA damage in -196°C storage

measured by SCGE(P=0.000**). (c) Effect of repeated freezing-thawing on sperm DNA damage in -80°C storage measured by SCSA(P=0.000**). (d) Effect of repeated freezing-thawing on sperm DNA damage in -196°C storage measured by SCSA(P=0.000**). Friedman nonparametric test was used.

Table 3: Correlations of sperm DNA damage with motility and morphology.

	Progressive motile(%)		total motility(%)		normal morphology(%)		Head defect(%)		Midpiece defect(%)		Tail defect(%)	
	r	P	r	P	r	P	r	P	r	P	r	P
%Tail DNA												
Fresh	-0.036	0.855	0.04	0.841	-0.219	0.283	0.016	0.939	0.514	0.007**	-0.071	0.729
-80°C/cycle1	0.036	0.855	0.086	0.663	0.135	0.51	-0.155	0.45	0.367	0.065	0.564	0.003***
-80°C/cycle2	-0.113	0.566	-0.15	0.446	-0.009	0.966	0.088	0.671	0.098	0.633	0.211	0.301
-196°C/cycle1	-0.318	0.099	-0.223	0.254	-0.039	0.851	0.376	0.059	-0.028	0.89	0.038	0.854
-196°C/cycle2	-0.267	0.165	-0.218	0.265	-0.029	0.889	0.384	0.023*	0.026	0.898	0.03	0.885
DFI												
Fresh	-0.25	0.2	-0.143	0.467	0.036	0.863	-0.1	0.627	0.098	0.636	0.112	0.584
-80°C/cycle1	-0.498	0.007**	-0.413	0.029*	-0.036	0.86	0.353	0.077	-0.013	0.948	0.026	0.898
-80°C/cycle2	-0.444	0.018*	-0.354	0.065	-0.054	0.793	0.214	0.294	-0.037	0.86	0.103	0.617
-196°C/cycle1	-0.522	0.004***	-0.429	0.023*	-0.144	0.482	0.425	0.031*	0.013	0.951	-0.046	0.823
-196°C/cycle2	-0.490	0.008**	-0.407	0.031*	-0.083	0.688	0.373	0.06	0.022	0.917	0.025	0.904

Note. Cycle 1, cycle2 mean the first freezing-thawing cycle and second freezing-thawing cycle respectively.

405 *P <0.05, **P <0.01, ***P <0.005.

406

407 **Table 4 :** Spearman rank correlations of the parameters derived from SCGE and SCSA

Group	r	P
Fresh	0.213	0.277
-80°C /cycle 1	0.318	0.099
-80°C /cycle 2	0.372	0.051
-196°C /cycle 1	0.604	0.001***
-196°C /cycle 2	0.555	0.002***

408 Note. Cycle 1, cycle2 mean the first freezing-thawing cycle and second freezing-thawing cycle
409 respectively.

410 ***P <0.005.