Routine cryopreservation of spermatozoa is safe — Evidence from the DNA methylation pattern of nine spermatozoa genes

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Abstract

Purpose Assess short- and mid-term impact of cryopreservation on DNA methylation status of different genes in spermatozoa.

Methods Semen samples from 10 healthy normozoospermic men were collected at the Department of Clinical Andrology of the Centre of Reproductive Medicine and Andrology (Muenster, Germany). Each was divided into four equal aliquots: 1) untreated, 2) diluted in cryoprotectant, 3) short term (2 days) cryopreserved and 4) mid term (4 weeks) cryopreserved. Samples were “swim-up” purified prior to analysis. DNA fragmentation was measured using comet assay and Flow cytometric evaluation with Acridine Orange (FCEAO). The degree of methylation of nine genes was determined by bisulfite pyrosequencing of genomic DNA.

Result(s) Analysis of three maternally imprinted genes (*LIT1*, *SNRPN*, *MEST*), two paternally imprinted genes (*MEG3*, *H19*), two repetitive elements (*ALU*, *LINE1*), one spermatogenesis-specific gene (*VASA*) and one gene associated with male infertility (*MTHFR*) in semen samples demonstrated no alteration in methylation pattern regardless of duration of cryopreservation.

Conclusion(s) The lack of any changes in the sub-fraction of the genome examined in our study, implies that sperm

DNA methylation is unaffected by cryopreservation and suggests that this daily clinical routine is safe in terms of DNA methylation.

Keywords Cryopreservation · DNA methylation · Spermatozoa · DNA fragmentation · ART

Introduction

Cryopreservation of spermatozoa is an important tool in daily clinical routine, in particular the preservation of spermatozoa of cancer patients prior to the gonadotoxic effects caused by irradiation or chemotherapy. Spermatozoal storage represents an important fertility reserve for further use in assisted reproductive techniques (ART) such as intrauterine insemination (IUI), in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [22,33]. Different reports describe successful pregnancies via ART with sperm from cancer patients which was collected before treatment and stored for up to 28 years [7,14,33]. Furthermore, cryopreservation is the foundation upon which donor services and sperm repositories function worldwide [36]. Current cryopreservation protocols involve the freezing of the ejaculate with cryoprotectants which when thawed are “swim-up” purified for use in ART. Previously, it has been described that cryopreservation causes DNA fragmentation in spermatozoa [8,12,42]. In particular, the effect of cryopreservation and thawing on DNA integrity was greater in infertile men than in fertile men [4,12]. As DNA fragmentation seems to be negatively correlated with embryo quality, implantation rates and miscarriages, ideally DNA fragmented sperm should be avoided when using ART [16,23,39,41].

“Epigenetics” are inheritable non-sequence based modifications such as DNA methylation, histone alterations and chromatin remodelling which regulate gene transcription.

Capsule Cryopreservation of spermatozoa in daily clinical routine can be considered safe with regard to DNA methylation, since neither short- nor mid-term cryostorage alters methylation patterns of spermatozoa.

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During spermatogenesis the genome undergoes massive epigenetic modifications which are crucial for fertilization and embryogenesis [11,17,30]. Early primordial germ cells (PGCs) possess a somatic-like methylation pattern with a high degree of genome-wide DNA methylation. When the PGCs enter the genital ridge a wave of genome-wide DNA demethylation occurs before the establishment of sex-specific epigenetic germline modifications [2,11]. In the male germline, remethylation is probably completed in pachytene spermatocytes and results in re-establishing the gender-specific imprints and methylation patterns [18,26,31]. After fertilization the genome undergoes a second genome-wide wave of demethylation followed by *de novo* methylation and the establishment of somatic methylation patterns around the time of implantation [28,37]. Only imprinted genes are unaffected by the demethylation wave after fertilization. These processes result in germ cell specific epigenetic signatures that are essential for fertility and dictate the expression profile of embryogenesis [37,44].

Recent studies have shown a strong association of aberrant DNA methylation patterns of spermatozoa with male infertility. In particular, oligozoospermia, abnormal morphology and decreased motility have all been found to be associated with abnormal DNA methylation of several imprinted genes [20,27,35]. As a consequence, it has been suggested that sperm from men with oligozoospermia carry a higher risk of transmitting aberrant imprints to their children [19]. These epimutations may be inherited via ART and are therefore potential risk factors for congenital diseases [35]. This assumption is corroborated by recent reports that show ART to be correlated with an increased frequency of congenital diseases associated with imprinting defects such as Beckwith-Wiedeman syndrome (BWS) and Angelman syndrome (AS) [1,3,9,24,25]. The increased frequency of miscarriages in ART could also be partly explained by these DNA methylation aberrations [5,15,46]. Considering the severity of the possible consequences to children born by ART, it is essential to assure that cryopreservation does not alter the DNA methylation patterns.

Thus far, little is known about the effect of cryopreservation on the epigenetic patterns of spermatozoa. Furthermore it is still unclear if there is a relationship between the extent of fragmented sperm DNA resulting from cryopreservation and any potential changes in the DNA methylation patterns. A study by Tunc and Tremellen [45] described a negative correlation of sperm DNA fragmentation by oxidative damage and DNA methylation [45], but a causative relation was not shown.

The aim of our study was to determine the impact of routinely used cryopreservation protocols on the DNA methylation status of spermatozoa of normozoospermic men. DNA fragmentation was assessed as a further clinical parameter.

Subjects and methods

Sperm samples from ten normozoospermic [47] healthy volunteers were collected at the Department of Clinical Andrology of the Centre of Reproductive Medicine and Andrology, Muenster, Germany. All volunteers provided written informed consent and agreed to the analysis of genetic material as approved by the Ethics Committee of the University and the state medical board (reference number of Institutional Review Board approval: 4 I Nie).

Each ejaculate was divided into four equal aliquots: 1) untreated, 2) diluted in SteriTec[®] medium (SteriPharm, Berlin, Germany), 3) diluted in SteriTec[®] medium and either short-term cryopreserved (2 days) or 4) mid-term cryopreserved (4 weeks). Each sample was “swim-up” purified after the dilution or thawing of the sample, directly prior to analysis. Measurements of sperm count, sperm motility and sperm morphology were carried out according to the guidelines of the WHO for the examination and processing of human semen [47].

In addition two fresh semen samples from two different volunteers were “swim-up” purified, analysed and prepared for the induction of DNA damage.

“Swim-up” purification

For the “swim-up” purification (see [47]) semen was diluted 1:1 in Sperm Preparation Medium (Origio, Måløv, Denmark). This suspension was centrifuged by 390 g for 10 min, the supernatant removed and the pellet washed in 2 ml Sperm Preparation Medium (390 g, 10 min). After washing 1 ml Sperm Preparation Medium was slowly given on the pellet and incubated for 1 h at 37 °C and 5 % CO₂. After incubation 500 µl of the supernatant containing motile spermatozoa was collected for subsequent analysis.

Cryopreservation

Semen was diluted 1:1 in SteriTec[®] and filled in straws (MTG, Bruckberg, Germany). The straws were heat sealed and the samples frozen using the Ice Cube 1810 (Sy-Lab, Purkersdorf, Austria) in 25.6 min cycle from 24 °C to −170 °C. Freezing program (according to manufacturer’s instructions): −3 °C/3 min, −0 °C/5 min, −4 °C/0.2 min, −1 °C/0.1 min, −4 °C/0.3 min, −22 °C/2.8 min, −60 °C/2.8 min, −30 °C/2.4 min, −10 °C/1.4 min, −4 °C/0.4 min, −56 °C/2.2 min, −0 °C/5 min.

Isolation of DNA

Genomic DNA from 1×10⁶ “swim-up” purified spermatozoa was isolated using the Master-Pure DNA Purification Kit (EPICENTRE Biotechnologies, Madison, WI, USA) according to the manufacturer’s instructions.

Bisulfite conversion

Genomic DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). In order to ensure that the bisulfite conversion was complete, the methylation levels of bisulfite converted control samples from different sources (blood, sperm) were compared to the expected methylation values. Furthermore, control dispensations at non CpG sites were used in all pyrosequencing assays to rule out insufficient bisulfite conversion.

Amplification of differentially methylated regions of interest

Regions of interest were two paternally imprinted genes *MEG3* (chromosome 14; 100,345,300–100,345,900 Ensembl version 54, May 2009) and *H19* (chromosome 11; 1977647–1977878, Ensembl version 54, May 2009), three maternally imprinted genes *LIT1* (chromosome 11; 2,677,751–2,677,873, Ensembl version 54, May 2009), *SNRPN* (chromosome 15; 22,751,105–22,751,342, Ensembl version 54, May 2009) and *MEST* (chromosome 7; 129919302–129919521, Ensembl version 54, May 2009), the spermatogenesis-specific gene *VASA* (chromosome 5; 55033903–55034028, Retrieved from NCBI build 37.1), the methylenetetrahydrofolate reductase (*MTHFR*) (chromosome 1; 11,865,619–11,865,654, Ensembl version 65, Dec 2011) and the repetitive elements *ALU* and *LINE1* (according to [5]).

PCR primers and the specific PCR thermocycling conditions are listed in supplemental material and methods section (Supplemental data S1).

Methylation analysis

Quantitative methylation analysis was performed by pyrosequencing (PyroMark Q24 System, Qiagen, Hilden, Germany). For each pyrosequencing assay the methylation levels of control samples of different sources (blood, sperm) and of control DNA (Qiagen, Hilden, Germany) were analysed in order to determine the sensibility and reproducibility of each pyrosequencing assay. Moreover, all pyrosequencing assays in this study used control dispensations at non CpG sites to technically monitor bisulfite conversion to rule out insufficient bisulfite conversion. The Pyromark Q24 software (PyroMark Q24 2.0.6.20, Qiagen) was used for analysis of DNA methylation. The sequencing primers are listed in supplemental material and method section (Supplemental data S2).

Comet assay

For the determination of DNA fragmentation the Alkaline comet assay (CometAssay[®] Kit, Trevigen, Gaithersburg, Maryland, USA) was used. Spermatozoa were lysed in a

special buffer (2.5 M NaCl, 100 mM Na₂ EDTA, 10 mM Tris, 10 % DMSO, 1 % TritonX-100, pH 10) then incubated with 10 mM DTT for 1 h at 4 °C followed by incubation with 200 µg proteinase K/ml for 1 h at 30 °C. After dark incubation with 300 mM NaOH and 1 mM EDTA at room temperature, electrophoresis was performed in alkaline electrophoresis solution pH >13 (300 mM NaOH, 1 mM EDTA) (300 mA, 30 min). Silver staining (CometAssay[®] Silver Staining Kit, Trevigen, Gaithersburg, Maryland, USA) allowed for visualization of the fragmented DNA. DNA damage was determined by measuring the length of the comet tails indicative for DNA fragmentation of 400 spermatozoa per sample. Spermatozoa damaged by Fenton's reaction were used as positive controls and ensured that the comet assays were performed well and worked as a reference for comet tail lengths of damaged spermatozoa.

Flow cytometric evaluation of DNA fragmentation with Acridine Orange (FCEAO)

FCEAO was performed as described previously [6]. Briefly, 200 µl TNE buffer including 2×10^6 sperm/ml were mixed with 400 µl of an acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1 % [vol/vol] Triton X-100, pH 1.2). After exactly 30 s, 1.20 ml of acridine orange (AO) staining solution (6 mg AO/ml AO buffer) was added. The AO buffer consisted of 0.037 M citric acid, 0.126 M Na₂HPO₄, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0. The samples were then placed in the flow cytometer Cytomics FC 500 (Beckman Coulter, Krefeld, Germany) containing an argon laser operated at 488 nm at 40 mW of power. After transiting a 560 nm short-pass dichroic mirror, the green fluorescence was detected through a 515- to 545-nm band-pass filter. After transiting a 640-nm long-pass filter, the red fluorescence was collected through a 650-nm long-pass filter. In total 5000 events were acquired. For the flow cytometer setup and calibration, a “reference” sample was used from a normal volunteer ejaculate sample.

Data were analysed with the FCS 3.0 software package (DeNovo software, 3250 Wilshire Blvd. Suite 803, Los Angeles, CA, 90010, USA). The mean levels of DNA fragmentation index (mean DFI) were calculated.

Induced DNA damage

In order to test if DNA damage could cause changes in the methylation patterns, DNA fragmentation was induced in control semen samples by UV-B light and by Fenton's reaction (FeSO₄/H₂O₂). From two normozoospermic healthy men “swim-up” purified semen samples were split into two aliquots, each diluted in phosphate-buffered saline (PBS), centrifuged at 400 g for 10 min, the supernatant was removed and the pellet resuspended in PBS. Of each semen

sample one aliquot served as untreated control. The second aliquot of one semen sample was exposed to 312 nm UV irradiation (Saur GmbH, Reutlingen, Germany) for 10 min. The second aliquot of the other semen sample was incubated in 21 mM FeSO₄ and 42 mM H₂O₂ for 30 min on ice, diluted in excess PBS, centrifuged at 400 g for 10 min, the supernatant removed and the pellet resuspended in PBS. The DNA fragmentation in these samples was measured only by FCEAO due to the limited amount of material.

Data analysis

All calculations were performed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Values were assessed for Gaussian distribution by D'Agostino & Pearson omnibus normality test. If not stated differently given data are expressed as mean $\pm$ SEM. Statistical differences were calculated by one-way ANOVA followed by Bonferroni's Multiple Comparison post-test. Differences were considered significant if $p < 0.05$.

Results

In order to assess the short- and mid-term impact of cryopreservation on DNA methylation status of spermatozoa we collected sperm of ten healthy normozoospermic volunteers. Each sample was divided into four aliquots and each aliquot was treated differently (untreated, diluted in cryoprotectant, diluted in cryoprotectant and short-term or rather mid-term cryopreserved). In Table 1 the mean semen parameters ($\pm$ SEM) of the native ejaculates and the sperm count and progressive sperm motility of all “swim-up” purified sperm samples are given. This “swim-up” purification leads to a

decreased number of sperm and progressive sperm motility after cryopreservation. However, only differences in sperm motility between the short-term cryopreserved sample and the untreated sample or the diluted sample were significantly different ($p < 0.05$).

The overall tendency of DNA fragmentation was similar in FCEAO and comet assay (Fig. 1), although the measured extent of fragmentation was higher when determined by FCEAO. Dilution of spermatozoa with the cryoprotectant and long-term cryopreservation did not significantly influence DNA integrity but short-term cryopreservation caused DNA fragmentation.

The DNA methylation patterns of the nine genes were stable in all four aliquots in all volunteers (Fig. 2). Neither dilution with cryoprotectant nor short- or mid-term cryopreservation had any effect on the DNA methylation patterns of the nine selected genes. DNA methylation levels of *MEG3*, *H19*, *LIT1*, *MEST*, *SNRPN*, *VASA* and *MTHFR* were similar amongst the individuals, showing only small variability. Concerning the repetitive elements, the inter-individual range of DNA methylation of *ALU* was identical in all volunteers, whereas *LINE1* methylation had large inter-individual variance. However, these remained unaffected after cryopreservation. DNA methylation of all 4 aliquots (untreated, diluted, short-term and mid-term cryopreservation) were 94.96 ($\pm$ 0.33) % for *MEG3*, 96.69 ($\pm$ 0.32) % for *H19*, 10.06 ($\pm$ 0.33) % for *LIT1*, 5.44 ($\pm$ 0.44) % for *SNRPN*, 14.37 ($\pm$ 0.25) % for *MEST*, 9.71 ($\pm$ 0.87) % for *VASA*, 6.73 ($\pm$ 0.4) % for *MTHFR*, 20.88 ($\pm$ 0.1) % for *ALU* and 65.28 ($\pm$ 1.64) % for *LINE1*.

Although DNA was damaged up to 100 % in control samples after treatment with UV-B light or with Fenton's reaction we could only detect marginal changes in the DNA methylation patterns of *MEG3*, *LIT1*, *ALU* and *LINE1* after DNA damages (Table 2).

Table 1 Sperm parameter of the different samples. Upper part: volume, sperm concentration, progressive sperm motility and sperm morphology of the native ejaculates. Lower part: sperm count and progressive sperm

motility of the four “swim-up”-purified samples (untreated, diluted, short-term and mid-term cryopreservation). Means ($\pm$ SEM) of ten volunteers are shown

Native ejaculate				
Volume (ml)				3.68 ($\pm$ 0.43)
Concentration (million/ml)				58.43 ($\pm$ 13.56)
Total sperm count (million)				235.28 ($\pm$ 83.9)
Progressive sperm motility (%)				52.4 ($\pm$ 1.5)
Normal sperm morphology (%)				5.86 ($\pm$ 0.55)
“Swim-up” purified spermatozoa				
	Untreated sample	Diluted sample	Short-term cryopres.	Mid-term cryopres.
Sperm count (million/sample)	9.23 ($\pm$ 2.11)	7.95 ($\pm$ 2.17)	2.48 ($\pm$ 0.77)	2.24 ($\pm$ 0.82)
Progressive sperm motility (%)	79.00 ($\pm$ 4.83)	79.56 ($\pm$ 2.33)	63.80 ($\pm$ 3.79)*	70.1 ($\pm$ 5.52)

* = significantly different ($p < 0.05$) to untreated sample and diluted sample. Statistical differences were calculated by one-way ANOVA followed by Bonferroni's Multiple Comparison post-test

Fig. 1 Analysis of DNA fragmentation of the four “swim-up”-purified samples (untreated, diluted, short-term and mid-term cryopreservation). The amount of DNA fragmentation of the untreated sample of each volunteer is set to 1 (as a reference value) and the other values are normalized to the value of the untreated sample. Mean ($\pm$ SEM) of ten volunteers (comet assay) or 6 volunteers (FCEAO) are shown. *** = significantly different ($p < 0.001$). Statistical differences were calculated by one-way ANOVA followed by Bonferroni’s Multiple Comparison post-test

Discussion

Cryopreservation of spermatozoa is a widespread and important method in the daily clinical routine of andrological and ART laboratories. Although epigenetic signatures have become a major issue in reproduction in recent years, the

impact of cryopreservation on DNA methylation has not yet been assessed despite its potential to influence ART outcome [5]. A negative correlation of sperm DNA fragmentation by oxidative damage and DNA methylation has already been described [45], however, it was not determined if the aberrations in DNA methylation patterns were induced by

Fig. 2 DNA methylation of nine selected genes of the four “swim-up”-purified samples (untreated, diluted, short-term and mid-term cryopreservation). The analysed genes were: two paternally imprinted genes *MEG3* and *H19*; three maternally imprinted genes *LIT1*, *MEST* and *SNRPN*; the spermatogenesis-specific gene *VASA*; the methylenetetrahydrofolate reductase (*MTHFR*) and the repetitive elements *LINE1* and *ALU*. The values are means ($\pm$ SEM) of ten volunteers. Statistical differences were calculated by one-way ANOVA followed by Bonferroni’s Multiple Comparison post-test

Table 2 Amount of DNA fragmentation and DNA methylation of selected genes of two control samples. Each sample was divided into two aliquots and used untreated or with induced DNA damage by UV-B light and Fenton's reaction ($\text{FeSO}_4/\text{H}_2\text{O}_2$), respectively

	DNA fragmentation ^c	DNA methylation			
		<i>MEG3</i>	<i>LIT1</i>	<i>LINE1</i>	<i>ALU</i>
Control 1 ^a	0.6 %	94.5 %	9.25 %	70.6 %	22.6 %
UV-B light	99.95 %	90 %	11.25 %	71.3 %	24.3 %
Control 2 ^b	5.46 %	94.5 %	9 %	75.33 %	23 %
$\text{FeSO}_4/\text{H}_2\text{O}_2$	97.79 %	94 %	8 %	76.67 %	21.67 %

Control 1 and control 2 were obtained from different volunteers.

a = control for UV-B light damage

b = control for $\text{FeSO}_4/\text{H}_2\text{O}_2$

c = measured by FCEAO

DNA fragmentation or the intrinsic effects of impaired spermatogenesis.

As there is a plethora of different DNA fragmentation assays with diverging results, we used two different methods (FCEAO and comet assay) to determine the amount of DNA fragmentation [34,38]. Our study confirmed that DNA fragmentation is increased after cryopreservation as previously described [8,12,42]. However, we also noted that the DNA fragmentation in “swim-up” purified samples is higher following short-term than after longer cryopreservation. This might be due to a selection bias in the quality of the “swim-up” purified spermatozoa but one which mimics the situation in the daily clinic routine. At first this finding appears counter intuitive, however it is reasonable to assume that after short-term cryopreservation some damaged sperm are still vital but following mid-term cryopreservation they lose their motility completely and hence do not “swim up”. This would also explain the increased progressive motility after mid-term cryopreservation (Table 1). Without the “swim-up” purification we would expect higher DNA fragmentation and decreased progressive motility after mid-term cryopreservation compared to

short-term cryopreservation as the process removes damaged, dead and immotile sperm. Our findings are in agreement with other studies which also showed a general improvement of sperm motility, nuclear maturity, morphology, kinetics and the exclusion of spermatozoa with nicked DNA and poorly condensed chromatin [21,29,40].

The different values obtained by FCEAO and the comet assay can be attributed to the FCEAO measuring susceptibility to DNA damage after treatment with mild acid whilst the comet assay detects DNA breaks [6,32,38]. It is not surprising therefore that FCEAO values are higher than comet. Furthermore, the FCEAO findings are consistent with the suggestion that damaged but still vital spermatozoa are present in the short-term cryopreserved aliquot, whilst in the mid-term cryopreservation the already compromised spermatozoa are further damaged and rendered incapable of swimming.

DNA methylation of our nine selected genes remained stable in “swim-up” purified spermatozoa and was neither affected by the cryoprotectant itself nor the duration of cryopreservation. However this analysis can only act as a representative sample of the genome as a whole. That said we

Table 3 List of analysed genes and reason for selection

Gene	Gene information	(Epi)genetic association with male infertility	Reference
<i>H19</i>	Paternally imprinted gene	Low methylation associates with low sperm concentration	[20,35]
<i>MEG3</i>	Paternally imprinted gene	Low methylation associates with poor semen parameters	[20]
<i>MEST</i>	Maternally imprinted gene	Hypermethylation at the imprinted loci associates with poor semen parameters	[35]
<i>SNRPN</i>	Maternally imprinted gene	Hypermethylation at the imprinted loci associates with poor semen parameters	[13,20]
<i>LIT1</i>	Maternally imprinted gene	Hypermethylation at the imprinted loci associates with poor semen parameters	[13,20]
<i>ALU</i>	Repetitive element, short interspersed elements (SINEs)	Repetitive element, gives information about genome-wide methylation	[20]
<i>LINE1</i>	Repetitive element, long interspersed elements (LINEs)	Repetitive element, gives information about genome-wide methylation	[20]
<i>VASA</i>	Spermatogenesis-specific gene	Decreased expression of <i>VASA</i> in sperm of oligozoospermic men	[10]
<i>MTHFR</i>	Methylenetetrahydrofolate reductase	DNA hypermethylation results in poor semen quality and infertility	[48]

carefully chose genes which provide as broad information about the status of DNA methylation as possible (Table 3). The two repetitive elements were chosen to define the genome-wide DNA methylation and the five imprinted genes indicate parent-of-origin expression and are essential for fertility. Recent studies have described an association of aberrant methylation of these selected imprinted genes and poor sperm quality [13,20,35]. Imprinted genes and repetitive elements were selected because of their wide range of inter-individual DNA methylation patterns allowing to detect individual variation. Furthermore, *VASA* is a spermatogenesis-specific gene for which decreased expression has been shown to be associated with oligozoospermia [10]. The expression of the ninth selected gene, *MTHFR*, has also been correlated with male infertility specifically the degree of its hypermethylation was found to be associated with poor semen quality and infertility [48].

To mimic ART procedures in our clinic, we confined our investigation to the DNA methylation pattern of “swim-up” purified spermatozoa (i.e. the “healthiest” and best). These sperm would be expected to be the most robust and thus more able to withstand the rigours of cryopreservation, while the effect on compromised and immotile sperm might be well different.

Undoubtedly, storage of spermatozoa for 4 weeks is a short time compared to the long-term storage used for cancer sufferers [7,14]. But as a study involving years of storage is practically unfeasible, the use of mid-term cryopreservation provided the means to detect whether DNA methylation was affected by the storage or the freezing-procedure.

Each obtained sample underwent multiple epigenetic analyses with nine different genes being assessed in ten individual samples, thus a good insight into the effect of cryopreservation on DNA methylation could be derived. Additionally, the genes were carefully chosen to represent different degrees of inter-individual DNA methylation variation. As we found no changes it is reasonable to conclude that cryopreservation is very unlikely to have an influence on the DNA methylation pattern.

As cryo-induced DNA damage is predominantly caused by oxidative stress [43] and a negative correlation of sperm DNA fragmentation by oxidative damage and DNA methylation has been described [45], we investigated whether induced oxidative damage (i.e. Fenton’s reaction) or UV-B light could disturb DNA methylation patterns. In contrast to the proposition of Tunc and Tremellen [45] we found no correlation between induced DNA fragmentation and DNA methylation. The epigenetic aberrations found by several other studies [20,27,35] do not seem to be due to DNA damage but rather reflect differing problems in spermatogenesis.

In summary, we found that the DNA methylation patterns in a selected sub-fraction of the genome were not altered by cryopreservation and that there was no correlation between

the induced DNA fragmentation and DNA methylation. These findings suggest that use of cryopreservation on the fraction of sperm routinely selected for ART appears to be safe with respect to DNA methylation. Further investigations need to be conducted to determine whether cryopreservation of spermatozoa from men with abnormal sperm quality, increased DNA fragmentation or aberrant DNA methylation has any effect on their methylation patterns. This would be of importance as these patients are potential candidates for cryopreservation and subsequent ART procedures.

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Conflict of interest The authors declare that they have no conflict of interest.

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