Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm

Gürler, H ; Malama, E ; Heppelmann, M ; Calisici, O ; Leiding, C ; Kastelic, J.P ; Bollwein, Heiner

Abstract: The objective was to examine if there are relationships between alterations in sperm viability, reactive oxygen species (ROS) synthesis, and DNA integrity induced by cryopreservation of bovine sperm. Four ejaculates were collected from each of six bulls. Each ejaculate was diluted and divided into two aliquots; one was incubated for 24 hours at 37 ℃, and the other frozen, thawed, and incubated for 24 hours at 37 ℃. Analyses of quality of sperm were performed after 0, 3, 6, 12, and 24 hours of incubation. Progressive motile sperm was determined with computer assisted sperm analysis. Percentages of plasma membrane- and acrosome-intact sperm, sperm with a high mitochondrial membrane potential, sperm showing a high degree of DNA fragmentation (%DFI), and their reactive oxygen species content were assessed with dichlorofluorescein-diacetate, dihydrorhodamine, diaminofluorescein diacetate, and mitochondrial superoxide indicator using flow cytometry. Although all other sperm parameters showed alterations ($P < 0.05$) during the 24-hour incubation time, %DFI stayed constant ($P > 0.05$, 0.91 ± 0.23) in nonfrozen sperm. Cryopreservation induced changes of all sperm parameters ($P < 0.05$). In contrast to all other sperm parameters, dichlorofluorescein-diacetate-fluoroescence indicating the synthesis of H2O2 showed a similar exponential rise ($P < 0.05$) like the %DFI values in frozen sperm. In conclusion, changes of DNA integrity in frozen sperm seem to be related to synthesis of H2O2 but not to sperm viability and synthesis of other reactive oxygen species.

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H. Gürler [a,*], E. Malama [b], M. Heppelmann [a], O. Calisici [c], C. Leiding [d], J.P. Kastelic [e], H. Bollwein [b]

[a] Clinic for Cattle, University of Veterinary Medicine Hannover Foundation, Hannover, Germany
[b] Vetsuisse Faculty, Clinic of Reproductive Medicine, Zürich, Switzerland
[c] Süt Kardesler A.S. Animal Breeding Center, Izmir, Turkey
[d] Besamungsvereins Neustadt Aisch, Neustadt an der Aisch, Germany
[e] Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada

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ABSTRACT

The objective was to examine if there are relationships between alterations in sperm viability, reactive oxygen species (ROS) synthesis, and DNA integrity induced by cryopreservation of bovine sperm. Four ejaculates were collected from each of six bulls. Each ejaculate was diluted and divided into two aliquots; one was incubated for 24 hours at 37 °C, and the other frozen, thawed, and incubated for 24 hours at 37 °C. Analyses of quality of sperm were performed after 0, 3, 6, 12, and 24 hours of incubation. Progressive motile sperm was determined with computer assisted sperm analysis. Percentages of plasma membrane- and acrosome-intact sperm, sperm with a high mitochondrial membrane potential, sperm showing a high degree of DNA fragmentation (%DFI), and their reactive oxygen species content were assessed with dichlorofluorescein-diacetate, dihydrorhodamine, diaminofluorescein diacetate, and mitochondrial superoxide indicator using flow cytometry. Although all other sperm parameters showed alterations ($P < 0.05$) during the 24-hour incubation time, %DFI stayed constant ($P > 0.05$, 0.91 ± 0.23) in nonfrozen sperm. Cryopreservation induced changes of all sperm parameters ($P < 0.05$). In contrast to all other sperm parameters, dichlorofluorescein-diacetate–fluoroescence indicating the synthesis of H_2O_2 showed a similar exponential rise ($P < 0.05$) like the %DFI values in frozen sperm. In conclusion, changes of DNA integrity in frozen sperm seem to be related to synthesis of H_2O_2 but not to sperm viability and synthesis of other reactive oxygen species.

1. Introduction

Although sperm cryopreservation is widely used, it is well-known that freezing and thawing processes damage sperm because of changes in temperature, induction of osmotic stress, and formation of ice crystals [1,2]. It is noteworthy that these alterations affect the plasma membrane, acrosomes, mitochondria, reactive oxygen species (ROS), and DNA integrity and reduce sperm motility and survival [3–6].

Sperm mitochondrial status is critical because of its relationship with the cell's energy status and motility and consequently has been related to fertility [7]. Sperm motility is important for transport from the site of ejaculation or insemination to the site of fertilization [8]. Sperm mitochondria, which is located in the midpiece, must produce energy (ATP) to power the flagellar motion that

* Corresponding author. Tel.: +49 0511 856 7184; fax: +49 0511 953 8504.
E-mail address: gurlerhakan@hotmail.com (H. Gürler).

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moves sperm to the site of fertilization [9,10]. Furthermore, it has been suggested that glycolysis is critical for psychological sperm function [11]. Likewise, intact plasma membranes and acrosomes are essential for sperm capacitation, acrosome reaction, and ultimately fertilization of the oocyte. In addition, DNA damage in sperm has been linked to a variety of adverse clinical outcomes, including reduced fertility [7]. Disturbances in DNA integrity are not only caused by impaired spermatogenesis but also by sperm cryopreservation [12]. In addition, there was a negative correlation between mitochondrial membrane potential and DNA integrity after thawing of frozen sperm [13].

Sperm spontaneously produce a variety of ROS, including superoxide anion, hydrogen peroxide, and nitric oxide [14]. Small amounts of ROS are functionally important to drive tyrosine phosphorylation cascades associated with sperm capacitation. However, when ROS production exceeds sperm's limited antioxidant defenses, oxidative stress is induced, characterized by peroxidative damage to the sperm plasma membrane and DNA strand breakage in the sperm nucleus. Such oxidative stress not only disrupts sperm fertilizing potential but also their ability to create a normal, healthy embryo [14]. Sperm ROS formation developed as a possible explanation of poor sperm quality, either in fresh collected semen or after processing for use in various reproductive technologies [15].

To be able to improve the process of cryopreservation of sperm, it is important to have a better understanding about the cellular and subcellular alterations of sperm induced by freezing and thawing of sperm. Therefore, the objectives of this study were to examine for the first time changes in sperm viability, ROS production and DNA integrity of bovine sperm before and after cryopreservation at different time points and to investigate if alterations of those parameters are related to each other.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich Co. (Steinheim, Germany), unless otherwise indicated.

2.2. Bulls

Ejaculates were collected twice weekly during two weeks from six Simmental bulls, aged 6.3 ± 0.4 years, maintained at the Besamungsverein Neustadt Aisch (Neustadt an der Aisch, Germany). The bulls were on a regular collection schedule, had passed a standard breeding soundness evaluation, and had produced sperm with acceptable postthaw characteristics (progressive motile sperm [PMS] >60%) and fertility (non return rate >65%). Sperm was collected twice weekly, with no apparent changes in animal health or semen quality throughout the semen collection interval.

2.3. Study design

Four ejaculates were collected from each of the six bulls (in total of 24 ejaculates). After sperm dilution, ejaculates were divided into two equal aliquots. The first aliquot was immediately cooled to 4 °C, maintained at that temperature for 24 hours, and then frozen. After cryopreservation, sperm samples were thawed and then examined immediately after thawing (0 hours) as well as after 3, 6, 12, and 24-hours incubation at 37 °C. The second diluted aliquot was examined immediately after dilution (0 hours), and after 3, 6, 12, and 24-hours incubation at 37 °C. The percentage of PMS was determined with a computer assisted sperm analysis system. Sperm quality was evaluated by measuring the percentage of sperm with both plasma membrane and acrosome intact (PMAI), the percentage of sperm with high mitochondrial membrane potential (HMMP), the amount of ROS synthesis of sperm (dichlorofluorescein-diacetate [DCFH-DA], dihydrorhodamine [DHR], diaminofluorescein diacetate [DAF-2DA], mitochondrial superoxide indicator [MITOSOX]), and the percentage of sperm with a high degree of DNA fragmentation (%DFI), using seven flow cytometric assays.

2.4. Semen collection, evaluation, dilution, and preservation

Semen was collected using an artificial vagina (Neustadt/Aisch, Müller, Nürnberg, Germany) and a mount animal. Only ejaculates with 70% greater than progressive motile sperm, estimated subjectively using a phase contrast microscope with × 100 magnification (Dialux 20, Leitz, Wetzlar, Germany), were used. Sperm concentration was determined using a Sperm Quality Analyzer (SQA-Vb, SION A.I. Company, Israel). A portion of each ejaculate was diluted to a final concentration of 60×10^6 sperm/mL with prewarmed (37 °C) AndroMed extender (Minitüb GmbH, Tiefenbach, Germany).

One aliquot was cooled at 4 °C for 24 hours. After 24 hours, sperm were packaged in 0.25 mL French straws (IMV Technologies; L'Aigle, France) at 4 °C using a fully automatic straw filling and sealing machine (MPP Quattro, Fa. Minitub, Tiefenbach, Germany). After filling and sealing, straws were transferred to the freezing chamber and frozen horizontally in racks. Straws were frozen in liquid nitrogen vapor at −95 °C for 9 minutes (NIFA Technologies BV, Leeuwarden, Niederlande). Thereafter, frozen samples were plunged into liquid nitrogen (−196 °C) and stored at least 24 hours before analysis. Twenty straws were frozen from each aliquot, and four straws were thawed for each set of analyses. Frozen samples were thawed by immersing straws in a water bath (37 °C for 30 seconds). Aliquots of the diluted semen were diluted to a concentration of 5×10^6 sperm/mL with prewarmed (37 °C) Tyrode's medium and kept at 37 °C until analyzed.

2.5. Computer assisted sperm analysis

The computer assisted sperm analysis system SpermVision version 3.0 (Spermvision; Minitube, Tiefenbach, Germany) equipped with a phase-contrast microscope (Olympus BX41, Olympus Europe GmbH, Hamburg, Germany) with a motorized microscope stage, a camera (Basler camera A301b, Basler AG, Ahrensburg, Germany), and video adapter (Olympus; magnification: × 0.75) were used. Chambers (20 μm; Leja, Nieuw Vennep, The

Netherlands) were loaded with semen and maintained at 37 °C. Settings were chosen for bull semen according to the manufacturers guidelines (Spermvision standard bulls, AOC < 2.5, STR < 0.5, LIN < 0.35, DSL < 4.5, DAP/Radius ≥ 3 and LIN < 0.5). The PMS was determined by observing greater than or equal to 1000 sperm in at least 10 microscopic fields per sample, with a frequency of 60 frames/s. Mean values of the results of all 10 examinations were determined and used for further analyses.

2.6. Flow cytometric analysis

Flow cytometry was performed using an Epics XL-MLC flow cytometer (Beckman Coulter, Fullerton, CA, USA). Cells were presented to a laser beam generated by a 488-nm (20-mW laser output) argon laser (Laser Components, Olching, Germany). Green fluorescence emissions were measured using a 530 ± 30-nm filter (FL 1), orange fluorescence emissions using a 590-nm filter (FL 2), and red fluorescence using a 650-nm filter (FL 3). Debris (nonsperm events) was gated out on the basis of forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest (Fig. 1). Data were collected from 10 $\times$ 10^3 events and saved as list mode files.

The fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA; green fluorescence)/PI (propidium iodide) dual-staining method was used to assess integrity of the plasma membrane and acrosome (Fig. 2), as described previously [16]. For this, 5 μL of FITC-PNA (100 μg/mL) and 3-μL PI (2.99 mM) were added to 492 μL of diluted sperm suspension.

The lipophilic cation 5,5′,6,6′-tetrachloro1,1′,3,3′-tetramethylbenzimidazolylcarbocyanine iodide (JC-1) was used to assess sperm mitochondrial status. This is a metachromatic stain, with orange and green fluorescence when the mitochondrial membrane potential is high and low (Fig. 3), respectively. In addition, PI was used to

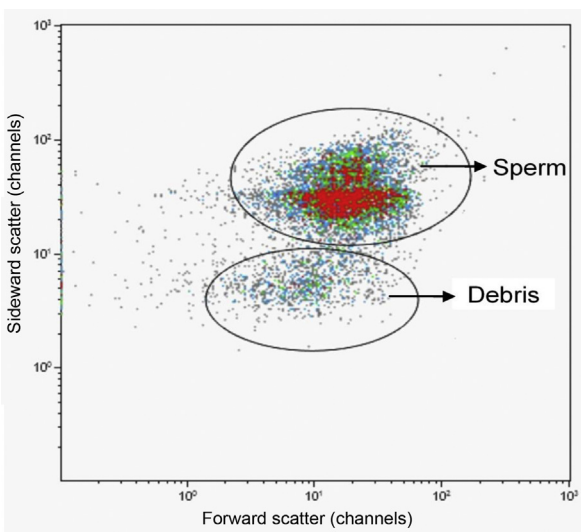

Fig. 1. Reactive oxygen species synthesis of sperm was analyzed after nonsperm events (Debris) were gated out using the characteristics of forward and sideward scatter.

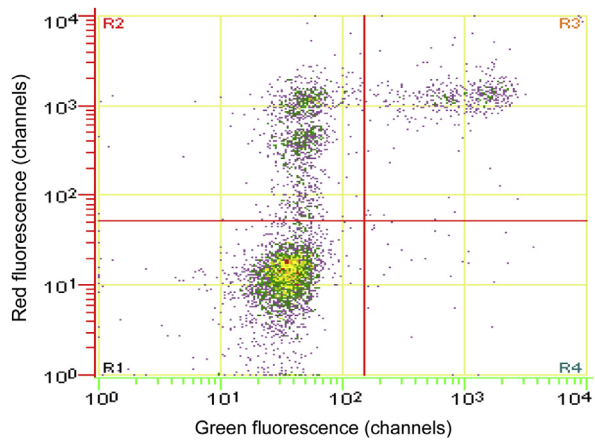

Fig. 2. Dot plot of a flow cytometric examination of bovine sperm after FITC-PNA/PI staining. R1: Plasma membrane and acrosome intact sperm, R2: Plasma membrane damaged sperm without acrosome staining, R3: Plasma membrane damaged sperm with acrosome staining, R4: Plasma membrane intact with acrosome staining. FITC-PNA/PI, fluorescein isothiocyanate–conjugated peanut agglutinin/propidium iodide, FL1, filter 1; FL2, filter 2.

differentiate between sperm with an intact versus damaged plasma membrane. For this, 10 μL of JC-1 (0.153 mM) and 3-μL PI (2.99 mM) were added to 487 μL of diluted sperm suspension.

The DCFH is widely used to measure ROS. The diacetate form of DCFH enters the cell and is hydrolyzed by intracellular esterases to liberate DCFH [17]. Although DCFH-DA is a nonfluorescent agent, it is converted by H_2O_2 into DCFH, which has a green fluorescence. To conduct this assessment, 5 μL of DCFH-DA (10 mM) and 3-μL PI (2.99 mM) were added to 492 μL of diluted sperm.

DHR is commonly used to detect hydroxyl-($OH^{\bullet}$), peroxynitrit ($ONOO^{-}$), nitrogendioxidradicals ($NO_2^{\bullet}$), or peroxidase-derived species [15]. In this assay, DHR is oxidized to Rhodamine 123, which produces green fluorescence. Rhodamine 123 is lipophilic and positively charged and tends to accumulate in mitochondria because of its high membrane potential. For this stain, 5 μL of DHR (40 mM) and 3 μL PI (2.99 mM) were added to 492-μL of diluted sperm.

Nitrogen oxide (NO) was detected and quantified using DAF–2DA, a cell-permeable derivative of DAF-2. On entry into the cell, DAF-2DA is transformed into the less cell-permeable DAF-2 by cellular esterases, thus preventing loss of signal because of diffusion of the molecule out of the cell [18,19]. Thereafter, NO was detected by monitoring emission fluorescence of DAF-2 at 522 nm. This was accomplished by adding 5 μL of DAF-2 DA (10 μM) and 3-μL PI (2.99 mM) to 492 μL of diluted sperm.

Intracellular generation of oxide radicals ($\bullet O_2^{-}$) was estimated using MitoSOX Red, a lipid-soluble, cell-permeable cation selectively targeted to the mitochondrial matrix [20]. The SYTOX Green stain only penetrates cells that have damaged plasma membranes and fluorescence green on binding to DNA. For this assay, 5 μL of MitoSOX Red (2 μM) and 3-μL SyTOX Green (0.05 μM) were added to 492 μL of diluted sperm suspension.

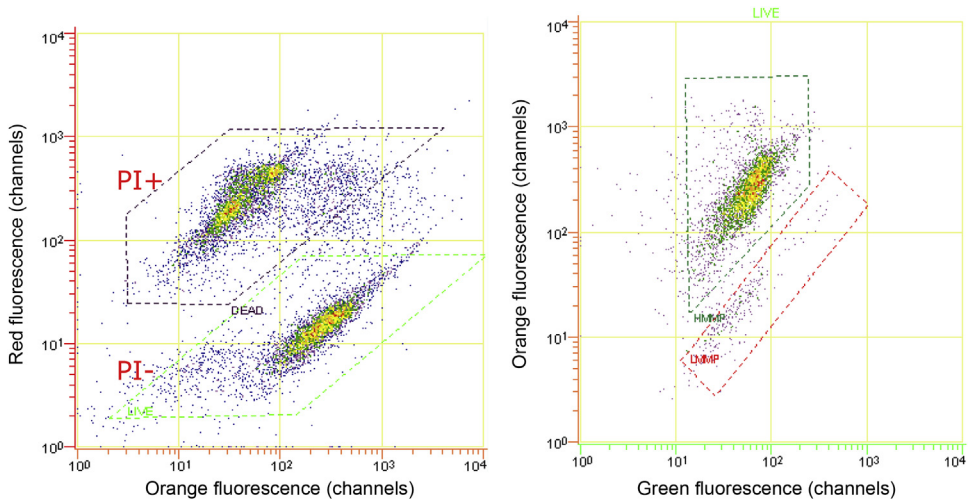

Fig. 3. Evaluation of the percentage of plasma membrane-intact sperm with a high mitochondrial membrane potential after staining with JC-1 and PI. Left panel: Dot plot showing sperm with a low red fluorescence (PI−: plasma membrane intact) and a high red fluorescence (PI+: damaged plasma membrane). Right panel: Dot plot showing plasma membrane-intact sperm (after gating using the dot plot of the left panel) with a low orange fluorescence (LMMP: low mitochondrial membrane potential) and a high orange fluorescence (HMMP: high mitochondrial membrane potential). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

After addition of fluorescence dyes, all sperm samples were incubated at 37 °C for 30 minutes and mixed just before measurement. In that regard, DCFH-DA, DHR, DAF-2DA, and MITOSOX were analyzed using (Fig. 4) EXPO32 ADC XL 4 Color software (Beckman Coulter, Fullerton, CA, USA); FITC-PNA and JC-1 assays were analyzed using FCS Express (De Novo Software, Los Angeles, CA, USA); and sperm chromatin structure assay (SCSA) was evaluated with Data Analysis Software (version 4.19, Germany).

2.7. Sperm chromatin structure assay

The %DFI values were determined by SCSA (Fig. 5), as described [21]. Sperm samples were diluted to a concentration of 2×10^6 sperm/mL with TNE buffer (stock solution; 0.1-M Tris–HCl, 0.15-M NaCl, and 1-mM EDTA, pH 7.4). The stock solution was diluted to 10x with distilled water and stored at 4 °C until measurements were done. The sperm suspension (200 μL) was treated with 400-μL acid-detergent solution (pH: 1.2, 0.08-N HCl, 0.15-M NaCl, and 0.1% Triton X-100) for 30 seconds and then stained with 1.2-mL (6 mg/L) purified acridine orange in a phosphate–citrate buffer (0.2-M Na_2HPO_4, 0.1-M citric acid, 0.15-M NaCl, and 1-mM EDTA, pH 6.0). Samples were examined after 3-minutes incubation. Each sample was examined twice and mean values were used for statistical analyses.

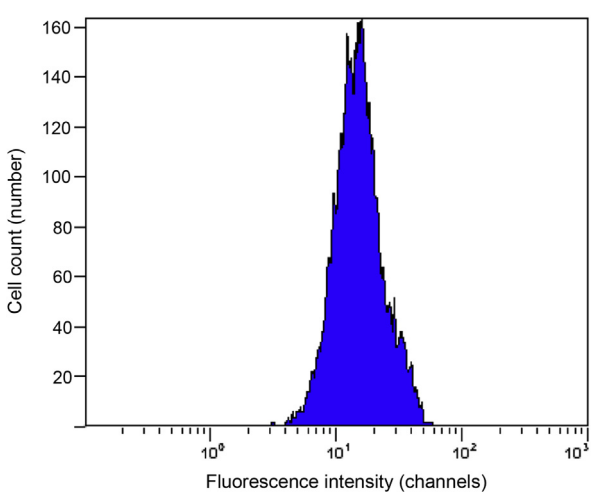

Fig. 4. Histogram for the quantification of mean fluorescence intensity of reactive oxygen species parameters.

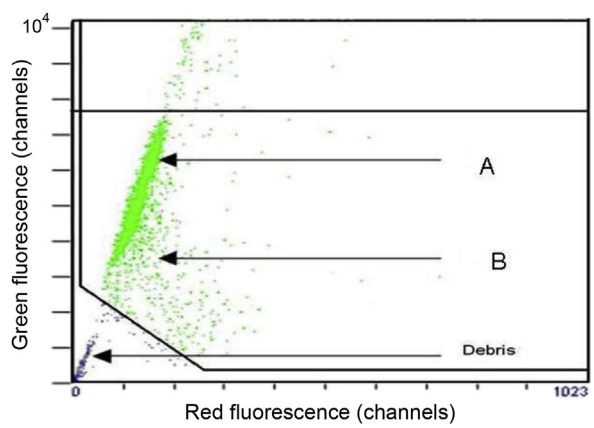

Fig. 5. Scattergram of red versus green fluorescence intensity of sperm examined by Sperm Chromatin Structure Assay. A: Sperm showing a double-stranded DNA with a high green fluorescence and a low red fluorescence fragmented DNA; B: Sperm showing single stranded DNA with a low green fluorescence and a high red fluorescence; Debris (bottom left corner) was excluded from the analyses. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.8. Statistical analyses

Mean value and standard deviation of sperm parameters, conditional on storage conditions and incubation time were calculated to describe the central tendency and dispersion of the data. Growth curve analysis was used to analyze sperm parameters over the course of a 24-hour incubation period. The relation of the outcome variables (PMS, PMAI, HMMP, DCFH, DHR, DAF-2DA, MITOSOX, and %DFI) to incubation time was assumed to be linear, quadratic, or cubic; thus, time curves were modeled using first-, second- and third-order orthogonal polynomials of incubation time. The fixed effect of cryopreservation was added in all time terms. Nonfrozen semen was set as the baseline condition (frozen vs. nonfrozen), and model parameters were estimated to describe the effect of cryopreservation on the rate of change of sperm parameters. Between-ejaculate variability was included as a random effect in a mixed-effects model structure; the intercept and slope of the models were allowed to vary across ejaculates nested within bull. The random effect of the ejaculate (nested within bull) as well as the fixed effects of cryopreservation, incubation time (time polynomials), and their interaction terms were added individually in a hierarchical order in the model structure. The subsequent improvement of model fit was evaluated by model comparisons on the basis of −2 log-likelihood ratio criterion (at 0.05 significance level). Parameter-specific P values were estimated using the normal approximation. The dependent *t* test (at 0.05 significance level) was used for the exploration of differences in sperm parameters between nonfrozen and frozen samples at 0 hours of incubation. Processing of the data, statistical analyses, and graphical illustration of the results were carried out using the nlme [22], lattice [23], and ggplot2 [24] packages in R version 3.1.3 [25].

3. Results

After sperm collection, all ejaculates were estimated with greater than 70% progressive motile sperm during the study period. Descriptive statistics of sperm parameters, in relation to semen storage conditions and incubation time, are presented in Table 1. Initial values of all sperm parameters (at 0 hours of incubation) differed between nonfrozen and frozen samples ($P < 0.05$ in all cases), with exception of DCFH ($t[23] = -0.968$, $P = 0.343$). The relation between outcome variables and time polynomials did not show a variance in intercept across ejaculates ($P > 0.05$ for the variance of intercept across ejaculates, in regard to all sperm parameters) (Table 2). The slope of curves describing the relation of PMS, PMAI, and HMMP with incubation time varied across ejaculates ($P < 0.001$ for the variance of slopes across ejaculates, Table 2) suggests that the rate of change of the previously mentioned sperm characteristics was affected by between-ejaculate variability. On the contrary, slopes of model curves describing the relation of % DFI and ROS-related parameters with the independent variables did not vary significantly across ejaculates ($P > 0.05$; Table 2); thus meaning that the previously mentioned sperm parameters showed a uniform response to incubation time between ejaculates.

Table 1
Changes in viability, DNA integrity, and ROS synthesis in nonfrozen and frozen-thawed sperm during a 24-hour incubation at 37 °C. Values are means ± standard deviation of 24 ejaculates from six bulls (four ejaculates from each bull).

Incubation time (hours)	Nonfrozen sperm					Frozen-thawed sperm				
	0	3	6	12	24	0	3	6	12	24
PMS (%)	73.28 ± 7.59	63.83 ± 8.97	27.91 ± 16.1	8.24 ± 6.82	0.8 ± 0.73	38.23 ± 11.48	32.38 ± 10.46	12.08 ± 8.75	3.64 ± 4.16	0.73 ± 0.38
PMAI (%)	81.37 ± 7.94	73.85 ± 9.44	43.42 ± 19.71	21.74 ± 13.51	7.07 ± 4.09	46.31 ± 11.78	39.8 ± 10.65	19.01 ± 9.95	11.75 ± 7.03	7.27 ± 3.41
HMMP (%)	76.52 ± 7.92	60.91 ± 9.46	25.3 ± 11.69	5.97 ± 3.8	0.79 ± 0.59	42.04 ± 10.12	29.38 ± 7.91	9.46 ± 4.86	2.51 ± 1.47	0.48 ± 0.23
DFI% (%)	0.99 ± 0.26	0.86 ± 0.3	0.88 ± 0.34	0.94 ± 0.24	1.13 ± 0.38	2.04 ± 0.81	3.46 ± 0.99	6.26 ± 2.3	10.41 ± 2.38	15.54 ± 4.17
DAF-2DA	20.75 ± 2.87	22.97 ± 2.18	24.32 ± 2.08	27.25 ± 3.08	30.69 ± 5.13	33.78 ± 2.79	35.79 ± 2.34	44.15 ± 3.5	45.27 ± 3.1	60.07 ± 5.43
DCFH	22.57 ± 3.9	22.32 ± 2.52	23 ± 3.29	22.59 ± 5.52	31.25 ± 10.46	23.35 ± 1.37	23.02 ± 2.85	32.83 ± 2.92	56.18 ± 4.62	80.09 ± 4.28
DHR	93.57 ± 11.05	99.1 ± 19.44	98.89 ± 23.43	112.17 ± 21.95	131.32 ± 11.96	103.4 ± 15.32	103.85 ± 19.55	114.16 ± 16.18	133.29 ± 13.51	141.89 ± 10.33
MITOSOX	56.55 ± 26.42	84.03 ± 28.79	137.42 ± 31.22	125.05 ± 19.91	164.9 ± 28.38	105.77 ± 23.69	159.65 ± 42.76	207.16 ± 71.33	214.15 ± 53.74	247.8 ± 64.37

Abbreviations: DAF-2DA, diaminofluorescein-diacetate; DCFH, dichlorofluorescein; DFI, DNA fragmentation index; DHR, dihydrodamine; HMMP, high mitochondrial membrane potential; MITOSOX, Mitosox; PMAI, Plasma membrane and acrosome intact; PMS, progressive motile sperm.

Table 2
Standard deviation (SD), χ^2, and P values of intercept and slope variance across ejaculates for the highest-order time polynomial applied to describe the relation between each sperm parameter and incubation time. Values were calculated from 24 ejaculates of six bulls (four ejaculates from each bull).

	Highest-order time polynomial	Intercept			Slope		
		SD	χ^2 test	P value	SD	χ^2 test	P value
PMS	2	4.525	0.009	0.925	0.012	38.602	<0.001
PMAI	2	5.972	0.000	1.000	32.713	63.265	<0.001
HMMP	2	3.798	0.009	0.926	0.012	38.602	<0.001
DFI	2	0.698	0.009	0.926	2.231	0.008	0.999
DCFH	2	0.339	0.009	0.926	10.838	0.009	0.999
DHR	1	1.250	0.009	0.926	0.325	2.745	0.254
DAF-2DA	1	6.209	0.668	0.414	0.052	0.003	0.999
MITOSOX	2	16.392	0.008	0.928	1.040	5.284	0.152

1, 2: first-, second-order polynomial, respectively.
Abbreviations: DAF-2DA, diaminofluorescein-diacetate; DCFH, dichlorofluorescein; DFI, DNA fragmentation index; DHR, dihydrodamine; HMMP, high mitochondrial membrane potential; MITOSOX, Mitosox; PMAI, Plasma membrane and acrosome intact; PMS, progressive motile sperm.

Sperm parameters PMS, PMAI, and HMMP were adversely affected by cryopreservation as dictated by the negative estimated coefficients for the fixed effect of cryopreservation (Table 3). Progressively motile sperm, PMAI, and HMMP of frozen samples were significantly lower than those of nonfrozen samples. Moreover, frozen semen showed higher values of %DFI and ROS-related parameters, compared to nonfrozen semen.

Sperm parameters of nonfrozen samples were assumed to have a linear, quadratic, or cubic relation with incubation time. Estimated coefficients and parameter-specific P values of first- and second-order time polynomials are presented in Table 3; none of the sperm parameters showed a cubic relation to incubation time ($P > 0.05$ in all cases), and thus, the respective coefficients are not included in Table 3. Progressively motile sperm, PMAI, and HMMP of nonfrozen semen quadratically decreased over time ($P < 0.001$ for the linear and quadratic time term), whereas %DFI values of these sperm remained unchanged during the 24-hour incubation period ($P > 0.05$ for the linear and quadratic time term). Growth curve model comparisons revealed that the increase of DCFH- and MITOSOX-emitted fluorescence was more adequately modeled as a function of a second-order polynomial, which implies a quadratic increase over time ($P < 0.001$ for the linear and quadratic time term). On the other hand, a positive linear relation was detected between DHR and DAF-2DA levels of nonfrozen semen and incubation time ($P < 0.001$ for the linear time term).

Differences in the rate of change of sperm parameters between nonfrozen and frozen semen were expressed as an interaction term of cryopreservation × polynomial time terms. Estimated coefficients and respective P values are presented in Table 3; the mean values (±standard error of the mean) of the experimental data and the model-predicted time curves of sperm parameters for nonfrozen and frozen samples are demonstrated in Figures 6 and 7. Frozen semen exhibited a slower rate of PMS, PMAI, and HMMP decrease compared to nonfrozen semen. Cryopreservation had a significant effect on the rate of change of %DFI ($P < 0.001$ for the effect of cryopreservation on the linear and quadratic time term; Table 2); unlike nonfrozen semen, %DFI of frozen semen significantly increased with time (Fig. 6). Dichlorofluorescein-diacetate and DAF-2DA levels of frozen samples increased faster compared to nonfrozen sperm (Fig. 7). On the contrary, the rate of change of DHR- and MITOSOX-emitted fluorescence did not differ significantly between nonfrozen and frozen sperm.

4. Discussion

The semen cryopreservation is influenced by several factors and is known to damage sperm in a variety of ways. Such damages lead to a decrease in survival rates, motility, and alterations of the plasma membrane, mitochondria, acrosomes, DNA integrity, and oxidative stress [3–6]. Therefore, it is not surprising that in our study values of sperm parameters PMS, PMAI, and HMMP at 0 hours of incubation were lower in frozen compared to nonfrozen samples. Furthermore, close associations between sperm motility, functional status of sperm mitochondria [16], and sperm plasma membrane integrity and/or function have been described [3–6]. However, it is initially surprising that during the 24-hour incubation time PMS, PMAI, and HMMP decreased faster in nonfrozen compared to frozen sperm samples [7]. This phenomenon could be explained given the higher initial values of these parameters in nonfrozen compared to frozen sperm. On the other side this result also shows that the resistance of these cellular functions against further damage during 24-hour incubation compared to nonfrozen semen is not impaired [26]. After 24 hours of incubation, at 37 °C, the values for PMS, PMAI, and HMMP were almost zero, both in nonfrozen and frozen sperm.

In our study, ROS parameters were increased immediately after thawing of frozen sperm compared to nonfrozen sperm with the exception of DCFH. An increase in ROS synthesis after the freezing and thawing processes has been described in several studies [27–29]. Ferrusola et al. (2009) [30] showed that nitric oxide is produced both in nonfrozen and frozen thawed of equine sperm. They used seven stallions, and nitric oxide values were higher in five stallions in frozen-thawed sperm compared to nonfrozen sperm detected by DAF-2DA staining using flow cytometry. Chatterjee and Gagnon (2001) [3] reported high levels of O_2^- in bull sperm after cryopreservation compared to nonfrozen sperm using spectrometry. Koppers et al. (2008) reported high levels of O_2^- after incubation time of 24 hours by MITOSOX staining using flow cytometry in human

Table 3
Estimated coefficients (±standard error of the mean) and the respective parameter-specific P values for the growth curve polynomial models applied for each sperm parameter. Values were calculated from 24 ejaculates of six bulls (four ejaculates from each bull).

	PMS	PMAI	HMMP	DFI	DAF-2DA	DCFH	DHR	MITOSOX
(Intercept)	34.81 ± 1.21 (P < 0.001)	45.49 ± 1.49 (P < 0.001)	33.90 ± 0.99 (P < 0.001)	0.91 ± 0.23 (P < 0.001)	21.58 ± 0.49 (P < 0.001)	24.09 ± 0.58 (P < 0.001)	92.72 ± 2.79 (P < 0.001)	114.61 ± 5.24 (P < 0.001)
Time	−397.80 ± 14.40 (P < 0.001)	−414.24 ± 14.93 (P < 0.001)	−401.80 ± 12.35 (P < 0.001)	1.23 ± 2.58 (P = 0.632)	0.40 ± 0.04 (P < 0.001)	49.13 ± 8.75 (P < 0.001)	1.58 ± 0.18 (P < 0.001)	535.11 ± 61.26 (P < 0.001)
$Time^2$	193.12 ± 11.89 (P < 0.001)	146.81 ± 14.62 (P < 0.001)	220.91 ± 9.62 (P < 0.001)	1.00 ± 1.65 (P = 0.544)	na	25.58 ± 6.43 (P < 0.001)	na	−177.25 ± 53.63 (P < 0.001)
Condition: Cryopreserved	−17.40 ± 1.09 (P < 0.001)	−20.66 ± 1.18 (P < 0.001)	−17.12 ± 0.85 (P < 0.001)	6.69 ± 0.24 (P < 0.001)	12.59 ± 0.68 (P < 0.001)	19.05 ± 0.75 (P < 0.001)	10.90 ± 2.92 (P < 0.001)	71.70 ± 5.86 (P < 0.001)
Time × Cryopreserved	196.01 ± 16.81 (P < 0.001)	206.68 ± 18.34 (P < 0.001)	194.32 ± 13.19 (P < 0.001)	74.74 ± 2.51 (P < 0.001)	0.67 ± 0.06 (P < 0.001)	288.40 ± 9.25 (P < 0.001)	0.17 ± 0.23 (P = 0.477)	153.27 ± 79.83 (P = 0.056)
$Time^2$ × Cryopreserved	−83.02 ± 16.81 (P < 0.001)	−38.63 ± 18.34 (P = 0.036)	−86.64 ± 13.19 (P < 0.001)	−10.39 ± 2.27 (P < 0.001)	na	−39.48 ± 8.65 (P < 0.001)	na	−137.90 ± 76.58 (P = 0.073)

na: term not included in the structure of the final model.
Abbreviations: DAF-2DA, diaminofluorescein-diacetate; DCFH, dichlorofluorescein; DFI, DNA fragmentation index; DHR, dihydrodamine; HMMP, high mitochondrial membrane potential; MITOSOX, Mitosox; PMAI, Plasma membrane and acrosome intact; PMS, progressive motile sperm.

sperm. One reason for the high ROS levels is the low antioxidative capacity of sperm as they discard most of their cytoplasm-containing antioxidants during the terminal stages of differentiation and therefore lack a significant cytoplasmic component that counteract the damaging effect of ROS. Dichlorofluorescein-diacetate values were not increased directly after cryopreservation. It can be speculated that the synthesis of hydrogen peroxide may begin not already immediately after thawing. The primary product of the spermatozoa system of generating free radicals appears to be the superoxide anion ($O_2\cdot-$), hydroxyl- (OH•), peroxynitrit (ONOO−), nitrogendioxide radicals (NO2•), which secondarily dismutate to H_2O_2 [3,31,32]. This might be the reason, why there was a delayed increase of H_2O_2 in frozen-thawed sperm compared to the other ROS.

In our study, DFI values were increased after cryopreservation. Several other studies have already shown that the cryopreservation process causes DNA damage to mammalian sperm in human [33,34], boar [35], bull [36], and ram [37]. There is strong evidence indicating that the freezing–thawing process induces oxidative stress associated with free radical–mediated damage to sperm nuclear DNA [27,28]. Amiri et al. (2007) [38] reported that high NO levels can cause DNA damage. A significant positive correlation between high semen oxidative stress levels depending on superoxide anion unbalance and an increase in sperm DNA fragmentation was reported in human sperm [39]. Sperm DNA damage induced by the hydroxyl radical is associated with nucleotide damage of the 8-OHdG type [14]. Because of the lack of functional DNA repair mechanisms [40], it is possible that frozen-thawed sperm are more vulnerable to oxidative attack compared to other cells [41].

All ROS parameters increased during incubation time before and after cryopreservation. These increases with incubation time were similar for NO, O2−, ONOO−, NO2•, and $OH^{\bullet}$ before and after cryopreservation. However, the rise was much more remarkable for H_2O_2 during incubation time in frozen compared to nonfrozen sperm. The reason for this distinct increase of H_2O_2 could be the reduced activity of glutathione peroxidase (GPx) after cryopreservation [42]. Because of the lower activity of this antioxidative enzyme, H_2O_2 could not be converted properly to H_2O after cryopreservation. The resistance to peroxidation of bovine spermatozoa seemed to be attributed to a factor in seminal plasma [43,44]. Bilodeau et al. (2000) [42] reported low levels of GPx and an absence of catalase, which reduce H_2O_2 to H_2O and O_2 in frozen-thawed bovine spermatozoa.

In our study, we did not find time-dependent changes in DNA damage of nonfrozen sperm, whereas ROS showed already a time-dependent rise before cryopreservation. Therefore, our results indicate that ROS can only affect DNA integrity, if this is already disturbed by other factors occurring during the processes of freezing and thawing. Like other cells, spermatozoa undergo chemical, osmotic, thermal, and mechanical shocks during freezing and then again during thawing [45,46]. A recent study performed by Johnston et al. (2012) [47] has demonstrated that osmotic changes provoke chromatin relaxation and DNA fragmentation in marsupial sperm. These authors showed that the water flux into the nucleus and general ice crystal damage to the sperm cell

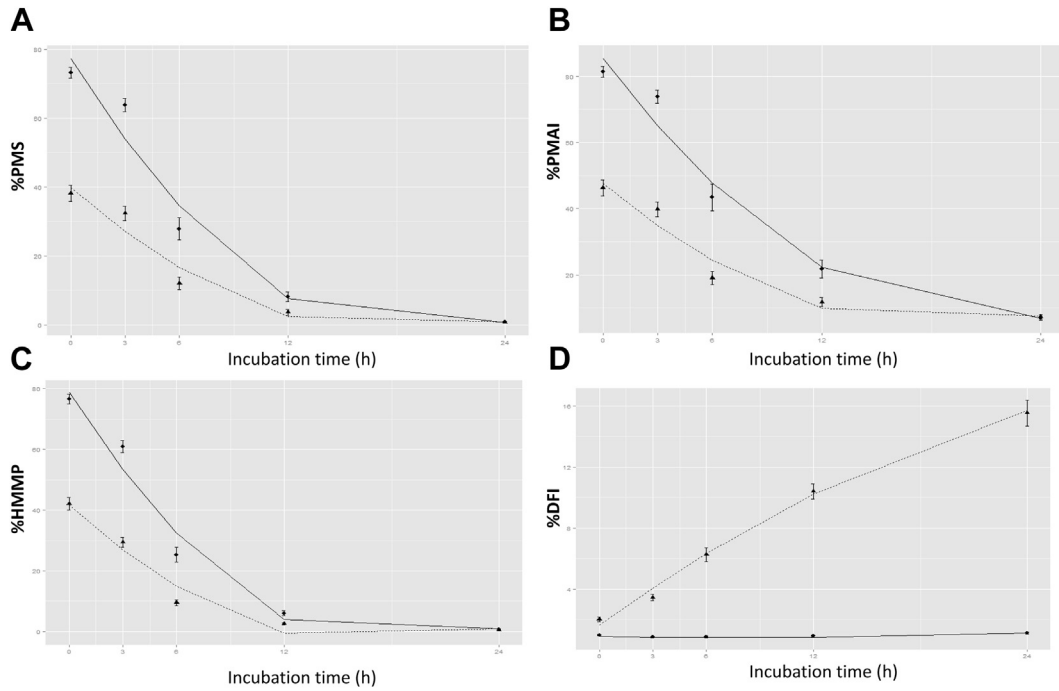

Fig. 6. Mean values (±standard error of the mean) and model-predicted time curves of sperm parameters (PMS, PMAI, HMMP, and DFI) conditional on semen storage (nonfrozen vs. frozen-thawed semen). In each of six bulls, four ejaculates were examined. ———: Before cryopreservation, ·····: After cryopreservation. DFI, DNA fragmentation index; HMMP, high mitochondrial membrane potential; PMAI, plasma membrane and acrosome intact; PMS, progressive motile sperm.

could result in a subsequent physical tearing of the tertiary structure of the DNA–protamine complex, leading to an increase in the incidence of relaxed chromatin.

Interestingly, the rise of the parameter for DNA damage (%DFI) was very similar to the increase H_2O_2 (DCFH) but different to those of the other ROS parameters in frozen

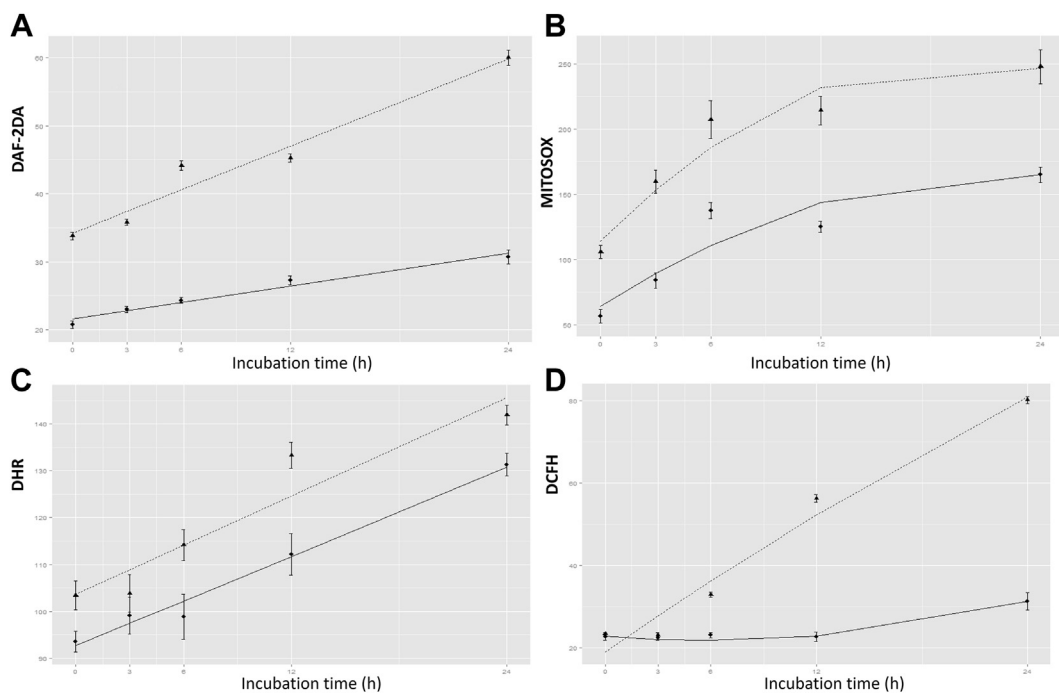

Fig. 7. Mean values (±standard error of the mean) and model-predicted time curves of sperm parameters (DAF-2DA, MITOSOX, DHR, and DCFH) conditional on semen storage (nonfrozen vs. frozen-thawed semen) In each of six bulls, four ejaculates were examined. ———: Before cryopreservation, ·····: After cryopreservation. DAF-2DA, diaminofluorescein-diacetate; DCFH, dichlorfluorescein; DHR, dihydrodamine; MITOSOX, Mitosox.

sperm. This result indicates that from the investigated ROS most likely H_2O_2 is responsible for further damage of DNA after cryopreservation. Hydrogen peroxide is a known genotoxic agent that causes DNA strand breakage [35]. Addition of increasing concentrations of H_2O_2 to human sperm suspensions caused DNA oxidation similar to endogenous higher concentrations of H_2O_2 [48]. Ball et al. (2000) [49] also reported that H_2O_2 is the major ROS responsible for damage to equine spermatozoa.

In addition, the decreases in PMS, PMAI, and HMMP were not related to DNA damage before and after freezing. Januskauskas et al. (2001) [50] and Kasimanickam et al. (2006) [51] reported a positive relationship between DFI and PMAI. One reason for these contradictory results could be the different extender used. Although Januskauskas et al. (2001) [50] and Kasimanickam et al. (2006) [51] carried out their investigations on semen extended in the egg-yolk medium, we used a custom-made extender containing soy-bean. Karabinus et al. (1991) [52] have shown differential extender effects on integrities of the plasma membrane and DNA. Giwercman et al. (2003) [53] reported a moderate correlation between sperm motility and SCSA parameters. Furthermore, this study supports the assumption that both SCSA and motility can be relatively independent predictors of male fertility. Bollwein et al. (2008) [54] reported no significant relationship between HMMP and %DFI directly after thawing but a negative relationship between these parameters after 3-hours incubation. Those results indicate that there is no relationship between the susceptibility of the DNA and the mitochondria of spermatozoa to the freezing-thawing process, but there is some interdependency between DFI and the loss of the mitochondrial function during the incubation time. It is well-known that the sperm parameters of PMS, PMAI, HMMP, and DFI have a pivotal importance for fertility. In accordance with our results it can be speculated that PMS, PMAI, HMMP, and DFI may have independent predictors of bull fertility.

Furthermore, %DFI was much lower than those of PMS, PMAI, and HMMP. This is in accordance with the findings of others [50,52,54] because bovine sperm DNA is organized in a specific manner that keeps the nuclear chromatin compact, stable, and protected [26].

Initial values of all tested parameters showed no significant variation across ejaculates in frozen sperm. In contrast, the response of PMS, PMAI, and HMMP to incubation time significantly varied between ejaculates, which implies differences in viability to incubation time between ejaculates. Similar variabilities between ejaculates within bulls were not detected for %DFI and the ROS-related parameters. The absence of variation between ejaculates in ROS changes could be related with antioxidant capacity of seminal plasma. Gürler et al. (2015) [55] reported that total antioxidant capacity varied among bulls but not between ejaculates within bulls. It can be speculated that this constant total antioxidant capacity of seminal plasma within animals is responsible for the absence of variability in ROS synthesis of sperm. This constant ROS synthesis between ejaculates might also be responsible for absence of the variation in DNA integrity between the ejaculates within the bulls. The positive effect of antioxidants in semen on DNA integrity has been reported in several studies [8,56–58]. However, PMS, PMAI, and HMMP showed different time-dependent alterations between ejaculates within bulls. This has also been shown in another study [59]. These parameters depend not only on the content of antioxidants but are also related to other factors like osmolarity [60] and protective proteins in seminal plasma [61].

In conclusion, the results show that cryopreservation has a negative effect on DNA integrity. Changes in DNA damage in sperm are related to H_2O_2 but not to alterations in other ROS and viability of sperm before and after cryopreservation of sperm.

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