Unravelling how in vitro capacitation alters ram sperm chromatin before and after cryopreservation

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Peris-Frau, P., Alvarez-Rodriguez, M., Martin-Maestro, A., Iniesta-Cuerda, M., Sanchez-Ajofrin, I., Medina-Chavez, D. A., Garde, J. J., Villar, M., Rodriguez-Martinez, H., Soler, A. J., (2020), Unravelling how in vitro capacitation alters ram sperm chromatin before and after cryopreservation, *Andrology*. <https://doi.org/10.1111/andr.12900>

Original publication available at:

<https://doi.org/10.1111/andr.12900>

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Journal:	<i>Andrology</i>
Manuscript ID	ANDR-2020-0031.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Major Area:	Basic Science
Clinical:	Assisted Reproduction
Basic Science:	Cell Biology
Specific Topics:	Spermatozoa

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1 Unraveling how *in vitro* capacitation alters ram sperm chromatin
2 before and after cryopreservation

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13 **Running title:** Chromatin changes during capacitation in ram spermatozoa
14
15 **Keywords:** chromatin condensation, DNA methylation, cryopreservation, capacitation,
16 ram spermatozoa
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ABSTRACT

Background: Sperm chromatin structure provides valuable information for the prediction of male fertility and can be altered during different procedures. Previous studies have shown that sperm chromatin condensation decreased during *in vitro* capacitation. Moreover, cryopreservation can affect sperm DNA integrity and chromatin compaction.

Objectives: This study aimed to investigate dynamic modifications produced in the chromatin structure of ram spermatozoa during *in vitro* capacitation before and after cryopreservation.

Materials and methods: Chromatin decondensation (AB+), DNA methylation, DNA fragmentation index (%DFI) and high DNA stainability (HDS) were evaluated in fresh and frozen-thawed ram spermatozoa incubated under capacitating (CAP) conditions at 1, 5, 15, 30, 60, 120, 180 and 240 min and under non-capacitating (NC) conditions at 0, 15 and 240 min.

Results: Incubation in NC conditions did not induce significant changes in chromatin condensation ($P > 0.05$; AB+ and HDS). However, incubation of fresh and cryopreserved ram spermatozoa under CAP conditions significantly increased chromatin decondensation ($P < 0.05$), reaching the highest percentage of AB+ and HDS from 180 to 240 min in fresh samples and from 5 to 30 min in cryopreserved samples. Both variables (HDS and AB+) were positively correlated with tyrosine phosphorylation, total motility, progressive motility, curvilinear velocity, and amplitude of lateral head displacement, as well as between them under CAP conditions in fresh and cryopreserved spermatozoa. DNA methylation significantly increased in cryopreserved spermatozoa ($P < 0.05$), but only after extended incubation under CAP

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3 50 conditions (60-240 min), while the %DFI, albeit higher in cryopreserved samples,
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5 51 remained constant under CAP and NC conditions in both types of sample ($P > 0.05$).
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8 52 **Discussion and conclusions:** Our results suggest that sperm chromatin condensation
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10 53 decreased progressively during *in vitro* capacitation of ram spermatozoa, while sperm
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12 54 DNA integrity remained intact. Such changes in chromatin condensation appeared faster
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15 55 after sperm cryopreservation.
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19 57 **INTRODUCTION**
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21 58 Recent discoveries have shown that the contribution of spermatozoa to offspring
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23 59 includes, besides the paternal genome, a complex set of RNAs, proteins and epigenetic
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25 60 marks such as DNA methylation and histone post-translational modifications.¹⁻³
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27 61 Moreover, the small nucleohistone fraction retained in mature spermatozoa, which is
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29 62 transmitted to the progeny, is associated with specific loci involved in embryogenesis.⁴⁻
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31 63 ⁶ Consequently, any significant perturbation in the sperm chromatin structure could lead
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33 64 to infertility, fertilization failure, spontaneous abortion, abnormal embryo development
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35 65 or even cause different disorders in the offspring.⁷⁻¹¹
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40 66 Mature spermatozoa have a highly compacted chromatin to protect paternal
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42 67 genetic and epigenetic inheritance during the spermatozoon's journey to the oocyte.
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44 68 Many histones are replaced by protamines during spermatogenesis, which increase the
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46 69 packaging and condensation of sperm chromatin.¹² This repackaging process finally
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48 70 ends in the formation of disulphide bonds between protamines during epididymal
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50 71 maturation.¹³
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53 72 Several studies have shown that abnormalities in sperm chromatin condensation
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55 73 may affect the fertilizing potential of spermatozoa.¹⁴⁻¹⁶ Furthermore, sperm chromatin
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57 74 must be completely decondensed at the appropriate velocity and time in the fertilization
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process for successful formation of the male pronucleus and zygote.¹⁷ Recently, the ability of human spermatozoa to decondense *in vitro* has been related with embryo quality, obtaining better embryo quality when sperm chromatin decondenses faster.¹⁸ This finding highlights the relevance of optimal chromatin decondensation for proper embryo development.

Some authors have investigated whether the acquisition of sperm fertilizing ability through the physiological process known as capacitation^{19,20} might prepare the sperm chromatin for optimal remodelling after oocyte penetration. In boar spermatozoa, a reduction in disulphide bonds between protamines was observed under capacitating conditions,²¹ while in bull and human spermatozoa, the degree of chromatin condensation decreased during *in vitro* capacitation without affecting DNA integrity.^{22–24} Such changes in nuclear compaction could facilitate the events surrounding and following fertilization, as has been suggested by De Lamirande et al.²² In line with these findings, Pérez-Cerezales et al.²⁵ demonstrated that a specific subpopulation of capacitated spermatozoa was selected by *in vitro* thermotaxis in humans and mice. These selected spermatozoa showed less condensed chromatin and greater DNA integrity than unselected spermatozoa, yielding better cleavage and pregnancy rates when used for intracytoplasmic sperm injection (ICSI) in mice. In ram spermatozoa, studies on the effect of capacitation on the chromatin structure are still required.

On the other hand, cryopreservation is another process that may affect the structure of sperm chromatin and at the same time accelerate sperm capacitation.^{26–32} Some studies revealed that cryopreservation reduced disulphide bonds between nuclear proteins in boar spermatozoa,³³ increased sperm DNA methylation in stallion³⁴ and modified the chromatin stability of diverse species due to membrane damage.³⁵

However, to the best of our knowledge, there is a lack of studies regarding the effect of cryopreservation and subsequent *in vitro* capacitation on sperm chromatin structure.

In the present study, sperm chromatin condensation, DNA integrity and DNA methylation were evaluated in fresh and cryopreserved ram spermatozoa during the time course of *in vitro* capacitation to verify whether capacitation induces chromatin changes in ram spermatozoa and how cryopreservation may affect these nuclear changes associated with sperm capacitation. We hypothesised that partial chromatin decondensation might take place during capacitation to ensure proper sperm chromatin remodelling after oocyte penetration, occurring in cryopreserved ram spermatozoa faster than in fresh spermatozoa and without inducing DNA damage.

MATERIALS AND METHODS

Semen collection and cryopreservation

Semen was collected at one-week intervals by artificial vagina from four mature Manchega rams (4-5 years old) housed at the UCLM experimental farm. The procedure was carried out by the UCLM Reproduction Biology Group which is officially authorized for collecting and storing sheep semen (ES07RS02OC), following RD 841/2011. The experiment was repeated three times using one pool of four ejaculates each time to avoid individual variability. A total of three ejaculates from each ram, with a minimum of 65% sperm motility and a wave motion value of 3.5, were used in the study. Sperm concentration ranged from 3,560 to 4,105 x 10⁶ sperm/ml and was calculated using a Makler counting chamber and the Sperm Class Analyzer software (SCA[®]) (Microptic, Barcelona, Spain).

Each pool was divided into two fractions, one processed as fresh semen, and the other cryopreserved in straws with Biladyl[®] (Minitube; Tiefenbach, Germany) and

thawed in a water bath at 37 °C for 30 s. Both fractions were processed at 30 °C until the incubation period.

For cryopreservation, semen diluted to 200×10^6 sperm/ml in Biladyl® (with 20% egg yolk and 7% glycerol) was slowly cooled from 30 °C (initial temperature) to 5 °C for 2 h and subsequently equilibrated at 5 °C for 2 h. Semen samples were then automatically packed into 0.25 ml straws, frozen in a programmable biofreezer (Planer Kyro 10 Series III; Planer PLC, London, United Kingdom) following a freezing curve (-20 °C/min from 5 °C to -100 °C and -10 °C/min from -100 °C to -140 °C) and stored in liquid nitrogen.

Sperm preparation and experimental design

Fresh and frozen-thawed semen were centrifuged through single columns of Percoll® 45% (Sigma-Aldrich; Madrid, Spain) (700 x g; 10 min) at room temperature (30 °C) to remove seminal plasma, extenders and debris. After centrifugation, sperm pellets were resuspended to a final concentration of 10×10^6 sperm/ml in synthetic oviductal fluid (SOF)³⁶ supplemented with 0.1% of polyvinyl alcohol (PVA) for non-capacitating conditions (NC) or in SOF supplemented with 2% of oestrous sheep serum³⁷⁻³⁹ for capacitating conditions (CAP). Then, fresh and cryopreserved spermatozoa were incubated up to 240 min in NC or CAP medium at 38.5 °C under 5% CO₂. *In vitro* capacitation of ram spermatozoa was confirmed by assessing the global increase in tyrosine phosphorylated proteins that characterizes this event (described below).^{40,41}

Chromatin decondensation (AB+), DNA methylation, DNA fragmentation index (%DFI) and high DNA stainability (HDS) were the nuclear variables evaluated in fresh and cryopreserved spermatozoa after 1, 5, 15, 30, 60, 120, 180 and 240 min under CAP conditions and after 0, 15 and 240 min under NC conditions. At the same time intervals,

mitochondrial activity, ROS production, apoptosis, protein tyrosine phosphorylation (pY) and diverse kinematic variables were investigated in fresh and cryopreserved spermatozoa to verify whether these variables were related to the nuclear variables evaluated under CAP and NC conditions, or not.

Aniline blue (AB) staining

Mature spermatozoa have a highly compacted chromatin after the replacement of many histones by protamines during spermatogenesis. Any change in the condensation of chromatin can be detected by aniline blue (AB) due to its ability to selectively stain lysine residues of persisting histones. This cytochemical stain has frequently been used to identify sperm samples from different individuals showing chromatin immaturity or lower chromatin condensation due to abnormal histone retention. However, reorganization of the sperm chromatin structure during physiological events, such as sperm capacitation, could also make histones more accessible to AB, revealing changes in chromatin condensation.²² Spermatozoa with a loose chromatin packaging are stained with AB because of their high histone levels, while spermatozoa with a tight chromatin packaging are not stained with AB because of their low histone levels.⁴² To assess aniline blue staining, air-dried smears (15 μ l sperm/sample) obtained at different incubation times were fixed with 3% (v/v) glutaraldehyde in PBS (pH 7.2) for 30 min. Then, slides were stained with 5% (v/v) aqueous aniline blue (Sigma-Aldrich) in 4% (v/v) acetic acid (pH 3.5) for 10 min, washed with water, allowed to dry and mounted with DPX (Sigma-Aldrich). As a positive control for chromatin decondensation, an air-dried smear from spermatozoa obtained at 0 min was incubated with 1% (w/v) SDS and 1 mM DTT for 15 min before following the staining protocol. Using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan), 300 spermatozoa were assessed per sample at 60x magnification. Spermatozoa

with unstained nuclei (AB-) indicated normal chromatin condensation while those blue stained indicated chromatin decondensation (AB+) (**Figure 1**). Results were expressed as the percentage of spermatozoa with AB+ heads.

Sperm chromatin structure assay (SCSA®)

Changes in chromatin integrity were evaluated by the sperm chromatin structure assay (SCSA®). This flow cytometry test measures the susceptibility of sperm DNA to acid-induced denaturation using the metachromatic properties of acridine orange (AO), a dye that emits green fluorescence when bound to intact DNA (double-stranded DNA) and red fluorescence when bound to denatured DNA (single-stranded DNA, obtained after denaturation of DNA at the sites of strand breaks). The metachromatic shift of AO is captured as fluorescence intensity cytogram patterns and then processed to obtain the extent of DNA fragmentation for each spermatozoon. SCSA® was performed following the procedure described by Evenson et al.⁴³ Samples taken at different time points of incubation were diluted to 2×10^6 cells/ml with TNE buffer and mixed with 400 µl of an acid-detergent solution for 30 s. Then, 1.2 ml of AO was added, and samples were evaluated 2 min later with a Gallios™ flow cytometer (Beckman Coulter; Bromma, Sweden). AO was excited with a 488nm argon laser. Red fluorescence was detected with FL3 (655SP; 620/30) and green fluorescence with FL1 (550SP; 525BP). FL1 and FL3 were adjusted to 475 ± 5 and 125 ± 5 , respectively, with a standard sample before running all the sperm samples. A total of 5,000 spermatozoa per sample were evaluated. Flow cytometry data (FCS) were acquired by the Navios software (Beckman Coulter; Bromma, Sweden) and processed by WEASEL v. 3.2 (WEHI; Melbourne, Australia). Further analyses of FCS using R software (package rflowcyt – Bioconductor) were done to obtain the DNA fragmentation index (DFI) in each sample, which is the ratio of red to total fluorescence intensity (red + green), the percentage of spermatozoa with a DFI

superior to 25% (%DFI) and the percentage of spermatozoa with high DNA stainability (HDS; spermatozoa with green fluorescence intensity above 600 in FL1).

5-Methylcytosine immunostaining

Variations in sperm DNA methylation were investigated by immunostaining.

The immunoassay was performed following the procedure described by Benchaib et al.⁴⁴, but with some modifications. Briefly, air-dried smears (15 µl sperm/sample) obtained at different incubation times were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) in PBS for 30 min. After washing twice with 0.5% (v/v) Tween 20 (Sigma-Aldrich) in PBS (PBS-T), DNA decondensation was carried out by incubation in 1 M Tris-HCl buffer (pH 9.5) supplemented with 25mM dithiothreitol (DTT) at room temperature (RT) for 20 min. Slides were washed three times with PBS-T and sperm DNA was denatured after 25 min with 6N HCL. Slides were then washed three times with PBS-T and incubated at RT in blocking buffer (Odyssey® blocking buffer; LI-COR Biosciences, Lincoln, NE, USA). After 1 h, spermatozoa were incubated with a monoclonal anti-5-methylcytosine antibody (Ab 10805; Abcam, Cambridge, UK) diluted 1:500 in blocking buffer at 4°C in a humidified chamber overnight. The slides were then washed in PBS-T and incubated with Alexa fluor488 labelled Goat anti-mouse IgG (Ab150113; Abcam, dilution 1:500 in blocking buffer) in a humidified chamber for 1 h. Spermatozoa incubated with buffer instead of the primary antibody were used as a negative control. The samples were washed with PBS-T, allowed to dry and mounted with anti-fade solution (Slowfade® Diamond; Life technologies, Linköping, Sweden). From each sample, 300 spermatozoa were analysed with a fluorescence microscope at 100x magnification (Eclipse Ti; Nikon). Spermatozoa showing green fluorescence at their heads were considered methylated (**Figure 2**) and results were expressed as the percentage of spermatozoa with DNA methylation.

Sperm kinematics

Diverse kinematic variables, including total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; $\mu\text{m s}^{-1}$), linearity (LIN; %) and amplitude of lateral head displacement (ALH; μm) were assessed at different time points using a computer-assisted sperm analysis (CASA) system. Mean values were depicted in **Table S1**. TM was defined as the percentage of spermatozoa with a VCL above $10 \mu\text{m s}^{-1}$, and PM as the percentage of spermatozoa with a VCL above $25 \mu\text{m s}^{-1}$ and straightness (STR) above 80%. Samples ($5 \mu\text{l}$ sperm/sample) were loaded on a pre-warmed (37°C) Makler counting chamber and examined in a phase contrast microscope (Nikon Eclipse 80i) equipped with a warmed stage (37°C) and a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany) connected to a computer by an IEEE 1394 interface. Images were captured and further analysed using SCA[®] software. At least ten fields or 400 spermatozoa were recorded and assessed in each sample. Settings were adjusted to ram spermatozoa (25 frames/s, $20\text{--}90 \mu\text{m}^2$ for head area and $\text{VCL} > 10 \mu\text{m s}^{-1}$ to classify spermatozoa as motile).

Mitochondrial activity, ROS production and apoptosis

Several sperm parameters (mitochondrial activity, ROS production and apoptosis) were evaluated at different incubation times by flow cytometry. Samples were diluted to 5×10^6 sperm/ml in SOF-PVA-HEPES and stained using combinations of two fluorochromes. Mitochondrial activity was evaluated with 25 nM of YO-PRO-1 and 100 nM of Mitotracker Deep Red; ROS production with $12 \mu\text{M}$ of PI and $5 \mu\text{M}$ of CM- H_2DCFDA and apoptosis with $15 \mu\text{M}$ of PI and 50 nM of YO-PRO-1. Apoptosis and ROS production were immediately assessed with a FlowSight[®] flow cytometer (Amnis; Merck-Millipore, Germany) controlled with the INSPIRE[®] software (v.3), while mitochondrial activity was evaluated after incubating sperm samples for 15 min

in the dark at 37 °C. All fluorochromes (Invitrogen; Barcelona, Spain) were excited with a 488 nm solid state laser (20 mW intensity), except Mitotracker Deep Red, which was excited with a 642 nm solid state laser (5 mW intensity). YO-PRO-1 and CM-H₂DCFDA fluorescence were detected on channel 2 (505-560 nm) while PI fluorescence was detected on channel 4 (595-642 nm) and Mitotracker Deep Red on channel 11 (642-740 nm). A dot plot with the parameters aspect ratio versus area was used to discriminate debris from spermatozoa during acquisition. The aspect ratio was adjusted to ≤ 0.35 and area to $\geq 50 - \leq 400 \mu\text{m}^2$ to acquire events compatible with ram sperm morphology. A total of 5,000 spermatozoa were evaluated per sample. Raw data were analysed with the IDEAS[®] software to obtain the proportion of apoptotic spermatozoa (YO-PRO-1+/PI-) and the proportion of viable spermatozoa with active mitochondria (Mitotracker+/- YO-PRO-1-) by scatter plots, and the mean fluorescence intensity (MFI) of CM-H₂DCFDA by histograms. This latter variable indicates ROS production in viable spermatozoa (PI-). Mean values were depicted in **Table S2**.

Protein tyrosine phosphorylation (pY)

Global protein tyrosine phosphorylation (pY) was assessed with the FlowSight[®] flow cytometer following the procedure described by Peris-Frau et al.²⁹ Briefly, samples collected at different incubation times were fixed with 2% (v/v) paraformaldehyde in PBS at RT for 10 min, washed and resuspended in 0.1% (v/v) BD FACST[™] Permeabilizing Solution for 10 min at RT. Spermatozoa were then washed and blocked with 10 % (w/v) BSA in PBS (blocking buffer) for 30 min at 38.5 °C. After washing again, spermatozoa were incubated with anti-phosphotyrosine monoclonal antibody Clone 4G10 (1:300 dilution in blocking buffer) for 1 h at 38 °C. Subsequently, samples were incubated with FITC-conjugated anti-mouse IgG antibody (1:300 dilution in blocking buffer) for 1 h in the dark at 38 °C and run through the flow cytometer. A

negative control was obtained by incubation with IgG1-FITC (isotype from murine myeloma, clone MOPC 21; 1:300 dilution) instead of the primary antibody. **At least 5,000 spermatozoa per sample were analysed and** the percentage of spermatozoa showing green fluorescence (tyrosine-phosphorylated spermatozoa) was recorded in each sample (**Figure S1**).

Statistical analysis

Statistical analyses were performed using SPSS v. 23.0 (IBM corp., Chicago, USA). Data were checked for normality by the Shapiro–Wilk test and logarithmic transformation was applied when required. Three biological replicates per sample were assessed. **AB+, DNA methylation, %DFI, HDS, apoptosis, mitochondrial activity, pY, TM, PM, VCL, LIN, ALH and ROS production were evaluated by a factorial ANOVA. The statistical model included the effect of treatments (incubation times in NC and CAP conditions), type of sample (fresh or cryopreserved spermatozoa) and replicates, as well as the interaction between treatments and type of sample. After obtaining significant interactions ($P < 0.05$) between the treatments and the type of sample in the different parameters studied, these factors were studied separately for a better interpretation of the main effects. When main effects were significant ($P < 0.05$), *post hoc* comparisons with Bonferroni correction were carried out. Values were expressed as non-transformed least square means $\pm$ SEM. Although the different incubation times in CAP and NC were statistically analysed all together to determine the significance between them, the results obtained during the incubation period under CAP conditions were divided from the results obtained under NC conditions in the different figures for a better visualization of the dynamics changes produced during CAP or NC conditions.**

Pearson correlation coefficients were calculated to estimate whether there were any association between the different sperm parameters evaluated. Correlations included

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299 all incubation times under CAP or NC conditions but were calculated separately for
300 fresh spermatozoa incubated under NC or CAP conditions and for cryopreserved
301 spermatozoa incubated under the same conditions. Significance was set to $P < 0.01$ for
302 correlations to avoid error type 1 due to the large number of correlations calculated at
303 once.

304 **RESULTS**

305 **Aniline blue staining only increased during incubation of fresh and cryopreserved**
306 **spermatozoa under capacitating conditions**

307 Incubation under NC conditions did not have any significant effect on chromatin
308 condensation in either fresh or cryopreserved spermatozoa over time (**Figure 3**). Under
309 CAP conditions, however, chromatin condensation decreased at certain incubation
310 times. The highest ($P < 0.05$) percentages of spermatozoa with decondensed chromatin
311 (AB+) were recorded between 5 and 30 min in cryopreserved samples and between 180
312 and 240 min in fresh samples. Additionally, significant differences were observed
313 between fresh and cryopreserved spermatozoa after 120 min under CAP conditions
314 (**Figure 3**).

315 **Chromatin integrity was not compromised during incubation of fresh and**
316 **cryopreserved spermatozoa under capacitating and non-capacitating conditions**
317 **but HDS increased during capacitation**

318 Neither media nor incubation time altered chromatin integrity, since the %DFI
319 remained constant ($P > 0.05$) throughout the entire incubation period under CAP and
320 NC conditions in both fresh and cryopreserved spermatozoa (**Figure 4**). However, when
321 the %DFI of both types of samples was compared at different time points of incubation
322 under CAP and NC conditions, cryopreserved samples showed, in general, a higher
323 %DFI ($P < 0.05$) than the fresh samples.

On the other hand, the percentage of HDS only changed during the incubation period under CAP conditions, depicting the highest values ($P < 0.05$) from 5 to 30 min in cryopreserved samples and from 180 to 240 min in fresh samples (**Figure 5**). At these incubation times, significant differences were found between the percentage of HDS in fresh and cryopreserved samples.

The proportion of methylated spermatozoa only increased in cryopreserved spermatozoa after prolonged incubations under CAP and NC conditions

In fresh samples, the proportion of methylated spermatozoa did not change ($P > 0.05$) throughout the incubation period under CAP and NC conditions (**Figure 6**). However, in the cryopreserved samples, prolonged exposure to CAP conditions significantly increased the proportion of methylated spermatozoa after 60 min. Although significant differences were not found under NC conditions ($P > 0.05$), an extended incubation of 240 min also intensified the proportion of methylated spermatozoa in the cryopreserved samples. Moreover, DNA methylation of cryopreserved spermatozoa was generally higher compared to fresh spermatozoa during incubation, being statistically significant ($P < 0.05$) after 30 min under CAP conditions and after 240 min under NC conditions.

Correlations between nuclear variables and sperm quality parameters

Chromatin decondensation (AB+) and HDS showed a positive correlation in fresh and cryopreserved spermatozoa with pY, total motility (TM), progressive motility (PM), VCL and ALH, but a negative correlation with LIN ($P < 0.01$) during the incubation period under CAP conditions (**Tables 1 and 2**). The strength of association between these variables was mostly moderate or strong and is depicted in **Table 1** for

AB+ and in Table 2 for HDS. Furthermore, a positive correlation between AB+ and HDS was observed under CAP conditions, being moderate in fresh samples and strong in cryopreserved ($P < 0.01$). Regarding mitochondrial activity, this variable only showed a positive and strong correlation with AB+ and HDS in cryopreserved spermatozoa incubated under CAP conditions. In contrast, ROS production and apoptosis were not significantly correlated ($P > 0.01$) with HDS or AB+ neither in fresh nor cryopreserved spermatozoa incubated under CAP conditions (Tables 1 and 2). Under NC conditions, all the sperm variables evaluated were not significantly correlated with AB+ or HDS ($P > 0.01$) neither in fresh nor cryopreserved spermatozoa (Tables 1 and 2).

The correlations between sperm DNA methylation and other sperm variables are shown in Table 3. No significant correlations ($P > 0.01$) were found between DNA methylation and nuclear variables (%DFI, HDS and AB+) or between DNA methylation and diverse sperm quality parameters (pY, TM, PM, VCL, ALH, LIN, mitochondrial activity and ROS production) in fresh and cryopreserved spermatozoa incubated under CAP and NC conditions. However, DNA methylation was positively correlated with apoptosis ($P < 0.01$) but only in cryopreserved spermatozoa incubated under CAP conditions.

DISCUSSION

Capacitation of mammalian spermatozoa is a complex event that involves a set of functional, structural, and molecular changes.^{19,20,45} However, only a few studies have investigated the effect of sperm capacitation on the chromatin structure. These studies found that spermatozoa showed a less compacted chromatin, sperm DNA integrity remained unchanged and the disulphide bonds between protamines decreased

during *in vitro* capacitation.^{21–24,46–48} Nevertheless, to the best of our knowledge, no previous studies have investigated sperm DNA methylation during this event. In line with these findings, our results revealed that fresh and cryopreserved ram spermatozoa exhibited a lower chromatin condensation at certain time points during *in vitro* capacitation, while DNA integrity remained constant during this event. In addition, DNA methylation only increased in cryopreserved ram spermatozoa incubated under capacitating conditions, but after 60 min.

Sperm chromatin condensation can be evaluated rapidly with aniline blue (AB). This cytochemical dye selectively stains persisting lysine-rich histones^{49,50} and has been widely used to identify an excessive histone retention in the chromatin of mature spermatozoa, which has been related to infertility, poor fertilization rates and morphological defects in the sperm head.^{10,14,51} However, a lower sperm chromatin condensation detected with AB does not always involve an abnormal histone content. De Lamirande et al.²² suggested that during capacitation, nuclear histones could be reoriented, facilitating AB access to them. Such chromatin decondensation should be moderate, because, beyond certain levels, a high or null chromatin condensation could compromise the fertilizing potential of spermatozoa.^{15,17,24} In line with previous studies conducted in bull and human sperm,^{22–24} our results for AB revealed that sperm chromatin condensation decreased during *in vitro* capacitation of ram spermatozoa. This variation in the chromatin condensation level was initiated in cryopreserved ram spermatozoa earlier than in fresh, reaching the highest percentages of spermatozoa with decondensed chromatin (AB+) at 5–30 min in cryopreserved samples and at 180–240 min in fresh samples. However, such changes in chromatin condensation were not observed under NC conditions. Earlier studies on sperm nuclear decondensation reported that it only occurs after changes in membrane fluidity and permeability, like

those observed after cryopreservation or during sperm capacitation.^{35,52–54} But apart from membrane destabilisation, decondensing factors are apparently required to induce sperm nuclear decondensation.^{35,54} Therefore, the differences found between CAP and NC conditions in our study could probably be explained by the presence of certain decondensing factors in the capacitating medium. In addition, we speculate that changes in membrane stability during cryopreservation might have accelerated the nuclear decondensation of cryopreserved ram spermatozoa during CAP conditions.

Romanato et al.⁵⁴ proposed that some glycosaminoglycans, specially heparan sulphate, which has been found in oocytes, could act as decondensing factors once spermatozoa penetrate the oocytes. Likewise, glycosaminoglycans have also been found in the oviductal fluid of many species,⁵⁵ participating in sperm capacitation under physiological conditions.⁵⁶ In small ruminants, *in vitro* capacitation is frequently attained after incubating spermatozoa in a capacitating medium supplemented with oestrous sheep serum (ESS).^{38,39,57} This capacitating agent (ESS) contains several glycosaminoglycans present in the oviductal fluid, including heparan sulphate⁵⁸ and was not added to the NC medium in our experiment, supporting the hypothesis that sperm chromatin decondensation could be induced by glycosaminoglycans under CAP conditions.

Incorrect or looser chromatin compaction usually entails an altered histone:protamine ratio and, more specifically, protamine deficiency, increasing sperm DNA damage.¹² However, when we evaluated sperm DNA integrity during the time course of *in vitro* capacitation with the SCSA, our results showed that the %DFI remained constant during the entire incubation period under CAP conditions either in fresh or cryopreserved spermatozoa. In line with our findings, earlier studies also reported that sperm DNA integrity was not adversely affected during *in vitro*

capacitation.^{22,47,48} These results suggest that this partial chromatin decondensation observed in fresh and cryopreserved ram spermatozoa takes place during physiological processes, such as sperm capacitation, without altering DNA integrity. Our results also revealed that cryopreserved spermatozoa showed a higher %DFI than fresh spermatozoa, which is not surprising because cryopreservation is known to exert a negative effect on sperm DNA integrity.^{59,60}

Another nuclear parameter evaluated with SCSA was HDS. According to Evenson,⁶¹ HDS represents the proportion of immature spermatozoa with a defective chromatin structure (abnormal histone-protamine content). This abnormal histone-protamine content implies a lower chromatin condensation, facilitating the access of AO staining to double-stranded DNA (intact DNA).⁶² However, chromatin decondensation of ram spermatozoa during *in vitro* capacitation could be more related to conformational changes in the chromatin structure rather than to abnormal histone-protamine content,⁶¹ as has been suggested by De Lamirande et al.²² Our results showed that similar to AB+, HDS increased at certain incubation times but only during *in vitro* capacitation, occurring in cryopreserved spermatozoa faster (at 5-30 min) than in fresh spermatozoa (at 180-240 min). Furthermore, a positive correlation was observed between HDS and AB+ under CAP conditions in fresh and cryopreserved spermatozoa. These findings suggest that HDS might represent, as AB+, the proportion of spermatozoa with a less condensed chromatin during *in vitro* capacitation. Although the mechanisms underlying sperm chromatin decondensation are yet unclear during capacitation, these changes in sperm nuclear compaction could facilitate the events surrounding and following fertilisation.²²

In addition, based on the different correlations found under CAP conditions between HDS, AB+ and the diverse sperm parameters assessed in this study, those

changes observed in the chromatin condensation were seemingly linked to changes in the motility pattern and in tyrosine phosphorylation during CAP conditions either in fresh or cryopreserved ram spermatozoa as well as to variations in mitochondrial activity in the case of cryopreserved spermatozoa, but not to apoptosis, oxidative stress, DNA damage or epigenetic changes. These findings suggest that during the sperm activation window, chromatin decondensation occurs in parallel to the other widely reported capacitation-associated changes.^{28,63–67} Moreover, our results differed from those related to male infertility, in which sperm samples had a high %DFI and DNA damage was linked to abnormal chromatin packaging.^{16,68,69}

We also explored whether cryopreservation and incubation under CAP conditions induced epigenetic changes. DNA methylation is an important epigenetic mechanism that is transmitted to the next generation and modulates gene expression during embryogenesis and gametic imprinting.² Previous studies have shown that freezing-thawing procedures can alter the pattern of sperm DNA methylation.^{34,70} Aurich et al.³⁴ reported that DNA methylation increased in stallion spermatozoa after cryopreservation, while other authors found that sperm DNA methylation decreased after cryopreservation in rooster spermatozoa.⁷⁰ In our study, sperm DNA methylation increased after cryopreserved ram spermatozoa were incubated for 60, 180 and 240 min under capacitating conditions, but not immediately after thawing. Differences between species, methodologies, cryopreservation media and sperm treatments could be the reason for such discrepancies. Perturbations in sperm DNA methylation have been associated with male infertility, embryogenesis defects and pregnancy loss.^{7,71} However, it is unclear whether these drastic changes in sperm DNA methylation imply only hypomethylation,⁴⁴ hypermethylation⁷² or even both alterations in different gene regions,⁹ which results in no differences in global methylation. Similar inconsistencies

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3 474 arise when methylation is correlated with other sperm quality parameters.^{73–75} Our
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5 475 results showed that sperm DNA methylation was only correlated with apoptosis in
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7 476 cryopreserved ram spermatozoa incubated under CAP conditions. A similar correlation
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9 477 was previously found in spermatozoa with poor quality, which could be indicative of
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11 478 defective spermatogenesis.⁷⁶ However, in the present study DNA methylation and
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13 479 apoptosis only increased after certain incubation times under CAP conditions in
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15 480 cryopreserved spermatozoa, probably as a result of an early response to oxidative
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17 481 stress,⁷⁷ but further investigations are required to better understand this correlation in
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19 482 our study.
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24 483 In summary, our results revealed that sperm chromatin condensation was
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26 484 progressively modified over the incubation period under CAP conditions in fresh and
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28 485 cryopreserved ram spermatozoa while DNA integrity remained constant. In addition,
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30 486 prolonged incubations (60–240 min) under CAP conditions resulted in epigenetic
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32 487 changes in cryopreserved ram spermatozoa. Cryopreserved spermatozoa exhibited less
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34 488 condensed chromatin earlier than fresh spermatozoa during capacitation probably as a
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36 489 result of those changes induced in membrane stability by the cryopreservation process.
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38 490 Finally, the correlations found in our study suggest that chromatin decondensation may
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40 491 occur simultaneously to other functional changes associated with sperm capacitation. In
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42 492 addition, these changes in sperm chromatin condensation do not seem to be related to
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44 493 apoptosis, oxidative stress or DNA damage. Further studies are required to better
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46 494 understand the mechanisms involved in these chromatin alterations observed during
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56 497 **ACKNOWLEDGEMENTS**

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3 498 PFP and DAMC were supported by a University of Castilla-La Mancha scholarship.
4
5 499 MIC and AMM were supported by a Ministry of Economy and Competitiveness
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7
8 500 scholarship. The study was supported by grants from ALF-Research (Region
9
10 501 Östergötland, LIO-698951), FORSS (Forskningsrådet i Sydöstra Sverige, 473121 and
11
12 502 745971), Lions Forskningsfond (DNRLIU-2016-00641), Linköping and the Swedish
13
14 503 Research Council FORMAS, Stockholm (Project 2017-00946 and Project 2019-00288)
15
16
17 504 and by the MINECO (AGL2013-48421-R). Sperm nuclear parameters were evaluated in
18
19 505 the Department of Biomedical and Clinical Sciences (BKV) from the Faculty of
20
21 506 Medicine in Linköping (Sweden) after preparing samples in the laboratory of SaBio
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23 507 IREC (ETSIAM, Albacete, Spain).
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28 509 **CONFLICT OF INTEREST**

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30 510 The authors declare that there is no conflict of interest.
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36 512 **AUTHOR CONTRIBUTIONS**

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38 513 Peris-Frau P, Álvarez-Rodríguez M, Martín-Maestro A, Iniesta-Cuerda M, Sánchez-
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40 514 Ajofrín I and Medina-Chávez DA took the samples, performed the experiments and
41
42 515 made the database. Garde JJ, Rodriguez-Martinez H, Villar M and Soler AJ performed
43
44 516 the statistical analyses and reviewed the final manuscript and Soler AJ designed the
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46 517 study.
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FIGURE LEGENDS

Figure 1. Ram spermatozoa stained with aniline blue (AB). (A) Unstained nuclei (AB-) indicated normal sperm chromatin condensation, while (B) blue-stained nuclei indicated chromatin decondensation (AB+).

Figure 2. Ram spermatozoa showing DNA methylation after 5-methylcytosine (5-mc) immunostaining. (A) Spermatozoa with detectable levels of DNA methylation showed green fluorescence (Alexa Fluor 488) at their heads at 100x magnification and were classified as methylated spermatozoa, while (B) spermatozoa showing no fluorescence revealed no detectable levels of DNA methylation.

Figure 3. Changes in the proportion of fresh and cryopreserved spermatozoa showing chromatin decondensation (AB+) during the incubation period under capacitating (CAP) and non-capacitating (NC) conditions. The mean percentage of AB+ spermatozoa $\pm$ SEM at each time point was illustrated in the figure. Different letters represent significant differences ($P < 0.05$) among incubation times. Uppercase letters were used for cryopreserved spermatozoa and lowercase letters for fresh spermatozoa. ‡ Denotes significant differences ($P < 0.05$) between fresh and cryopreserved samples.

Figure 4. Changes in the proportion of fresh and cryopreserved spermatozoa with DNA fragmentation (%DFI) during the incubation period under capacitating (CAP) and non-capacitating (NC) conditions. The mean percentage of %DFI $\pm$ SEM at each time point was depicted in the figure. ‡ Indicates significant differences ($P < 0.05$) between fresh and cryopreserved spermatozoa. Statistical differences were not ($P > 0.05$) found among incubation times neither in fresh nor cryopreserved samples.

Figure 5. Changes in the proportion of fresh and cryopreserved spermatozoa with high DNA stainability (HDS) during the incubation period under capacitating (CAP) and

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779 non-capacitating (NC) conditions. The mean percentage of HDS $\pm$ SEM at each time
780 point was illustrated in the figure. Different letters indicate significant differences ($P <$
781 0.05) among incubation times. Uppercase letters were used for cryopreserved
782 spermatozoa and lowercase for fresh spermatozoa. ‡ Denotes significant differences (P
783 < 0.05) between fresh and cryopreserved spermatozoa.

784 **Figure 6.** Changes in the proportion of fresh and cryopreserved spermatozoa showing
785 DNA methylation during the incubation period under capacitating (CAP) and non-
786 capacitating (NC) conditions. The mean percentage of DNA methylated spermatozoa $\pm$
787 SEM at each time point was depicted in the figure. Different letters represent significant
788 differences ($P < 0.05$) among incubation times in cryopreserved samples. In fresh
789 samples, DNA methylation did not change significantly ($P > 0.05$) over time. ‡
790 Indicates significant differences ($P < 0.05$) between fresh and cryopreserved
791 spermatozoa.

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TABLES

Table 1. Correlation coefficients between chromatin decondensation (AB+) and several variables in fresh and cryopreserved spermatozoa incubated under capacitating (CAP) and non-capacitating (NC) conditions. Correlations were estimated considering all incubation times under CAP or NC conditions.

Variables ^a	AB+ (%) ^b			
	Fresh sperm		Cryopreserved sperm	
	CAP	NC	CAP	NC
HDS (%)	0.49*	0.27	0.75*	0.24
DFI (%)	-0.46	-0.33	-0.44	-0.28
pY (%)	0.87*	0.45	0.50*	0.24
TM (%)	0.46*	-0.70	0.83*	-0.78
PM (%)	0.50*	-0.62	0.81*	-0.76
VCL ($\mu\text{m s}^{-1}$)	0.82*	-0.13	0.72*	0.16
LIN (%)	-0.76*	-0.52	-0.70*	-0.21
ALH (μm)	0.76*	-0.10	0.72*	0.26
Mitochondrial activity (%)	0.50	-0.47	0.79*	-0.43
ROS (MFI)	-0.33	-0.56	-0.32	-0.08
Apoptotic sperm (%)	-0.20	-0.31	-0.14	0.05

* $P < 0.01$

^a HDS: percentage of spermatozoa with high DNA stainability; DFI: percentage of spermatozoa with DNA fragmentation index values higher than 25%; pY: percentage of tyrosine phosphorylated spermatozoa; TM: percentage of total motility; PM: percentage of live spermatozoa with progressive motility; VCL: curvilinear velocity; LIN: linearity coefficient; ALH: amplitude of lateral head displacement; Mitochondrial activity: percentage of live spermatozoa with active mitochondria; ROS: mean fluorescence intensity (MFI) of reactive oxygen species produced by live spermatozoa; Apoptotic sperm: percentage of spermatozoa showing apoptosis.

^b AB positives: percentage of spermatozoa showing chromatin decondensation.

Table 2. Correlation coefficients between spermatozoa with high DNA stainability (HDS) and several variables in fresh and cryopreserved spermatozoa incubated under capacitating (CAP) and non-capacitating (NC) conditions. Correlations were estimated considering all incubation times under CAP or NC conditions.

Variables ^a	HDS (%) ^b			
	Fresh sperm		Cryopreserved sperm	
	CAP	NC	CAP	NC
AB+ (%)	0.49*	0.27	0.75*	0.24
DFI (%)	-0.42	-0.41	-0.44	-0.17
pY (%)	0.64*	0.40	0.52*	0.29
TM (%)	0.41*	0.48	0.84*	-0.79
PM (%)	0.51*	0.46	0.86*	-0.67
VCL (µm s ⁻¹)	0.71*	0.45	0.79*	0.34
LIN (%)	-0.73*	-0.31	-0.64*	-0.10
ALH (µm)	0.78*	0.34	0.79*	0.16
Mitochondrial activity (%)	0.59	-0.19	0.87*	-0.35
ROS (MFI)	-0.36	-0.60	-0.35	-0.08
Apoptotic sperm (%)	0.14	-0.14	-0.17	0.18

* P < 0.01

^aAB+: percentage of spermatozoa showing chromatin decondensation; DFI: percentage of spermatozoa with DNA fragmentation index values higher than 25%; pY: percentage of tyrosine phosphorylated spermatozoa; **TM: percentage of total motility; PM: percentage of live spermatozoa with progressive motility**; VCL: curvilinear velocity; LIN: linearity coefficient; ALH: amplitude of lateral head displacement; Mitochondrial activity: percentage of live spermatozoa with active mitochondria; ROS: mean fluorescence intensity (MFI) of reactive oxygen species produced by live spermatozoa; Apoptotic sperm: percentage of spermatozoa showing apoptosis.

^bHDS: percentage of spermatozoa with high DNA stainability.

Table 3. Correlation coefficients between global DNA methylation and several variables in fresh and cryopreserved spermatozoa incubated under capacitating (CAP) and non-capacitating (NC) conditions. Correlations were estimated considering all incubation times under CAP or NC conditions.

Variables ^a	DNA methylation (%) ^b			
	Fresh sperm		Cryopreserved sperm	
	CAP	NC	CAP	NC
AB+ (%)	0.04	0.06	-0.18	-0.47
HDS (%)	0.08	0.07	-0.26	-0.46
DFI (%)	0.09	-0.31	0.20	0.18
pY (%)	0.01	0.23	-0.23	-0.04
TM (%)	0.22	0.57	-0.62	-0.54
PM (%)	-0.31	0.06	-0.06	-0.05
VCL ($\mu\text{m s}^{-1}$)	0.01	0.03	0.05	-0.04
LIN (%)	-0.04	0.06	0.03	0.07
ALH (μm)	0.05	0.09	0.12	-0.21
Mitochondrial activity (%)	-0.13	-0.29	-0.09	-0.23
ROS (MFI)	0.19	-0.55	0.39	0.27
Apoptotic sperm (%)	0.28	0.14	0.61*	0.21

* $P < 0.01$

^a AB+: percentage of spermatozoa showing chromatin decondensation; HDS: percentage of spermatozoa with high DNA stainability; DFI: percentage of spermatozoa with DNA fragmentation index values higher than 25%; pY: percentage of tyrosine phosphorylated spermatozoa; TM: percentage of total motility; PM: percentage of live spermatozoa with progressive motility; VCL: curvilinear velocity; LIN: linearity coefficient; ALH: amplitude of lateral head displacement; Mitochondrial activity: percentage of live spermatozoa with active mitochondria; ROS: mean fluorescence intensity (MFI) of reactive oxygen species produced by live spermatozoa; Apoptotic sperm: percentage of spermatozoa showing apoptosis.

^b DNA methylation: percentage of spermatozoa showing detectable levels of DNA methylation.

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Figure 2.

1 **Figure 3.**

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Supplementary Figure 1. Changes in the proportion of fresh and cryopreserved ram spermatozoa showing tyrosine phosphorylation during the incubation period under capacitating (CAP) and non-capacitating (NC) conditions. The mean percentage of spermatozoa showing protein tyrosine phosphorylation $\pm$ SEM at each time was represented in the figure. Different letters illustrate significant differences ($P < 0.05$) among incubation times. Uppercase letters were used for cryopreserved spermatozoa and lowercase letters for fresh spermatozoa.

Supplementary table 1. Kinematic changes in fresh and cryopreserved ram spermatozoa incubated under CAP and NC conditions from 0 min to 240 min.

Values are expressed as means $\pm$ SEM.

Treatments	Fresh sperm					Cryopreserved sperm				
	TM (%)	PM (%)	VCL ($\mu\text{m s}^{-1}$)	LIN (%)	ALH (μm)	TM (%)	PM (%)	VCL ($\mu\text{m s}^{-1}$)	LIN (%)	ALH (μm)
0 NC	54.96 $\pm$ 0.58 ^{ab}	44.74 $\pm$ 5.33 ^{ab}	32.33 $\pm$ 0.48 ^a	86.50 $\pm$ 1.56 ^a	1.35 $\pm$ 0.07 ^a	36.77 $\pm$ 3.68 ^{ab}	26.09 $\pm$ 3.13 ^a	35.60 $\pm$ 4.00 ^a	87.35 $\pm$ 1.87 ^a	1.36 $\pm$ 0.23 ^a
1 CAP	53.15 $\pm$ 3.00 ^{ab}	41.45 $\pm$ 1.23 ^{ac}	45.89 $\pm$ 2.93 ^a	72.18 $\pm$ 1.94 ^b	1.76 $\pm$ 0.18 ^a	34.28 $\pm$ 3.50 ^{ab}	25.24 $\pm$ 3.70 ^a	67.07 $\pm$ 3.36 ^b	45.31 $\pm$ 2.16 ^{bc}	1.71 $\pm$ 0.28 ^a
5 CAP	51.98 $\pm$ 2.47 ^{ab}	45.48 $\pm$ 1.40 ^{ab}	47.84 $\pm$ 0.84 ^a	62.98 $\pm$ 2.15 ^c	1.62 $\pm$ 0.39 ^a	33.22 $\pm$ 4.23 ^{ab}	31.29 $\pm$ 1.71 ^{ab}	115.17 $\pm$ 10.39 ^c	37.70 $\pm$ 1.51 ^d	3.05 $\pm$ 0.08 ^b
15 CAP	57.06 $\pm$ 1.14 ^a	36.80 $\pm$ 2.25 ^{ac}	87.18 $\pm$ 3.05 ^{bc}	64.91 $\pm$ 1.06 ^c	1.55 $\pm$ 0.24 ^a	42.79 $\pm$ 2.00 ^a	36.41 $\pm$ 2.43 ^b	160.54 $\pm$ 7.68 ^d	35.83 $\pm$ 1.56 ^d	4.17 $\pm$ 0.19 ^c
15 NC	25.05 $\pm$ 1.83 ^c	18.69 $\pm$ 4.59 ^d	40.76 $\pm$ 6.54 ^a	66.47 $\pm$ 0.23 ^{bc}	1.41 $\pm$ 0.25 ^a	12.76 $\pm$ 2.52 ^{cd}	11.35 $\pm$ 1.14 ^{cd}	33.29 $\pm$ 1.17 ^a	58.86 $\pm$ 1.74 ^e	1.08 $\pm$ 0.26 ^a
30 CAP	46.64 $\pm$ 4.05 ^b	44.92 $\pm$ 0.76 ^{ac}	83.85 $\pm$ 4.00 ^b	66.44 $\pm$ 2.65 ^{bc}	1.89 $\pm$ 0.55 ^a	30.73 $\pm$ 1.26 ^{bc}	22.22 $\pm$ 6.10 ^a	131.17 $\pm$ 0.15 ^c	41.13 $\pm$ 1.95 ^{dc}	3.61 $\pm$ 0.29 ^{bc}
60 CAP	46.05 $\pm$ 1.06 ^b	42.53 $\pm$ 5.66 ^{dc}	104.52 $\pm$ 9.72 ^{cd}	51.31 $\pm$ 2.55 ^d	1.77 $\pm$ 0.35 ^a	19.59 $\pm$ 4.32 ^{cc}	12.50 $\pm$ 1.79 ^c	75.66 $\pm$ 4.00 ^b	49.28 $\pm$ 2.58 ^b	1.95 $\pm$ 0.55 ^a
120 CAP	46.11 $\pm$ 2.26 ^b	41.60 $\pm$ 4.02 ^{ac}	119.23 $\pm$ 4.97 ^d	45.69 $\pm$ 2.39 ^d	3.08 $\pm$ 0.33 ^b	12.72 $\pm$ 1.88 ^{cd}	5.83 $\pm$ 1.30 ^{cc}	41.95 $\pm$ 2.29 ^a	49.92 $\pm$ 0.67 ^b	1.51 $\pm$ 0.24 ^a
180 CAP	58.24 $\pm$ 1.84 ^a	37.28 $\pm$ 1.13 ^a	157.69 $\pm$ 1.99 ^c	37.88 $\pm$ 2.06 ^e	3.58 $\pm$ 0.43 ^c	3.66 $\pm$ 2.20 ^d	2.84 $\pm$ 1.13 ^{de}	42.18 $\pm$ 1.55 ^a	48.15 $\pm$ 3.31 ^{bc}	1.58 $\pm$ 0.11 ^a
240 CAP	58.87 $\pm$ 1.57 ^a	29.58 $\pm$ 3.95 ^b	165.27 $\pm$ 4.34 ^e	38.40 $\pm$ 1.17 ^e	4.33 $\pm$ 0.33 ^c	4.01 $\pm$ 0.95 ^d	3.33 $\pm$ 1.38 ^{cc}	34.50 $\pm$ 2.03 ^a	45.68 $\pm$ 0.29 ^{bc}	1.18 $\pm$ 0.44 ^a
240 NC	15.66 $\pm$ 2.41 ^d	5.65 $\pm$ 1.44 ^e	31.97 $\pm$ 1.90 ^a	61.95 $\pm$ 1.51 ^c	0.79 $\pm$ 0.12 ^a	4.31 $\pm$ 2.67 ^d	0.60 $\pm$ 0.12 ^c	22.03 $\pm$ 4.74 ^a	48.90 $\pm$ 0.59 ^b	0.75 $\pm$ 0.08 ^a

Abbreviations: TM (%), total motility; PM (%), progressive motility; VCL ($\mu\text{m s}^{-1}$), curvilinear velocity; LIN (%), linearity coefficient; ALH (μm), amplitude of lateral head displacement.

Different letters indicate significant differences ($P < 0.05$) among incubation times for each parameter.

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11 **Supplementary table 2.** Changes in viability, apoptosis, mitochondrial activity and ROS production in fresh and cryopreserved ram spermatozoa incubated
12 under CAP and NC conditions from 0 min to 240 min. Values are expressed as means ± SEM.

Treatments	Fresh sperm				Cryopreserved sperm			
	Viability (%)	Apoptosis (%)	Mitochondrial activity (%)	ROS (MFI)	Viability (%)	Apoptosis (%)	Mitochondrial activity (%)	ROS (MFI)
0 NC	54.88 ± 4.62 ^a	19.41 ± 3.60	36.15 ± 3.88 ^a	68.10 ± 1.80 ^a	39.25 ± 1.34 ^a	13.18 ± 2.21 ^a	24.15 ± 0.16 ^{ab}	100.53 ± 4.93 ^a
1 CAP	50.13 ± 6.57 ^{ab}	20.26 ± 5.62	39.77 ± 4.89 ^{ab}	39.33 ± 1.47 ^b	34.90 ± 2.18 ^a	16.91 ± 1.44 ^{ab}	28.16 ± 4.25 ^{ab}	66.47 ± 1.76 ^b
5 CAP	50.51 ± 7.35 ^{ab}	18.77 ± 4.52	40.09 ± 5.39 ^{ab}	42.13 ± 4.22 ^b	31.54 ± 3.56 ^a	17.24 ± 0.44 ^{ab}	32.11 ± 0.52 ^{ac}	63.50 ± 3.05 ^b
15 CAP	46.99 ± 5.65 ^{ac}	17.84 ± 4.43	40.28 ± 5.72 ^{ab}	37.43 ± 1.73 ^b	38.18 ± 2.98 ^a	14.09 ± 2.40 ^{ab}	38.34 ± 4.34 ^c	68.10 ± 8.18 ^b
15 NC	25.95 ± 4.42 ^c	19.83 ± 4.23	25.00 ± 3.20 ^c	75.53 ± 1.50 ^{ac}	15.78 ± 1.95 ^b	19.96 ± 4.01 ^{bc}	13.02 ± 0.39 ^d	97.33 ± 5.19 ^a
30 CAP	40.68 ± 1.59 ^{bc}	15.26 ± 1.47	28.31 ± 3.39 ^c	40.80 ± 2.22 ^b	32.24 ± 2.66 ^a	16.07 ± 1.50 ^{ab}	35.32 ± 4.56 ^c	59.03 ± 6.98 ^b
60 CAP	40.45 ± 8.38 ^c	14.53 ± 2.66	23.47 ± 8.01 ^c	49.93 ± 2.07 ^b	20.32 ± 5.96 ^{ab}	24.71 ± 1.81 ^c	17.53 ± 4.70 ^b	52.33 ± 1.16 ^b
120 CAP	36.99 ± 2.76 ^c	15.36 ± 0.25	30.72 ± 7.50 ^c	49.33 ± 7.90 ^b	13.07 ± 2.16 ^b	28.77 ± 0.79 ^c	9.95 ± 1.28 ^d	67.17 ± 1.92 ^b
180 CAP	44.05 ± 2.18 ^{ac}	15.59 ± 1.90	45.39 ± 1.90 ^b	42.23 ± 1.94 ^b	4.94 ± 1.45 ^c	32.99 ± 0.59 ^d	8.68 ± 1.31 ^d	73.33 ± 1.69 ^c
240 CAP	44.13 ± 3.72 ^{ac}	19.04 ± 7.40	45.87 ± 2.30 ^b	50.80 ± 1.34 ^b	3.51 ± 0.13 ^c	32.88 ± 0.42 ^d	7.03 ± 1.25 ^{de}	84.60 ± 2.97 ^c
240 NC	13.26 ± 1.52 ^d	20.75 ± 6.34	14.08 ± 1.81 ^d	83.97 ± 1.76 ^c	4.08 ± 0.83 ^c	16.51 ± 0.69 ^a	2.82 ± 1.29 ^e	116.80 ± 4.33 ^d

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14 Abbreviations: Viability (%), percentage of live spermatozoa; Apoptosis (%), percentage of apoptotic spermatozoa; Mitochondrial activity (%), percentage of
15 live spermatozoa with active mitochondria; ROS (MFI), mean fluorescence intensity (MFI) of reactive oxygen species (ROS) produced by live spermatozoa.
16 Different letters indicate significant differences (P < 0.05) among incubation times for each parameter.