Deliverable 5.3
GSC storage and evaluation of GSC cryo-damage

Version 1

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Deliverable 5.3
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Contents

Objective

Evaluation of spermatogonia cryodamage.

Background

Provide some background to the report and the subject area

Methodology

1.1. Cryopreservation procedure

Senegalese sole testes fragments were cryopreserved into L-15 based medium supplemented with 0.5% of bovine serum albumin and 5.5 mM glucose with 1.5 M DMSO. Samples were loaded into a portable programmed biofreezer (Asymptote EF600, Grant Instruments, Cambridge, UK) and the freezing rate was set as the one described in Cabrita et al. (2023). Afterwards, cryovials were introduced directly in liquid nitrogen and stored in a liquid nitrogen container until further procedures. Cryovials were thawed in a water bath at 40 °C for 140 s and testes fragments were washed in L-15 to eliminate the cryoprotectant. Testes were then dissociated to obtain germ cells.

1.2. Post-thaw quality assessment

1.2.1. Plasma membrane integrity

Plasma membrane integrity analysis was performed using propidium iodide and SYBR-14 (PI/SYBR-14) staining. Briefly, 20 µL of the fresh and cryopreserved cell suspensions were mixed with 0.1 µL SYBR-14 working solution (0.25 µM) and 0.5 µL propidium iodide (24 µM). Cells were incubated for 5 min and then observed in a fluorescence microscope (Nikon Eclipse E200) with an excitation filter of 450 nm at 200x magnification. Images were captured and recorded with a digital camera (VisiCam 5 Plus, VWR). Plasma membrane integrity was calculated as the proportion of cells non-permeable to PI relative to the total number of cells. At least 100 cells per sample were counted in triplicate.

1.2.2. DNA integrity

DNA integrity was determined by the comet assay following the protocol described by Cabrita et al. (2023). Fresh and cryopreserved cell suspensions were diluted in L15 to attain a final concentration of approximately $1x10^6$ cells per 50 µL. After dilution, cells were embedded in 0.5 % low melting point agarose and placed in agarose pre-coated slides. The slides were introduced into a lysis solution for 1 h at 4 °C. To decondense the DNA, DTT was added to the lysis buffer, at the final concentration of 10 mM, and the slides were immersed for 30 min at 4 °C. After lysis, the slides were placed horizontally in an electrophoresis cube filled with electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) for 30 min at 4 °C to allow DNA to unwind. Electrophoresis was conducted for 10 min at 25 V and 300 mA at 4 °C. Afterwards, the slides were neutralized (0.4 M Tris, pH 7.5, 5 min x2) and left to dry before observation. Visualization of the comets was carried out using PI staining (19.2 µM) and a fluorescence microscope. Approximately 100 cells from each slide (50 per replicate) were captured with a digital camera (VisiCam 5 Plus, VWR). Comet analysis was performed with the Kinetic Imaging Komet v6.0 software (Andor Technology, UK). The percentage of tail DNA (% DNA_t) was used to determine the amount of DNA fragmentation.

1.2.3 Lipid peroxidation

Lipid peroxidation were determined by quantifying the concentration of malondialdehyde (MDA) using BIOXYTECH colourimetric assay (OxisResearch), following the protocol described by Martínez-Páramo et al. (2012) and adapted by Cabrita et al. (2023) for spermatogonia. Fresh and cryopreserved cell suspensions were incubated in 10 µL of 200 µM sodium ascorbate containing 40 µM ferrous sulphate for 30 min at 37 ºC in the dark. An MDA calibration curve was prepared by diluting MDA standard solution (20 µM) in MilliQ water. Subsequently, reagents provided in the kit were added to 100 µL of the cell suspension, following manufacturer instructions, and samples were incubated at 45ºC in the dark. After 1 h, samples were centrifuged at 10 000 g for 10 min at 4 ºC, and 200 µL of each supernatant were transferred to a 96-well flat-bottom transparent plate (Nunc). The absorbance was read in a microplate reader (Synergy 4, Biotek Instruments. Inc.) at 586 nm. MDA concentrations were calculated from a standard curve and presented as µM of MDA per million spermatogonia.

1.2.4 Methylation profile

Genomic DNA extraction, library construction and whole genome bisulfite sequences

DNA was extracted from fresh and cryopreserved testicular cells (45-60% spermatogonia) using a QIAamp genomic DNA kit (Qiagen, Germany). DNA was checked using nanodrop (NanoDrop™ One/ OneC, Thermo Scientific) where the quality absorbance ratios were assessed (A280/260 > 1.8; A230/260 > 1.8). Whole genome bisulfite sequencing (WGBS) was performed by NOVOGENE (UK). The quality control was also checked by the company. Accel-NGS Methyl-Seq DNA Library Kit for Illumina was used to prepare the libraries. Bisulfite-converted DNA fragments were sequenced in paired ends of 150 bp read length, with an expected sequencing depth of 35X (considering a genome size of 612.3 Mb for *Solea senegalensis*).

Bioinformatics

Whole genome modifications related to the methylated region were analysed according to Nilsson et al. (2021). Briefly, data quality control (FASTA/FASTQ format) was performed using the FastQC program [28]. Then, low-quality reads and bases were trimmed and filtered to remove low-quality bases (Phred quality score < 30), and inserts shorter than 20 bp. The bisulfite conversion efficiency provided in the NOVOGENE quality report was > 98 %. The bisulfite sequencing reads for each sample were mapped to the Senegalese sole reference genome (Solea_v4.1) using default parameters of Bismark software (Krueger et al., 2011,2012), except for the smoothing function that was disabled according to the choices developed in El Kamouh et al. (2023). This step relied on read 1 and read 2 to ensure proper alignment of both sequences. All strict duplicates originating from PCR bias were removed after alignment, as well as reads whose best alignment scores were found in more than one location. For CpG sites analysis, reads from both strands were combined to calculate the methylation levels using the "bismark_methylation_extraction". Each cytosine position in a CpG dinucleotide context was identified, and the number of methylated and unmethylated cytosine reads at each position was counted. These steps are available in the workflow which agrees to FAIR principles and is accessible online (https://forgemia.inra.fr/lpgp/methylome). The count files generated by Bismark for each condition were processed for the characterisation of cytosines whose average methylation status was different between treatments, using the DSS program (Feng et al., 2014). These differentially methylated cytosines (DMCs) characterization was conducted while the smoothing option of DSS was disabled to avoid some isolated CpGs modified by cryopreservation being undetected. Smoothing is more relevant when studying biological processes, where the methylation changes always affect a broad span of CpGs. From DMC characterization, a search for regions enriched in DMC (DMRs) was performed with DSS. It was based on the search for 5 consecutive CpGs (in a sliding frame of 50 bp) which had at least 75 % of significant DMCs (false discovery rate – FDR- < 5 %).

Results and Discussion

Spermatogonia cryopreservation is a method to preserve valuable genomes from both maternal and paternal origin. The damage associated with the application of this technology on post-thaw cell quality is important to assess, including at the epigenetic level. This study aimed to assess post-thawed spermatogonia quality by evaluating alterations in plasma membrane integrity, DNA integrity (fragmentation and apoptosis), lipid peroxidation (malondialdehyde levels) and epigenetic modifications (DNA methylation profile). We observed that plasma membrane integrity (fresh 78.98% ± 5.66; cryopreserved 62.81% ± 3.25) and DNA integrity (fresh 32.95% ± 2.28; cryopreserved 37.28% ± 1.87) were affected by cryopreservation, while no difference in lipid peroxidation was observed (fresh 1.13% ± 0.45; cryopreserved 0.91% ± 0.96). While global levels of DNA methylation were unaffected by cryopreservation (fresh 82.80% ± 0.47; cryopreserved 83.32% ± 0.81), some differentially methylated cytosines (DMC) were observed in cryopreserved versus fresh spermatogonia (156 DMC). These results corroborate the fact that cryopreservation only affected spermatogonia DNA methylation at a slight level. The absence of differentially methylated regions (DMR) is a direct consequence of the low number of DMCs obtained, and of the fact that the distribution of these DMCs was not concentrated in some regions to form a DMR. This indicates that the effect of cryopreservation was scattered throughout the genome and was not restricted to specific areas. Additionally, the fact that up to 156 DMCs correspond to CpG information reproducible among samples (detected in at least 3 samples per group), indicates that, despite spermatogonia being sampled from different males and testis, cryopreservation triggered a homogeneous effect between samples making them replicates. Our results also revealed that spermatogonia cryopreservation with DMSO induced DNA hypermethylation of the sensitive sites. However, the mechanism involved in how the cryoprotectant, and cryopreservation constraints may modify DNA methylation is still unclear. Some studies report no straightforward effect with cryopreservation which induced both hypermethylation and hypomethylation of specific CpG sites (Kamouh et al., 2023) while others report global hypomethylation with a less suitable cryoprotectant or no alteration on global DNA methylation of fresh sperm after exposure to cryoprotectant (Depincé et al., 2020).

This study showed that spermatogonia cryopreserved according to our protocol provides a good supply of undamaged cells for several applications. Although the global DNA methylation was not affected in the present study, indicating a relative safety of the procedure, it might still be relevant in further studies to try to understand the epigenetic risk taken when using chemically active molecules that are permeant cryoprotectants, and whether the few identified DMCs may have biological significance during gametogenesis and embryo development. This question also deserves better knowledge on the DNA methylation reprogramming events during gametogenesis (reviewed in Alonso et al. 2023), as it is uncertain to what extent incorrect DNA methylation will be reprogrammed.

Conclusion

We confirmed that cryopreservation affects plasma membrane and DNA integrity in spermatogonia of Senegalese sole, while no difference in lipid peroxidation was observed. No global methylation changes were observed, despite some scattered genomic sites undergoing mostly hypermethylation after cryopreservation, which also indicates the global safety of the cryopreservation procedure

regarding this epigenetic factor. These results contribute to the understanding of cryoinjuries in Senegalese sole spermatogonia. Notably, this study is one of the first to analyze the impact of cryopreservation on the methylation profiles of fish spermatogonia (especially in Senegalese sole), filling a gap in the current literature and providing new insights into the cryopreservation of germ cells in fish.

Appendix

Use when applicable

References

M.L. Alonso, A. Laurent, M.P. Herráez, C. Labbé, Epigenetics in Sexual Maturation and Gametes of Fish, in: Epigenetics in Aquaculture, Wiley, 2023: pp. 175–191.

S. Martínez-Páramo, P. Diogo, J. Beirão, M.T. Dinis, E. Cabrita, Sperm lipid peroxidation is correlated with differences in sperm quality during the reproductive season in precocious European sea bass (Dicentrarchus labrax) males, Aquaculture 358–359 (2012) 246–252.

E. Cabrita, T. Pacchiarini, E. Fatsini, C. Sarasquete, M.P. Herráez, Post-thaw quality assessment of testicular fragments as a source of spermatogonial cells for surrogate production in the flatfish Solea senegalensis, Fish Physiol Biochem (2023). https://doi. org/10.1007/s10695-023-01232-2.

M. El Kamouh, A. Brionne, A. Sayyari, A. Laurent, C. Labbé, Cryopreservation effect on DNA methylation profile in rainbow trout spermatozoa, Sci Rep 13 (2023) 19029.

A. Depincé, A. Gabory, K. Dziewulska, P.Y. Le Bail, H. Jammes, C. Labbé, DNA methylation stability in fish spermatozoa upon external constraint: Impact of fish hormonal stimulation and sperm cryopreservation, Mol Reprod Dev 87 (2020) 124–134.

E. Nilsson, I. Sadler-Riggleman, D. Beck, M.K. Skinner, Differential DNA methylation in somatic and sperm cells of hatchery vs wild (natural-origin) steelhead trout populations, Environ Epigenet 7 (2021) 1–17.

F. Krueger, S.R. Andrews, Quality Control, trimming and alignment of Bisulfite-Seq data (Prot 57), Https://Api.Semanticscholar.Org/CorpusID:12843502 (2012).

F. Krueger, S.R. Andrews, Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications, Bioinformatics 27 (2011) 1571–1572.

H. Feng, K.N. Conneely, H. Wu, A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data, Nucleic Acids Res 42 (2014) e69–e69.

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