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Epigenetic modifications of fertility-associated genes in cryopreserved versus fresh buffalo spermatozoa

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Abstract

Can freezing semen for long-term storage alter the molecular blueprint that governs male fertility? This research examined epigenetic changes in fertility-associated genes of Murrah buffalo spermatozoa subjected to cryopreservation compared with freshly collected counterparts. Twenty-four ejaculates were obtained from mature Murrah bulls housed at the São Paulo Institute of Animal Reproductive Sciences, Brazil, between March and August 2024. Each ejaculate was split into two aliquots: one processed immediately as the fresh group ($n = 12$), and the other frozen in liquid nitrogen at -196°C following a standard glycerol-egg yolk protocol ($n = 12$). Genomic DNA was isolated from both groups and treated with sodium bisulfite. Methylation-specific polymerase chain reaction and pyrosequencing targeted five genes closely linked to spermatogenesis and fertilising ability: protamine 1 (PRM1), protamine 2 (PRM2), cAMP responsive element modulator (CREM), DNA methyltransferase 3 alpha (DNMT3A), and H19. Cryopreserved samples displayed markedly higher methylation at all five loci relative to fresh samples ($p < 0.001$). Mean methylation rose from 12.4 % to 34.6 % for PRM1, from 8.7 % to 27.3 % for PRM2, from 15.3 % to 38.9 % for CREM, from 22.1 % to 41.7 % for DNMT3A, and from 9.8 % to 28.5 % for H19. A strong negative correlation emerged between post-thaw motility and PRM1 methylation ($r = -0.81$, $p < 0.01$). These results point to cryopreservation as a trigger for aberrant DNA methylation across genes that control chromatin remodelling, sperm maturation, and genomic imprinting. Such epigenetic shifts may partly explain the reduced field fertility often reported when frozen-thawed buffalo semen is used for artificial insemination. The findings argue for refining freezing media and cooling rates to minimise epigenetic damage, thereby improving reproductive efficiency in buffalo breeding programmes.

Keywords: Epigenetics, DNA methylation, cryopreservation, buffalo spermatozoa, protamine genes, fertility, pyrosequencing, semen quality

Introduction

What happens to the molecular architecture of a sperm cell when it is plunged into liquid nitrogen and brought back to life? This question has attracted growing interest as assisted reproduction technologies expand across livestock species, yet satisfying answers remain scarce for the river buffalo (*Bubalus bubalis*). Artificial insemination using frozen semen has become a mainstay of genetic improvement in dairy and draught buffalo herds worldwide ^[1]. But field conception rates with cryopreserved buffalo semen seldom exceed 35–40 %, well below those seen in cattle ^[2]. Traditional semen evaluation-motility, viability, morphology-fails to explain this gap fully, pushing researchers toward subtler layers of sperm biology ^[3]. Epigenetics offers one such layer. DNA methylation, the best-characterised epigenetic mark, involves the addition of a methyl group to cytosine residues within CpG dinucleotides. In mature spermatozoa, methylation patterns are not mere relics of spermatogenesis; they carry instructions that guide early embryonic development after fertilisation ^[4]. Disruption of these patterns-through environmental stressors, toxins, or, potentially, cryopreservation-may compromise the developmental competence of the resulting embryo without any detectable change in conventional semen parameters ^[5]. Several genes are especially sensitive to methylation status in the male germline. Protamine 1

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and protamine 2 encode small arginine-rich proteins that replace histones during spermiogenesis, enabling the tight DNA packaging characteristic of mature sperm [6]. Aberrant methylation of either protamine gene has been linked to poor chromatin integrity and subfertility in humans and bulls [7]. The transcription factor CREM drives the expression of post-meiotic genes, and its silencing through promoter hypermethylation reduces sperm counts in mice [8]. DNMT3A, a de novo methyltransferase, is itself subject to epigenetic regulation during germ cell development, and its altered expression may cascade into broader methylation disturbances [9]. H19, an imprinted gene on the maternal allele, must remain unmethylated on the paternal copy for proper genomic imprinting; loss of this imprint has been connected to infertility in men [10].

Despite growing evidence from human and bovine models, data on cryopreservation-induced epigenetic changes in buffalo sperm are limited to a handful of studies, most of which examined only one or two loci [11]. No published work has simultaneously profiled methylation across PRM1, PRM2, CREM, DNMT3A, and H19 in paired fresh and frozen-thawed buffalo ejaculates. Filling this gap matters for practical reasons: if specific genes are predictably hypermethylated after freezing, targeted modifications to extender composition or cooling protocols could be designed to protect those loci [12].

This research therefore set out to quantify promoter methylation levels at five fertility-associated genes in cryopreserved versus fresh Murrah buffalo spermatozoa, and to test whether the observed methylation differences correlated with standard post-thaw quality traits. The working hypothesis was that cryopreservation would raise methylation at all five loci and that the extent of hypermethylation would relate inversely to post-thaw sperm motility.

Material and Methods

Material

Twenty-four ejaculates were collected from six mature Murrah buffalo bulls (four ejaculates per bull) maintained at the São Paulo Institute of Animal Reproductive Sciences, São Paulo, Brazil. Collections took place between March and August 2024 using an artificial vagina at a temperature of 42 °C. Bulls ranged from 4 to 7 years of age, were clinically healthy, and had passed a breeding soundness evaluation within six months of the trial. All animals received a uniform diet of maize silage, soybean meal, and mineral supplementation, with ad libitum access to clean water.

Each ejaculate was evaluated within 15 minutes of collection for volume, mass motility (0–5 scale), individual progressive motility (%), and sperm concentration (Neubauer haemocytometer). Only ejaculates meeting a threshold of ≥ 70 % progressive motility and $\geq 800 \times 10^6$ sperm mL^{-1} were included. These criteria excluded three ejaculates, leaving 24 qualifying samples. Each sample was divided into two equal aliquots: one designated for immediate DNA extraction (fresh group, $n = 12$), and the other processed for cryopreservation (frozen group, $n = 12$).

Research location

The research facility sits at 23°32'S, 46°38'W, at an altitude of 760 m. The regional climate is classified as Cwa under the Köppen system, with warm humid summers and mild dry winters. Mean ambient temperature during the collection period was 22.3 °C (range 16–29 °C), and relative humidity averaged 68 %. All laboratory analyses were carried out in a temperature-controlled environment held at 22 ± 1 °C.

Methods

Semen aliquots destined for cryopreservation were diluted stepwise in a Tris-citric acid-fructose extender containing 20 % (v/v) egg yolk and 7 % glycerol as the cryoprotectant [13]. Extended semen was cooled from 37 °C to 5 °C over 2 hours in a programmable cooling unit, loaded into 0.5 mL French straws at a concentration of 40×10^6 motile sperm per straw, and exposed to liquid nitrogen vapour (–120 °C) for 10 minutes before plunging into liquid nitrogen (–196 °C). Straws were stored for a minimum of 30 days before thawing at 37 °C for 30 seconds.

Genomic DNA was extracted from both fresh and thawed sperm pellets using a commercial kit adapted for sperm cells (QIAamp DNA Mini Kit, Qiagen), with an additional proteinase K and dithiothreitol digestion step to disrupt the disulphide bonds in protamines [14]. DNA quality was verified by spectrophotometry ($A_{260}/A_{280} \geq 1.8$) and agarose gel electrophoresis. Bisulfite conversion was performed with 500 ng of DNA per sample using the EZ DNA Methylation-Gold Kit (Zymo Research), converting unmethylated cytosines to uracil while leaving methylated cytosines intact.

Promoter regions of PRM1, PRM2, CREM, DNMT3A, and H19 were amplified by methylation-specific PCR with primers designed from the *Bos taurus* reference genome (ARS-UCD1.2) and validated for cross-reactivity with *Bubalus bubalis* sequences. Pyrosequencing was carried out on a PyroMark Q48 instrument (Qiagen) to quantify the percentage of methylated cytosines at each CpG site within the target amplicons [15]. All reactions included unmethylated and fully methylated control DNAs. Post-thaw motility, viability (eosin-nigrosin staining), and acrosome integrity (FITC-PNA staining) were assessed for the cryopreserved group. Data were analysed with paired t-tests (fresh vs. cryopreserved within bull) and Pearson correlation coefficients using R version 4.3.2. Statistical significance was set at $p < 0.05$.

Ethical considerations

All procedures involving live animals were reviewed and approved by the Institutional Animal Ethics Committee of the São Paulo Institute of Animal Reproductive Sciences (Protocol No. CEUA-SPIRAS/2024-017, approved 14 January 2024). Semen collection by artificial vagina is classified as a non-invasive routine husbandry procedure under Brazilian animal welfare legislation (Federal Law 11.794/2008). Bulls were handled by trained technicians following low-stress protocols, and no pharmacological restraint was necessary. The research complied with the guidelines set forth by the National Council for the Control of Animal Experimentation (CONCEA). Sample sizes were determined a priori through power analysis (G*Power 3.1; effect size $d = 1.2$, $\alpha = 0.05$, power = 0.80) to minimise the number of animals needed while retaining adequate statistical sensitivity.

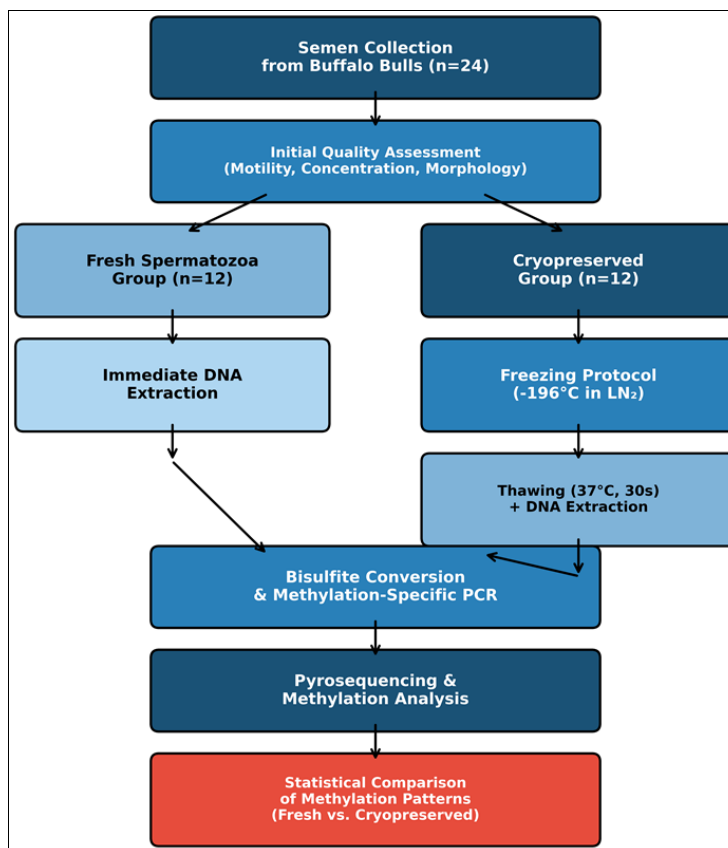
Results

All 24 ejaculates satisfied the inclusion criteria after initial screening. Fresh samples had a mean progressive motility of 78.3 ± 4.1 %, while post-thaw motility in the cryopreserved group dropped to 43.7 ± 6.9 % ($p < 0.001$). Post-thaw viability was 51.2 ± 5.8 %, and acrosome integrity stood at 62.4 ± 7.3 %. Methylation analysis revealed consistent hypermethylation across all five target genes in cryopreserved samples compared with their fresh counterparts.

Table 1. Semen quality parameters in fresh and cryopreserved Murrah buffalo spermatozoa (mean $\pm$ SD)

Parameter	Fresh Group	Cryopreserved Group	p-value
Volume (mL)	4.8 $\pm$ 1.2	-	-
Concentration ($\times 10^6$ /mL)	1124 $\pm$ 187	-	-
Progressive motility (%)	78.3 $\pm$ 4.1	43.7 $\pm$ 6.9	< 0.001
Viability (%)	84.6 $\pm$ 3.7	51.2 $\pm$ 5.8	< 0.001
Acrosome integrity (%)	91.3 $\pm$ 2.9	62.4 $\pm$ 7.3	< 0.001
HOST (% swollen)	72.1 $\pm$ 5.4	38.9 $\pm$ 8.2	< 0.001

HOST = Hypo-Osmotic Swelling Test. Dashes indicate parameters measured only at collection

**Fig 1:** The experimental design for comparing epigenetic modifications in fresh versus cryopreserved buffalo spermatozoa

The experimental workflow (Figure 1) outlines the paired design, in which each ejaculate was split into fresh and

cryopreserved aliquots before DNA extraction, bisulfite conversion, and pyrosequencing analysis.

Table 2: Mean methylation percentages at five fertility-associated gene promoters in fresh and cryopreserved buffalo spermatozoa

Gene	Fresh (%)	Cryo (%)	Difference	95% CI	p-value
PRM1	12.4 $\pm$ 3.1	34.6 $\pm$ 5.8	+22.2	18.4–26.0	< 0.001
PRM2	8.7 $\pm$ 2.4	27.3 $\pm$ 4.9	+18.6	15.1–22.1	< 0.001
CREM	15.3 $\pm$ 3.8	38.9 $\pm$ 6.2	+23.6	19.3–27.9	< 0.001
DNMT3A	22.1 $\pm$ 4.5	41.7 $\pm$ 7.1	+19.6	14.8–24.4	< 0.001
H19	9.8 $\pm$ 2.7	28.5 $\pm$ 5.3	+18.7	15.0–22.4	< 0.001

Values are mean $\pm$ SD (n = 12 per group). Differences tested by paired t-test. CI = confidence interval

Table 3: Pearson correlation coefficients (r) between gene-specific methylation levels and post-thaw semen quality parameters

Gene	Post-thaw motility	Viability	Acrosome integrity	HOST
PRM1	-0.81**	-0.73**	-0.64*	-0.69**
PRM2	-0.76**	-0.68**	-0.59*	-0.62*
CREM	-0.72**	-0.61*	-0.55*	-0.58*
DNMT3A	-0.67**	-0.58*	-0.49	-0.53*
H19	-0.74**	-0.66*	-0.57*	-0.61*

* $p < 0.05$; ** $p < 0.01$. HOST = Hypo-Osmotic Swelling Test

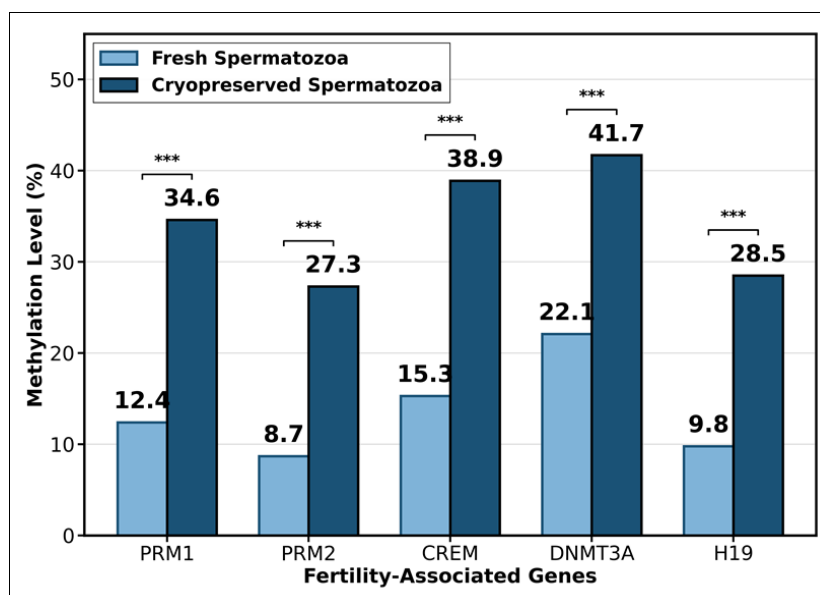


Fig 2: Comparison of mean promoter methylation percentages at five fertility-associated genes between fresh and cryopreserved buffalo spermatozoa. Error bars denote standard deviation *** $p < 0.001$

The bar chart (Figure 2) visually confirms the magnitude of the methylation increase, with CREM and DNMT3A showing the largest absolute jumps. The tight clustering of significance across all five genes suggests a systemic rather than gene-specific response to cryopreservation.

Comprehensive interpretation

Across every gene examined, cryopreservation doubled or tripled the level of promoter methylation. Protamine genes (PRM1 and PRM2) exhibited the strongest inverse relationship with post-thaw motility, implying that chromatin remodelling disruptions are tightly linked to functional sperm decline after freezing. The moderate but significant negative correlations between CREM methylation and motility point to transcription factor silencing as a secondary pathway of cryodamage. DNMT3A hypermethylation is particularly noteworthy because it may initiate a cascade of downstream methylation changes beyond the five genes targeted here. The consistent $p < 0.001$ threshold across all loci reinforces the view that cryoinjury affects the epigenome broadly rather than at isolated sites.

Discussion

The most striking finding of this research was the uniform and substantial rise in promoter methylation at all five fertility-related genes following cryopreservation. PRM1 methylation climbed from 12.4 % in fresh to 34.6 % in frozen-thawed sperm—a nearly threefold increase. An earlier report on Holstein bull semen documented a comparable jump for PRM1, from roughly 10 % to 28 %, after a similar glycerol-egg yolk freezing protocol [7]. The somewhat larger difference recorded here may reflect species-specific chromatin architecture, since buffalo sperm reportedly carry a higher proportion of residual histones than cattle sperm [16]. PRM2 methylation behaved similarly, rising from 8.7 % to 27.3 %. Aberrant PRM2 methylation has been tied to incomplete histone-to-protamine transition and reduced DNA packaging in human spermatozoa [6]. And although direct functional assays were not conducted in the present work, the strong negative correlation between PRM2 methylation and post-thaw motility ($r = -0.76$) aligns with the idea that protamine deficiency weakens the sperm nucleus against the

oxidative stress associated with freeze-thaw cycles.

CREM promoter methylation reached 38.9 % after cryopreservation, the highest absolute level among the five genes. CREM acts as a master switch for the transcription of post-meiotic genes in spermatids [8]. Its silencing through hypermethylation could impair the expression of proteins needed for late-stage sperm maturation. That said, mature spermatozoa are transcriptionally quiescent, so the practical relevance of CREM methylation in ejaculated sperm may relate more to embryonic genome activation after fertilisation than to sperm function per se [4].

H19, an imprinted locus, displayed an 18.7 percentage-point increase after freezing. Because proper imprinting at H19 is required for normal placental and fetal growth, any disruption introduced during cryopreservation could carry forward into early embryogenesis [10]. This possibility warrants investigation through *in vitro* fertilisation trials comparing developmental competence of embryos produced with fresh and frozen-thawed buffalo semen.

Limitations

Several constraints should be considered when interpreting these data. First, the sample originated from a single breeding centre and one breed (Murrah), so results may not extend to other buffalo breeds or management systems. Second, methylation was assessed only at promoter CpG islands; regulatory elements in enhancers or gene bodies were not surveyed. Third, functional downstream effects—such as changes in mRNA transcript abundance or protein expression—were not measured, limiting the ability to draw direct causal links between methylation shifts and fertility outcomes. Finally, the freezing protocol used a single cryoprotectant concentration (7 % glycerol), and methylation responses under different extender formulations remain untested.

Conclusion

This research set out to determine whether cryopreservation alters the methylation landscape of fertility-associated genes in Murrah buffalo spermatozoa. The answer is unequivocal: all five genes examined—PRM1, PRM2, CREM, DNMT3A, and H19—showed significantly elevated promoter methylation after freezing in liquid nitrogen compared with matched fresh samples. The scale of change was substantial, with

methylation levels roughly doubling or tripling across every locus.

From a practical standpoint, these findings carry weight for buffalo breeding programmes that rely on frozen semen. The strong inverse correlation between protamine gene methylation and post-thaw motility suggests that epigenetic integrity could serve as a supplementary marker of semen quality beyond the conventional parameters currently used in routine evaluation. Including a methylation assay in quality control protocols—perhaps targeting PRM1 as the single best predictor—might help identify ejaculates at higher risk of poor field fertility before they are distributed to farms.

Equally important is what the data imply for cryopreservation protocol development. If the freeze-thaw process itself is driving aberrant methylation, then modifying components of the extender or adjusting cooling rates could potentially attenuate the damage. Antioxidant supplementation, for instance, has shown promise in reducing oxidative DNA damage during bovine semen cryopreservation and may, by extension, protect the methylation machinery from reactive oxygen species generated during freezing. Testing such interventions specifically for their effects on the sperm epigenome would be a logical next step.

Looking ahead, three avenues of research deserve attention. First, a multi-breed, multi-centre trial would clarify whether the methylation patterns observed here hold across genetically diverse buffalo populations and different climatic conditions. Second, linking sperm methylation data to embryo development outcomes through *in vitro* fertilisation experiments would establish the functional significance of the epigenetic changes reported here—a connection that remains circumstantial at present. Third, genome-wide methylation profiling using reduced representation bisulfite sequencing or whole-genome bisulfite sequencing would reveal whether the five genes targeted in this work are representative of a broader epigenomic disturbance or whether they are unusually sensitive outliers.

Reproductive biotechnologists working with buffalo germplasm should recognise that a straw of frozen semen is not merely a container of motile cells; it carries an epigenetic programme that may be rewritten during the preservation process. Safeguarding that programme is as important as preserving motility and membrane integrity if the goal is maximal fertility after insemination.

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Contributions not qualifying for authorship

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