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Molecular basis of fertility variation in cryopreserved bull semen: Functional evaluation and expression analysis of *IZUMO1*, *PLCζ*, and *CatSper* genes

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Semen cryopreservation serves as a pivotal biotechnological tool implemented in artificial insemination programs to accelerate genetic progress in cattle breeding. However, the freeze-thawing cycle can heavily compromise fertilization efficiency due to sublethal cellular, functional, and molecular injuries. This study aimed to evaluate the impacts of cryopreservation on conventional semen parameters and the relative transcript abundance of fertility-associated target genes (*CatSper*, *IZUMO1*, and *PLCζ*) utilizing straws from high-fertility and low-fertility breeding bulls. A retrospective cohort comprising 92 post-thaw semen samples (50 high-fertility and 42 low-fertility) was investigated. Sperm motility kinetics were quantified via Computer-Assisted Sperm Analysis (CASA), while structural viability, plasma membrane integrity, and normal cytomorphology were determined using Eosin-Nigrosin exclusion, hypoosmotic swelling tests, and Diff-Quik staining, respectively. Quantitative real-time PCR (RT-qPCR) was executed to evaluate relative gene expression normalized against the reference gene *GAPDH*, based on the comparative $2^{-\Delta\Delta Ct}$ methodology. The spermiogram analysis demonstrated a statistically significant downregulation in directional progressive motility (62.7 % vs. 65.4 %; P=0.011), structural viability (78.5 % vs. 80.2 %; P=0.045), and membrane permselectivity (72.2 % vs. 74.6 %; P=0.045) in the low-fertility group, accompanied by a suppression in normal sperm morphology (73.7 % vs. 78.1 %; P=0.0001). Conversely, overall total sperm motility displayed no significant statistical variance between the cohorts (74.0 % vs. 77.9 %; P=0.063). At the molecular level, all three target transcripts exhibited profound downregulations in the subfertile group: a 2.43-fold decrease for *IZUMO1*, a 2.78-fold decrease for *PLCζ*, and a 3.45-fold decrease for *CatSper*, establishing a distinct hierarchy of transcript instability (P<0.001). The study demonstrates that cryopreservation induces high transcript degradation that directly compromises flagellar hyperactivation, gamete fusion, and post-fertilization oocyte activation pathways. These findings suggest that integrating quantitative gene expression analysis with conventional spermiograms offers an invaluable diagnostic modality to diagnose cryptic male subfertility, optimize semen straw selection, and enhance reproductive efficiency in assisted breeding programs.

Keywords: Bull semen cryopreservation, Gene expression, *CatSper*, *IZUMO1*, *PLCζ*, RT-qPCR.

Молекулярні основи варіації фертильності у кріоконсервованій спермі бугаїв: функціональна оцінка та аналіз експресії генів *IZUMO1*, *PLCζ* та *CatSper*

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Кріоконсервація сперми є ключовим біотехнологічним інструментом, який застосовують у програмах штучного осіменіння для покращення генетичних ознак великої рогатої худоби. Проте цикл заморожування-розморожування може суттєво знижувати ефективність запліднення через сублетальні клітинні, функціональні та молекулярні пошкодження. Метою цього дослідження було оцінити вплив кріоконсервації на традиційні параметри спермограми та відносний вміст транскриптів, пов'язаних із фертильністю генів-мішеней (*CatSper*, *IZUMO1* та *PLCζ*) у паєтах із замороженою спермою бугаїв-плідників із високою та низькою фертильністю. У межах ретроспективного дослідження було проаналізовано 92 зразки розмороженої сперми (50 від високофертильних і 42 від низькофертильних тварин). Кінетичні характеристики рухливості сперматозоїдів визначали за допомогою комп'ютерного аналізу CASA, тоді як структурну життєздатність, цілісність плазматичної мембрани та нормальну цитоморфологію оцінювали відповідно методом виключення еозину-нігрозину, гіпоосмотичним тестом (HOST) та фарбуванням Diff-Quik. Для визначення відносної експресії генів, нормованої за референсним геном *GAPDH*, виконували кількісну ПЛР у реальному часі (RT-qPCR) за порівняльним математичним методом $2^{-\Delta\Delta Ct}$. Аналіз спермограми продемонстрував статистично значуще зниження спрямованої прогресивної рухливості (62,7 проти 65,4 %; P=0,011), структурної життєздатності (78,5 проти 80,2 %; P=0,045) та функціональної цілісності мембран (72,2 проти 74,6 %; P=0,045) у групі з низькою фертильністю, що супроводжувалося пригніченням показника нормальної морфології сперматозоїдів (73,7 проти 78,1 %; P=0,0001). Навпаки, загальна рухливість сперматозоїдів не виявила значущої статистичної різниці між вибірками (74,0 проти 77,9 %; P=0,063). На молекулярному рівні всі три досліджувані транскрипти продемонстрували глибоке пригнічення в субфертильній групі: зниження експресії *IZUMO1* у 2,43 раза, *PLCζ* у 2,78 раза та *CatSper* у 3,45 раза, що підтвердило чітку ієрархію нестабільності кількості транскриптів (P<0.001). Дослідження доводить, що кріоконсервація зумовлює виражену деградацію мРНК, яка безпосередньо порушує процеси гіперактивації джгутика, злиття гамет та активації ооцита в процесі запліднення. Отримані результати свідчать, що інтеграція кількісного аналізу експресії генів з метою оцінки якості сперми забезпечує високу точність діагностики прихованої субфертильності плідників, оптимізує добір паєт зі спермою і підвищує ефективність програм штучного осіменіння.

Ключові слова: кріоконсервація сперми бугаїв, експресія генів, *CatSper*, *IZUMO1*, *PLCζ*, RT-qPCR.

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Introduction

Artificial insemination techniques serve as a cornerstone for improving livestock production and are widely implemented in breeding programs to enhance genetic traits and boost productivity, as this approach provides an effective method for the long-term preservation of genetic material, while facilitating its transfer and utilization in genetic improvement programs [1].

However, numerous studies have demonstrated a significant decrease in fertility rates when utilizing cryopreserved semen compared to fresh samples, even in cases where spermatozoa exhibit normal motility and morphology. These outcomes strongly indicate the involvement of underlying molecular factors that evade detection by traditional assays. Because routine laboratory tests are limited to evaluating baseline parameters such as sperm motility, morphology, concentration, and viability, they remain insufficient for the accurate prediction of actual fertilization efficiency [2].

In this context, recent studies have focused on molecular markers related to the fertilization process, given that sperm functionality depends on the proper expression of several genes regulating different stages of the fertilization cascade. Among the most critical of these markers are *IZUMO1*, which mediates sperm-egg fusion; *PLCζ*, responsible for post-fertilization oocyte activation; and the CatSper gene, which regulates the influx of calcium ions into the spermatozoon – a process essential for inducing the hyperactivation necessary to penetrate the outer layers of the oocyte and successfully complete fertilization [3].

The freezing and thawing process induces severe structural and functional alterations in spermatozoa, including the disruption of plasma membrane integrity, elevated oxidative stress, and impaired mitochondrial function. These changes can detrimentally affect messenger RNA (mRNA) stability and the relative expression of genes involved in fertilization, thereby compromising the sperm's capacity to interact with the oocyte. However, these cryopreservation-induced molecular shifts remain insufficiently explored, particularly in bovines [4].

Recent investigations have demonstrated that cryopreservation workflows lead to a significant downregulation in the gene expression of several proteins associated with the sperm membrane, particularly those orchestrating sperm-egg interactions. For example, transcript levels of mRNA for the *IZUMO1* gene were significantly lower in cryopreserved semen compared to fresh samples, resulting in an impaired capacity of the spermatozoa to bind to and fuse with the oolemma [5].

Similarly, a significant downregulation in the expression of the *PLCζ* gene, the pivotal factor responsible for stimulating calcium waves within the oocyte post-fertilization, has been observed. This molecular deficiency leads to oocyte activation failure and the subsequent disruption of early embryonic cleavages [6].

Despite significant progress in understanding the impact of cryopreservation on semen quality, most studies have focused on conventional parameters or examined each gene individually. Investigations that simultaneously link the relative gene expression of *IZUMO1*, *PLCζ*, and *CatSper* to the actual fertilization efficiency of cryopreserved bovine semen remain limited.

The aim of the study

The aim of this study was to compare the relative gene expression of *CatSper*, *IZUMO1*, and *PLCζ* genes in the cryopreserved semen of high-fertility and low-fertility bulls to assess their potential as molecular biomarkers that enhance the accuracy of fertilization efficiency predictions and improve the outcomes of artificial insemination programs.

Problem statement. Artificial insemination (AI) centers and in vitro fertilization (IVF) laboratories routinely rely on traditional assays to assess the quality of cryopreserved semen. However, the outcomes of these baseline tests do not always correlate with actual field fertility rates in cows. Certain samples may exhibit acceptable motility, viability, and normal morphology, yet yield low fertilization rates, strongly suggesting the presence of underlying molecular or functional deficits that evade detection by routine screenings.

Research hypothesis. This study hypothesizes that low fertility in cryopreserved bovine semen is closely associated with a reduced relative expression of the *CatSper*, *IZUMO1*, and *PLCζ* genes, and that analyzing their transcript abundance provides a more precise and reliable assessment of fertilization efficiency compared to conventional semen testing.

Study objectives:

1. To evaluate the quality of cryopreserved bull semen using conventional laboratory assays.
2. To measure the transcript expression levels of the *CatSper*, *IZUMO1*, and *PLCζ* genes using quantitative RT-qPCR.
3. To compare relative gene expression profiles between high-fertility and low-fertility semen samples.
4. To evaluate the potential of using these specific genes as functional molecular indicators to forecast fertilization efficiency.

Materials and methods

Study area, design, and sample classification

A retrospective review of the reproductive records at the artificial insemination center in Diwaniyah Governorate, Iraq, revealed a notable discrepancy in field pregnancy rates. This variation occurred despite the fact that all utilized semen straws strictly met established quality standards during routine testing both pre-freezing and post-thawing. Furthermore, no technical anomalies were recorded regarding storage conditions or liquid nitrogen levels.

In this study, cryopreserved semen straws served as the primary unit for molecular analysis, whereas field pregnancy outcomes were utilized to stratify the samples into high-fertility and low-fertility groups, accounting for

the bull effect in the subsequent statistical modeling. The semen straws were classified into two distinct cohorts based on the documented reproductive outcomes of the inseminated cows:

- High-Fertility Group (n = 50 semen samples): Semen samples where pregnancy was successfully confirmed via manual rectal palpation at 45 and 60 days following the initial insemination.

- Low-Fertility Group (n = 42 semen samples): Semen samples from cows that failed to conceive after at least three consecutive inseminations, despite identical storage, handling, and thawing conditions across both cohorts.

All samples were coded and classified based on post-thaw fertility indicators. Standard semen analysis – including the baseline evaluation of motility and morphology – showed statistical similarity between the two groups within acceptable thresholds, highlighting the critical limitations of conventional criteria in elucidating variations in fertilization efficiency. Notably, a subset of cows that had previously failed to conceive successfully achieved pregnancy when re-inseminated with semen from the high-fertility cohort, strongly suggesting the involvement of cryptic molecular or functional factors that evade routine laboratory screening.

To ensure high reproducibility, a standardized operating protocol was strictly implemented across all stages of the study, including sample thawing, conventional laboratory assays, and quantitative gene expression analysis via RT-qPCR. All procedural steps were performed identically, ensuring that any observed differences between the samples resulted strictly from biological variations rather than procedural discrepancies.

Cow selection and exclusion criteria

To minimize the confounding effects of female reproductive factors on the field pregnancy outcomes, all cows included in the retrospective cohort underwent a comprehensive pre-insemination reproductive screening. The specific parameters for animal enrollment were established based on the following selection criteria:

Inclusion criteria: Local, multiparous cows aged between 3 and 7 years; Body condition score (BCS) between 2.5 and 3.5 at the time of insemination; Regular estrous cycles confirmed by two consecutive progesterone measurements or direct behavioral observation; Transrectal palpation demonstrating a dominant follicle with a minimum diameter of 12 mm at the time of insemination, accompanied by a normal uterine wall tone and the complete absence of ovarian cysts or a persistent corpus luteum; No clinical history of reproductive pathology, such as metritis or pyometra, within the preceding 90 days.

Exclusion criteria: Cows presenting with endometritis; Cows with abnormal peripheral progesterone profiles indicative of ovulatory disturbances or luteal phase insufficiency; Cows that had received any hormonal therapy within 30 days prior to artificial insemination; Cows with a body condition score (BCS) below 2.0 or exceeding 4.0.

Based on the strict fulfillment of all inclusion criteria, the enrolled cows were considered reproductively sound. Consequently, any failure to conceive within the low-fertility cohort was primarily attributed to sperm-related factors, thereby validating the classification of semen straws as the primary independent variable in this study.

Thawing and sample preparation

Cryopreserved semen straws were retrieved from liquid nitrogen storage (–196 °C) and thawed in a digital water bath at 37 °C for 30–40 seconds. After being thoroughly dried, the straws were opened under aseptic conditions, and their contents were transferred into a sterile 15-mL conical centrifuge tube. The samples were subsequently incubated at 37 °C for 10 minutes prior to downstream analysis. The individual straw identification number, assigned fertility cohort, and exact thawing date were recorded for each sample.

Laboratory tests

The operational protocols for assessing baseline sperm quality parameters were adapted from the WHO Laboratory Manual for the Examination and Processing of Human Semen [7], with minor modifications to accommodate the specific requirements for evaluating cryopreserved bovine spermatozoa. To ensure strict methodological alignment with veterinary medicine standards, the guidelines of the Society for Theriogenology [8], which are widely implemented in assessing the fertility potential of farm animals, were also integrated into the experimental workflow.

Sperm evaluation using CASA

Sperm motility characteristics were evaluated using a Computer-Assisted Sperm Analysis (CASA) system as the primary quantitative methodology. A 5–10 µL aliquoted semen sample was loaded onto a pre-heated microscope slide maintained at 37 °C, and a minimum of 200 spermatozoa were tracked and analyzed per individual sample. The assessed kinematic parameters included total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, µm), and beat cross-frequency (BCF, Hz) [9].

Sperm morphology assessment

For morphological evaluation, a total of 200 spermatozoa per sample were stained using the Diff-Quik methodology and examined under a light microscope at 1000× magnification using an oil immersion lens. Structural abnormalities localized in the sperm head, midpiece, and tail were recorded according to standardized veterinary and theriogenology criteria for bovine semen, with the results expressed as percentages [10].

Gene expression analysis by RT-qPCR

Quantitative gene expression analysis was performed according to the methodology described by [11], with minor modifications.

Total RNA extraction

Total RNA from the thawed spermatozoa was isolated using the GeneJET RNA Purification Kit (Thermo Scientific, USA) in strict accordance with the manufacturer's operational instructions. On-column

DNase I digestion was implemented to eliminate any residual genomic DNA contamination. Total RNA concentration and purity were assessed using a NanoDrop Spectrophotometer based on the 260/280 nm absorbance ratio, with values ranging strictly between 1.8 and 2.0 considered acceptable for downstream applications. Additionally, RNA structural integrity was verified via horizontal gel electrophoresis on a 1 % agarose gel supplemented with SafeRed dye, run at 90 V for 20 minutes.

cDNA synthesis

For reverse transcription, 1 µg of isolated total RNA was converted into complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) in strict accordance with the manufacturer's operational manual. The amplification protocol involved the initial denaturation of RNA secondary structures at 65 °C for 5 minutes, followed by the reverse transcription reaction at 42 °C for 60 minutes. The process was finalized by enzyme thermal inactivation at 70 °C for 5 minutes. The resulting cDNA templates were stored at –20 °C until further utilization in downstream quantitative RT-qPCR assays.

Primer Design. Specific oligonucleotide primer sequences for the target genes (*IZUMO1*, *PLCζ*, and *CatSper*) and the internal reference gene (*GAPDH*) were designed based on the GenBank database (NCBI) using Primer3 software. Primer specificity, thermodynamic characteristics, and the prevention of self- or cross-dimer formations were thoroughly verified utilizing the PCR PrimerStats software application. The optimized primer sequences, target gene configurations, and specific melting temperatures are summarized in ***Table 1***.

Table 1

Characteristics and oligonucleotide sequences of the primers used for RT-qPCR

Target gene	Primer sequence (5'–3')	NCBI Gene ID	Amplicon size (bp)	Tm (°C)
IZUMO1	F: 5′-TTCCAGAAACTCAGGGAAGG-3′ R: 5′-CATTCAGCTTTCCGGTCTTC-3′	618057	134	59.8
PLCζ	F: 5′-AGGCTGAAGTTGGGGATTTC-3′ R: 5′-ACCTTCAGCCAACAACATCC-3′	514590	123	60.0
CatSper	F: 5′-GTCCATTGTGTTGGTGATGC-3′ R: 5′-ATACTTTGGCCACCTGATGC-3′	616691	130	59.8
GAPDH	F: 5′-CTCCCTCTTTTCCAGCAATG-3′ R: 5′-TTCAAGTTTCCCACCACTCC-3′	281181	120	59.9

Quantitative real-time PCR (RT-qPCR)

Transcript expression analysis was executed via quantitative real-time PCR utilizing an Exicycler™ 96 Real-Time Quantitative Thermal Blocker (Bioneer, Korea) and Maxima SYBR Green Master Mix (Thermo Scientific, USA). The reaction mixture was prepared to a final volume of 20 µL, comprising 10 µL of SYBR Green Master Mix (2×), 1 µL of each forward and reverse primer (10 pmol/µL), 2 µL of cDNA template, and supplemented to volume with nuclease-free water.

The RT-qPCR amplification was performed using a optimized thermal profile consisting of an initial denaturation stage at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, primer annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. To verify the thermodynamic specificity of the amplification products, a post-amplification melting curve analysis was executed within a temperature range of 65–95 °C, with a continuous heating ramp of 0.5 °C every 5 seconds. All reactions were performed in triplicate per biological sample to ensure high analytical reproducibility. Each operational run included a no-template negative control (NTC) to monitor for potential contamination, alongside a positive control utilizing cDNA derived from healthy bovine testicular tissue to validate amplification efficiency.

The cycle threshold (Ct) values were automatically determined by the Exicycler™ 96 system software. To control for variations arising from differences in initial RNA quantity or reverse transcription efficiency across individual samples, target gene expression levels were normalized against the internal reference gene (*GAPDH*). The relative fold change in transcript abundance was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak methodology) [12]:

$$\text{Fold change}=2^{-\Delta\Delta Ct}$$

The high-fertility sample cohort served as the experimental calibrator, with its baseline relative gene expression level standardized to 1.00. The final quantitative outcomes were expressed as mean ± standard deviation (Mean±SD) calculated from three independent replicates for each sample to minimize experimental variance (***Fig. 1***).

The flowchart illustrates the sequential methodology, starting with the classification of cryopreserved semen straws based on field fertility levels. This is followed by the evaluation of conventional sperm parameters, total RNA extraction, and the relative gene expression analysis of *CatSper*, *IZUMO1*, and *PLCζ* transcripts via RT-qPCR. The process concludes with the comprehensive statistical analysis of the quantitative results.

Statistical analysis

All statistical evaluations were performed using IBM SPSS Statistics software, version 26.0 (IBM Corp., Armonk, NY, USA). Quantitative data are expressed as mean ± standard deviation (Mean ± SD). The normality of the data distribution was verified using the Shapiro-Wilk test. To determine significance between the high-fertility cohort and the low-fertility (cryopreserved semen) group, an independent-samples Student's t-test was implemented.

The relative gene expression profiles of the *IZUMO1*, *PLCζ*, and *CatSper* genes were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak methodology), with the high-fertility group serving as the experimental calibrator (standardized to 1.00) to determine the relative fold change in transcript abundance. A probability value of $P<0.05$ was adopted as the threshold for statistical significance, whereas $P<0.001$ was considered highly statistically significant [13].

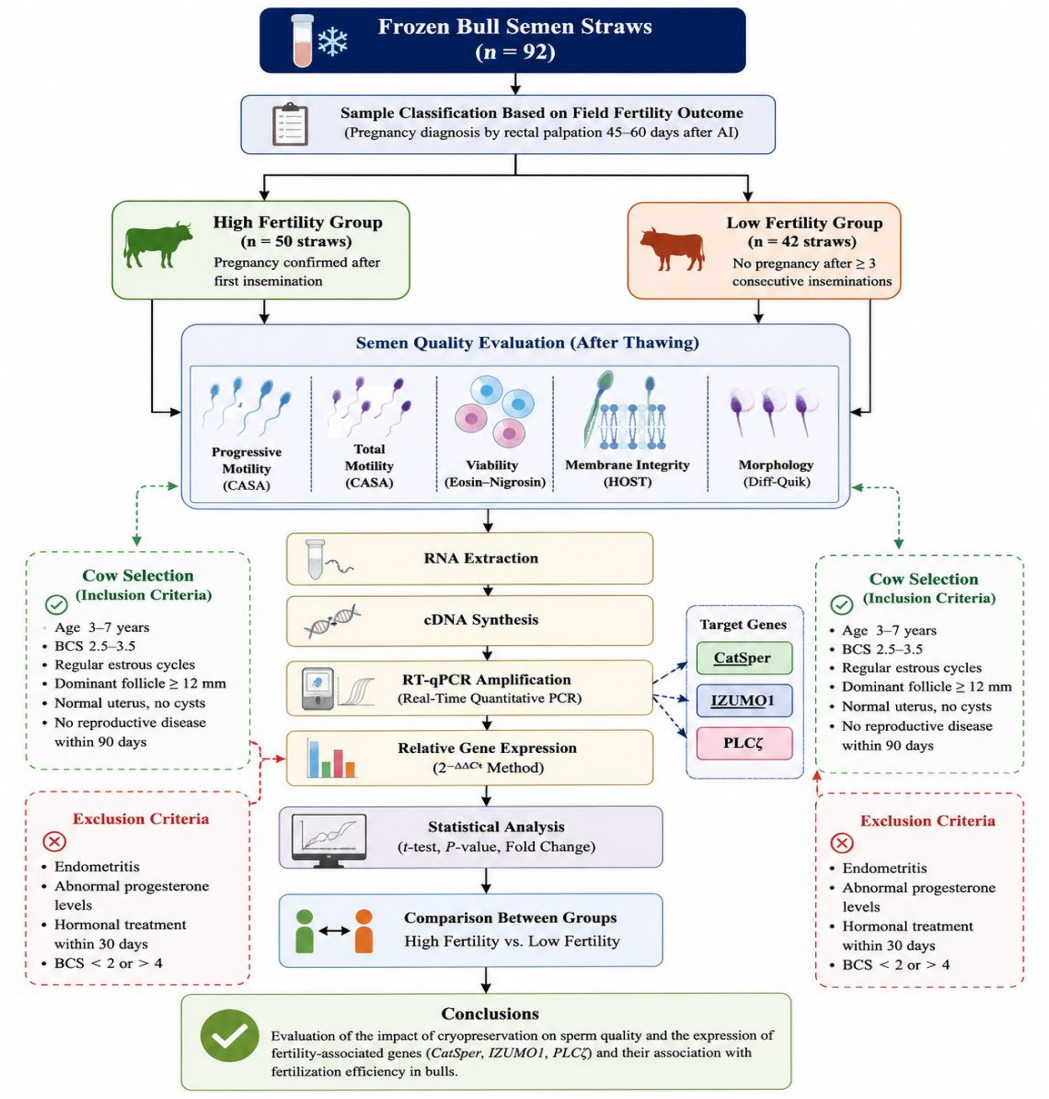

Fig. 1. Schematic workflow of the experimental study design

Results and discussion

Conventional semen profile

To establish a baseline functional baseline, a comparative evaluation of conventional semen characteristics was executed between the high-fertility and low-fertility cohorts. The quantitative parameters, including sperm motility kinetics, structural viability, membrane permselectivity, and cytomorphological profiles, are summarized in ***Table 2***.

Table 2

Comparative analysis of conventional semen parameters and sub-cellular structural integrity between high- and low-fertility cryopreserved bull straws, Mean ± SD

Sperm quality parameter	High-fertility group (n = 50)	Low-fertility group (n = 42)	Relative reduction (%)	P-value
Progressive motility (PR, %)	65.4±5.2	62.7±4.8	4.13	0.011
Non-progressive motility (NP, %)	12.5±2.1	11.3±3.0	9.60	0.031
Total motility (%)	77.9±4.5	74.0±5.1	5.20	0.063
Sperm viability (Eosin-nigrosin, %)	80.2±3.7	78.5±4.2	2.12	0.045
Membrane integrity (HOST, %)	74.6±5.0	72.2±6.1	3.22	0.045
Normal sperm morphology (Diff-Quik, %)	78.1±4.8	73.7±5.5	5.63	0.0001

Notes: An independent-samples Student's t-test was utilized for statistical comparisons between the experimental cohorts. Statistical significance was defined at $P<0.05$, whereas highly statistically significant differences were defined at $P<0.001$.

Quantitative assessment of semen parameters

The spermiogram outcomes revealed that cryopreservation workflows induced a significant degradation of directional kinetic traits in the low-fertility cohort. Specifically, a statistically significant decrease was observed in progressive sperm motility within the low-fertility group compared to the high-fertility control (62.7±4.8 % vs. 65.4±5.2 %, respectively; P=0.011), accompanied by a significant reduction in non-progressive motility (P=0.031). Conversely, the variance in overall total sperm motility between the two experimental cohorts did not reach the threshold for statistical significance (74.0±5.1 % vs. 77.9±4.5 %; P=0.063).

Furthermore, sub-cellular structural traits were compromised in the low-fertility group; both structural sperm viability, assessed via Eosin-Nigrosin exclusion, and functional plasma membrane integrity, determined through the hypoosmotic swelling test (HOST), exhibited significant reductions (*P*=0.045 for both parameters). Additionally, the incidence of normal sperm morphology was significantly suppressed in the low-fertility group (73.7±5.5 % vs. 78.1±4.8 %; P=0.0001), reflecting a highly significant increase in overall structural abnormalities. Taken together, these data indicate that while baseline total motility remained statistically equivalent, the specific functional and structural attributes of the spermatozoa were heavily impaired in the low-fertility cohort.

Relative transcript expression profile

To elucidate the molecular mechanisms underlying the observed variations in field fertility, a quantitative real-time PCR (RT-qPCR) analysis was executed for three critical fertility-associated target genes. The relative transcript abundance levels of *IZUMO1*, *PLCζ*, and *CatSper* were normalized against the internal reference gene (*GAPDH*), and the comparative data across the high-fertility and low-fertility cohorts are summarized in ***Table 3***.

Table 3. Comparative assessment of relative transcript abundance and fold changes for fertility-associated genes between high- and low-fertility bull semen cohorts

Target gene	High-fertility calibrator group (Mean±SD)	Low-fertility / cryopreserved group (Mean±SD)	Fold change analysis	P-value
IZUMO1	1.00±0.08	0.41±0.09	↓ 2.43	<0.001
PLCζ	1.00±0.06	0.36±0.07	↓ 2.78	<0.001
CatSper	1.00±0.05	0.29±0.06	↓ 3.45	<0.001

Notes: The high-fertility cohort served as the experimental calibrator, with its baseline relative gene expression level normalized to 1.00. Relative transcript expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ methodology (Livak method). Downward arrows (↓) indicate significant transcript downregulation relative to the calibrator group.

Quantitative assessment of relative gene expression

The molecular outcomes demonstrated a profound and highly significant suppression of mRNA transcript stability across all investigated target loci in the low-fertility group compared to the high-fertility control (***Table 3***, ***Figs. 2, 3***).

Regarding the *IZUMO1* locus, the mean relative gene expression dropped to 0.41±0.09 within the low-fertility cohort relative to the baseline calibrator value (1.00±0.08), representing a highly statistically significant 2.43-fold downregulation (P<0.001). Similarly, the transcript level for the *PLCζ* gene was significantly reduced to 0.36±0.07 in subfertile samples compared to the control group (1.00±0.06), yielding a highly significant 2.78-fold decrease (P<0.001).

The *CatSper* gene exhibited the most severe suppression among all analyzed molecular markers. Its mean relative expression level dropped to 0.29±0.06 in the low-fertility group compared to 1.00±0.05 in the high-fertility control cohort. This reduction represents a highly statistically significant 3.45-fold downregulation (P<0.001).

Taken together, these results indicate a coordinated downregulation of fertility-associated transcripts in cryopreserved semen straws from subfertile bulls, with the magnitude of suppression establishing a distinct hierarchy: *CatSper* > *PLCζ* > *IZUMO1*. This consistent transcript degradation implies a direct functional link between compromised mRNA stability and reduced fertilization efficiency, given the critical physiological roles these specific genes play in modulating sperm hyperactivation, post-fertilization oocyte activation, and gamete membrane fusion pathways.

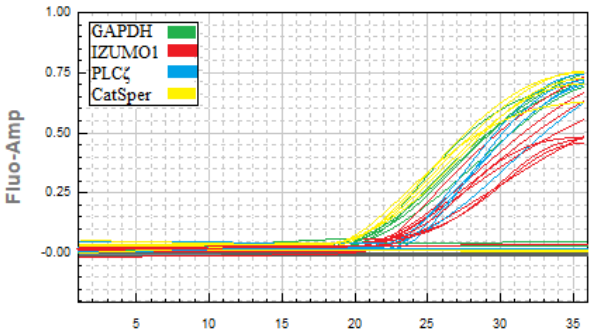

Fig. 2. Real-time PCR amplification curves of the target genes in the high-fertility group

The representative plots demonstrate the fluorescence intensity shifts across 36 amplification cycles. Variation in the amplification onset cycles reflects distinct quantification baseline thresholds (Ct values). The *CatSper* gene recorded the lowest Mean Ct value (20.75), indicating high transcript abundance, followed sequentially by *IZUMO1* (23.04) and *PLCζ* (24.24), which exhibited relatively delayed amplification curves indicative of lower relative expression levels.

The representative plots demonstrate a general delay in the amplification onset cycle for all investigated transcripts compared to the control cohort, corresponding to higher mean Ct values. Specifically, the *IZUMO1*

locus recorded a mean Ct value of 24.33, and *CatSper* recorded a mean Ct value of 25.04, whereas *PLCζ* exhibited the highest threshold detection value (25.74). The overall rightward shift of the amplification trajectories indicates that a higher number of replication cycles was required to detect target fluorescence signals, confirming a severe suppression of mRNA stability, with the most pronounced downregulation observed in the *CatSper* gene.

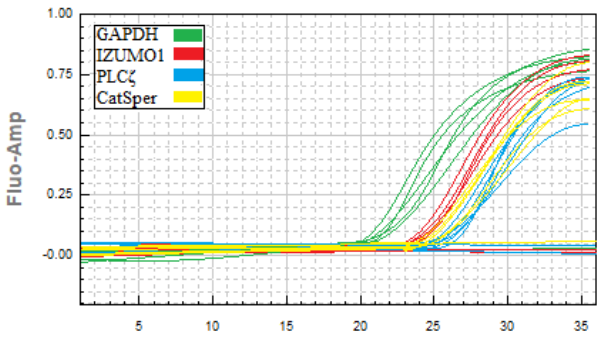

Fig. 3. Real-time PCR amplification curves of the target genes in the low-fertility group

The findings presented in ***Table 2*** indicate limited discrepancies in certain conventional semen parameters, contrasting sharply with the pronounced downregulation observed in fertility-associated gene expression. This is consistent with previous reports highlighting that relying solely on routine semen indicators is insufficient for a comprehensive assessment of fertilization efficiency, and that integrating molecular markers provides a more accurate evaluation of bull fertility [14].

Our results demonstrate a significant decrease in progressive sperm motility within the low-fertility group compared to the high-fertility cohort (62.7 % vs. 65.4 %, respectively; P=0.011). This decline likely reflects an impaired capacity of spermatozoa to efficiently reach the site of fertilization, given that progressive motility strictly depends on mitochondrial integrity, energy production efficiency, and the maintenance of intracellular ionic balance [15]. Such downregulation can be attributed to cryopreservation-induced cellular damage, which suppresses ATP production and accelerates reactive oxygen species (ROS) formation, ultimately disrupting directional sperm movement [16].

Notably, while no statistically significant difference was observed in total sperm motility between the two cohorts (P=0.063), progressive motility was significantly compromised in the low-fertility group (P=0.011). This underscores that progressive motility is more intimately linked to fertilization success, as it dictates the sperm's capacity to navigate toward and penetrate the oocyte. Consequently, evaluating total motility alone proves to be an inadequate standalone metric for predicting the fertilization potential of cryopreserved semen straws [17].

Our results also demonstrate a significant reduction in both sperm viability and plasma membrane integrity within the low-fertility group compared to the high-fertility cohort (P=0.045 for both parameters). This decline is primarily attributable to cryopreservation-induced injuries, including osmotic shock, intracellular ice crystal formation, and oxidative stress, which collectively compromise membrane permselectivity and disrupt transmembrane ion transport mechanisms. Such structural alterations detrimental to sperm metabolic activity, survival, and capacitation pathways have been extensively documented [18]. Furthermore, the proportion of morphological abnormalities was markedly elevated in the low-fertility group, showing a highly significant difference (P=0.0001). This structural deterioration suggests that freezing-thawing cycles cause severe physical damage to sperm sub-cellular components, thereby impairing sperm-egg interactions and paternal genetic material transfer, ultimately leading to reduced fertilization efficiency [19].

In summary, while most conventional semen characteristics were inferior in the low-fertility group, the magnitude of these reductions remained relatively narrow, and the variance in total motility lacked statistical significance ($P = 0.063$). These modest discrepancies reinforce the premise that routine semen profiles fail to comprehensively capture the cellular and molecular deficits governing fertilization success. Consequently, these findings validate the clinical necessity of supplementing traditional assays with functional molecular biomarkers to ensure precise fertility forecasting in artificial insemination programs utilizing cryopreserved semen straws [20].

The analysis of transcript abundance revealed that the *IZUMO1* gene was markedly downregulated in the low-fertility group compared to the high-fertility control cohort, exhibiting a highly statistically significant 2.43-fold decrease (P<0.001) (***Table 3***, ***Figs. 2***, ***3***). This prominent transcript degradation aligns with the molecular patterns observed by previous investigators, who demonstrated that *IZUMO1* protein abundance – measured via Western blot and immunohistochemical staining – strongly correlates with field fertility rates and reproductive outcomes [21]. Consequently, the marked suppression of *IZUMO1* transcripts identified in our low-fertility cohort directly points to a compromised capacity of the spermatozoa to bind to and fuse with the oolemma, thus explaining the reduction in actual fertilization efficiency.

As an indispensable transmembrane component localized on the sperm head, the *IZUMO1* protein plays a mandatory role in mediating gamete membrane fusion. Our findings align with the data reported by [22], who observed that suppressed *IZUMO1* levels strongly correlate with reduced reproductive outcomes, suggesting that quantification of this marker serves as a robust diagnostic tool for evaluating male fertility potential. Furthermore, recent structural investigations underscore the collaborative nature of the sperm-egg interaction cascade, emphasizing that while *IZUMO1* is a critical executioner of fusion, the process dynamically requires the synergistic participation of other membrane-bound molecules [23]. The pronounced transcript degradation identified in our subfertile sample cohort may be fundamentally linked to cryopreservation-induced oxidative stress. Extensive reviews confirm that elevated

reactive oxygen species (ROS) trigger rapid mRNA instability and oxidative damage to crucial paternal surface proteins, whereas mitigating these oxidative stressors represents a primary pathway for optimizing reproductive efficiency [24].

The *PLCζ* gene exhibited a distinct 2.78-fold downregulation in the low-fertility group compared to the high-fertility cohort (P<0.001) (***Table 3***). Phospholipase C zeta (*PLCζ*) serves as the critical sperm-borne oocyte activation factor (SOAF) responsible for triggering intracellular calcium (Ca^{2+}) oscillations post-fusion – a biochemical prerequisite for meiotic resumption, cortical granule exocytosis, and the initiation of early embryonic development. Functional validation trials have confirmed that PLCζ absolute deficiencies, structural mutations, or transcription blocks are heavily implicated in male-factor subfertility and total fertilization failure due to failed oocyte activation [6]. Consequently, the significant suppression of *PLCζ* expression demonstrated in the current study provides a robust molecular explanation for the compromised fertilization outcomes observed in the low-fertility group.

The simultaneous downregulation in the gene expression of both *IZUMO1* and *PLCζ* highlights coordinated alterations in transcripts governing distinct, sequential stages of the fertilization cascade. Regarding *PLCζ*, loss-of-function investigations have demonstrated that spermatozoa deficient in this specific phospholipase fail to trigger essential intracellular Ca^{2+} oscillations post-fusion, resulting in severe subfertility or complete developmental arrest [25]. Thus, the concurrent suppression of *IZUMO1* and *PLCζ* transcripts observed in our current study provides a compelling molecular rationale for the reduced fertilization outcomes, even in instances where baseline spermiogram characteristics remained within clinically acceptable thresholds. These outcomes strongly suggest that field fertility potential is not merely dictated by cellular traits visible under light microscopy, but is fundamentally influenced by the structural and functional integrity of downstream molecular mechanisms.

Consequently, the quantification of *IZUMO1* and *PLCζ* mRNA levels emerges as a promising diagnostic modality for assessing cryopreserved semen quality, revealing critical sub-cellular anomalies that evade detection by conventional laboratory screenings. Furthermore, integrating these functional molecular markers into routine breeding evaluations could significantly enhance the precision of semen straw selection and optimize the overall efficiency of artificial insemination and assisted reproductive workflows [26].

The *CatSper* gene exhibited the most pronounced suppression of transcript abundance among all investigated target loci within the low-fertility cohort, dropping 3.45-fold compared to the high-fertility control (P<0.001) (***Table 3***). CatSper functions as a principal, sperm-specific multi-subunit cation channel essential for regulating transmembrane influxes of calcium ions, a process tightly linked to the modulation of flagellar beat asymmetry and sperm motility profiles. Structural or functional disruptions within this ion channel complex have been thoroughly associated with idiopathic male infertility [27]. Our findings align with previous investigations highlighting the indispensable role of CatSper channels during fertilization and its potential utility as a high-resolution molecular marker for uncovering hidden subfertility, an area of reproductive biology that remains insufficiently explored in bovines [3]. Therefore, the critical loss of *CatSper* transcripts identified in the low-fertility group directly points to an impaired capacity for flagellar hyperactivation, reinforcing the clinical necessity of supplementing routine motility assays with precise molecular transcript profiling.

This sharp reduction in *CatSper* gene expression can be further elucidated by sub-cellular damage inherent to the cryopreservation cycle. Sperm freezing-thawing cycles induce severe molecular changes that compromise post-thaw semen functionality. Recent high-throughput proteomic and transcriptomic profiles have confirmed widespread freeze-related alterations in structural proteins and critical signaling molecules that dynamically regulate sperm function. These cryo-injuries directly influence the spermatozoa's capacity to withstand thermal shock and maintain normal functional traits [21]. Consequently, the observed 3.45-fold downregulation of *CatSper* transcripts (P<0.001) serves as a critical molecular hallmark of sublethal cryo-injury within the low-fertility cohort.

The simultaneous downregulation of *CatSper*, *IZUMO1*, and *PLCζ* transcripts reflects a comprehensive cascade failure across key sequential stages of fertilization – spanning from flagellar hyperactivation and gamete membrane fusion to oocyte activation [3, 6]. These cumulative findings firmly corroborate that relying solely on conventional spermiogram profiles is insufficient for a comprehensive prediction of actual field fertilization outcomes. Integrating quantitative gene expression analysis into routine workflows offers a high-resolution diagnostic tool to optimize semen straw selection and enhance the overall reproductive efficiency of artificial insemination and assisted reproduction programs [2, 4]. Ultimately, these results provide a foundational molecular baseline for developing advanced, targeted cryopreservation protocols designed to preserve transcript stability and protect fertility-associated target genes.

Conclusions

The present study established that while cryopreserved bovine spermatozoa from the low-fertility cohort exhibited minor reductions in conventional semen quality parameters, the most profound and critical alterations occurred at the molecular level. Freezing-thawing workflows induced a highly significant downregulation in the transcript levels of all investigated fertility-associated target genes (P<0.001). The magnitude of this suppression followed a distinct hierarchical order, with the largest decrease identified in *CatSper* expression (3.45-fold), followed sequentially by *PLCζ* (2.78-fold) and *IZUMO1* (2.43-fold).

These cumulative findings reinforce that routine spermiograms fail to comprehensively mirror actual field fertilization potential. Supplementing traditional laboratory assays with quantitative gene expression

profiling provides a high-resolution diagnostic approach to precisely evaluate cryopreserved semen quality. Ultimately, *CatSper*, *PLCζ*, and *IZUMO1* are determined to be highly promising functional molecular biomarkers capable of identifying cryptic male subfertility and optimizing semen straw selection for cattle breeding and assisted reproduction programs.

DECLARATIONS

Ethical Statement

The authors declare that this study was conducted in strict accordance with national and institutional ethical guidelines governing the handling and utilization of biological materials of animal origin. The experimental procedures were performed exclusively on commercially available cryopreserved bull semen straws; therefore, no direct interventions, manipulations, or experimental protocols were executed on live animals, and obtaining formal institutional bioethics committee approval was not required for this research.

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Conflict of Interest

The authors declare no conflicts of interest.

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Declaration of AI and AI-assisted technologies

The authors declare that ChatGPT (OpenAI) was utilized solely to assist with linguistic editing, structural formatting, and the refinement of specific manuscript paragraphs, as well as to assist in designing the conceptual layout of the experimental flowchart. Artificial intelligence technologies were strictly not employed for the generation, rendering, or formatting of scientific data plots, nor for any statistical analyses or interpretation of the biological results. The authors maintain full accountability for the integrity of the content, data accuracy, and final conclusions presented in this study.

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