JAST (Journal of Animal Science and Technology) TITLE PAGE

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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Trequinsin mitigates heat stress–induced impairment of bovine sperm function and embryo development
Running Title (within 10 words)	Trequinsin protects sperm under heat stress
Author	Hyeonguk Baek[1+], Jihwan Lee[1+], Adel R. Moawad[2], Kwanghyeon Cho[3*], Inchul Choi[4*]
Affiliation	1 Dairy Science Division, National Institute of Animal Science, RDA, Cheon-an, 31000, Republic of Korea 2 Division of Animal Science, College of Agriculture, Family Sciences, and Technology, Fort Valley State University, Fort Valley, GA 31030, USA 3 Department of Beef and Dairy Science, Korea National University of Agriculture and Fisheries, Jeonju-si, Jeollabuk-do 54874, Republic of Korea 4 Department of Animal and Dairy Sciences, Chungnam National University, 34134, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Hyeonguk Baek (http://orcid.org/0009-0007-6761-5711) Jihwan Lee (http://orcid.org/0000-0002-0040-3104) Adel R. Moawad (http://orcid.org/0000-0001-5011-2658) Inchul Choi (https://orcid.org/0000-0001-5011-2658) Kwanghyeon Cho (https://orcid.org/0000-0003-1564-5656)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was partially supported by the Tech. Incubator Program for Startup (TIPS; G21002584702) funded by the Ministry of SMEs and Startups(MSS, Korea).
Acknowledgements	
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Baek H, Lee J, Moawad AR, Choi I Data Curation: Baek H, Lee J Formal Analysis: Baek H, Lee J Funding acquisition: Cho K, Choi I Investigation: Baek H, Lee J Methodology: Baek H, Lee J Project administration: Cho K, Choi I Resources: Choi I Software: Baek H, Lee J Supervision: Cho K, Choi I Validation: Baek H, Lee J, Choi I Visualization: Baek H, Lee J Writing - original draft: Baek H, Lee J, Moawad AR, Cho K, Choi I Writing - review & editing: Baek H, Lee J, Moawad AR, Cho K, Choi I
Ethics approval and consent to participate	All experimental protocols performed on the animals were approved by the National Institute of Animal Science Animal Care and Ethics Committee in South Korea (approval number: NIAS-2026044).

Abstract

Global climate warming is increasingly associated with reduced reproductive efficiency in cattle, as elevated temperatures impair sperm function and subsequent embryo development. During summer breeding, artificially inseminated spermatozoa may be exposed to hyperthermic conditions within the female reproductive tract, highlighting the need for strategies that mitigate heat stress–induced functional decline. Because cyclic nucleotide signaling and CatSper-mediated Ca^{2+} influx—key regulators of sperm motility—are susceptible to thermal disruption, we evaluated trequinsin, a phosphodiesterase-3 inhibitor, as a potential protective agent. Dose–response testing using swim-up–selected bovine sperm demonstrated that trequinsin did not alter total or progressive motility under normothermic conditions (38.5 °C), whereas under acute heat stress (41 °C) it improved motility across tested concentrations, with maximal recovery observed at 20 µM and no additional benefit at 50 µM. Accordingly, 20 µM was used for subsequent analyses. A computer-assisted sperm analysis revealed that heat stress markedly reduced curvilinear velocity and straight-line velocity over 2 h, while trequinsin significantly attenuated these declines and maintained higher movement efficiency. Functional relevance was confirmed by in vitro fertilization, where trequinsin-treated heat-stressed sperm yielded higher cleavage and blastocyst rates than heat-stressed controls, although not fully reaching normothermic levels. Gene expression analysis showed reduced stress and pro-apoptotic signatures in resulting blastocysts, with profiles approaching those of non-heat-stressed controls. Collectively, trequinsin partially preserves sperm fertilizing capacity and embryo developmental competence under thermal stress, suggesting its potential as a pharmacological strategy to mitigate summer infertility in cattle.

Keywords: Heat stress, Bovine spermatozoa, Trequinsin, Phosphodiesterase inhibitor, Embryo development.

Introduction

Global climate change has emerged as a critical threat to the sustainability of the livestock industry, as rising ambient temperatures increasingly compromise the health and productivity of cattle worldwide [1,2].

Heat stress (HS), commonly quantified using the Temperature–Humidity Index (THI), induces a cascade of adverse physiological responses, resulting in substantial economic losses in both the dairy and beef sectors [3,4]. These challenges are particularly pronounced in the Republic of Korea, where summer conditions are characterized by persistently high temperatures combined with extreme humidity. Recent studies have shown that local climatic conditions in South Korea frequently exceed the physiological THI thresholds of lactating cows, inducing severe thermal stress [5]. Moreover, retrospective analyses of bovine reproduction on the Korean peninsula have demonstrated a clear negative correlation between elevated THI levels and conception rates, identifying summer hyperthermia as a major driver of reduced fertility [6,7].

In modern livestock production systems, artificial insemination (AI) using frozen–thawed semen is the predominant reproductive strategy. Maintaining stable breeding schedules through summer AI programs is therefore essential for production continuity [8]. However, hyperthermic conditions within the female reproductive tract following insemination—particularly during heatwaves—pose a direct environmental stressor to introduced spermatozoa [9]. Although maternal HS is known to impair reproductive performance, it remains difficult to distinguish whether fertility declines arise from oocyte dysfunction, sperm impairment, or adverse changes in the maternal environment, as these components are often simultaneously exposed to elevated temperatures [10,11]. Consequently, experimental models that isolate heat exposure specifically to spermatozoa during the peri-fertilization period are required to delineate sperm-specific effects of thermal stress [10,12].

At the molecular level, intracellular calcium concentration ($[Ca^{2+}]_i$) plays a central role in regulating sperm function by modulating soluble adenylyl cyclase activity and the production of cyclic nucleotides, including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). These second messengers are essential for sperm motility, capacitation, and the acrosome reaction, and their intracellular levels are tightly regulated by phosphodiesterases (PDEs) [13,14]. Calcium influx in sperm is primarily mediated by the cation channel of sperm (CatSper), a sperm-specific calcium channel localized to the principal piece of the flagellum, whose activation is indispensable for hyperactivated motility and

fertilization competence [15,16]. Disruption of CatSper signaling—through genetic abnormalities or environmental stressors—leads to impaired sperm motility and male infertility [17,18]. Notably, emerging evidence indicates that thermal stress negatively affects CatSper expression and calcium signaling, suggesting a mechanistic pathway by which heat exposure compromises sperm function [19,20].

Given the pivotal role of cyclic nucleotide–calcium signaling in sperm physiology, pharmacological modulation of these pathways represents a rational strategy to counteract heat stress–induced sperm dysfunction. Trequinsin, a selective phosphodiesterase-3 (PDE3) inhibitor, has been shown to enhance human sperm motility and fertilization potential by elevating intracellular cAMP and cGMP levels, increasing $[Ca^{2+}]_i$, and activating CatSper channels [21,22]. These properties position trequinsin as a promising candidate for mitigating thermal stress–induced impairment of sperm function. However, its effectiveness in bovine spermatozoa and its impact on subsequent embryo developmental competence remain poorly defined.

In the present study, we aimed to elucidate the effects of acute high-temperature exposure on bovine spermatozoa and to evaluate whether trequinsin hydrochloride treatment can alleviate heat-induced functional impairment. To isolate sperm-specific responses, thermally challenged and trequinsin-treated sperm were used to fertilize in vitro–matured oocytes maintained under physiological temperature conditions. Treatment efficacy was assessed by analyzing sperm kinematic parameters, in vitro embryo development rates, and the expression of genes associated with embryonic developmental competence. Collectively, this study establishes a controlled framework for investigating sperm-specific responses to HS and proposes a targeted pharmacological approach to improve bovine reproductive performance under increasingly severe summer conditions.

Materials and Methods

Animal Ethics and Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All experimental protocols performed on the animals were approved by the National Institute of Animal Science Animal Care and Ethics Committee in South Korea (approval number: NIAS-2026044).

Preparation of Bovine Spermatozoa and Swim-up Selection

Frozen Hanwoo semen straws (Korean Proven Bull) were thawed in a 37 °C water bath for 30 s. The semen was diluted in SP-TALP medium supplemented with 0.6% (w/v) bovine serum albumin (BSA) and centrifuged at 700 × g for 5 min to remove extenders and cryoprotectants. The pellet was gently resuspended in 0.3 mL SP-TALP and carefully overlaid with 1.5 mL pre-warmed SP-TALP in a 15-mL conical tube. Tubes were incubated at 38.5 °C in 5% CO_2 for 45 min at a 45° angle to allow highly motile spermatozoa to swim up into the upper layer. The upper 0.8 mL fraction was collected and centrifuged at 500 × g for 5 min. The final pellet was resuspended in IVF-TALP medium, and sperm concentration was adjusted to 1×10^6 spermatozoa/mL for subsequent experiments.

Optimization of Trequinsin Concentration

To determine the optimal concentration for mitigating thermal stress, sperm motility was evaluated under normothermic (38.5 °C) and hyperthermic (41 °C) conditions in the presence of trequinsin (0, 10, 20, and 50 μM of trequinsin hydrochloride). Total motility (TM) and progressive motility (PM) were assessed after 2 h incubation using a computer-assisted sperm analysis (CASA) system (ISAS v1.1, PROISER, Valencia, Spain). The concentration that best preserved sperm motility under HS was selected for subsequent experiments.

Assessment of Sperm Kinematic Parameters

Sperm movement characteristics were analyzed using the ISAS CASA system. Kinematic parameters were recorded at baseline (0 h) and after 0.5, 1, and 2 h of incubation under HS (41 °C) with or without 20 μM trequinsin treatment. Sperm kinematic parameters were evaluated by measuring curvilinear velocity (VCL),

straight-line velocity (VSL), and average path velocity (VAP), as well as the derived indices linearity (LIN = VSL/VCL) and straightness (STR = VSL/VAP). These parameters were used to assess changes in sperm kinematic quality and movement efficiency under thermal stress conditions.

In Vitro Fertilization and Embryo Culture

To evaluate sperm-specific responses to HS, heat-stressed sperm with or without trequinsin treatment were used for in vitro fertilization (IVF). Bovine ovaries were obtained from a local abattoir, and cumulus–oocyte complexes (COCs) were aspirated from 3–8 mm follicles. Oocytes were matured in vitro for 22–24 h at 38.5 °C in 5% CO_2. For fertilization, spermatozoa were co-incubated with matured oocytes at a final concentration of 1×10^6 sperm/mL for 18 h. After fertilization, presumptive zygotes were denuded and cultured in micro-drops of modified synthetic oviductal fluid (mSOF) supplemented with 0.5% (v/v) non-essential amino acids, 4.5% (v/v) amino acids and 10% (v/v) FCS under mineral oil at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Embryo development was assessed by recording cleavage rates on Day 2 and blastocyst formation rates on Day 7.

Gene Expression Analysis

Groups of 10 blastocysts per biological replicate were used for RNA extraction using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA, USA). Complementary DNA (cDNA) was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with gene-specific primers (Table 1). Relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method, with GAPDH as the reference gene.

Table 1. Bovine specific sequences of primers for qRT-PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)

POU5F1	AGAAGCTGGAGCCGAACC	CTGCTTTAGGAGCTTGGCAAA
NANOG	TCAGCTACAAGCAGGTGAAGAC	GCATGCCATTGCTATTCCTC
SOX2	CATGGCAATCAAAATGTCCA	AGACCACGGAGATGGTTTTG
CDX2	CAGAGAGGCAGGTTAAAATTTGGT	CTGCTGTTGCAACTTCTTCTTGTT
HSPA1A	GAGTCGTACGCCTTCAACAT	ATGATGGGGTTACACACCTG
HSP90AA1	AGAGCCCTTCTTTTTGTCCC	TCCTCAGAATCCACCACACC
SOD2	CAGGCAGCTGGCTCCGCTCT	CCACCGACAGGCCTTGGACC
GPX4	CGTGTGCTAGGGCTTTGTCC	GTAGGCACACGCACTTGTCC
BAX	TGTCGCCCTTTTCTACTTTG	GCCACAAAGATGGTCACTGT
BCL2	AGGCCACCAAGATACCTGAA	TGGGCCATATAGTTCCACAA
GAPDH	CCCACTCCCAACGTGTCTGT	CCTGCTTCACCACCTTCTTGAT

Statistical Analysis

All experiments were conducted with at least three independent biological replicates. Data were analyzed using GraphPad Prism software (Version 5.03; GraphPad Software, San Diego, CA, USA). Differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. When only two groups were compared, Student's t-test was used. Results are presented as mean ± standard error of the mean (SEM), and differences were considered statistically significant at $P < 0.05$.

Results

Optimization of Trequinsin for Heat Stress–Induced Motility Impairment

We first evaluated the effects of trequinsin on bovine sperm motility under physiological temperature conditions (38.5 °C). Treatment with trequinsin at concentrations of 10, 20, or 50 μM resulted in no significant changes in either TM or PM compared with the untreated control group (0 μM; $P > 0.05$) (Figure 1). Conversely, exposure to acute hyperthermic stress (41 °C) for 2 h caused a clear reduction in sperm motility, with TM and PM decreasing by approximately 25–40% relative to the normothermic control. Under these heat-stressed conditions, trequinsin treatment improved sperm motility at all tested concentrations. Specifically, treatment with 10 μM trequinsin led to a partial recovery of TM and PM, whereas the 20 μM dose further enhanced motility, yielding values that were statistically indistinguishable

from those observed in the 38.5 °C control group ($P > 0.05$). However, increasing the concentration to 50 μM did not provide additional significant benefits, indicating a plateau in the protective effect at higher dosages. Based on these findings, 20 μM trequinsin was selected as the optimal concentration for subsequent IVF and embryo development assays.

Figure. 1

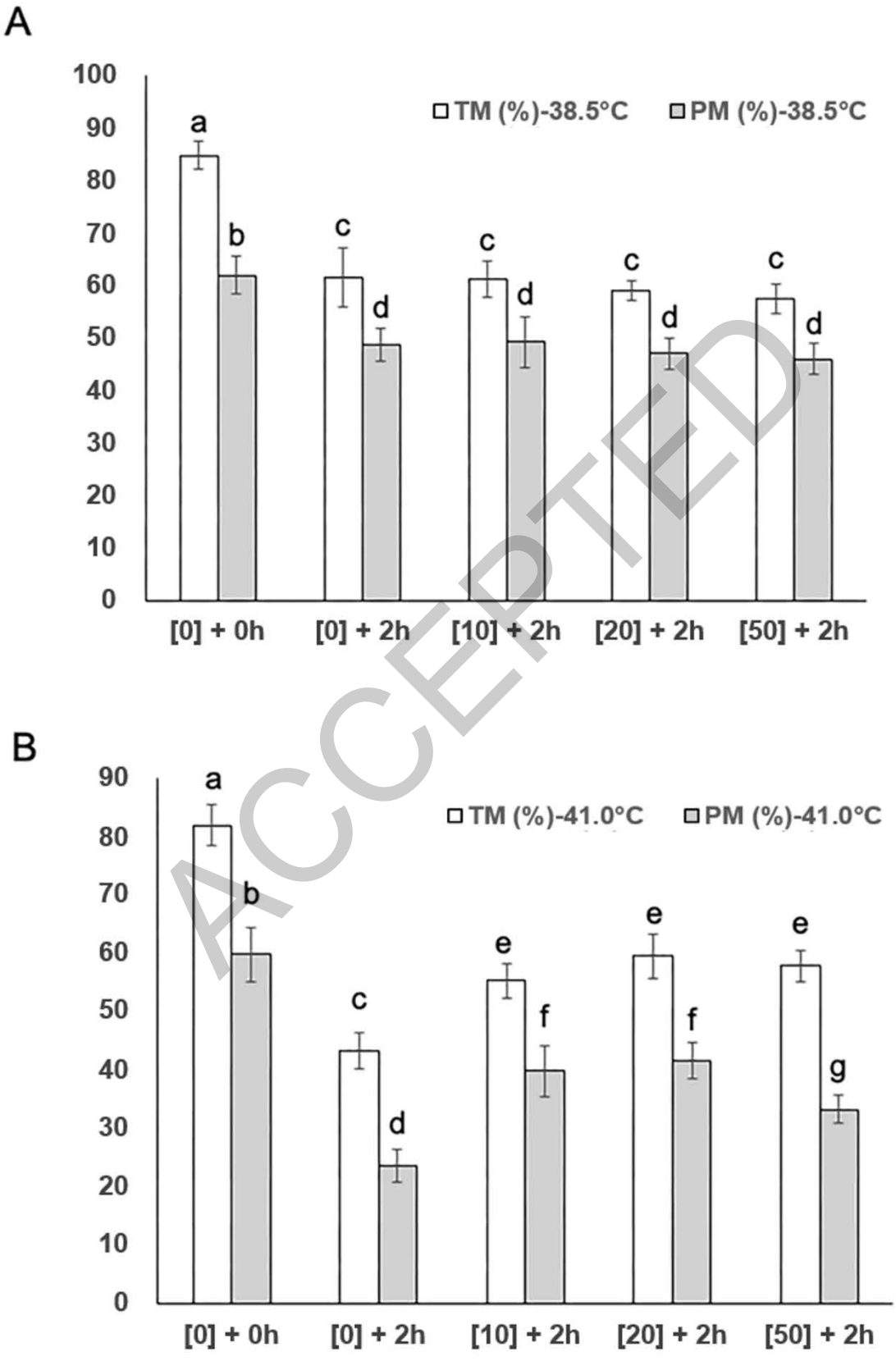

Figure 1. Effects of Trequinsin on bovine sperm motility under normothermic and hyperthermic conditions. Frozen–thawed bovine spermatozoa were incubated with Trequinsin at the indicated concentrations for 2 h at either 38.5 °C (normothermic condition) or 41 °C (hyperthermic condition). (A) Total motility and (B) progressive motility were assessed using computer-assisted sperm analysis. Data are presented as mean ± SEM. Different letters above bars indicate statistically significant differences among treatment groups within the same temperature condition ($P < 0.05$). TM, total motility; PM, progressive motility.

Effects of Heat Stress and Trequinsin on Sperm Kinematic Parameters

To evaluate the effects of thermal stress on bovine sperm motility and the potential protective role of trequinsin, sperm kinematic parameters were analyzed using a CASA system over a 2 h incubation period. Measurements at 0 h represent baseline values obtained immediately after the swim-up procedure, prior to HS exposure. As shown in Figure 2A, exposure to HS resulted in a marked decline in all velocity-related parameters, including VCL, VSL, and VAP, over time. In contrast, the heat-stressed sperm treated with trequinsin (HS-TQ) group maintained consistently higher velocity values throughout the incubation period. By the 2 h time point, sperm in the HS-TQ group exhibited substantially higher velocities than those in the HS group, with VCL and VSL remaining approximately 60–65% and 50–55% higher, respectively, relative to heat-stressed sperm without trequinsin treatment.

Analysis of movement indices further demonstrated the protective effects of trequinsin under HS conditions (Figure 2B). Both LIN and STR progressively declined in the HS group following the baseline measurement. In contrast, the HS-TQ group showed a more stable kinetic profile, maintaining higher values for both indices over time. At the 2 h time point, LIN and STR in the HS-TQ group remained approximately 10% and 20–25% higher, respectively, than those observed in the HS group. Notably, the preservation of STR was particularly evident at the final time point, indicating improved directional movement in trequinsin-treated sperm under thermal stress.

Figure. 2

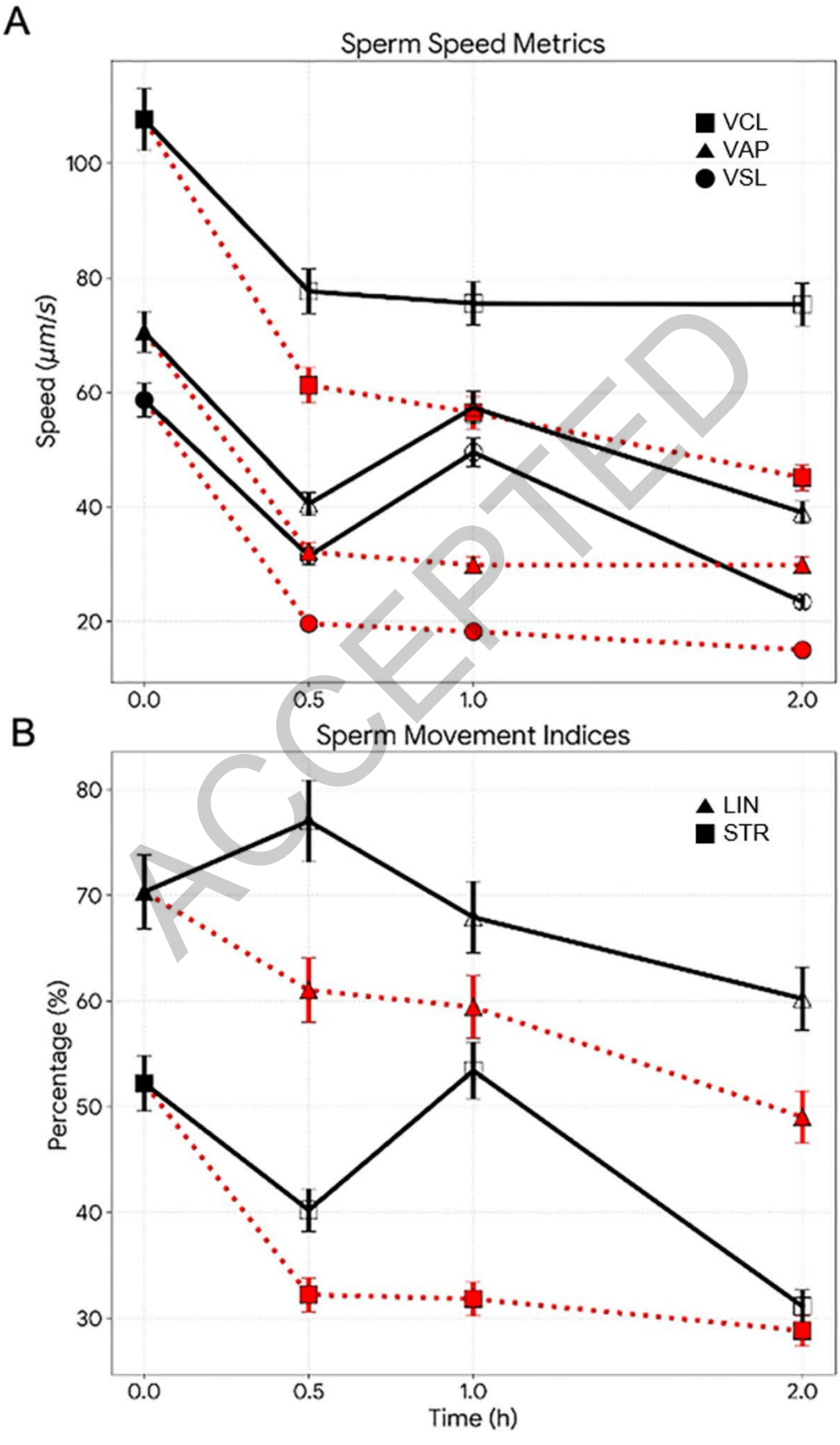

Figure 2. Effects of heat stress and Trequinsin on bovine sperm kinematic parameters over time. Frozen–thawed bovine spermatozoa were subjected to heat stress (41 °C) in the absence (HS) or presence of Trequinsin (HS-TQ) and analyzed at 0, 0.5, 1, and 2 h using a computer-assisted sperm analysis system. (A) Velocity parameters, including curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). (B) Movement indices, including linearity (LIN) and straightness (STR). Filled black symbols at 0 h indicate baseline values obtained after swim-up and prior to heat stress exposure. Red filled symbols with dotted lines represent heat-stressed sperm (HS), whereas open symbols with solid black lines represent heat-stressed sperm treated with trequinsin (HS-TQ). Data are presented as mean ± SEM.

Impact of Restored Sperm Kinetics on Embryo Development and Quality

To examine whether the restoration of sperm kinematic parameters was associated with improved fertilization outcomes and early embryo development, IVF was performed using sperm from each treatment group. Embryo developmental competence was assessed by monitoring cleavage and blastocyst formation (Figure 3A).

The heat-stressed control group without trequinsin treatment (HS-Ctrl) exhibited the lowest developmental performance, particularly at the blastocyst stage, with a blastocyst-to-cleavage rate of approximately 13%. In contrast, embryos derived from trequinsin-treated heat-stressed sperm (HS-TQ) showed improved developmental progression, with the blastocyst rate increasing to approximately 21%. Although this level remained lower than that observed in the non-heat-stressed control group (Non-HS-Ctrl; ~33%), trequinsin treatment partially restored embryo developmental competence under HS conditions.

To further assess embryo quality, the expression of genes associated with lineage specification, stress response, antioxidant defense, and apoptosis was analyzed in blastocysts (Figure 3B). Gene expression levels were quantified relative to the non-heat-stressed control group. Embryos generated using heat-stressed control sperm (HS-Sperm-IVF) displayed elevated expression of stress-response and pro-apoptotic genes, including HSPA1A (RQ ~ 2.7) and BAX (RQ ~ 2.2). In embryos derived from trequinsin-treated sperm (HS-TQ-Sperm-IVF), the expression of these genes was reduced and closer to baseline levels. In addition, the HS-TQ group maintained higher relative expression of the anti-apoptotic marker BCL2

compared with the HS control group, suggesting an improved molecular profile associated with embryo viability.

Figure. 3

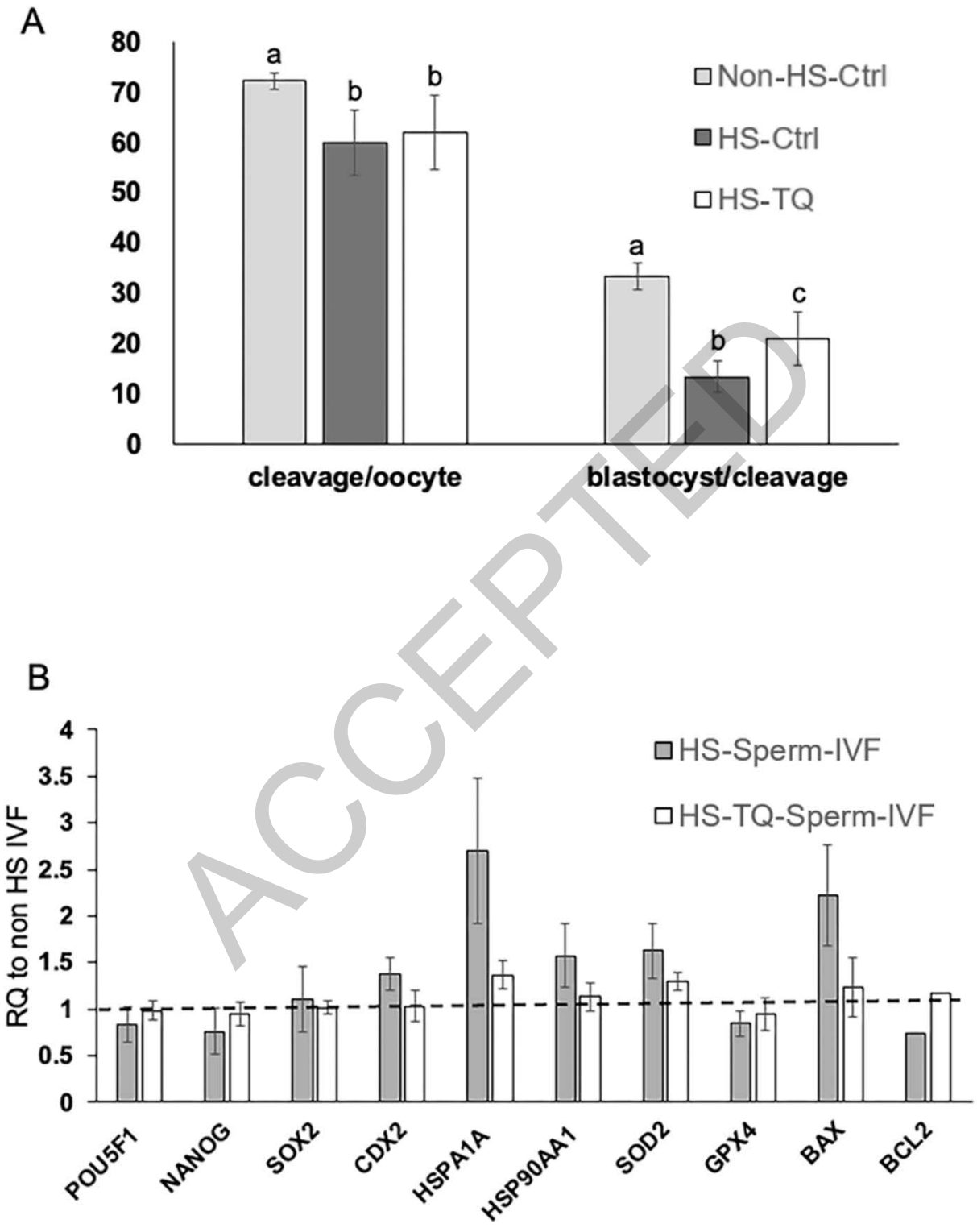

Figure 3. Effects of Trequinsin-treated heat-stressed sperm on embryo development and gene expression following IVF. (A) Cleavage and blastocyst development rates of embryos generated using sperm from the non-heat-stressed control group (Non-HS-Ctrl), heat-stressed control group without

Trequinsin (HS-Ctrl), and Trequinsin-treated heat-stressed group (HS-TQ). (B) Relative gene expression profiles of blastocysts derived from IVF using heat-stressed sperm without (HS-Sperm-IVF) or with Trequinsin treatment (HS-TQ-Sperm-IVF). Gene expression levels are presented as relative quantification (RQ) normalized to the non-heat-stressed control group (baseline = 1.0). Data are expressed as mean ± SEM.

Discussion

This study demonstrates that acute thermal stress markedly compromises bovine sperm kinematic performance and subsequently impairs early embryonic development, whereas pharmacological modulation with trequinsin, a selective phosphodiesterase (PDE) inhibitor, partially alleviates these detrimental effects. By restoring sperm velocity profiles and improving embryo developmental competence under HS conditions, trequinsin emerges as a targeted intervention acting primarily at the level of sperm functional resilience rather than as a general motility enhancer.

Heat Stress–Induced Sperm Dysfunction and Conditional Efficacy of Trequinsin

A key finding of this study is that the beneficial effect of trequinsin in bovine spermatozoa is conditional and becomes evident predominantly under hyperthermic stress. Unlike human sperm, in which PDE inhibitors can enhance motility even under normothermic conditions [22], trequinsin did not significantly alter bovine sperm motility at physiological temperature. This suggests that bovine sperm may possess sufficient basal cyclic nucleotide homeostasis as PDE activity during capacitation appears largely constitutive and maintains intracellular cAMP levels [23]. Furthermore, the motility-enhancing effects of PDE inhibitors are known to be species- and condition-dependent, with several studies reporting minimal or no improvement in sperm motility following caffeine or related treatments [24].

In contrast, exposure to acute HS (41 °C) resulted in pronounced reductions in velocity and movement efficiency, consistent with previous reports showing that elevated temperature disrupts mitochondrial activity, increases oxidative stress, and diminishes the fertilizing capacity of bovine sperm [25–27]. Under

these compromised conditions, trequinsin treatment became effective, indicating that thermal stress likely shifts sperm signaling toward a state in which PDE-mediated degradation of cyclic nucleotides becomes a limiting factor [28].

Mechanistically, HS has been associated with accelerated cAMP depletion, impaired mitochondrial protein import, and activation of stress-related kinases such as GSK3α, all of which converge on reduced flagellar ATP availability and inefficient motility [26,29]. By inhibiting PDE3, trequinsin likely stabilizes intracellular cAMP and cGMP pools, sustaining protein kinase A (PKA)–dependent phosphorylation cascades and dynein ATPase activity required for flagellar propulsion [30,31]. This interpretation aligns with prior evidence that PDE inhibition can preserve sperm motility under stressful or suboptimal conditions rather than enhance motility beyond physiological limits [14,32].

Kinematic Efficiency and Functional Interpretation of Linearity

Beyond absolute velocity, sperm fertilizing competence depends critically on movement efficiency. LIN provides an integrated index of how effectively sperm translate energy expenditure into forward progression. In the present study, HS disproportionately reduced VSL relative to VCL, leading to a marked decline in LIN. Such a pattern reflects erratic, non-productive trajectories that waste limited energetic reserves—an interpretation consistent with earlier observations of heat-induced mitochondrial dysfunction and oxidative damage in bovine spermatozoa [26,33–35]. Trequinsin treatment partially restored both VSL and VCL, resulting in higher LIN and STR values over time. Importantly, this improvement does not imply hyperactivation per se, but rather preservation of coordinated flagellar beating and directional persistence. Thus, trequinsin treatment appears to support “kinematic efficiency” rather than merely increasing speed, enabling sperm to maintain productive forward motion under thermal stress.

Embryo Development and the “Survivor Sperm”

Improved sperm kinematics translated into higher cleavage and blastocyst formation rates following IVF. Nevertheless, developmental outcomes in the HS-TQ group did not fully reach those of the non-heat-stressed control, indicating that trequinsin-mediated rescue is partial. This finding is consistent with the

notion that HS induces multifaceted sperm damage, including oxidative stress, altered RNA cargo, and latent DNA or chromatin defects that cannot be completely reversed by restoring motility alone [36]. Interestingly, embryos that did reach the blastocyst stage in the HS-TQ group exhibited gene expression profiles closer to non-heat-stressed controls, particularly with respect to stress-response (HSPA1A) and apoptosis-related markers (BAX, BCL2). Similar observations have been reported following sperm heat shock, where reduced blastocyst yield coexists with relatively normal transcriptional profiles among surviving embryos [37,38]. This supports a “survivor sperm” model, whereby only a resilient subpopulation of sperm—either inherently resistant or functionally rescued—successfully contributes to embryo development, resulting in embryos of comparatively preserved quality despite reduced overall efficiency [39,40].

From an applied perspective, declining fertility during periods of high THI remains a major constraint in dairy production systems. Retrospective analyses in Korea and other regions consistently demonstrate a strong negative association between HS and conception rates, even when AI or embryo transfer is employed [7]. Our findings underscore that paternal factors remain vulnerable to thermal insult and should be considered in summer fertility management strategies.

Nonetheless, several limitations should be acknowledged. This study did not directly assess acrosome integrity, mitochondrial membrane potential, or DNA fragmentation, all of which are known to be sensitive to HS and critically influence fertilization and embryo quality [38]. Moreover, emerging evidence suggests that HS alters sperm-borne small RNAs and epigenetic marks with potential long-term developmental consequences [41,42]. Future studies integrating functional, molecular, and epigenetic endpoints will be necessary to determine whether pharmacological restoration of sperm motility fully translates into safe and sustainable reproductive outcomes.

In summary, acute thermal stress significantly compromises bovine sperm kinematic performance and reduces subsequent embryo developmental competence. Pharmacological inhibition of PDE activity with trequinsin partially mitigated these detrimental effects, restoring sperm movement efficiency and improving fertilization outcomes under HS conditions. Although developmental rates in the trequinsin-

treated group did not fully reach those observed under normothermic conditions, blastocysts that developed exhibited molecular profiles closer to non-stressed controls, suggesting preservation of embryo quality among successfully fertilizing sperm. These findings indicate that the beneficial effect of trequinsin is conditional and becomes evident primarily under thermal challenge, supporting its role as a targeted intervention that enhances sperm functional resilience rather than a general motility enhancer.

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