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Evaluating Thermal Stress Responses in Bovine Adipose-Derived Stromal Cells: Implications for Viability, Oxidative Status, and Differentiation

Abstract

Rising global temperatures pose significant threats to livestock production through heat stress-induced impairments in growth, reproduction, and tissue maintenance. While systemic responses to hyperthermia are well-documented, cellular mechanisms underlying thermal injury in bovine stem cell populations remain insufficiently characterized. This study systematically evaluates the impact of defined heat stress conditions on bovine adipose-derived stromal cells (bADSCs) by assessing viability, oxidative stress responses, and multilineage differentiation potential. Cells were exposed to acute (2 h) and chronic (24 h) heat stress at 37°C, 39°C, and 41°C. Our findings demonstrate that mild hyperthermia (39°C) transiently enhances metabolic activity (262.50 ± 32.35% viability at 2 h) and induces adaptive stress responses, while severe stress (41°C) triggers antioxidant exhaustion (SOD activity: 1.74 ± 0.22 U/10^4 cells at 24 h) and significant *HSP70* upregulation (2.28-fold at 2 h; 1.82-fold at 24 h). Notably, chronic severe stress fundamentally alters differentiation potential: osteogenesis is markedly suppressed (*OPN* expression: 0.28-fold at 41°C), while adipogenic signaling trends upward (*PPAR*-γ: 1.77-fold). These findings provide crucial cellular evidence for heat stress-related impairments in cattle and establish a systematic framework for investigating thermal tolerance and regenerative resilience in livestock species.

Key words:

Heat stress, Bovine ADSCs, Cellular viability, Oxidative stress, Stem cell differentiation, *HSP70* expression

Introduction

Rising global temperatures and increasing frequency of heat waves represent pervasive environmental challenges for livestock production. When ambient heat load exceeds an animal's thermoregulatory capacity, heat stress (HS) ensues, disrupting metabolic balance and compromising physiological function. In cattle, HS impairs growth, reproductive efficiency, milk yield, meat quality and immune competence, threatening global food security and agricultural sustainability.

While systemic and physiological responses to HS have been widely documented, the cellular mechanisms underlying thermal injury and adaptation in bovine tissues remain insufficiently characterized. At the cellular level, elevated temperature disrupts proteostasis, destabilizes lipid membranes, and enhances reactive oxygen species (ROS) generation, leading to oxidative stress and mitochondrial dysfunction. Cells activate conserved stress-response pathways, including heat shock proteins (HSPs) and antioxidant defense systems, to restore protein folding and redox balance (Slimen et al. 2014). However, sustained severe stress overwhelms these protective mechanisms, resulting in DNA damage, cell cycle arrest, and apoptosis (Lukácsi et al. 2024).

Mesenchymal stromal/stem cells (MSCs) are key mediators of tissue maintenance and repair. Adipose-derived stromal cells (ADSCs) serve as accessible in vitro models due to their well-characterized functional properties (Choudhery et al. 2015). Previous studies in mammalian MSCs demonstrate that heat stress can induce premature senescence, alter transcriptional profiles, and reduce differentiation potential (Shimoni et al. 2020). Despite these observations, studies examining bovine MSC responses to defined thermal stress remain limited, and experimental variability hampers direct comparison across reports.

A deeper understanding at the cellular and molecular levels is essential for developing targeted

mitigation and adaptation strategies. This study aims to systematically evaluate the impact of defined heat stress conditions on bovine adipose-derived stromal cells by assessing cell viability, oxidative stress responses, and multilineage differentiation potential, providing a foundation for understanding cellular mechanisms of heat tolerance in cattle.

Materials and methods

Materials

Collagenase type I, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), antibiotic–antimycotic solution, osteogenic and adipogenic differentiation media, MTT reagent, and dimethyl sulfoxide (DMSO) were procured from HiMedia Laboratories Pvt. Ltd., India. Trypan blue dye and agarose were purchased from Sigma-Aldrich (USA). Superoxide dismutase (SOD) assay kit, RNA isolation kit, cDNA synthesis kit, and PCR master mix were obtained from Origin Diagnostics and Research, India. Real-time PCR master mix was purchased from Bio-Rad Laboratories, USA.

Methods

Isolation and culture of bovine adipose-derived stromal cells

Bovine subcutaneous adipose tissue was aseptically collected in sterile PBS containing 1% antibiotic–antimycotic solution. Tissue was washed thoroughly, minced into fine fragments, and digested in DMEM supplemented with 0.01% Type I collagenase at 37°C for 3 h with manual agitation. The enzymatic reaction was stopped by adding complete culture medium (DMEM with 15% FBS and 1% antibiotic–antimycotic solution). The cell suspension was filtered through a 100-µm cell strainer and

centrifuged at 900 rpm for 10 min. The stromal vascular fraction pellet was resuspended in complete medium and seeded into six-well culture plates. The summarized protocol is mentioned in Fig. 1. Cells were cultured at 37°C in a humidified incubator with 5% CO_2, with medium replaced every three days. At 80–90% confluency, cells were detached using Accutase ® and sub cultured.

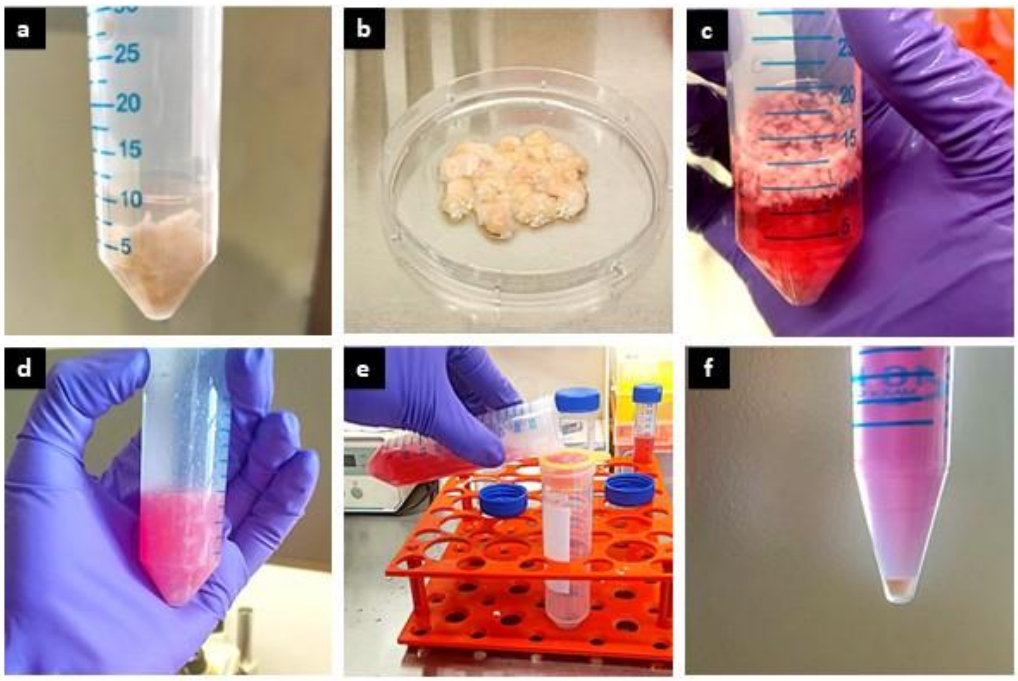


Fig. 1 Isolation of bADSCs. (a). Adipose tissue sample collected in PBS. (b). Tissue minced into small pieces. (c). Enzymatic digestion with 0.01% Type I collagenase for 3 h. (d). Tissue after digestion. (e). Digested contents filtered through a 100-µm cell strainer. (f). Stromal vascular pellet obtained after centrifugation of the filtrate.

Heat stress treatment

At third passage, cells at 80–90% confluence were divided into six experimental groups. Heat stress experiments were performed in two independent sets: acute exposure (2 h) and chronic exposure (24 h) at 37°C, 39°C and 41°C. In each set, control cells were maintained at 37°C, while HS groups were exposed separately to 39°C or 41°C, all under 5% CO_2.

Cell viability assay (MTT assay)

Cells were seeded at 5,000 cells per well in 96-well plates. After 24 h incubation at 37°C, medium was replaced and cells were exposed to HS at 37°C, 39°C, or 41°C for 2 h or 24 h. Following exposure, medium was replaced with 100 µL plain DMEM and 20 µL MTT solution (5 mg/mL) added. Plates were incubated at 37°C for 4 h in the dark. Formazan crystals were dissolved in 200 µL DMSO, and absorbance was measured at 540 nm using a microplate reader. Cell viability was determined from optical density values relative to controls.

Superoxide dismutase activity

Cellular SOD activity was determined using a Superoxide Dismutase Activity Assay Kit (OriginLab, India). Briefly, 5×10^6 cells were harvested, resuspended in extraction solution, and lysed by ultrasonication on ice. Lysates were centrifuged at 8000 rpm for 10 min at 4°C, and supernatant collected. The assay measures SOD-mediated inhibition of NADPH oxidation. Absorbance was measured at 340 nm before and after β-mercaptoethanol addition using a UV–VIS spectrophotometer. SOD activity was expressed as units per 10^4 cells.

Osteogenic and adipogenic differentiation

Cells were seeded into six-well plates and cultured until 70–80% confluency. Following HS exposure, culture medium was replaced with osteogenic or adipogenic differentiation media. Media were refreshed every three days. After three weeks, cells were harvested for staining and gene expression analysis. Von Kossa staining (5% w/v) was performed to detect mineralization, and Oil Red O staining (0.5% w/v in isopropanol) to visualize lipid droplets.

175

176 ***Gene expression studies***

177 Total RNA was extracted using Total RNA isolation kit (Origin) according to manufacturer's
178 instructions. RNA quality and concentration were evaluated spectrophotometrically (NanoDrop™2000).
179 cDNA was synthesized using Origin cDNA synthesis kit. Quantitative real-time PCR (qRT-PCR) was
180 performed using SYBR® Green Master Mix (Bio-Rad) on a real-time PCR system with the following
181 program: initial denaturation at 95°C, followed by 40 cycles of 95°C denaturation, 60°C annealing, and
182 72°C extension. Gene-specific primers (Table I) were designed using NCBI Primer-BLAST. Relative
183 expression was calculated using the $2^{-}\Delta\Delta CT$ method with *β-actin* as internal reference.

184

185 **Table I**: Primer sequences for qRT-PCR.

GENE	GENE ACCESSION NO. (NCBI)	PRIMER NAME	PRIMER SEQUENCE (5'----3')	PRIMER LENGTH (bases)	PRODUCT SIZE (bp)
HSP70	GenBank: **U09861**	*HSP70* F	TACGTGGCCTTCACCGATAC	20	171
		HSP70 R	GTCGTTGATGACGCGGAAAG	20	
OPN	GenBank: **M66236.1**	*OPN* F	GCCTGACCCATCTCAGAAGC	20	117
		OPN R	TGCTCAGGGCTTTCATTGGA	20	
PPAR-γ	GenBank: **AY179866.1**	*PPAR-γ* F	GACGGGAAAGACGACAGACA	20	100
		PPAR-γ R	CAGGGGACTGATGTGCTTGA	20	
β-actin	NCBI Ref. Sequence: **NM_173979.3**	*β-actin* F	GCACCACACCTTCTACAACG	20	102
		β-actin R	GTCATCTTCTCACGGTTGGC	20	

HSP70 – Heat shock protein 70 gene
OPN – Osteopontin gene [Osteogenic marker]
PPAR-γ – Peroxisome Proliferator-Activated Receptor Gamma gene [Adipogeneic marker]
β-actin – Beta actin [housekeeping gene]

186

187 ***Statistical analysis***

188 Results are expressed as mean ± standard error. Statistical analysis was performed using SPSS

version 24.0. One-way ANOVA was used for viability and SOD assays, while independent t-test was used for gene expression studies, with *β-actin* as reference gene. Significance levels: $p < 0.05$ (*), $p \leq 0.01$ (**), ns = non-significant.

Results

Morphology of cultured bADSCs

Following seeding, cells initially appeared heterogeneous with rounded or irregularly shaped cells and tissue debris. Within 24–48 h, cells attached to the plastic surface and exhibited characteristic spindle-shaped, fibroblast-like morphology (Fig. 2a). Between days 3 and 5, cells became more homogeneous with uniform spindle morphology. Active proliferation and colony formation were evident, with cultures reaching 80–90% confluency within 13–14 days (Fig. 2b). Cells retained morphological consistency for up to eight-ten passages; beyond this stage, proliferative capacity declined with noticeable morphological changes.

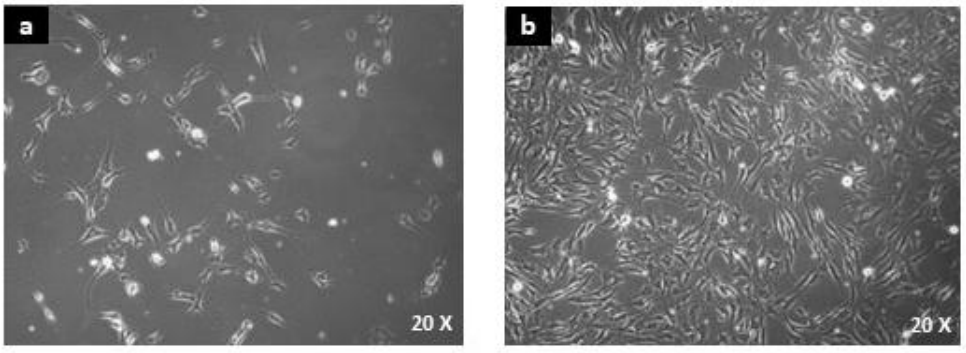
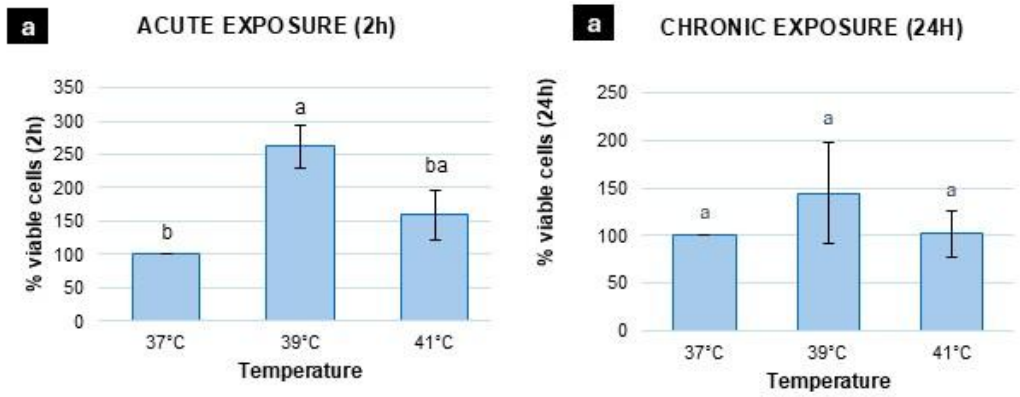


Fig. 2 Stages of attachment of freshly isolated bADSCs (a). Attachment of bADSCs cells observed on day 4 (b). Cells at day 15 reaching approximately 70–80% confluency

Impact of heat stress on cell viability

Cell viability assessed by MTT assay is presented in Fig.3. During acute exposure (2 h), a

statistically significant temperature-dependent effect was observed ($p \leq 0.01$). Cells at 39°C exhibited highest viability (262.50 ± 32.35%) compared to 37°C control (100.00 ± 0.00%). Exposure to 41°C resulted in 159.65 ± 37.53% viability, marginally higher than control but not significantly different from 39°C. Following 24 h exposure, no significant differences were detected ($p > 0.05$): 100.00 ± 0.00% at 37°C, 144.48 ± 53.14% at 39°C, and 101.70 ± 24.79% at 41°C, despite a numerically higher mean at 39°C.



c

Group	Mean ± SE (% viable)			p value
	37° C	39° C	41° C	
2 h (acute)	100^{b} ± 0.00	262.50^{a} ± 32.35	159.65^{ba} ± 37.53	0.009**
24 h (chronic)	100^{a} ± 0.00	144.48^{a} ± 53.14	101.7^{a} ± 24.79	0.59^{ns}

Fig. 3 Effect of heat stress on cell viability of bADSCs exposed for 2 h and 24 h at different temperatures. Comparison of cell proliferation (% viable) of bADSCs using MTT assay after exposure to 2 h (a) and 24 h (b) HS and their mean values as Mean ± SE (c). [Mean values within a row with different superscripts differ significantly; **$p \leq 0.01$, ns = non-significant.]

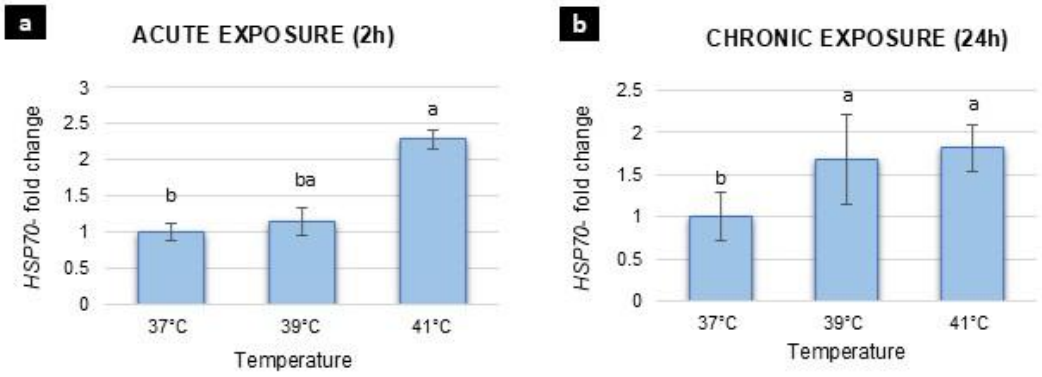
***HSP70* expression**

Under acute exposure (Fig. 4a), *HSP70* expression showed non-significant increase at 39°C (1.15-fold) and significant upregulation at 41°C (2.28-fold; $p \leq 0.05$) relative to 37°C. After 24 h exposure (Fig.

223 4b), *HSP70* expression was significantly increased at both 39°C (1.68-fold; $p \leq 0.05$) and 41°C (1.82-

224 fold; $p \leq 0.05$), with no significant difference between stress temperatures.

225



226

227 **Fig. 4** Impact of heat stress on expression of *HSP70* in bADSCs . Fold change in *HSP70* expression in

228 cells exposed to heat stress (HS) for 2 h (a) and 24 h (b)

229

230 **Superoxide dismutase activity**

231 Superoxide dismutase (SOD) activity was markedly influenced by both temperature and duration

232 (Fig. 5). Under acute HS, SOD showed temperature-dependent increase, with maximum activity at 41°C

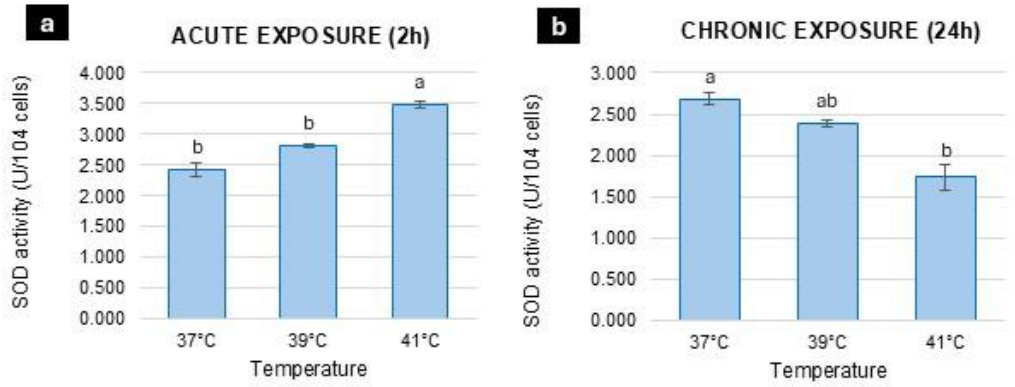
233 (3.48 ± 0.09 U/10^4 cells), significantly higher than 39°C (2.80 ± 0.04) and 37°C (2.42 ± 0.14) ($p = 0.01$).

234 Conversely, prolonged exposure (24 h) resulted in significant reduction at 41°C (1.74 ± 0.22 U/10^4 cells)

235 compared to 37°C (2.68 ± 0.10) ($p = 0.04$). The 39°C group showed marginal non-significant decrease

236 (2.38 ± 0.05).

237



c

Groups	Mean ± SE (U/104 cells)			p value
	37° C	39° C	41° C	
2 h (acute)	2.42b ± 0.14	2.80b ± 0.04	3.48a ± 0.09	0.01**
24 h (chronic)	2.68a ± 0.10	2.38ab ± 0.05	1.74b ± 0.22	0.04*

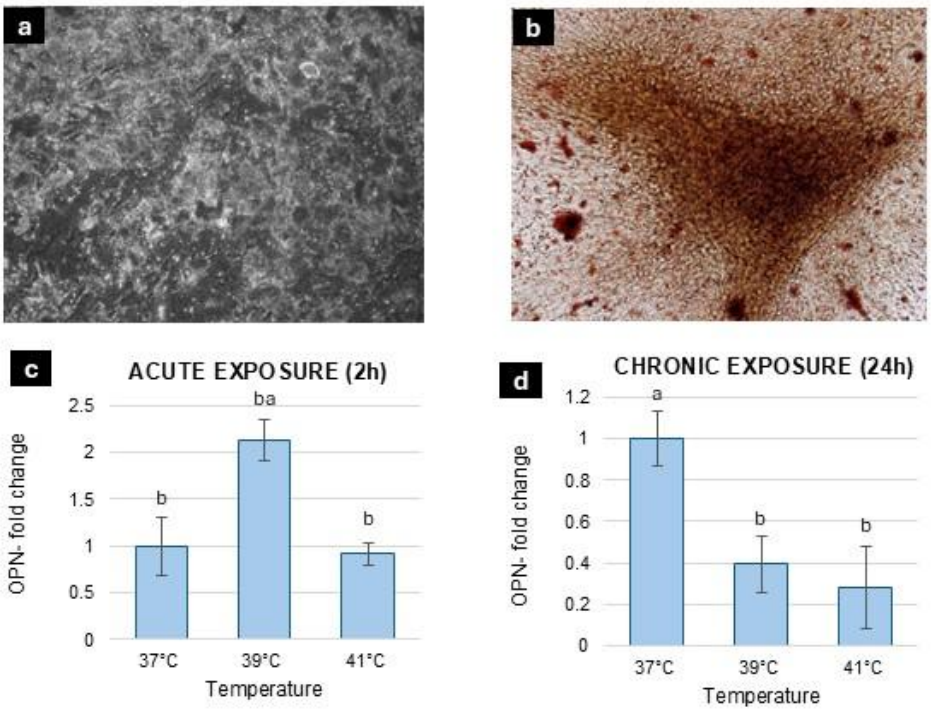
Fig. 5 Effect of heat stress on SOD activity (U/104 cells) of bADSCs cells. Comparison of SOD activity (U/104 cells) of bADSCs after exposure to 2 h (a) and 24h (b) HS and their mean values as Mean ± SE (c)

Differentiation potential under heat stress

Cells exhibited typical multilineage differentiation capacity. Under osteogenic conditions, early mineralized nodules appeared between days 4–8, progressing through day 21 (Fig. 6A). Von Kossa staining confirmed mineralization as dark brown–black deposits (Fig. 6B). Notably, cells exposed to 41°C for 24 h showed extensive detachment with only scattered adherent cells remaining, suggesting impaired osteogenic progression. Under acute exposure (Fig. 6C), *OPN* expression showed non-significant increase at 39°C (2.13-fold) and decrease at 41°C (0.91-fold). Under chronic exposure (Fig.6D), *OPN* expression was significantly reduced at both 39°C (0.39-fold; $p \leq 0.05$) and 41°C (0.28-fold; $p \leq 0.05$) relative to control.

Under adipogenic conditions, cells gradually became rounded with small cytoplasmic vacuoles

253 appearing from day 8 onward, increasing in size and number through day 21 (Fig. 7A). Oil Red O staining
254 confirmed intracellular neutral lipid accumulation as red-orange droplets (Fig. 7B). Under both acute and
255 chronic HS, *PPAR-γ* expression showed consistent pattern: mild downregulation at 39°C (0.65-fold acute;
256 0.91-fold chronic) followed by upregulation at 41°C (1.66-fold acute; 1.77-fold chronic) relative to 37°C
257 (Fig. 7C & 7D). Although differences were not statistically significant, expression consistently trended
258 upward at higher temperature.
259



260
261 **Fig. 6** Impact of heat stress on osteogenic differentiation potential of bADSCs. (a). Increased calcium
262 deposition detected microscopically, indicating osteogenic differentiation. (b). Von Kossa staining of
263 osteogenically differentiated cells showing dark brown to black areas, indicating extracellular matrix
264 mineralization. Fold change in *OPN* expression in adipogenically differentiated cells subjected to HS for
265 2 h (c) and 24 h (d).
266

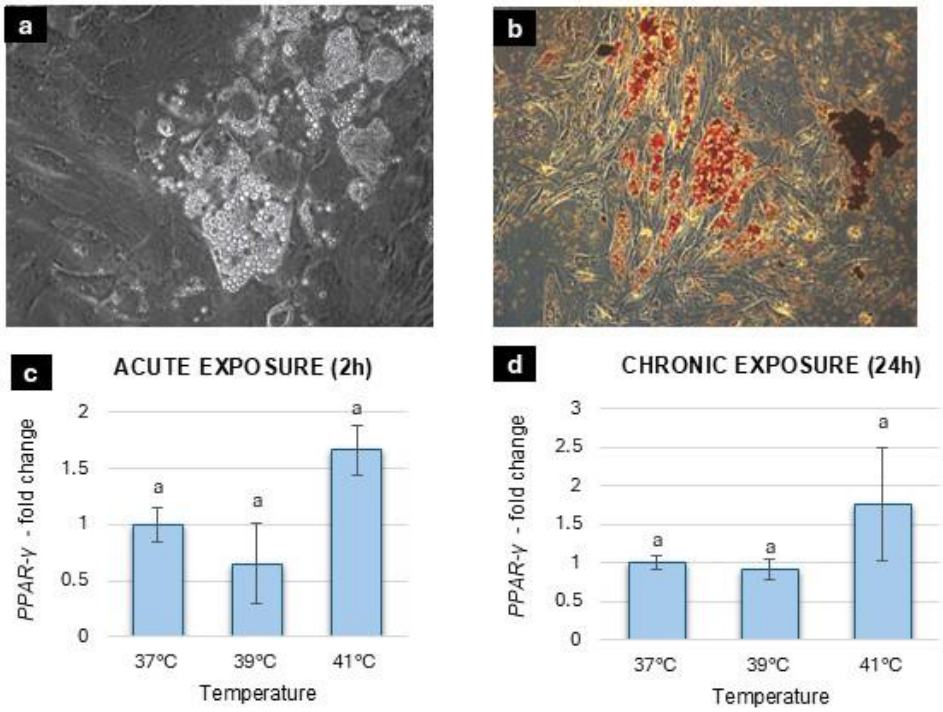


Fig. 7 Impact of heat stress on adipogenic differentiation potential of bADSCs. (a). Prominent intracellular lipid vacuoles visible at higher magnification during the later stages of differentiation. (b). Oil Red O staining of adipogenically differentiated cells showing intracellular lipid accumulations appearing as red droplets within the cytoplasm. Fold change in *PPAR- γ* expression in adipogenically differentiated cells subjected to HS for 2 h (c) and 24 h (d).

Discussion

This study demonstrates that heat stress exerts profound, differential effects on bovine adipose-derived stromal cells, with outcomes critically dependent on both temperature magnitude and exposure duration. Our findings reveal that mild hyperthermia (39°C) falls within the adaptive cellular range, while severe stress (41°C) drives cells toward a critical threshold where protective mechanisms are ultimately overwhelmed.

The growth kinetics and morphology of bADSCs in the present study align with previously reported characteristics (Cebo 2017; Bukowska et al. 2021; Maldonado et al. 2024). The early heterogeneous

283 population and subsequent transition to a spindle-shaped, fibroblast-like morphology confirm the typical
284 behavior of adipose-derived stromal cells. Robust proliferative capacity and colony formation in early
285 passages indicate strong expansion potential (Pittenger et al. 1999). However, the observed decline in
286 growth rates and morphological changes in later passages are consistent with culture-induced senescence
287 and pro-inflammatory alterations described by Pokrywczynska et al. (2020).
288 The MTT assay revealed a striking 2.6-fold increase in metabolic activity at 39°C (2 h), which returned
289 toward baseline at 41°C. Because MTT reduction reflects NAD(P)H-dependent dehydrogenase activity
290 rather than cell number (Ghasemi et al. 2021), this transient increase may reflect early metabolic
291 reprogramming and enhanced mitochondrial respiration (Marquez-Acevedo et al. 2023) required to
292 support cellular heat shock responses, including *HSP70*-mediated protein stabilization and refolding.
293 This interpretation is consistent with reports demonstrating adaptive and cytoprotective effects of mild
294 hyperthermia in MSCs (Ryu et al. 2023). The decline at 41°C suggests bioenergetic impairment under
295 severe stress, consistent with findings from Shimoni et al. (2020) and Lu et al. (2023).
296 The differential induction of *HSP70* provides mechanistic insight into stress thresholds. At 39°C,
297 transcript levels showed modest non-significant increase, whereas 41°C caused pronounced 2.28-fold
298 upregulation (2 h). Heat Shock Factor 1 (HSF1) is the master transcriptional regulator of *HSP70*
299 expression during cellular heat stress responses (Kmiecik and Mayer 2022). At 39°C, proteotoxic stress
300 is likely insufficient to robustly activate HSF1 trimerization, while 41°C generates greater protein
301 unfolding, creating stronger chaperone demand and driving efficient transcriptional induction. Sustained
302 elevation at 24 h (1.68–1.82-fold) likely reflects continued demand for molecular chaperones involved
303 in stabilization, refolding, and maintenance of proteostasis under stress conditions (Singh et al. 2024).
304 Superoxide dismutase (SOD) catalyzes the conversion of superoxide radicals (O_2^-) into H_2O_2, thereby

playing a vital role in protecting cells against oxidative damage and reducing the formation of highly reactive hydroxyl radicals (•OH) through the Fenton reaction (Fridovich 1981). Here, the SOD activity exhibited a biphasic response i.e. acute elevation at 2 h exposure reflecting robust antioxidant defense activation, followed by significant depletion after 24 h. This pattern indicates initial compensatory upregulation of the primary antioxidant enzyme (Choudhery et al. 2015) giving way to exhaustion under sustained thermal challenge. Similar progressive decline in antioxidant competence under chronic heat stress has been documented in vivo, where prolonged exposure reduced total SOD activity and increased oxidative injury in lambs (Shi et al. 2020).

Most significantly, heat stress fundamentally altered differentiation potential. *OPN* expression (osteogenic marker) was markedly suppressed under chronic stress at both 39°C (0.39-fold) and 41°C (0.28-fold), with significant reduction between acute 39°C and 41°C groups. This suppression likely reflects cellular prioritization of survival pathways over energy-demanding differentiation programs under severe stress (Shimoni et al. 2020). The extensive cell detachment observed at 41°C (24 h) in osteogenic cultures further supports impaired osteogenic progression, potentially associated with heat-induced oxidative imbalance and cytoskeletal disruption (Almeida 2011).

Conversely, *PPAR-γ* expression (adipogenic marker) showed consistent upward trend at 41°C (1.66–1.77-fold) in both acute and chronic exposures, suggesting temperature-specific induction of adipogenic signaling. This finding aligns with Bernabucci et al. (2009), who demonstrated *PPAR-γ* induction in 3T3-L1 adipocytes at 41°C but not at 37°C or 39°C. Given *PPAR-γ*'s multifunctional role in both adipogenesis and cell survival under oxidative stress (Blanquicett et al. 2010), it remains unclear whether this activation directly drives adipose expansion or primarily supports cellular stress resilience. The inherent adipogenic bias of AD-MSCs compared to bone marrow-derived MSCs (Mohamed-Ahmed

et al. 2018) may further amplify this response under thermal challenge.

These findings provide crucial in vitro evidence for cellular mechanisms underlying heat stress-related impairments in cattle. The pronounced suppression of osteogenesis coupled with enhanced adipogenic signaling suggests that heat stress may fundamentally alter the regenerative function of MSC populations in vivo, potentially compromising tissue repair processes critical for maintaining productivity and welfare in livestock exposed to rising global temperatures.

Conclusion

This study demonstrates that heat stress exerts profound, differential effects on bovine adipose-derived stromal cells. Mild hyperthermia (39°C) falls within the adaptive range, stimulating transient metabolic activity and provoking protective HSP responses. In contrast, severe stress (41°C), particularly when prolonged, drives cells toward a critical threshold: initial protective responses (SOD, *HSP70* upregulation) are ultimately overwhelmed, leading to antioxidant exhaustion, metabolic decline, and pronounced bias in differentiation potential. The significant suppression of osteogenesis coupled with enhanced adipogenic signaling suggests that heat stress may fundamentally alter MSC regenerative function in vivo. These findings highlight the vulnerability of tissue repair processes to elevated temperatures and suggest that strategies enhancing cellular stress resilience such as mild preconditioning or antioxidant support could mitigate adverse effects of climate change on livestock production and welfare.

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Statements and Declaration

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The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

The study was designed, experiments conducted, data analyzed, and the original draft written by Dr. Harippriya Kanagaraj. Project supervision, conceptualization, and manuscript revision were provided by Dr. Pratheesh Mankuzhy. Protocol development and technical expertise were contributed by Dr. K. Raji. Administrative and resource oversight were provided by Dr. K. Karthiayini. Sample collection assistance and manuscript review were provided by Dr. T. Sathu and Dr. Reji Varghese. Technical assistance and experimental work contributions were provided by Dr. S. Abishek. The final version was approved by all authors.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The adipose tissue samples were collected from cattle processed for routine slaughter at a licensed slaughterhouse and were obtained solely as post-slaughter tissue by-products. As no animals were specifically procured, sacrificed, or subjected to any experimental intervention for this study, institutional animal ethical approval was not required for the sample collection.