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Influence of heat stress on the proliferation of bovine adipose-derived stromal cells#

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Abstract

Heat stress is a critical environmental factor influencing cellular physiology and has implications for livestock cell biology and regenerative research. This study evaluated the effects of acute (2 h) and chronic (24 h) heat exposure on proliferation and morphology of bovine adipose-derived stromal/stem cells (bADSCs). Bovine ADSCs were isolated from subcutaneous adipose tissue (~5 g) by collagenase digestion and in vitro cultured in DMEM supplemented with 15 per cent FBS and one per cent antibiotic–antimycotic. Cells attached within 24–48 h, adopted a spindle-shaped fibroblastic morphology by days 3–5, and reached 80–90 per cent confluence by 13–14 days; morphology and growth remained stable through passages 6–8. For heat stress experiments, third-passage cells were seeded (5×10^4 cells/well) and exposed to 39°C or 41°C (controls at 37°C). Proliferation was assessed by Trypan Blue dye exclusion and expressed as $\times 10^3$ cells/mL. Under acute exposure, proliferation at 39°C (68.0 ± 3.60) exceeded that at 37°C (55.0 ± 1.00) and 41°C (54.0 ± 6.92), but differences were not statistically significant ($p = 0.13$). After 24 h, proliferation modestly increased across groups with values of 72.0 ± 5.20 (39°C), 68.0 ± 4.46 (37°C) and 62.0 ± 6.08 (41°C) ($p = 0.45$). Overall, 39°C produced the highest proliferation at both time points, though effects did not reach significance. These findings suggest that mild hyperthermia (39°C) may transiently stimulate bADSC metabolic activity and proliferation, possibly via. pro-survival signalling and induction of heat shock responses, whereas higher thermal load (41°C) tends toward neutral or slightly adverse effects.

Keywords: *Bovine adipose-derived stromal cells, heat stress, cell morphology, proliferation, Trypan Blue*

Climate change, as repeatedly highlighted by the Inter-governmental Panel on Climate Change (IPCC), is a major threat to ecosystems and food security worldwide. Rising global temperatures are leading to more frequent, intense, and longer heatwaves. The adverse effects of heat stress on livestock performance such as reduced growth, impaired reproductive efficiency, decreased milk yield and disrupted acid-base balance (Mathew *et al.,* 2024) can be attributed to disruptions in fundamental cellular processes. At the cellular level, thermal challenge causes oxidative and mitochondrial dysfunction, endoplasmic reticulum stress and DNA/epigenetic damage that inhibit cell proliferation and

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promote senescence or apoptosis (Ribarski-Chorev *et al*., 2023). Cell proliferation plays a pivotal role in development, tissue maintenance, and repair. Impairment of proliferative capacity was observed under heat stress (Lu *et al*., 2023), which compromises tissue growth, delays wound healing, and weakens immune competence, thereby contributing to production losses. Understanding these outcomes requires a focus on stem cells, the primary proliferative population in the body. As key mediators of tissue regeneration and repair, stem cells are central to an animal's resilience and ability to recover from stress-induced damage.

This study aims to determine how heat stress affects the proliferative capacity of stem cells, using adipose-derived stromal cells (ADSCs) as an *in vitro* model. ADSCs are a plentiful source of multipotent mesenchymal progenitors that play key roles in metabolism and tissue repair (Gimble *et al*., 2007). They are highly sensitive to environmental stress, readily obtained from adipose tissue, and therefore represent a practical and physiologically relevant system for studying cellular responses to thermal challenge. Trypan blue dye exclusion method was used to quantify proliferation of ADSCs under heat stress (Strober, 2001), providing insight into cytotoxic mechanisms that can guide interventions to increase livestock heat tolerance.

Materials and methods

Isolation and Culture of bADSCs

Bovine subcutaneous adipose tissue (~5 g) was collected aseptically during slaughter (Meat Technology Unit, CVAS, Mannuthy) in sterile PBS containing one per cent Antibiotic-Antimycotic solution and transported to the laboratory. Samples were washed repeatedly with PBS to remove blood and debris, minced into fine fragments, and digested in DMEM containing 0.01 per cent Type I collagenase at 37°C for approximately 3 h with intermittent agitation. Digestion was halted by adding an equal volume of complete culture medium, prepared using DMEM supplemented with 15 per cent FBS and 1 per cent Antibiotic-Antimycotic solution (100 U/mL penicillin and 100 µg/mL streptomycin), sterilised through a 0.22 µm filter and pre-warmed to 37°C before use. The suspension was filtered through a 100 µm strainer and centrifuged at 900 rpm for 10 min. The stromal vascular pellet was collected, resuspended in complete medium, and seeded into six-well plates for primary culture.

Cells were maintained at 37°C in a humidified incubator with 5 per cent CO_2. After 24–48 h, non-adherent cells were removed, and the medium was refreshed every three days. Cultures were expanded until reaching 80–90 per cent confluence, at which point they were passaged using 0.25 per cent trypsin-EDTA. Detached cells were neutralized with complete medium, centrifuged at 900 rpm for 10 min, and resuspended in fresh medium for reseeding. For downstream experiments, viable cells were counted using a hemocytometer and seeded in equal numbers in triplicates.

Heat exposure protocol

At the third passage, once cells attained 80–90 per cent confluence, they were seeded in triplicates into suitable culture plates and divided into six groups, including controls, to study the effect of heat stress (HS). Cells were maintained either at 37°C (control) or exposed to elevated temperatures of 39°C and 41°C for two different durations—2 h representing acute exposure and 24 h representing chronic exposure. Control cultures were kept in a standard incubator at 37°C with 5 per cent CO_2, whereas HS treatments were carried out in a separate incubator pre-set at the respective temperatures with identical CO_2 conditions. After the designated exposure periods, the cells were harvested for further assessing their proliferation.

Cell proliferation assay (Trypan blue dye exclusion method)

Cell proliferation was assessed using the Trypan Blue dye exclusion method, which differentiates live (unstained) from dead (blue-stained) cells. bADSCs were seeded in triplicates into 24-well plates at a density of 5×10^4 cells per well and allowed to attach for 24 h at 37°C. Plates were then subjected to heat stress by incubation at 39°C or 41°C for the desired durations, while control cells were maintained at 37°C. After treatment, cells were trypsinised, pelleted, and resuspended in 1 mL of complete medium. For counting, 10 µL of the suspension was mixed with an equal volume of 0.4 per cent Trypan Blue and loaded onto a haemocytometer. Live(unstained) cells were enumerated in the four large corner squares under a light microscope. The cell concentration was calculated as:

$$\text{Live cells/mL} = \text{Average live cell count} \times \text{dilution factor} \times 10^4$$

where, the dilution factor was 2 (1:1 mixing with Trypan Blue), and the constant 10^4 corresponds to haemocytometer chamber dimensions (1 $mm^2 \times 0.1$ mm depth).

Statistical analysis

Results are reported as mean ± SE. One-way analysis of variance (ANOVA) was performed using SPSS v24.0 to evaluate differences among the treatment groups.

Results and discussion

Culture and Morphology of bADSCs

Cells isolated from bovine subcutaneous

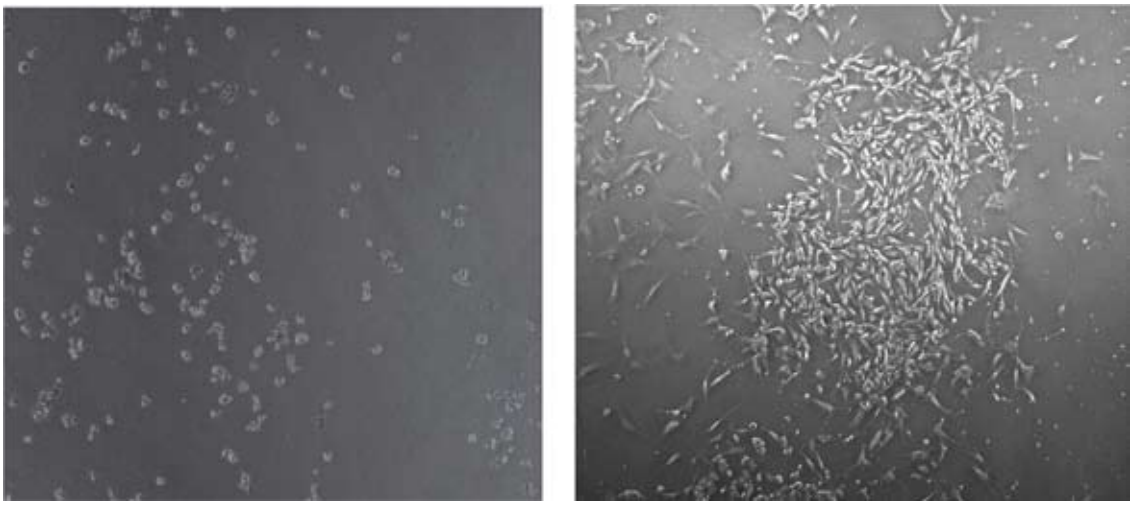

Fig. 3.1. Rounded, floating cells during seeding (A) and characteristic fibroblast like morphology of cells observed during active proliferation (B).

adipose tissue attached to culture plates within 24–48 h, while non-adherent debris and rounded cells were gradually removed during medium changes. The adherent population displayed a spindle-shaped, fibroblast-like morphology (Fig. 3.1), which became more uniform by days 3–5 of culture. Cultures reached 80–90% confluence within 13–14 days, showing active proliferation and colony formation. Morphological features remained stable up to the sixth to eighth passage; however, beyond these passages, a decline in proliferation and noticeable alterations in morphology were observed.

The rapid attachment of cells within 24–48 h and the emergence of a spindle-shaped, fibroblast-like adherent population are consistent with the canonical behaviour of mesenchymal stromal/stem cells (MSCs). Plastic adherence and a fibroblastic morphology are among the primary culture characteristics used to identify MSCs, as set out by the International society for cell therapy position statement. (Dominici *et al*., 2006). The observed homogeneity of spindle morphology by days 3–5 and the subsequent colony formation and attainment of 80–90% confluence within ~2 weeks align with early

Table 3.1 Effect of heat stress on cell proliferation (×10^3 cells /ml) in cells exposed for 2 h and 24 h at different temperatures (Mean ± SE)

Group	Mean ± SE (×10^3 cells /ml)			p value
	37°C	39°C	41°C	
2 h (acute)	55^a ± 1.00	68^a ± 3.60	54^a ± 6.92	0.13^{ns}
24 h (chronic)	68^a ± 4.46	72^a ± 5.20	62^a ± 6.08	0.45^{ns}

Mean values within a row bearing the same superscript (a) do not differ significantly (p > 0.05), ns- non-significant at 0.05 level.

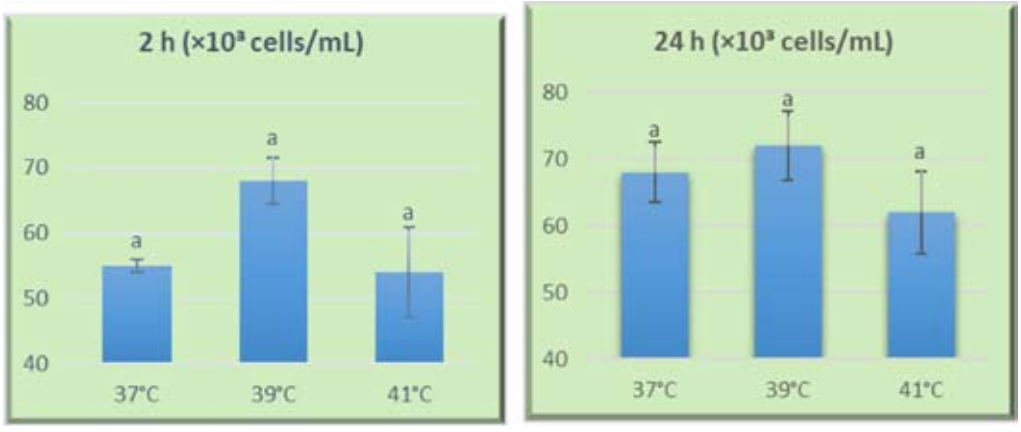

Fig. 3.2. bADSC viability (×10^3 cells/mL) measured after 2-h and 24-h heat stress at three temperatures (37°C, 39°C, 41°C)

descriptions of multipotent mesenchymal cells and their *in-vitro* expansion kinetics. (Pittenger *et al*., 1999)

Cell proliferation assay (Trypan blue dye exclusion test)

Cell proliferation, measured by the Trypan Blue assay and expressed as $\times 10^3$ cells/mL (Table 3.1; Fig. 3.2), showed the following pattern. Under acute exposure (2 h), proliferation was highest at 39°C ($68.0 \pm 3.60 \times 10^3$ cells/mL), followed by 37°C ($55.0 \pm 1.00 \times 10^3$ cells/mL) and 41°C ($54.0 \pm 6.92 \times 10^3$ cells/mL); these differences were not statistically significant ($p = 0.13$). After chronic exposure (24 h), proliferation showed a modest rise at 39°C ($72.0 \pm 5.20 \times 10^3$ cells/mL) compared with 37°C ($68.0 \pm 4.46 \times 10^3$ cells/mL), while it was lower at 41°C ($62.0 \pm 6.08 \times 10^3$ cells/mL). In summary, cultures exposed to 39°C exhibited the highest proliferation at both time points, although none of the between-group differences reached statistical significance ($p > 0.05$).

A small, non-significant rise in cell proliferation was seen at 39°C following acute exposure compared with the 37°C control, which may reflect a transient stimulatory effect of mild hyperthermia on cellular metabolism. Mild heat can briefly activate pro-survival pathways such as ERK and Akt, promoting metabolic activity and cell-cycle progression in various cell types (Hossain *et al*., 2017). By contrast, exposure to 41°C for 2 h produced no notable change in proliferation, suggesting that a higher thermal load may trigger compensatory stress responses that negate any short-term metabolic benefit.

After 24 h of exposure, the modest increase in proliferation at 39°C persisted but remained statistically non-significant, indicating possible cellular adaptation to prolonged mild heat—potentially *via.* induction of heat shock proteins (e.g., HSP70) that preserve protein homeostasis and protect cells from damage (Choudhery *et al*., 2015). The slight decline at 41°C after chronic exposure points toward accumulating proteotoxic stress; sustained elevated temperature can impair mitochondrial function, raise mitochondrial ROS production and dissipate membrane potential, ultimately compromising ATP generation and cell health (Shimoni *et al*., 2020).

Conclusion

Bovine adipose-derived stromal cells were reliably isolated and expanded *in vitro*, exhibiting the expected spindle-shaped, fibroblast-like morphology and stable growth through early-to-mid passages, with decline only after extended culture. Exposure to mild heat stress produced a tendency toward maintained or slightly enhanced proliferation, suggesting adaptive, pro-survival responses, whereas higher thermal challenge activated stress pathways that restrained proliferative advantage. These findings underscore the importance of using early-passage cells and clearly reporting culture conditions in functional studies. Future work should examine heat shock protein induction, mitochondrial function and differentiation capacity under varied heat regimes to delineate adaptive versus deleterious thresholds and improve relevance to livestock physiology and regenerative applications.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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