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SHORT COMMUNICATION

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Heat stress induced in vitro affects cell viability and gene expression of dermal fibroblasts from bovine and buffalo

Natasha P. BORGES[1], Eduardo B. SOUZA[2], Simone S. D. SANTOS[2], Otávio M. OHASHI[2†], Priscila P. B. SANTANA[3] and Ednaldo SILVA-FILHO[3,*]

[1] Post-graduate Programme of Health and Animal Production. Federal Rural University of Amzonia - UFRA, Belém, Pará, Brazil. [2] Biologic Science Institute. Federal University of Pará - UFPA, Belém, Pará, Brazil. [3] Post-graduate Programme of Applied Biotechnology in Agrarian Science. Federal Rural University of Amazonia - UFRA, Belém, Pará, Brazil.

*Correspondence should be addressed to Ednaldo Silva-Filho: silva.filho@ufra.edu.br

[†] *In memoriam*

Abstract

Aim of the study: To evaluate the response of dermal fibroblasts to heat stress and different time exposures on the cell survival and gene expression.

Area of study: Belém city, Pará state. Brazil.

Material and methods: Fibroblasts were isolated from ear skin of bovine (n= 4) and buffalo (n= 4), cultured in vitro until the 3rd passage and submitted to heat stress at 42°C for 3, 6 and 12 h, except for the negative control (38.5°C for 24 h). Cell survival was measured using Trypan blue, and RNA isolation was performed using Trizol method following qRT-PCR to quantify the relative expression of the inducible heat shock protein *HSPA1A*, the pro-apoptotic *BAX* and pro-inflammatory *IFN-γ* genes.

Main results: Heat stress induced in vitro affected the cell viability and gene expression in a time-dependent manner. Gene expression was relatively lower in buffalo ($p<0.05$) than in bovine. Until 3 h of heat stress, *HSPA1A* showed a slight increase in both bovine and buffaloes, and *BAX* was 5.82-fold greater in bovine ($p<0.05$). After 6 h, HSPA1A was 75.81-fold ($p<0.0001$) and *INF-γ* was 20.15-fold greater ($p<0.05$) in bovine than buffalo. Only after 6 h the cell viability started to decrease significantly ($p<0.05$) in both species.

Research highlights: Dermal fibroblasts of buffaloes and bovine were sensitive to heat stress induced in vitro, which was most detrimental to cell survival after 6 h. The expression of *HSPA1A*, *BAX* and *INF-γ* genes in response to heat stress indicate a slight sensibility of the dermal fibroblasts of bovine compared to their buffalo counterpart.

Additional key words: *HSPA1A*; cell survival; qRT-PCR.

Abbreviations used: *BAX* (BCL2 Associated X gene); *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase gene); *HSPA1A* (Heat Shock Protein A1A gene); *HSP* (heat shock protein); *INF-γ* (Interferon Gamma gene); PBS (phosphate-buffered saline); qRT-PCR (quantitative real time polymerase chain reaction).

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Introduction

Global climate changes and increasing temperatures are currently the most challenging and harmful threats to animal production. In response to the heat stress livestock animals usually show a reduction in performance and reproductive parameters to ensure survival causing, in extreme situations, an increase in mortality (Bishop-Williams et al., 2015; Cheng et al., 2022).

Due to this global scenario, the selection of heat-tolerant animals has been developed aiming at the increment of livestock productivity (Hassan et al., 2019). The selection for heat tolerance relies on the use of appropriate biomarkers, such as heat shock proteins (HSPs), mainly the HSP70s family which emerged as potential markers to select heat-tolerant animals (Basiricó et al., 2011; Kumar et al., 2015; Hyder et al., 2018; Hassan et al., 2019). HSPs are chaperones that play a role in the rescue of cells damaged by a variety of environmental stresses (Bhat et al., 2016; Hyder et al., 2018; Mishra, 2022).

Nevertheless, animal susceptibility to heat stress depends on environmental and individual variables (Collier et al., 2008), as well as on the production system, nutrition, species, genetics and age (Das et al., 2016). Hence, animals present many responses to heat stress which may differ according to the breed and species (Mishra, 2021). At the molecular level, previous studies reported that heat stress elicits different HSPs gene expression in dermal fibroblasts of buffalo and cattle (Singh et al., 2014, 2020; Shandilya et al., 2020). Also, influence the HSPs levels in fibroblasts and the immune response in broiler (Siddiqui et al., 2020; 2022).

In this regard, dermal fibroblasts culture cells have been related as an experimental model, instead of living animals, to investigate the effects of heat stress in vitro at the cellular level given its easy isolation and culture (Madelaire et al., 2022). At the physiological point of view, the fibroblasts from skin play a role in the first defense barrier against environmental factors and trigger responses to temperature, humidity, and radiation changes (Sriram et al., 2015).

Herein, we aimed to investigate the cellular response to heat stress induced in vitro of dermal fibroblasts derived from skin of bovine and buffalo animals. Also, we aimed to address a putative species-specific mechanism of heat tolerance in the cells. For that, the cell survival and relative gene expression of *HSPA1A*, the pro-apoptotic (*BAX*), and pro-inflammatory (*IFN-γ*) genes were evaluated in dermal fibroblasts submitted to prolonged heat stress. The *BAX* and *IFN-γ* genes are associated to the progression of apoptosis in cells, albeit through different pathways, and altogether with cell viability analysis may indicate the cell survival. The study of the cell response to heat stress may help to improve selection of heat-tolerant animals.

Material and methods

Animals and experimental design

The ear biopsies were obtained postmortem through donation of the Cooperative of Livestock Industry of Pará Ltda (SOCIPE) slaughterhouse. All experiments were performed following the recommendations of the National Council for the Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee of Federal Rural University of Amazonia (CEUA number 2820150222, UFRA, Belém, Pará, Brazil).

Skin biopsies were taken from four bovine, and four buffalo crossbred, all healthy males, aged between 18-24 months. Based on previous studies which reported that temperatures above 40°C induced heat stress in fibroblasts cultured in vitro (Singh et al., 2014; Shandilya et al., 2020), we submitted the cells to 42°C, according to the following treatments: negative control (38.5°C/24 h), and 3, 6 and 12 h at 42°C. All the treatments were repeated 4 times, each time using one different individual of both species, thus performing 4 biological replicates which were evaluated for cell viability and gene expression.

Isolation, culture and heat stress of dermal fibroblasts

Skin biopsies fragments measuring approximately 4 × 2 cm were collected aseptically from the ear skin, cleaned with 70% ethanol, and washed twice in sterile phosphate buffer saline (PBS) supplemented with 0.5% of streptomycin-penicillin (SP). Tissue was held in sterile PBS with 1% SP and gentamicin for transport to the laboratory. The samples were washed in 70% ethanol again, following two washes in PBS with 0.5% SP, the aseptic removal of the edges, and the fragmentation of the resulting piece into 1 × 2 mm fragments (explants) using a sterile surgical blade. Explants were placed in 35-mm Petri dish containing Dulbecco's Modified Eagle Medium® (DMEM) medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and 50 mg/mL of gentamicin, and cultured in an incubator at 38.5°C in 5% CO_2, 20% O_2 and 75% N_2 in humidified air atmosphere for 48 h. The primary cultures were observed at every 48 h for medium replacement and the explants were removed in the 7th day of culture when a monolayer of adherent cells was visualized. As fibroblast proliferation reached 70-80% confluence, cells were dissociated from the Petri dish using 0.25% Trypsin-EDTA, then quantified and subcultured in a concentration of 1×10^5 cells in 65-mm Petri dishes. The cells were continuously cultured until the 3rd passage.

Briefly, the third passage fibroblasts were cultured for 24 h at 38.5°C in a humidified air, after that the cells were transferred to another incubator adjusted with the same

Table 1. Designed primer sequences for qRT-PCR analysis.

Gene	Species	Primer sequences (5’→3’)	NCBI ID
HSPA1A	*Bos taurus*	F: AGGACTTCGACAACAGGCTG R: TGCTGGACGACAAGGTTCTC	282254
HSPA1A	*Bubalus bubalis*	F: AGGAAGAGATCGAGCGCATG R: AGGCCTTCATCACTCACAGC	102409533
BAX	*Bubalus bubalis*	F: TGTGGACACAGACTCTCCCC R: GGCAAAGTAGAAAAGGGCGAC	102400076
INF-γ	*Bubalus bubalis*	F: TTCAGAGCCAAATTGTCTCCT R: TCTCAGAGCTGCCATTCAAGA	102416494
GAPDH	*Bubalus bubalis*	F: ACCCAGAAGACGGTGGATG R: CCGTTGAGCTCAGGGATGA	102404028

F: primer forward; R: primer reverse.

conditions except for the temperature of 42°C. Incubation at 42°C followed for 3, 6 and 12 h, except the control group which remained at 38.5°C. At the end of each time exposure (3, 6 or 12 h), one Petri dish from buffalo and other from bovine were collected from the incubator to follow evaluations. Firstly, cells were detached by trypsinization and washed by centrifugation at 300 g for 5 min. For cell viability evaluation, cells were stained with 0.4% Trypan blue solution and counted in Improved Neubauer’s chamber. For gene expression analysis, approx. 2×10^6 cells were storaged in cryogenic tubes containing RNAlater™ stabilization solution (Invitrogen, Carlsbad, CA, USA) at -80º C in the Indrel Ultra Freezer until RNA isolation.

RNA isolation and real-time reverse transcription polymerase chain reaction (qRT-PCR)

Prior to RNA isolation, firstly we merged the replicates of the same experimental group to obtain an RNA sample merged. After that, RNA isolation was carried out using TRIzol LS® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and total RNA was quantified in a BioDrop spectrophotometer (Thermo Scientific, Wilmington, UK) by reading the absorbances at 260 nm and 280 nm, and their purity was determined by the ratio A260/A280. Before the mRNA quantification by qRT-PCR, the initial amount of sample was adjusted, thus each treatment was measured as a pool containing 100 ng of RNA.

Total RNA was amplified in qRT-PCR using the CFX96 Touch™ Real-Time Detection System thermocycler (Bio-Rad, Hercules, CA, USA) and the Master Mix Power SYBR Green One-Step PCR® kit (Applied Biosystems, Woolston Warrington, UK) according to the manufacturer’s instructions, in a final volume of 10 µL. Four replicates were performed for each treatment. The primers (Table 1) were designed using the Prime3Plus platform. All the primers were designed to amplify conserved regions displaying 100% homology between buffalo and bovine, except for *HSPA1A* gene, where primers were designed separately because their alignment showed slight nucleotide differences in the exon 1 region.

Statistical analysis

The relative gene expression was determined by the $2^{-\Delta Ct}$ method using *GAPDH* as housekeeping gene. Data were tested for normality by the Kolmogorov-Smirnov test and further for significance test using ANOVA considering the species and treatment effects. Means were compared using the SNK (Student-Newman-Keuls) test. Treatment effects were validated through linear and quadratic regression analyses, with a significance level of 0.05. All analyses were performed using the SAS program (onDemand version).

Results and discussion

This study investigated the cellular response of dermal fibroblasts from bovine and buffalo to the heat stress induced in vitro. The *HSPA1A* is an inducible member of the family HSP70 which is shown to be expressed in response to high temperatures (Singh et al., 2014, 2020; Archana et al., 2016, 2017; Kapila et al., 2016; Maibam et al., 2017; Abdelnour et al., 2019; Shandilya et al., 2020; Mishra, 2022). Singh et al. (2014) reported different responses of dermal fibroblasts from zebu (Tharpakaar) and crossbred (Karan-Fries) bovine that were cultured in vitro up to 40°C for 3 h; the inducible *HSPA1A* and *HSPA2* gene expression was relatively lower in zebu than in bovine, while the constitutive *HSPA8* was higher in zebu. Maibam et al. (2017) reported a similar result in skin biopsies

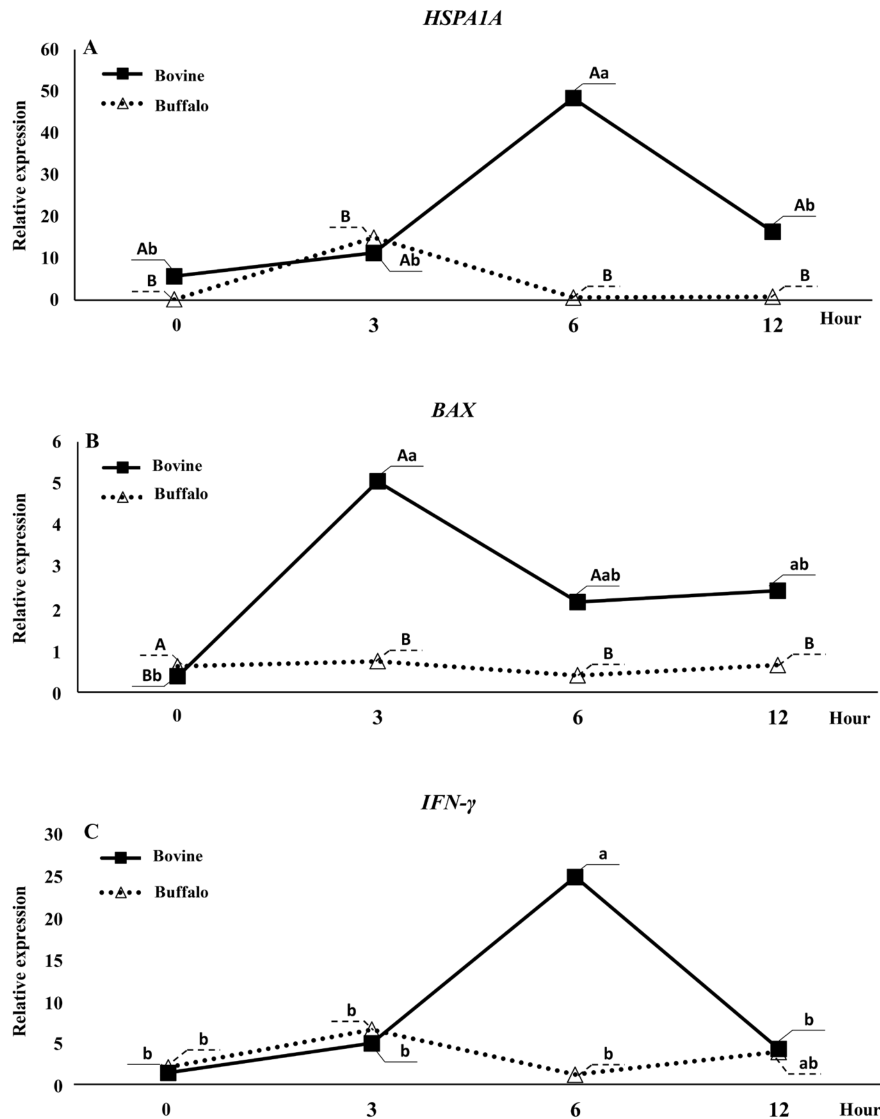

Figure 1. Relative expression of *HSPA1A* (A), *BAX* (B) and *IFN-γ* (C) genes in dermal fibroblasts derived from ear skin of bovine (continuous line with squares) and buffaloes (dashed line with triangle). Treatments were 0 h (negative control, 38.5°C for 24 h), and heat stress induced in vitro at 42°C for 3, 6 and 12 h. Relative expression was measured using GAPDH as a housekeeping gene. The uppercase letters indicate a significant difference (p<0.05) between species and lowercase letters among treatments into the same species.

isolated at the summer season, suggesting again that zebu was more heat-tolerant than bovine. It was corroborated in a microarray analysis of dermal fibroblasts under heat stress which showed that zebu presented a higher number of chaperone genes expressed than bovine (Singh et al., 2020).

HSPs can stabilize protein conformations and maintain cell survival (Scieglinska et al., 2019), thus their increased expression may work as a cytoprotection mechanism against heat stress (Kumar et al., 2015; Mishra, 2022). On the other hand, increased expression of inducible HSPs can also be an indication of more injuries due to heat stress. In this study, heat stress modified gene expression in fibroblasts of buffalo and bovine. *HSPA1A*, *BAX* and *IFN-γ* gene expression in buffalo fibroblasts were relatively lower (p<0.05) than in bovine counterparts. *HSPA1A* expression was also different between treatments (p<0.05). Dermal fibroblast exposed to heat stress at 42°C after 3 h showed a slight increase of *HSPA1A* gene expression in both species, and after 6 h increased significantly (p<0.0001) in bovine, which was 75.81-fold greater than buffalo cells (Fig. 1). These results may indicate a cellular effort to maintain homeostasis and that bovine is more sensitive to heat stress than buffalo fibroblasts.

Concomitantly, no difference in cell viability (p>0.05) was observed between bovine and buffaloes (Fig. 2) showing

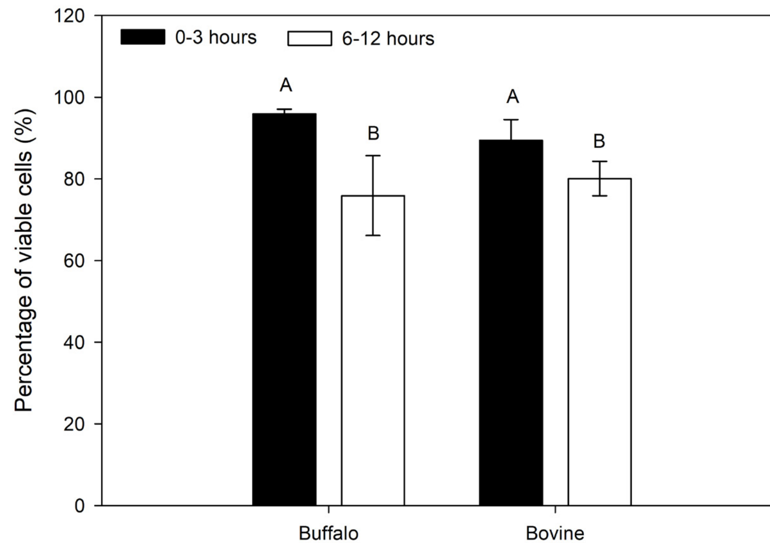

Figure 2. Cell viability rates in dermal fibroblasts from bovine and buffaloes under heat stress at 42°C for periods of 0 (negative control group) to 3 h and 6 to 12 h. Experimental data were expressed as the mean % ± SD. The uppercase letters indicate a significant difference (p<0.05) between treatments into the same species.

that HSPs mechanism against heat stress was effective in protecting dermal fibroblasts in both species, despite their differential gene expression. Heat stress induces different levels of HSPs expression according to tissue (Rout et al., 2016), breed (Yamashita et al., 2004; Singh et al., 2014, 2020; Pires et al., 2019) and species (Guerriero & Raynes, 1990; Yamashita et al., 2004; Siddiqui et al., 2020), thus it is expected to find a variability of heat tolerance into breeds, species and even individuals. Shandilya et al. (2020) reported a differential HSPs expression which also decreases cell viability in dermal fibroblasts from bovine and buffaloes albeit to a different extent. The fibroblasts were exposed at 41°C for 1 hour, following an incubation at 37°C to enable the cells to recover from the heat stress. Herein, from the total number of cells counted in buffalos (n= 8270) and bovine (n= 7505) the mean percentage of live cells decreased after 6-12 h of heat stress compared to the 0-3 h groups in both buffalo (75.9% vs 95.9%, respectively, p<0.001) and bovine (80% vs 89.4%, respectively, p=0.007) counterparts. The decrease in cell survival after 6 h of heat stress exposure showed that heat stress affected the cell viability in a time-dependent manner and that there is a time-limit when cells cannot be protected against the injuries and avoid apoptosis.

It is known that chaperones help in adaptation and heat tolerance (Patir & Upadhyay, 2010) by enhancing cellular resistance, inhibiting the formation of proteasomes (Filipczak et al., 2012) and eliminating damaged proteins during hyperthermia (Kapila et al., 2016). However, HSP70 proteins cannot protect cells against damage induced by the continuous increase in temperature that causes the imbalance between physiological and cellular functions (Patir & Upadhyay, 2010). Besides the heat stress, cells are exposed to the accumulation of reactive oxygen species which triggers oxidative stress and activates apoptosis (Petitjean et al., 2019). Thus, the regulation of pro-apoptotic proteins such as Bax and Bak leads to the permeabilization of the outer mitochondrial membrane and apoptosis induced through the release of cytochrome c (Opferman & Kothari, 2018). Herein, after 3 hours of heat exposure, pro-apoptotic *BAX* gene expression was 5.82-fold higher (p<0.05) in bovine than in buffalo, also the cell viability decreased. In buffalos, however, *BAX* expression did not differ after 3, 6, and 12 hours of heat stress (p>0.05), although cell viability decreased after 6 hours of treatment.

When fibroblasts are exposed to high temperatures, they increase the synthesis of cytokines and cellular matrix proteins (Liu et al., 2015), triggering an inflammatory process in response to cellular damage caused by hyperthermia (Pasparakis & Vandenabeele, 2015). The production of pro-inflammatory cytokines such as *IFN-γ* induces cellular apoptosis via the mitochondrial pathway (Grunnet et al., 2009; Cao et al., 2015) through STAT1-mediated regulation of *BCL-2* and *BAX* gene expression, which increase the pro-apoptotic *BAX,* leading to cytochrome c release and caspase activation in mitochondria, ultimately resulting in cell death (Zhou & Chang, 2008). *IFN-γ* gene expression did not differ between buffalo and bovine until 3 hours (p>0.05), however, after 6 hours of heat stress *IFN-γ* increased 20.15-fold in bovine compared to buffalo fibroblasts (p<0.05), thereafter decreased again at 12 hours (p<0.05) in bovine. In this study, *IFN-γ* and *BAX* expressions were higher in bovine than buffalo, suggesting

the activation of apoptosis via mitochondrial pathway in fibroblasts of bovine but not in buffalo.

In summary, both buffalo and bovine dermal fibroblasts were sensitive to heat stress-induced in vitro, which was most detrimental to cell survival after 6 hours. The *HSP1A1*, *BAX* and *IFN-γ* gene expression seem to indicate a slight sensitivity of bovine compared to buffalo counterparts. Also, the molecular mechanisms that heat stress triggers apoptosis in dermal fibroblasts may be different in both species. However, the activated heat tolerance mechanism in dermal fibroblasts under heat stress shows variability in terms of gene expression possibly influenced by genetic, environmental and evolutionary factors.

Authors' contributions

Conceptualization: O. M. Ohashi.
Data curation: Not applicable.
Formal analysis: N. P. Borges, E. B. Souza, S. S. D. Santos.
Funding acquisition: E. Silva-Filho.
Investigation: N. P. Borges.
Methodology: N. P. Borges, E. B. Souza, P. P. B. Santana.
Project administration: Not applicable.
Resources: Not applicable.
Software: Not applicable.
Supervision: E. Silva-Filho.
Validation: Not applicable.
Visualization: Not applicable.
Writing - original draft: N. P. Borges.
Writing - review & editing: P. P. B. Santana.

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