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Antioxidants Affect Heat Stress Proteins Reactivity on Dermal Fibroblast Cells of Buffalo *In Vitro*

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Abstract | High ambient temperatures adversely affect buffalo production and reproduction. Dermal fibroblasts are essential for regulating temperature homeostasis in the skin. This study aimed to evaluate the mRNA expression profile of different heat shock proteins (HSPs) in dermal fibroblast cells due to heat stress and examine the effect of inorganic zinc and vitamin C in this condition. For this study, the primary fibroblast cells of the Murrah Cross were used and identified by FSP1 and FN_1 primers. The pretreated cultured cells with zinc and vitamin C were kept at 42°C for three hours, as buffaloes in tropical areas experience such temperatures in the summer. The culture cells were subsequently allowed to recuperate at 37°C and collected at various time points along with control samples (unstressed), and cellular viability was determined using the trypan blue dye exclusion method. Heat stress significantly ($p<0.05$) decreased cell viability. Zinc-propagated cells had higher cell viability ($p<0.05$) and proliferation rate than vitamin C-propagated cells across post-heat stress, representing zinc's protective nature against heat stress-induced cell apoptosis and cell injury. HSPs expression studies revealed that heat-responsive genes (HSPA1A, HSPA2, HSPA8, and HSP90AA1) were induced after heat stress and remained upregulated during the post-heat stress periods. Among them, the HSPA2 gene displayed maximal induction across post-heat shock periods. Zinc and vitamin C treatments showed a significant ($p<0.05$) reduction in most HSPs' expression across different post-heat stress periods, which may be due to their pivotal role in the body's immune system and oxidative stress management.

Keywords | Dermal fibroblast, Heat stress, Heat shock proteins, Cell viability, Zinc, Vitamin C

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INTRODUCTION

Extreme heat waves and climate change are a burning concept worldwide. Heat waves lead to a situation where animals are unable to adapt to a sudden increase in ambient temperatures, which causes heat stress (Belhadj Slimen *et al.*, 2016; Bharati *et al.*, 2017). In many tropical countries, heat stress becomes an economic challenge for

animal production. 97.1% of buffaloes are mainly found in Asian countries, with the remaining 2.9% located in other parts of the world (Hansen, 2009; Kapila *et al*., 2016). India, Pakistan, and China have the highest buffalo populations, with India and Pakistan collectively making up approximately 65% of the global buffalo population (Kapila *et al*., 2018). According to Mauger *et al*. (2015), economic losses in the USA are predicted to rise from US$670 million per year in 2014 at current milk prices to US$2.2 billion annually in 100 years due to climate change. Global warming will lead to a decline in total milk production in India, resulting in a loss of approximately 3.2 million tons of milk by 2020 and over 15 million tons by 2050 (Das *et al*., 2016; Purohit *et al*., 2020). Bangladesh is a tropical country, faces similar crisis where summer temperature ranging from 32 to 40 °C and relative humidity levels between 50 and 65% (Alam *et al*., 2010). Islam *et al*. (2024) classified heat stress levels in ruminant animals in Bangladesh, as ranging from normal to extremely severe based on a Temperature-Humidity Index (THI) of 71 to 90 and identified June as the hottest month with a THI of 90. Heat stress is a crucial environmental stimulus that affects milk yield, meat quality, growth rate, and fertility of buffaloes (Kapila *et al*., 2016). The milk production, growth rate, and reproductive performance of buffaloes decline due to elevated ambient temperatures since they absorb heat due to their black skin and thin coat or hair (Marai and Haeeb, 2010).

A complex system of gene expression and adaptive biochemical processes was activated by heat stress (Singh *et al*., 2020). HSPs (Heat stress proteins) work as molecular chaperones in regaining cellular balance and improving cell viability (Collier *et al*., 2008). HSPs are highly conserved proteins that are fundamentally expressed in cells and have housekeeping functions. The synthesis of inducible forms of HSP is enhanced during stress and operated by protecting the cell by refolding denatured, damaged, or degraded proteins and preventing protein aggregation (Malyshev, 2013). HSPs have an enormous role in the case of environmental stress tolerance and thermal adaptation (Maibam *et al*., 2017; Sørensen *et al*., 2003). However, except for HSPs, several other biomolecules play a role in cellular reactions due to heat stress (Kregel, 2002). HSP70 (HSPA1A, HSPA2, and HSPA8) is the most temperature-sensitive and influenced by various physiological, pathological, and environmental stressors (Beckham *et al*., 2004). Throughout protein folding and translocation, HSPA1A and HSPA2 have a significant role in regulating protein structure (Arya *et al*., 2007; Singh *et al*., 2020).

Heat stress in buffalo leads to a range of significant alterations in biological functions, including reduced feed intake efficiency and utilization, imbalances in water and protein metabolism, modifications in enzymatic activities, hormonal levels, blood metabolites, as well as energy and mineral balances (Kapila *et al*., 2016; Marai and Haeeb, 2010). Dairy animals under heat stress had higher oxygen demands and metabolic needs, resulting in the increased production of oxygen-derived free radicals (Sheikh *et al*., 2017; Yuan *et al*., 2012). Antioxidants protect the body's immune system against excessively produced free radicals. Antioxidant supplementation, particularly vitamins (C, A, and E), zinc, and chromium, can mitigate the negative effects of environmental stress in bovine (B.v *et al*., 2011; Kumar *et al*., 2015; Sheikh *et al*., 2016). Trace minerals are vital for reproduction, vitamin production, oxidative metabolism, enzyme formation, and energy metabolism in dairy animals (Overton and Yasui, 2014; Rabiee *et al*., 2010). Being a key trace element, zinc downregulates the production of reactive oxygen species (ROS) by inhibiting the oxidation of macromolecules like proteins and DNA and suppressing inflammatory reactions (Prasad and Bao, 2019). Sheikh *et al*. (2016) found that treating heat-stressed lymphocytes of dairy animals with zinc lessened the negative impacts of heat stress and enhanced their immunological condition. Though dairy animals can synthesize vitamin C, it is rapidly depleted during heat stress, leading to lower plasma vitamin C concentrations (Padilla *et al*., 2006). Supplementation of vitamin C to heat-stressed animals can alleviate stress-induced metabolic changes, enhance immune function, improve oxygen consumption regulation, and lower mortality rates (Abidin and Khatoon, 2013; Minka and Ayo, 2012). Vitamin C supplementation reduced glutathione peroxidase activity in buffaloes exposed to heat stress in a climatic chamber (Kumar *et al*., 2010). Antioxidant dietary supplements administered prior to the onset of heat stress months and throughout the stress period alleviated infertility caused by heat stress and reduced oxidative stress in buffaloes (Megahed *et al*., 2008). To avoid severe production losses and maintain animal health, technical modification of environmental conditions (i.e., shading, fans, etc.) and optimized feeding can be the best options for the short-term management of the upcoming climatic challenges for farmers (Lamp *et al*., 2015).

Although our major segment of the skin is composed of dermal fibroblast cells, little is known about the behavior of dermal fibroblasts in buffalo during heat stress, particularly at the molecular level. A better understanding of the physiological role of skin will assist in modulating buffalo's ability to withstand heat and mitigate adverse effects during various heat stress conditions. Dermal fibroblasts are a major component of skin and are involved in various physiological and pathological conditions. It is the primary active cell within connective tissue, providing advantages for therapeutic research due to its capability to grow easily in culture. Fibroblasts can be utilized for both primary and permanent cell lines, and express numerous genes coding for immune mediators and proteins (Afroz *et al*., 2023). Moreover, from a physiological perspective, skin fibroblasts

play a role in the body's initial defense against environmental challenges and trigger responses to changes in temperature, humidity, and radiation. Because of these properties, dermal fibroblasts can serve as a suitable cellular model to investigate the effects of heat stress (Shandilya *et al*., 2020; Sriram *et al*., 2015). Additionally, the skin constitutes the external layer of animals, and heat load is taken in directly through its skin. Therefore, to protect themselves from rising oxidative stress, skin cells undergo numerous alterations, probably by triggering their antioxidant systems (Singh *et al*., 2020). Several researchers have documented the presence of HSPs genes in the dermal fibroblasts of cattle and buffalo (Shandilya *et al*., 2020; Singh *et al*., 2020, 2014). Though some research has been done on supplementing dairy animal feed with antioxidants to mitigate heat stress effects, little information is available on its effect on dermal cells during this situation. The present research was conducted to observe the cellular and molecular changes in buffalo during heat stress in buffalo during heat stress and investigate the effects of vitamin C and zinc on heat-stressed dermal fibroblast cells.

MATERIALS AND METHODS

Experimental Animal

The research was carried out using the ear tissue from the ear pinna of Murrah Cross (Murrah X Indigenous), which was collected from a slaughterhouse in Mohammadpur, Camp Bazar, Dhaka, under aseptic conditions and promptly transported into a flask containing saline solution. The buffalo weighed about 450 kg and was 2.5 years old without having any disease record. Then, the sample was kept in the laboratory with a saline solution at 4°C before skin biopsies. Norms for the ethical treatment of animals were meticulously followed throughout the process and approved by the Bangladesh Livestock Research Institute.

DNA Extraction from the Ear Tissue

The samples were cleaned and cut into pieces in the presence of Dulbecco's phosphate buffer saline (DPBS). The Wizard Genomic DNA Purification Kit (Promega, USA) was used to isolate the total DNA from the samples. The DNA was extracted and then rehydrated at 4°C overnight in 100 µl of DNA Rehydration Solution. A nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the concentration and purity of DNA. After measuring the amount, DNA was promptly kept at -20°C.

Isolation and Culture of Fibroblast Cells

Skin biopsy samples were collected aseptically from the ear pinna of a buffalo in DPBS (Dulbecco's Phosphate-Buffered Saline) solution and sent to the laboratory for other processing. After rinsing the samples with DPBS, the fat layer and hair were removed meticulously. The tissue was cut into tiny pieces and washed several times with DPBS. The chopped homogenized tissues were incubated in PBS containing trypsin-EDTA for 5 min. After that, DMEM containing 10% Fetal Bovine Serum was added to inactivate enzymatic activity and spun at 800 rpm for 5 min. The supernatant was thrown away, and tissues were rinsed in PBS several times before placing in the culture flasks. The primary fibroblast cells were developed using an established (Afroz *et al*., 2023) fibroblast cell culture method. Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin solution was used to culture the primary fibroblast cells and incubated at 37°C with 5% CO2. Tissue explants were frequently monitored to observe the growth of fibroblasts and aseptically removed when a monolayer had formed on the cell culture dishes. The dermal fibroblasts were routinely evaluated to check cell viability. When fibroblast cells reached 70–80% confluence, they were subcultured using partial trypsinization. The process was repeated until a homogeneous population of dermal fibroblasts was obtained.

DNA Extraction from Fibroblast Culture Cells

Cultured cells were detached from the culture flask using trypsinization. The Wizard Genomic DNA Purification Kit (Promega, USA) was used to isolate the total DNA from the samples. DNA concentration and purity was determined using nanodrop spectrophotometer and kept at -20°C for further use.

Fibroblast Cell Identification

PCR reactions were performed using a Q-Cycler 96 PCR machine (Hain Lifescience, Germany). All PCR reactions were conducted with a total volume of 25 µl, including Master Mix (Promega, USA), forward and reverse primers (Table 1), nuclease-free water, and 2 µl DNA.

Table 1: Primers were used for PCR analysis.

Genes	Role of genes	Primer sequences
FSP1	Fibroblastic hallmarks	F-GATGAGCAACTTGGACAGCAA
		R-CTGGGCTGCTTATCTGGGAAG
FN_1	Fibroblastic hallmarks	F-CGGTGGCTGTCAGTCAAAG
		R-AAACCTCGGCTTCCTCCATAA

The following conditions were used to validate the PCR results: 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes using the DNA (from tissue and cultured cells) and primers. After that, 2% agarose gel electrophoresis was performed. 5 µl of DNA ladder, 1 µl of 6X loading dye (Promega, USA) were mixed with PCR product and loaded into the holes of the agarose gel. After electrophoresis, the agarose gel was carefully removed from the chamber and placed beneath a

UV transilluminator for observation.

Table 2: Primers were utilized for real-time PCR analysis.

Genes	Primer sequence	Accession no.	Size (bp)
HSPA1A	F-TCATCAACGACG-GAGACAAGCCTA R-TTCATCTTGGT-CAGCACCATCGAC	GU_183097.1	103
HSPA2	F-AAGCACAAGAAG-GACATTGCACCC R-AAGTGTAGAAATC-CACGCCCTCGT	NM_174344.1	130
HSPA8	F-CGGTGATGCAG-CAAAGAACCAAGT R-CACCACCATGAA-GGGCCAATGTTT	NM_174345.3	133
HSP90AA1	F-ATGAGCAG-TATGCCTGGGAGTC R-CCCATTGGT-TCTCCTGTGTCAG	M_ 001012670.2	73
GAPDH	F-CCAACGTGTCT-GTTGTGGATCTGA R-GAGCTTGACAAA-GTGGTCGTTGAG	NM_ 001034034.2	218

Heat and Antioxidants Treatment on Buffalo Fibroblast Cells

The cells after the fourth passage were used for antioxidants and heat treatments. The four culture flasks were pretreated with zinc (0.01 mM), four with L-ascorbic acid (20 µM), and others without zinc and L-ascorbic acid supplementation. Then the culture flasks were placed in a 5% CO2 incubator and kept at 37˚C for a week. Zinc sulfate heptahydrate (Sigma Aldrich, USA) and L-ascorbic acid (Sigma Aldrich, USA) were utilized for cell culture after adjusting their concentrations to effectively stimulate fibroblast cell culture. A solution of 1% penicillin-streptomycin was used to keep the culture free of contamination. After seven days, the culture flasks were kept at 42˚C for three hours and collected at various time points, except for control cells (without zinc and L-ascorbic acid pretreatment). After that, the cells were subsequently allowed to recover at 37˚C and harvested by trypsinization at different time points (30 minutes, 2 hours, 4 hours, and 6 hours) along with control samples (unstressed). The culture plate marked as control was maintained at 37˚C during the experiment and was collected at the same time intervals as the treated samples.

Cell Viability

Cell viability was recorded during post-heat stress periods using the trypan blue exclusion method to evaluate the impact of heat stress on fibroblast cells. The cells' viability was assessed with 10 µl of cell solution and an equal volume of 0.04% Trypan blue. The cell number and viability were determined using a hemocytometer under an inverted microscope (10X). The stressed and unstressed (control) cells were checked at various time intervals after heat stress (30 minutes, 2 hours, 4 hours, and 6 hours) to check the cell viability.

RNA Purification and cDNA Synthesis

Total RNA extraction from all culture cells with and without treatments was harvested at different time intervals (30 minutes, 2 hours, 4 hours, and 6 hours) after heat stress using the Monarch Total RNA Miniprep Kit (New England Biolabs, USA). A NanoDrop spectrophotometer was used to measure the concentration and purity of the RNA and stored at -20 ˚C until further usage. cDNA synthesis was performed using the ProtoScript II first-strand cDNA synthesis kit (New England Biolabs, USA). 2 µl RNA, 10 µl 2X reaction buffer, 2 µl 10X Oligo d(T), 2 µl 20X enzyme mix, and RNase-free water were used for a 20 µl solution. At first, It was left to incubate at 65 ˚C for 5 minutes. After that, the master mix was kept at 42˚C for 1 hour. Next, the enzyme was deactivated by heating to 80˚C for 5 minutes. Reactions were performed using a Q-Cycler 96 PCR machine (Hain Life Science, Germany).

Real-Time PCR

The Applied Biosystems Real-Time PCR system was used for this study. SYBR green was our targeted dye in this experiment. The RT-PCR master mix was prepared using 2.0 µl of diluted cDNA, 10 µl of 2X master mix (Addbio, Korea), 10 µM primers, and nuclease-free water to achieve a total volume of 20 µl. GAPDH was used as a reference gene. Forward and reverse-specific primers for HSP genes and the reference gene were used for the real-time PCR presented in Table 2. Data was collected in the form of C_T values (Threshold Cycle). On different plates, three technical replications were carried out. Since each plate featured a representation of each sample, plates functioned as technical replicates (Derveaux *et al*., 2010).

Statistical Analysis

The normalization of the gene expression data was carried out using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The data was analyzed using the SPSS statistics 25.0 version. Data from the experiment are presented as mean ± SD. One-way ANOVA and Duncan's multiple-range tests (DMRT) were used for comparison. $P<0.05$ was considered to be statistically significant. The statistical model was utilized to determine the effect of treatments and their interactions with heat shock proteins expression in dermal fibroblast cells.

RESULTS AND DISCUSSION

Establishing Primary Culture of Buffalo Fibroblast Cells

The present study utilizes buffalo fibroblast cells as an in vitro model to investigate the changes in mRNA expression of different heat shock proteins (HSPs) in response to heat stress. Fibroblast cells initially grew slowly, but after 8 days, they formed a vigorous culture. Within 13 days, the culture flasks were fully covered by cells (Figure 1). Once the fibroblast cells reached 70-80% confluence, they were subcultured by partial trypsinization. Skin temperature varies with physical, environmental, and physiological conditions, as well as the optical qualities of the hair coat. Cattle and buffaloes experienced higher skin temperatures during the hot, dry season (Aggarwal and Singh, 2008; Koga *et al*., 2002). The skin temperature of Egyptian buffalo exposed to THI 94 was found to be elevated (Hafez *et al*., 2011). Some researchers used ear tissue from the ear pinna of buffalo like us to develop fibroblast culture (Afroz *et al*., 2023; Borges *et al*., 2023) to observe heat stress effects on dermal fibroblast cells. Singh *et al*. (2020, 2014) used ear tissue from the ear pinna of Tharparkar and Karan-Fries cattle to understand the heat stress effects on cells. They also want to observe the heat stress effect on dairy animals using dermal fibroblast cells like us. Due to their ease of isolation and culture, dermal fibroblast culture cells are a suitable experimental model for studying the impacts of environmental stress at the cellular level *in vitro* rather than using live animals (Madelaire *et al*., 2022).

Identification of Fibroblast Cells to Evaluate the Purity of Cultured Fibroblast Cells

The presence of fibronectin (FN_1) and fibroblast-specific protein 1 (FSP1) in tissue and cultured cells was detected through PCR to confirm the purity of fibroblast cells. A total of eight samples were used for PCR. The total DNA extracted from individual cell samples exhibited an A260/A280 ratio of 1.85-1.9, indicating moderately high DNA quality in each sample. Among them, four DNA samples were extracted from tissue, and others were extracted from culture cells. 2% agarose gels were used to separate the PCR products, observed with ethidium bromide staining under a UV transilluminator at 254 nm, and a photograph was taken for further analysis (Figure 2). The desired product size was less than 200 bp. The size of the band was determined using a 100 bp DNA ladder. In addition to purified DNA and other chemicals, the optimization of PCR conditions was crucial for achieving results. Generally, primary fibroblasts expressed types I and III collagen (Col1 and Col3), fibronectin (FN), and fibroblast-specific protein 1 (FSP1) (Yoon *et al*., 2018; Lee *et al*., 2010). Based on that fact, identifying the presence of fibronectin (FN_1) and fibroblast-specific protein 1 (FSP1) in DNA via the PCR technique can be the best alternative to the immunostaining technique for identifying the fibroblast cells. PCR can be more sensitive than immunohistochemistry, as analysis of DNA can reveal more alternations in the DNA sequence, and sometimes immunohistochemistry gives equivocal results (Awan *et al*., 2017). Immunohistochemistry is a type of immunostaining. We generally agree with Yoon *et al*. (2018), that Col1 and Col3, FN, and FSP1 primers gave satisfactory results in the fibrotic differentiation identification of hESC-MSCs (human embryonic stem cell-derived mesenchymal stem cells).

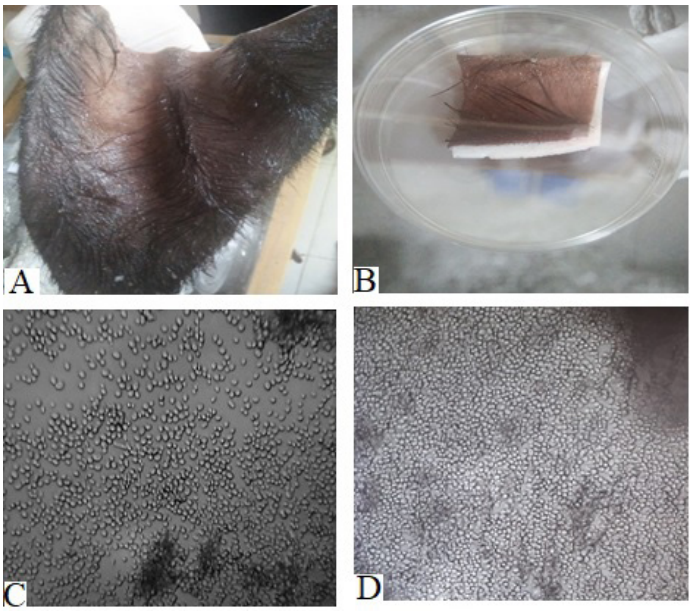


Figure 1: Dermal fibroblast cell culture of buffalo. (A) and (B) Sample for skin biopsy; (C) Growth of cells after 7 days (10x); (D) Growth of fibroblast cells with 70–80% confluence (10x).

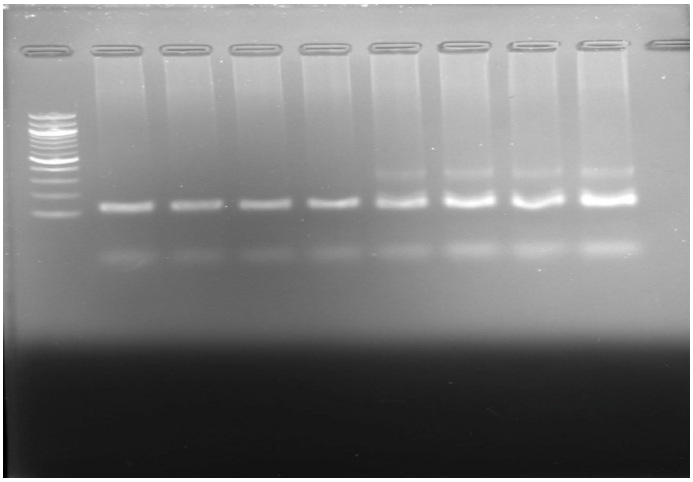
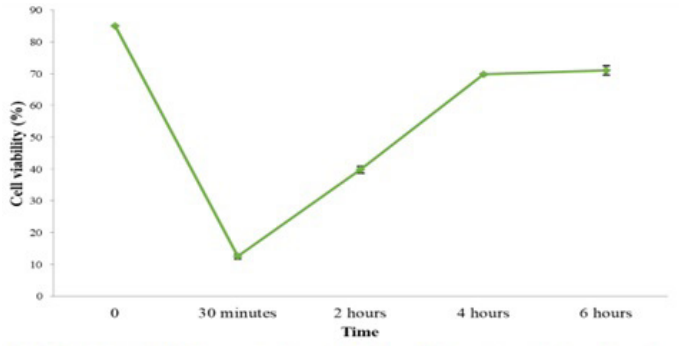


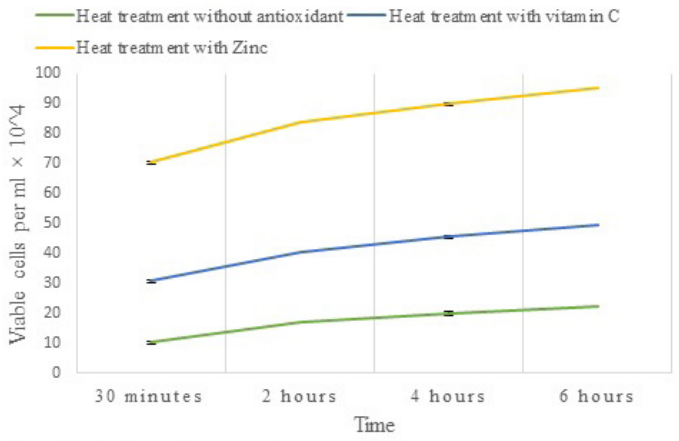
Figure 2: Fibroblast cell confirmation using the polymerase chain reaction (PCR). Here, from the DNA ladder, the first four are DNA samples from tissue with FN_1 and FSP1 primers, and the other four are DNA samples from cultured cells with FN_1 and FSP1 primers.

Heat Stress Effects on Cell Viability and Growth Pattern

Cell viability and number were significantly lower (P<0.05) in cells exposed to high temperature across various time intervals of post-heat stress (30 minutes, 2 hours, 4 hours, and 6 hours) compared to unstressed cells kept at 37°C, which did not exhibit any decrease in cell viability. A significant decrease (P<0.0001) in viable cells was exhibited right after heat stress; however, cell viability recovered (P<0.05) during later stages for all treated cells (Figure 3). Singh *et al.* (2020) observed the effects of heat stress by culturing dermal fibroblasts from cattle at 42 °C for three hours, which is similar to our approach. Kapila *et al.* (2016) exposed mammary epithelial cells of riverine buffalo to thermal stress at 42 °C for one hour to observe the heat stress effect.



A) Cell viability pattern (%) without antioxidants



B) Viable cells number per ml

Figure 3: Viable cell growth pattern in heat-stressed buffalo fibroblast cells with and without antioxidant treatment across post-heat stress using the trypan blue dye exclusion method. All data are presented as mean ± SD. All experimental data represent means at P < 0.05. Cell viability after 30 min P< 0.001; after 2h P< 0.001; after 4h P< 0.005; after 6h P <0.006. Live cell numbers after 30 min P< 0.0001; live cells after 2h P< 0.0001; live cells after 4h P< 0.0001; live cells after 6h P <0.0001.

The cells pretreated with zinc showed less viable cell growth fluctuation (P< 0.0001) in post-heat stress conditions. Kapila *et al.* (2016) observed that buffalo MECs upregulate anti-apoptosis genes expression (BAX and BCL2 genes) during post-heat stress periods (30 minutes to 48 hours). Cell viability decreased (P< 0.001) significantly right after the heat stress, possibly due to the negative impact of thermal stress on cell membrane integrity, cell necrosis, or impairment of cell growth. The results of our study have similarities with Singh *et al.* (2020), Adachi *et al.* (2009) and Zhao *et al.* (2006), who reported that temperatures between 42–46 °C lead to cell death in various cell types. In contrast, cell viability significantly increased (P < 0.05) during the subsequent stages might be because of the recovery of cell survival mechanisms by fibroblast cells. This non-linear pattern of cell viability to thermal stress that was found in our study has aligned with Kapila *et al.* (2016), Kapila *et al.* (2013), Collier *et al.* (2006), Du *et al.* (2008) and Du *et al.* (2007), where a similar pattern of cell viability due to high temperature was examined in various *in vitro* cell culture models. Environmental factors like temperature, radiation, and lack of oxygen induce cell cytotoxicity, resulting in higher membrane permeability, decreased pH within the cell, and disruptions in ion balance (Singh *et al.*, 2014; Piper *et al.*, 2003). Moderate stress triggers appropriate protective cellular responses, but extreme stress causes irreversible cellular damage (Singh *et al.*, 2014). Heat stress leads to oxidative stress in cells, which indicates an imbalance in the antioxidant system. Oxidative stress has the potential to harm membranes, proteins, RNA, and DNA (Singh *et al.*, 2014; Pryor *et al.*, 2006). ROS damages DNA, producing hydroperoxides and peroxides (Kastan and Bartek, 2004). Heat exposure can lead to oxidative stress in dairy animals by reducing antioxidant activity (Megahed *et al.*, 2008; Singh *et al.*, 2014).

The cells propagated with antioxidants had higher cell viability (P<0.05) than untreated cells under 42 °C heat stress. Zinc showed notable cell viability (P<0.05) across post-heat stress periods and promoted higher cellular growth of fibroblasts. Sheikh *et al.* (2016) also reported that zinc increased cell viability in heat-stressed peripheral blood mononuclear cells (PBMC) of Sahiwal and Karan Fries cows. Li *et al.* (2015) found that adding zinc supplementation to the diet of Bama miniature pigs impacted the growth of their reproductive system, immune response, and red blood cell production but did not enhance their ability to withstand heat. Vitamin C also promoted the fibroblast cell proliferation rate in heat-stressed buffalo fibroblast cells. Some existing studies found that vitamin C affects the synthesis of inflammatory cytokines, monocytes, lymphocytes, and cell adhesion factors in phagocytes (Sorice *et al.*, 2014). Kuo *et al.* (2012) reported that cells propagated with vitamin C have a higher cell proliferation rate in mouse embryonic fibroblast cells in normal conditions.

Real Time-PCR Data Analysis

The total RNA isolated from each fibroblast cell samples showed an A260/A280 ratio of 1.91-2.06, indicating

Table 3: Differentially expressed genes in dermal fibroblast cells of Murrah Cross buffalo with the presence and absence of antioxidants after 3 hours of heat stress at 42°C *in vitro* at different time points compared with controls.

Gene Name	Time points (Post heat stress)	Antioxidants	No. of replications	Fold change (mean ± SD)	P-value
HSPA1A	30 minutes	Vitamin C	3	14.16±1.85[b]	0.0001
		Zinc	3	8.15±1.36[a]	
		Without antioxidants	3	19.38±1.69[c]	
	2 hours	Vitamin C	3	7.96±3.05[ab]	0.011
		Zinc	3	3.72±1.36[a]	
		Without antioxidants	3	12.39±2.20[b]	
	4 hours	Vitamin C	3	4.71±1.42	0.112
		Zinc	3	2.62±0.67	
		Without antioxidants	3	4.15±0.88	
	6 hours	Vitamin C	3	0.73±0.34[a]	0.001
		Zinc	3	0.39±0.10[a]	
		Without antioxidants	3	3.16±0.78[b]	
HSPA2	30 minutes	Vitamin C	3	18.62±3.34[b]	0.0001
		Zinc	3	6.36±2.31[a]	
		Without antioxidants	3	37.60±5.42[c]	
	2 hours	Vitamin C	3	5.81±3.01	0.77
		Zinc	3	5.85±2.35	
		Without antioxidants	3	7.29±3.00	
	4 hours	Vitamin C	3	7.78±1.13[c]	0.0001
		Zinc	3	2.12±0.19[a]	
		Without antioxidants	3	4.99±0.73[b]	
	6 hours	Vitamin C	3	3.79±1.84	0.281
		Zinc	3	1.87±0.59	
		Without antioxidants	3	3.74±1.75	
HSPA8	30 minutes	Vitamin C	3	7.49±2.05[a]	0.023
		Zinc	3	5.69±1.19[a]	
		Without antioxidants	3	13.35±3.66[b]	
	2 hours	Vitamin C	3	2.49±0.34[a]	0.008
		Zinc	3	0.22±0.09[a]	
		Without antioxidants	3	4.94±2.01[b]	
	4 hours	Vitamin C	3	0.18±0.05[a]	0.0001
		Zinc	3	0.16±0.07[a]	
		Without antioxidants	3	4.24±0.91[b]	
	6 hours	Vitamin C	3	0.06±0.02[a]	0.002
		Zinc	3	0.05±0.01[a]	
		Without antioxidants	3	1.99±0.74[b]	
HSP90AA1	30 minutes	Vitamin C	3	13.03±1.65[b]	0.0001
		Zinc	3	2.49±0.60[a]	
		Without antioxidants	3	13.37±1.79[b]	
	2 hours	Vitamin C	3	4.76±1.85[ab]	0.021
		Zinc	3	2.30±0.67[a]	
		Without antioxidants	3	6.48±1.12[b]	
	4 hours	Vitamin C	3	4.26±1.09[b]	0.009
		Zinc	3	1.01±0.49[a]	
		Without antioxidants	3	5.29±1.56[b]	

6 hours	Vitamin C	3	0.29±0.11[a]	0.0001
	Zinc	3	0.28±0.07[a]	
	Without antioxidants	3	3.11±0.47[b]	

[1] In Table 3, all experimental data has shown represented by means ± S.D. at P < 0.05; [a,b,c] Means with distinct superscript letters denote significant differences in treatments at the same time point.

moderately high RNA quality in each sample. The cDNA A260/A280 ratio differed from 1.8 to 1.83. Every examined molecular chaperone had an intensive response within 30 minutes, and the majority of them continued to be upregulated until 6 hours post-heat stress periods (Table 3). The elevation in the expression of HSP at the mRNA level in buffalo fibroblast cells indicated their sensitivity to high temperatures in vitro. The upregulation of molecular chaperone activity in buffalo fibroblast cells demonstrated a thermoregulatory response to heat shock in the initial cellular stages. Without any antioxidant supplementation in the culture flask, the hierarchy of HSP expression ranked from highest to lowest was HSPA2>HSPA1A>HSP90AA1>HSPA8. For all HSP genes, the greatest relative expression of mRNA was observed after 30 minutes of the heat shock. Zinc and vitamin C-pretreated cells had significantly lower levels of HSP expression than cells without any antioxidant supplementation. At 30 minutes, the vitamin C and zinc treatment of thermally stressed cells significantly decreased (P<0.0001) the HSPA1A levels (14.16 ± 1.85 and 8.15 ± 1.36). A similar trend could be seen in the following hours. After six hours, HSPA1A expression in the heat-stressed dermal fibroblast cells without antioxidants remained higher (P<0.0001) than in antioxidant-treated cells. HSPA2 was identified as the prime thermoregulatory induction mechanism across post-heat stress. Vitamin C (18.62 ± 3.34) and zinc (6.36 ± 2.31) showed fewer (P<0.0001) effects on HSPA2 at 30 minutes (post-heat stress period) than other heat stress genes at the same time point. In the absence of antioxidants, the heat-stressed dermal fibroblast cells showed non-significant elevated HSPA2 expression levels (3.74 ± 1.75) even after six hours. Cells that were pretreated with vitamin C and zinc showed a notable decrease in the concentration of HSPA8 (7.49 ± 2.05, 5.69 ± 1.19 v/s 0.06 ±0.02, 0.05±0.01) at 30 minutes and at 6 hours (P<0.002) in heat-stressed fibroblast cells. The concentration of HSPA8 in vitamin C and zinc propagated cells at 2 hours (P<0.008) and 6 hours (P<0.0001) were (2.49 ± 0.34, 0.22 ± 0.09 v/s 0.18 ± 0.05, 0.16 ± 0.07). HSP90AA1 expression in heat-stressed dermal fibroblast cells significantly (P < 0.05) reduced during post-heat stress periods after antioxidant treatment. After six hours, vitamin C and zinc significantly (P<0.0001) reduced the HSP90AA1 concentration to 0.29 ±.11 and 0.28 ±0.074, respectively, in heat-stressed dermal fibroblast cells, whereas without antioxidants the HSP90AA1 concentration was 3.11 ±.47. Zinc displayed a notable reduction of HSPA1A, HSPA8, and HSP90AA1 mRNA expression in heat stress cells. HSPA1A, HSPA2, and HSPA8 are the representative members of the HSP70 family. The two major HSPs selected for this study are responsible for proper protein folding and apoptosis regulation under stressful conditions (Kapila *et al.*, 2016; Collier *et al.*, 2008). HSP 70 could be a genetic indicator for choosing animals with improved ability to withstand harsh climates, strong immune systems, and outstanding performance (Hassan *et al.*, 2019). Detecting heat stress in dairy cows through measuring HSP 70 expression in cow's milk is a promising method that is simple, non-invasive, and accurate (Rakib *et al.*, 2024).

There are several studies on bovine species where HSP70 and HSP90 expressions were observed during heat stress (Collier *et al.*, 2006; Hu *et al.*, 2016; Singh *et al.*, 2014). Singh *et al.* (2020) observed higher expression of HSPA1A, HSPA8, and HSPA2 in cattle at 44˚C for 3 hours. According to Kapila *et al.* (2013), HSP70 was the most commonly induced predominant chaperone in riverine buffalo MECs because of in vitro thermal stress. Singh *et al.* (2014) and Kapila *et al.* (2013) worked on dermal fibroblasts in cattle and riverine buffalo, who examined heat shock protein expression at different time intervals after heat stress in vitro like us. Banerjee *et al.* (2014) reported that HSPA1A, HSPA8, and HSPA6 showed high levels of expression in the goat blood leukocytes in the summer season. Heat stress leads to a rise in glucocorticoid secretion, which suppresses pro-inflammatory cytokines like TNF-α, IL-6, and IL-8, triggering innate immune responses by blocking the p38 MAPK pathway (Abraham *et al.*, 2006). Nutrients, metabolism, inflammation, and oxidative stress are interconnected, and these interactions are affected by heat stress (Sordillo and Mavangira, 2014). Feeding antioxidants can enhance cellular immune function, especially during heat stress, by preserving antioxidant levels in plasma and tissues, modulating pro-inflammatory cytokines and NF-κβ transcription factors, and reducing oxidative stress (Rooke *et al.*, 2004; Zhang *et al.*, 2020). Pearce *et al.* (2015) and Sheikh *et al.* (2017) found that zinc supplementation in heat-stressed pigs decreased the adverse effects. Our finding is quite similar to Sheikh *et al.* (2017), who found that zinc supplementation at the 0.01 mM concentration had positive effects on mitigating HSPs expression and immune system responses in heat-stressed bovine PBMC. Kuo *et al.* (2012) used vitamin C at a 20 µM concentration in mouse embryonic fibroblast cells to check

its effect on cellular senescence. Yin *et al.* (2018) observed that pretreatment H9C2 cells with 20 µg/ml of vitamin C reduced the production of Hsp70. Sahin *et al.* (2009) found that dietary vitamin C supplementation significantly reduced the expression of HSP70 and oxidative stress in quails. In the present study, we also found antioxidants' ability to reduce HSP protein expression in heat-stressed fibroblast cells of buffalo. In this study, we observed cellular and molecular changes in buffalo dermal fibroblast cells during post-heat stress periods from 30 minutes to 6 hours. If we could observe changes in a broader range of temperatures beyond 42°C and additional post-heat stress periods beyond six hours, we might get more information about heat stress effects on cellular and molecular levels. We used only vitamin C and zinc. If we could use more antioxidants, we might get to know about more potential antioxidants capable of mitigating the negative impacts of high temperature in animals.

CONCLUSIONS AND RECOMMENDATIONS

This study represents heat stress-induced changes in the gene expression of buffalo fibroblast cells. Cell viability had fallen overall for all culture cells due to in vitro heat stress. However, cultured cells propagated with zinc showed noteworthy cell viability and proliferation rates across post-heat stress periods. Heat stress proteins were expressed in buffalo dermal fibroblast cells during post-heat stress periods. Zinc and vitamin C were effective in alleviating heat stress effects on fibroblast cells. The reactivity of buffalo fibroblast cells to high temperature in this study suggested it can serve as a suitable *in vitro* model for investigating the way buffalo react to being exposed to high ambient temperatures. Further investigation is needed to elucidate more gene functions and regulation in heat-stressed cells and their relation to buffalo production and reproduction systems.

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NOVELTY STATEMENTS

This study represents the molecular activity of dermal fibroblast cells after exposure to heat stress. Some limited evidence has been found regarding the molecular responses of dermal fibroblast cells during heat stress. Antioxidants were effective in reducing HSPs expression. These results provide important insights for mitigating the heat stress effects of buffalo through nutritional management. Especially, adding antioxidants to their feed may be a good choice to lessen the negative effects of heat stress.

AUTHOR'S CONTRIBUTIONS

Bipasha Mazumder and Khadija-Tut-Tahira- Writing, original draft preparation, methodology, software, editing, Conceptualization and investigation, Both authors contributed equally as 1st author; Khondoker Moazzem Hossain- Review, supervision, Conceptualization; Gautam Kumar Deb- Review, supervision, conceptualization, resources and editing; Md. Ashadul Alam: Review, software, editing; S. M. Jahangir Hossain- Resources. Each author has reviewed and approved the final version of the manuscript for publication.

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Data Availability Statement

This article contains the data used to support the study's conclusions.

Conflict of Interest

No conflicts of interest are disclosed by the authors.

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