EFFECTS OF HEAT STRESS AND ANTIOXIDANTS ON DERMAL FIBROBLAST CELLS OF BUFFALO

A Thesis Submitted by

Bipasha Mazumder
Student ID: MS 190702
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Supervisor
Professor Dr. Khondoker Moazzem Hosssain
Biotechnology and Genetic Engineering Discipline
Khulna University, Khulna-9208
Bangladesh

Co-Supervisor
Dr. Gautam Kumar Deb
Principal Scientific Officer
Biotechnology Division
Bangladesh Livestock Research Institute
Savar, Dhaka-1341
Bangladesh

Professor Dr. Sheikh Julfikar Hossain
Chairman of the Examination Committee
Biotechnology and Genetic Engineering Discipline
Life Science School
Khulna University, Bangladesh

BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL
KHULNA UNIVERSITY, BANGLADESH

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DECLARATION

This thesis is the original research work of the undersigned author, except as otherwise appropriately cited. The materials presented have not been submitted either in whole or in part for a degree for this or any other university. The methods used were designed and implemented according to institutional and established international biosafety and bioethical guidelines. I also declare that the findings of the research have not been/will not be published anywhere or disseminated otherwise without the concurrence of the concerned supervisors.

Bipasha Mazumder

Bipasha Mazumder

Examination Roll No: MS 190702

DEDICATED

TO

MY BELOVED PARENTS

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ABSTRACT

High ambient temperature affects buffalo production and reproduction notably. Dermal fibroblasts are the most common cell element of the dermis that is crucial for temperature homeostasis. A limited number of data is available now on the heat stress response of fibroblast cells in buffaloes. The present research aimed to evaluate the mRNA expression pattern of various heat stress proteins (HSPs) in response to heat stress and to study the effects of zinc and vitamin C activities regarding thermal stress in Murrah buffalo. The primary fibroblast cells of Murrah buffalo were identified by FSP1 and FN_1 primers using the PCR method. The buffalo fibroblast cells were pretreated with zinc and vitamin C and exposed to heat stress conditions at 42^0C for three hours. To assess post-heat stress effects in buffalo fibroblast cells, cells were harvested at different time intervals. The heat stress resulted in an overall decrease in cell viability of culture, and the test was highly significant. Zinc propagated cells had higher cell viability than the cells pretreated with vitamin C. The expression studies of HSPs indicated that all HSPs (HSPA1A, HSPA2, HSPA8, and HSP90AA1) were induced after heat stress and remained upregulated during the post-heat stress periods. Among them, the HSPA2 gene depicted maximal induction across post-heat stress periods. Zinc treatment caused a fall in HSPA1A, HSPA8, and HSP90AA1 concentration in dermal fibroblast cells compared to vitamin C.

Keywords: Buffalo dermal fibroblast cells, Heat stress, Heat shock proteins, Antioxidants.

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LIST OF ABBREVIATION

IPCC	Intergovernmental Panel on Climate Change
HSPs	Heat stress proteins
ANS	Autonomic nervous system
HPA	Hypothalamic-pituitary-adrenal axis
DMI	Dry matter intake
ROS	Reactive oxygen species
SOD	Superoxide dismutase
GSH	Glutathione
GPX	glutathione peroxidase
CAT	Catalase
HS	Heat stress
HSF	Heat shock transcription factors
WBCs	White blood cells
RBCs	Red blood cells
Hb	Hemoglobin
PCV	packed cell volume
THI	Temperature-humidity index
T3	Triiodothyronine
T4	Tetraiodothyronine
Glg	Glucagon
DNA	Deoxyribonucleic Acid
RNA	Ribosomal RNA
DPBS	Dulbecco's phosphate buffer saline
RT-PCR	Real- Time Polymerase Chain Reaction
µl	Microliter
µM	Micrometre
Tm	Melting temperature
DMEM	Dulbecco's Modified Eagle's medium
FSH	Follicle-stimulating hormone

CHAPTER ONE
INTRODUCTION

1. Introduction

1.1 Background of the study

Presently sudden and extreme heat waves are burning concept throughout the world. Heat weaves led to the situation where animals unable to adapt to a sudden increase in temperatures, which causes heat stress. According to Intergovernmental Panel on Climate Change (IPCC), the earth temperature has been elevated 1.0°C over the past 150 years and also assumed that global warming is likely to reach 1.5°C- 2°C between 2030 and 2052 if it continues to increase at the current rate which is approximately 0.026°C per year (Masson-Delmotte et al., 2018). Heat stress becomes an economic burden on an animal production system in most tropical countries (Hansen, 2009). Bangladesh as a tropical country faces a similar crisis. The accepted level of air temperature for dairy animals 25.0–26.0 °C (West, 2003) or 24.0–27.0 °C (Broucek et al., 2009), above it is called the critical temperature. Due to heat waves, in the USA industry faced annual losses averaged $897 million, $369 million, $299 million, and $128 million for dairy, beef, swine, and poultry industries respectively (St-Pierre, 2003). During 2014 losses were estimated in the European present-day milk prices which were US$670 million/year, and this will probably rise to US$2.2 billion/year by the end of the century (Mauger et al., 2015). 97.1% of buffaloes are distributed predominantly in Asian countries, while only 2.9% are found in the rest of the world. Milk yield, growth rate, and fertility of buffaloes decrease due to high ambient temperature as they soak up the heat because of their dark skin and sparse coat or hair (Marai et al., 2010).

An intricate program of gene expression and biochemical adaptive responses were triggered by thermal stress. HSPs (Heat stress proteins) work as molecular chaperones in regaining cellular homeostasis and bettering cell survival rate (Collier et al., 2008). HSPs are extremely conserved proteins that are fundamentally expressed in cells and have housekeeping functions. Synthesis of inducible forms of HSP is enhanced during stress and operated by protecting the cell by refolding denatured, taking off damaged and degraded proteins, and also preventing protein aggregations (Malyshev, 2013). HSPs have an enormous role in the case of environmental stress tolerance and thermal adaptation (Sorensen et al., 2003). However, except HSPs several other biomolecules are involved in cellular response due to heat stress (Kregel, 2002). HSP70 (HSPA1A, HSPA2 and, HSPA8) is the most temperature-sensitive and persuaded by different physiological stressors, pathological stressors, and environmental stressors (Beckham et al., 2004). Throughout the protein

folding and translocation, HSPA1A and HSPA2 perform a key role in guiding the conformational status of the proteins (Arya et al., 2007). A series of drastic changes in biological functions such as lessened intake efficiency and utilization of feed, disturbances in the metabolism of water and protein, changes in enzymatic reactions, hormonal secretions, blood metabolites, energy and mineral balances are induced due to heat stress in buffalo ((Marai et al., 2010). So, farmers face a great crisis to maintain buffaloes' health and production. To alleviate the heat stress susceptibility of buffalo genetically is time-consuming. 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 option for the short-term management of the upcoming climatic challenges for farmers (Lamp et al., 2015). Antioxidants are free radical scavengers that protect the body's defense system against excessively produced free radicals and stabilize the health status of the animal. Antioxidant supplementation particularly, vitamins (C, A, and E), zinc, and chromium can be suitable for mitigating the unfavorable effects of environmental stress in bovine (Sheikh et al., 2016, Kumar et al., 2015, Kumar et al., 2011). Dietary supplements with antioxidants before the beginning of months of heat stress and also during the stress period may correct infertility due to heat stress and can decrease oxidative stress in buffaloes (Megahed et al., 2008).

The skin is the largest organ of the body that leads to protect the internal body structures from a hostile external environment. Although our major segment of skin is composed of dermal fibroblast cells, the information on fibroblasts and their repercussion to heat stress, especially on the molecular behavior of dermal fibroblast in livestock, are insufficient. A better understanding of the physiological role of skin will help in modulating heat tolerance and improve functions under different heat stress conditions.

1.2 Objectives

The present study will provide information about the thermotolerance ability of dermal fibroblast cells and the role of antioxidants, especially zinc and vitamin C, during heat stress in buffalos. Therefore, the present study was undertaken with the following objectives:

- To examine heat shock proteins (HSPs) gene expression in dermal fibroblast of buffalo.
- To investigate the thermotolerance ability of buffalo through the analysis of heat shock proteins (HSPs).

- To study the effects of zinc and vitamin C on heat stress fibroblast cells of buffalo.

CHAPTER TWO
REVIEW OF LITERATURE

2. Review of literature

2.1 Heat stress

Heat stress is a kind of hyperthermia (upraised body temperature). As a consequence, the physiological systems of the body give out to regulate the body temperature within a normal range. Initially, symptoms like increased panting, hypersalivation, gastrointestinal problems (vomiting, diarrhea) might be noticeable. Behavioral changes like seeking shade, crowding towards shady areas, orientation avoiding contact with solar radiation, standing in or next to a water source may observe in animals having the onset of heatstroke (Nardone et al. 2010). According to Robinson, 2013 and Scharf et al. 2010, heat stress in mammals is characterized by increasing respiration rate, rectal temperature, and sweating due to changes from sensible heat dissipation to evaporative cooling in an attempt to thermoregulate, and by the loss of the equilibrium between metabolic heat production and heat dissipation to the environment.

2.2 Physiologic consequences of heat stress on buffaloes

2.2.1 Stress response

The degree of heat stress stimulation dictates the intensity of the stress response as well as the consequences they bring to the organism. In that sense, physiological and behavioral disturbances are only mechanisms of adaptation of animals to a threat to the animals' homeostasis (Adamczyk et al. 2015). Stress responses to high ambient temperature and humidity involve mainly autonomic responses via activation of the autonomic nervous system (ANS) mediated by catecholamines (adrenaline and noradrenaline) including increased respiration and heart rate, elevated body temperature, redistribution of blood flow from the viscera to the skin for thermoregulation, and promotion of energy utilization from body reserves, accelerating muscle glycogenolysis and suppressing energy storage (Afsal et al. 2018, Gregory, 2010). According to Kadim et al. (2006), acute and chronic heat stress also increases glucocorticoids plasma concentrations levels via activation of the hypothalamic-pituitary-adrenal (HPA) axis. Acute thermal stress prompts an elevation in glucocorticoids more than chronic thermal stress. The noteworthy signs of thermal stress in buffalo include reduced feed intake, escalated reddening of hiding, protruded tongue, panting, bloodshot eyes, and increased rectal temperature (Marai et al. 2010).

2.2.2 Feed intake and rumen physiology

Feed intake is a natural, protective, and adaptive mechanism in animals that get hampered under high ambient temperatures due to a rise in metabolic heat production. However, the reduction in feed intake explains only 50 % of the physiological and metabolic effects of heat stress in animals, while metabolic and hormonal changes and variations in energy partitioning explain the rest (Odongo et al. 2006). Feed intake initiates to decline at air temperatures of 25-26°C in lactating cows and reduces more rapidly above 30°C in temperate climatic conditions, and at 40°C it may decline by as much as 40%, 22-35% in dairy goats or 8-10% in buffalo heifers (Rhoads et al., 2013, Hamzaoui et al. 2012, Hooda et al. 2010). According to Nardone et al. (2010) and Soriani et al. (2013), the basic physiological mechanisms of rumen alter due to high temperature, which negatively affects the ruminants with increased risk of metabolic disorders and health problems. As a response, animals consumed fewer roughages which changes the rumen microbial population, pH from 5.82 to 6.03, and rumen motility and rumination. Subsequently, other effects like low saliva production, variation in digestion patterns, and dry matter intake (DMI) decrease occur. Moreover, heat stress also results in hypofunction of the thyroid gland and effects on metabolism patterns of the animal to reduce metabolic heat production.

2.2.3 Increase in water requirements

Water requirements of heat-stressed animals rise twofold during heat stress compared with thermoneutral conditions. To counteract water evaporation via panting and sweating and to reduce body temperature via direct cooling of the reticulum-rumen water intake increase is pivotal for thermoregulation (Bewley et al. 2008, Marai et al. 2007). The failure to provide enough water to heat-stressed animals will lead to dehydration and enhancement of adrenergic stress responses. The rectal temperature of buffaloes fluctuates more than tropical cattle with changes in ambient temperature. The water secretion rate through urine in buffaloes is higher than in cattle, and buffaloes have a greater dependence on external water because of their evolutionary adaptation in a wet environment (Koga et al. 2002). For this reason, they need more water in heat stress conditions than in cattle.

2.3 Metabolic consequences of heat stress on buffaloes

2.3.1 Acid base balance

Respiration rate and sweating increase due to heat stress, which results in increased body fluid loss that needs to control dehydration and blood homeostasis. Since respiration rate increases, the expiration of CO2 via the lungs increases, which results in respiratory alkalosis, as blood carbonic acid concentration decreases. Therefore, the animal needs to recompense for higher blood pH by excreting bicarbonate in the urine to maintain the carbonic acid: bicarbonate ratio (Schneider et al. 1984). Chronic hyperthermia arises to critical or lengthen appetite which provokes the rised up the supply of total carbonic acid in the rumen, and lessens ruminal pH (Kadzere et al. 2002)

2.3.2 Oxidative stress

Oxidative stress causes by the increase in reactive oxygen species (ROS) in different cells and tissues of heat stress animals that have negative effects on the body's normal physiology and metabolism. Oxidative stress causes by the increase in reactive oxygen species (ROS) in different cells and tissues of heat stress animals that have negative effects on the body's normal physiology and metabolism. Heat stress increases the production of reactive oxygen species (ROS) and may decrease the natural antioxidant capabilities, both of which can induce oxidative stress (Shakeri et al. 2018, Chauhan et al. 2014, Liu et al., 2016, Mujahid et al. 2007). However, the body has antioxidants in the form of enzymatic (superoxide dismutase [SOD], glutathione (GSH) peroxidase and catalase), non-enzymatic (albumin, L-cysteine, homocysteine, melatonin, and protein sulfhydryl groups), and non-enzymatic low molecular weight antioxidants (ascorbic acid, GSH, uric acid, α-tocopherol, β-carotene, pyruvate, and retinol), which increases as a result of heat stress to protect cells from excess exposure of ROS. Significantly, higher levels of catalase, SOD, GSH reductase, and malondialdehyde were observed in lactating and non-lactating buffaloes and cattle during summer compared to spring seasons. The increased antioxidant enzymes activities in response to the increased ROS levels aim to maintain the steady-state concentrations of generated free radicals (Chaudhary et al. 2015, Lallawmkimi et al. 2013, Yatoo et al. 2014). Heat stress effects on antioxidant enzymes activities, namely superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), are summarized in Table 2.1.

Table 2.1: Variation of the activity of antioxidant enzymes during the summer season in bovine

Antioxidant enzymes	Animals	Activity during summer	References
Superoxide dismutase(SOD)	Heifers	+++	Chandra and Agarwal, (2009)
	Dairy cows	+++	Lallawmkimi, (2009), Bernabucci et al., (2002)
	Heifer and lactating Murrah buffaloes	+++	Chaudhary et al., (2015), Yatoo et al., (2014), Lallawmkimi et al., (2013)
Catalase(CAT)	Heifer and lactating Murrah buffaloes	+++	Yatoo et al., (2014), Lallawmkimi et al., (2013)
	Cattle	+++	Lallawmkimi, (2009), Bernabucci et al., (2002)
Glutathione peroxidase (GPX)	Cattle	+++	Chaudhary et al., (2015), Lallawmkimi, (2009)

(Source: Belhadj et al., 2016)

2.3.3 Heat stress protein expression

Cells generate heat shock proteins (HSPs) upon encountering physiological stress that are major proteins induced during cellular responses to various stresses such as heat shock and oxidative stress. HSPs are responsible for protein folding, assembly, translocation, and degradation during ordinary cellular growth and development (Mayer et al. 2006). Cellular exposure to heat stress (HS) creates a number of anomalies in the functioning of cells which alters the biological molecules, disturbs cell functions, modulates metabolic reactions, induces oxidative cell damage, and activates both apoptosis and necrosis pathways, ultimately leading to cell survival, acclimation, or cell death depending on the time and success of these alterations (Belhadj et al. 2016, Pandey et al. 2012, Du et al. 2008). During summer, oxidative stress observed in livestock

animals is attributed to HS. The ultimate response of thermo-tolerant gene expression and elevated HSP levels through which the cell sustains the impact of HS makes it a full-proof biomarker for the condition (Collier et al. 2008). The cellular response to thermal stress in mammalian organisms is controlled at the transcription level and is mediated by a family of heat shock transcription factors (HSF) which are regulated by the inducible expression of HSF genes. The HSF1 to HSF4 has been reported to have a direct correlation with thermo-tolerance in livestock. The HSF1 is mainly correlated with induction of HSP70 gene expression (Dikmen et al., 2008).

Table 2.2: Different heat stress proteins studied in farm animals

Species	Type of HSP	Organ/Blood	References
Murrah buffalo	HSP 70	Serum lymphocytes, PBMC	Mishra et al., 2011
Tarai buffalo	HSP 70	Serum lymphocytes	Manjari et al., 2015
Tharparkar Cattle	HSP 70	Intesine, kidney and embryo	Bhat et al., 2016
Beef Cattle	HSP 27	Skeletal Muscle	Shibata et al., 2014
Sahiwal heifer	HSPA1A	Testes	Mehla et al., 2014
Nellore and Jersey	HSP70	Embryo	Silva et al., 2012
Osamanabadi goats	HSP70	Plasma and PBMC	Shilja et al., 2016
Chicken	HSP70	Brain	Tamzil et al., 2013
Broiler	HSP25, HSP90AA1, HSPA2	Plasma	Gade et al., 2010

Based on molecular weight, HSP proteins have been classified into two major families; High molecular weight heat shock proteins such as HSP120, HSP90, HSP70, HSP60, HSP40, and small molecular weight heat shock proteins like HSP27, HSP26, HSP16. In farm animals, HSP70 and

HSP90 elevation was observed in sheep, buffalo, cattle, broilers, and goats (Archana et al., 2017, Belhadj et al. 2016, Shilja et al. 2016). The molecular characterization of HSP70-1 gene in goats revealed that at the nucleotide level, there was 96–99% similarity with that of sheep, cattle, and buffalo whereas 95-100% similarity at the amino acid level (Gade et al. 2010). The amino acid sequence analysis of HSP70 in buffalo lymphocytes showed 98% identity with *Bos taurus*, *Bos indicus*, Yak, *Capra hircus* and 90–95% identity with *Camelus dromedaries*, *Sus scrofa*, and *Homo sapiens* and it reported 1,926bp long open reading frame of HSP70 gene encoding 641 a8mino acids in buffalo (Pawar et al. 2014). Mishra et al. (2011) found that the concentration of 8HSP70 in the serum lymphocytes of Murrah buffalo was high following dry heat exposure compare8d with the controlled condition. Significantly higher expression values of HSP 70 were seen in Tarai buffalo during the summer season (2.37±0.12) compared to winter (0.29±0.04) (Manjari et al. 2015). Increased HSP70 and HSP90 levels during heat stress are probably due to the increased number of damaged proteins, as HSPs work for the cellular defense. Heat stress was shown to increase both HSP-70 and HSP-90 in buffalo (Kishore et al. 2014, Kapila et al. 2013).HSPs play regulatory roles in various types of immunity. Mishra et al. (2011) revealed that the augmentation of HSP-derived peptides in buffalo lymphocytes increased the innate and adaptive immune effectors.

2.3.4 Immune system

The immune system is the vital body defense system to protect and fight against environmental stressors. Heat stress causes functional and metabolic alterations in cells and tissues including cells of the immune system. Primary indicators of immunity response include white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), glucose, and protein concentration in the blood get altered on thermal stress. WBC (leukocytes) count increased by 21-26% and RBC count decreased by 12-20% in thermally stressed bovine species which could be due to thyromolymphatic involution or destruction of erythrocytes (Habeeb et al. 1987). The blood glucose significantly decreased in heat stress farm animals following greater blood insulin activity. The release of plasma cortisol rises in stressed animals, which causes down-regulation or suppression of L-selectin expression on the neutrophils surface. Further, this poor L-selectin expression is accountable for weak neutrophils function by failing to move into the tissue being invaded by pathogens and resulting in the clinical outcome of disease following exposure to an

infective organism. Cellular levels of heat shock proteins (HSPs) are also increased by increased circulating cortisol that function as a dangerous signal to the immune system to encourage the increased killing of pathogenic bacteria by neutrophils and macrophages against invading bacteria (Do et al. 2011, Rhoads et al. 2009, Srikandakumar et al. 2004). Thornton et al. (2009) reported that climate change may create substantial shifts in disease distribution and outbreaks of disease prevalence in previously unexposed animal populations possibly with the breakdown of endemic stability and changes in rainfall and temperature regimes may also affect both the distribution and the abundance of disease-causing vectors. Jingar et al. (2014) observed that the increase in the temperature-humidity index (THI) resulted in an increased incidence of mastitis in cows ($p<0.01$), while Murrah buffaloes were less affected.

2.3.5 Hormones

Heat stress causes changes in some hormones levels in buffalo. The thyroid gland is closely related to thermogenesis in mammals and heat stress normally induces reductions in triiodothyronine (T3) and tetraiodothyronine (T4) plasma concentration. This response is considered an adaptive mechanism to avoid extra heat load, by reducing metabolic heat production, the maintenance energy requirements and increasing fat deposition by reducing lipolysis (Collin et al. 2005). Decrease in thyroid hormones occurs during heat acclimation and those mammals acclimated to warmer climate adopt this pattern. Chaudhary et al. (2015) observed that Surti buffaloes exposed to summer solar radiation showed a significant decrease in plasma concentrations of Thyroxine (T4). The thyroid hormones, T4 and T3, provide a major mechanism for acclimation, and hence they have known indicators of heat stress in buffalo. This can be explained by the findings of Silanikove, (2000) who has reported that thyroid hormones take a long time to achieve new steady levels in response to heat stress. Meghad et al. (2008) reported that oestradiol, progesterone levels also decrease due to heat stress in Egyptian buffaloes. Acute exposure to elevated ambient temperature increased the secretion of cortisol in buffaloes (Ahmed et al. 2010). High temperature lowered the level of Triiodothyronine (T3), thyroxine (T4), insulin (Ins) and 8glucagon (Glg) in Egyptian buffalo-calves (Omran et al. 2011).

Table 2.3: The correlation coefficient between hormones and antioxidant and oxidative stress during summer and winter season

Seasons	Oestradiol/ superoxide dismutase	Oestradiol/ lipid peroxides	Oestradiol/ nitric oxide	Cortisol/ superoxide dismutase	Cortisol/ lipid peroxides	Cortisol/ nitric oxide
Summer	+0.12**	-0.03**	-0.23*	-0.06**	+0.03**	+0.058**
Winter	+0.56*	0.27 ns	-0.01*	-0.17 ns	+0.25 ns	+0.07 ns

Here, ns= non-significant

*Significant at $p < 0.05$.

**Significant at $p < 0.01$.

(Source: Megahed et al., 2008)

Table 2.4: Variation of hormones levels in buffaloes under heat stress and comfort state (Mean ±SE).8

Hormones	Heat stress (HS) 40°C	Comfort state (CS) 25°C
Triiodothyronine (ng/dl)	114.73 ±5.02	233.641±5.77
Thyroxine (µg/dl)	2.59±0.20	6.03±0.16
Insulin (µlU/ml)	19.37±1.17	48.30±2.25
Glycogen (pg/ml)	86.44±3.06	56.82±2.17

*Significant (P<0.05)

(Source: Omran et al., 2011)

2.4 Heat stress effects on production and reproduction performance of buffalo

2.4.1 Milk production and composition

Due to increased global temperatures, concerns regarding heat stress and thermoregulation in dairy animals, including buffaloes, have been extended to tropical countries like Bangladesh. It is well documented that stress induced by hot environments lowers productive efficiency in dairy animals (Das et al. 2016, Bernabucci et al. 2002) The effects of environmental temperature and humidity on milk-related performance, fertility, and welfare have been widely studied in dairy cattle (Nasar et al. 20817, Polsky et al. 2017), while little information we get about buffalo milk quality and production. The distribution of sweat glands and the dark skin color of buffaloes can negatively affect heat tolerance. The high temperature increases body heat leading to low heat dissipation from the body surface, which leads to stress.

Upadhyay et al. (2007) reported that a sudden change (rise or fall) in Maximum/Minimum temperature during summer and winter was observed to affect milk production in Murrah buffaloes. The decline in minimum temperature (>3°C) during winter and increase (>4°C) during summer than normal was observed to negatively impact milk production up to 30% on the next or subsequent days after the extreme event. Up to 50% drop in milk production in dairy animals is due to reduced feed intake and rest due to metabolic adaptations to heat stress as heat stress response markedly changes post-absorptive nutrient metabolism (Baumgard et al. 2013). Heat stress significantly reduces the production of milk, the percentage of milk fat, and the percentage of proteins, but it does not affect the content of lactose in milk. This stress reduced butterfat by 20–40%, non-fat solids by 10–20%, a8nd total milk protein by 10–20% (Zheng et al. 2009, Singh et al. 2005). Upadhyay et al. (2009) reported that the annual total milk loss due to heat stress in India was 1.8 million tonnes, and the negative impact of global warming on total milk production in India is also estimated to be about 3.28 million tons by 2020 and more than 15 million tons by 2050. A reduction in casein percentage and casein index (casein/total proteins ratio) can be seen in summer (2.18% vs. 2.58% and 72.4% vs. 77.7%, respectively) compared to the spring season (Bernabucci et al. 2002).

2.4.2 Effects on reproductive performance

2.4.2.1 Estrous period and follicular growth

Thermal stress reduces the length and intensity of estrus besides increasing the incidence of anestrous and silent heat in buffaloes. It rises ACTH and cortisol synthesis and prevents estradiol-controled sexual behavior (Singh et al. 2013). Developed follicles bear damage and become non-viable when the body temperature cross 40°C (Roth et al. 2000). A temperature rise of more than 2°C in buffaloes may create adverse impacts due to poor or desynchronized endocrine activities particularly, pineal-hypothalamic-hypophyseal-gonadal axis altering specific hormone functions (Upadhyay et al. 2009). They also reported that low estradiol levels on the day of estrus during the summer period may be the likely factor for the poor expression of heat in Indian buffaloes. Silent estrus is the most crucial limiting factor, especially during hot months, which leads to poor reproductive efficiency in buffaloes. There is a decrease in the concentration of oestradiol-17 beta in summer which reduces the intensity of estrus manifestation and results in silent heat in buffaloes (Singh et al. 2013). Roy and Prakash, (2007) reported that the mean plasma prolactin concentration was significantly higher in summer than in winter which may cause acyclicity or infertility in buffaloes.

2.4.2.2 Fertility rate

Seasonal reproductive behavior incidence is more common in buffaloes. Shortening day length and temperature sexually activated buffaloes. During the winter and the lowest in the summer season the highest breeding frequency can be seen in buffaloes. 80% of estrus of dairy animals may be unnoticeable during summer, which reduces fertility (Rutledge et al. 2001). Heat stress results in to increase secretion of endometrial PGF-2α, thereby threatening pregnancy maintenance leads to infertility (Bilby et al. 2008). Follicle-stimulating hormone (FSH) secretion is elevated under heat stress conditions, probably due to reduced inhibition of negative feedback from smaller follicles which ultimately affect the reproductive efficiency of dairy animals (Roth et al., 2000). The temperature-humidity indexes (THIs) play a crucial role in the reproductive functions of buffaloes, and it is suggested that THI >75 has a bad effect on the reproductive performances of buffaloes in the tropical areas of the Amazon basin in Brazil (Vale, 2007). Similarly, Dash et al. (2015) observed a distinct relationship between THI and pregnancy rate in Murrah buffaloes. The

average pregnancy rate of Murrah buffaloes was found to decline from 0.41 to 0.25 with the onset of THI ≥75. When the monthly average pregnancy rate of Murrah buffaloes was analyzed with monthly average THI values, then the lowest average pregnancy rate was observed as 0.25 in the month of July with a corresponding THI of 80.88, and the highest average pregnancy rate was obtained as 0.58 in the month of November at average THI value 66.09.

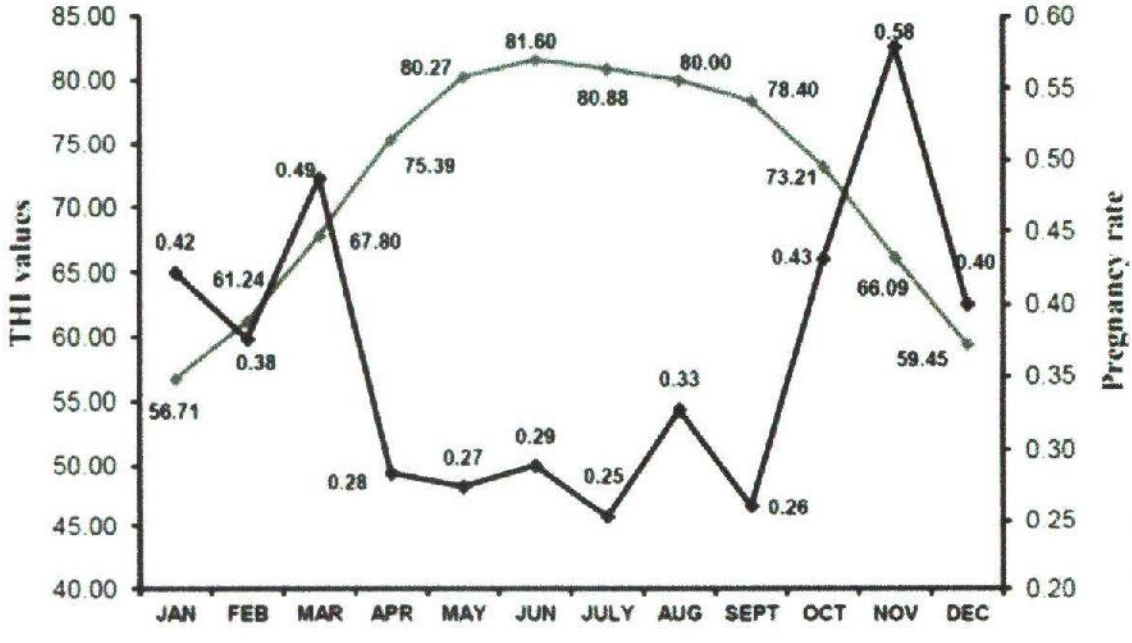


Figure 2.1: Month wise average pregnancy rate and temperature humidity index (THI) values during 1993 through 2012 (Dash et al., 2015).

The threshold THI for pregnancy rate in buffaloes was determined as 75 above which the detrimental effects of heat stress are affecting fertility are observed in buffaloes (Dash et al. 2016). Zoheir et al. (2007) investigated the effects of season on the quality of oocytes of Egyptian buffalo and reported that in spring and winter, the percentage of good quality oocytes was (71 and 74.6%), denuded oocytes were (12.8 and 9.1%) and the fair type of oocytes was (16.3 and 16.3%) respectively. Consequently, the maturation rate was 85.5 and 92.5% respectively. While in summer and autumn, the percentage of good quality oocytes was (50 and 56.9%) instead of denuded oocytes was (25.5 and 18.5%), and the fair type of oocytes was (24.4 and 21.5%) respectively. Consequently, the maturation rate was 59.6 and 74.5% respectively. Heat stress increases scrotal surface temperature and reduces semen quality in buffalo bulls. Ahirwar et al. (2018) reported that

the scrotal temperature gradient was low when Temperature Humidity Index (THI) was high. Sperm abnormalities were highest when the THI reached 81.66. Fewer sperm abnormalities were observed when THI was lower or medium.

Table 2.5: Classification of zones based on THI values in buffalo

Temperature humidity index (THI)	Stress level	Symptoms in buffalo
<72	None	Optimum productive and reproductive performance
72-78	Mild	Elevation in rectal temperature and respiration Rate
79-88	Moderate	Respiration rate is significantly increased. Dry matter intake of buffalo is decreased and ratio of forage to concentrate intake is decreased. Water intake in buffalo is significantly increased
89-98	Severe	Excessive panting and restlessness are observed. Rumination and urination are lowered along with a negative impact on reproductive performances in buffaloes
>98	Danger	Heat stress is extreme and buffaloes may die

(Source: Dash et al., 2016)

2.4.3 Meat quality

The most frequent problems caused by heat stress in dairy animals are weight loss, carcass injuries, and alteration of meat quality, particularly due to an increase in pH (>5.8), affecting meat tenderness and color (dark meat) (Lomiwes et al. 2014).

Table 2.6: Values of blood parameters indicative of stress in dairy animals by pH group

Parameter	pH<5.8	pH≥5.8
PCV (Packed cell volume)	36.71±0.73[a]	35.28±0.23[a]
NLR(Neutrophil/lymphocyte ratio)	0.76±0.06[a]	0.80±0.02[a]
Glucose (mmol/L)	6.82±0.34[a]	5.64±0.10[b]
Cortisol (ng/mL)	75.33±6.06[a]	63.89±1.93[a]

([a,b] Least squares means within a row with different letters differ ($p<0.05$) between pH values.)

(Source: Carrasco-Garcia et al., 2020)

Meat pH is a consequence of the amount of glycogen present in the muscles before slaughter, which depends greatly on the factors responsible for physical and psychological stress (Amtmann et al. 2006).

2.5 Fibroblast cells as a cellular model for heat stress

Fibroblasts are the most common connective cells in animals that synthesizes the extracellular matrix and collagen. Fibroblasts from different anatomical sites in the body express many genes that code for immune mediators and proteins (Krausgruber et al. 2020). Skin is the first defense mechanism of the immune system that allows itself to respond to and resist the hostile environment of temperature, humidity, and radiation. Dermal fibroblasts are one of the main constituents of skin, contributing to homeostasis of the skin, and also involved in various physio-pathological conditions. Fibroblast cells are spindle-shaped cells and are actively involved in interactions with other cell types by cell-matrix or direct cell interactions (Sriram et al. 2015). Several anatomical and physiological factors control the thermoregulation ability of animals. Properties of skin, length of hairs, the relationship between surface area per unit of body weight, and metabolic heat production are some of the well-known factors that influence heat stress response (Singh et al. 2013). For this reasons, dermal fibroblast could be employed as a cellular model to observe the impact of heat stress. Further, it has been reported that differences in functional and morphological characteristics of skin could help explain differences in heat tolerance among species and breeds (Shandilya et al. 2020, Singh et al. 2014). Although few studies reported the response of fibroblast to heat stress, still the information on the cellular and transcriptional level in livestock especially dairy animals is meager (Shandilya et al. 2020).

2.6 Antioxidants effects on heat stress dairy animals

Antioxidants have attracted widespread interest in nutrition research, biology and medicine. Antioxidants prevent or inhibit the oxidation of the substrate (lipids, protein, or DNA) by donating electrons. Antioxidants can be divided into two types like enzymatic and non-enzymatic. Enzymatic antioxidants consists of superoxide dismutase, catalase, glutathione peroxidase, and glutathione oxidase, whereas the non-enzymatic group consists of vitamins A, C, and E, beta-carotene, and some trace elements such as copper, manganese, selenium, and zinc, which are cofactors of antioxidant enzymes (Agarwal et al. 2012, Pisoschi and Pop, 2015). Heat stress

accelerates plasma reactive oxygen species levels. Reactive oxygen species (ROS) are naturally present in the organism, but they are mainly confined to cell compartments and counterbalanced by natural antioxidant molecules such as glutathione, glutathione peroxidase, superoxide dismutase (SOD), vitamin E, and vitamin C, acting as free radical scavengers (Aruoma et al. 1994). Du Preez et al. (1999) found that heat stress is one of the most important factors that decrease the conception rate in animals living in hot and humid areas, and treating animals with antioxidants will elevate the pregnancy rate in the heat-stressed animals.

Kumar et al. (2010) reported that supplementation of vitamin C lowered glutathione peroxidase activity in buffaloes exposed to heat stress in a climatic chamber. The lower levels of glutathione peroxidase activity in buffaloes may be the response to minimizing cell damage caused due to oxidative stress during summer in buffaloes supplemented with vitamin C. Heat stress accelerates plasma reactive. Oxidative stress arises during various periods in animal systems, and antioxidants can help to improve reproductive performance (Nayyar and Jindal, 2010). Megahed et al. (2008) reported that the treatment of buffalo and cow with antioxidants containing vitamin E and sodium selenite reduced the free radicals production due to heat stress and reduced cortisol production and consequently increases the fertility of these animals. Oxidative stress with its oxidant/antioxidants imbalance and elevation of cortisol can be corrected or decreased by the administration of antioxidants in dairy animals (Megahed et al. 2003). Dietary supplements such as vitamins, trace elements and minerals are exclusively utilized to alleviate the unfavorable effects of thermal stress (Tawfeek et al. 2014). Zinc has a crucial role in cell-mediated immunity, and it also acts as antioxidant and anti-inflammatory agent. Various supplements and additives, zinc being one amongst them are used in Murrah buffaloes during the periparturient period to maintain their productive and reproductive performance (Deka et al. 2014). Zinc treatment to heat-stressed lymphocytes of dairy animals can alleviate the negative effects of heat stress and improve the immune status (Sheikh et al. 2016). Khorsandi et al. (2016) found that supplementing heat-stressed transition cows with ruminal bolus leads to a higher milk yield which suggests that it mitigates the adverse effects of thermal stress. Nutritional supplementation includes dietary chromium picolinate, betaine, and antioxidant supplementation can alleviate some of the negative impacts of thermal stress on farm animals (Dunshea et al. 2013).

CHAPTER THREE
MATERIALS AND METHODS

3. Materials and Methods

3.1 Collection of sample

The research had been conducted with ear pinna of Murrah buffalo, which was collected from a slaughter house of Mohammadpur, Camp Bazar, Dhaka, under aseptic condition and promptly transported into a flask containing saline solution. The buffalo was apparently healthy, age 2.5 years, and body weight was around 450 kg. The sample was then kept in the laboratory at 4°C before skin biopsy. Norms regarding the ethical treatment of animals during the whole operation were strictly followed.

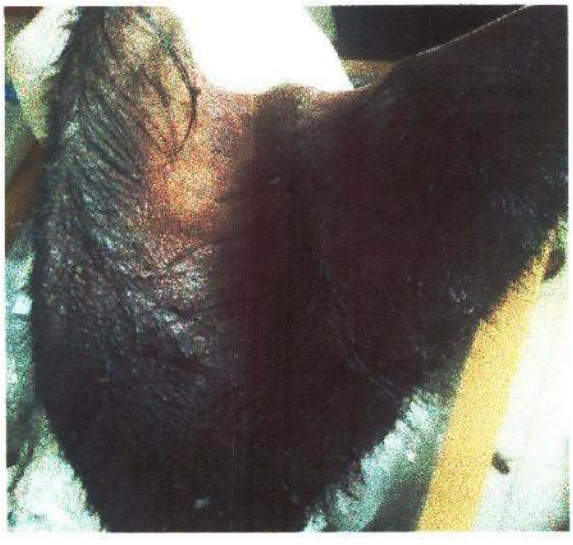


Figure 3.1: Ear pinna of Murrah buffalo.

3.2 Experimental site

The experiment was performed in the molecular lab of the biotechnology division of Bangladesh Livestock Research Institute, Savar, Dhaka.

3.3 Preparation of specimens

The samples were rinsed and cut into square shapes for proper chopping in the presence of Dulbecco's phosphate buffer saline (DPBS). The hair and extra fat that was attached to the desired sample were cleaned meticulously. The tissue was minced into small pieces (about 1 mm in size)

using a sterile surgical blade. Again, tissues were washed 3 times with DPBS and some pieces were used for DNA extraction besides transferring into cell culture flask.

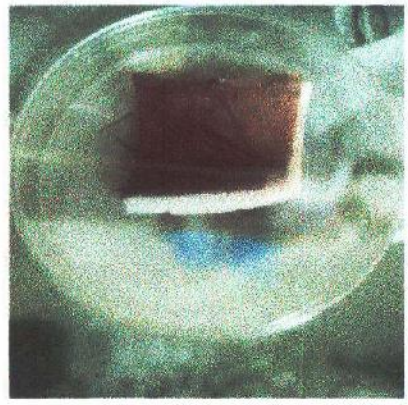
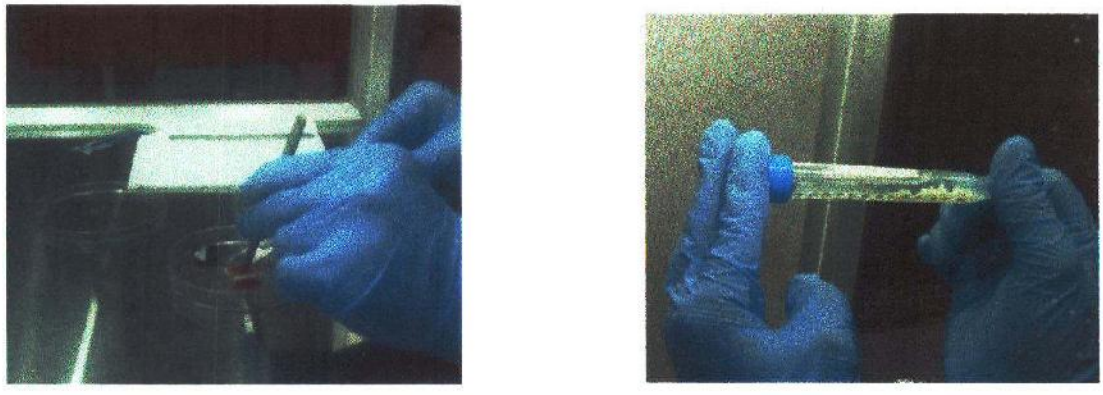


Figure 3.2: Sample from ear pinna of buffalo for performing skin biopsy.



A B

Figure 3.3: Skin biopsy procedure of sample. (A) Removal of extra fats and hair from the sample. (B) Chopped pieces of skin tissues with DPBS in a centrifuge tube.

3.4 DNA extraction from tissue of ear pinna

Total DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA). For maximal DNA recovery, tissues were mechanically homogenized using mortar and pestle to make powder. Then, 600µl of chilled Nuclei Lysis Solution was added to the pellet and homogenized for 10 seconds. 3µl of RNase Solution was added to the animal tissue nuclei lysate and mixed properly. Next, the mixture was incubated for 20 minutes at 37°C and cooled to room temperature. 200µl of Protein Precipitation Solution was added and was mixed by. vortexing and chilled on ice

for 5 minutes. After that, the tube was centrifuged at 16,000 × g* for 4 minutes. The supernatant was transferred to a fresh tube containing 600µl of room temperature isopropanol. It was mixed gently by inversion and centrifuged at 16,000 × g* for 1 minute. The supernatant was removed, and 600µl of room temperature 70% ethanol was added. The tube was centrifuged at 16,000 × g* for 4 minutes. The supernatant was discarded. Ethanol was aspirated, and the pellet was air dried for 15 minutes. The DNA was rehydrated in 100µl of DNA Rehydration Solution overnight at 4°C.

3.5 DNA quantification

DNA concentration and purity was determined by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared by taking 2 µl nuclease free water. At first, the blank was placed in the measurement pedestal using pipette and was adjusted at value 0. Then, 2 µl sample was pipetted onto the measurement pedestal. After measuring the amount, DNA was promptly kept at -20^{0}C.

Figure 3.4: Quantification of DNA by using Nanodrop Spectrophotometer.

3.6 Primary culture of buffalo fibroblast cell

After that, chopping pieces were put into a centrifuge tube containing 10 ml DPBS solution and centrifuged at 37^{0}C, 2000 RPM for 5 minutes. The supernatant was removed and again 10 ml DPBS was added to the Eppendorf tubes. These steps were repeated three times, and then 10 ml Trypsin solution was added to the centrifuge tubes containing desired samples.

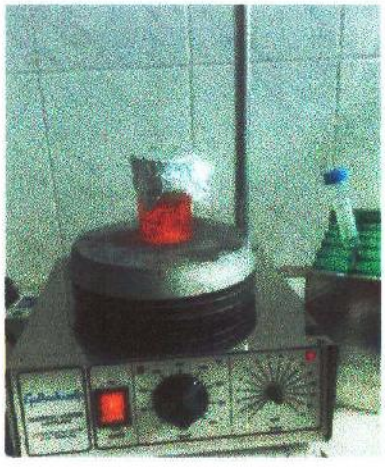


Figure 3.3: Cell culture media ($_p$H 7.2) mix thoroughly by using vortex machine.

Next, the tubes were incubated for 1hour. After that, the tubes were placed in a centrifuge machine for centrifugation at 37^{0}C for 5 minutes, 2000RPM. The supernatant was discarded and washed properly using 4 ml Dulbecco's Modified Eagle's medium (DMEM). Finally, the cells were seeded in a tissue culture flask containing DMEM media supplemented with 1% penicillin-streptomycin solution, 10% fetal bovine serum and incubated at 37^{0}C with 5% CO2. Tissue explants were regularly observed for the proliferation of fibroblasts and were removed aseptically when a sufficient number of cells had proliferated and formed a monolayer on the cell culture dishes.

Figure 3.4: Chopping skin tissues of buffalo ear pinna with media seeded in tissue culture flask.

Cells were harvested when they reached 70-80% confluence. After reaching 70-80% confluence, the fibroblast cells were sub-cultured by partial trypsinization. The cells were subjected to 3-4

continuous passages for selection of homogeneous population of dermal fibroblast. The dermal fibroblasts were routinely evaluated for checking cell viability.

3.7 DNA extraction from fibroblast culture cells

Total DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA). Cultured cells were detached from the culture flask using 0.0.2 % trypsinization and centrifuged at 16,000 × g* for 4 minutes. The supernatant was discarded. Then, 600µl of chilled Nuclei Lysis Solution was added to the pellet and homogenized for 10 seconds. 3µl of RNase Solution was added to the animal tissue nuclei lysate and mixed properly. Next, the mixture was incubated for 20 minutes at 37°C and cooled to room temperature. 200µl of Protein Precipitation Solution was added and was mixed by. vortexing and chilled on ice for 5 minutes. After that, the tube was centrifuged at 16,000 × g* for 4 minutes. The supernatant was transferred to a fresh tube containing 600µl of room temperature isopropanol. It was mixed gently by inversion and centrifuged at 16,000 × g* for 1 minute. The supernatant was removed, and 600µl of room temperature 70% ethanol was added. The tube was centrifuged at 16,000 × g* for 4 minutes. The supernatant was discarded. Ethanol was aspirated, and the pellet was air dried for 15 minutes. The DNA was rehydrated in 100µl of DNA Rehydration Solution overnight at 4°C.

3.8 Quantification of DNA from culture cells

DNA concentration and purity was determined by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared by taking 2 µl nuclease free water. At first, the blank was placed in the measurement pedestal using pipette and was adjusted at value 0. Then, 2 µl sample was pipetted onto the measurement pedestal. After measuring the amount, DNA was kept at -20^{0}C.

3.9 Polymerase Chain Reaction (PCR)

PCR reactions were performed by Q-Cycler 96 PCR machine (Hain Lifescience, Germany). All amplifications were conducted in a final reaction mixture (25 µl) containing GoTaq G2 Green Master Mix (Promega, USA), forward and reverse primers, Nuclease free water and 2 µl DNA. These all mixture was taken into 0.2 ml PCR tube. Then, it was mixed by gentle vortexing, and immediately brief centrifugation was performed. Finally, the tubes were loaded in the PCR

machine. PCR results were validated under the following conditions: 95° C for 5 min; denaturation at 95° C for 30 s, annealing at 58° C for 30 s for 20 cycles, elongation for 72° C for 30 s, and a final extension at 72° C for 5 min using the DNA and primers.

Table 3.2: Primers were used for PCR analysis

Primers	Sequence	GC content (%)	Melting temperature, Tm
FSP1	F-GATGAGCAACTTGGACAGCAA	48	59.4^{0}C
	R-CTGGGCTGCTTATCTGGGAAG	57	63.3^{0}C
FN_1	F-CGGTGGCTGTCAGTCAAAG	58	59.5^{0}C
	R-AAACCTCGGCTTCCTCCATAA	48	59.4^{0}C

3.10 Agarose Gel Electrophoresis

Ensuring the presence of DNA, agarose gel electrophoresis was performed. For preparing 2% agarose gel, 90 ml distilled water and 2 % agarose were taken into a conical flask. After that, the mixture was boiled by using a micro-oven to dissolve the agarose. 5µl of ethidium bromide was put in the mixture. The solution was poured on the gel casting tray when the agarose gel temperature was reduced to almost 60^{0}C. The comb was set in gel solution for loading sample and was kept at room temperature for polymerization. 5 µl PCR products were loaded in holes of gel carefully by using pipettes. 5 µl of DNA ladder was taken in a clean sterile 0.2 ml PCR tube and 1 µl of Blue/Orange 6X loading dye (Promega, USA) was added into the tubes. Then, it was mixed by gentle vortexing, and immediately brief centrifugation was performed and loaded into the holes of agarose gel. After loading samples, the gel with the tray was put in the electrophoresis chamber containing 1X TAE buffer. The power supply was switched on and connected with the electric source at 120V and was applied for 15 minutes. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed under a UV transilluminator for observation.

3.11 Heat and antioxidants treatment to buffalo fibroblast cells

The cells after the 2nd passage were used for antioxidants and heat treatments. The culture flasks, four pretreated with zinc (0.01 mM) and four pretreated with L-ascorbic acid (20 μM), and four without zinc and L-ascorbic acid, were incubated at 37^0C in a 5% CO2 incubator for seven days. Zinc was used in its salt form, Zinc sulfate heptahydrate (Sigma Aldrich, USA), and Vitamin C was used in form of L- ascorbic acid (Sigma Aldrich, USA), which were suitable for cell culture after standardizing its concentration to provide optimal stimulation of fibroblast cell culture. The culture was maintained free from contamination using a 1% Penicillin Streptomycin solution. After seven days, the culture flasks were exposed to various levels of heat exposure for three hours.

a) 37^0C (Flask without zinc and L-ascorbic acid pretreatment, kept as Control)

b) 42^0C (Flask pretreated with zinc pretreatment, kept as Treatment I)

c) 42^0C (Flask pretreated with L-ascorbic acid, kept as Treatment II)

d) 42^0C (Flask without zinc and L-ascorbic acid pretreatment as Treatment III)

After that, the cells were subsequently allowed to recover at 37^0C and harvested by trypsinization at different time points (30m, 2h, 4h, and 6h) along with control samples (un-stressed). The culture plate marked as control was kept at 37°C throughout the study and was harvested at the same time points corresponding to the treated samples. The cells were subjected to the determination of cell viability. After that, the cells were processed for extraction of total RNA.

3.12 Cell viability

To evaluate heat stress effects on fibroblast cells, cell viability was recorded using the trypan blue exclusion method. The cells were taken from the culture flask by trypsinization and centrifuged at 16,000 × g* for 2 minutes. The obtained cell pellet was diluted with the DEME medium. 10 μl cells were taken to a microfuge tube. An equal amount of 0.04% Trypan blue was added to the microfuge tube. The cell number and viability were determined by Hemocytometer under the inverted microscope (10X). The stressed and unstressed (control) cells were checked across different time intervals after heat stress (30 m, 2 h, 4 h, and 6 h) to check the cell viability.

3.13 RNA extraction from treatment and control cells

RNA was extracted from all culture cells with treatments and without treatments. Total RNA was isolated using the Monarch Total RNA Miniprep Kit (New England Biolabs, USA). Cultured cells were removed from the culture flasks using 0.0.2 % trypsinization and centrifuged at 16,000 × g* for 4 minutes. The supernatant was discarded. Pellet cells were washed with ice-cold PBS and centrifuged at 16,000 x g for 1 minute. Again, the supernatant was discarded, and this process was repeated twice. The pellets were suspended in 600 µl RNA Lysis Buffer by pipetting. 800 µl of the sample from the tubes were transferred to a gDNA removal Columns fitted with a collection tube were labeled and spun at 16,000 x g for 30 seconds to remove most of the gDNA. The flow-through was saved, and the gDNA Removal Column was discarded. An equal volume of ethanol was added to the flow-through and mixed thoroughly by pipetting.

Then, the mixtures were transferred to an RNA purification Columns fitted with a collection tube and was pinned 16,000 x g for 30 seconds. The flow-through was discarded. 500 µl RNA priming buffer was added and spun at 16,000 x g for 30 seconds. The flow-through was discarded. 500 µl RNA wash buffer was added to the mixture and spun at 16,000 x g for 30 seconds. Again, the flow-through was discarded. Another 500 µl RNA wash buffer was added and spun at 16,000 x g for 2 minutes. The columns were transferred to the RNase-free microfuge tubes. 50 µl nuclease-free water was added directly to the center of the column matrix and spun at 16,000 x g for 30 seconds. Then, the tubes were incubated at room temperature for 1 minute and centrifuged the tubes at 16,000 x g for 2 minutes at room temperature. Finally, RNA was placed at -20°C for short-term storage.

3.14 RNA quantification

RNA concentration and purity from stressed and unstressed samples were determined by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared by taking 2 µl nuclease free water. At first, the blank was placed in the measurement pedestal using pipette and was adjusted at value 0. Then, 2 µl sample was pipetted onto the measurement pedestal. After measuring the amount, RNA was kept at -20^{0}C.

3.15 cDNA synthesis

2 µl RNA was used for cDNA synthesis using ProtoScript II first-strand cDNA synthesis Kit (New England Biolabs, USA) by PCR according to the manufacturer's protocol. RNA was stored previously at -20^{0}C temperature. PCR master mix was prepared for 20 µl. Then, the RNA template was denatured at 65°C for 5 minutes. After that, the master mix was incubated at 42°C for 1 hour. Next, the enzyme was inactivated at 80°C for 5 minutes. Reactions were performed using a Q-Cycler 96 PCR machine (Hain Lifescience, Germany).

Table 3.1: PCR reaction mixture and concentration of reagents for cDNA synthesis

Serial no	Reagents	Volume for per sample
1	2 X reaction buffer	10 µl
2	10 X Oligo d(T) 23VN	2 µl
3	Nuclease-free Water	4 µl
4	20 X Protoscript II Enzyme mix	2 µl
5	RNA	2 µl

3.16 cDNA quantification

cDNA concentration and purity were determined by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared by taking 2 µl of nuclease-free water. At first, the blank was placed in the measurement pedestal using a pipette and was adjusted at a value of 0. Then, a 2 µl of cDNA sample was pipetted onto the measurement pedestal. After measuring the amount, cDNA was promptly kept at -20^{0}C.

3.17 Real-time quantitative PCR (RT-qPCR)

The RT-PCR reaction was accomplised in Applied Biosystems® 7500 Real-Time PCR systems. SYBR green was our targeted dyes in this experiment.

center of the column matrix and spun at 16,000 x g for 30 seconds. Then, the tubes were incubated at room temperature for 1 minute and centrifuged the tubes at 16,000 x g for 2 minutes at room temperature. Finally, RNA was placed at -20°C for short-term storage.

3.14 RNA quantification

RNA concentration and purity from stressed and unstressed samples were determined by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared by taking 2 µl nuclease free water. At first, the blank was placed in the measurement pedestal using pipette and was adjusted at value 0. Then, 2 µl sample was pipetted onto the measurement pedestal. After measuring the amount, RNA was kept at -20^{0}C.

3.15 cDNA synthesis

2 µl RNA was used for cDNA synthesis using ProtoScript II first-strand cDNA synthesis Kit (New England Biolabs, USA) by PCR according to the manufacturer's protocol. RNA was stored previously at -20^{0}C temperature. PCR master mix was prepared for 20 µl. Then, the RNA template was denatured at 65°C for 5 minutes. After that, the master mix was incubated at 42°C for 1 hour. Next, the enzyme was inactivated at 80°C for 5 minutes. Reactions were performed using a Q-Cycler 96 PCR machine (Hain Lifescience, Germany).

Table 3.2: PCR reaction mixture and concentration of reagents for cDNA synthesis

Serial no	Reagents	Volume for per sample
1	2 X reaction buffer	10 µl
2	10 X Oligo d(T) 23VN	2 µl
3	Nuclease-free Water	4 µl
4	20 X Protoscript II Enzyme mix	2 µl
5	RNA	2 µl

3.16 cDNA quantification

cDNA concentration and purity were determined by Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The blank was prepared by taking 2 µl of nuclease-free water. At first, the blank was placed in the measurement pedestal using a pipette and was adjusted at a value of 0. Then, a 2 µl of cDNA sample was pipetted onto the measurement pedestal. After measuring the amount, cDNA was promptly kept at -20^{0}C.

3.17 Real-time quantitative PCR (RT-qPCR)

The RT-PCR reaction was accomplished in Applied Biosystems® 7500 Fast Real-Time PCR systems. SYBR green was our targeted dyes in this experiment.

Figure 3.5: 7500 Fast Real-Time PCR machine with loading sample

RT-PCR master mix was prepared using 2.0 µl of diluted cDNA, 10 µl of 2X Add SYBR Master Mix (Addbio, Sweden), and 0.5 µl of 10 µM forward and reverse primers, and the final volume of 20 µl was prepared with nuclease-free water. Forward- and reverse-specific primers for HSPs genes and positive control genes that were used for the RT-PCR are shown in Table 3.3 and Table 3.4.

Table 3.3: Targets primers were used for RT-qPCR analysis

Primers	Sequence	GC content (%)	Melting temperature, Tm
HSPA1A	F-TCATCAACGACGGAGACAAGCCTA R-TTCATCTTGGTCAGCACCATCGAC	50	65.3^{0}C
HSPA2	F-AAGCACAAGAAGGACATTGCACCC R-AAGTGTAGAAATCCACGCCCTCGT	50	65.3^{0}C
HSPA8	F-CGGTGATGCAGCAAAGAACCAAGT R-CACCACCATGAAGGGCCAATGTTT	50	65.3^{0}C
HSP90AA1	F-ATGAGCAGTATGCCTGGGAGTC R-CCCATTGGTTCTCCTGTGTCAG	55	64^{0}C

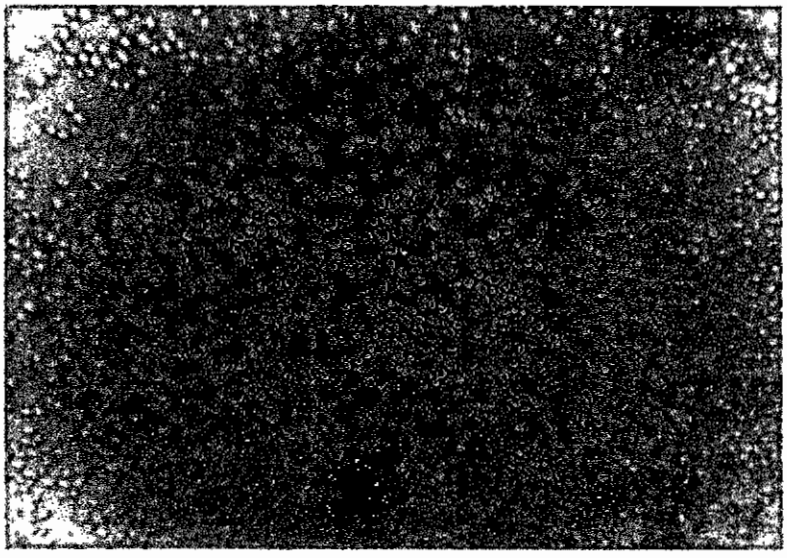
Table 3.4: Control primers were used for RT-PCR analysis

Primers	Sequence	GC content (%)	Melting temperature, Tm
GAPDHx	F-AGGTCGGAGTGAACGGATTC R-GGAAGATGGTGATGGCCTTT	55	59.3^{0}C
ACTB	F-ATTTTGAATGGACAGCCATC R-TGTACAGGAAAGCCCTGACT	50	57.3^{0}C

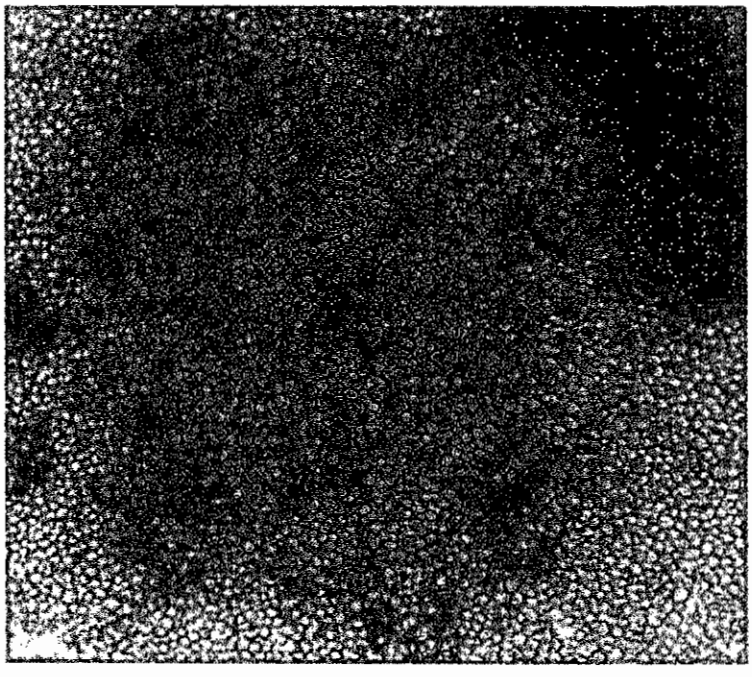
The RT-PCR program comprised initial heating at 60 °C for 1 min, followed by 95 °C for 15 sec, and samples were amplified for 40 cycles. The final extension at 72 °C incubation was continued for a further 10 min.

3.18 Data analysis

Data was collected from RT-PCR in the form of C_T value. (Threshold Cycle). By analyzing the C_T values, mRNA expression levels were determined.



(A)



(B)

Figure 4.1: Dermal fibroblast cell culture of buffalo. (A) Growth of cells after 7 days (B) Growth of fibroblast cells with 70-80 % confluence after 14 days.

4.3 DNA concentration of fibroblast culture cells

DNA purification kit improved the quality and quantity of DNA. It also saved time. Some steps of extraction process was carried out in the presence of ice to minimize DNA degradation. These steps also helped to improve the yield of DNA.

Table 4.2: The quantity of DNA from buffalo fibroblast cell culture using Nanodrop spectrophotometer

Serial no	Tag no	ng/ml	A260/A280
1	BC1	31.3	1.85
2	BC2	28.3	1.85

Total DNA extraction from culture cells displayed a high A260/A280 ratio, indicating the high-quality DNA sample from culture cells (Table 4.2). Here, DNA yield (31.3 ng/ml) was higher in sample BC1.

4.4 Buffalo fibroblast cells identification

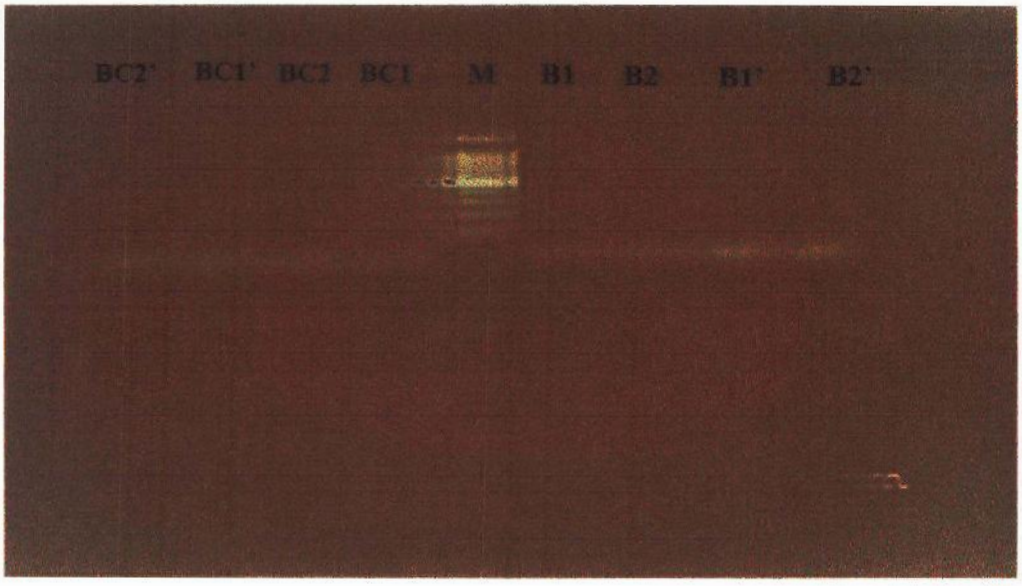


Figure 4.2: Fibroblast cells identification from ear tissue and culture cells of buffalo by the polymerase chain reaction (PCR). Here, B1 and B2 depict DNA samples from tissue with fibronectin (FN_1) primer; B1'and B2' represent DNA samples from tissue with fibroblast-specific protein 1 (FSP1) primer, whereas BC1 and BC2 depict DNA samples from culture

cells with fibronectin primer and BC1'and BC2' represent DNA sample from culture cells with fibroblast-specific protein 1. 'M' depicts DNA ladder.

For identifying the fibroblast cells, fibronectin (FN_1) and fibroblast-specific protein 1 (FSP1) were used as primers. A total of eight samples were used for PCR. Among them, four were DNA samples from tissue, and others were extracted from cell culture. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining under a UV trans-illuminator at 254 nm, and the photograph was taken for further analysis (Figure 4.2). Desired product sizes were less than 100bp (100 bp DNA ladder was used to get the information about the size of the band).

The present research utilizes the fibroblast as in vitro model to examine the direct temporal changes in the mRNA expression pattern of various heat shock proteins (HSP) in buffalo fibroblast cells during heat stress conditions and the activities of antioxidants to alleviate these heat stress effects on cells. Skin temperature is dynamic, which is affected by physical, environmental, and physiological factors and the hair coat's optical properties The cell culture of fibroblast specifically, in bovine species, has been explored by a few workers like us to understand the environmental stress effects on cells (Singh *et al.* 2020, Singh *et al.* 2014). Generally, primary fibroblasts expressed types I and III collagen (Col1 and Col3), fibronectin (FN), and fibroblast-specific protein 1 (FSP1) (Lee *et al.* 2010). Based on that fact, we used fibronectin (FN_1) and fibroblast-specific protein 1 (FSP1) as primers for identifying the fibroblast cells. In this aspect, we are in general agreement with Yoon *et al.* (2018), who reported that Col1 and Col3, FN, and FSP1 primers gave satisfactory results in the fibrotic differentiation identification of human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs).

4.5 Cell viability pattern of stressed and unstressed fibroblast cells

The Trypan Blue dye exclusion test is used to determine the number of viable cells present in a cell suspension. Live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. The cell viability evaluation by trypan blue exclusion method depicted decreased cell number and viability in heat-stressed cells across a different time intervals of post heat stress (30 minutes, 2 hours, 4 hours, and 6 hours) (Figure 4.3),

whereas the unstressed (control) cells maintained at 37°C did not exhibit any reduction in cell viability. The cells pretreated with zinc exhibited more cell viability in heat shock at 42 °C for 3 hours compared with the cells pretreated with vitamin C (Figure 4.4). Cells pretreated with zinc also depicted more cellular growth/ ml than control cells and vitamin C pretreated cells (Figure 4.5).

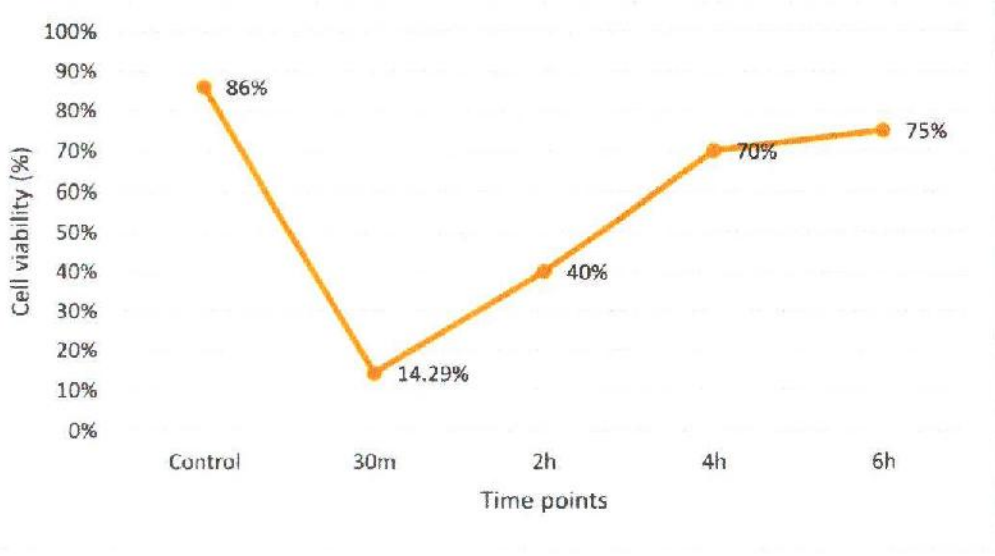
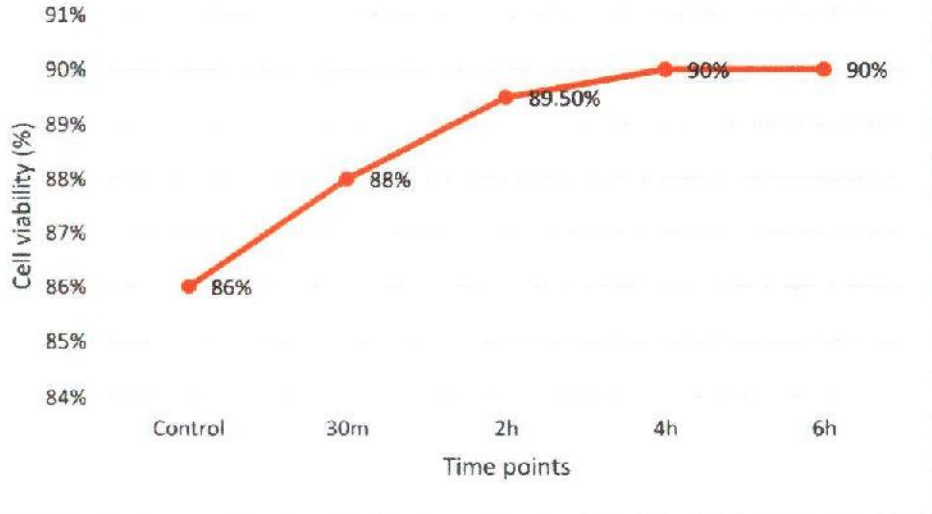
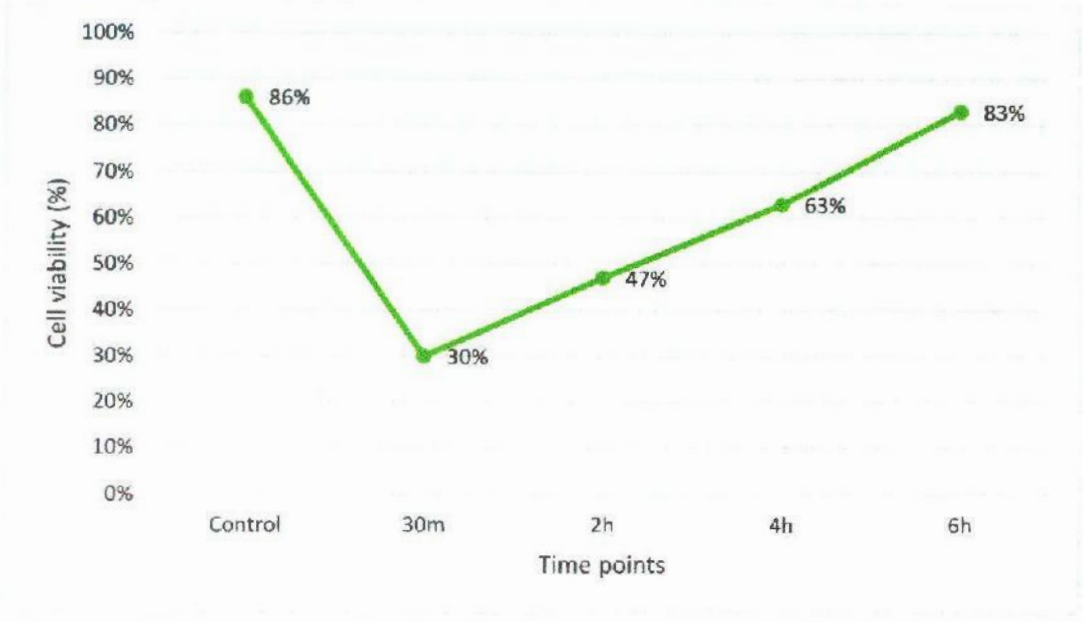


Figure 4.3: Cell viability pattern in heat stressed buffalo fibroblast cell without antioxidants treatment using trypan blue dye exclusion method



(A)



(B)

Figure 4.4: Cell viability pattern in heat stressed buffalo fibroblast cells culture with zinc and vitamin C treatment using trypan blue dye exclusion method. (A) Viability of cells pretreated with Zinc after heat stress at 42 °C for 3 hours (B) viability of cells propagated with vitamin C after heat stress at 42°C for 3 hours

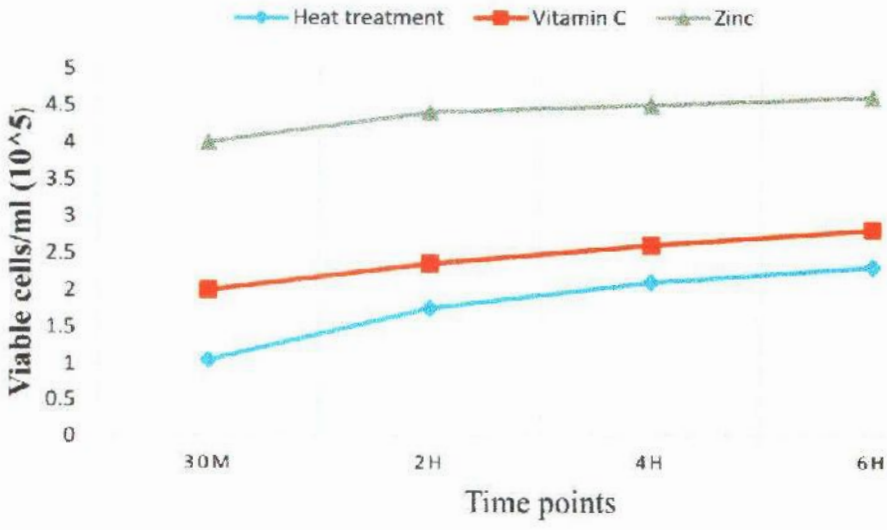


Figure 4.5: Viable cells growth pattern in heat stressed buffalo fibroblast cells with and without antioxidants treatment across post heat stress

The low percentage of viable cells observed immediately after the heat stress, while the recovery in cell viability during the later stages, exhibited all treatment cells. The cells pretreated with zinc showed less viable cell growth fluctuation in post-heat stress conditions.

Table 4.3: Viability of fibroblast cells for different treatments at different time intervals with and without antioxidants treatment across post heat stress

Comparison with	Groups	After 30 min, P value of live cells	After 2h, P value of live cells	After 4h, P value of live cells	After 4h, P value of live cells
Control	Vitamin C pretreated cells	0.0001	0.001	0.0001	0.001
	Zinc pretreated cells	0.0001	0.001	0.001	0.0001
	Cells without antioxidants	0.001	0.001	0.001	0.001

P-value analysis was done using one-way ANOVA.

Table 4.4: Viability of fibroblast cells for different treatments at different time intervals with and without antioxidants treatment across post heat stress

Comparison with	Groups	After 30 min, P value of live cells	After 2h, P value of live cells	After 4h, P value of live cells	After 6h, P value of live cells
Control	Vitamin C pretreated cells	0.001	0.001	0.001	0.001
	Zinc pretreated cells	0.001	0.0001	0.001	0.0001
	Cells without antioxidants	0.001	0.001	0.001	0.001

P-value analysis was done using one-way ANOVA.

Table 4.3 and Table 4.4 showed the significance level of the cell viability test for different treatments at different time intervals using one-way ANOVA. Cell viability decreased notably immediately after the heat stress might be attributed to the adverse effect of thermal stress on cell membrane structure or/and cell necrosis or impairment of cell growth. The results of our study have similarities with Adachi *et al.* 2009 and Zhao *et al.* 2006, who reported that hyperthermia at 42–46 °C causes cell death in many types of cells.

In the contrast, the recovery in cell viability during the later stages could be because of the recovery of cell survival mechanism by fibroblast cells. This pattern of response of the cell viability to thermal stress observed in our study have similarities with Collier *et al.* 2006 and Du *et al.* 2006, Du *et al.* 2007 works, where a similar response of the cell viability to heat stress in different *in vitro* cell culture models was examined. However, the cells propagated with antioxidants have higher cell viability than untreated cells under 42 °C heat stress. Zinc showed notable cell viability across post-heat stress periods and promoted higher cellular growth of fibroblast cells. Sheikh *et al.* (2016) reported that zinc increased cell viability in heat-stressed peripheral blood mononuclear Cells (PBMC) of dairy cows. This finding is quite similar to our work on buffalo fibroblast cells. Vitamin C also promoted fibroblast cell proliferation rate in heat-stressed buffalo fibroblast cells. In this case, our study has fewer similarities with Kuo *et al.* 2010 work, which reported that cells propagated with vitamin C have a higher cell proliferation rate in mouse embryonic fibroblast cells in normal condition.

4.6 Concentration of RNA from cells of treatment and control groups

RNA extraction was performed to isolate RNA from other cellular components. RNA was extracted from the sub-culture of fibroblasts cells. RNA extraction kit helped to improve the quantity and quality of RNA. As RNA is less stable and has more chance of degradation, the total extraction process was performed meticulously on ice. RNA was extracted from total 13 samples of stressed and unstressed cells (Table 4.5).

Table 4.5: RNA concentration of control and treatment cells after 3 hours of thermal stress at 42°C

Serial no	Tag no	ng/ml	A260/A280
1	Control	4.8	2.1
2	Heat treatment (30 minutes)	5.4	2.04
	Heat treatment (2 hours)	6.0	1.98
	Heat treatment (4 hours)	4.4	2.1
	Heat treatment (6 hours)	5.1	1.92
3	Vitamin C treatment (30 minutes)	6.3	2.03
	Vitamin C treatment (2 hours)	5.4	2.02
	Vitamin C treatment (4 hours)	6.0	2.1
	Vitamin C treatment (6 hours)	6.1	2.06
4	Zinc treatment (30 minutes)	6,7	2.02
	Zinc treatment (2 hours)	6.2	1.91
	Zinc treatment (4 hours)	6.1	2.01
	Zinc treatment (6 hours)	6.0	2.1

The total RNA extracted from individual fibroblast cells samples exhibited the A260/A280 ratio of 1.91-2.06, indicating moderately high RNA quality from each sample. The RNA quantity of stressed and unstressed cells varied from 4.4- 6.3 ng/ml

4.7 cDNA quantity

RNA is more fragile than DNA, which makes it tough to handle and analyze. cDNA is a more convenient way to work with the coding sequence than RNA. For this reason, the RNA was converted into complementary DNA sequences or cDNA. cDNA first strand synthesis kit helped to improve its quality and quantity.

Table 4.6: cDNA concentration of control and treatment cells after 3 hours of thermal stress at 42°C

Serial no	Tag no	ng/ µl	A260/A280
1	Control	1241.7	1.83
2	Heat treatment (30 minutes)	1226.2	1.80
	Heat treatment (2 hours)	1221.4	1.8
	Heat treatment (4 hours)	1223.0	1.81
	Heat treatment (6 hours)	1284.3	1.83
3	Vitamin C treatment (30 minutes)	1213.3	1.80
	Vitamin C treatment (2 hours)	1242.3	1.82
	Vitamin C treatment (4 hours)	1229.2	1.82
	Vitamin C treatment (6 hours)	1216.8	1.81
4	Zinc treatment (30 minutes)	1234.7	1.80
	Zinc treatment (2 hours)	1254.8	1.80
	Zinc treatment (4 hours)	1212.9	1.80
	Zinc treatment (6 hours)	1211.5	1.81

The number of total RNA samples were 13. All RNA samples were converted into first-strand cDNA using the cDNA synthesis kit. The quantity of cDNA varied from 1211.5-1284.3 ng/ µl, and the A260/A280 ratio differed from 1.8-1.83 (Table 4.6).

4.8 Real time qPCR data analysis

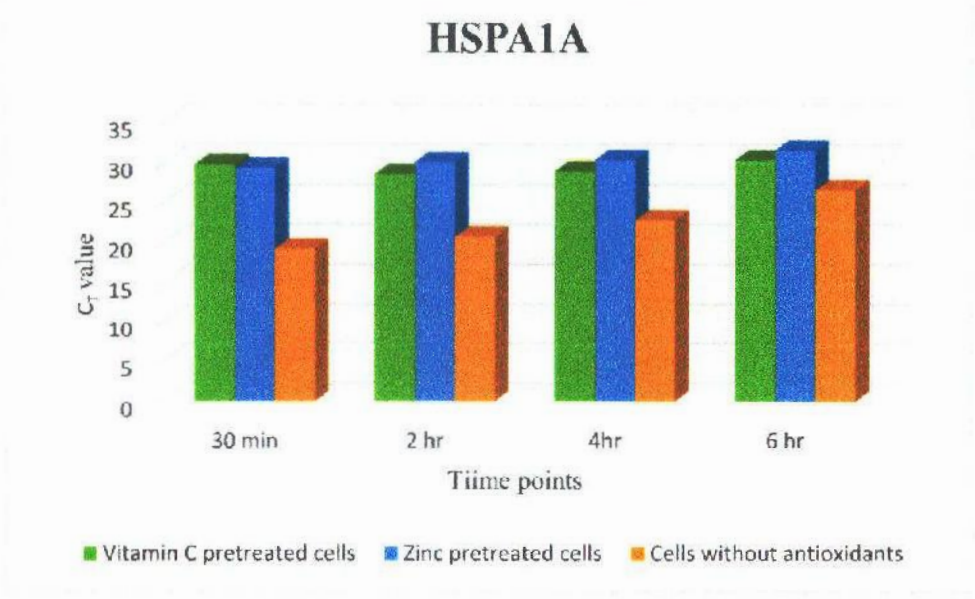


Figure 4.6: Expression profile of HSPA1A gene in buffalo dermal fibroblast cells with or without vitamin C and zinc in response to heat stress

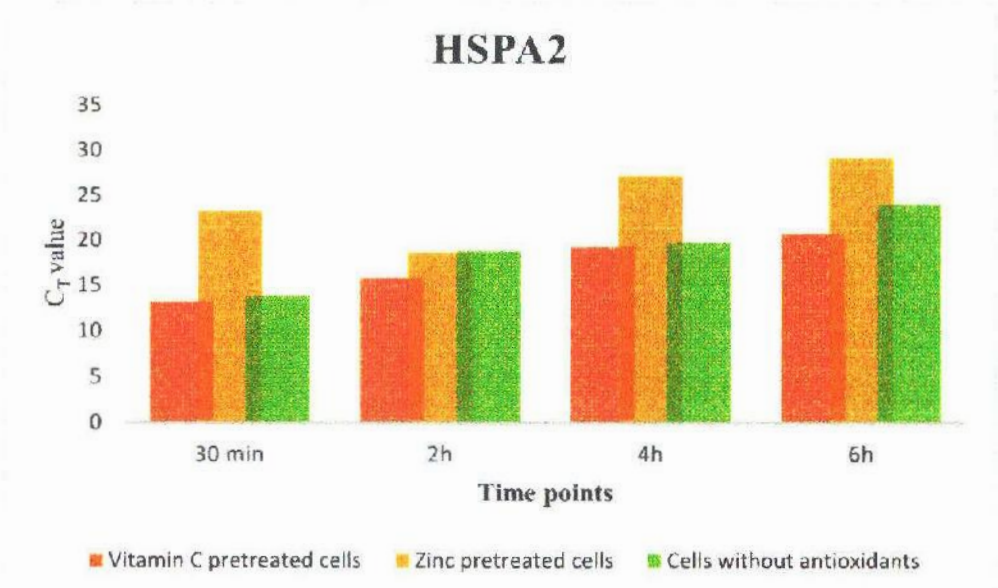


Figure 4.7: Expression profile of HSPA2 gene in buffalo dermal fibroblast cells with or without vitamin C and zinc in response to heat stress

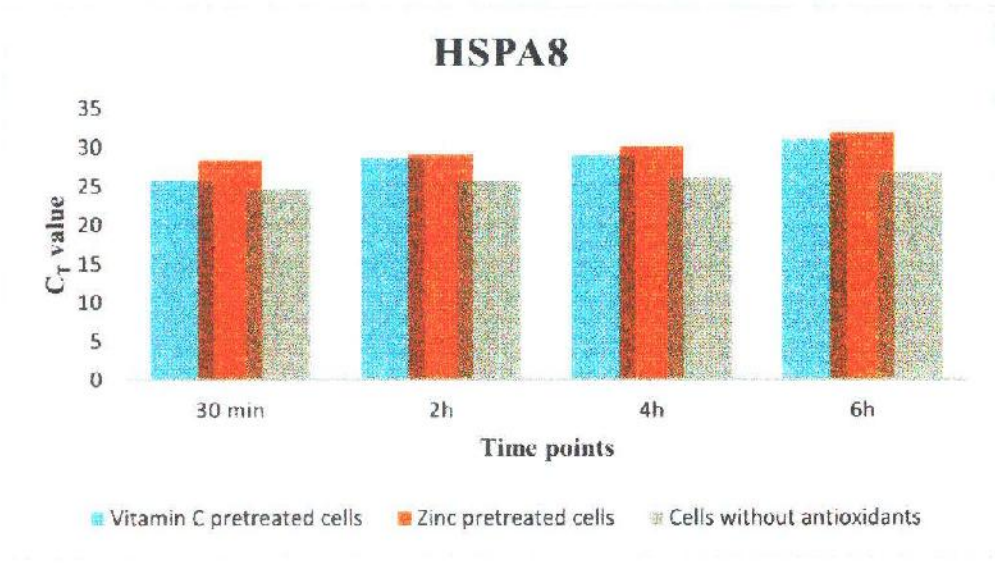


Figure 4.8: Expression profile of HSPA8 in buffalo dermal fibroblast cells with or without vitamin C and zinc in response to heat stress

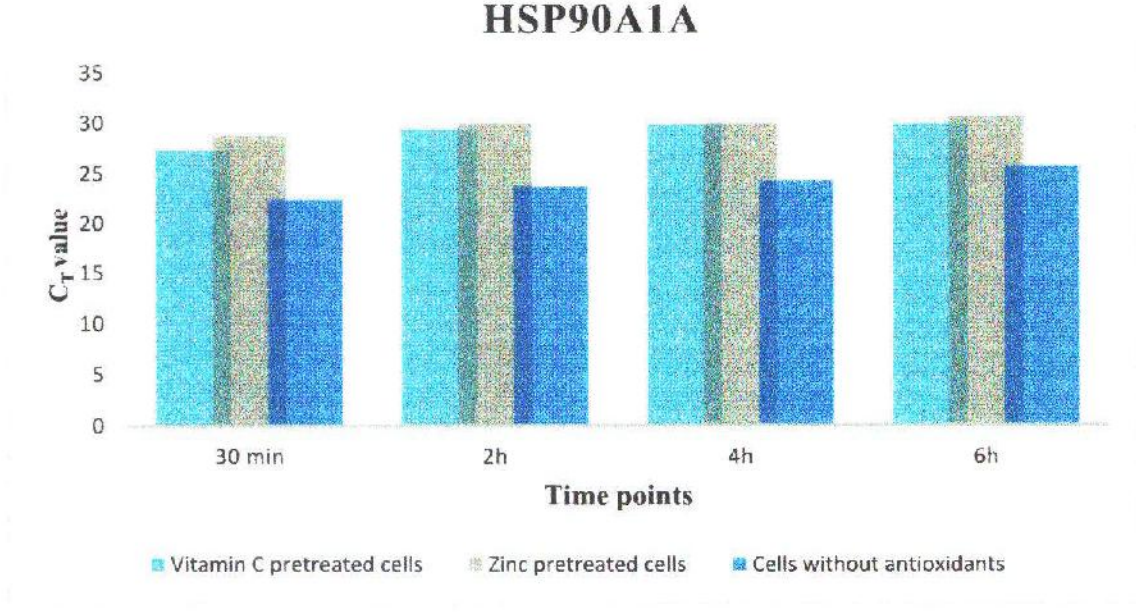


Figure 4.9: Expression profile of HSP90AA1 in buffalo dermal fibroblast cells with or without vitamin C and zinc in response to heat stress

C_T (Threshold cycle) has a reverse relationship with mRNA expression in RT-PCR. C_T values in depicts that overall, cells without antioxidants have low C_T values (Figure 4.6, Figure 4.7, Figure 4.8 and Figure 4.9). Among them, HSPA2 was identified as the prime of thermoregulatory induction mechanism across post heat stress. Zinc propagated cells have a low level of HSP genes expression than vitamin C pretreated cells. For zinc treated cells, highest C_T value was observed in expression profile of HSP90AA1 gene, and the highest C_T value of vitamin C propagated cells displayed in HSPA8 mRNA expression across post heat stress. For getting clear idea about their expression level targeted cells compared with control cells (37^0C) and control genes (GAPDHx, ACTB). Each member of the examined molecular chaperons (HSP family) responded intensively within 30 min, and most of them remained upraised till 6h post-heat stress. Most of the proteins displayed a maximum increase in mRNA expression at 30 min-4 hr post-heat stress. The elevation in the expression of HSP genes suggested the responsiveness of buffalo fibroblast cells to heat stress *in vitro*. The upregulation of molecular chaperones activity in buffalo fibroblast cells demonstrated the presence of a thermoregulatory mechanism during the early stages of cellular response to heat shock. Vitamin C showed fewer effects on HSPA2 mRNA level expression alleviation. Zinc displayed an effective role to minimize HSPA1A, HSPA8 and HSP90AA1 genes expression in heat stress cells, but it could not work well against HSPA2 gene.

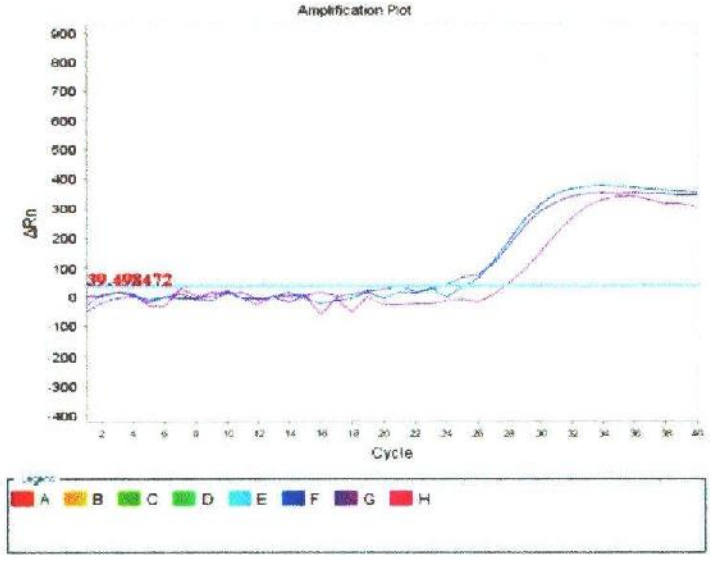


Figure 4.10: PCR amplification cycle graph for HSPA2 primer

In our present study, major groups of heat stress proteins were selected. All cells induced heat stress protein expression after heat shock at 42^0C for 3 hours. Singh *et al.* 2020, examined that HSPA8 expression was higher in cattle. However, at 40°C and 44°C, the expression of HSPA1A and HSPA2 was observed to be higher in cattle. We observed HSPA2 gene higher expression. In this perspective, our findings differ a little from their work, as we observed HSPs expression on buffalo. HSPA1A, HSPA2, and HSPA8 are representative members of the HSP70 family. According to Kapila *et al.* (2016), HSP70 was the most induced predominant chaperones in buffalo MECs because of thermal stress. Our findings were quite similar to their work. Our work are in general agreement with Singh *et al.* 2014 and Kapila *et al.* 2016, who examined heat shock protein expression at different time intervals after heat stress in dermal fibroblasts of dairy cows. Banerjee *et al.* (2014) reported that HSPA1A, HSPA8, and HSPA6 were highly expressed in the blood leukocytes of goats during summer season. There are several studies on bovine species where HSP70 and HSP90 expressions were observed (Collier *et al.* 2006, Singh *et al.* 2020, and Singh *et al.* 2014). Across the post-heat stress time interval, the HSP gene expression was high from 30 minutes to 6 hours, these findings were a little bit different from Kapila *et al.* 2016 study, where they examined the periods from 2 hours to 6 hours. We used vitamin C and zinc as antioxidants to observe their activity across post-heat stress. Sahin *et al.* 2009 reported that dietary zinc supplementation significantly reduced the HSP70 expression level and oxidative damage in quails. We also observed zinc reactivity for decreasing the HSP expression in fibroblast cells. Pearce *et al.* 2015 also reported that dietary zinc supplementation in heat-stressed pigs decreased the heat stress effects. Our finding about zinc is in accordance with Sheikh *et al.* 2017 work, who found inorganic zinc supplementation had positive effects on mitigating heat shock and immune response in heat-stressed peripheral blood mononuclear cells of cattle. Vitamin c exhibited its effects on reducing HSP expression. Kumar *et al.* 2010 reported that supplementation of vitamin C lowered glutathione peroxidase activity in buffaloes exposed to heat stress in a climatic chamber. But in our study, we found zinc more effective than vitamin C in the case of alleviating heat stress effects on fibroblast cells.

CHAPTER FIVE
SUMMARY AND CONCLUSIONS

Chapter 5. Summary and Conclusions

5.1 Conclusions

For observing the effects of thermal stress on buffalo, *in vitro* buffalo fibroblast culture cells were used. PCR was performed using FSP1 and FN_1 primers for distinguishing fibroblast cells. The desired gene size was smaller than 100 base pairs. The cell viability fell overall for all culture cells. However, Zinc pretreated culture cells' cell viability and proliferation rate across post-heat stress were noteworthy. The cells propagated with vitamin C had less cell viability than zinc pretreated cells. All fibroblast cells induced heat stress proteins (HSPA1A, HSPA2, HSPA8, and HSP90AA1) expression across post-heat stress. HSPA2 was identified as the most predominant form of heat stress proteins across post-heat stress periods. Zinc mitigated heat stress affects more than vitamin C.

5.2 Limitations

In the present study, the time limitation was the more crucial fact otherwise, more replication of treatments could be applied.

5.3 Recommendations

The present study used fibroblast *in vitro* cell culture to examine thermal stress effects on buffaloes using RT-PCR. Besides this, microarray from extracted RNA can give more details information about genes and their roles in handling heat stress events. We used antioxidants treatment on fibroblast cell culture, but the appropriate effects of antioxidants on heat-stressed buffaloes might be evaluated properly after live animal trials.

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APPENDICES

Appendix 1

For live cells

Comparison with	Groups	Standard deviation	Standard error	F	Significance
Control	Vitamin c pretreated cells (30 min)	1.414	1.000	1360.375	0.0001
	Vitamin c pretreated cells (2h)	1.414	1.000		
	Vitamin c pretreated cells (4h)	1.414	1.000		
	Vitamin c pretreated cells (6h)	1.414	1.000		
Control	Zinc pretreated cells (30 min)	1.414	1.000	1397.5	0.0001
	Zinc pretreated cells (2 hr)	1.414	1.000		
	Zinc pretreated cells (4 hr)	2.828	1.000		
	Zinc pretreated cells (6 hr)	1.414	2.000		
Control	Cell without antioxidants (30 min)	1.414	1.000	875.20	0.001
	Cell without antioxidants(2hr)	1.414	1.000		
	Cell without antioxidants(4hr)	1.414	1.000		
	Cell without antioxidants(6hr)	1.243	1.000		

For death cells

Comparison with	Groups	Standard deviation	Standard error	F	Significance
Control	Vitamin c pretreated cells (30 min)	1.414	1.000	39.179	0.001
	Vitamin c pretreated cells (2h)	1.414	1.000		
	Vitamin c pretreated cells (4h)	1.414	1.000		
	Vitamin c pretreated cells (6h)	1.414	1.000		
Control	Zinc pretreated cells (30 min)	1.340	1.000	644.562	0.0001
	Zinc pretreated cells (2 hr)	1.340	1.000		
	Zinc pretreated cells (4 hr)	1.340	1.000		
	Zinc pretreated cells (6 hr)	1.340	1.000		
Control	Cell without antioxidants (30 min)	1.414	1.000	367.649	0.001
	Cell without antioxidants(2hr)	1.414	1.000		
	Cell without antioxidants(4hr)	1.414	1.000		
	Cell without antioxidants(6hr)	1.414	1.000		

Appendix 2

1. List of instruments and apparatus used in experiment:

Instruments and apparatus

Test tubes

Blades

Scissors

Scalpels

Forceps

Eppendorf tubes

Petri dishes

Beakers

Conical flasks

Autoclave

Pipettes

Micropipettes

Micro oven

Electrical analytical balance

Incubator

Measuring cylinder

Aluminium foil

Water bath

Refrigerator

UV transilluminator

Centrifuge machine

Spectrophotometer

Shaker

PCR machine

RT PCR machine

DNA measuring machine

Mortar and pestle

Composition of solution that was used in skin biopsies, cell culture, DNA extraction, PCR and RT-qPCR

1. Digestion media (10 ml)

Composition	Amount
Mili Q water	8 ml
Trypsin	2 ml

2. DPBS solution (500 ml)

Composition	Amount
DPBS	4.8 gm
Calcium chloride	0.05 gm
Penicillin-Streptomycin	5 ml
Mili Q water	495 ml

The solution was stored at -20^{0}C temperature.

3. 10% fetal bovine serum

Composition	Amount
Fetal bovine serum	10 ml
Mili Q water	90 ml

The solution was stored at -20^{0}C temperature.

4. 10X TAE buffer (500 ml)

Composition	Amount
Tris HCL	24.2 gm
0.5M EDTA	1.87 gm
Glacial Acetic acid	5.7 ml
Mili Q water	500 ml

The solution was stored at room temperature.

5. 1X TAE buffer

Composition	Amount
10X TAE buffer	100 ml
Mili Q water	900 ml