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Molecular characterization of *IGF-1* gene expression in crossbred calves exposed to acute heat stress in a climate-controlled environment

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Abstract

Heat stress in livestock causes tremendous impacts on their overall production, productivity and health. Cattle are homeotherms, initiate various thermo-regulatory mechanisms to maintain homeostasis. Molecular adaptations are considered as dominant mechanism in thermoregulation. There are changes in the expression patterns of certain genes that are fundamental for thermo-tolerance at the cellular level in cattle. The present study was conducted exclusively to characterize the Insulin-like Growth Factor-1 (*IGF-1*) gene expression in heat-stressed calves under controlled climatic conditions using thermo-neutral zone as control. Venous blood samples with anti-coagulant were collected for gene expression studies in the peripheral blood leukocytes of calves in both the groups by quantitative real-time polymerase chain reaction (qRT-PCR) using *GAPDH* as housekeeping gene. The present study shown that the *IGF-1* gene expression shown a non-significant down regulation under heat stress when compared to thermo-neutral zone. It was clear from the findings that crossbred calves were trying to maintain thermal homeostasis by employing molecular adaptations when they were exposed to controlled environmental challenges and gave a focus on energy conservation for broader shift of gene expression towards thermo-regulation.

Keywords: Heat stress, thermo-neutral zone, crossbred calves, *IGF-1* gene, climatic chamber, molecular adaptations

1. Introduction

Heat stress in livestock causes tremendous impacts on their overall production, productivity and health. Cattle are homeotherms, maintains their thermal homeostasis by initiating various thermo-regulatory and adaptive mechanisms which promote their welfare and survival under stressful conditions (Indu and Pareek, 2015) [8]. Thermo-neutral zone (TNZ) or comfort zone of cattle is the zone where there is no energy expenditure for thermoregulation. If the temperature exceeds its upper critical limit, the animal experiences HS (Bagath *et al.*, 2019) [2]. Molecular adaptations are considered as dominant mechanism in thermoregulation. There are changes in the expression patterns of certain genes that are fundamental for thermo-tolerance at the cellular level in cattle (Gupta *et al.*, 2021) [7]. Insulin-like Growth Factor-1 (*IGF-1*) also called Somatomedin, is a hormone similar in structure to insulin, has its significant role in regulating growth, metabolism, and thermoregulation in cattle. The insulin-like growth factors (IGF) which includes IGF-1 and IGF-2 are single chain, ubiquitously expressed polypeptides plays a pivotal role in the regulation of cell growth, cell differentiation and maintenance of differentiated cell function (Jones and Clemmons, 1995; Cohick, 1998) [10, 4]. The *IGF-1* gene in cattle is located on chromosome five and is expressed in various tissues including the liver, muscle and mammary gland, influencing growth traits and milk production. Heat stress can reduce *IGF-1* expression in cattle, which can subsequently affect their growth. The expression of the *IGF-1* gene is influenced by multiple factors, mainly temperature-humidity challenges during HS, which poses a significant challenge to livestock productivity, especially in tropical and subtropical regions (Lima *et al.*, 2017; Barrera *et al.*, 2023) [13, 3]. Heat stress in cattle disrupts the homeostatic balance, leading to alterations in molecular and biochemical pathways to mitigate its effects (Collier *et al.*, 2008) [5].

IGF-1 is primarily produced in the liver under the influence of growth hormone, acts as a key mediator of anabolic processes, supporting growth and tissue repair. During HS, changes in *IGF-1* gene expression act as an adaptive response aimed at conserving energy while mitigating physiological stress (Gupta *et al.*, 2013) [7]. The down regulation of *IGF-1* gene expression during HS is considered as broader shift of gene expression towards thermoregulation rather than towards metabolic functions (Rhoads *et al.*, 2009) [16]. Studies have shown that *IGF-1* expression is often downregulated during acute HS due to reduced feed intake, altered growth hormone signaling and metabolic shifts prioritizing thermoregulation over anabolic functions (Pragna *et al.*, 2018) [15]. These changes can impair growth rates, reproductive performance, and overall health. Additionally, *IGF-1* interacts with other regulatory pathways involving heat shock proteins (HSPs) and oxidative stress markers, highlighting its role in the complex network of heat stress adaptation (Fabris *et al.*, 2020) [6]. Understanding *IGF-1* gene expression during HS is crucial for developing strategies to enhance cattle resilience, such as genetic selection for heat tolerance and nutritional interventions. Measuring these bio-markers played a significant role in evaluating the impact of high temperature-humidity-index (THI) on growth as well as metabolism in heat-stressed cattle. In view of this, the present study was conducted with the objective of exploring the molecular adaptation exhibited by crossbred (Holstein Friesian × Sahiwal) calves exposed to acute heat stress in a climate-controlled environment, by characterizing *IGF-1* gene expression using *GAPDH* as a reference gene.

2. Materials and Methods

2.1 Ethical approval of experiment

The research was conducted at the climatic chamber present at the Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS), Kerala Veterinary and Animal Sciences University. The experimental protocol was approved by the Committee for the Control and Supervision of Experimentation on Animals (CCSEA), New Delhi.

2.2 Experimental layout

Six healthy crossbred (Holstein Friesian × Sahiwal) female calves of eight to twelve months age were selected randomly from University Livestock Farm, Mannuthy and the same animals were distributed in control group or thermo-neutral zone (TNZ) and acute heat stress or treatment group. The selected animals were given an acclimatisation period of 10 days each initially in the animal holding facility and in the environmentally controlled chamber (Fig.1) in TNZ for three hours a day for adaptation of the animals to novel environmental conditions of the chamber. This was followed by experimental period of 10 days each under TNZ and in climatic chamber for acute HS study. In TNZ, the comfort chamber was maintained at a temperature of 27°C and relative humidity of 45-55 per cent (THI < 72). For acute HS study, maximum temperature of 40°C and relative humidity of 55-65 per cent (Maximum THI of 95.04) was simulated in the climatic chamber for three hours a day (from 11 a.m. to 1 p.m.) for 10 days to assess the impact of HS on *IGF-1* expression in crossbred calves. The animals were re-shifted for rehabilitation and

reuse to the respective sheds of the cattle farm after the experiment, as per the recommendations of CCSEA.



Fig 1: Experimental animals kept in the climate-controlled chamber

2.3 Collection of blood samples and procedure of RNA isolation

Isolation of RNA from blood sample was done using commercial RNA isolation kit supplied by Origin Diagnostics and Laboratory, Kerala (ORlonX Total RNA Isolation Kit, Origin). Fifteen mL of venous blood sample was collected on day 10 of the experiment at 2 p.m. from each of the experimental animals in both the control and treatment groups in a 15 mL centrifuge tube using EDTA (1mg/mL of blood) as anticoagulant.

The collected blood samples were centrifuged at 3000 rpm for 8 min and plasma is discarded. Two to three volumes of “ice-cold RBC lysis buffer” were then added to the remaining erythrocytes and leukocytes in centrifuge tube and incubated the mixture in ice with occasional shaking for 10 min for the complete lysis of erythrocytes. After ten min, it was centrifuged at 6000 rpm for eight min to pellet the contents. Pellet of leukocytes was recovered after decanting the red coloured supernatant, containing the lysed erythrocytes and haemoglobin. This procedure was repeated until pellet is clear without any unlysed erythrocytes. After centrifugation one mL of TRI reagent was added and mixed well by pipetting. This was then transferred into a two mL RNase free Eppendorf tube and again mixed by pipetting and vortexing. Vortexed sample was then incubated for five min at room temperature to permit complete dissociation of nucleoprotein complex. After incubation 200 µL of chloroform per mL of TRI Reagent was added for homogenization and vortexed vigorously for five min and incubated again for 10 min at room temperature. The sample was then centrifuged at 12000 rpm for 15 min at 4 °C. The mixture separates into a lower red phenol chloroform phase, an interphase and a colourless upper aqueous phase in which RNA is present. Aqueous phase was removed carefully by angling the tube at 45° and pipetting the solution out into a new RNase free spin column provided in the kit. 500µL of Buffer RD is added to the RNase-Free spin column and centrifuged at 12,000 rpm for 1 min at 4°C. Flow-through was discarded and 700 µL Buffer RW was added to the RNase-Free spin column and centrifuged again at 12,000

rpm for one min at 4 °C. Flow-through was discarded and 500 µL Buffer RW was added to the RNase-Free spin column and centrifuged at 12,000 rpm for one min at 4 °C. Flow-through was discarded and dry spinning was done at 12,000 rpm for 2 min at 4 °C. After dry spinning RNase-Free spin column was placed in a new 1.5mL RNase-Free Eppendorf tube and 35µL of RNase-Free water directly to the spin column membrane. It was then incubated in room temperature for two min and centrifuged at 12,000 rpm for two min at 4 °C to elute the RNA. Concentration and purity of RNA was determined immediately for complementary DNA (cDNA) synthesis.

2.4 Determination of concentration and purity of total RNA

The quantity and purity of total RNA were estimated by Nanodrop (Thermo-Scientific NanoDrop™2000 Spectrophotometer) method spectrophotometrically. The concentration of total RNA per microliter of the sample was quantified by placing 1 µL of the sample in the Nano-Drop spectrophotometer after calibrating it with the blank solution, nuclease-free water. The purity of total RNA was checked by A260/A280 and A260/A230 ratio using Nano-Drop spectrophotometer. A ratio of 1.8 or above for both A260/A280 and A260/A230 was preferred for cDNA synthesis.

2.5 Checking the quality of RNA by agarose gel electrophoresis

The quality and integrity of total RNA were analysed electrophoretically using agarose gel (0.8%) electrophoresis. Agarose gel (0.8%) was prepared in 1X Tris Acetate EDTA buffer (TAE), which was prepared using diethyl pyrocarbonate (DEPC) treated water. Ethidium bromide (to a final concentration of 0.5g/mL) was added into the molten agarose, mixed thoroughly and poured into gel casting tray to polymerise. After solidification, the comb and the seal were removed. The gel with the tray was immersed in 1X TAE buffer in the electrophoresis tank. RNA sample (5 µL) was mixed with 1µL of RNA gel loading dye in a 0.25mL microcentrifuge tube and denatured at 70°C for 10 min, snap chilled in ice and loaded into the wells along with a molecular size ladder. Horizontal electrophoresis unit was set at 70 V for 50 min and the gel was visualized under gel documentation system (BioRad, Gel Doc 2000™). After analysing the quantity and integrity, the RNA samples were taken for cDNA synthesis.

2.6 Preparation of complementary DNA (cDNA)

Complementary DNA is the DNA copy of RNA molecules, synthesized by the enzyme RNA dependent DNA

polymerase (Reverse transcriptase). The total RNA was reverse transcribed into DNA using Origin cDNA synthesis kit with RNase inhibitor (Origin Diagnostics and Laboratory, Kerala) as per manufacturer's protocol using oligo (dT)₁₈ and random hexamer primers. For cDNA synthesis, 500 ng of RNA was reverse transcribed. The RNA samples were set up as per the standard protocol of the cDNA synthesis kit.

Protocol

First strand cDNA synthesis (reaction volume 20 µL)

1. Prepared the following mixture in a PCR tube
2. Heated the mixture at 65 °C for 5 min and cooled down immediately on ice for 1 min Spun down the tube by centrifugation briefly.
3. Added the following components to the same tube.
4. Mixed gently.
5. Incubated the combined reaction mixture (Priming) at 25 °C for 5 min.
6. Incubated at 50 °C for 60 min.
7. Inactivated the reaction mixture by heating at 95 °C for 5 min in the PCR machine (Biorad, Thermal cycler)

The product of first strand cDNA synthesis was stored at -80 °C until use. The 1µL of cDNA was used for further qRT-PCR experiments.

Table 1: Master mix for cDNA synthesis

Components	Volume (µl)
Template RNA	To get 500 ng
Primer	0.5 µL oligo (dT) ₁₈ and 0.5 µL Random hexamer
dNTP Mix (10 mM)	1
Nuclease-free water	Added to make 14.5µl of the total mix

Reagents	Volume
5X RT Reaction buffer	4 µL
RTase	0.5 µL
RNase inhibitor	1 µL

2.7 Designing and selection of primers

Exon-spanning primers (Table 2) were designed using online NCBI primer design software (Primer3, <http://bioinfo.ut.ee/primer3/>) and specificity of the primers was checked using BLAST. Designed primers were assessed for quality using PCR primer stats (Bioinformatics.org). The primers purchased in the lyophilized form was reconstituted with nuclease-free water to make a concentration of 100 pM/µL for the stock and then stored at -20 °C. Working solution of each primer was prepared by diluting the stock to 10 pM/µL using nuclease free water.

Table 2: Description of primers designed for qPCR reaction

Gene	Gene Accession no. (NCBI)	Primer (F/R)	Primer Sequence (5'-----3')	Primer Length (Bp)	Product Size (Bp)
<i>IGF-1</i>	NCBI reference sequence NM_001077828.1	<i>IGF-1</i> F	CAACAAGCCCCACGGGGGTAT	19	99
		<i>IGF-1</i> R	TCCAGCCTCCTCAGATCACA	20	
<i>GAPDH</i>	NCBI reference sequence NM_001034034.2	<i>GAPDH</i> F	TGGAGAAACCTGCCAAGTATG	21	127
		<i>GAPDH</i> R	TGAGTGTCTGCTGTTGAAGTC	20	

2.8 Standardization for quantitative real-time PCR (qRT-PCR) conditions by gradient PCR using Thermal cycler: Complementary DNA samples with good purity and concentration determined by Nano drop spectrophotometer (Fig.3) were used as templates for PCR. The polymerase

chain reaction (PCR) was carried out to amplify *IGF-1* (target gene) and *GAPDH* (house-keeping or reference gene) genes. The conditions were optimized by setting different temperatures for annealing processes that was calculated by using thermal melting temperatures (T_m) of

forward and reverse primers. The optimum annealing temperature for the primers was determined by gradient PCR (Biorad, Thermal cycler, Fig.2) and in quantitative real-time PCR system using SYBR green chemistry. The gradient PCR reaction mix and cyclic conditions used are given in Table 3.

Table 3: Reaction mix for standard PCR reactions

Components	Volume (μ L)
Taq PCR Smart mix (2x) w/o dye	25.00
Forward primer (10 pM/ μ L)	2.00
Reverse primer (10 pM/ μ L)	2.00
Template DNA	1.00
Nuclease-free water	20.00
Total	50.00

The tubes were spun briefly and placed in a thermal cycler. The following Table 4 describes the details of PCR conditions used for the reaction.

Table 4: PCR conditions for amplification

Steps	Temperature ($^{\circ}$ C)	Time
Initial denaturation	95	2 min
35 cycles of	Denaturation	95
	Annealing*	63.3 $^{\circ}$ C for <i>IGF-1</i> 60.5 $^{\circ}$ C for <i>GAPDH</i>
	Extension	72
	Final extension	72
		5 min

* Based on gradient PCR results, temperature set for the target and reference genes



Fig 2: Thermal cycler (Bio-Rad) for performing cDNA synthesis and gradient PCR

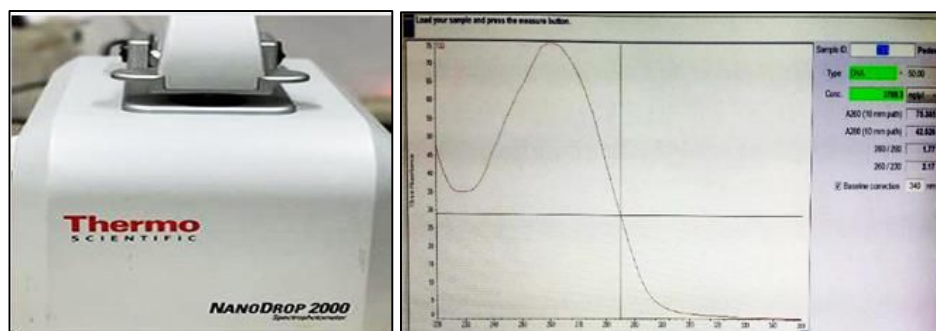


Fig 3: Purity and concentration of cDNA determined by Nanodrop spectrophotometer before qPCR

The PCR amplified products were stored at -20° C till further processing. The amplified products were used in 2% agarose gel [1X Tris-Borate-EDTA buffer (TBE)] electrophoresis. Five microlitre of the product was mixed with one microliter of 6 X gel loading buffer and loaded into the wells carefully. As a molecular size standard, 1 μ L of 100 bp DNA ladder (Thermo scientific, USA) was also loaded into one of the wells. Electrophoresis was then

carried out at 70 V for 50 min or until bromophenol blue dye migrated more than 4 cm length of gel in 1X TBE buffer. The gel was documented in a gel documentation system (BioRad, Gel Doc 2000TM). After detecting the bands by electrophoresis, the product size of *IGF-1* was confirmed. Annealing temperature at good dark bands for each gene were standardised for real-time PCR reaction.

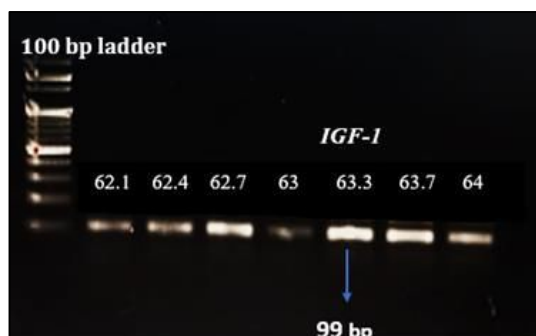


Fig 4: Gel documentation images of standardisation of annealing temperature for *IGF-1* gene

2.9 Quantitative real-time PCR amplification of genes

After validation of primer annealing temperature, the cDNA of target gene along with the reference gene was studied for mRNA expression by Real Time PCR (Biorad, USA) and

analysed using CFX Maestro software. The expression of *IGF-1* and *GAPDH* was studied using iTaq™ Universal SYBR Green Supermix, Bio-Rad Laboratories, USA (Table.5).

Table 5: Optimized concentrations of qRT-PCR Master Mix (10 µL) for *IGF-1* and *GAPDH* genes

Components	Volume (µl)
Template (cDNA)	1.00
iTaq™ Universal SYBR Green Supermix (2 x)	5.00
Forward Primer (10pM/ ul)	0.50
Reverse primer (10pM/ul)	0.50
Nuclease-free water	3.00

Table 6: qRT-PCR conditions for *IGF-1* and *GAPDH* genes

Steps	Temperature	Time
Polymerase activation	95 °C	2 min
35-40 cycles of Denaturation	95 °C	5 sec
Combined Annealing & Extension *	63.3 °C for <i>IGF-1</i> , 60.5 °C for <i>GAPDH</i>	30 sec

2.10 Relative quantification of genes

Relative quantification describes the change in expression of the target sample relative to some reference group such as an untreated control. *IGF-1* gene quantification was done by C_T (Cycle Threshold) comparative method and was expressed as 'n' fold up-regulation of the transcribed gene in relation to the calibrator (Untreated control). For relative quantification by comparative method, the values were expressed relative to control sample called calibrator. *GAPDH* was used as an internal control. The C_T of the target gene and C_T of control gene were determined for each sample and calibrated ($\Delta\Delta C_T$ method) (Livak and Schmittgen, 2001) [14]. Statistically analyzed the data using SPSS version 24.0 software.

$$\Delta C_T = C_T (\text{target gene}) - C_T (\text{reference gene})$$

$$\Delta\Delta C_T = \Delta C_T (\text{test sample}) - \Delta C_T (\text{reference sample})$$

$$RQ = 2^{-\Delta\Delta C_T}$$

3. Results

The mean values of C_T , ΔC_T , $\Delta\Delta C_T$ and fold change ($2^{-\Delta\Delta C_T}$) in *IGF-1* gene expression of crossbred calves in thermo-neutral zone (control) and acute heat stressed conditions (treatment) are presented in Table 7. The fold change of *IGF-1* gene expression is presented pictorially in Fig. 5 and the amplification plot and melt peak obtained by qRT-PCR is depicted in Fig.6. Relative quantification of *IGF-1* gene expression in peripheral blood leukocytes shown a non-significant ($p>0.05$) 0.71 fold down regulation in crossbred calves exposed to acute heat stress when compared to those under TNZ.

Table 7: Cycle threshold values and fold change in *IGF-1* expression in crossbred calves under thermo-neutral zone and acute heat stressed conditions (Mean $\pm$ SE, N=6)

Groups	Mean $C_T \pm$ SE		ΔC_T	$\Delta\Delta C_T$	Fold Change ($2^{-\Delta\Delta C_T}$)	P-Value
	<i>IGF-1</i>	<i>GAPDH</i>				
Control (TNZ)	30.48 $\pm$ 0.21	22.75 $\pm$ 0.25	7.40 $\pm$ 0.16	0.00 $\pm$ 0.16	1.00 ^A	P=0.077 ^{ns}
Treatment (HS)	30.10 $\pm$ 0.44	22.18 $\pm$ 0.37	7.92 $\pm$ 0.51	0.48 $\pm$ 0.51	0.71 ^A	

Means bearing same superscript within columns did not differ significantly, ns – non-significant at 0.05 level

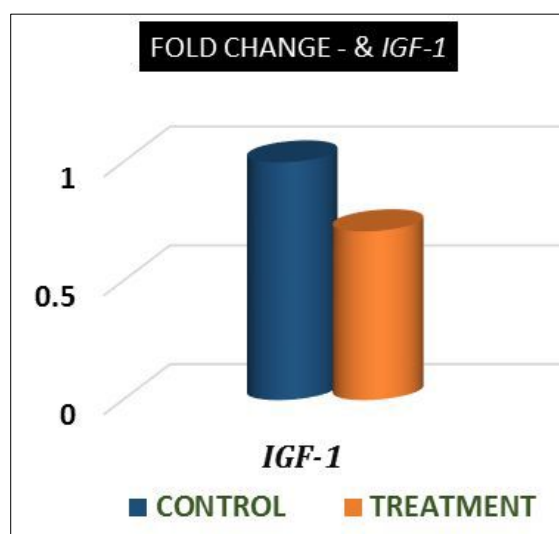


Fig 5: Fold change in *IGF-1* expression in crossbred calves under thermo-neutral zone and acute heat stressed conditions.

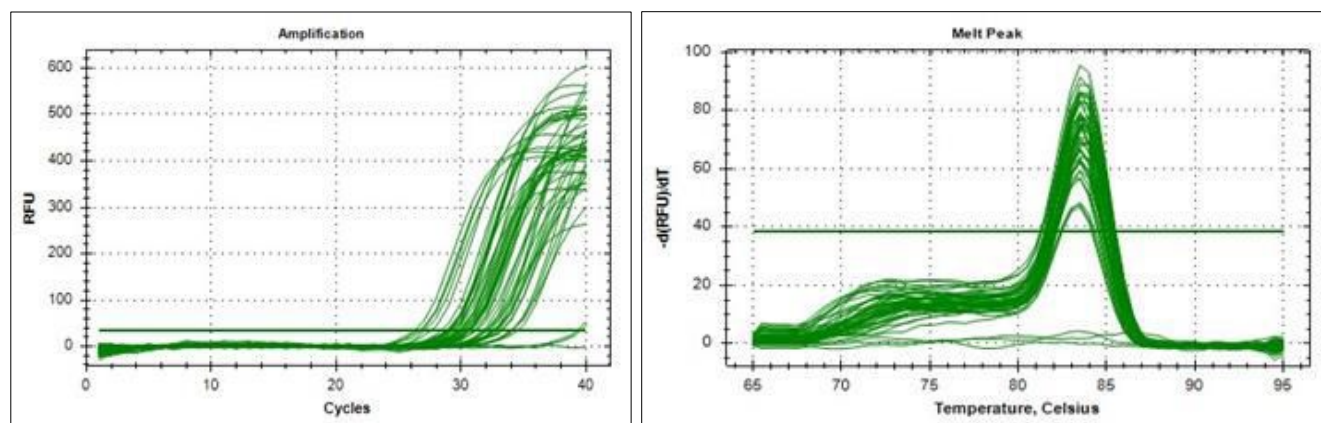


Fig 6: Amplification plot and melt peak of *IGF-1* gene in crossbred calves under thermo-neutral zone and acute heat stressed conditions

4. Discussion

The present molecular study of relative quantification of *IGF-1* gene expression in peripheral blood leukocytes showed a non-significant ($p>0.05$) 0.71-fold down regulation in crossbred calves exposed to acute HS when compared to TNZ which is in accordance with the results reported by Pragna *et al.* (2018) [15] and Shashank *et al.* (2023) [18]. Pragna *et al.* (2018) [15] found a significantly lower expression of *IGF-1* mRNA at high THI during hotter periods of summer stress which validates the current findings. Fabris *et al.* (2020) [6] evidenced that Holstein cows exposed to HS had down regulation of *IGF-1* ($p\leq 0.10$) when compared to cows provided with cooling further supported the present findings. Kim (2023) [12] found that extreme heat stress of 41°C upregulated ($p<0.01$) the expression of *IGF-1* in bovine myocytes when compared to the control group (38°C) which is contradictory to the present findings. The down regulation of *IGF-1* in the present study might be involved in energy conservation for thermoregulation. The down regulation of *IGF-1* and upregulation of *HSP-70* expressions during HS could also be considered as a broader shift in gene expression towards thermo-regulatory adaptation. During HS, there might be decline in growth hormone (GH) levels which could down regulate *IGF-1* expression (Aggarwal and Upadhyay, 2013; Pragna *et al.*, 2018) [1, 15]. It might also be due to low GH during HS that might not be sufficient to induce hepatic GH-IGF axis thereby reducing GH-dependent hepatic IGF-I hormone production which could down regulate its mRNA expression in heat-stressed calves.

5. Conclusion

It was evident from the current findings that down regulation of *IGF-1* gene expression in peripheral blood leukocytes of crossbred calves (Holstein Friesian × Sahiwal) exposed to acute HS under climate-controlled environment thrown light on molecular adaptation mechanisms exhibited by them when exposed to a maximum temperature of 40°C and relative humidity of 55-65 per cent, contributing in energy conservation for heat stress adaptation due to temperature-humidity challenges.

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7. Conflict of Interest

The authors declared no conflict of interest.

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