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Thermal apoptosis causes reduction in peripheral blood mononuclear cells in Indian Gir cattle breed when exposed to high temperatures for a long time

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Abstract

Background and Objective: The changing effect of climate has grave consequences on livestock production. Hence, the goal of this study is to conduct an *in vitro* thermal stress stimulation on Indian Gir cattle peripheral blood mononuclear cells (PBMCs) by exposing them to a range of temperatures and time, in order to determine how PBMCs react to different levels of heat shock.

Methodology: Fresh blood (10 mL) was collected from each of the 45 Indian Gir cattle, and PBMCs were separated. PBMCs were divided into nine groups; each group had 5 PBMC samples. Aliquots of 500 µL of PBMCs were stressed by exposing them to different temperatures (normal: 37°C and extreme: 45°C) for durations of thermal exposure (DTEs) of 0, 1, 2, 3, and 4 h. The control samples, which were not stressed

(500 µL aliquot of PBMCs), were exposed to no temperature (0°C) and zero DTE (0 h).

Main Results: A significant positive relationship between increasing temperature and PBMC count was observed ($P < 0.01$). Also, the viability of PBMCs was negatively impacted ($P < 0.01$) by heat shock, which accounted for the exponential decrease in PBMCs as TACs/heat shock toughened. *In vitro* study of thermally stressed PBMCs provides insight into the response of cellular systems to heat shock. We discovered a significantly strong regression coefficient (b) of 92.0%, predicting a 92.0% response of PBMCs to an increase in temperature. After heat shock stimulation, we also calculated the coefficient of determination (R^2), and we discovered that R^2 (84.7) accounted for a decrease in PBMC count due to heat shock. Further to the above, we detected a 92.0% relationship ($P < 0.01$) between an increase in temperature (heat shock) and PBMC count.

Conclusions: This study proved that PBMCs can be employed as a cellular system to learn about how Gir cattle responds to assaults of thermal conditions for improved management and performance.

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INTRODUCTION

Global warming occasioned by climate change has had grave consequences on livestock production systems over the past few decades and has had a negative impact on animal output in general (Onasanya *et al*., 2021). In light of the existential threat presented by climate change, previous authors reported that environmental temperature has made it challenging for both humans and other mammalian species to live. This has had several negative impacts on food security, productivity, and output (Onasanya *et al*., 2020). Thermal stress (TS) can have a devastating effect on cattle, thereby making them susceptible to hyperthermia if assaults of thermal stress are not ameliorated, and these result in reduced feed intake, low milk yield, poor growth, reduced physiological activity, and performance (Onasanya *et al*., 2017). Furthermore, livestock animals in the tropics are forced to elevate their respiratory rate and peripheral blood flow as a result of harsh climatic conditions, which has a negative impact on their physiology and performance, including reduced milk yield (Onasanya *et al*., 2022). Changes in environmental temperatures and relative humidity impair the ability of cattle and other animals to maintain homeostasis, forcing them to actively maintain the internal body temperature required for survival and productivity (Habeeb *et al*., 2018). When body temperature of an animal is elevated over its typical physiological range, thermoregulation of internal body temperature or homeothermy, is This

is true regardless of the external thermal challenges (Onasanya *et al*., 2022). Economic loss results from these harsh conditions raise the cost of management and production with a negative impact on food security and income (Kishore *et al*., 2016).

Recently, studies showed that thermal necrosis seriously caused cell death and exponential reduction in peripheral blood mononuclear cells (PBMCs) among Indian Zebu-Jersey crossbreds when exposed to high TS for a long time as the thermal assault conditions (TACs) toughened (Onasanya *et al*., 2022). Different TACs and heat stress were found to affect cellular viability, integrity, and proliferation, thereby causing an impaired immune response, which exposes the animals to opportunistic infections with a decrease in production performance (Gao *et al*., 2015; Wang *et al*., 2017; Siddiqui *et al*., 2020; 2021). Additional reports demonstrated that extreme environmental TACs can degrade nucleotides and induce mutational damage to the structure of nucleic acids, which can hamper the synthesis and function of nucleic acids and hinder cell proliferation (Wang *et al*., 2017; Simoni *et al*., 2020; Siddiqui *et al*., 2021).

For instance, a moderate *in vitro* temperature of 37°C mimics the body temperature of mammals and increases cell survival and DNA synthesis, which aids the growth of cells (Onasanya *et al*., 2020; Simoni *et al*., 2020; Siddiqui *et al*., 2021). Circulating leukocytes, including PBMCs, can be utilized as a biological system to study how livestock animals react to different TACs, particularly in the tropics. Reduced effects of harsh environmental conditions are required for better performance of livestock animals in production, including fertility (Sheikh *et al*., 2017).

Onasanya *et al*. (2022) reported that Indian Zebu-Jersey crossbreds were vulnerable and largely impacted by the assaults of thermal conditions due to changing effect of climate occasioned by global warming, the authors found that heat shock (HS) greatly challenged Zebu-Jersey crossbred cattle especially when exposed to assaults of thermal condition for a long time or long durations of thermal exposure (DTEs). Therefore, the purpose of this work was to conduct an *in vitro* thermal stress stimulation (TSS) of purebred Indian Gir Zebu cattle PBMCs and investigate how PBMCs react to different combinations of HS/TACs. The hypothesis/goal of this study seeks to investigate the effect of TACs/HS on the viability of PBMCs/cellular system of Indian Gir cattle upon exposure to in vivo thermal stress simulation (exposure to different doses of temperature and time). This current work will allow additional *in vivo* investigations on the potential for thermo-tolerance in Indian Gir cattle in real-life conditions. In our future works, the information and results obtained from this study will be used to understand the differession of bovine heat shock protein genes, such as *HSPs* 70, 90, and *sHSPs*, to different TACs *in vivo*. Furthermore, RNA-seq technology will be used to investigate how heat shock proteins (HSPs) and cytokines, and other biomolecular species collectively influence thermotolerance and immune response, particularly in Indian Zebu cattle breeds, as well as other Zebu cattle, when exposed to HS or the thermal conditions of the tropics.

MATERIALS AND METHODS

Location of Study, Description of the Study Area, Meteorological Data, and Ethical Approval

This study was conducted between the period of May 2022 to February 2023, including the collection of blood samples, laboratory studies, and statistical analyses. The study animals were kept and raised at the University Farm (Instructional Livestock Farm Complex (ILFC), Madhavaram), Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. Longitude and latitude of the study area are 27.2046°N 77.4977°E. Blood collection was between the hours of 2 and 12 p.m. when the mean ambient

temperature was 43°C. The study area experiences mean annual rainfall that ranges from around 640–920 mm, with a mean precipitation of 887 mm. The climate is tropical, and it is hot all year round, including wet season and dry season, as obtained in tropical environments. The mean annual temperature ranges between 33 and 45°C. The mean wind speed ranges between 6 and 13 km/h, while ambient humidity ranges between 67% and 95%. The above meteorological data were obtained from the Agromet Unit of University Farm, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India (Onasanya *et al*., 2022). The study was reviewed on October 12, 2021, and approved with Decision No: TWAS-DBT/FRnumber :3240313769/PDF/2019 by the post-doctoral research supervisory team led by Prof. A.K. Thiruvenkadan (host supervisor) before embarking on the various activities reported here and this met laid down ethics for carrying our animals' research by the institution.

Study Animals and Collection of Blood Samples

The animals were kept in an intensive system and were fed forages and concentrates. Cool and clean water was provided ad libitum. Blood samples (10 mL per animal) were randomly collected from 45 Indian Gir Zebu cattle, aged 5–6 years, as shown in the cattle experimental shed (Figure 1). The blood samples were carefully collected via the jugular vein into a 10 mL vacuum blood collection tube treated with ethylenediaminetetraacetic acid (EDTA) anticoagulant. The collected blood samples were immediately transported in a cooled iced packed box within 5 min to the Translational Research Platform for Veterinary Biologicals (TRPVB) Laboratory at Tamil Nadu Veterinary and Animal Sciences University, Chennai, India where laboratory analyses such as isolation of PBMCs, TSS procedure including measurement of cell viability were immediately performed.

Figure 1 India Gir Zebu cattle kept in the open-ended cattle experimental pen

Fractional Separation of Peripheral Blood Mononuclear Cells from Fresh Whole Blood

Five (5) milliliters of fresh animal blood samples were homogenized and adequately mixed. Then, homogenized blood was added in an equal V/V ratio to 5 mL of previously prepared phosphate-buffered saline (PBS) pH 7.4 (8.58 g of PBS powder (HiMedia Laboratories, Mumbai, India) was dispensed into a

measuring cylinder containing 1,000 mL of double-distilled water and was allowed to thoroughly dissolve. A moderate homogenization and thorough mixing with pipetting up and down of the Blood-PBS mixture came next. A 5 mL of blood-PBS mixture was fetched and carefully added to 3 mL of Histopaque®-1077 having a density of 1.077 g/mL (Sigma-Aldrich Co. LLC, Darmstadt, Germany) in a new 10 mL conical tube and was immediately spined.

This was done in a REMI R-4C laboratory centrifuge at 400 x g (3,000 rpm) for 20 min (Goregaon East, Mumbai, India). Four distinct layers, including the top yellowish plasma layer, the milky PBMCs layer directly below the plasma layer, the histopaque layer, and the bottom layer containing erythrocytes and granulocytes, were obtained during fractional separation of fresh whole blood using Histopaque®-1077 (Sigma-Aldrich Co. LLC, Darmstadt, Germany) as shown in Figure 2B. After centrifugation, PBMCs were collected with the least amount of plasma (and Histopaque®-1077) feasible and put into a new 15 mL conical tube.

The PBMC suspension was then thoroughly homogenized by pipetting up and down after adding 10 mL of PBS, followed by 10 min of centrifugation at 100 x g (1,500 rpm). After that, the supernatant was thrown away in order to recover the PBMC pellet. The technique (washing with PBS, mixing, and centrifuging at 100 x g (1,500 rpm) for 10 min was done three times) was performed on the recovered PBMCs pellet. The PBMC pellets were then added with 1 mL of nutrient media consisting of fetal bovine serum (100 mL) and RPMI-1640 (900 mL) (HiMedia, Laboratories, Mumbai, India), and cells were gently resuspended by up and down pipetting the mixture. The trypan blue dye exclusion method was used to count and assess the viability of the fractionated PBMCs, and TSS was performed on the PBMCs immediately (Onasanya *et al*., 2022).

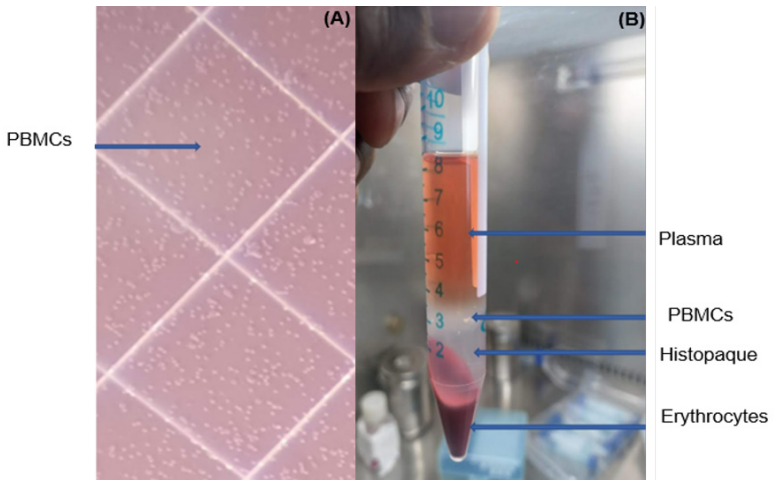

Figure 2 Detection of PBMCs using hemocytometer viewed under microscope (A) and fractional separation of bloods sample showing PBMCs and other fractional layers (B). PBMCs = peripheral blood mononuclear cells.

Different Thermal Assault Conditions and Durations of Exposure

The procedures were carried out in accordance with earlier published procedures for different temperatures and durations/time (Onasanya *et al*., 2022). In this study, 45 animals were placed into nine treatment groups in a factorial design such that all PBMC samples were randomly allocated to different

dosages of temperature (37 and 45ºC) and time (1, 2, 3, and 4 h) without preference except for control which received zero temperature (0ºC) for 0 h-DTE, with five animals per group. Blood samples of the 45 Indian Gir cattle were used to obtain 45 aliquots of PBMCs, both stressed and unstressed cells included. Each of the PBMCs received two temperatures (37ºC: normal temperature and 45ºC: extreme temperature) for 1, 2, 3, and 4 h, except for control, which received zero temperature (0ºC) for 0 h-DTE as shown in Figure 3. 37ºC is the average temperature considered as normal temperature for mammalian species for proper body functions (Onasanya *et al*., 2022), hence we used extreme 45ºC to study how HS provoke thermal apoptosis within the cellular systems of Indian Gir cattle while zero temperature (0ºC) was used to monitor extent to which HS caused cell death in PBMCs of Indian Gir cattle.

Before the TSS procedure, the average number of viable cells before TSS was between 7.655 and 8 × 10^7 cells in 500 µL. All PBMC aliquots were first stabilized by being incubated for 30 min at 37°C in a basic nutrient media containing RPMI 1640 (900 mL) and fetal bovine serum (100 mL). After 30 min of initial stabilization of both stressed and unstressed samples in nutrient media at 37°C in 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, Massachusetts, USA), the control samples labelled unstressed were immediately harvested for cell counting and viability checked (Onasanya *et al*., 2022).

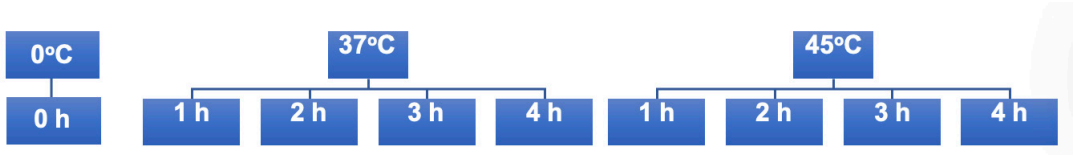

Figure 3 Thermal stress stimulation design showing different TACs durations for thermally stressed and Unstressed PBMCs of Indian Gir cattle. TACs = thermal assault conditions, PBMCs = peripheral blood mononuclear cells.

Estimation of the Cell Number and Viability

The estimation of cell viability and number was done in accordance with previously published procedures and methods (Onasanya *et al*., 2022). Following PBMC isolation, the trypan blue dye exclusion method was used to evaluate PBMC quantity and viability. The trypan exclusion dye approach involved staining PBMCs with trypan blue dye, excluding the live cells while staining the dead cells, which could then be seen on a hemocytometer (Microyn Improved Neubauer Hemocytometer, Maryland, USA) under a microscope (Euromex Microscope bv, Papenkamp 20, 6836BD Arnhem, Holland, Netherlands) as shown in Figure 2A. The average number of viable cells contains about 8.00–7.655 × 10^7 cells/500 µL before TSS.

Thermal Stress Stimulation

The processes for thermal stimulation were carried out in accordance with previously published techniques described by Onasanya *et al*. (2022). Separated PBMCs were split into two groups, one of which was exposed to TS and the other was not. For stabilization, each aliquot of PBMCs (500 µL) in a clear 1.5 mL microcentrifuge tube was first cultured in a nutrient media (RPMI 1640 and fetal bovine serum), HiMedia, Laboratories, Mumbai, India) at 37°C for 30 min in an incubator with 5% CO_2.

Temperature and DTEs were used to perform an *in vitro* TSS on the separated PBMCs. The PBMC aliquot (500 µL) was labelled and treated to three different temperatures considered as no TS (0°C),

normal (37°C), and extreme (45°C) in a circulating REMI RSB-12 water bath (REMI Lab World, Mumbai, India): three treatment groups (no TS (°C): control, 37°C: normal and 45°C: extreme) and four DTEs (1, 2, 3, and 4 h) as shown in Figure 3. After completion of TSS, the stressed PBMCs were allowed to recover at 37°C for 30 min in a 5% CO_2 incubator before being harvested by trypsinization, and quantity of viable PBMCs and viability were immediately estimated via the Trypan blue exclusion dye method as earlier described above. In contrast, unstressed control samples (500 µL) were exposed to no TS (0°C) and 0 h-DTE and were harvested immediately after 30 min of stabilization in a 5% CO_2 incubator.

Estimation of Viable PBMCs Isolated from Indian Gir Cattle

The viable PBMCs were counted, and viability was estimated according to Onasanya and his co-workers using various parameters as shown in Equations [1]–[4] (Onasanya *et al*., 2022). The 50 dilution factors (df) were used for the estimation of the PBMC count and viability test.

% Viability of PBMCs = [Total viable PBMCs/Total number of PBMCs (Viable + Dead)] × 100 --- [1]

Average number of viable PBMCs per square = Total number of viable PBMCs in 4 squares/4 --- [2]

Dilution factor = Total volume (Volume of PBMCs + Volume of trypan blue dye/Volume of PBMCs) --- [3]

Concentration of viable PBMCs/square = Average number of viable PBMCs × Dilution factor × 10 --- [4]

Statistical Analyses

Data generated for analysis of variance (one-way analysis of variance) were analyzed using the generalized linear model of the statistical analysis system (SAS) Software Version 9.2 (SAS Institute Inc., Cary, North Carolina, USA). The model (Equation 5) included parameters such as PBMC dependent variables, while temperatures and DTEs depicted TACs was independent variable. the effects of TACs on PBMC count were considered significant at $P < 0.01$. The mean comparison was performed using Duncan's Multiple Range Test.

The yield equation:

$$y_i = \mu + S_i + e_i \quad \text{--- [5]}$$

where y_i = observations of thermal-related performance of PBMC count, μ = overall mean, S_i = i^{th} effect of TACs on performance bovine PBMC count, and e_i = random error associated with y_j observations for thermal-related performance of bovine PBMCs.

Further to the above, the association analyses using Pearson's correlation analysis of Statistical Package for the Social Sciences Statistics (SPSS) version 20 (IBM, Chicago, IL, U.S.) was performed to test the strength of the relationship between an increase in temperature (HS) and PBMC count. The correlation coefficient (r) was considered significant for $P < 0.01$ at two-tailed.

Finally, regression analysis was performed to predict the reduction of PBMC count that can be accounted for by heat shock (HS) or attributed to thermal assault (increase in temperature). The regression coefficient (b) was considered significant at $P < 0.01$. According to Onasanya *et al*. (2022), both equations of regression (Equation 6) and correlation coefficients (Equation 7) are shown below:

Linear regression equation

$$y = a + b(x) \quad \text{--- [6]}$$

where x = predictor variable (TACs, an independent variable), y = response variables (PBMCs, a dependent variable), a = TACs intercept, which is the plot on the x (where there is zero TAC/ no temperature), b = slope of regression line.

Pearson's correlation coefficient equation

$$r = \frac{n\sum xy - (\sum x)(\sum y)}{\sqrt{[n\sum x^2 - (\sum x^2)][n\sum y^2 - (\sum y^2)]}} \quad \text{--- [7]}$$

where n = number of observations, $\sum x$ = total values of TACs, $\sum y$ = total values of PBMCs count, $\sum xy$ = sum of the product TACs count and PBMCs count, $\sum x^2$ = sum of the squares of TACs, $\sum y^2$ = sum of the squares of PBMCs count.

RESULTS AND DISCUSSION

Number and Viability of PBMCs of Indian Gir Cattle Upon Exposure to TACs

The viability and numbers of PBMCs were estimated before and after exposure to TS. The 100% PBMC viability was obtained, indicating that all PBMCs were viable. After the exposure of PBMCs to TACs, the estimated number of viable PBMCs ranged from 7.66×10^7−1.24×10^6 cells/mL.

Effects of Different Temperatures-DTEs Combinations on PBMC Count of Indian Gir Cattle

The summary of mean performance values for PBMC count exposed to different temperature and DTEs is presented in Table 1. In all, our findings showed that tougher temperature and longer DTEs negatively impacted PBMC count ($P < 0.01$). For instance, the highest PBMC count ($7.66 \times 10^7 \pm 0.12$ cells/mL) and the highest performance obtained during the no-stress and zero-time DTE (control). Suffice it to say that, in the control experiment, PBMCs did not receive heat shock. Therefore, they were not affected by thermal apoptosis or cell death.

However, PBMCs that received extreme temperature (45°C) for 4 h-DTE were the most negatively impacted by HS, as evident in the low PBMC count after the TSS procedure ($1.24 \times 10^6 \pm 0.32$ cells/mL (Table 1). The other temperature-DTEs and their corresponding effects are shown as presented in Table 1, and they follow a similar pattern. These results demonstrated that, as the temperature with DTE progresses on the increase, the numbers of thermally shocked PBMCs exponentially decrease. The summary of the PBMCs performance is as shown in Table 1.

Table 1 Least square mean values for interaction effect of different temperatures and time/duration of exposure on PBMC count of Indian Gir cattle

Thermal assault conditions (°C/h)	PBMC count (cells/mL)	P-value
0°C/0 h	$7.66 \times 10^7 \pm 0.12^a$	0.00004
37°C/1 h	$4.25 \times 10^7 \pm 0.11^b$	0.00002
37°C/2 h	$3.58 \times 10^7 \pm 0.22^b$	0.00001
37°C/3 h	$2.76 \times 10^7 \pm 0.33^b$	0.00003
37°C/4 h	$2.89 \times 10^7 \pm 0.45^b$	0.00004
45°C/1 h	$2.09 \times 10^6 \pm 0.34^c$	0.00001
45°C/2 h	$1.97 \times 10^6 \pm 0.22^d$	0.00002
45°C/3 h	$1.25 \times 10^6 \pm 0.12^e$	0.00005
45°C/4 h	$1.24 \times 10^6 \pm 0.32^f$	0.00001

Note: Means within the same column followed by different superscript letters (a, b, c, d, e, f) are significantly different at $P < 0.05$. Values are presented as least square mean ± standard error.

A Pearson correlation analysis (r) revealed a very strong positive association ($P < 0.01$) between different TACs and PBMC count. The correlation coefficients (r) are summarized as shown in Table 2. The correlation coefficient obtained from this study ranged from 84.5 to 92.6 (Table 2). Overall, we detected a 92.0% relationship in both correlation coefficient and regression coefficient between an increase in

temperature/time and PBMC count/cell viability, as presented in Table 2 for correlation coefficient and Figure 4 for regression coefficient. Correlation coefficients and regression coefficients are both measure of the degree of association between a predictor variable temperature/time (TACs) and response variable (PBMC count). Regarding this study, correlation coefficients and regression coefficients were used to estimate the degree of association between changes in doses of temperatures/time and viability of PBMCs in Indian Gir cattle upon exposure to HS/thermal stress. Biologically, this means that r and *b* were used to estimate the degree to which an increase in temperature and time cause PBMCs death in Gir zebu cattle upon exposure to heat shock. In this study, both correlation coefficients and regression coefficients were estimated for every unit increase in temperature/time. There was a corresponding 92% loss in PBMCs of Indian Gir cattle due to heat shock or harsh assault of thermal conditions (Onasanya *et al*., 2022).

Table 2 Association (correlation coefficient) analysis between PBMC count of Indian Gir cattle and different thermal assault conditions (TACs)

Thermal assault conditions	Correlation coefficient (*r*) for PBMC count	P-value
0°C/0 h	0	0.073
37°C/1 h	85.1	0.005
37°C/2 h	84.5	0.008
37°C/3 h	86.3	0.007
37°C/4 h	88.5	0.008
45°C/1 h	92.6	0.006
45°C/2 h	92.5	0.009
45°C/3 h	92.4	0.001
45°C/4 h	92.3	0.001
Overall TACs on PBMC counts	92.0	0.004

Note: Correlation coefficient (*r*) is significant at $P < 0.01$. $P > 0.05$ = not significant, PBMCs = peripheral blood mononuclear cells.

A normal distribution curve indicating that the regression model is a good fit for predicting the response variable (PBMC count) to HS/TS was observed (Figure 5). It also depicts that the residual error in the regression model is not sufficient enough to affect the validity and reliability of the prediction model (linear regression model). There is a very strong relationship between the predictor variable (TACs/HS) and the PBMCs death due to HS (Figure 6). The straight line that runs through the scattered plot shows that the prediction model (linear regression model) is fit for the data, simply put it's a measure of how data fits the linear regression model. Our analyses showed that the data is fit for the regression model, as shown in normal distribution curve and the regression model is fit for the data as shown in the scattered plot. These imply that the data obtained, and linear regression model used in this study are suitably reliable enough to predict the relationship/association between PBMCs death and thermal assault conditions.

Figure 4 shows the prediction of PBMCs response to an increase in temperature regarding the number of PBMCs deaths that resulted from elevated temperature (thermal apoptosis/HS) using linear regression analysis. We found a very strong regression coefficients (b) of 92.0% reduction in PBMC count and a high coefficient of determination ($R^2 = 84.7$). Increase in temperature (TACs) as a predictor variable

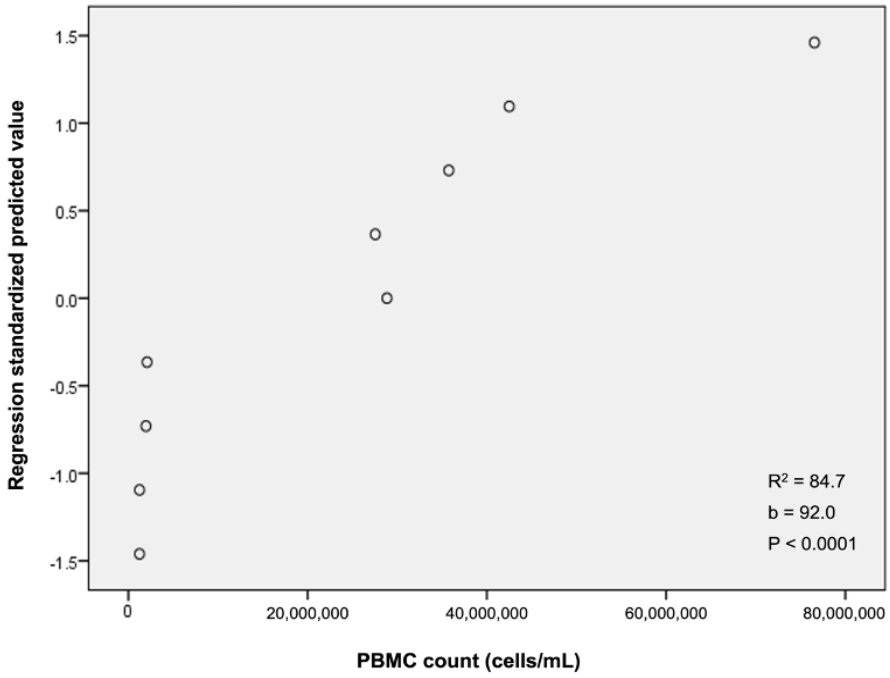

Figure 4 Graphical representation of the R-squared showing how increase in temperature caused loss in PBMC count due to thermal assault conditions/heat shock. PBMCs = peripheral blood mononuclear cells.

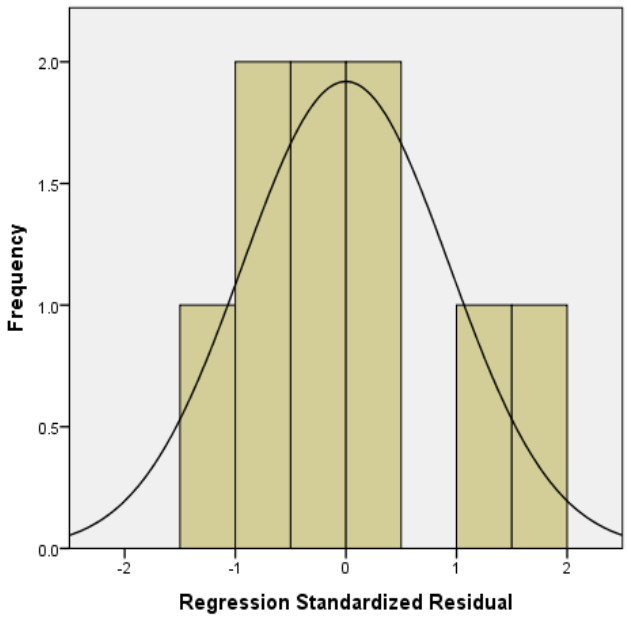

Figure 5 Histogram showing regression standardized residuals for PBMC count (response/dependent variable) due to the effect of TACs/heat shock (independent/predictor variable). PBMCs = peripheral blood mononuclear cells, TACs = thermal assault conditions.

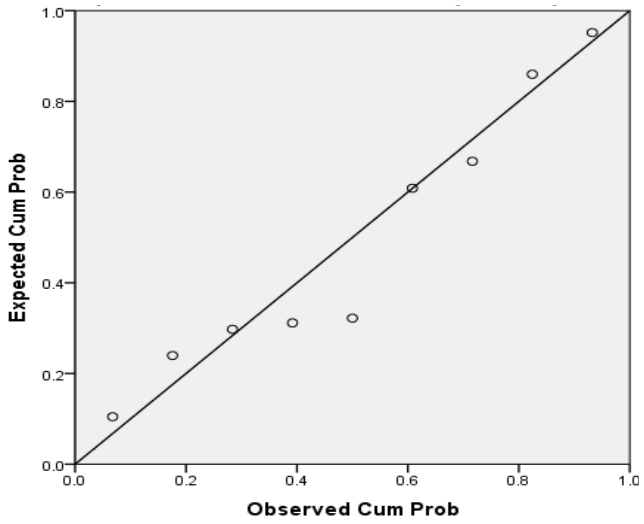

Figure 6 Regression plot showing normal P-Plot of regression standardized residuals for PBMC count (response variable) due to the effect of TACs/heat shock. PBMCs = peripheral blood mononuclear cells, TACs = thermal assault conditions.

accounted for PBMCs death. The R^2 is also a measure of goodness-of-fit for linear regression model for the data or how data fits the linear regression model. This test statistic predicts the quantity of the changes/ variation in the response/dependent variable that can be accounted for by the predictor/ independent variables. In the context of this study, the R^2 is used to estimate the quantity of PBMCs' death that can be accounted for by HS. In the context of this study, it means that for every 100 PBMCs exposed to thermal assault conditions, HS/thermal stress accounted for 84.7 PBMCs deaths within the cellular system of the studied animals. The findings of this study demonstrated that an increase in temperature (HS) resulted in an exponential loss in PBMC count and viability, where we reported the highest PBMC death at an elevated temperature of 45°C, whereas PBMCs exposed to no (zero) temperature had the highest PBMC abundance, viability, and survivability. The details of these results are graphically presented in Figure 7.

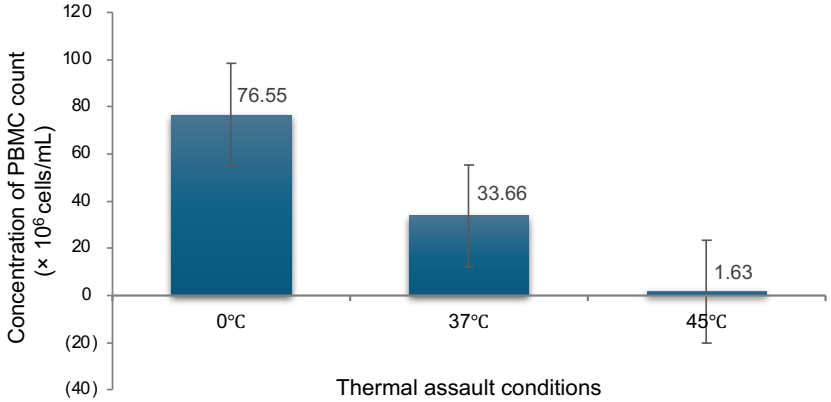

Figure 7 Overall effect of temperature on PBMC count of Indian Gir cattle. Reduction in cell count and viability of PBMCs at overall different TACs were significantly different. Error bars represent the standard deviation of the mean value at $P < 0.0001$ level of significance between the treatment and control groups. PBMCs = peripheral blood mononuclear cells.

The performance and the survival of livestock animals are both negatively impacted by climate change, which has grave consequences. Understanding how animals react to different temperatures and DTEs is crucial to achieve this goal. Recent reports from the literature confirm that PBMCs are a useful biological model for analyzing how livestock animals react to HS (Kheirandish *et al*., 2017). Thermal assaults/HS associated with senescence have been shown to cause loss of the ability of cells to proliferate and damage cellular processes, including the immunomodulatory activity of immune response cells (Kim *et al*., 2020). Circulating leukocytes, including peripheral mononuclear cells, can be used as a cellular model to learn more about how HS affects the animals' bodies and how HS causes chronic inflammation and raises the chance of provoking a wide range of diseases in bovines and other mammalian species (Kheirandish *et al*., 2017; Sheikh *et al*., 2017; Habeeb *et al*., 2018; Dado-Senn *et al*., 2020).

According to Simoni *et al*. (2020), during severe TACs, the cellular system experiences HS, and as a result, its defense mechanisms are rendered ineffective in combating TS and opportunistic infections. In contrast to control samples subjected to no TACs, we discovered in the current study that harsher *in vitro* TACs were directly correlated with a smaller number of PBMCs. Kim *et al*. (2020) reported that tougher TACs impede cell proliferation, lead to cellular failure and a loss in cellular functioning and induce thermal apoptosis. HS has been reported to cause cell death and a decline in PBMC counts by preventing cell proliferation and lowering cellular activities that support cell proliferation (Li *et a*l., 2014; Bhanuprakash *et al*., 2016; Li *et al*., 2016; Sun *et al*., 2018; Kim *et al*., 2020). Costa *et al*. (2011) revealed that heat shocks or harsher TACs block the distribution of the macromolecules needed for cell proliferation by inhibiting the pathways that lead to cellular proliferation.

From our study, regression standardized residuals, as depicted in the histogram and regression plot, showed a normal distribution curve, indicating that the regression model is a good fit for predicting the response variable (PBMC count) to HS (Kiernan, 2014). This implies that the regression model is valid, reliably suitable and a good fit for the prediction of PBMC reduction due to heat shock. Also, the regression plot depicts a normal distribution profile, indicating that the prediction model/regression model is fit for the data (Kiernan, 2014). Furthermore, we tested for a relationship using correlation analysis between temperatures-DTEs and PBMC count/viability and found a significantly strong correlation coefficient (*r*) of 92.0%. Meaning that the apoptotic effect of heat shock/thermal assault on PBMCs of the Indian Gir cattle was very profound (Onasanya *et al*., 2022). This implies that, as TACs toughen, more PBMCs perished.

Based on our analysis, we quantitated and regressed PBMC count over different doses of temperature (TACs/heat shock), and we discovered a significantly strong regression coefficient (*b*) of 92.0%, predicting a 92.0% response of PBMCs (response variable) to heat shock/thermal assault (predictor variable). This implies that there is strong relationship between an increase in temperature and the decrease in the proportion of PBMCs that perished due to an increase in temperature. The biological implication of this is that out of the total PBMC count exposed to TAC 92.0% of them were perished by HS as temperature/DTE increases. This suggests that a long-term increase in heat shock/TACs accounted for a 92.0% decrease in PBMC count (Kiernan, 2014; Onasanya *et al*., 2022).

However, there was no discernible difference between the heat-shocked or thermally stressed PBMCs of the control because they were not exposed to *in vitro* heat shock or TSS (Onasanya *et al*., 2022).

After heat shock, we also calculated the R^2, and we discovered that R^2 was 84.7 for a decrease in PBMC count. This implies that 84.7 PBMCs per 100 PBMCs perished as a result of heat shock, meaning that for every 100 PBMCs, heat shock accounted for 84.7 PBMCs' deaths (Onasanya *et al*., 2022). Our results revealed a regression coefficient that is greater than the values obtained for the R^2; this indicated a very significant association between the predictor variable (HS/TACs) and the response variable (Kiernan, 2014; Onasanya *et al*., 2022).

According to the results of our investigation, PBMCs subjected to mild/moderate thermal conditions of 37°C experienced the least heat shock or thermal assault compared to those exposed to 45°C. Next, to control samples that weren't exposed to heat shock, this temperature-time of exposure (37°C) produced the highest PBMC viability. This situation further demonstrates that a moderate *in vitro* temperature setting (37°C) mimics the body temperature of mammals and provides the body temperature necessary for healthy cellular system functioning and survival (Siddiqui *et al*., 2020; Onasanya *et al*., 2022). The results of the current study demonstrate that PBMCs functioned better under a mild *in vitro* thermal condition of 37°C, further demonstrating that 37°C is the typical body temperature needed for optimal biological processes. This is possible because cells thrive better at a temperature of 37°C *in vitro*, which mimics mammalian body temperature (Olson *et al*., 2011; Wang *et al*., 2017).

The duration of the cell cycle, the production of nucleic acids during the S phase of the cycle, and the breakdown of nucleic acids can all be compromised by harsher thermal conditions or thermal assaults on cells (Siddiqui *et al*., 2020). Additionally, a prolonged "S" phase suggests a greater potential for cell proliferation, which often takes place at 37°C under moderate temperature circumstances. Similarly, we reported greater PBMC count/viability under a normal temperature condition of 37°C; this is consistent with recently published works of previous authors (Onasanya *et al*., 2020). In those works, authors reported that thermal conditions at 37°C mimic typical body temperature and provided a favorable atmosphere for PBMCs of Indian Zebu-Jersey crossbred with higher PBMC count and viability than those exposed to harsher TACs.

From our results, we observed that as the temperature with DTE progresses on the increase, the numbers of thermally shocked PBMCs exponentially decrease as a result of thermal apoptosis. This is in line with earlier research on PBMCs from Indian Zebu-Jersey crossbred, in which the authors reported that an increase in temperature and exposure duration subjects PBMCs to a harsher thermal assault and heat shock, leading to prompt apoptosis or cell death (Onasanya *et al*., 2022).

Due to the activation of extracellular protein kinase and phosphoinositide 3-kinase/Akt signal transduction during transcription, nucleic acid synthesis is increased in normal body temperature (37°C) circumstances (Roux and Topisirovic, 2018; Xie *et al*., 2019). Additionally, it was discovered that prolonged S and G2/M stages of the cell cycle led to an increase in cell viability, cell proliferation, and nucleic acid production (Gao *et al*., 2015; Siddiqui *et al*., 2021). Additionally, a recent study found that TAC exposure made cells at the G1/S or G2/M checkpoints, as well as those in the S phase, more susceptible to cell death, including proliferative arrest (Simoni *et al*., 2020).

Lymphocyte functions were impaired during cellular failure caused by different temperatures and durations because thermal assaults result in a drop in lymphocyte numbers. A thermal assault/heat shock also reduces the survival of PBMCs while increasing the production of several immune response genes,

including toll-like receptors, interleukins, and cytokines that support the body's defense against infectious pathogens (Coleman and Tsongalis, 2017). Heat shock impairs the ability of immune cells, especially lymphocytes, to combat foreign objects, leaving animals more susceptible to harmful infections and resulting in poor health, lower productivity, and financial loss (Dado-Senn *et al*., 2020).

The results of a previous *in vivo* TS study on PBMCs of Bama miniature pigs and an earlier *in vitro* investigation on cell proliferation in native and crossbred dairy cattle demonstrated that exposure of animals to severe heat shock for a long time resulted in a reduction in the reactivity of immune cells, such as lymphocytes. This could increase the likelihood of some pathogenic illnesses in environments with extreme temperatures (Ju *et al*., 2014). Studies have proven that exposing cells to mild or low temperatures, such as 37°C, boosts their growth rate, viability, and survival and influences embryo development (Vergara *et al*., 2014; Bharati *et al*., 2017). This is possible because Taq polymerase, which has a significant amount of enzymatic activity for DNA synthesis, has a favorable physiological environment at the typical body temperature (37°C). Moreover, it has been demonstrated that exposing cells to moderate or mild TACs promotes cell proliferation, including survival (Choudhery *et al*., 2015; Wang *et al*., 2017).

The current *in vitro* TS on PBMCs of Indian Gir cattle confirmed the susceptibility of the cellular systems of bovine species to thermal assault. At higher or more extreme temperatures, the PBMCs were fewer in number and experienced thermal apoptosis. Tough TACs/heat shock were found to harm PBMCs in an *in vitro* study comprising bovine and human species (Bhanuprakash *et al*., 2016; King *et al*., 2020). According to Bhanuprakash *et al*. (2016) and King *et al*. (2020), long-lasting and severe TACs reduced lymphocytes' ability to respond to mitogens exponentially. This suggests that subjecting animals to the intense sunlight or hot temperatures of tropical conditions causes catastrophic cellular failure, resulting in a low cell count, cellular inhibition, impaired optimum performance, and possibly thermal apoptosis (Kim *et al*., 2020).

Compared to other TACs and TSS procedures studied, heat shock/thermal assault had the greatest influence on the PBMCs of the Indian Gir cattle at 45°C for 4 h-DTE, resulting in higher cell death/thermal apoptosis. The findings of this study are similar to previously published reports of Onasanya *et al*. (2022) in Indian Zebu-Jersey crossbred cattle, where PBMCs exposed to extreme temperature for a long time suffered thermal apoptosis and decline in viability. It's important to note that PBMCs that were exposed to no HS had the highest cell count and viability. This further confirmed earlier published works of Onasanya *et al*. (2021; 2022) on PBMCs of Indian Zebu-Jersey crossbred cattle, where PBMCs exposed to no HS had higher abundance of cell count and viability. Further to the above, when cellular systems of mammalian species are exposed to heat shock for a long time, such as harsher tropical temperatures prevent normal cell performance and may result in cell death/thermal apoptosis. Therefore, toughened temperatures-DTEs combinations (HS) have deleterious consequences on the cellular system and survival of Zebu cattle in the tropics. Biological information obtained from this study offers a better understanding of the potential of thermo-tolerance in bovine species, including other mammalian species raised under the assaults of thermal conditions of real-life scenarios for improved adaptation, survival, and performance.

Finally, this study will be validated in our future works using RNA-seq technology to further investigate the responses of heat shock protein genes and cytokines to different combinations of temperature and time (DTE) where RNA-seq will be used to perform

differential expression of mRNA transcripts of these candidate genes including gene ontology (molecular function, biological process and cellular component) and protein-protein interaction (cytoscape analyses) which will reveal how graphical network of molecular species such as HSPs and cytokines intervene thermotolerance and immune response potentials in zebu cattle raised under assault of thermal conditions.

CONCLUSIONS

The findings of this study demonstrated that an increase in temperature (HS) resulted in an exponential loss in PBMC count and viability, as we reported the highest PBMC death at elevated temperature of 45°C for a longer time while PBMCs exposed to no (zero) temperature had highest PBMC abundance, viability and survivability. This study provided an insight and understanding into how pure bred Gir cattle respond to heat shock when exposed to different TSS *in vitro*, and biological information obtained from this study will be helpful in management of heat stress in the studied animals and other mammalian species raised under assault of thermal conditions of tropical environment/climate change.

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