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Assessing viability of peripheral blood mononuclear cells of Vechur and Holstein friesian crossbred cattle in response to *in-vitro* thermal stress

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Abstract
Climate change has profound effects on global weather patterns, with rising temperatures and altered precipitation contributing to shifts in ecosystems. In the Indian context, climate change has manifested in decreased monsoon circulation, increased heat waves, and altered cold wave occurrences. Livestock, particularly dairy cattle, are susceptible to these climatic changes, affecting their productivity and health. This study investigates the cellular stress response of peripheral blood mononuclear cells (PBMCs) from Vechur cattle (*Bos indicus*) and Holstein Friesian (HF) crossbred cattle (*Bos indicus* × *Bos taurus*) exposed to *in vitro* thermal stress temperatures (42 °C for heat and 25 °C for cold) compared to the control temperature (37 °C). The methodology involves PBMC isolation, culturing, and exposure to thermal stress, with subsequent assessment of cell viability. Results indicate a significant decrease in the viability of PBMCs after thermal stress exposure, with a more pronounced decline observed in that of crossbred animals. This study contributes to understanding the impact of climate-induced thermal stress on cellular function in livestock, emphasising the importance of considering genetic variability in heat-tolerant cattle breeds.

Keywords: Climate change, peripheral blood mononuclear cells (PBMCs), thermal stress, cellular stress response, genetic variability

Introduction
Climate change, as defined by the United Nations, refers to long-term shifts in temperatures and weather patterns within a region [1]. The fourth assessment report of the Intergovernmental Panel for Climate Change (IPCC) in 2007 unequivocally concluded that the climate system is undergoing unprecedented warming, experiencing large-scale changes not observed over millions of years [2]. Most of these changes can be attributed to human activities – greenhouse gas production, emission of aerosols, changes in land use via urbanization, etc. The expected increase in global surface temperature over 2021-2040 is at an average of 1.5 °C relative to the period of the beginning of the industrial revolution [3]. Under the Indian scenario, climate change has caused a gradual decline in the monsoon circulation and the amount of rain [4]. India is experiencing a notable rise in the occurrence of heat waves and a decline in cold waves during the respective hot and cold seasons. Projections indicate a significant increase in heat waves, a decline in cold waves, and a rise in warm nights and days by the end of the 21st century [5].

In harsh cold conditions, animals undergo physiological changes such as muscle shivering, elevated heart rate, deeper breathing, and an increased metabolic rate, leading to higher nutrient and energy requirements. Dairy cattle are presumed to thrive within a neutral temperature range of -5 °C to 25 °C, and temperatures below -0.5 °C have been shown to impact milk production negatively. Exposure to cold stress in dairy cows can result in heightened plasma free fatty acid levels and reduced milk yield. Additionally, heifers exposed to cold stress during the breeding program initiation exhibit delayed pregnancy rates compared to those in milder temperatures [6]. In elevated temperatures, animals exhibit a decrease in feed intake of approximately 3–5% for each additional degree rise, leading to a decline in productivity. Heat stress contributes to heightened respiration and mortality rates, diminished fertility, alterations in animal behavior, and suppression of the immune and endocrine systems. Consequently, this heightened susceptibility to certain diseases can have significant impacts on the economic performance of both dairy and beef production systems [7].

Indian cattle - *Bos indicus* (*B. indicus*) have an added advantage in being more thermotolerant than *Bos taurus* cattle (*B. taurus*) due to the genetic variability conferred to them through evolutionary processes. [8] Studies in the peripheral blood mononuclear cell (PBMC) model have been conducted to understand the underlying differences (genetic and physiological) in the thermal stress response of different breeds [9]. To contribute to the collective knowledge on cellular stress in animals, the present study was conducted. It aimed at replicating and testing whether the PBMC cultures from Vechur cattle (*B. indicus*) and Holstein Friesian crossbred cattle (CBHF) (*B. indicus* × *B. taurus*) differ in their response upon *in vitro* exposure to two different thermal stress temperatures – heat (at 42 °C) and cold (25 °C) – when compared to the control temperature (37 °C).

2. Materials and methods

2.1 Collection of Samples

The trial involved conducting experiments on peripheral blood mononuclear cells (PBMCs) obtained from the whole blood of CBHF and Vechur cattle housed at the University Livestock Farm and Fodder Research Development Scheme (ULF & FRDS), Mannuthy, and Vechur Cattle Conservation Unit, Mannuthy, respectively, of the Kerala Veterinary and Animal Sciences University.

The selected CBHF animals had HF crossbred animals as their sires for at least two generations. The Vechur cattle were accommodated in a semi-intensive shed that included both a shaded area and an open space. In contrast, the crossbred animals were housed in an intensive system with a head-to-head arrangement.

Blood samples were taken from six apparently healthy heifers randomly chosen from each genetic group for the isolation of PBMCs. At the time of blood collection, the rectal temperature and respiration rates of all experimental animals were recorded. The entire process of PBMC isolation and culturing was conducted within a laminar air flow hood (Labline Instruments, Kochi) to ensure a sterile environment. Additionally, precautions were taken to utilize sterile chemicals, plastic wares, and glass wares.

Thirty millilitres of blood were collected aseptically from the jugular vein of every animal into vials containing sodium heparin solution (10 IU/ml blood) as an anticoagulant. These samples were promptly transported to the laboratory, and the isolation of PBMCs commenced within one hour of collection. The PBMCs were isolated through density gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich, Cat. No. 10771), following a modified version of the manufacturer's protocol.

2.2 Isolation and culturing of PBMCs

Initially, the whole blood (2 × 15 mL) was centrifuged at 2500 rpm for 10 minutes to separate it into plasma, buffy coat, and a layer of red blood cells (RBCs). Subsequently, the buffy coat, accompanied by a small amount of plasma and RBCs, was collected into a separate 15 mL centrifuge tube. To dilute the contents, an equal quantity of 1X Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich, Cat. No. D8537) was added. The diluted buffy coat was then gently layered over an equal volume of Histopaque®-1077, ensuring careful prevention of mixing between the two layers. Centrifugation at 2500 rpm for 30 minutes at 20 °C facilitated the separation of the buffy coat into an upper layer of plasma, a thin white layer of PBMCs, and a clear layer of Histopaque®-1077, with RBCs settled at the tube's bottom.

The PBMC layer, along with a small quantity of plasma, was cautiously aspirated and transferred to a new sterile 15 mL centrifuge tube. Care was taken to avoid aspirating the underlying Histopaque®-1077 layer. Subsequently, the PBMCs underwent two washes with 1X DPBS through centrifugation at 2500 rpm at 20 °C, with the pellet being resuspended each time to ensure thorough removal of any remaining plasma and RBCs. Finally, the PBMC pellet was suspended in one millilitre of the prepared RPMI 1640 culture medium for cell counting and plating.

The culture medium was formulated by supplementing RPMI 1640 medium (Sigma Aldrich, Cat. No. R8578) with 10 per cent heat-inactivated fetal bovine serum (Sigma-Aldrich, Cat. No. F7524), and a 10 µL/mL antibiotic antimycotic solution (Sigma-Aldrich, Cat. No. A5955) containing 100 U of penicillin, 100 µg streptomycin, and 0.25 µg of Amphotericin B per millilitre.

The determination of cell count per millilitre and cell viability was conducted using the Trypan blue dye exclusion method on the Invitrogen™ Countess™ automated cell counter. The PBMC suspension underwent a ten-fold, and this dilution was employed for the cell counting process. For this, 5 µL of the PBMC suspension was combined with an equivalent amount of 0.04 per cent Trypan blue dye (Sigma-Aldrich, Cat, No. T8154). The resulting mixture was then loaded into the counting chamber of the Invitrogen™ Countess™ automated cell counter (Thermo Scientific, Cat. No. C10227).

The volume of the cell suspension required for seeding was calculated as shown below:

1. Concentration of cells in suspension = C_1 viable cells/mL (value obtained from the automated cell counter)
2. Volume before dilution = V_1 mL = 1 mL
3. Concentration of cells for seeding = 1×10^6 viable cells/mL
4. Final volume after dilution = V_2 mL

V_2 is calculated as follows: $C_1V_1 = C_2V_2$

∴ Volume required for dilution, $V = V_2 - V_1$

The cell suspension prepared for each sample was diluted by adding V millilitres of prepared RPMI 1640 culture medium. Seeding was done in triplicates for each sample, in sterile 6-well plates, at a concentration of 1×10^6 viable cells/mL. The PBMCs of each animal were divided into three sets and were incubated at 37 °C for 24 h in a CO_2 incubator (Thermo Scientific, Cat. No. 371) at 5% CO_2 level and 95% relative humidity. The cells were cultured initially at 37 °C for 24 hours following which they were exposed to thermal stress treatment.

2.3 Exposure of PBMCs to thermal stress treatment

Following 24 hours of culturing at 37 °C, the thermal stress treatment was administered as follows: one set of cell cultures underwent exposure to 25 °C (cold stress treatment), the second set to 42 °C (heat stress treatment) for one hour, and the third set was subjected to 37 °C (control) for one hour, as outlined by Fang *et al.* (2021) [12]. Consequently, cells from each genetic group had two treatment groups and one control group.

After exposure to treatment, the cells from both the control and treatment sets were extracted from the CO_2 incubator. Cell viability was assessed through the Trypan blue dye

exclusion method utilizing the Invitrogen™ Countess™ automated mammalian cell counter (Thermo Scientific, Cat. No. C10227).

The paired t-test was applied to the viability per cent data of both genetic groups obtained before culturing and after thermal stress treatment. The null hypothesis was that there would be no significant difference in the viability (both percentage and absolute count) of PBMCs before and after exposure to thermal stress temperatures in each genetic group.

4. Results

The respiration rates and rectal temperatures of the animals at the time of sample collection are given in Table 1. These values were within normal range, indicating that the animals were apparently healthy.

The concentration and viability of the PBMCs extracted from the blood of Vechur and CBHF animals, as measured before seeding and culturing are given in Table 2. The concentration and viability data of PBMCs after exposure to control and thermal stress treatment for one hour are given in Tables 3.

The results indicate no significant difference was observed in the cell viability (both percentage and absolute viable cell count) control set of both genetic groups. However, a significant difference was observed in the PBMC cultures of both genetic groups after the heat and cold stress treatment. The cell viability of the cultures in both stress treatments had decreased compared to the pre-seeding viability. The percent decline was higher in CBHF animals compared to Vechur animals.

Table 1: Rectal temperatures and respiration rates of the experimental animals as recorded before collection of blood samples

S. No.	Animal	Rectal Temperatures (°F)	Respiration rates (breaths/min)
1.	V1	99.9	19
2.	V2	99.8	23
3.	V3	99.9	20
4.	V4	100.8	22
5.	V5	99.8	18
6.	V6	101.2	30
7.	C1	99.8	20
8.	C2	100.5	16
9.	C3	99.9	18
10	C4	100.6	35
11	C5	101.8	15
12	C6	100.6	20

5. Discussions

The serious repercussions of climate change extend to livestock production, adversely impacting animal farming. Consequently, it becomes imperative to address and alleviate the adverse impacts of climate change on the well-being and sustainability of livestock animals. The results obtained in the present study are indicative of the response of PBMCs in culture to cold and heat shock.

The PBMCs have been reported as a suitable model for conducting thermal stress (cold and heat shock) studies in livestock animals [9]. The PBMCs are circulating lymphocytes and monocytes which respond to thermal stress by differences in their cell proliferation and activation of various genes and pathways at the molecular levels [10].

Cell viability was found to decrease significantly in heat-stress samples compared to control samples, *in vitro*. This was also true for cold-stress samples compared to control, *in vitro* [11, 12, 13].

Additionally, an experiment was conducted by Kamwanja *et al.* (1993) [14] to assess the viability response of PBMCs from Angus (*B. taurus*), Brahman (*B. indicus*), and Senepol (*B. taurus*) breeds, to heat shock. The decrease in viability at 45 °C and 42 °C was reported to be highest in Angus heifers when compared to Brahman cattle. Similar results were noticed between Sahiwal and Frieswal cattle [15], and Sahiwal and HF cattle [9], where the viability of the *B. indicus* breeds was found to be less affected than the *B. taurus* breed/crossbreds. The present study also showed the same pattern, where the decline in the viability of cells was significant when thermally stressed PBMCs when compared to PBMCs at control temperature in both genetic groups. The decline was noticed to be higher for CBHF animals than that of Vechur animals.

Table 2: Concentration and viability of cells after 10-fold serial dilution in RPMI-1640 culture medium

Sample ID	Total cells/ml	Viable cells/ml	Viability (%)
V1	3.6×10^6	2.8×10^6	77
V2	1.3×10^6	1.1×10^6	86
V3	9.4×10^6	7.9×10^6	84
V4	6.8×10^6	5.8×10^6	84
V5	5.7×10^6	4.1×10^6	71
V6	6.2×10^6	5.4×10^6	86
C1	9.1×10^6	8.1×10^6	88
C2	5.4×10^6	4.5×10^6	84
C3	6.4×10^6	6×10^6	92
C4	1.1×10^7	1×10^7	95
C5	4.7×10^6	4.1×10^6	86
C6	1.2×10^7	1.1×10^7	94

Table 3: Concentration and viability of cells after stress and control treatments

Sample ID	T1 (37 °C)			T2 (25 °C)			T3 (42 °C)		
	Total cells/ml	Viable cells/ml	Viability (%)	Total cells/ml	Viable cells/ml	Viability (%)	Total cells/ml	Viable cells/ml	Viability (%)
V1	5×10^5	3.9×10^5	70	7.6×10^5	5.7×10^5	75	1×10^6	7.8×10^5	76
V2	4.9×10^5	3.3×10^5	67	6.2×10^5	4.1×10^5	66	7.1×10^5	4.2×10^5	59
V3	9.4×10^5	7.1×10^5	77	1.2×10^6	8.1×10^5	70	1.5×10^6	1×10^6	77
V4	2.5×10^6	2.1×10^6	85	8.3×10^5	5.1×10^5	62	2.1×10^5	1.5×10^5	71
V5	5.1×10^6	4.3×10^6	84	4.3×10^5	3.2×10^5	74	2.7×10^5	2×10^5	74
V6	4.7×10^5	3.9×10^5	82	8.8×10^5	6.2×10^5	70	3.2×10^5	2.5×10^5	78
C1	6.9×10^5	5.4×10^5	78	5.1×10^5	4.1×10^5	81	8.9×10^5	7.5×10^5	85
C2	8.6×10^5	7.3×10^5	85	8.8×10^5	6.3×10^5	71	1×10^6	7.3×10^5	70
C3	6.2×10^5	5.8×10^5	94	1×10^6	6.6×10^5	65	8.2×10^5	6.4×10^5	78
C4	1.8×10^6	7.7×10^5	43	2.2×10^6	9.9×10^5	44	3.6×10^6	1.7×10^6	58
C5	1.1×10^6	8.8×10^5	79	1×10^6	8.9×10^5	86	7.6×10^5	5.8×10^5	76
C6	1.5×10^6	5.9×10^5	40	2.9×10^6	5.9×10^5	21	2.5×10^6	9.5×10^5	38

The decrease in cell viability in response to heat stress can be attributed to its effect on the morphology and membrane structure [18]. Elevated temperature exposure in cells triggers various anomalies, such as inhibited protein synthesis, structural defects, morphological changes, altered metabolism, and reduced cell proliferation. These anomalies prompt significant changes in gene transcription and protein synthesis, collectively referred to as the heat shock response [16, 17]. The timing and effectiveness of these changes determine the cell's fate, whether it survives and acclimates or undergoes cell death [18]. This suggests that subjecting animals to intense sunlight or the thermal conditions characteristic of tropical climates can lead to severe cellular damage or malfunction. Such exposure may result in a decreased cell population or low cell counts, cellular suppression, hindered peak functioning, and could potentially trigger cell mortality or induce rapid apoptosis [13].

Studies on the response of PBMCs to cold stress are limited. In the present study, a significant decline in viability was also noticed in both genetic groups in response to cold stress. This result is in agreement with the findings of Fang *et al.* (2021) [12]. Acute cold stress is reported to decrease the rate of protein synthesis and metabolic rate, suppressing the cell's active functioning [19]. The reduced viability, in this case, can be a result of such effects of cold stress as a response to the disruption of cellular cytoskeletal elements, changes in the membrane permeability, and a generalised cold-induced inhibition of transcription and translation leading to changes in gene expression pattern.

Several research findings indicate that subjecting cells to mild or low temperatures, like 37 °C, can boost their growth rate, enhance survival and viability, and influence embryo development [19,20]. This effect is likely due to the optimal physiological conditions provided by the normal body temperature (37 °C), which favour the activity of Taq polymerase, an enzyme crucial for DNA synthesis [21]. The present study showed no significant differences in the viability of cells before and after 25 (24+1) hours of exposure to 37 °C, indicating that this temperature setting replicates the inherent body temperature of mammals, ensuring the presence of necessary conditions for proper cellular operations and survival [22].

6. Conclusion

From the present study, it can be concluded that PBMCs are significantly affected when subjected to heat and cold stress. Their viability was found to be significantly reduced in response to thermal stress when the stress group was compared with the control group at 37 °C. However, the viability of cells at the control temperature was not significantly affected after 25 hours in culture at 37 °C, indicating that this can be used as an effective control temperature for thermal stress studies in PBMCs in mammals. This study, in agreement with other previous reports, also suggests that PBMCs can be used as a model to study thermal stress in animals without physical stress to the animal to carry out further investigations at cellular and molecular levels regarding gene expressions and pathways involved in thermal stress response.

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