Impacts of chronic heat stress on Lymphocytic proliferation and phagocytic assayin Sahiwal and Tharparkar Cattle

Abstract

High temperatures causes many immune and physiological alterations in livestock, making them more vulnerable to a wide range of diseases. Lymphocyte proliferation assay and neutrophil phagocytic assay are frequently employed to evaluate cell-mediated immunity.The present study used in vitro culture of blood polymorphonuclear cells after animals were exposed to in vivo heat stress in order to examine the differing effects of heat stress on defensive responses in Sahiwal and Tharparkar calves. The objectives of this research were to contrast the thermo-adaptability of Sahiwal and Tharparkar breedson the basis of their immune responses. In the present study, ten male cattle aged between 1 to 1.5 years were selected and divided into two groups with five animals, each of Sahiwal and Tharparkar cattle. Animals were maintained inside a psychrometric chamber under the following conditions: 7 days acclimatization period at a thermo-neutral zone, 38°C temperature exposure for 6 hours upto 49 days, followed by seven days recovery period. Blood was collected once a week on the following days: -7, 0, 7, 14, 21, 28, 35, 42, 49 and 56. Physiological responses such as rectal temperature and respiration rate were measured daily.THI was calculated by temperature and relative humidity (RH). Following their isolation from blood, the polymorphonuclear cells were cultured at 38°C. Then, using NBT and MTT tests, respectively, phagocytosis and lymphocyte proliferation were assessed. The entire mean THI was significantly ($p< 0.5$) higher during heat exposure period (88.41± 1.54) when compared to the control period (64.75 ± 0.97). There is a significant high lymphocyticproliferation in Tharparkar Group when compared to Sahiwal Group cattle. Additionally, the level of PA significantly decreases during heat exposure period when compared to control period in both the breeds and in Sahiwal group Ithere is significantreductionin LPA during heat stress. The current study's findings imply that during the heat stress period, Tharparkar showed greater tolerance to heat stress than Sahiwal group.

Keywords: Cattle, Heat Stress, Immune Response, PMN, Lymphocyte proliferation assay, Phagocytic Assay, Sahiwal, Tharparkar

Abbreviations: LPA (Lymphocyte proliferation assay), PA (Phagocytic assay), PMN (polymorphonuclear cells)

Introduction

Global warming directly impacts livestock welfare by increasing incidence of heat stress. Dairy cows have been identified as the livestock species most sensitive to elevated temperatures and

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UNDER PEER REVIEW

humidity due to their high metabolic heat load and therefore are quite susceptible to heat stress (Das *et al*., 2016).Acute heat stress may have a stimulatory effect on the immune system, however chronic heat stress may have an inhibitory role on the capacity of the immune system to maintain homeostasis (Cantetet al., 2021). Heat stress inhibits the expression of genes involved in T-cell activation and cytokine production, which compromises cellular immunological capabilities through the release of cortisol (Bagath et al., 2019). Heat stress is well documented to reduce dry matter intake (DMI) in cattle, which consequently has a negative influence on nutrient absorption overall impacting the immune system and inflammatory response of cattle (Yadav et al., 2016). According to Dahl et al. (2020), heat stress has a detrimental impact on the health and productivity of dairy cattle. It also raises the risk of disease and renders the immune system. Heat stress impacts the cellular immune response by increasing the cortisol concentrations, as it binds to DNA and suppresses the expression of genes related to cytokine production and T-cell activation (Caroprese et al., 2014). The anti inflammatory properties of corticosteroids results in a decrease in phagocytic cell activity and alter lymphocyte function (Caropreseet al., 2012).Heat stress increases the secretion of glucocorticoids which acts as inhibitor of the pro-inflammatory cytokines such as TNF-α, IL-6, IL-8 initiating the innate immune responses by the inhibition of the p38 MAPK pathway which maintains the stability of the immune system in animals (Abraham et al. 2006).Stimulation of the HPA axis results in the production of cortisol, which is associated with a suppression of the immune system in cattle (Grandin, 2018).Glucocorticoids influence the balance of T-helper 1 (Th1) and T-helper 2 (Th2) through the inhibition of IL-12, whereas catecholamines inhibit IL-12 and enhanced IL-10 production (Inbaraj et al., 2016). Therefore, glucocorticoids and catecholamines may suppress cellular immunity and result in a preferential shift toward Th2-mediated HI (Elenkovet al., 2000).These health-related issues could be due to the impaired immune function observed in heat stressed dairy cattle. Heat stress can impact both the innate and adaptive arms of the immune system. The disruption of the balance between T-helper 1 (TH1) and T-helper 2 (TH2) responses is one of the impacts of heat stress on the adaptive immune response, causing a shift towards a TH2 response (Cartwright et al., 2021). This bias can lead to impaired cellmediated immune response (CMIR). In similar circumstances, it has been demonstrated that heat stress in dairy cattle decreases lymphocyte proliferation (Cartwright et al., 2023). Lymphocytes, which include B and T-cells, rapidly proliferate in response to invading pathogens in order to facilitate clearance. Accordingly, if lymphocyte proliferation is reduced it is more difficult for cattle to defend against invading pathogens.Several studies have assessed immunological changes in Holstein dairy cows under heat stress conditions. For example, the conditions associated with high temperature and dry environmental seasons have been found to affect immune responses, including reduction in lymphocyte proliferation, neutrophil, phagocytosis, and cytokine expression in Holstein dairy cows (Do Amaral et al. 2010, 2011). Moreover, heat-stressed Holstein cattle reportedly show reduced cellular immunity and enhanced humoral immune responses under prolonged heat stress (Laceteraet al. 2006). It is imperative to take prompt and suitable measures to conserve indigenous bovine genetic resources; otherwise, irrecoverable

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damage is likely to occur in the subsistence agriculturesystem of India. However, scientific information regarding immune systems alteration under heat-stressed environment is rather limited, particularly when it pertains to cattle. Not enoughfield works on the aspect of theadaptive capability of indigenous cattle,especially to the extreme climatic conditions that are predominant in a tropical climate like India, have been accomplished so far.

Material and methods

All the experiments, procedures, and protocols on animals wereconducted following the approval of the Ethics Committee,Indian Veterinary Research Institute, No.F.1-53/2012-13/J.D(R).

Experimental animals and site

The experiment involved 10 healthy Sahiwal and Tharparkar cattle of the same age group between 1 and1.5 years maintained at, Indian Veterinary Research Institute, Izatnagar, UP,India. The institute is situated in a subtropical climate at a height of 564 feet above mean sea level, with latitudes of 28°21′N and longitudes of 79°24′E.

Experimental design

Temperature and~~Relative~~relative humidity of the animal sheds were recorded daily. There were two different types of immunity tests used to assess the immunological status during the study period by using PBMCs and Neutrophils isolated from the blood within two hours of sample collection.

Blood sampling

Sterilized vacutainers were used to collect blood samples (spray-coated lithium heparin and clot activators containg tubes) at the seven day interval and during acclimatization period. During the fifty six days experiment period at 38°C, blood collection was done on day 0, 7, 14, 21, 28, 35, 42, 49, 56 days. On 56th day blood sample was collected as recovery sample. The blood sample was collected immediately after the heat exposure period in each day.

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Measurement of meteorological variable

The minimum temperature, maximum temperature, relativehumidity (RH) were recorded at the experimental shed dailyduring the experiment period using dry and wet bulb thermometer, and then the THI, a measure of thermal load onanimals, was calculated from a formula by McDowell *et al.*,1976.

Assessment of lymphocyte proliferation assay (LPA) and phagocytic assay (PA)

Peripheral blood mononuclear cells were isolated using Histopaque according to the method described by English andAndersen (1974), and The lymphocyte proliferation and

UNDER PEER REVIEW

Phagocytic assays were done as per the method described by Khatti*et al.* (2017).The phagocytic assay (PA) and lymphocyte proliferation assay (LPA) were used to estimate the phagocytic activity of neutrophils and proliferation of lymphocytes in vitro respectively. Peripheral blood samples were subjected to hypotonic lysis of erythrocytes followed by isolation of neutrophils. The neutrophils were pipetted in RPMI 1640 (Sigma -Aldrich, USA) culture media containing 10 % FB S (Gibco). Cell viability was determined using trypan blue and coun ting was done thereafter cell concentration was adjusted to 10^5 -10^6 live cells/ml.

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Lymphocyte proliferation assay (LPA)

After centrifugation of blood at 1000g for time period of 30 min; the buffy coat was collected and resuspended in phosphate buffer saline (PBS). The entire content was precisely layered on a lymphocyte separation medium in sterile 15ml polypropylene centrifuge tube and centrifuged at 700g for 30 min at room temperature Khatti et al., (2017). The layer rich in lymphocytes was removed and washed twice with PBS. The washed cells were then resuspended in RPMI 1640 culture media with supplementation of 10% FBS and antibiotics. The lymphocytes suspension was adjusted to 5×10^6 live lymphocytes per ml. A 96-well tissue culture plate with a flat bottom was filled with 200µl of diluted cell suspension per well, made in triplicate. The mitogen used for study was concanavalin A (5µg/ml), which stimulated T lymphocytes. During LPA, concanavalin A (5 µ g /ml), a mitogen was used to stimulate T lymphocytes, and B lymphocytes were stimulated by lipopolysaccharide (5 0 µ g /ml) (Dang et al., 2013). The cells were allowed to incubate with and without mitogen, to determine the difference in cell proliferation at 37°C in a humidified CO2 incubator (5% CO2 and 95% air) for 24 to 72 hours. The proliferative response of lymphocyte was estimated using the colorimetric MTT (Mosmann*et al.*, 1983). Approximately 50µl/ml of MTT solution to each well was added after culturing lymphocytes for the standard incubation period. The further incubation of plates at 38.5°C for 4 hours in humidified CO_2 incubator was done. This results in the formation of formazan crystals at bottom of each well during this period. The residual supernatant along with the suspended cells was pipette out without disturbing the forazman crystal layer. Then 100µl DMSO in each well was added and thoroughly mixed in order to dissolve the dark blue crystal. The optical density was read using ELISA reader in the dual wavelength measuring system, at a test wavelength of 503nm and reference wavelength of 630nm after incubating the plate at room temperature for 15 min. Plates were read within 1 hour of adding DMSO. Stimulation index (SI) or proliferation index calculated by using the formula:

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SI= Average OD with mitogen / Average OD without mitogen

Phagocytic activity or assay (PA)

Isolation of neutrophills from peripheral blood using Granulosep and Histopaque according to the methods described by English and Anderson (1974) and modified by Khatti et al (2017).The neutrophils cell suspension was adjusted to 1 to 5×10 live cells per ml by culture

UNDER PEER REVIEW

media containing 10% FCS and antibiotics. 200 µl of the diluted cell suspension per well, in triplicate, were added to a 96-well flat-bottomed tissue culture plate.Seeding was done at the rate of 20 0 µL /well in triplicate. DuringPA , incubationneutrophils with Zymosan (650 µg /ml) and Nitro blue Tetrazolium (NBT , 25 0 µg /ml)at 37 °C (Dang et al., 2013) for 2 hours. The quantity of phagocytosed zymosan was utilized as a PA indicator.The OD values were taken at 540 nm in Absorbance reader (Bio - rad). In a humidified CO2 incubator (5% CO2 and 95% air) all cultures are allowed to incubate at temperature of 37°C. The amount of zymosan which was phagocytozed was used as Phagocytic activity indicator. The yellow color of Nitro-blue tetrazolium changes to blue formazan after phagocytosis, which is measured spectrophotometrically (Simchoiet al., 2006). OD was taken using ELISA reader at 540nm.

Resultsand Discussion

Figure 1 shows the changes in the temperature-humidity index (THI) over a period of different weeks. During the period of experiment it was observed that THI was lowest during control period (25^{o}C) and was more during heat stress period (38oC). During the period of heat stress, the overall mean THI was observed to be significantly ($p<0.05$) higher as compared to control period. The differences in lymphocyte proliferation and phagocytic activity index among the groups during summer were presentedin Figures 2and 3, respectively.Both the breeds when compared on heat exposure period showed significantly ($p<0.05$) decreased in value of LPA during heat stress (38^{o}C) as compared to control period (25^{o}C). In both the breeds LPA values reduced significantly ($p<0.05$) up to 28 days of heat exposure and then started to increase. In case of Tharparkar cattle significantly ($p<0.05$) decrease was observed at 21st day of heat stress whereas in Sahiwal significant ($p<0.05$) reduction was observed on 14th and 21st day of heat exposure. Both the breeds when compared during heat exposure period (38^{o}C), significant difference was observed on 21st, 35th and 42nd day of heat exposure.

While comparing both the breeds, significant ($p<0.05$) decline in values of PA during heat stress (38^{o}C) as compared to control period (25^{o}C) was observed. Significant difference was observed between both on day 7 and 28 day.On comparing both the breeds, significant ($p<0.05$) difference in phagocytic activity was observed on all the days of heat exposure period, whereas no significant ($p>0.05$) difference was observed in control period between both the breeds.

In some cases, in vivo heat stress has been found to be associated with reduction in the levels of neutrophil phagocytosis (Tejaswi *et al.* 2020).LPA is used for assessment of proliferation ability of peripheral blood mononuclear cells (PBMCs) in response to antigen stimuli like concanavalin A. Al-Busaidi*et al.*,(2008) reported significant increase in lymphocytes during summers as compared to winters in goats. Decline in lymphocyte proliferation in reported by Elvinger *et al.*, (1991) in cows exposed to high temperature. In the study conducted, it was observed that LPA value of both Tharparkar and Sahiwal breeds significantly ($p<0.05$) decreased during heat exposure as compared to control period. The reduction in LPA values was more evident in Sahiwal than Tharparkar breed during heat exposure period as compared to control period. The observations were found to be similar with the observations of Mukherjee *et al.*,

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(2011) who reported, during heat exposure in buffaloes there was decrease in mitogen induced lymphocyte proliferation response.

The initial line of defense against pathogen invasion by phagocytic cells is crucial.In-vitro assessment of phagocytosis by engulfment of substrate by phagocytic cells. According to Callahan et al., 1999 heat shock response enhanced during heat stress and exerts anti-inflammatory action.In the experiment conducted, It was noted that phagocytosis rate was significantly ($p<0.05$) reduced in both Tharparkar and Sahiwal breed cattle during heat exposure (38^oC) as compared to control period (25^oC).The results were found to be similar with Lecchi *et al.*, (2016).

Conclusion

In contrast to Tharparkar cattle, Sahiwal cattle exhibit decreased lymphocyte proliferation and phagocytic activity (LPA and PA), which explain the decline in immune cell reactivity and increased susceptibility to infections during heat stress. Furthermore, it can be inferred from the study that Tharparkar has higher heat stress resistance than Sahiwal.

Declaration

Data availability The data will be made available and would be shared on reasonable request.

Ethics approval All the animal experiments had prior approval from the animal ethics committee of the Indian Veterinary Research Institute (IVRI), Izatnagar, UP, India.

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UNDER PEER REVIEW

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Figure Legends

Fig1. Temperature Humidity Index (THI) before, during and after heat exposure (38°C). All the values are shown as Mean±SE.

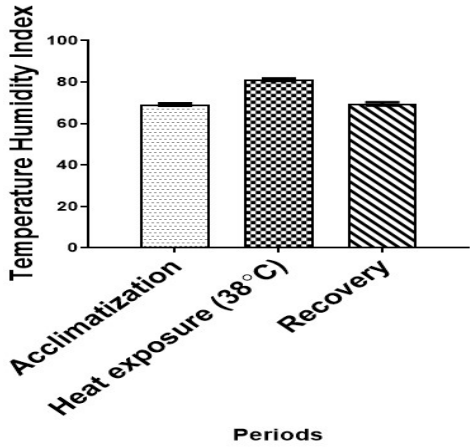


Fig2. Lymphocyte Proliferation Assay (SI) in serum of Sahiwal and Tharparkar breeds compared before, during and after heat exposure (38°C). Samples collected at -7 day, before heat exposure was taken as control. All the values are shown as Mean±SEM.Different superscripts denotes statistically different values (P<0.05).

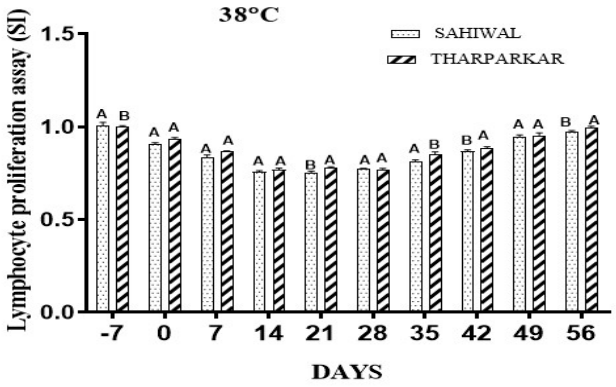
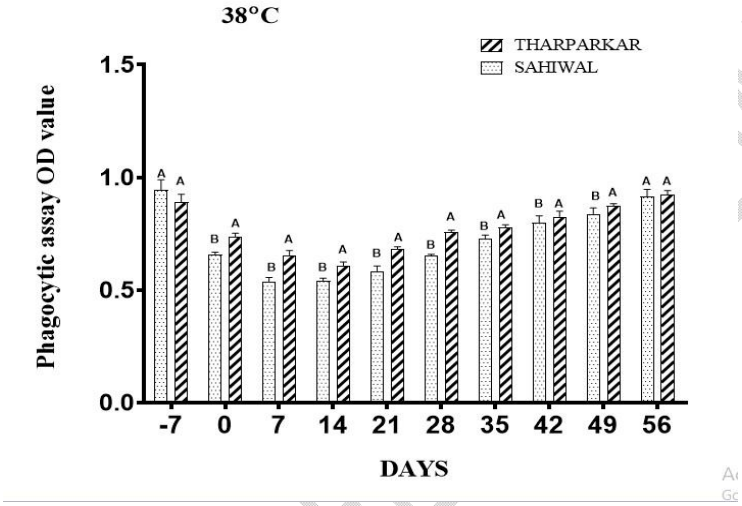


Fig3. Phagocytic Assay (OD value) in serum of Sahiwal and Tharparkar breeds compared before, during and after heat exposure (38°C). Samples collected at -7 day, before heat exposure was taken as control. All the values are shown as Mean±SEM.Different superscripts denotes statistically different values (P<0.05).



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