

Research Article

In Vitro Immune Competence of Buffaloes (*Bubalus bubalis*) of Different Production Potential: Effect of Heat Stress and Cortisol

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Twelve healthy lactating Murrah buffaloes of similar parity (3rd) between 90 and 120 days of lactation, selected from the herd of National Dairy Research Institute (Karnal, India) and maintained at managemental practices as followed at the Institute they were included in this experiment. The animals were divided into two groups based on their production level in previous lactation. The average milk production level of group I and II was 9.3 and 6 lit/day, respectively. Blood was collected from these buffaloes on three occasions 10 days apart. The lymphocytes were separated and cultured in RPMI 1640 medium with PHA-P for 24 h at 37°C in a humidified CO₂ incubator (95% air and 5% CO₂). The lymphocyte responsiveness was also evaluated in response to the *in vivo* heat stress and *in vitro* cortisol. Mitogen-induced stimulation index was not affected by production level ($P < .01$). Stimulation index was significantly reduced ($P < .01$) in both the groups when cortisol was added at 2.0 ng level in the culture. However, in heat-stressed buffaloes stimulation index did not vary despite increasing levels of cortisol, thus indicating that lymphocyte may become cortisol resistant during periods of acute heat stress. The results showed that lymphocyte proliferation response can be effectively used to study buffalo cell-mediated immunity *in vitro*.

1. Introduction

Induction of lymphocyte proliferative response induced by antigen/mitogen *in vitro* has been shown to be representative of cellular immunocompetence. This measure can potentially be used as an indicator of an individual's ability to mount an immune response to specific pathogen or immunomodulators. A series of *in vitro* studies in dairy cattle have demonstrated that exposure of bovine peripheral blood mononuclear cells to short and severe heat shock reduced their responsiveness to mitogens or decreased the number of viable cells [1, 2]. These authors suggested that exposure to high temperature *in vitro* can depress responses of lymphocytes; apparent adaptive mechanisms induced by *in vivo* heat stress provide protection from effects of high temperature seen *in vitro*. But so far no studies on validation have been conducted on the *in vitro* immune competence of buffaloes in response to heat stress. Therefore the present study has been designed to evaluate the potential of using *in vitro* lymphocyte proliferation assay for determining the cellular

immunocompetence of lactating buffaloes in response to *in vivo* heat stress and *in vitro* cortisol.

2. Materials and Methods

Twelve lactating Murrah buffaloes, apparently healthy and of similar parity (3rd) between 90 and 120 days of lactation were selected from the herd of National Dairy Research Institute (Karnal, India) and were divided into two groups according to milk production during previous lactation that is, average lactation yield \pm 1SD. Based on this, the production average of group I and II corresponded to 9.33 and 6.0 lit/day, respectively. All these buffaloes were maintained under general managemental practices as followed at the institute. The feed and water were available ad lib.

Buffaloes of both the groups were used as lymphocyte donors. Blood (15 mL/buffalo) was drawn in sterile heparinised vacutainer tube from jugular vein puncture, posing minimum disturbance to the animal during collection, on three occasions 10 days apart, at 6.00 AM in the morning.

Buffaloes of high yielding groups were also exposed to acute heat stress (45°C , 55% RH) for 3 h. The blood samples were collected before exposure and after 3 h of exposure from these heat exposed buffaloes ($n = 6$). Immediately after collection the samples were transported to the laboratory in ice for further processing.

Blood was centrifuged at 3000 rpm for 30 min; the buffy coat was harvested and resuspended in 1:1 v/v Dulbeccos Phosphate Buffer Saline (DPBS). The total contents were carefully layered on lymphocyte separation medium (Histopaque 1077) at concentration of 4:1 v/v in sterile 15 mL polypropylene centrifuge tube and centrifuged at 2500 rpm for 30 minutes at room temperature. The lymphocyte rich layer was removed and washed twice with DPBS. The contents were centrifuged at 1200–1500 rpm for 15 min. each time washing with DPBS. Contaminating erythrocytes were then lysed with addition of few drops of distilled water and again washed with DPBS. The washed cells were then resuspended in RPMI 1640 media (3-4 mL) supplemented with antibiotics and 10% FCS and centrifuged at 1200–1500 rpm for 15 min. After the final wash, the lymphocyte rich pellet was resuspended in 5 mL of the culture medium.

Trypan blue exclusion method was used to determine the proportion of viable cells in the separated lymphocytes.

The lymphocyte suspension was adjusted to 5×10^6 live lymphocytes/mL by the culture media (RPMI 1640) containing 10% FCS. 200 μL of the diluted cell suspension per well in triplicate was placed in a 96 well flat bottomed tissue culture plate. The mitogen employed in the present study was phytohemagglutinin (PHA-P) at the concentration of 5 $\mu\text{g}/\text{mL}$ of the final culture volume (200 μL), a concentration that had been determined previously to provide maximal stimulation of bovine lymphocyte [3]. The cells were allowed to proliferate with and without mitogen (PHA-P) to determine the difference between cell proliferations. Cortisol, dissolved in culture media was tested at concentrations of 0, 0.5, 2, and 10 ng/mL. All cultures were allowed to incubate at 37°C in a humidified CO_2 incubator (95% air and 5% CO_2) for 24 h. The proliferative response of lymphocyte was estimated using the colorimetric MTT (tetrazolium) according to the procedure given by Mosmann [4]. Lymphocyte blastogenic response was expressed as stimulation index (SI) and was calculated as follows.

$$\text{Stimulation Index (SI)} = \frac{\text{OD of the stimulated cells}}{\text{OD of the unstimulated cells}}, \quad (1)$$

where OD Optical Density at 503 nm with reference to 630 nm. All analyses was done using Windstat 8.0 software package. Data from different experiments are presented as mean \pm SE. Analysis of variance of the data was done using RBD factorial design to see the effect of milk production and different levels of cortisol on lymphocyte blastogenesis. The responses have been compared with respect to unstimulated cells for different experiment involving the mitogen and cortisol. The interaction effects including animals \times group, group \times treatment, animals \times treatment \times group were also calculated. To see the effect of *in vivo* heat stress and *in vitro* cortisol treatment on lymphocyte blastogenesis effect of

heat stress, effect of cortisol and interaction effect (group \times treatment) were calculated.

3. Results

Mitogen-induced stimulation index in buffaloes exhibiting different levels of milk production have been presented in Figure 1. Mitogen-induced lymphocyte blastogenesis was significantly ($P < .01$) different in different buffaloes in both the groups. There were no significant differences between the groups. The variations observed were independent of milk production potential of these buffaloes.

Mitogen-induced stimulation index at different levels of cortisol in different groups of buffaloes have been presented in Figure 2. The stimulation indices in group I were significantly different ($P < .01$) between cortisol treatments. The interaction effects of group \times treatment ($P < .01$) were also significant. The stimulation index of group II tended to differ significantly between treatments ($P < .05$). The interaction effect as mentioned earlier was also significant ($P < .001$) for this group. But, when the two groups were compared with respect to blastogenic response, the differences were not found to be significant. This indicated that the animals of both the groups did not react identically to different levels of cortisol. While cortisol at the level of 0.5 ng/mL did not affect the lymphocyte blastogenesis, it significantly reduced blastogenesis at 2 ng/mL in both the groups. Due to animal variations, the responses at different levels of cortisol supplementation were not found to be linear.

The blastogenic response was further tested in buffaloes of group I ($n = 6$) in response to imposition of *in vivo* acute heat stress and *in vitro* cortisol (Figure 3). There was significant ($P < .01$) decrease in lymphocyte blastogenic response after exposure of heat stress. The stimulation index prior to exposure was significantly different between treatments ($P < .01$). During pre-exposure the lymphocyte blastogenesis reduced significantly ($P < .01$) at 2 ng level of cortisol and remain unaltered with the increasing levels of cortisol. After the exposure of acute heat stress the lymphocyte blastogenesis followed similar trends as pre exposure therefore, it indicated that upon exposure to heat stress the lymphocytes become either nonresponsive or cortisol resistant and therefore, do not respond to exogenous cortisol (Figure 4).

4. Discussion

Lymphocyte stimulation is widely used to measure (i) immune competence by stimulation of lymphocytes with phyto-mitogens [5, 6] (ii) histocompatibility by mixed leukocyte culture [7] and (iii) exposure to infectious agents by stimulation of lymphocytes with specific antigens [8]. Zimmerman et al. [9] associated the mitogen stimulated lymphocyte blastogenesis to host immunosuppression induced by parasites in sheep. In this investigation, cell-mediated immunity was assessed by measuring the proliferation of mitogen-stimulated lymphocytes in buffaloes under different experiments. The response was not found to be related to milk

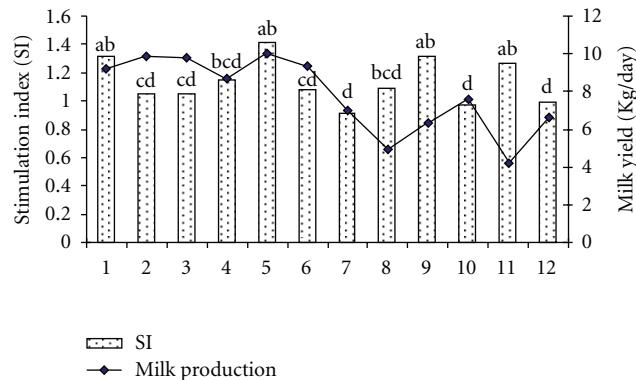


FIGURE 1: Mitogen-induced stimulation index in buffaloes exhibiting different levels of milk production. Values are mean \pm SEM. Bars with values in different groups that lack a common letter are significantly different ($P < .05$).

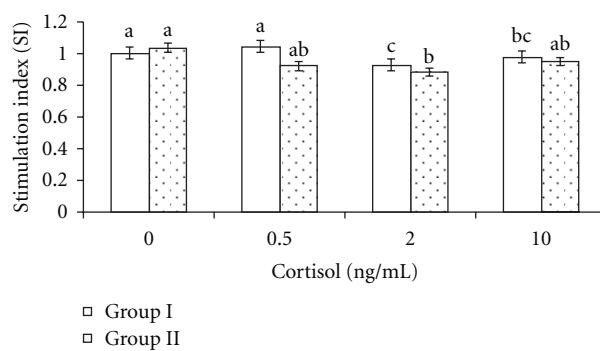


FIGURE 2: Mitogen-induced stimulation index at different levels of cortisol in different groups of buffaloes. Values are mean \pm SEM. Bars with values in different groups that lack a common letter are significantly different ($P < .05$).

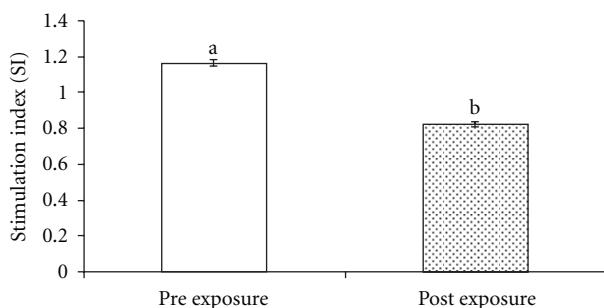


FIGURE 3: Mitogen-induced stimulation index in buffaloes after exposed to acute heat stress. Values are mean \pm SEM. Bars with values in different groups that lack a common letter are significantly different ($P < .05$).

production potential of these buffaloes. This was realized from the fact that rate of lymphocyte blastogenesis was similar in buffaloes having a potential of 6.0 and 9.33 lit/day. Variations in the rate of blastogenesis from animal to animal have also been previously reported for outbred populations of cattle and for lactating Holsteins [10]. These variations have also been reported to be influenced by season and the age of the bovine [3].

Glucocorticoids are suppressive to a variety of lymphocyte functions. During the past several years, a number of

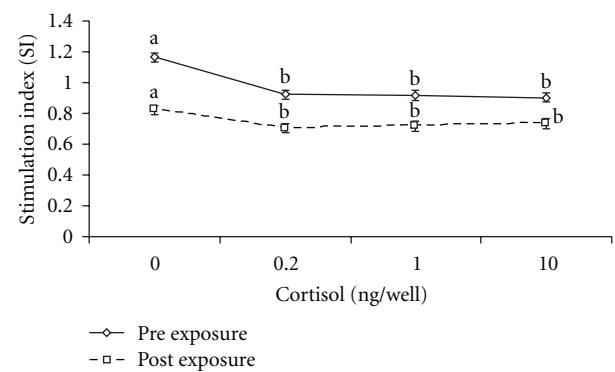


FIGURE 4: Stimulation index at different levels of cortisol in heat stressed buffaloes. Values are mean \pm SEM. Bars with values in different groups that lack a common letter are significantly different ($P < .05$).

studies have indicated that the glucocorticoids are inhibitory to lymphocyte functions *in vitro* when administered at pharmacological levels [11–15]. Segel et al. [16] found inhibition of human lymphocyte blastogenesis by cortisol only when suboptimal PHA levels were used. Chenault, [17] demonstrated that increasing concentration of cortisol inhibited Con-A-induced blastogenesis such that from 10 to 10^4 nM little reactivity could be measured in Holstein cattle,

but did not affect blastogenesis when PHA-P was used in place of Con-A. In the present study, cortisol at the level of 0.5 ng/mL, however, did not affect the PHA-P induced lymphocyte blastogenesis; it significantly reduced blastogenesis at 2 ng/mL in both the groups of buffaloes.

Mitogen-induced lymphocyte blastogenesis upon exposure to acute heat stress was significantly reduced in this investigation. A series of *in vitro* studies have demonstrated that exposure of bovine peripheral blood mononuclear cells to short and severe heat shock reduce responsiveness to mitogens or decrease the number of viable cells [1, 2]. Lacetera et al. [18] reported that different incubation temperatures simulating hyperthermia impaired DNA synthesis in mitogen-stimulated PBMC *in vitro*. Studies conducted *in vivo* have provided contradictory results regarding the effects of air temperatures above upper critical temperature on immune cell functions in bovine. Some authors reported an improvement [3] some described impairment [1, 2] and others indicated no effects [19] of heat exposure on lymphocyte functions. The large variety of experimental conditions (e.g., *in vivo* versus *in vitro*, field versus controlled environment, animals from different breeds or different ages, duration and intensity of heat exposure, lymphocyte function studied) likely explains the discrepancy among results. The differential effects of heat on proliferative response of mitogen-stimulated PBMC from Brown Swiss and Holstein cows. Lacetera et al. [18] further suggested that body temperature increase under heat-stress conditions might not be associated with the same degree of alteration of immune cell efficiency in two breeds. Generalization of this concept indicates that impairment of cell functions may start at a different level of body hyperthermia in different animals as seen in this study also. Further, prolonged exposure to severe heat stress [18] is responsible for a decline of immune cells' reactivity, which may contribute to the higher occurrence of some infections during summer [20].

5. Conclusion

Major findings of the present investigation permitted us to conclude that lymphocyte proliferation assay could be used as one of the measures to determine cell-mediated immunity of the Murrah buffaloes *in vitro*. The stimulation index significantly differed between animals, albeit not affected by production levels. Stimulation index was affected by *in vitro* cortisol. The blastogenesis was reduced significantly at 2.0 ng level. Mitogen-induced lymphocyte proliferation response significantly decreased in buffaloes when exposed to heat stress. However, addition of cortisol at different levels *in vitro* produced no further change in lymphocyte proliferation response therefore, indicating that upon exposure to heat stress the lymphocytes may become cortisol resistant.

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