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ORIGINAL RESEARCH

Molecular insights into the neutrophils activation in zebu cattle during seasonal variation

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ABSTRACT

Earlier, we reported the impact of season on neutrophils' functional competence and also hypothesized that it could be the impact of different seasons on neutrophils' activation. In cerebration, the present study aimed to provide insights into neutrophils' activation in terms of phosphorylation of tyrosine containing proteins during different seasons. Ten *Hariana* cows participated in the study and eight times blood samples were collected from each animal (twice in each month) during each season. Phosphorylation of tyrosine proteins was evaluated using western blotting and immunolocalization using a fluorescent microscope. Immuno blotting identified six tyrosine-phophorylated proteins p28, p42, p44, p58, p84, and p104 in winter and rainy seasons, whereas, p84 protein was absent in summer season. Immunolocalization revealed positive immune reactivity (IR) for tyrosine-phosphorylated proteins and significantly ($p < 0.05$) lower percent of neutrophils showed positive IR during the summer season as compared to winter and rainy seasons. The results of the study evidently indicate the activation of neutrophils is mediated through tyrosine phosphorylation and this may be a probable reason behind the decreased neutrophils' functional competence during the summer. Further studies are warranted to decipher the possible association between tyrosine phosphorylation and expression of surface receptors required for the recruitment of neutrophils.

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Immunolocalization; summer season; neutrophils' functional competence; tyrosine phosphorylation; tyrosine-phosphorylated proteins

Novelty of the research

(1) The first study to report the molecular basis of neutrophils activation during seasonal variation in zebu cattle.
(2) The first report demonstrating the insights into tyrosine phosphorylation and neutrophils activation during seasonal variation in zebu cattle.

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1. Introduction

The neutrophils are terminally differentiated cells, recruited to the site of pathogen entry after activation through complex series of signaling mechanisms (Sadik et al. 2011). Activation of the cells and regulation of the cell physiological processes are mostly arbitrated by the phosphorylation of tyrosine containing proteins could be one of the key mechanisms to regulate the neutrophils activation (Nathan 2006). Neutrophils activation leads to several processes like surface expression of cell adhering molecules, tethering, degranulation, and free radical-mediated cell killing (Segal 2005). Moreover, neutrophils apoptosis as well as expression of toll-like receptors are regulated via the activation of protein kinases and phosphorylation of tyrosine containing proteins (Bratton & Henson 2011).

Recently, we have demonstrated the impacts of seasonal variations on the neutrophil functional dynamics of zebu cattle (Swain et al. 2016c) and in compliance various studies have also demonstrated the impacts of seasonal variations on the neutrophils competence as well as function and thereby differential susceptibility of hosts to various diseases. Surface expressions of toll-like receptors significantly decrease during hot humid season, whereas, apoptosis of neutrophils significantly increases during hot humid summer season (Swain et al. 2016c)**.** We hypothesized that it could be mediated trough which reduced neutrophils activation during the summer season compared to the winter and rainy seasons. Literatures are meager in terms of molecular basis of neutrophils activation and particularly in terms of the tyrosine containing proteins phosphorylation during the seasonal variations in zebu cattle. Theretofore, the present study aimed to provide insights into the possible involvement of tyrosine-phosphorylated proteins with neutrophils activation in different seasons; secondly if there are any variations among the tyrosine-phosphorylated proteins, and thirdly whether different seasons are having any impact on the process of tyrosine phopshorylation in relation to neutrophils functional competence.

2. Materials and methods

2.1. Selection of experimental animals and sampling

The proposed study was carried out at institutional livestock farm complex of the university in *Hariana* cows of 2nd to 3rd parity who are apparently free of infections and are reared under standard nutritional practices. Blood samples were collected from *Hariana* ($n = 10$) throughout the year to evaluate the effects of season (rainy, winter, and summer) on neutrophils functional competence. In each season, eight blood samples (9 mL/animal) were collected in sterile heparinized vacutainer tubes by jugular venipuncture from each animal having a frequency of twice in a month. Immediately after collection, blood samples were transported to the laboratory in ice for further processing and were used for the isolation of neutrophils and further processing for study.

2.2. Time of blood collection

Blood samples were collected with minimal disturbance to the animals during the three seasons. Following Table 1 depicts the range of environmental temperature (°C) along with relative humidity (%) in the three different seasons of blood collection.

2.3. Isolation of neutrophils from blood samples

All materials and reagents used for the isolation of blood polymorphonuclear neutrophils (PMN) were sterile and of cell culture grade. Isolation of PMN from peripheral blood samples was performed as described earlier (Swain et al. 2014, 2015a, 2015b, 2016a, 2016b, 2016c). Briefly, 9 ml of blood was poured into the Falcon tubes and centrifugation was carried out at 2000 RPM at room temperature for 20 min. The upper plasma layer and the buffy coat were removed carefully. The haematocrit at the bottom of the falcon tube served as the source of PMN. Three ml of the haematocrit was taken in a falcon tube and was slowly mixed with equal volume of 1.5% ammonium chloride. The tube was allowed to stand still for 5 min followed by centrifugation at 1000 × *g* for 10 min at room temperature. The supernatant was discarded and the cell pellet was collected. The cell pellet was dissolved in 3 ml of PBS and the cells were resuspended. Three ml of *Histopaque* 1077 (Sigma, Germany) was taken and over this 3 ml of the cell suspension was layered. Centrifugation was carried out at 2000 × *g* for 20 min at room temperature. The layer formed above the Histopaque 1077 was considered as any of the left lymphocytes in the cell suspension. The cell pellet formed at the bottom of the falcon was considered as PMNs. The collected cell pellet was washed three times with PBS and centrifugation was carried out at 500 × *g* for 5 min. The pellet so formed after washing was resuspended in RPMI medium for further analysis and served as the source of blood PMN. The purity of the blood PMN was found to be more than 90% as evaluated by Field's stain under oil immersion (100×). Different types of blood and milk PMNs were estimated by Field's stain and were observed under oil immersion (100×).

2.4. Immunoblotting of tyrosine-phosphorylated proteins

Immunoblotting for tyrsoine phosphorylation was evaluated using the protocol described earlier (Chauhan et al. 2017). In brief, total protein was extracted from blood neutrophils using commercial total cell lysis protein isolation buffer and the isolated proteins were quantified using commercial kit. To the protein isolation buffer, Dithiothreitol (DTT, 5µL/500µL of isolation buffer) and sodium orthovanadate were added to inhibit the autophosphorylation of proteins along with protease inhibitor cocktail (5µL/500µL of isolation buffer) to prevent the activities of the proteases. After quantification, 20 µg of protein sample (1 µg/1µL) was loaded in each well of stacking gel for SDS-PAGE for separation using 10% resolving gel. The proteins were resolved using constant current and 60 V of voltage for 4 h.

After completion of the SDS-PAGE, the gel was transferred to the transfer buffer [25 mM Tris, 192 mM Glycine, 20% Methanol (v/v), and 0.1% SDS 3.5 mM, pH 8.3 (*Towbin buffer*)] for equilibration for 10 min and the protein transfer on to the membrane was carried out using a semidry blotting unit (*Invitrogen*, USA) at a voltage of 25 V for 1 h 15 min. After

Table 1. Seasonal variations in environment temperature (°C) and relative humidity (%).

	Summer season		Rainy season		Winter season	
Parameters	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Environment temperature (°C)	34.90–36.80	35.98 ± 0.12^{c}	28.85–30.64	29.88 ± 0.10^{a}	6.54–16.86	11.80 ± 0.46^{b}
Relative humidity (%)	42–63	49.98 ± 1.38^{c}	77–86	81.18 ± 0.60^{a}	76–95	84.16 ± 0.76^{b}

Note: Different superscripts show significant difference between the rows.

transfer, the membrane was transferred to the blocking solution (2% of skim milk powder prepared in Tris-buffered saline with Tween-20, TBST: 20 mM Tris, 150 mM NaCl, pH 7.6; 0.1% (v/v) Tween-20, TBST) and kept overnight with slow agitation at 4 °C. After blocking, the membrane was removed and washed twice with TBST. Then, the membrane was treated with the primary antibody (Monoclonal antiphosphotyrosine antibody, Sigma, P1869; Clone PT-154, diluted 1:50 in TBST) for overnight at 4 °C. After washing, it was treated with the secondary antibody (goat anti mouse IgG HRP, 1:1000 in TBST) for 2 h at 37 °C. Then the membrane was washed with TBST and protein bands were developed using DAB system. The relative molecular weights and relative intensities were determined using broad-range molecular weight markers and using gel documentation and analysis system (Gel- Doc. Model-Alpha Imager TM1220; Alpha Innotech Corporation, Santa Clara, CA, USA).

2.5. Immunolocalization of tyrosine-phosphorylated proteins in neutrophils

Immunolocalization of tyrosine-phosphorylated proteins were carried out by employing indirect immune fluorescence technique as described earlier (Swain et al. 2017) with little modifications for neutrophils. The technique employed two antibodies to localize the tyrosine-phosphorylated proteins on neutrophil surface. At the end of neutrophils incubation at different temperatures, neutrophils suspensions were smeared on Polyprep glass slides (Sigma, Saint Louis, USA) and allowed to air dry. The air-dried smears were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 30 min and followed by rinsing with PBS. The rinsed smeared slides were permeabilized with cold methanol for 30 min at −20 °C and again rinsed with PBS. Nonspecific sites on the slides were blocked with 5% BSA in PBS for 2 h at room temperature. The smeared slides were incubated with monoclonal antiphosphotyrosine antibody (Sigma; P1869; Clone PT-154, diluted (1:50) in 1% BSA in PBS) for 4 h. After incubation with primary antibody, the slides were rinsed with PBS and incubated with secondary antibody (anti mouse IgG- FITC conjugate, Sigma; F4018; diluted (1:50) in 1% BSA in PBS for 2 h at 37 °C in dark. Following two hrs of incubation, slides were rinsed with PBS three times and then processed for examination under microscope. Cover slip was mounted on the smeared slide with antifading medium (1.5% w/v DABCO, Sigma Aldrich, St Louis, MO, USA in 90% v/v glycerol). A total of 400 neutrophils per slide were observed and different patterns of neutrophil were evaluated as per the site of fluorescence on neutrophils.

2.6. Statistical analysis

Statistical analysis was carried out using the Sigma Plot software package version 7.01. Data from two breeds from three different seasons are presented as mean ± SE. Significance was tested by employing one-way ANOVA, considering seasons as a factor. Tukey's post hoc test was used to compare all pair wise differences in mean. The differences was considered as significant if $p < 0.05$.

3. Results

Immunoblotting revealed the presence of six tyrosine-phosphorylated proteins namely p28, p42, p44, p58, p84, and p104 in the neutrophils lysate isolated from the *Hariana* cows in winter and rainy seasons, however, summer season revealed the absence of p84

tyrosine-phosphorylated protein (Figure 1). However, the expression cum relative densities of the tyrosine-phosphorylated proteins did not show any variation. From the results, it was not clear which tyrosine-phosphorylated protein is specifically involved in neutrophils activation during winter and rainy seasons. Proteins like p28, p42, and p44 were found in all the three seasons and were assumed to be involved in neutrophil activation during different seasons.

Immunolocalization of tyrosine-phosphorylated proteins revealed significantly ($p < 0.05$) lower percent of neutrophils showing positive immune reactivity for tyrosine-phosphorylated proteins in summer compared to winter and rainy seasons (Figures 2 and 3) in both breeds of zebu cattle. Percent neutrophils showing highest positive reactivity for tyrosine-phosphorylated proteins were found in rainy season.

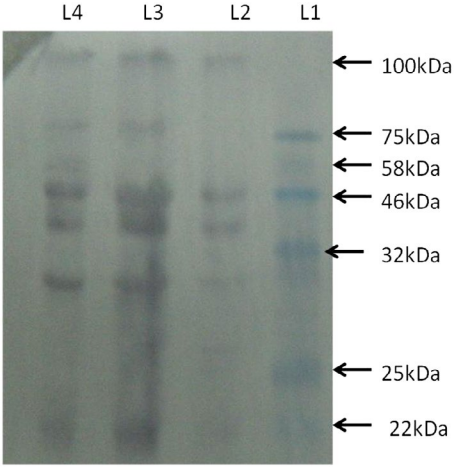

Figure 1. Immunoblot showing tyrosine phosphorylated proteins during different seasons.
Note: L1: molecular weight marker; L2: summer season; L3: rainy season; L4: winter season.

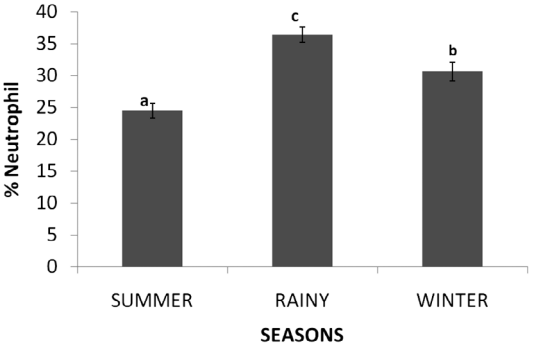

Figure 2. Per cent neutrophil showing positive reactivity for tyrosine phosphorylated proteins in different seasons (different superscripts differed significantly $p < 0.05$).

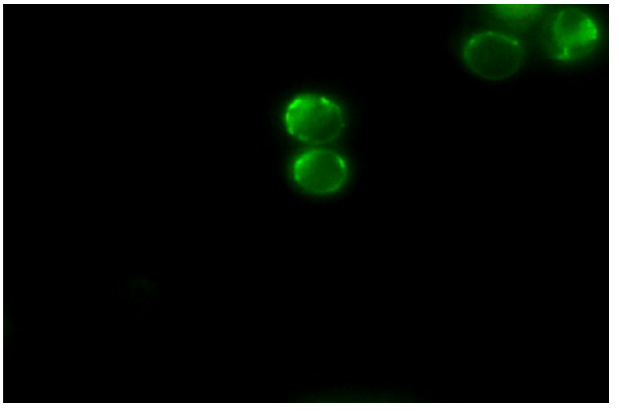

Figure 3. Photomicrograph showing immunolocalization of tyrosine phosphorylated proteins on neutrophils of zebu cows (40×).

4. Discussion

Recruitment of neutrophils at the site of pathogens entry is orchestrated with the activation of neutrophils, neutrophils mediated cell killing, apoptosis, and eventually neutrophils clearance from the site of inflammation. All these cellular events are tightly regulated by the intracellular machinery of the neutrophils and the extracellular signaling molecules which are recognized by the neutrophils (Borregaard 2010). The result of the present study demonstrates the presence of six tyrosine-phopshorylated proteins in Hariana cattle neutrophils, thus indicating the involvement of tyrosine phosphorylation in the process of neutrophils activation. These proteins were found to be phosphorylated in all the three seasons, indicating their possible roles in the regulation of neutrophils function. It has been known that tyrosine phosphorylation is one of the key events in the process of neutrophils activation in human blood neutrophils isolated in various pathological states. Several *in vitro* systems have been shown to activate the neutrophils via the activation of tyrosine phosphorylation and thereby mediate their functions (Berkow & Dodson 1990).

Neutrophils isolated from synovial fluid of patients suffering from rheumatoid arthritis showed increased tyrosine phosphorylation of two proteins having molecular weight 42 and 74 kDa. Interestingly, neutrophils isolated from patients without rheumatoid arthritis showed lower degree of tyrosine phosphorylation (Lloyds et al. 1996). *In vitro* stimulation of human blood neutrophils showed proteins of 120, 89, 84, 75, 70, 57, 52, and 44 kDa which were tyrosine containing phosphorylated proteins indicating their evident involvement during neutrophils activation (Berkow & Dodson 1990). In compliance to aforesaid studies, the result of the present study also demonstrates the existence tyrosine phosphorylated p28, p42, p44, p580, p84, and p104 proteins in neutrophils of *Hariana* cows in different seasons. From the results it is evident that phosphorylation of tyrosine containing proteins vary from species to species in activating neutrophils. Secondly, in different conditions, the degree of stimulation of blood neutrophils is different; thirdly, different external factors are required for the activation of neutrophils and hence different tyrosine phosphorylating proteins are involved in neutrophils activation.

The present study clearly demonstrates a remarkable reduction in percent of neutrophils showing tyrosine phosphorylation during the summer indicating reduced functional

activation of the neutrophils. This could be the possible mechanism behind reduced expression of TLR2 and TLR4 in zebu cattle during hot and humid climate, a key finding of our previous study (Swain et al. 2016c). In compliance with the results of the present study, neutrophils isolated from rabbit peritoneal flushing exhibited tyrosine phosphorylation when treated with chemotactic factors indicating the role of tyrosine phosphorylation as a key process during activation of neutrophils (Huang et al. 1988, 1989). We have also reported previously, a significant increase in per cent of neutrophils showing apoptosis during hot and humid climate (Swain et al. 2016c). Tyrosine phosphorylation is one of the determinants for the degree of neutrophils apoptosis. It has been well demonstrated that an activation of tyrosine containing phosphoproteins induces the death of the immune cells (Yousefi et al. 1994). Therefore, this might be a probable mechanism corroborating the findings of our previous study (Swain et al. 2016c) but still requires its validation in large numbers of zebu cattle.

Neutrophils' activities in terms of release of granules, reduction in molecular oxygen to superoxide anion, chemotaxis, and phagocytosis are regulated by phosphorylation of proteins and in specific phosphorylation of tyrosine containing proteins (Huang et al. 1988, 1989). The activation of phosphorylation events are mediated by calcium-dependent protein kinases (PKC-C), chemotactic peptides FMLP, leukotriene B4, phorbol myristate acetate (PMA), calcium ionophore A23187 induce phosphorylation of tyrosine containing proteins in human neutrophils (Berkow & Dodson 1990). Studies have shown that protein tyrosine phosphorylation is an early event in the activation of human neutrophils through a variety of receptor-dependent and receptor-independent agonists. In the present study, seasonal variation in terms of temperature and humidity had played role in the activation of tyrosine containing proteins in terms of their phosphorylation in neutrophils of zebu cattle. Therefore, it is evident from the present study that, high temperature downregulates tyrosine phosphorylation of neutrophils, thereby result in reduction in neutrophils activation and signal transduction.

Phosphotyrsoine containing proteins form less than 1% of total cellular phosphoproteins and serve as the major candidates for regulation of cellular events. These proteins have been reported to be present in non proliferative cells like mature lymphocytes, erythrocytes, and platelets. These tyrosine kinase proteins are activated during the process of stimulus response coupling (Sadik et al. 2011). During hyperthermia and febrile states, human blood neutrophils exhibit faster apoptosis (Salanova et al. 2005). In our previous study, we observed lower tyrosine phosphorylation during summer season, but higher apoptosis of neutrophils (Swain et al. 2016c). These contrasting findings indicate involvement of some other pathways regulating neutrophils apoptosis and activation during different seasons.

In India, summer season is a combination of high temperature and high humidity and a consequent high temperature humidity index (THI) which has been considered as highly critical for animal production as well as reproduction. Incidence of increase in diseases due to high THI has been reported earlier (Morse et al. 1988; Cook et al. 2002; Mukherjee et al. 2015). High THI also activates sympatho-adrenal axis and thereby increases secretion of glucocorticoids and in specific cortisol (SunilKmar et al. 2011). High cortsiol may serve as one of the significant factors for lower activation of neutrophils and may have resulted in altered as well as decreased tyrosine phosphorylation in neutrophils which require further investigation. Febrile temperature also lowers signal transduction in neutrophils during their activation (Salanova et al. 2005) and this may be linked to lower tyrosine phosphorylation in neutrophils proteins.

5. Conclusion

Neutrophils activation is the key event which mediates neutrophils' functional competence. It can be concluded from the present study that neutrophils activation might be mediated through phosphorylation of tyrosine containing proteins. Different seasons have a significant impact on the neutrophils activation and thereby regulate the neutrophils function. Moreover, hot humid environmental conditions could downregulate phosphorylation of tyrosine containing proteins and hence could blunt the functional competence of neutrophils in zebu cattle. Further studies are required to explore other possible signaling pathways involved in the activation of neutrophils and their functional competence during seasonal variations in zebu cattle.

Ethical compliance

The study was in compliance with the Institutional Animal Ethics Committee, DUVASU, Mathura.

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Disclosure statement

No financial or any other conflicts have been reported by the authors with anyone.

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