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RESEARCH ARTICLE

Changes occurring in the receptors of blood neutrophils during implantation in Sahiwal cows

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Abstract Neutrophils are considered to be the first line of defence against any external invasion. A study was undertaken to evaluate the number of blood neutrophils released and expression of adhesion receptors on them during implantation of an embryo in the mothers endometrium. For this, blood samples were collected from 15 Sahiwal cows on the day of Artificial Insemination (A.I.) (day 0), and on day 18 post A.I. (day of implantation). Total leucocyte counts (TLC), number and type of neutrophils were observed under light microscopy, whereas, neutrophils receptors were observed by flow cytometry. Cows were divided into pregnant (P) and non-pregnant (NP) groups once they were confirmed pregnant after estimation of plasma progesterone (on day 18) and also by rectal palpation (on day 60). Higher ($P<0.05$) TLC and percentage neutrophil were observed during early pregnancy in P cows on day 18th of A.I. The surface receptor expression of CD62L and CD11B by flow cytometry showed significantly lower ($P<0.05$) expression of these receptors on day 18th in P cows. From this study it was concluded that more neutrophils are released during circulation in both P and NP cows, but more surface receptor on the surfaces of NP cows increase the overall activity of their neutrophils and thus prevents implantation of the embryo in them.

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Introduction

Neutrophils are key blood cells during the initial inflammatory response and are commonly referred to as the "first line of defense" against infection. Neutrophils are able to integrate complex arrays of adhesion, chemoattractant and cytokine driven signals and are able to synthesize cytokines in response to inflammatory stimuli. During inflammation, these are the first cells, recruited to the injury or infection site and undergo major cytoskeletal changes and differential gene expression (Kobayashi, 2005) that mediate their directional migration to sites of injury and ultimately trigger bactericidal reactions. Recently neutrophils have also been used as the first cells to detect an implanting embryo (Kizaki *et al.*, 2013). Neutrophils have various receptors on their surface which are broadly classified into surface adhesion molecules, signalling molecules, pattern recognition molecules and terminal receptors (Kebir and Filep, 2013). The signalling receptors are associated with the recognition of the chemokine's and cytokines released by an invading agent or an implanting embryo.

The neutrophils exhibit presence of surface adhesion receptors. These receptors are classified as Integrins (CR3) and L-Selectin (CD62L). Both the selectins and integrins possess Ca2+binding domains and their binding to the ligands are dependent on the Ca2+(Zarbock *et al.*, 2013). Upon activation, neutrophils marginate and position themselves adjacent to the blood vessel endothelium. Here they undergo selectin-dependent capture followed by integrin-dependent adhesion, after which they migrate into the areas or tissues in demand. The intimacy of the attachment between the neutrophils and endothelial surfaces during rolling ultimately leads to the removal of the invading agent.

During the process of implantation, when the bovine embryo is ready to attach to the dam, it signals its presence by a glycoprotein, interferon-tau, which is secreted by embryonic

trophoblastic cells. This IFN-τ is produced in large amounts by the embryo after day 14, signals to the mother and establishes the pregnancy (Roberts, 2007). Embryonic interferon-tau not only causes inhibition of luteolytic mechanism but also stimulates expression of its target genes both in intra-uterine and extra-uterine tissues. Being a cytokine, interferon-tau is released in circulation and stimulate leukocyte/ neutrophilic activity (Gifford *et al.*, 2007; Shirasuna *et al.*, 2012). It is not clear till now, how the neutrophils behave in response to an invading embryo which tries to implant itself in the mothers (dam's) endometriium. Therefore the present study was undertaken to evaluate how the neutrophilsregulate their surface receptors in pregnant and non-pregnant cows.

Materials and Methods

Selection of Animals

Fifteen Sahiwal cows were selected from the Institute's dairy herd, that were randomly coming on heat in readiness for A.I. Artificial insemination was performed on the day of estrus using frozen semen. Grouping of the cows were done into two; pregnant (n = 5) and non-pregnant (n = 10) cows. Cows in each group were validated by estimating their plasma progesterone on day 18th of A.I. and further confirmation by rectal palpation on the 60th day of gestation. The animals were apparently free of clinical infections as per the records of the dairy firm during the entire experimental period.

Ethical Permission for the animals for experimentation

The guidelines for animal experiments outlined by the Institutional Animal Ethics Committee which approved this study, and the ethical guidelines of the National Dairy Research Institute, Karnal (Haryana, India) were followed during all the animal experimentations.

Blood Sampling

Blood (10 ml) was drawn in vacutainer tubes from jugular vein posing minimum disturbance to the animal during collection. As pregnancy recognition signaling occurs on day 12 in pigs, days 13-14 in sheep, and days 16-17 in cattle (Spencer, 2013). Therefore, blood sampling was carried out on days 0 (day of A.I.) and day 18 post A.I. (day of implantation). Total leucocyte counts were counted using a haemocytometer. A smear of blood was prepared on a clean glass slide. It was stained with Leishman stain and observed under the microscope to see any change in the nucleus of neutrophils (band and segmented) and the total number of neutrophils present both on day 0 and 18 of blood sampling.

Chemicals for flow cytometry

Flow cytometry was carried out to study the degree of expression of surface receptors on blood neutrophils. Bovine specific primary antibodies were procured from Bio-Rad Company which were tagged with FITC probe. Details of the antibodies are given in Table 1.

Evaluation of surface receptors of blood neutrophils by flowcytometry isolated from pregnant and non-pregnant group of cows

The surface receptor expressions were quantified by using Flow Cytometry. The results obtained from the study are depicted in dot plots and histograms. Fluorescence was quantified using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, BD Bioscience, USA, FACS ARYA and QUANTO II). The instrument used the Diva software for the analysis of the data. PMNs were gated on dot plots representing cell size based on forward light scattering and granularity based on side light scattering. 50,000 events were acquired for the evaluation of the relative expression of neutrophil surface receptors in pregnant and non-pregnant group of cows.

During the study, forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1) parameters of blood PMNs were quantified by using the Flow Cytometry. The neutrophils were identified and gated on the basis of their size (FSC) and granularity (SSC). The neutrophils were gated to quantify the percentage of expression of surface receptors.

Results and Discussion

The results of TLC in P and NP cows during the day of A.I. and on day 18 post A. I. have been presented in figure 1. These leucocyte counts increased significantly in pregnant cows, whereas, non-pregnant cows showed a non-significant decline in the TLC values. The results of neutrophil percentage have been presented in Figure 2. There was an increase in the

Table 1 List of Primary antibodies tagged with FITC used in flow cytometry

Name of the antibody	Catalogue No	Concentration
CD62L (Bovine)	MCA1649F	0.1mg/ml
CD11b (Bovine)	MCA1425F	0.1mg/ml

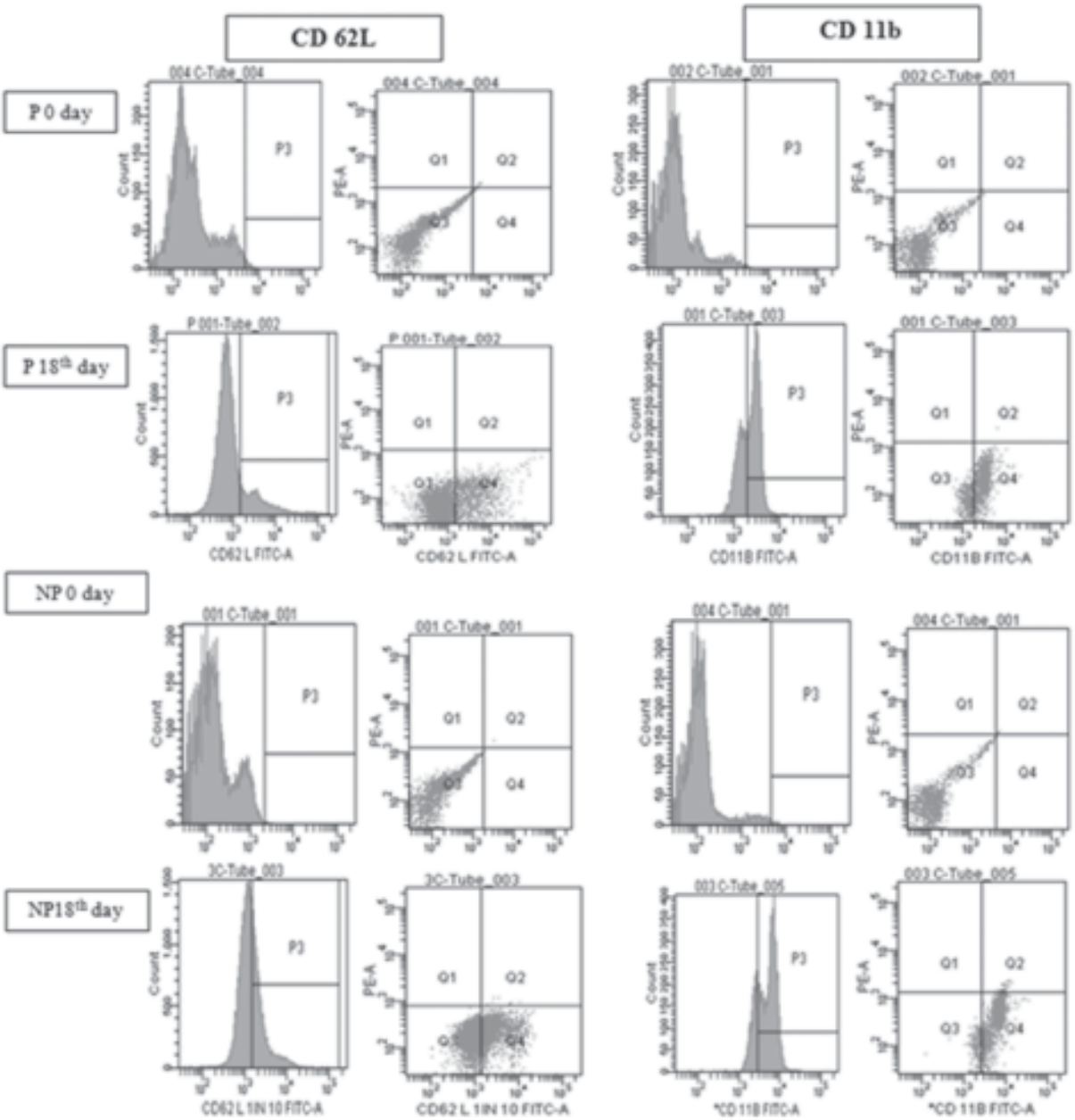

Plate 1: Expression histograms of CD 62 L and CD 11b on the neutrophils of Pregnant (P) and Non-pregnant (NP) cows on day of A.I. (day 0) and on the day of implantation (day 18)

number of neutrophils obtained per ml of blood in pregnant as compared to the non-pregnant cows. Under the light microscopy 98% of the neutrophils were segmented and about 2% were found to be of band shape in both the group of cows.

Majority of neutrophils circulate in the bloodstream under resting conditions, microbial invasion or other inflammatory stimuli trigger the extravasation of neutrophils to the inflamed interstitium. This process is mediated by a multistep cascade of neutrophil adhesion to, and transmigration through, the vessel wall (Ley *et al*. 2007). The number of neutrophils increases in pregnancy and these cells undergo functional and metabolic changes (Taniguchi *et al.* 2004). We also found an increased amount of blood leucocyte and neutrophils/ ml

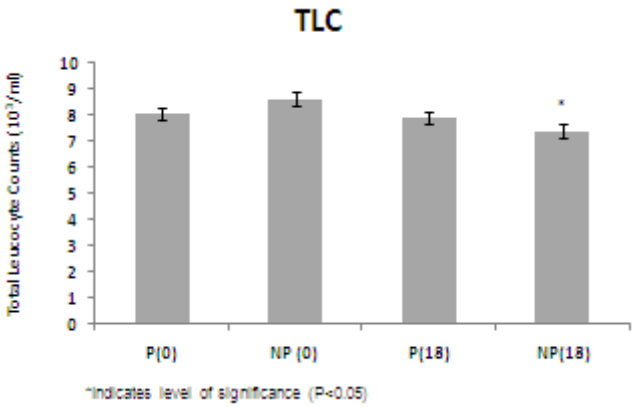

Fig 1: Total leucocyte counts on the day of A.I. and day 18 post A.I. in pregnant and non-pregnant cows

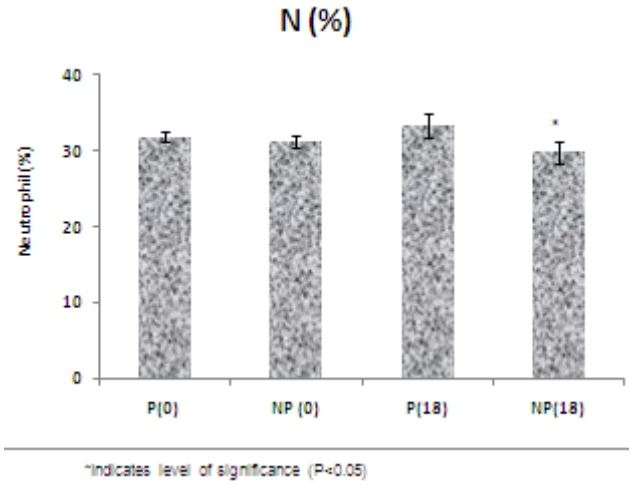

Fig 2: Percent neutrophil on the day of A.I. and day 18 post A.I. in pregnant and non-pregnant cows

of blood samples collected from non-pregnant cows. Sacks *et al.* (1998) found that delayed apoptosis of neutrophils in normal pregnancy promotes inflammation to persist and may contribute to pregnancy-associated neutrophilia and pregnancy-induced inflammatory changes in neutrophils in the peripheral blood are akin to those of sepsis. Therefore, the function of neutrophils in pregnancy has to be very tightly controlled since enhanced inflammatory responses have been linked to pregnancy complications such as preeclampsia in humans.

The expression histograms obtained from the analysis is presented in Plate 1. In the pregnant cows, the CD62L expression was significantly (P<0.05) lower as compared to the non-pregnant cows. The results also revealed a significant (P<0.05) down regulation of CD11B positive cells in the pregnant cows as compared to control/non-pregnant cows.

Flow cytometry was used to study the adhesion receptors present on the surface of blood neutrophils. The Selectin and Integrin receptors on neutrophils mainly help in catching of the neutrophil during an invasion and migrate them at the site of invasion. Flow cytometric analysis provides a rapid screen for activities and abnormalities of neutrophil function and reflects more accurately their behavior *in vivo* in humans (Nicholson *et al.*, 2007). An advantage of flow cytometry is that the majority of neutrophil functions can be measured using a small volume of whole blood that reduces artifactual changes in function caused by purification procedures. Kizaki *et al.* (2013) reported for the first time by flow cytometric analysis that neutrophils respond to positive signals of pregnancy. We also found that blood neutrophils change their receptors in response to an invading embryo.

The two major groups of neutrophil adhesion receptors are selectins/selectin ligands and integrins. Selectins are single-chain transmembrane glycoproteins that recognize carbohydrate moieties and mediate transient interactions between leukocytes and the vessel wall (Sperandio *et al.*, 2009). Integrins are heterodimerictrans membrane glycoproteins present on virtually all mammalian cells. As the leukocyte rolls along the blood vessel wall, the distal lectin-like domain of the selectin binds to certain carbohydrate groups presented on proteins (such as PSGL-1) on the leukocyte, which slows the cell and allows it to leave the blood vessel and enter the site of infection. The most important integrins expressed on leukocytes belong to the β_2 integrin family, formed by the β_2 (CD18) integrin chain and a unique α chain (Schymeinsky *et al.*, 2007). On engagement by their ligands, integrins activate a wide range of signaling pathways that regulate a broad array of leukocyte functions such as chemotaxis, cytokine responsiveness, phagocytosis, and gene expression.

Higher expression of CD62L receptors on the blood neutrophils of non-pregnant cows indicate that more and more neutrophils are being mobilized during implantation. Whereas, an increase in CD11B receptors in non-pregnant cows indicate that more and more neutrophils are marginating towards the implanting embryo and may be one of the cause of non-pregnancy in these animals. According to Dominguez *et al.* (2005), a clear parallelism occurs between the different steps in human embryo-endometrialapposition/adhesion/ invasion and leukocyte-endothelium rolling/adhesion/ extravasation. They reported that during human implantation and leukocyte trafficking, a wave of soluble mediators regulates the expression and functional activity of adhesion molecules such as L-selectin and integrins, which mediate both the processes of embryonic implantation and leukocyte transendothelial migration. However our results could not be

compared in cows due to non-availability of literature on selectins and integrins in these species.

From the study, it can be concluded that the neutrophils sense the implanting embryo and their number also increases. But, before they can act on an implanting embryo, the mother (dam) down regulates selectins and integrins receptors on neutrophils. The down regulation of these surface adhering receptors causes a reduction in the migration of neutrophils towards the implanting embryo and thereof allows the successful process of implantation. However, more studies carried out on other receptors like chemokine and toll like receptors on neutrophils present of pregnant versus non-pregnant cows may further give a clear and better picture.

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