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Fluctuation in the number, type and activity of blood neutrophils in cows exhibiting successful and unsuccessful completion of gestation cycle

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ABSTRACT
Neutrophils (first line of cellular defense) are capable of detecting presence of foreign genome in the mother's womb. Role of neutrophils during full gestation cycle of ruminants and the difference in their number, type, and activity in successful and unsuccessful pregnancies is not known. To evaluate this, blood samples were collected at artificial insemination (0 day) and on days 10, 14, 16, 18, and 21 in non-pregnant (NP) cows. However in pregnant (P) cows, samples were collected as indicated above and every 30 days for the complete gestation. In aborted cows, samples were collected as above till abortion. Higher total leukocyte counts were observed in NP and aborted cows at abortion. Neutrophil: lymphocyte ratio increased significantly ($p < 0.05$) in NP and aborted cows. Phagocytic activity (PA) and myeloperoxidase concentrations were significantly higher ($p < 0.05$) on day 18 post insemination in NP cows. PA and myeloperoxidase also increased significantly ($p < 0.05$) at abortion in aborted cows. Neutrophils exhibited limited decrease in their number and activity in successful pregnancies during implantation. After that their number and activity were constantly maintained throughout the gestation cycle. Any increase in the number and inflammatory activity of neutrophils may lead to non-pregnancy or loss in pregnancy.

KEYWORDS
Blood neutrophil; number; activity; pregnancy; abortion; cow

Novelty of the work

(1) First report about infradian rhythmicity of blood neutrophils throughout the gestation cycle in cows.
(2) Fluctuation in the neutrophils number, type, and activity compared between successful and unsuccessful pregnancies.
(3) Neutrophils display circadian rhythmicity in their activity for successful implantation and growth of the fetus.

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(4) Any increase in the inflammatory activity of neutrophils may lead to non pregnancy or abortion.
(5) Understanding the circadian rhythm of blood neutrophils in pregnant cows may help us to better manage those cows during the critical periods of pregnancy.

1. Introduction

Neutrophils are the oldest known phagocytes and have traditionally been thought of as simple foot soldiers of the innate immune system. More recently, it has become apparent that neutrophils are complex cells capable of a vast array of specialized functions (Kolaczkowska & Kubes 2013). Being the first line of cellular defense, they can sense two classes of signals, tissue injury, and presence of foreign genome (Nagahata et al. 2011; Kizaki et al. 2013; Manjari et al. 2016).

During the initiation of pregnancy, neutrophils sense the molecular crosstalk between the trophoblast and maternal immune cells of bovine endometrium (Oliveira et al. 2012) and help in the maternal recognition of pregnancy after getting stimulated by interferon tau which is released by the trophoblast cells of the embryo (Shirasuna et al. 2015). It is the differential expression and secretion of chemokines that induces selective trafficking of leukocyte subsets to the maternal fetal interface and regulates normal pregnancy (Du et al. 2014).

Key parameters of the immune system in the blood exhibit circadian rhythms, most strikingly the number of circulating hematopoietic cells, as well as hormones and cytokines (Haus & Smolensky 1999). These factors oscillate according to the rest–activity phase of the species, whether the species is diurnal (e.g. humans) or nocturnal (e.g. rodents). A pregnant cow also alters immune cell functionality, and it is a combined effect of various hormones, growth factors, cytokines, extracellular matrix, and nutrients which determines the successful outcome of pregnancy (Bazer 2013). Over the years, most of the literature regarding immune activities of pregnant cows has been focused only during the periods of early and late pregnancy (Mateus et al. 2002; Kim et al. 2005; Pathan et al. 2015). How various peripheral blood leukocytes behave in the presence of conceptus during the whole gestation period in ruminants is still unknown (Ott et al. 2014). Therefore, the present study was carried out to record the number, type, and activity of neutrophils throughout pregnancy, our main aim was to observe the circadian rhythmicity of blood neutrophils throughout the gestation cycle and compare them between successful and unsuccessful pregnancies.

2. Materials and methods

2.1. Study site

The present study was conducted at the Livestock Research Centre of ICAR-National Dairy Research Institute, Karnal, India. This institute is situated 250 m above mean sea level, latitude 29.43°N and longitude 77.2°E.

2.2. Selection of experimental animals and design of experiment

Multiparous Karan Fries (KF) crossbred cows having good health condition and free from any reproductive and anatomical disorders were selected. Blood sampling was done from those cows which naturally came to heat and were brought for artificial insemination (AI). Blood samples were collected on the day of AI (0 day) and on days 10, 14, 16, 18, and 21 in non pregnant (NP) cows. Whereas, pregnant (P) cows were followed throughout the gestation period and blood samples were taken as indicated above and then every 30 days for the complete gestation period i.e. on the day of AI (0 day), 10, 14, 16, 18, 21, 30, 60, 90, 120, 150, 180, 210, 240. Observation was also recorded on the day of calving i.e. 270 ± 10 days but have not been discussed in this manuscript. In the aborted cows, samples were collected as given above and till the day of abortion. About 9-ml blood was collected in sterile heparinized vacutainer tubes (greiner bio-one, Vacuette) from jugular vein puncture posing minimum disturbance to the animal. Immediately after collection, the blood samples were transported to the laboratory in ice box for further processing. All the cows were fed with balanced ration and provided with *ad libitum* water during all times of the day. Regular examination of physiological responses was done to ensure healthy status of the cows during the entire duration of the study. Pregnancy was confirmed by non-return to heat, estimating plasma progesterone level on day 18 post AI by bovine specific ELISA test kit CUSABIO (Cows having progesterone levels <2 ng/ml were categorized as NP and those having values >2 ng/ml were considered as P). Ultrasonography was done on day 30 and pregnancy was also reconfirmed by rectal palpation at day 60. Finally, a total of 26 KF cows were taken i.e. 10 P, 10 NP, and 6 aborted. Out of the six aborted animals, three aborted on 110 ± 10 and three on 170 ± 10 days. All procedures were performed according to approved animal care and use protocols of the institutional ethics committee and to good veterinary practice for animal welfare (Ethics approval IAEC dated 4 January 2014).

2.3. Total leukocyte counts and neutrophils: lymphocyte (N:L) ratio of blood

Total leukocyte counts (TLC) of blood were enumerated by a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany) as per standard haematological procedure. Blood N:L ratio was evaluated microscopically (Olympus iX51; Olympus, Tokyo, Japan). Segmented neutrophils were having divided or multilobed nucleus, whereas band neutrophils were characterized as having a curved nucleus which was not lobar in form.

2.4. Isolation of blood neutrophils

Isolation of neutrophils from peripheral blood samples was performed as per the protocol described by Mehrzad et al. (2002). Briefly, 9 ml of blood was poured into the falcon tubes and centrifugation was carried out at 1200 × g at room temperature for 20 min. The upper plasma layer and the buffy coat were removed carefully. The hematocrit at the bottom of the falcon tube was considered for further processing. Three ml of the hematocrit was taken in a falcon tube and was slowly mixed with Three ml 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 Dulbecco's phosphate-buffered Saline (PBS) and the cells were suspended. Three ml of Histopaque1077 (Sigma, Germany) was taken and over this 3 ml of the cell suspension was layered. Centrifugation was carried out at 1800 × g for 20 min at room temperature. The cell pellet formed at the bottom of the falcon tube was considered for further processing. The collected cell pellet was washed three times with PBS and centrifugation was carried out at 400 × g for 5 min. The pellet formed after washing was resuspended in PBS for further analysis and served as the source of blood neutrophils. The purity of the blood PMN was found to be more than 95% as checked by counting the cells in smear stained with May-Grunwald Giemsa stain under oil immersion (100×). Viability of the blood neutrophils was about 98% as evaluated using Trypan Blue method (Sigma Chemical Co) (Dang et al. 2010).

2.5. In vitro *phagocytic activity of blood neutrophils*

In vitro phagocytic activity of blood neutrophils was estimated by calorimetric method using nitro blue tetrazolium (NBT) assay as described by Choi et al. (2005). Briefly, the cell suspension of neutrophils was adjusted to 5×10^6 live cells/ml by the culture media (RPMI 1640). Triplicate of 200 µl of the diluted cell suspension per well was placed in a 96-well flat-bottomed tissue culture plate. The cells were allowed to proliferate with (650 µg/ml) of zymosan and (250 µg/ml) of NBT. All cultures were incubated at 37 °C in a humidified CO_2 incubator (95% air and 5% CO_2) for 3 h.

2.6. Estimation of myeloperoxidase enzyme

Concentration of myeloperoxidase enzyme was measured from neutrophils cell lysate using bovine-specific ELISA test kit (Uscn life science, USA). For preparing lysate of neutrophils, the isolated cells were dissolved in 1 ml PBS. Neutrophil suspension was mixed with glass beads and shock was given for 25 s by bead beater (Unigenetics Instrument Pvt. Ltd., India). The lysate were then put on ice for 1 min, and shock was used again for 25 s. The sample was centrifuged at 1000 × g for 10 min and supernatant was taken in 2-ml eppendorf tubes and stored at −80 °C till further estimation.

2.7. Statistical analysis

All data were presented as means ± SEM. All data were analyzed by repeated measures one-way ANOVA for within-group analysis and repeated measures two-way ANOVA (mixed model) for between-group (P, NP and aborted) analysis and hypothesis testing was done at 5% significance level. This followed by Fischer's multiple comparison test using SAS software, version 9.1 of SAS system for window, copyright© (2011), SAS Institute Inc., CARY, NC, USA.

3. Results

3.1. Blood total leukocyte count

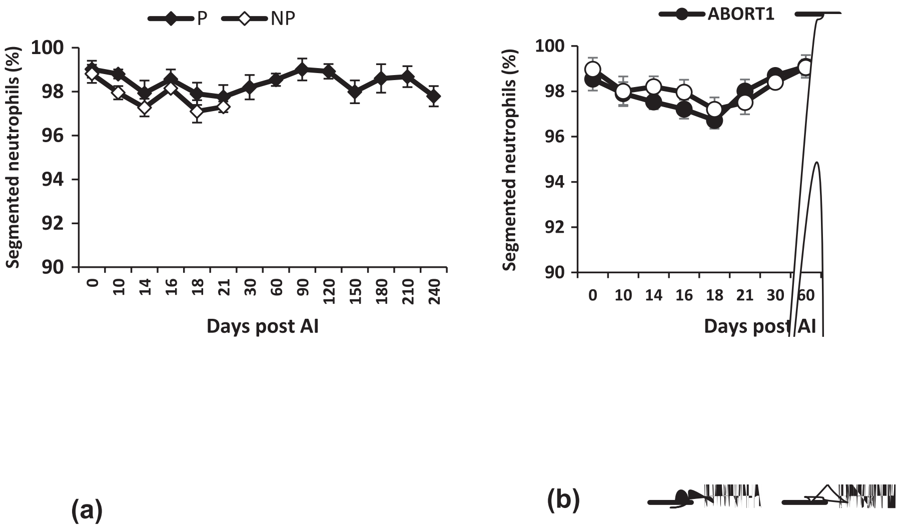
The results of TLC in blood samples collected from various group of P, NP, and aborted cows have been presented in (Figure 1). Total leukocyte count increased on day 10 in all the 3 group of cows. Blood TLC increased significantly ($p < 0.05$) till day 18 and then decreased on day 21 in both P and NP cows and then showed constant level throughout the gestation cycle. Although fluctuation in the TLC in aborted groups was minimum throughout the gestation period, however, a higher TLC ($p < 0.05$) was observed on the day of abortion in both groups.

3.2. Neutrophil: lymphocyte (N:L) ratio

The results of N:L ratio in blood samples collected from various group of P, NP, and aborted cows has been presented in (Figure 2). N:L ratio was always significantly higher ($p < 0.05$) in NP cows as compared to P cows i.e., from day 14 to 21 post AI with the highest N:L ratio observed on day 18 post AI in NP cows. On comparison between NP and aborted cows, N:L ratio was higher ($p < 0.05$) in NP cows from day 14 to 18 post AI. However, day 21 post AI onwards N:L ratio decreased and became almost constant throughout the gestation period in P cows. A significant ($p < 0.05$) increase in N:L ratio was observed on the day of abortion. A very high and significant ($p < 0.05$) change in the N:L ratio was seen in the cows which had aborted on day (170 ± 10 days).

3.3. Segmented and band neutrophils

The results of segmented and band neutrophils in blood samples collected from various group of P, NP, and aborted cows are presented in Figures 3 and 4, respectively. Both segmented and band neutrophils displayed infradian rhythmicity throughout the gestation cycle. Segmented neutrophils were always higher in P cows as compared to NP cows i.e.,



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and aborted 2 (170 ± 10 days) cows post artificial insemination (AI).

from day 14 to 21 post AI and remained after that almost constant with minor fluctuations throughout the gestation cycle. A significant ($p < 0.05$) decrease in the percentage of segmented neutrophils was noticed around the day of abortion in both groups. Percentage of band neutrophils increased significantly ($p < 0.05$) around the period of implantation in all groups of cows, however, the major increment in the percentage of band cells was observed at the day of abortion. Interestingly, an inverse circadian rhythmicity was observed between the percentage of both segmented and band cells around the most critical periods of gestation cycle just like implantation and abortion.

3.4. Phagocytic activity of blood neutrophils

[redacted] NP and aborted cows.

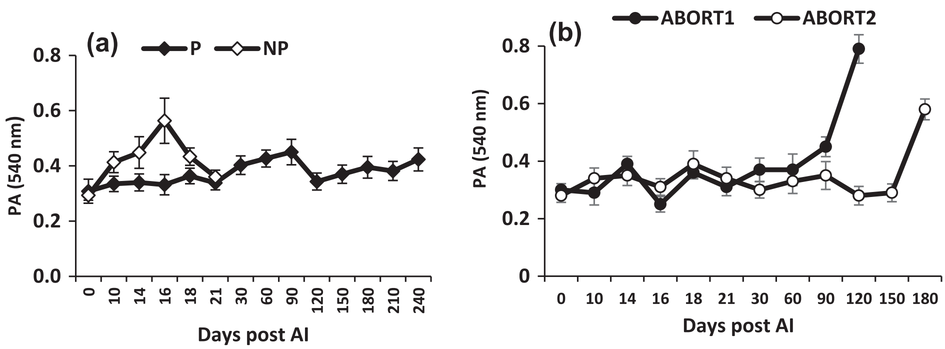
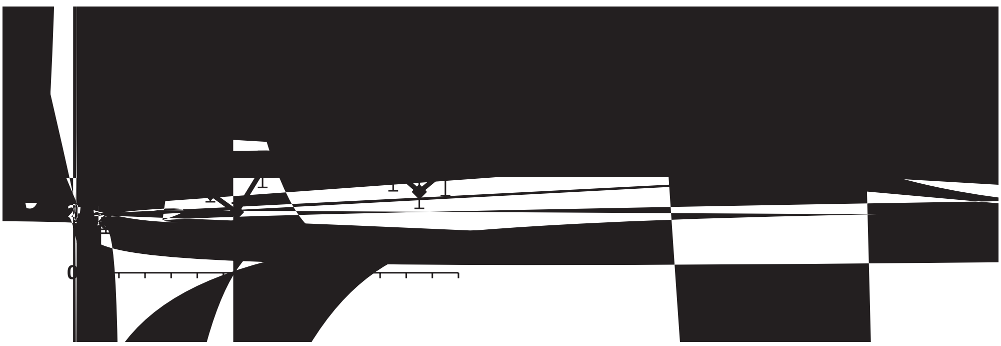


Figure 5. Phagocytic activity (5a, 5b) in pregnant (P), non-pregnant (NP), aborted 1 (110 ± 10 days) and aborted 2 (170 ± 10 days) cows post artificial insemination (AI).



3.5. *Myeloperoxidase enzyme of blood neutrophils*

Concentration of myeloperoxidase enzyme in blood neutrophils collected from various group of P, NP, and aborted cows has been presented in (Figure 6). Concentration of myeloperoxidase enzyme increased on day 10 in all the 3 group of cows. But in NP cows, the concentration increased significantly ($p < 0.05$) and remained high from day 14 to 21. It has also been observed that the level of this enzyme was always higher in samples collected from NP cows as compared to P cows. In P cows, it started declining till day 21 and again increased significantly ($p < 0.05$) till 150 days of the gestation period with subsequent decrease during the remaining gestation cycle. In aborted groups, the level of myeloperoxidase enzyme showed circadian rhythmicity until the termination of pregnancy with maximum values ($p < 0.05$) recorded at the day of abortion in both groups.

4. Discussion

The present study was carried out to see how the circadian rhyhmicity in the number, type, phagocytic activity, and myeloperoxidase enzyme concentration of blood neutrophils would differ under successful and unsuccessful termination of the gestation cycle. Successful delivery of pregnant cow once in a year is an essential step to maintain the production cycle on farms. During pregnancy, there is a change in immune cells which is driven by local signal received from the conceptus as well as hormonal changes mediated by the placental or maternal system (Gifford et al. 2007). Various observations recorded about the total leukocyte count as well as type and activities of blood neutrophils in P, NP, and aborted cows have been discussed below.

The circadian variations in the percentage of circulating immune cells are not only related to rhythmic variations in cell proliferation in bone marrow but also depending on apoptosis and clearance of these cells at tissue level (Tripathi et al. 2015). Total leukocyte count and N:L ratio measured in blood samples of crossbred cows during initial stage of pregnancy were found to be within the normal range as reported for exotic cows (Meglia et al. 2001). There was an increase in blood TLC and N:L ratio during early pregnancy in NP cows which may be an indicator of the level of stress faced by these cows. The presence of semi allogenic embryo causes the influx of neutrophils as these are the first immune cells to sense the presence of a foreign genome. An increase in TLC and N:L ratio observed at the time of abortion may be due to the presence of infection which may have induced abortion in cows. Neutrophil: lymphocyte ratio is a marker of stress in animals (Davis et al. 2008; Lynch et al. 2010). Increase in N:L ratio gives an indication that the cows were in stress during the period of implantation and abortion or it may be possible that implantation or abortion itself was the cause of stress which resulted in rejection of embryo/fetus. On the other hand, in case of P cows no change in N:L ratio indicates that dam was able to sense the embryo and regulated its immune cells in favor of embryo implantation.

We have observed more immature, band-shaped neutrophils around implantation and abortion. This indicates a high level of stress during these critical periods mainly around abortion where the maximum band cell percentage was seen. At the time of high systemic demands or stress, more neutrophils have to be sent to the circulation and as result bone marrow releases more immature band neutrophils (Alhussien et al. 2015). Neutrophils migrate to the site of infection and perform phagocytosis which is mediated through lysosomal enzymes and respiratory burst (Dang et al. 2013; Alhussien et al. 2016). We observed an increase in phagocytic activity of neutrophils in NP cows during peri-implantation period as compared to P cows. This increase in phagocytic activity in NP cows may influence the Neutrog(s ha)-

The enzyme myeloperoxidase was estimated as it is abundantly expressed in neutrophil granulocytes and forms a complex with H_2O_2 that attacks the pathogen directly or reacts with nitrogen intermediates to form highly reactive compounds (Shepherd 1986). Myeloperoxidase enzyme concentration increased in NP cows from day 14 to 21 post AI as compared to P and aborted cows. This may be due to the increase in both the number of neutrophils and phagocytic activity in NP cows. High levels of myeloperoxidase at the time of abortion indicate high degranulation and bactericidal activity of blood neutrophils at that particular time which might be the reason for sudden loss of pregnancy.

Role of immune cells like maternal macrophage and dendritic cells in the establishment of pregnancy particularly, development of the corpus luteum and maternal immune response to the embryo is well documented (Fair 2015), but studies on neutrophils are scanty. One thing common for all the studies is that it is the local adaptation of the cow's immune system that allows and helps in the co-existence between the mother and semi-allograft. In other words we can say that it is the receptivity, balance in the function of various immune cells and alertness of the maternal immune system along with external environmental factors which may influence the outcome of pregnancy. Also according to Scheiermann et al. (2013), circadian oscillations of immune mediators coincide with the immune system activity, possibly allowing the host to handle microbial infections more efficiently. These oscillations may also help to enhance tissue recovery and the removal of potentially harmful cellular elements from the circulation.

5. Conclusions

This is the first study carried out to explore the activity of blood neutrophils throughout the full pregnancy in cows. Based on our findings, it can be said that blood neutrophils display circadian rhythmicity throughout the gestation cycle. Neutrophils exhibit a limited decrease in their number, phagocytosis, and myeloperoxidase concentration in case of successful implantation. During the remaining period of gestation cycle, neutrophils of the pregnant cows regulate their type and activity at optimum levels until the fetus is delivered successfully. However, an increase in the percentage of immature neutrophils (band) and inflammatory activity may lead to loss of pregnancy. Further, recording the blood neutrophils rhythmicity of pregnant cows at timely intervals may help us to better manage these cows during the critical periods of pregnancy.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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