Theriogenology 157 (2020) 140–148

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

A comparative study on various immunological parameters influencing embryo survivability in crossbred dairy cows

Bibhudatta S.K. Panda [a], Sunil Kumar Mohapatra [b], Arvind Kumar Verma [c], Aarti Kamboj [a], Mohanned Naif Alhussien [d], Ajay Kumar Dang [a, *]

[a] Lactation and Immuno-Physiology Laboratory, ICAR-National Dairy Research Institute, Karnal, Haryana, 132001, India
[b] Department of Animal Biochemistry, ICAR-National Dairy Research Institute, Karnal, Haryana, 132001, India
[c] Department of Animal Biotechnology, ICAR-National Dairy Research Institute, Karnal, Haryana, 132001, India
[d] Animal Production Division, Aleppo University, Aleppo, Syria

ARTICLE INFO

Article history:
Received 15 January 2020
Received in revised form
26 May 2020
Accepted 28 May 2020
Available online 24 July 2020

Keywords:
Embryonic mortality
Pregnancy
Blood neutrophils
Cytokines
CD-Molecules

ABSTRACT

Since long embryonic mortality has remained an area of concern affecting the reproduction, production, and profitability of dairy cows. We investigated the possible interaction between interleukins, hormones, and neutrophil associated CD markers during the implantation window in Karan Fries (KF) cows naturally coming to heat. Blood collection was done on days 0 i.e. day of Artificial Insemination (AI), 10, 18, 21, 30 and on day 40 post-AI. Total leucocyte count (TLC) and neutrophil to lymphocyte (N:L) ratio were recorded. Blood neutrophils were isolated and their number, phagocytic activity (PA), myeloperoxidase (MPO) concentration and relative mRNA expression of cell adhesion molecules (CD-11b, CD-31, CD-44, CD-62L) as well as progesterone-inducing-blocking-factor (PIBF) and glucocorticoid receptor alpha (GRα) were examined. Plasma progesterone, cortisol, IL-2, IL-8, IL-6, and IL-10 were also measured. Pregnancy was confirmed by non-return to heat, ultrasonography and per rectal examination along with progesterone assay. Cows were further divided into pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) groups. Embryonic losses cows showed lower plasma concentration of IL-10 (<100 pg/ml) and a higher concentration of IL-2 (>500 pg/ml). Also, a 4 fold increase in the relative mRNA expression of CD-11b and 2.5 fold changes in CD-44 expression were observed in embryonic mortality. We observed a 1.5 fold increase in the relative mRNA expression of PIBF and a 0.5 fold increase in GRα expression in pregnant cows compared to EEM (on day 21) and LEM (on days 30 and 40) cows. Our results depicted that the hyperimmune status of the dam which could be due to multifactorial events that led to the pregnancy failure. The above basic values may be used for checking the immune status and thus timely management strategies can be taken to prevent embryonic losses.

1. Introduction

To achieve one calf per year per cow successful embryonic implantation followed by complete fetal development and calf survival is required. About 20–50% of embryonic and fetal deaths have been recorded in normal healthy bovines during the early days of pregnancy [1]. Diskin et al. reported that compared to the fertilization rate of 90%, the average calving rate in cattle is only 55% and rest 35% reflects the occurrence of dreadful embryonic/fetal mortality [2]. Approximately 80% of embryonic losses happen between 8 and 16 days, 10–15% of losses between days 17–42 and around 5% after day 42 of post-AI [3]. Genetical, nutritional, climatic, endocrine, improper uterine environment and some disease-causing agents are major predisposing factors of pregnancy loss during the first 42 days of calving [4,5]. However, in addition to these factors, the immune-physiological cause/improper immune system activation might be one aspect of pregnancy loss which is yet to be fully explored in bovine [6,7] and this is the major focus of our study.

Specific changes occur in the maternal immune system which begin right after fertilization and are tightly regulated to protect both the mother and the growing fetus from any type of infection [8]. Fine orchestration between the immune and endocrine system

* Corresponding author. Lactation and Immuno-Physiology Laboratory, ICAR-NDRI, India.
E-mail addresses: rajadang@gmail.com, rajadang@rediffmail.com (A.K. Dang).

https://doi.org/10.1016/j.theriogenology.2020.05.041
0093-691X/

and the secreted cytokines (pro and anti-inflammatory) not only help in embryo implantation but also aid in successful placentation and parturition [9]. Cytokines play a critical role in signaling between different cells of the immune system by influencing their recruitment, activation, stimulation, killing or suppression. Being the regulator of cell apoptosis, proliferation and differentiation, they also help in feto-maternal tissue growth [10]. In humans, studies suggest that improper regulation of cytokines leads to various pregnancy-related complications like pre-eclampsia, miscarriage and pre-term labor [11,12]. However, in the bovines study of cytokine patterns during early pregnancy in context for successful implantation or failure still needs to be explored.

Kizaki et al. observed that among all the blood cells, neutrophils are the first cells to sensitize the embryo in the uterus. They participate in maternal recognition of pregnancy (MRP) after getting induced by interferon tau (IFNτ) which is exclusively secreted by trophoectodermal cells of developing embryo in cows [13,14]. After getting influenced by IFNτ, neutrophils along with IL-8 aid in increase progesterone secretion, hence pregnancy establishment as well as the survival of the embryo. In human deciduous, a recent study indicates that the presence of neutrophils with pro-angiogenic factors has an impact on pregnancy outcomes [15]. In pregnancy-associated disorders like infertility, miscarriage, pre-eclampsia and fetal loss in human, the role of neutrophil have been thoroughly discussed with the context of pregnancy failure [16]. Additionally, depletion of neutrophils aggravates pregnancy complications in mice [17]. However, in bovine the role played by neutrophils during the establishment of pregnancy is still unclear and a largely neglected topic. Moreover, the emerging view on neutrophils states that being a part of innate immunity may also portray the adoptive immune tolerance during pregnancy [18]. Thus, understanding the differential role of neutrophils in pregnancy establishment as well as in failure in bovine is definitely the need of the hour. Therefore, the present study was undertaken to study and compare the alteration in the activity of neutrophils with various cytokines and associated hormones which may lead to either successful establishment of pregnancy or embryonic mortality in cows.

2. Materials and methods

2.1. Study site and selection of cows

The present study was conducted at the Livestock Research Centre of National Dairy Research Institute, Karnal, India, situated 250 m above mean sea level, latitude 29.43°N & longitude 77.2°E. The temperature-humidity index (THI) during the study period ranged from 60 to 64 (Thermo-neutral condition). Twenty-six healthy multiparous cows (KF breed) were selected after obtaining approval from the Institute's animal ethics committee (IAEC) as per rules and guidelines framed and communicated by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), laid down by the Government of India. The selected cows were aged between 5 and 7 years, with regular cyclicity and in their early lactation, without any history of reproductive ailment and that having not more than two services per conception. The length of estrous cycle was 15.1 ± 4.4 days with fertility rate 85–90% and conception rate of 45–50%. All the cows were fed with balanced ration and provided with *ad-lib* water during all times of the day. Regular examination of physiological responses was done to ensure the health status of the cows under study.

2.2. Samples collection and grouping of animals

The study was carried out during the onset of the winter season (October and November) to provide a thermo-neutral and comfortable condition to the animals. Blood samples were collected from the cows that exhibited natural heat and were brought for AI.

Forty animals were taken at the beginning of the experiment. Blood samples were collected on day 0, 10 and 18 (without AI) from the animals which exhibited signs of heat like mucus discharge, swelling and reddening of the vulva and other behavioral signs of estrus. Their plasma progesterone concentration and total leucocyte counts (TLC) were estimated using bovine specific ELISA test kit and haemocytometer respectively. The animals were then subjected to AI in their next heat and blood samples were collected on day 0 (day of estrus in which blood samples were collected before the animals were subjected to AI which served as non-pregnant control), 10, and 18 post-AI. The differential plasma concentrations of progesterone of each animal without AI and after AI on different days were estimated. Out of 40 animals post AI, 10 animals did not return to heat and subsequently became pregnant. Pregnancy confirmation was carried out by ultrasonography on day 30. Positive diagnosis of pregnancy was based on the presence of fluid filled allantoic cavity termed as embryonic vesicle. The embryonic heart beats were used as the main criteria to assess the viability of the embryo. Reconfirmation of pregnancy was also performed by transrectal palpation at day 60.

The cows which return to heat on 21 days and after 21 days were separated. The cows which returned to heat on day 21 but showed increased plasma progesterone levels to those of normal estrous cows (without AI) on days 10 and 18 were placed in EEM group. AI failure was ruled out by comparing the plasma progesterone concentration of estrous animals subjected to AI with the estrous animals which were not subjected to AI. Thus, the period from 14 ± 7 days was considered as period of EEM.

The animals which displayed approximately similar progesterone concentration on day 21 but returned to heat after 21 days were placed in the LEM group. The animals which were pregnant on day 30 (confirmed by ultrasonography) but were non-pregnant on day 40 (confirmed by the changes in uterine content and loss of fetal fluid from the uterus previously diagnosed gravid on day 30 post AI) were also placed in LEM group. Thus, the period from 35 ± 10 days was considered as period of LEM.

About 10 ml of blood was collected in sterile K_2 EDTA vacutainer tubes from jugular vein puncture in the morning (around 10 a.m.), posing minimum disturbance to the animal during collection. Immediately after collection, the blood samples were transported to the laboratory in ice for further processing. A total of 26 KF cows were used in the study i.e. 10 P, 10 EEM and 6 LEM. Out of the 6 LEM cows, 3 aborted on 30 ± 5 and 3 on 40 ± 5 days.

2.3. Isolation of plasma from whole blood

Freshly collected blood samples were centrifuged in 15 ml polypropylene Falcon tubes (Tarsons) @ 2500 × rpm for 25 min to separate the plasma which was stored in storage vials at −20 °C for the analysis of IL-2, IL-6, IL-8, IL-10, cortisol and progesterone.

2.4. Quantification of plasma cortisol, progesterone, and interleukins levels

Plasma cortisol, progesterone and interleukins (IL-2, IL-6, IL-8 and IL-10) levels were estimated using bovine specific ELISA Test Kits according to the manufacturers' protocols. The optical density (OD) was measured by ELISA reader (Multiskan Go, Thermo Scientific, Finland).

2.4.1. Quantitative sandwich enzyme immunoassay for the determination of IL-2, IL-6, IL-8 and IL-10

Plasma IL-2, IL-6, IL-8 and IL-10 were estimated by "Bovine specific ELISA Kits" (Cloud-Clone Corp., Houston, USA). The minimum detectable dose of bovine IL-2 and IL-8 were 5.2 pg/ml and 6.8 pg/ml respectively. The detection range of the assays was 15.6 pg/ml to 1000 pg/ml for both IL-2 and IL-8. The minimum detectable limit of bovine IL-6 and IL-10 were 3.2 pg/ml and 2.8 pg/ml respectively. The detection range of the assays was 7.2 pg/ml to 500 pg/ml for both IL-6 and IL-10. The intra and inter-assay CV were 10% and 12%, respectively for all the interleukins.

2.4.2. Competitive enzyme immunoassay for the quantification of plasma cortisol and progesterone levels

Plasma cortisol and progesterone were quantified by "Bovine specific ELISA kits" (Cusabio Biotech co., Ltd, Wuhan, China). The minimum detectable limit of bovine cortisol was 0.049 ng/ml with a detection range of 0.049 ng/ml to 200 ng/ml. The intra and inter-assay CV were 8% and 10%, respectively. The minimum detectable dose of bovine progesterone was 0.2 ng/ml with a detection range of 0.5 ng/ml to 30 ng/ml. The intra and inter-assay CV were 15%.

2.5. Total leucocyte count and neutrophil-lymphocyte ratio of blood

TLC of blood was enumerated by a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany) as per standard hematological 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.

2.6. Isolation of neutrophils from whole blood

Neutrophils isolation was performed by density gradient centrifugation method using Histopaque solutions 1077 (Catalogue No. 10,771, Sigma Aldrich, St. Louis, MO, USA) and 1119 (Catalogue No. 11,191, Sigma Aldrich, St. Louis, MO, USA) in 15 ml polypropylene Falcon tubes, as reported by Sheikh et al. [19].

2.7. Quantitative sandwich enzyme immunoassay for the determination of MPO levels in blood neutrophils

The concentration of MPO was measured from neutrophils cell lysate using a bovine-specific ELISA test kit (USCN life science, USA). Briefly, the isolated neutrophils were dissolved in 1 ml PBS and the suspension was mixed with glass beads and shock was given for 25 s by bead beater (Unigenetics Instrument Pvt. Ltd., India). The lysate was 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 the supernatant was taken for MPO estimation. The minimum detectable dose of bovine MPO was 0.65 ng/ml, and the detection range of the assay was 1.56 ng/ml to 100 ng/ml. The intra and inter-assay CV were 10% and 12%, respectively.

2.8. In vitro phagocytic activity (PA) of blood neutrophils

PA of blood neutrophils was estimated by colorimetric method using nitro blue tetrazolium (NBT) assay as described by Ref. [20]. Briefly, the cell suspension of neutrophils was adjusted to 5×10^6 live cells/ml by the culture media (RPMI 1640). 200 μl of the diluted cell suspension per well was plated in triplicate 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.

2.9. RNA isolation and real-time PCR

The RNA isolation from the neutrophils was done immediately within 2 h of blood collection. The RNA was extracted and purified by Trizol method [21] and subjected to DNase treatment by DNase I, RNase-free (Thermo Scientific, USA) as per the manufacturer's protocol. Purified RNA was dissolved in nuclease-free water (Thermo Scientific, USA) and its concentration was determined by measuring absorbance at 260 nm. The purity of RNA was verified by optical density (OD) absorption ratio 260 nm/280 nm using BioSpec-nano (serial no., A116449; Biotech). 1 μg of RNA was electrophoresed through a 2% agarose gel in Tris–EDTA buffer (0.002 M EDTA) with ethidium bromide (0.5 μg/ml) to verify the integrity of each sample. Two intact bands of 28s and 18s without smearing indicated good quality and intactness of RNA. Total RNA from neutrophils was then transcribed into cDNA by using Thermo Scientific Revert Aid First Strand RT-PCR kit (Thermo Scientific, USA). The cDNA was amplified in a Light Cycler 480 Instrument (Roche, Switzerland) using the Thermo Scientific Maxima SYBR Green qPCR Master Mix kit (Thermo Scientific, USA) and specified primers for CD-11b, CD-31, CD-44, CD-62L, PIBF and GRα (Table 1). The following protocol and program were used: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s and then 72 °C for 5 min and final holding temperature was 4 °C. GAPDH and β-actin were also analyzed and used as internal control housekeeping genes. Each PCR 96-well plate contained a "no template control" (water substituted for cDNA in the reaction) to ensure that there was no background contamination. Samples were assayed in triplicate. Probabilities less than 5% ($P < 0.05$) were considered significant.

F: forward; R: reverse; CD-11b, CD-31, CD-44 and CD-62L: Cell adhesion molecules; PIBF: Progesterone induced blocking factor; GRα: Glucocorticoid receptor alpha; GAPDH and β -Actin: housekeeping genes.

2.10. Statistical analysis

All data were presented as mean ± standard error mean (SEM) and were analyzed by repeated-measures two-way ANOVA (mixed model). Hypothesis testing was done at a 5% level of significance 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. The following statistical model was

Table 1
Details of various primers used in the study.

Genes	Sequence (5′→3′)	Acc. No.	Annealing Temp (°C)
CD11b	F: TAAGAAGAGCCCGGTGCTGAAC R: TGGGATGGCACACTGGATTCTC	NM_001039957.1	58
CD-31	F: TCGGCAGGGTGTTCAAGAGAAG R: CTGGGCTTGGAGAGCATTTCAC	NM_174571.3	58
CD44	F: CTGTCAACAGTAGGAGAAGGTGTG R: TCCTCCATGGTTCCATTCCCATTG	NM_174013.3	58
CD62L	F: TCCAGAACCAACCTGTCGAGTG R: TCCATGGTTCCCAAATCGGGTTC	NM_174182.1	58
PIBF	F: GATGCACTTCACCAGAAGCA R: CAAAGTCACGCAGGTTTCGAC	XM_015465716.1	58
GRα	F: GGCCAGATGTACCACTACGAC R: CCAGGGCTTGAATAGCCGTTAGAA	AY238475.2	58
β-Actin	F: CATCGCGGACAGGATGCAGAAAGC R: GCGCGATGATCTTGATCTTCATTG	NM_173979.3	59
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001034034	59

used to estimate the effect of the groups, days and their interactions;

$$Yijk = \mu + Gi + Dj + (GD)ij + eijk$$

Where Yijk is a dependent variable, μ is the population mean, Gi is the effect due to groups (i = 3), Dj is the effect due to days (j = 6), and GDij is the effect due to groups in different days, and eijk is the residual error.

3. Results

Levels of plasma progesterone and cortisol have been presented in Fig. 1 and Fig. 2 respectively. Plasma progesterone increased significantly ($P < 0.05$) in the pregnant group of cows till day 21 and then remained constant. Progesterone levels were found to be significantly ($P < 0.05$) lower on days 21 and 40 in EEM and LEM cows respectively. However on comparing the differential plasma progesterone levels in normal estrous cows (without AI) with estrous cows (with AI), it was observed that the progesterone levels started to increase from day 10 in estrous with AI group compared to normal estrous without AI cows (Supplementary Fig. 1). This increased plasma progesterone levels were maintained in animals which subsequently became pregnant, whereas the plasma progesterone levels declined gradually to the basal level in animals which returned to heat after day 21 (non-pregnant and EEM). Plasma cortisol increased significantly ($P < 0.05$) on day 18 in all the groups of cows. In P and EEM group a decrease in plasma cortisol concentration was observed during the subsequent days of sampling. Cortisol values decreased significantly ($P < 0.05$) on day 21 and then remained higher on days 30 and 40 in the LEM group of cows.

TLC was similar in all the groups of cows (Fig. 3). The values of TLC increased on days 10 and 18 in both P and EEM cows. TLC which showed a decreasing trend again increased on days 30 and 40 respectively. A significant ($P < 0.05$) increase in TLC was observed on days 18 and 21 (day of embryonic mortality) in EEM cows. Values of TLC remained unchanged till day 21 in LEM cows but were found to be significantly ($P < 0.05$) higher on days 30 and 40 (day of embryonic mortality). The TLC values estimated in cows exhibiting estrous with AI and without AI groups were found to be statistically non-significant when compared between these two groups (Supplementary Fig. 2).

The N:L ratio ranged between 0.47 and 0.48 in all the groups of cows (Fig. 4). An increase in N:L ratio was observed in P cows on day 18 but their values were then decreased significantly ($P < 0.05$) on day 21 and remained constant thereafter. The ratio of N:L showed a significant ($P < 0.05$) increase on day 18. N:L ratio in LEM increased on days 10 and 18, decreased significantly ($P < 0.05$) on day 21 and then showed a significant ($P < 0.05$) increase on day 30.

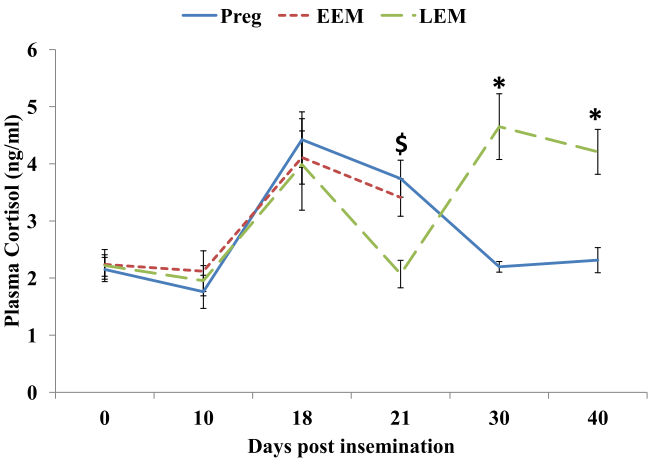

Fig. 2. Plasma cortisol concentration (ng/ml) in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

PA of blood neutrophils isolated from all the groups of cows have been presented in Fig. 5. PA was similar in all the cows on the day of AI. These values decreased on days 18 and 21 in pregnant cows. However, the PA of neutrophils increased significantly ($P < 0.05$) on day 18 and day 40 in EEM and LEM cows respectively. Changes observed in the enzyme MPO have been presented in Fig. 6. All the cows showed a significant ($P < 0.05$) increase in MPO levels on day 10 post-AI. These values decreased subsequently in the pregnant group whereas the levels first decreased and then increased many times in the cows exhibiting embryonic mortality.

Pro-inflammatory cytokines (IL-2, IL-6, and IL-8) have been shown in Figs. 7–9 whereas, values of anti-inflammatory cytokine (IL-10) has been depicted in Fig. 10. Plasma IL-2 levels were initially higher in P cows but then decreased during the subsequent days of sampling. These values started to increase on day 18 and on day 30 in EEM and LEM groups respectively. IL-2 value increased ($P < 0.05$) significantly on the day of embryonic mortality in the NP group of cows. Plasma IL-6 values started to increase on various days post-AI to up to day 18 in all the cows. P cows showed significantly

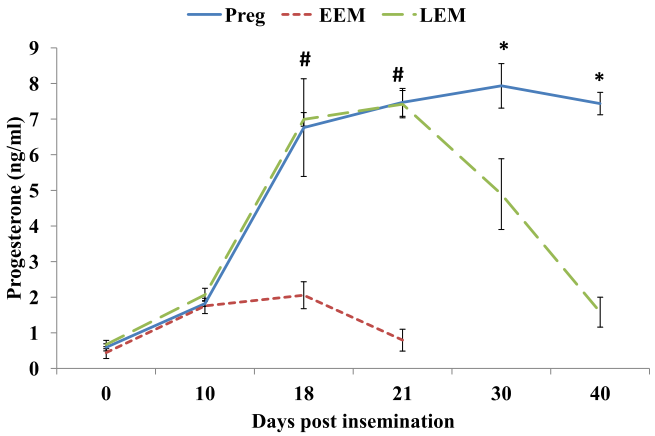

Fig. 1. Plasma progesterone concentration (ng/ml) in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

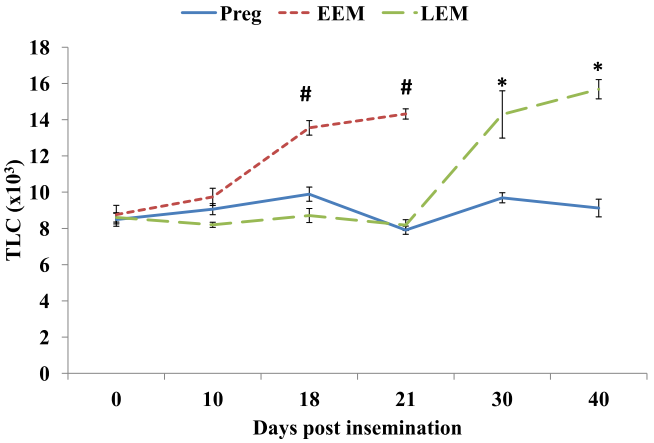

Fig. 3. Total leucocyte count in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

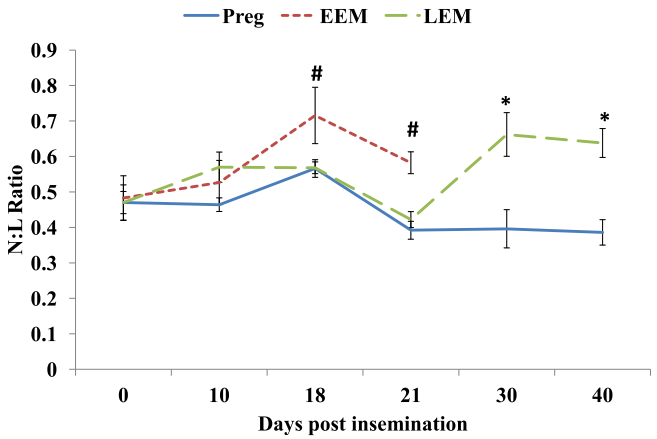

Fig. 4. Neutrophil: Lymphocyte ratio in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

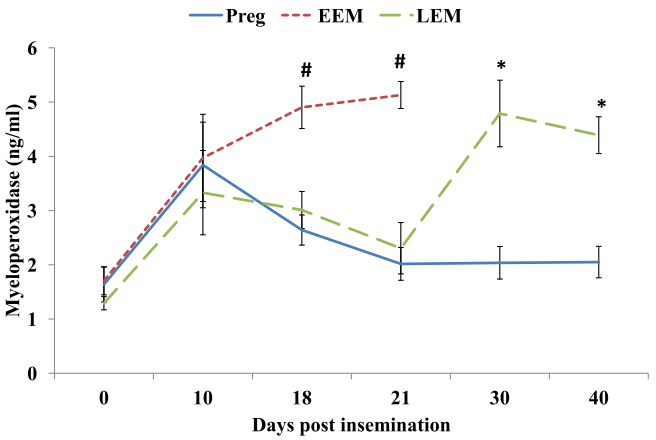

Fig. 6. Myeloperoxidase level (ng/ml) of neutrophils in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

($P < 0.05$) lower IL-6 levels subsequently on various days of sampling. A decrease was observed in the plasma IL-6 levels on day 21 in both the embryonic mortality groups which then increased significantly ($P < 0.05$) in the LEM group.

Plasma IL-8 level decreased significantly ($P < 0.05$) in the P group and then remained lower afterward. IL-8 levels first increased on day 18 and then decreased in the EEM group. In the LEM group, IL-8 increased on day 10 then decreased up to day 21 and then again increased on day 30 and day 40 of blood sampling. Of all the interleukins studied, maximum levels were recorded for the IL-8 cytokine. Plasma IL-10 levels increased significantly ($P < 0.05$) on day 21 in both P and LEM groups of cows as compared to the day of AI and then decreased subsequently. In P cows the levels become constant afterward whereas, it decreased further in the LEM group cows on day 40 of blood sampling. Some increase in plasma IL-10 was observed in EEM cows on day 10 which then decreased on days 18 and 21 respectively. On the comparison between P, EEM and LEM group of cows it was found that more than 500 pg/ml plasma concentration of IL-2 and lesser than 100 pg/ml of IL-10 may result in embryonic death.

The relative mRNA expressions of cell adhesion molecules (CD-62L, CD-11b, CD-44, CD-31) have been portrayed in Figs. 11–14 respectively. The expressions of CD-62L and CD-11b showed almost similar trends on all the sampling days when compared between all the groups. The real-time expressions of both genes in EEM cows increased from day 10 to day 18. In contrast to LEM cows and P cows, the expression of CD-62L and CD-11b was found to be significantly ($P < 0.05$) more on day 18 and day 21 post-AI. P cows displayed almost similar expression of CD-62L and CD-11b on all the sampling days. On day 30 and day 40, LEM cows exhibited significantly ($P < 0.05$) higher mRNA transcripts of CD-62L and CD-11b than in P cows. qPCR analysis of CD-44 revealed that in all the groups mRNA expression started to increase from day 10 onwards. However, on day 18 and day 21, the expression of CD-44 was significant ($P < 0.05$) among all. The LEM cows disclosed significantly higher mRNA transcripts of CD-44 on days 30 and 40 post-AI in comparison to P cows whereas, P cows indicated almost similar expression on all the sampling days. The relative mRNA expression of CD-31 in all 3 groups started to increase from day 10 onwards. In EEM, the expression of CD-31 was marked to be significantly ($P < 0.05$) more on day 18 than LEM and P cows. However, on day 21, its expression was significant ($P < 0.05$) among all the groups. In LEM cows, the mRNA transcripts of CD-31 started to increase from day 21 and on days 30 and 40, the expression was noted to be significantly ($P < 0.05$) higher than that of P cows. On comparison of the mRNA expression of all the studied CD molecules, we found that more than 4 fold changes in the relative mRNA expression of CD-11b and 2.5 fold changes in CD-44 expression may be one of the causes of embryonic death.

The relative mRNA expressions of PIBF and GRα have been presented in Figs. 15 and 16. The mRNA expression of PIBF started to

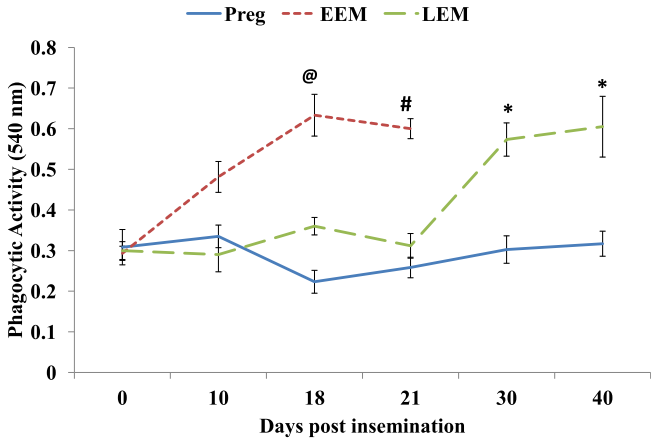

Fig. 5. Phagocytic activity of neutrophils in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

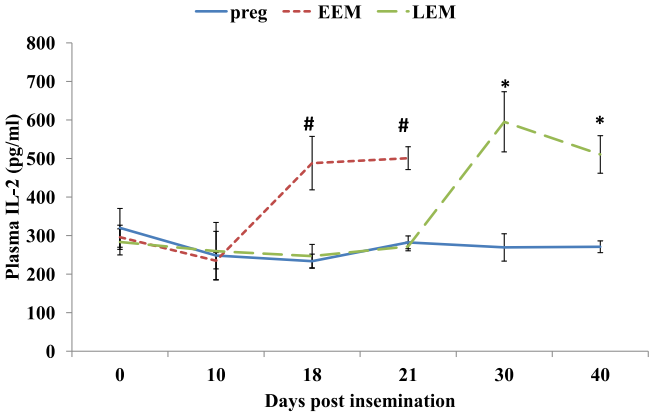

Fig. 7. Plasma IL-2 concentration (pg/ml) in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

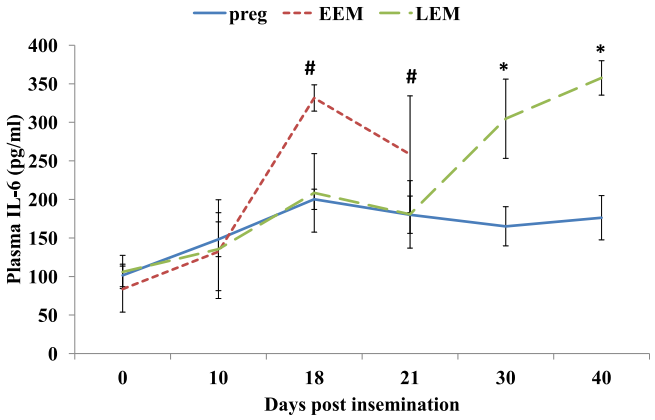

Fig. 8. Plasma IL-6 concentration (pg/ml) in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

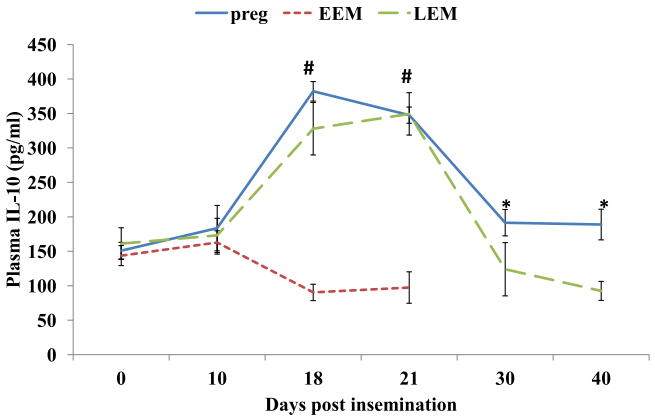

Fig. 10. Plasma IL-10 concentration (pg/ml) in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

rise from day 10 onwards in all groups. Then, the PIBF expression decreased subsequently after day 18 post-AI in EEM. However, in LEM and P groups, the relative mRNA expression of PIBF remained significantly ($P < 0.05$) high on day 18 and day 21 post-AI, when compared with EEM cows. Subsequently, the mRNA transcripts abundance of PIBF in LEM followed a decreasing pattern after 21 days post-AI whereas, in the P group, the values manifested a similar pattern on consecutive days of sampling. P cows demonstrated significant PIBF ($P < 0.05$) expression on day 30 and 40. The relative mRNA expression of GRα began to shoot up in LEM and P cows after day 10 post-AI, but decreased in EEM cows. LEM cows depicted a decreasing trend of GRα mRNA expression after day 21 whereas, P cows showed constant expression after day 18. However, on days 30 and 40, GRα expression revealed a significant difference between P and LEM cows where the P cows had significantly ($P < 0.05$) higher GRα mRNA expression. On comparison with EEM and LEM, we found that pregnant healthy cows had more than 1.5 fold increase in the relative mRNA expression of PIBF and 0.5 fold increase in GRα expression.

4. Discussion

During establishment of pregnancy, the maternal immune system experiences a number of challenges starting from insemination (i.e., introduction of semen into the female reproductive tract), blastocyst hatching, elongation, implantation, placentation up to parturition [9]. The interaction between the maternal immune system and the developing semi-allogeneic embryo can either be harmful or beneficial. However, the conceptus signals its presence to its dam by secreting some chemokines like IFNτ (cow), estradiol (sow), HCG (human), etc. for maternal recognition of pregnancy (MRP). In the bovine embryo, synthesis of IFNτ initiates around the time of blastocoel formation (around 8 days) and goes up to 21–25 days with a peak around 14–18 days post fertilization [22]. It prepares the endometrium for a successful implantation. Besides this, IFNτ is anti-luteolytic, antiviral, immunomodulatory, anti-proliferative and non-viral inducible [23]. Prolonging maintenance of corpus luteum because of IFNτ enables sustained progesterone production which is essential for the establishment and maintenance of pregnancy. From regulating endometrial secretions to the growth and differentiation of conceptus, progesterone plays a crucial role during early pregnancy in cows [24]. There is a positive correlation between the rise in plasma progesterone concentrations post-AI and embryonic development in cattle [25]. Similarly in our study, the plasma progesterone concentration was significantly ($P < 0.05$) higher in P cows on days 30 and 40 compared to LEM cows. On comparison with EEM cows, P and LEM cows showed a significantly ($P < 0.05$) higher plasma progesterone concentrations on day 18 and 21. Therefore, optimum progesterone level is necessary for embryonic survival and immunologically it prevents allograft rejection by suppressing different immune cell migration including the neutrophils towards the developing embryo in uterus [26]. Besides IFNτ and progesterone, plasma cortisol is also having

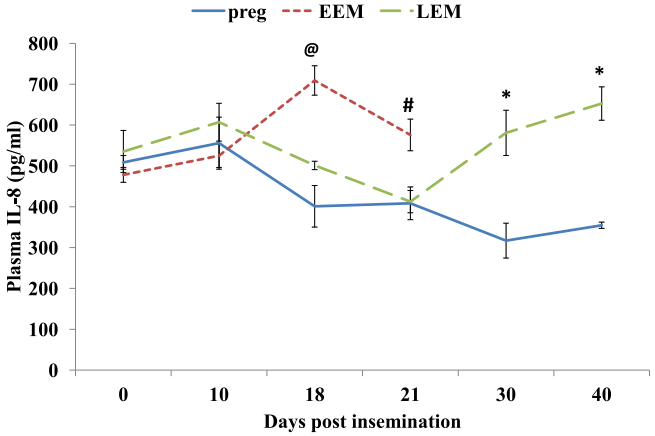

Fig. 9. Plasma IL-8 concentration (pg/ml) in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

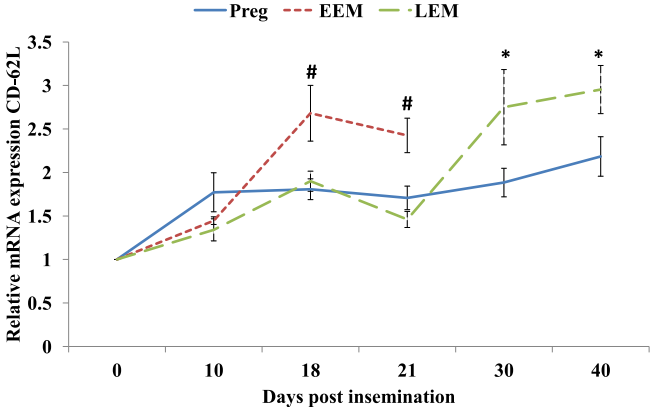

Fig. 11. Relative mRNA expression level of CD-62L in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

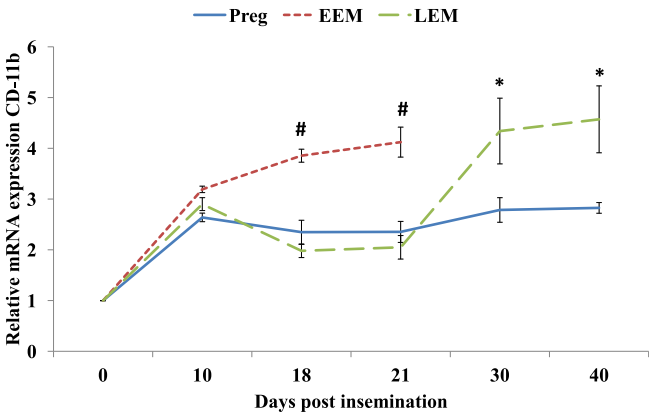

Fig. 12. Relative mRNA expression level of CD-11b in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

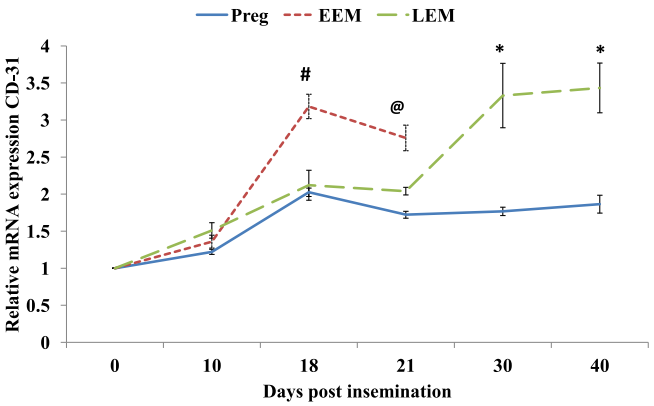

Fig. 14. Relative mRNA expression level of CD-31 in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

certain role in conceptus elongation in ruminants [27] and according to Duong et al. cortisol has positive impact on corpus luteum function during early pregnancy and it also supports embryonic implantation and by doing this it influences higher pregnancy rate in heifers [28]. It is well known that the optimum concentration of cortisol is necessary for successful embryo implantation as well as growth, development and maturation of fetal organs and placenta. In our study, we also found significantly ($P < 0.05$) higher concentration of cortisol on 30th and 40th day of pregnancy in LEM cows as compared to P cows. The possible reason might be due to some stress faced by the mother, although the reasons for stress have not been investigated.

During early pregnancy, signals from conceptus (IFNτ) and hormones of maternal origin like progesterone and cortisol etc. modulate the migration of different immune cells towards the gravid uterus. Studies on hematological changes during pregnancy reflect physiological status of animals. In our study, we have taken TLC and N:L ratio as hematological parameters. The overall TLC values of EEM cows was significantly ($P < 0.05$) more on day 18 and 21 and that of LEM cows was higher on day 30 and 40 in comparison to P cows. The high level of TLC in NP cows might be due to some infection or stress and the low number of TLC in P cows could be due to higher progesterone levels. Higher TLC might also be associated with an increase in the influx of neutrophils to sense the foreign genome. It could be justified with the N:L ratio which indicates stress [29] in animals. Significantly ($P < 0.05$) high N:L ratios in EEM cows on days 18 and 21 and in LEM cows on day 30 and 40 compared to P cows demonstrates that the animals might be incurring immunological challenges from the embryo and hence implantation failure might happen. On the contrary, P group displayed no change in N:L ratio which might have favored embryo implantation and our results are in accordance with Mohammed et al. where high TLC and N:L ratio resulted in non-pregnancy. Thus, a cutoff N:L ratio could be helpful for the diagnosis of non-pregnancy status [6].

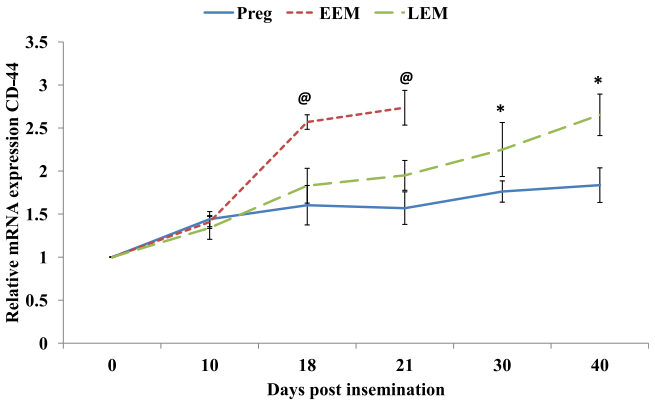

Fig. 13. Relative mRNA expression level of CD-44 in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

Neutrophils migrate to the site of inflammation and carry out phagocytosis as a killing mechanism to eliminate invaders. Significantly ($P < 0.05$) higher PA in EEM cows on day 21 compared to LEM cows and significantly ($P < 0.05$) increased PA in LEM cows on day 30 and 40 compared to P cows indicates a possible immune response during that period. However, another killing mechanism that neutrophils used to adopt is the process of degranulation. Our observations delineated significantly ($P < 0.05$) higher MPO concentration in the neutrophil lysate of EEM cows on days 18 and 21 compared to LEM and P cows whereas, significantly ($P < 0.05$) increased MPO concentration in LEM cows compared to P cows on day 30 and 40. NP cattle showed the highest concentration of MPO. Similar to our results, pregnant neutrophils exhibited circadian rhythm and lower PA contrast to nonpregnant neutrophils [6]. In addition to this, they observed the highest concentration of MPO in neutrophils obtained from non-pregnant cattle than pregnant during day 14 to day 21 post-AI. According to them, MPO reflects bactericidal activities and a higher-level may hamper pregnancy. Contrary to this Kindzelskii et al. were able to localize MPO at the surface of pregnant neutrophils of the human but failed to do in non-pregnant neutrophils [30]. This may be a point to debate because MPO has a role in inflammation as well as in neutrophil metabolism based on these two observations and long-lasting concept suggests that pregnant neutrophils undergo metabolic changes along with functional changes [31]. Thus, we proposed thorough research on this signature molecule as MPO is the hallmark of neutrophil.

Cytokines are soluble proteins produced by leucocytes and other cell types which are instrumental in maternal tolerance against the semi-allergenic embryo during establishment of pregnancy. We observed significantly ($P < 0.05$) higher plasma pro-inflammatory cytokines (IL-2, IL-6 and IL-8) levels on days 18 and 21 post-AI in EEM cows compared to LEM and P cows. In LEM cows on days 30 and 40 the plasma levels of all 3 cytokines were found to be significantly ($P < 0.05$) more compared to P cows. IL-2 is known to be a T-cell growth factor and a potent pro-inflammatory cytokine. However, it activates neutrophils and increases their adherence to human umbilical vein endothelial cells by increasing the expression

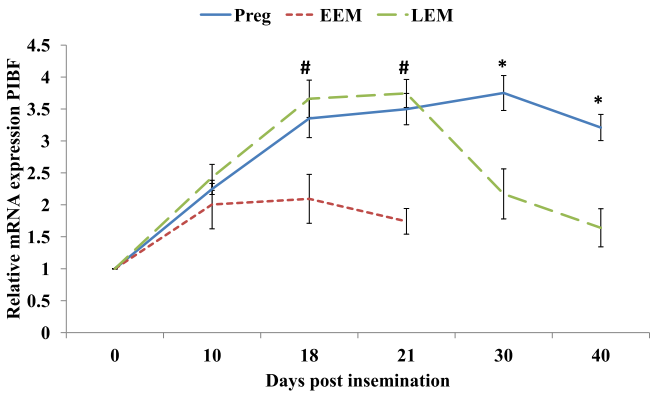

Fig. 15. Relative mRNA expression level of PIBF gene in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

of CD-18 [32]. IL-8 is also a pro-inflammatory cytokine produced by many cell types including neutrophils, macrophages and lymphocytes and is known to be a potent chemo-attractant and activator of neutrophils to the inflamed site and subsequent phagocytosis and degranulation. Another side of IL-8 function includes they are the potent promoter of angiogenesis along with neutrophil infiltration in developing corpus luteum [33]. So, monitoring of IL-8 could be an aid to predict the outcome of implantation. IL-6 cytokine helps in neutrophil production from bone marrow and influences the neutrophil trafficking during inflammation [34] and enhances the elastase as well as PA of neutrophils [35]. Anti-inflammatory cytokines like IL-10 also modulates neutrophils chemotaxis during disease condition [36]. We noticed significantly ($P < 0.05$) low plasma levels of IL-10 on days 18 and 21 in EEM cows in contrast to LEM and P cows. In LEM cows also we found significantly ($P < 0.05$) low IL-10 levels on days 30 and 40 comparison to P cows. Thus, non-pregnant cows might be in hyper-immune state during that period and in P cows IL-10 could have inhibited the innate and adaptive immune responses against inflammation and curtails the potential tissue damage during the process. Studies on the human model demonstrate that deficiency of IL-10 along with IL-4 could lead to infertility and spontaneous abortion [37].

Whenever there is an immunological challenge, out of all leucocytes, neutrophils quickly and firstly respond to the inflammatory signals and move towards the site of inflammation by the process of tethering, rolling, adhesion, crawling and transmigration [20,38,39]. Molecules like selectin (CD-62L), CD-44 mediate the beginning of migration after activation of neutrophils by different

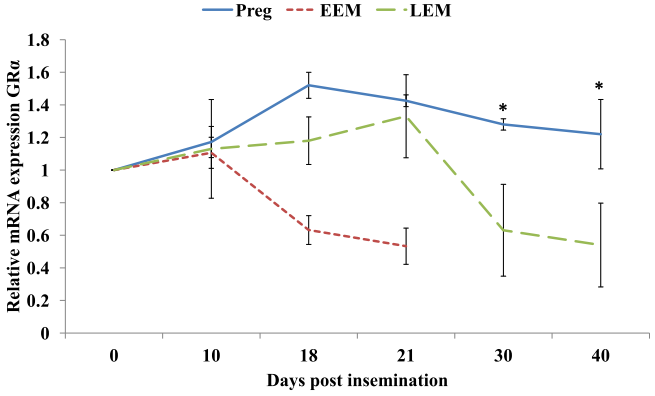

Fig. 16. Relative mRNA expression level of GRα gene in pregnant (P), early embryonic mortality (EEM) and late embryonic mortality (LEM) cows.

chemokines like IL-8 which leads to a conformational change of neutrophils and expression of integrin molecules like CD-11b or β integrin [40]. The initial loose attachment to the endothelium is mediated through selectin molecules followed by firm adhesion by integrin molecules. Finally, the neutrophils cross the endothelial layer via trans-endothelial migration in order to crawl to the inflammation area which is regulated by CD-31 molecules. Similar to the inflammatory condition due to any invading pathogens, drifting of neutrophils towards the developing embryo could be possible. To verify the hypothesis, we framed to study the expression of different CD molecules like CD-62L, CD-11b, CD-31, CD-44 on neutrophils during pregnant and non-pregnant condition. Our findings revealed that relative mRNA expression of all 4 genes on days 30 and 40 in LEM cows was more significant ($P < 0.05$) than P cows. CD-62L and CD-11b in EEM cows showed significantly ($P < 0.05$) higher expression on days 18 and 21 in contrast to LEM and P cows. The relative abundance of CD-44 was found to be significantly ($P < 0.05$) more in all the 3 groups on day 18 and 21 post-AI. Higher expression of CD molecules in different embryonic mortality cows might be suggesting more migration of neutrophils towards the uterus creating adverse condition for the implanting embryo and our study also indicated that regulated movement of neutrophils is all-important for the pregnancy establishment and aberration in this can lead to pregnancy disorder [41]. However, lower expression of CD molecules in pregnant cows might be due to either IFNτ secretion [42] or progesterone or cortisol or might be some other factors which could be the key molecules controlling the neutrophil trafficking towards the implanting embryo. Cortisol exerts its various effects through glucocorticoid receptors present in the cytoplasm of various immune cells like neutrophils. Glucocorticoid receptor alpha (GRα) inhibits NF-kB signaling and mediate anti-inflammatory responses [43]. In neutrophils, it is associated with a reduction in phagocytic activity. In our study, we found significantly ($P < 0.05$) higher expression of GRα in P group around 30th and 40th days. At the molecular level, effects of progesterone are governed via PIBF which has a multi-faceted role during pregnancy [44]. PIBF up regulates Th2 cytokine production and inhibits NK cell activity. Our findings suggest that PIBF expression is in harmony with plasma progesterone concentration and significantly requisite for embryonic survival.

5. Conclusions

Our study highlights the possible immunological mechanism involved during the establishment of pregnancy or the loss of bovine conceptus during the implantation period. We observed higher neutrophil number, MPO concentration and increased neutrophil trafficking (expression of CD molecules) in cows exhibiting embryonic mortality as compared to pregnant cows. Higher progesterone concentration and lower pro-inflammatory cytokines, PA and cortisol plasma values help in the establishment of pregnancy. All the above immunological parameters indicate that any failure in the bidirectional communication between the implanting conceptus and the dam may lead to inappropriate immune response and ultimately embryonic loss in cows. However, to investigate the possibility of using these immunological parameters as biomarkers for embryo mortality, large scale experiments needs to be carried out. Finally, selection of females with high immune regulatory competence before breeding and their optimum nutrition may also reduce early embryonic mortality and thus improve the overall productivity of the animals.

CRediT authorship contribution statement

Bibhudatta S.K. Panda: Conceptualization, Writing - original

draft. **Sunil Kumar Mohapatra:** Visualization, Formal analysis, Writing - original draft. **Arvind Kumar Verma:** Investigation. **Aarti Kamboj:** Conceptualization, Investigation. **Mohanned Naif Alhussien:** Conceptualization, Investigation. **Ajay Kumar Dang:** Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they don't have any competing interests.

Acknowledgement

The authors are thankful to the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India for providing financial support to conduct this study (Grant number BT/PR23570/AAQ/1/691/2017; Dated: May 10, 2018).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2020.05.041.

References

[1] Noakes DE, Parkinson TJ, England GC. Arthur's Veterinary reproduction and Obstetrics-E-Book. Elsevier Health Sciences; 2018.
[2] Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim 2008;43:260–7.
[3] Parmar SC, Dhami AJ, Hadiya K, Parmar CP. Early embryonic death in bovines: an overview. Raksha Tech Review 2017;4.
[4] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, Ochoa JC, Santos JE, Sartori R. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 2016;86:239–53.
[5] Rani P, Dutt R, Singh G, Chandolia RK. Embryonic mortality in cattle-A review. Int J Curr Microbiol App Sci 2018;7:1501–16.
[6] Mohammed S, Aarti K, Kalyan A, Alhussien MN, Manjari P, Yadav ML, Sheikh AA, De S, Mohanty AK, Dang AK. Fluctuation in the number, type and activity of blood neutrophils in cows exhibiting successful and unsuccessful completion of gestation cycle. Biol Rhythm Res 2017;48:855–65.
[7] Manjari P, Kapoor S, Hyder I, De S, Mohanty AK, Dang AK. A study of altered cytokine rhythms associated with successful implantation in cows. Biol Rhythm Res 2018;49:329–33.
[8] Ott TL, Dechow C, O'connor ML. Advances in reproductive management: pregnancy diagnosis in ruminants. Anim Reprod 2018;11:207–16.
[9] Fair T. The contribution of the maternal immune system to the establishment of pregnancy in cattle. Front Immunol 2015;6:7.
[10] Yockey LJ, Iwasaki A. Interferons and proinflammatory cytokines in pregnancy and fetal development. Immunity 2018;49:397–412.
[11] Raghupathy R, Kalinka J. Cytokine imbalance in pregnancy complications and its modulation. Front Biosci 2008;13. 985-4.
[12] AlJameil N, Tabassum H, AlMayouf H, Alshenefy A, Almohizea MM, Ali MN. Identification of serum cytokines as markers in women with recurrent pregnancy loss or miscarriage using MILLIPLEX analysis. 2018.
[13] Kizaki K, Shichijo-Kizaki A, Furusawa T, Takahashi T, Hosoe M, Hashizume K. Differential neutrophil gene expression in early bovine pregnancy. Reprod Biol Endocrinol 2013;11:6.
[14] Shirasuna K, Matsumoto H, Matsuyama S, Kimura K, Bollwein H, Miyamoto A. Possible role of interferon tau on the bovine corpus luteum and neutrophils during the early pregnancy. Reproduction 2015;150:217–25.
[15] Croxatto D, Micheletti A, Montaldo E, Orecchia P, Loiacono F, Canegallo F, Calzetti F, Fulcheri E, Munari E, Zamò A, Venturini PL. Group 3 innate lymphoid cells regulate neutrophil migration and function in human decidua. Mucosal Immunol 2016;9:1372.
[16] Hahn S, Giaglis S, Hoesli I, Hasler P. Neutrophil NETs in reproduction: from infertility to preeclampsia and the possibility of fetal loss. Front Immunol 2012;3:362.
[17] Higashisaka K, Nakashima A, Iwahara Y, Aoki A, Nakayama M, Yanagihara I, Lin Y, Nagano K, Tsunoda SI, Saito S, Yoshioka Y. Neutrophil depletion exacerbates pregnancy complications, including placental damage, induced by silica nanoparticles in mice. Front Immunol 2018;9:1850.
[18] Nadkarni S, Smith J, Sferruzzi-Perri AN, Ledwozyw A, Kishore M, Haas R, Mauro C, Williams DJ, Farsky SH, Marelli-Berg FM, Perretti M. Neutrophils induce proangiogenic T cells with a regulatory phenotype in pregnancy. Proc Natl Acad Sci Unit States Am 2016;113:e8415–24.
[19] Sheikh AA, Hooda OK, Dang AK. JAK3 and PI3K mediate bovine Interferon-tau stimulated gene expression in the blood neutrophils. J Cell Physiol 2018;233:4885–94.
[20] Panda BS, Mohapatra SK, Alhussien MN, Dang AK. Amount of milk neutrophil percentage and associated CD molecular changes on the compositional and technological properties of milk. Open Biotechnol J 2019;13.
[21] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
[22] Mor A, Mondal S, Reddy IJ, Soumya NP. Genes regulating maternal recognition of pregnancy in domestic animals: an update. Braz Arch Biol Technol 2015;58:854–63.
[23] Roberts RM. Interferon-tau, a Type 1 interferon involved in maternal recognition of pregnancy. Cytokine Growth Factor Rev 2007;18:403–8.
[24] Lonergan P, Forde N, Spencer T. Role of progesterone in embryo development in cattle. Reprod Fertil Dev 2016;28:66–74.
[25] Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF, Lonergan P. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev 2008;20:368–75.
[26] Soloff MS, Jeng YJ, Izban MG, Sinha M, Luxon BA, Stamnes SJ, England SK. Effects of progesterone treatment on expression of genes involved in uterine quiescence. Reprod Sci 2011;18:781–97.
[27] Brooks K, Burns G, Spencer TE. Conceptus elongation in ruminants: roles of progesterone, prostaglandin, interferon tau and cortisol. J Anim Sci Biotechnol 2014;5:53.
[28] Duong HT, Piotrowska-Tomala KK, Acosta TJ, Bah MM, Sinderewicz E, Majewska M, Jankowska K, Okuda K, Skarzynski DJ. Effects of cortisol on pregnancy rate and corpus luteum function in heifers: an *in vivo* study. J Reprod Dev 2011:1112060422.
[29] Lynch EM, Earley B, McGee M, Doyle S. Characterisation of physiological and immunological responses in beef cows to abrupt weaning and subsequent housing. BMC Vet Res 2010;6:37.
[30] Kindzelskii AL, Clark AJ, Espinoza J, Maeda N, Aratani Y, Romero R, Petty HR. Myeloperoxidase accumulates at the neutrophil surface and enhances cell metabolism and oxidant release during pregnancy. Eur J Immunol 2006;36:1619–28.
[31] Kindzelskii AL, Huang JB, Chaiworapongsa T, Fahmy RM, Kim YM, Romero R, Petty HR. Pregnancy alters glucose-6-phosphate dehydrogenase trafficking, cell metabolism, and oxidant release of maternal neutrophils. J Clin Invest 2002;110:1801–11.
[32] Li J, Gyorffy S, Lee S, Kwok CS. Effect of recombinant human interleukin 2 on neutrophil adherence to endothelial cells *in vitro*. Inflammasome 1996;20:361–72.
[33] Jiemtaweeboon S, Shirasuna K, Nitta A, Kobayashi A, Schuberth HJ, Shimizu T, Miyamoto A. Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. Reprod Biol Endocrinol 2011;9:79.
[34] Fielding CA, McLoughlin RM, McLeod L, Colmont CS, Najdovska M, Grail D, Ernst M, Jones SA, Topley N, Jenkins BJ. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. J Immunol 2008;181:2189–95.
[35] Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol 2003;24:25–9.
[36] Peñaloza HF, Nieto PA, Muñoz-Durango N, Salazar-Echegarai FJ, Torres J, Parga MJ, Alvarez-Lobos M, Riedel CA, Kalergis AM, Bueno SM. Interleukin-10 plays a key role in the modulation of neutrophils recruitment and lung inflammation during infection by *Streptococcus pneumoniae*. Immunol 2015;146:100–12.
[37] Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. Front Immunol 2014;5:253.
[38] Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013;13:159.
[39] Alhussien MN, Dang AK. Potential roles of neutrophils in maintaining the health and productivity of dairy cows during various physiological and physiopathological conditions: a review. Immunol Res 2019;67:21–38.
[40] McEver RP, Zhu C. Rolling cell adhesion. Annu Rev Cell Dev Biol 2010;26:363–96.
[41] Giaglis S, Stoikou M, Grimolizzi F, Subramanian BY, van Breda SV, Hoesli I, Lapaire O, Hasler P, Than NG, Hahn S. Neutrophil migration into the placenta: good, bad or deadly? Cell Adhes Migrat 2016;10:208–25.
[42] Manjari P, Kapoor S, Hyder I, De S, Mohanty AK, Dang AK. A study of altered cytokine rhythms associated with successful implantation in cows. Biol Rhythm Res 2018;49:329–33.
[43] Cruz-Topete D, Cidlowski JA. One hormone, two actions: anti-and pro-inflammatory effects of glucocorticoids. Neuroimmunomodulation 2015;22:20–32.
[44] Szekeres-Bartho J, Sucurovic S, Mulac-Jericevic B. The role of extracellular vesicles and PIBF in embryo-maternal immune-interactions. Front Immunol 2018;9:2890.