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Pregnancy stage-dependent modulation of neutrophil function may impact embryo survivability and pregnancy outcome in crossbred cows

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ABSTRACT

Pregnancy is a complicated physiological process that involves synchronized coordination between immune and endocrine systems. Neutrophils have been suggested as a critical immune cell for embryo implantation and pregnancy maintenance. The present study was conducted to evaluate the dynamic changes in the mRNA expressions of the cluster of designation (CD11b, CD31, CD44 and CD62L) molecules and interferon-stimulated genes (ISG15, MX1 and OAS1) in blood neutrophils throughout pregnancy in dairy cows and correlate them with the outcome of pregnancy. Blood samples were taken from negative control (NC) group, and non-pregnant (NP) group at the time of artificial insemination (AI, day zero) and on days 10, 14, 16, 18, and 21 post-AI. In pregnant (P) cows, samples were taken as described above and after every 30 days until the time of parturition. In aborted cows, samples were collected until the time of the abortion. Comparison between pregnant, non-pregnant and aborted cows revealed that the expression of CD molecules increased ($p < 0.05$) on days 14, 16, 18 and 21 post-AI only in NP cows as compared to other groups. Although the expression of CD molecules remained constant throughout the study period in pregnant and aborted cows, the expression of CD11b, CD31 and CD62L increased ($p < 0.05$) on the day of abortion and parturition. Unlike CD molecules, the expression of CD44 decreased significantly ($p < 0.05$) at the time of abortion. There was a significant ($p < 0.05$) increase in the expression of interferon-stimulated genes including MX1, OAS1 and ISG15 during the peri-implantation period in pregnant cows, and at the time of abortion in aborted cows. However, the expression of ISGs was lower ($p < 0.05$) in non-pregnant cows as compared to the other groups. The results revealed the critical role played by neutrophils during pregnancy and form the basis to unravel the underlying mechanism for neutrophil associated immunological infertility in bovines.

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1. Introduction

The profitability of dairy farms depends on producing one calf per cow annually, which depends on the successful implantation of the embryo, the proper development of the fetus and the maintenance of pregnancy. Although the fertilization rate is quite high (70–75%), the calving rate is considerably lower which reflects the higher rate of embryonic losses in cattle [1]. The majority (20–50%) of embryonic deaths occur during the early stage of pregnancy, however, a considerable number of fetal death or abortion occurs during the mid and late stages of pregnancy in bovines [2]. Multifactorial components such as stressful climate, improper nutrition, genetic background, hormonal imbalance, and disturbance in the uterine environment may lead to abortions in cows [3,4]. Besides, improper activation of the immune system is another important factor that can lead to embryonic losses at any stage of pregnancy [5–7]. The impact of the immune-physiological factor on implantation, embryonic development, pregnancy maintenance, and completion of healthy pregnancy has not been fully addressed in bovines, and this is the major focus of the present study.

Neutrophils, the oldest known phagocytes of the innate immune system, are sophisticated cells that can perform a wide range of

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specialised tasks [8,9]. Neutrophils can detect two types of signals: tissue damage and the presence of alien DNA such as the semi-allogeneic fetus [10,11]. Indeed, our group and others reported significant differences in the mRNA expression profile of neutrophils between pregnant (P) and non-pregnant (NP) cows during the peri-implantation phase which has a great impact on the outcome of pregnancy establishment [12–14].

The regulation of neutrophil function is mediated by a wide variety of receptors and genes expressed by them such as cluster of designations (CDs), interferon stimulated genes, Fc receptors, adhesion receptors, cytokine receptors, etc. [15]. Selectin (CD62L), Integrin (CD11b) and cluster of designation 31 (CD31) are key molecules for the transmigration of activated neutrophils from circulation to the inflamed tissue to maintain homeostasis. Control of conformation and clustering of these adhesion molecules is essential for proper adhesion, migration, and the clearance of source of the inflammation by neutrophils [16–18]. CD44 is a multifunctional cell surface molecule involved in proliferation, differentiation and migration of various immune cells, angiogenesis, presentation of chemokines and cytokines to the corresponding receptors [19]. Besides, CD44 mediates the engulfment of apoptotic neutrophil by macrophages which is critical step for the resolution of inflammation and maintenance of tissue homeostasis [20].

As pregnancy is instigated and triggered by interferon tau (IFNτ), which is released by the embryo's trophoblast cells, neutrophils perceive the molecular crosstalk between the trophoblast and maternal immune cells of the bovine endometrium and aid in the maternal recognition of pregnancy [14,21,22]. Interferon tau's major function is to suppress prostaglandin F2 (PGF2) synthesis and

immersion (100 X, Olympus IX51 microscope) revealed that the purity of the neutrophils was greater than 95%. Trypan Blue (0.4%, Sigma, USA) technique revealed that the neutrophils were 98% viable.

2.5. Isolation of RNA and real-time PCR

Within 2 h of blood collection, RNA was isolated from neutrophils using RNeasy Mini Kit (Qiagen, India Pvt. Ltd.) as per the manufacturer's guidelines. The contamination due to genomic DNA was removed using the RNase-Free DNase Set (Qiagen, India Pvt. Ltd.) as per the manufacturer's protocol. Agarose gel (1.8%) electrophoresis was used to evaluate the integrity of RNA by observing RNA bands (28S and 18S). Using Biospec-nano Spectrophotometer, the purity of RNA was confirmed using an optical density (OD) absorption ratio at λ260/λ280 with a ratio of 1.9–2.0 considered as "pure".

The isolated RNA was kept at −80 °C until further processing. Thermo Scientific RevertAid First Strand cDNA synthesis kit was used to convert RNA (1 μg) into cDNA (Thermo Scientific, USA). The cDNA was amplified using the Thermo Scientific Maxima SYBR Green qPCR Master Mix kit in a Light Cycler 480 Instrument (Roche, Switzerland) and specified primers for CD62L, CD11b, CD31, CD44, ISG15, OAS1, MX1 (Sigma, USA) are shown in Table 1. The protocol of RT-PCR consisted of initial denaturation at 95 °C for 5 min was followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 5 min and final holding temperature of 4 °C. Internal control housekeeping genes (GAPDH and β-Actin) were employed, and the mRNA abundance of the day of AI (day 0) was considered as a calibrator with which the relative expression of each gene at different time intervals was calibrated. A "non-template control" (water substituted for cDNA in the reaction) was included in each PCR 96-well plate to make sure that there is no background contamination. Triplicate samples were analyzed. The relative quantification of target genes was calculated by the $2^{-\Delta\Delta CT}$ method [32].

2.6. Statistical analysis

Using SAS software, version 9.1 of SAS system for window, copyright© (2011), SAS Institute Inc., Cary, NC, USA, all data were analyzed using repeated measures two-way ANOVA analysis (mixed model), followed by Tukey multiple comparison tests. The results are presented as means ± SEM. p values less than 5% were judged statistically significant.

3. Results

3.1. Plasma progesterone

The concentration of plasma progesterone was less than 1 ng/ml on the day of AI (day 0) in all the groups (Fig. 1). Thereafter, it increased significantly ($p<0.01$) starting from day 10 post-AI in all the groups as compared to the day of AI. The concentration of plasma progesterone remained below 2 ng/ml in the NC group and declined to 0.5 ng/ml on day 21 post-AI. Although the concentration of progesterone increased significantly ($p<0.01$) in NP cows starting from day 10 and reached to 3.6 ng/ml on day 14, it declined again to 0.8 ng/ml on day 21 post-AI. In pregnant and aborted groups, the concentration of progesterone remained high throughout the study and declined ($p<0.01$) on the day of calving and at the time of abortion, respectively (Fig. 1).

3.2. Relative mRNA expression of cluster of designation (CD) molecules

The relative mRNA expression of CD molecules (CD62L, CD11b, CD31, CD44) are shown in Fig. 2 and presented with detailed information in the Supplementary Tables 1–4, respectively. The relative mRNA expression of L-selectin (CD62L) and integrin (CD11b) increased in all three groups of cows on day 10 post-AI. Comparison between P, NP, and aborted cows showed that the expression of both CD62L and CD11b increased significantly ($p < 0.05$) on days 14, 16, 18 and 21 post-AI only in NP cows. Expression of CD62L and CD11b in both pregnant and aborted cows remained almost constant during the period of mid-gestation. Whereas a four fold increase in the expression of CD62L was observed at the time of abortion. The expression of CD31 increased in all groups of cows on day 10 post-AI. On comparison between the three groups of cows, NP cows showed a significant ($p < 0.05$) increase in the expression of CD31 on days 14, 16, 18 with a significant ($p < 0.05$) decrease observed on day 21 post-AI. There was no difference in the expression of CD31 between pregnant and aborted cows throughout the gestation period, but there was a five fold

Table 1
Details of various primers used in the study.

Genes	Sequence (5′→3′)	Accession number	Size (bp)	Annealing Temp (°C)
CD62L	F: TCCAGAACCAACCTGTCGAGTG R: TCCATGGTTCCCAAATCGGGTTC	NM_174182.1	66	58
CD11b	F: TAAGAAGAGCCCGGTGCTGAAC R: TGGGATGGCACACTGGATTCTC	NM_001039957.1	63	58
CD31	F: TCGGCAGGGTGTTCAAGAGAAG R: CTGGGCTTGGAGAGCATTTCAC	NM_174571.3	76	58
CD44	F: CTGTCAACAGTAGGAGAAGGTGTG R: TCCTCCATGGTTCCATTCCCATTG	NM_174013.3	73	58
ISG15	F: ACTCCATGACGGTATCCGAG R: ACCCTTGTCGTTCCTCAC	NM_174366	203	58
MX1	F: GTACGAGCCGAGTTCTCCAA R: ATGTCCACAGCAGGCTCTTC	AF_047692	197	58
OAS1	F: TCATCCGCCTGGTGAAGCACTGG R: TTGCTCCCAGGCATAGACCGTCAG	NM_001040606.1	107	58
ß-Actin	F: CATCGCGGACAGGATGCAGAAAGC R: GCGCGATGATCTTGATCTTCATTG	NM_173979.3	71	58
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001034034.1	176	58

F: forward; R: reverse; CD62L, CD11b, CD31, CD44: cluster of designation molecules; ISG15, MX1, OAS1: interferon-stimulated gene; GAPDH and β-actin: housekeeping genes.

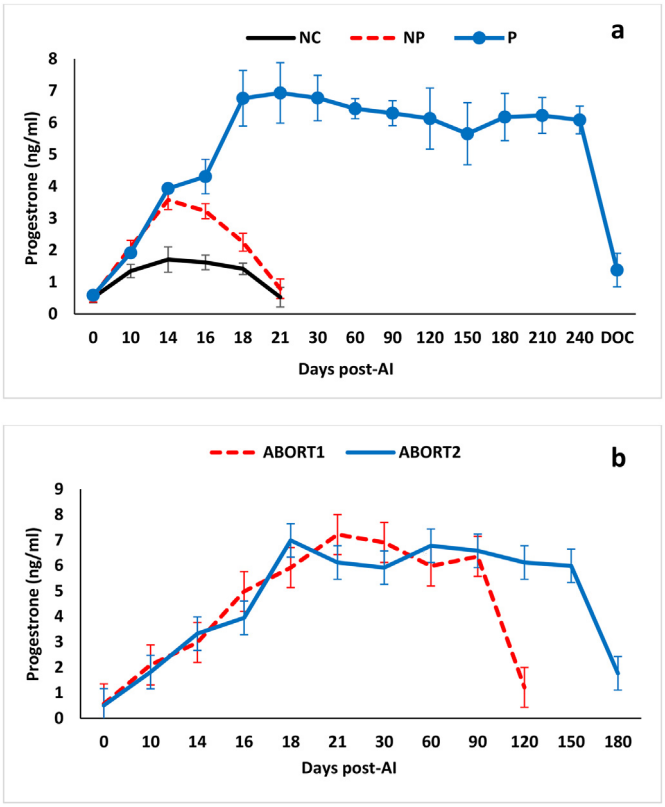

Fig. 1. Changes in plasma progesterone (ng/ml) in negative control cows (NC, estrous cycle without AI), pregnant (P) and non-pregnant (NP) cows (estrous cycle with AI) (a), aborted 1 (ABORT1, 110 ± 10 days) and aborted 2 (ABORT2, 170 ± 10 days) groups of cows post artificial insemination (AI) (b). Data were expressed as mean ± SEM. P values less than 0.05 were considered statistically significant.

increase ($p < 0.05$) on the day of abortion. Relative mRNA expression of CD44 increased in all groups of cows on day 10 post-AI, but in NP cows it continued to increase ($p < 0.05$) and remained higher up to day 18 and then dropped on day 21. There was no difference in the expression of CD44 between pregnant and aborted cows until the time of abortion where there was a significant ($p < 0.05$) decrease in the aborted groups. At the time of calving, the relative mRNA expression of CD62L decreased ($p < 0.05$) whereas the expression of CD11b, CD31, CD44 increased ($p < 0.05$) in the cows that completed their gestation successfully (Fig. 2, Supplementary tables 1- 4).

3.3. Relative mRNA expression of interferon-stimulated genes

The relative mRNA expression of various interferon-stimulated genes (MX1, ISG15 and OAS1) in blood neutrophils of different groups of cows has been depicted in Fig. 3 and presented with detailed information in the Supplementary Tables 5–7, respectively. The relative mRNA expression of interferon-stimulated genes (ISGs) increased in all groups from day 10–21 post-AI and was significantly lower in NP cows compared to the other groups ($p < 0.05$). Subsequently, the expression of ISGs returned to normal levels and remained constant for the rest of the study in pregnant and aborted groups. However, there was a significant increase ($p < 0.05$) in the expression of ISGs at the time of abortion in aborted groups and during calving in the pregnant group. The overall mean values showed maximum expression of OAS1, followed by ISG15 and MX1. The highest expression ($p < 0.05$) was recorded on day 18 for OAS1 and day 21 for ISG15 post-AI in all the groups (Fig. 3, Supplementary tables 5- 7).

4. Discussion

During pregnancy, there is a nexus of physiological events that occur in the uterus and impact the maternal immune system starting from insemination, blastocyst hatching, implantation, placentation and up to calving [33]. Local signals from the developing semi-allogeneic conceptus, as well as hormonal changes mediated by the placental or maternal systems, leads to activation of various immune cells including the first line of cellular defence, i.e., neutrophils [11,25]. Dairy cattle abortions can be idiopathic or caused by metabolic or hormonal disorders, dietary deficits, trauma, toxicities, or infectious pathogens [3–5,34]. Christiansen et al. [35] have speculated that inflammatory response caused by a viral infection or autoimmune disorder can lead to dysregulation of some immune cells and spontaneous abortion in both animals and humans. A study survey of the many causal agents that cause abortion in African cattle from mid-to-late gestation has been published [36]. Although there are many factors related to either mother or embryo that may lead to pregnancy loss or abortion, there is rising interest in the role of the maternal immune system in this phenomenon [6,7,13,22]. Neutrophils are a major maternal innate immune cell that presents abundantly at the maternal-fetal interface, but their role in relation to fetal development from implantation to birth is largely elusive. In this novel study, we have studied the dynamic changes in the mRNA expression of blood neutrophils throughout the pregnancy (270 days) and compared them between the cows that experienced abortion and the cows that completed their pregnancy successfully. Indeed, the modulation in the expression of CD molecules and ISGs in blood neutrophils could significantly impact the process of implantation, embryo development and pregnancy maintenance.

Many molecules are involved in the process of the attachment and migration of activated neutrophils to the site of inflammation. The first step in neutrophil migration depends on the coordinated function of selectins and β2-integrins, i.e., adhesion molecules situated on leukocytes and endothelial cells [16]. L-selectin (CD62L) mediates the process of neutrophils rolling on the endothelial cells, whereas integrins (CD11b) expressed by neutrophils change shape in response to activating signals such as chemokines, causing the neutrophil to decelerate from rolling to a stable arrest [9,15]. CD31 regulate the process of transendothelial migration and subsequent movement in the inflamed area. After neutrophils perform their function, CD44 act as an apoptotic signal to mediate the elimination of neutrophils by macrophages which is essential to prevent unnecessary inflammatory reaction and tissue damage [20]. In this study, we have tried to figure out whether these CD molecules possess similar roles in response to the semi-allogenic embryo during different stages of pregnancy. The relative mRNA expression of CD molecules was lower in pregnant cows as compared to the cows that experienced pregnancy disorders. Lowering the activity of neutrophils in pregnant cows is an immunomodulatory mechanism that facilitates maternal tolerance of fetal tissues and provides optimum conditions for embryonic development and pregnancy maintenance. The controlled functions of neutrophils can be attributed to interferon tau secreted by embryo trophoblast, pregnancy-associated hormones (progesterone, cortisol) or/and other unknown factors that regulate neutrophil migration towards the uterus [14,22,37].

The endometrium expresses chemokines in a highly regulated temporal and spatial pattern during implantation. This not only leads to precise neutrophil recruitment and activation but also appropriate placentation and angiogenesis coordination [38]. Up-regulation of CD62L and CD11b expression in NP cows at the time of implantation was observed. Besides, the relative mRNA expression of the studied CD molecules remained constant from day 21

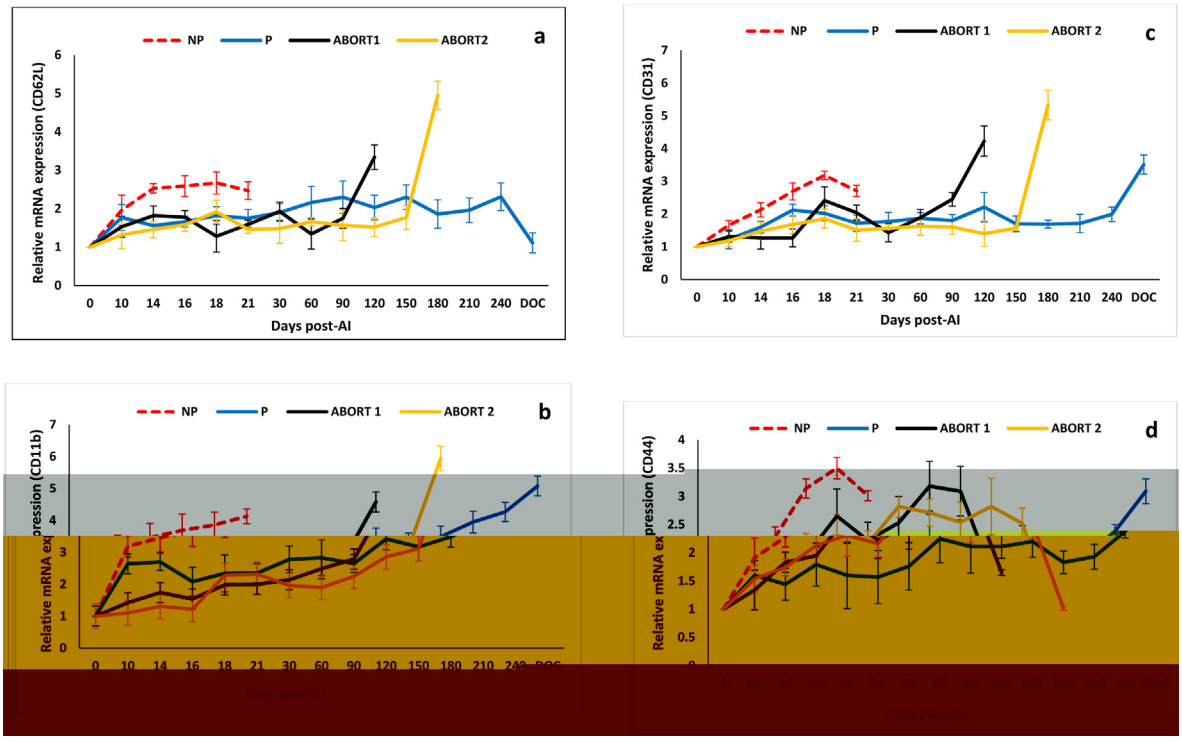

Fig. 2. Relative mRNA expression of CD62L (a), CD11b (b), CD31 (c) and CD44 (d) in blood neutrophils isolated from pregnant (P), non-pregnant (NP), aborted 1 (ABORT1, 110 ± 10 days) and aborted 2 (ABORT2, 170 ± 10 days) groups of cows post artificial insemination (AI). GAPDH and β-Actin were used as Internal control housekeeping genes. The mRNA abundance of the day of AI (day 0) was considered as a calibrator for the relative expression analysis. For the P and NP groups, six cows per group with three replicates (n = 18) was used for the gene expression study. For the ABORT1 And ABORT2 groups, three cows per group with three replicates (n = 9) was used. Values are expressed as mean ± SE. P values less than 0.05 were considered statistically significant.

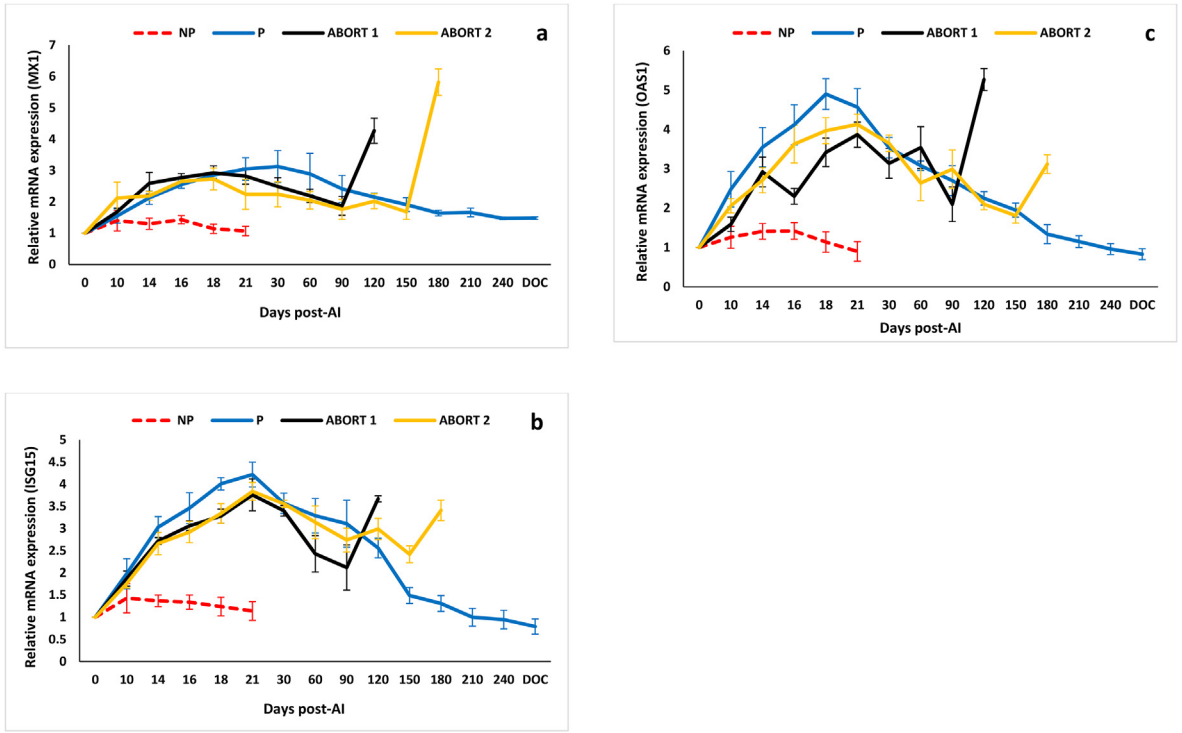

Fig. 3. Relative mRNA expression of MX1 (a), ISG15 (b) and OAS1 (c) in blood neutrophils isolated from pregnant (P), non-pregnant (NP), aborted 1 (ABORT1, 110 ± 10 days) and aborted 2 (ABORT2, 170 ± 10 days) groups of cows post artificial insemination (AI). GAPDH and β-Actin were used as Internal control housekeeping genes. The mRNA abundance of the day of AI (day 0) was considered as a calibrator for the relative expression analysis. For the P and NP groups, six cows per group with three replicates (n = 18) was used for the gene expression study. For the ABORT1 And ABORT2 groups, three cows per group with three replicates (n = 9) was used. Values are expressed as mean ± SE. P values less than 0.05 were considered statistically significant.

post-AI until calving in pregnant cows. The aborted cows showed a significant increase in the expression of CD molecules at the time of abortion except for CD44 which displayed a considerable decrease. This indicates that once successful maternal recognition and implantation occur, the activity of CD molecules remains constant for protecting the growing fetus and maintaining pregnancy with the background support of the increasing pregnancy maintaining hormone i.e., progesterone. An increase in the expression of CD molecules at the time of abortion indicates an inflammatory response associated with a health disorder that resulted in abortion. The exceptional decrease in the expression of CD44 at the time of abortion is essential for the clearance of apoptotic neutrophils in peripheral tissue to prevent oxidative stress and tissue injury. Except for CD62L, the expression of CD molecules (CD11b, CD31 and CD44) increased at the time of calving. Induced expression of these CD molecules is pivotal for increased adhesive ability to endothelium and explain the higher percentage of circulating neutrophils at the time of calving [9]. Higher expression of CD11b is required during infection so that more neutrophils are recruited towards the inflamed tissue such as the mammary gland [39]. However, higher levels of expression of CD11b in pregnant cows after mid-gestation don't necessarily indicate higher adhesiveness by integrins because neither the constitutive nor the induced integrins are fully functional until stimulated by chemoattractants [40]. Studies carried out in our laboratory on mastitis cows have shown that CD44 potentially regulates the removal of the neutrophils from the site of infection and a lower expression of CD44 on the surface of neutrophils can be a major cause behind the secondary inflammation as well as tissue damage during clinical mastitis due to their delayed removal [41]. Lower expression of CD44 at the time of abortion indicate excessive inflammation and uncontrolled immune reaction in the aborted cows. Increased expression of CD62L, CD11b, and CD33 along with decreased expression of CD44 in cows that experienced embryonic mortality at different stages of pregnancy is indicative of excessive stimulation and migration of neutrophils towards the uterus. Besides, our findings reveal that regulated activation and migration of neutrophils is critical for embryo implantation and pregnancy maintenance.

The relative mRNA expression of interferon-stimulated genes i.e., ISG15, OAS1 and MX1 were higher in pregnant cows on days 14–21 post-AI. This signifies that a higher expression of ISGs is indispensable for the successful establishment of pregnancy [42]. The changes in the expression levels of these genes might be related to changes in the concentration of IFNτ during gestation as also reported by Austin et al. [43]. Our result is supported by Kizaki et al. [11] who reported that ISG15, MX1, MX2, and OAS1 displayed significantly higher expression levels in the granulocyte fraction, especially from days 14–21 of gestation in exotic cows. Surprisingly the mRNA expression of ISGs did not drop immediately after peri-implantation period and remained comparatively high up to 90 days post-AI. The changes in the expression of ISGs might be stimulated by another type I IFN such as IFN-α/β since the secretion of IFN-τ is restricted to the peri-implantation period. Similarly, Shirozu et al. [44] reported that the expression of MX gene, IFN signaling pathway-related genes, and *IFN-α* were significantly higher in the endometrium and fetal placenta after implantation and up to day 150 of pregnancy. Fetal immunity is not fully functional until day 120 of gestation which suggest that maternal immune response must be more active during this period to provide an adequate protection for the undeveloped fetal immune system [44,45].

Recently, we have demonstrated that IFNτ induces the expression of various ISGs in bovine neutrophils through JAK3 and PI3K leading to impaired neutrophil extracellular trap formation and successful embryo implantation [13]. This was also highlighted in the present study in which higher expression of ISGs during implantation was associated with suppressed expression of CD molecules that are essential for neutrophil activation and migration. However, higher expression of ISGs observed at the time of abortion doesn't indicate an immunomodulatory mechanism mediated by IFNτ but rather mediated by other immunological factors in response to some reproductive complications. This is because abortion occurred around mid-pregnancy in the absence of IFNτ which is limited to the peri-implantation period. Besides, ISGs is also activated by other classes of interferon such as IFN-α and IFN-λ. These classes of bovine interferons have been reported to play major roles in the defence mechanisms against viral infections such as the bovine-viral-diarrhea virus (BVDV) which is a common virus responsible for abortion in cattle [46–48]. Therefore, an increase of ISGs expression in neutrophils of aborted cows may be due to the presence of some microbial agents, although, we could not verify our results with the type of stress that caused abortion. Indeed, the induced ISGs expression at the time of abortion was associated with higher neutrophil activity as reflected by the increased expression of various CD molecules which subsequently led to inflammatory immune response and adversely impacted the pregnancy outcome.

In the first part of this study, we have reported an increased number, phagocytic activity, and myeloperoxidase concentration in the blood neutrophils during early embryonic mortality around implantation and at the time of abortion [49]. Besides, these changes in the activity of neutrophils were associated with higher concentration of plasma pro-inflammatory cytokines (IL-2, IL-6, IL-8) and lower concentration of the anti-inflammatory cytokine (IL-10) [49]. The increased concentration of pro-inflammatory cytokines and decreased concentration of IL-10 and progesterone can justify the observed modulation in the expression of ISGs and CD molecules in neutrophils during embryonic mortality and pregnancy loss.

5. Conclusions

Successful establishment of pregnancy in cows was associated with a decreased relative mRNA expression of various CD molecules and increased expression of ISGs in blood neutrophils around the peri-implantation period. In mid-pregnancy, lower expression of CD molecules and ISGs indicates maternal immune tolerance for the semi-allogenic embryo. Around the end of the gestation period, the expression of CD molecules (except CD62L) reached its peak in association with lower expression of ISGs. At the day of abortion, the expression of both ISGs and CD molecules in neutrophils increased significantly. Further studies are warranted to unravel cell-to-cell communication between neutrophils and other immune cells during pregnancy disorders such as implantation failure and abortion. This is essential to identify the underlying mechanisms for immunological infertility and develop immunomodulatory strategies to reduce pregnancy loss and enhance reproductive efficiency in bovines.

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CRediT authorship contribution statement

Seid Mohammed: Conceptualization, Investigation, Visualization, Formal analysis, Data curation, Methodology, Writing - original draft. **Mohanned Naif Alhussien:** Conceptualization, Visualization, Formal analysis, Investigation, Methodology,

Software, Validation, Writing - review & editing. **Ajay Kumar Dang:** Conceptualization, Supervision, Formal analysis, Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.08.020.

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