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Implantation associated changes in expression profile of indoleamine-2, 3-dioxygenase 1, Th1-Th2 cytokines and interferon-stimulated genes on neutrophils and peripheral blood mononuclear cells of crossbred cows

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ABSTRACT

Effective bidirectional communication between the embryo and dam improves the reproductive efficiency of dairy cows. Possible role of immunosuppressive indolamine-2, 3-dioxygenase 1 (IDO1) enzyme in the regulation of maternal systemic cytokine balance/shift during early pregnancy establishment along with various interferon-stimulated genes (ISGs) expression in neutrophils and peripheral blood mononuclear cell (PBMCs) were investigated in crossbred cows. Blood was collected on days 0 i.e. day of Artificial Insemination (AI), 10, 18 and 36 post-AI followed by isolation of neutrophils and PBMCs for gene expression study of IDO1, anti-inflammatory cytokines (IL-4, IL-10 and TGFβ1), pro-inflammatory cytokines (IFNγ and TNFα) and ISGs (ISG15, MX1, MX2, OAS1) in pregnant and non-pregnant cows. Cows were grouped as pregnant and non-pregnant after pregnancy confirmation by non-return to heat, ultrasonography, per rectal examination along with progesterone and IFNτ assay. Significantly ($P < 0.05$) higher relative mRNA expression of IDO1 and anti-inflammatory cytokines on days 10 and 18 post-AI were observed in both neutrophils and PBMCs of pregnant cows. Pregnant cows showed significantly ($P < 0.05$) higher mRNA transcripts of IFNγ and TNFα genes on days 18 post-AI in both neutrophils and PBMCs. Expression of ISGs was higher ($P < 0.05$) on day 10^{th} and 18^{th} post AI in both the neutrophils and PBMCs of pregnant cows. The study indicates that systemic immune regulation by IDO1 (through cytokine shift) and ISGs in peripheral immune cells are essential for the establishment of pregnancy and may be targeted in future as biomarkers for pregnancy diagnosis.

1. Introduction

Reproduction is an essential biological process for species conservation where pregnancy demonstrates an immunologically unique and challenging period for the dam. In order to achieve successful pregnancy, the maternal immune system has to be precisely regulated so that it accommodates the semi-allogeneic embryo without compromising with its own defense system. The exact underlying mechanism behind such precise and controlled activation of the maternal immune system during early pregnancy establishment in bovines still remains elusive. Reports suggest that the alloantigens of conceptus impart specific changes in the maternal immune function both locally at the feto-maternal juncture (Bulla et al., 2004) as well as systemically in peripheral blood circulation to prevent immunological rejection of the fetus (Bianchi et al., 1996). Circulating immune cells migrate from peripheral circulation towards the feto-maternal interface and positively contribute towards maternal tissue remodeling and embryo-maternal cross-talk around the implantation period which is regulated under the influence of endocrine system (Fujiwara, 2009). Prevention of semi-allogeneic fetus from undergoing rejection is taken care of by the modulation of various maternal immune cells which drive the cytokine balance towards the anti-inflammatory Th2 pathway (Morelli et al., 2015). This immune tolerance character exhibited by the immune cells might be triggered via several molecules like hormones, cytokines and enzymes (Bonney, 2016).

One important molecule is indoleamine-2, 3-dioxygenase 1 (IDO1) which is the first and rate-limiting enzyme of tryptophan catabolism along the kynurenine pathway and is instrumental for pregnancy

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establishment (Munn et al., 1998). Several reports confirm that IDO1 is expressed at the maternal-fetal interface including trophoblast cells, decidual stromal cells, decidual immune cells (e.g., natural killer cells, T cells, and macrophages), vascular endothelial cells of decidua and chorion in humans (Von Rango et al., 2007; Ligam et al., 2005). However, very few literatures are available in relation to IDO1 expression in peripheral blood immune cells (Miwa et al., 2005; Grozdics et al., 2014). IDO1 causes maternal immunosuppression at the feto-maternal interface by inhibiting alloreactive T-cells and thus, protects the fetus from undergoing rejection during establishment of pregnancy (Munn et al., 1998, 2002). However, the role of IDO1 in systemic immunosuppression and thereby prevention of undue maternal immune system activation during pregnancy establishment in bovines is still unexplored.

Shifting of pro-inflammatory cytokines (Th1 cytokines) towards anti-inflammatory side (Th2 cytokines) i.e., Th1-Th2 shift at the feto-maternal interface is another important mechanism adopted by the maternal immune system to prevent the fetus from undergoing maternal rejection during pregnancy establishment (Wegmann et al., 1993). Cytokines secreted by the mother at the time of embryo implantation aids in successful placentation and parturition (Robertson et al., 1994). In this context the role of indoleamine 2, 3-dioxygenase 1 (IDO1) enzyme is considered as pivotal as IDO1 is responsible for immune-suppression at the maternal-fetal interface (Mezrich et al., 2010). IFNτ being the only agent for maternal recognition of pregnancy (MRP) in cattle stimulates the IDO1 expression at the feto-maternal interface (Groebner et al., 2011) and skew the differentiation of T-cells into Th2 cells during implantation (Mezrich et al., 2010). However the role of systemic IFNτ on IDO1 expression and associated Th1-Th2 regulation in peripheral immune cells (neutrophils and PBMCs) still needs to be explored in bovines.

Apart from IDO1 expression and Th1-Th2 cytokine shift, interferon-stimulated genes (ISGs) like ISG15, MX1, MX2 and OAS1 also play a significant role in successful pregnancy establishment in bovines (Manjari et al., 2016; Panda et al., 2020). The expression pattern of these ISGs has been observed to be influenced under IFNτ stimulation both at the maternal fetal interface (Bauersachs et al., 2006) as well as in the peripheral immune cells during the implantation window (Shirasuna et al., 2011; Manjari et al., 2016). Several studies suggest that dysregulation in IDO1 and ISGs expression along with improper cytokines regulation is associated with various pregnancy-related complications like pre-eclampsia, miscarriage and pre-term labor in humans and mice (Clark et al., 2005; Kose et al., 2016; Daher et al., 2004). Therefore, the present study tested the hypothesis that under the influence of systemic IFNτ, IDO1 expression might be one of the key agents which regulate the Th1-Th2 cytokine shift along with the complementation of various ISGs expression in bovine neutrophils and PBMCs and thus might be helping in embryo survival during early pregnancy establishment.

2. Materials and methods

2.1. Location of the study

The present study was carried out at Livestock Research Centre of ICAR-National Dairy Research Institute, Karnal, Haryana, India. This institute is situated at 250 m above mean sea level, in the Indo-Gangetic plains on 29°43″ N altitude and longitude 77°2″ E.

2.2. Selection of cows, ethics approval and consent to participate

A total of twenty four (n = 24) multiparous, Karan Fries (KF) crossbred (Holstein Friesian x Tharparkar) cows of third to fifth parity, aged between 5–7 years with regular cyclicity and good health condition were selected from the Institute herd. Clearance for conducting the present study was taken from the Institute's Animal Ethics Committee (Approval No. 41-IAEC-18-32) as per the rules and guidelines framed and communicated by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), a statutory Committee, which is established under Chapter 4, Sect. 15(1) of the Prevention of Cruelty to Animals Act 1960, laid down by the Government of India. Only those cows which had no history of reproductive ailment and those which required not more than two services per conception were included in the study. The length of estrus cycle was 15.1 ± 4.4 days with the fertility rate of 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 as per the recommendation of National Research Council. The study was undertaken during the onset of the winter season (October and November) to provide thermo-neutral conditions (temperature 18−25 °C) to the animals. Regular examination of physiological responses like respiration rate, heart rate, per-rectal temperature etc. were performed to ensure the health status of all the experimental cows.

2.3. Sample collection, pregnancy confirmation and grouping of experimental animals

Blood samples were collected from the cows naturally coming to heat and exhibiting signs of estrus like mucus discharge, swelling and reddening of the vulva and other estrus behavioural signs. Around 18 mL blood was collected on days 0 (day of AI), 10, 18 and 36 post-AI in sterile K_2 EDTA vacutainer tubes (Greiner bio-one, Vacuette) via jugular vein puncture in the morning hours (around 9 AM) for neutrophils and PBMCs isolation. Additional 8 mL blood was collected in sterile K_2 EDTA vacutainer tubes (Greiner bio-one, Vacuette) for plasma separation. Immediately after collection the blood samples were transported to the laboratory in ice for further processing. Pregnancy confirmation was done by observing non-return to heat on day 21 post-AI and by estimating plasma progesterone concentration on day 18 post-AI using bovine specific ELISA test kit Cloud-Clone, USA (Cows having progesterone levels ≤2 ng/mL were categorized as non-pregnant and those having values >2 ng/mL were considered as pregnant). Pregnancy was also confirmed 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 carried out by transrectal palpation on day 60. Based on the pregnancy confirmation, the cows were categorized into pregnant (n = 10) and non-pregnant (n = 14) groups.

2.4. Isolation of plasma from whole blood

Freshly collected blood samples were centrifuged in 15 mL polypropylene falcon tubes (Tarsons) @ 1200 X g for 25 min to separate the plasma which was stored in storage vials at −20 °C for the analysis of IFNτ and progesterone levels.

2.5. Quantification of plasma IFNτ and progesterone levels

Plasma IFNτ and progesterone levels were estimated using bovine specific ELISA Test Kits purchased from Cloud-Clone Corp., USA according to the manufacturers' protocols. The optical density (OD) was measured by ELISA reader (Multiskan Go, Thermo Scientific, Finland). The average coefficient of variation (CV) was calculated for inter assay and intra assay. The inter-assay CV% was found to be 2.63 and the intra-assay CV% was found to be 0.06.

2.6. Isolation of neutrophils and peripheral blood mononuclear cells (PBMCs) from whole blood

All materials and reagents used for the isolation of neutrophils and PBMCs were sterile and of cell culture grade. Blood neutrophils and PBMCs were isolated through density gradient centrifugation using Histopaque 1119 (Sigma Aldrich, Darmstadt, Germany) and Histopaque

1077 solution (Sigma Aldrich, Darmstadt, Germany) in 15 mL polypropylene falcon tubes. Briefly, 3 mL of Histopaque 1119 solution was taken at the bottom of the tube and over this 3 mL of Histopaque 1077 solution was carefully layered. Equal volume of whole blood i.e., 6 mL of whole blood was taken and carefully layered over the Histopaque 1077 solution gently through the wall of the tube. Centrifugation was carried out at 900 × g for 40 min at room temperature (RT). PBMCs were separated as a layer of ring just above the Histopaque 1077 solution and neutrophils were separated at the interface of the Histopaque 1119 and Histopaque 1077 layers. Neutrophils (3 mL) and PBMCs (3 mL) were carefully transferred to new 15 mL falcon tubes and were slowly mixed with equal volume of RBC lysis buffer (HiMedia Laboratories, Pennsylvania, USA) to remove any residual red blood cells present with the cells. 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 pellets were washed 3 times in DPBS (HiMedia Laboratories, Pennsylvania, USA) at 300 × g for 10 min at 4 °C and suspended in Roswell Park Memorial Institute (RPMI) media (HiMedia Laboratories, Pennsylvania, USA) for further analysis. The purity of the isolated neutrophils were found to be more than 90 % as evaluated by May-Grunwald and Giemsa stain (HiMedia Laboratories, Pennsylvania, USA) under oil immersion (100 X).

2.7. Total RNA extraction from neutrophils and PBMCs

Neutrophils and PBMCs isolated from each blood sample were adjusted to 1×10^6 live cells/mL for carrying out expression studies. The RNA was extracted and purified by TRIzol reagent (Invitrogen, Carlsbad, CA) as prescribed by manufacturer's protocol. Briefly, cells were lysed by adding 1 mL of TRIzol reagent and the cell lysate was passed several times through a pipette followed by vortex for 5 min at RT. The samples were incubated for 5 min at RT, followed by addition of 0.2 mL of chloroform (HiMedia Laboratories, Pennsylvania, USA) per 1 mL of TRIzol and centrifuged at 12,000 × g for 15 min at 2 − 8 °C. The aqueous phase was transferred to a new tube and 0.6 mL of isopropyl alcohol (Sigma Aldrich, Darmstadt, Germany) was added per mL of TRIzol, mixed and incubated for 10 min at RT. The samples were centrifuged at 12,000 × g for 10 min at 2 − 8 °C and the RNA pellets were washed with 1 mL of 75 % ethanol, vortexed and centrifuged at 8500 × g for 5 min at 2 − 8 °C. The ethanol was removed and the pellets were left to air dry for 15 min. The dried RNA pellet was dissolved in 25 µl nuclease-free water (Thermo Scientific, USA) and its concentration was determined by measuring absorbance at 260 nm. The purity of RNA was evaluated by Biospec-nano Spectrophotometer (Shimadzu Corp., Japan) and judged by OD ratio at 260:280 nm. A ratio of (1.8 − 2.0) was considered for further processing. The integrity of each RNA sample was evaluated by agarose gel (2%) electrophoresis in Tris-EDTA buffer (0.002 M EDTA) with (0.5 mg/mL) of ethidium bromide. Two intact bands of 28 s and 18 s without smearing indicated good quality and intactness of RNA. The RNA was then stored at −80 °C until further use.

2.8. cDNA synthesis and real-time PCR (qPCR)

The extracted and purified RNA was subjected to DNase treatment by DNase1, RNase free (Thermo Scientific, USA) as prescribed by the manufacturer's protocol. Total 1 µg of RNA from each sample was transcribed into complementary DNA (cDNA) by using Thermo Scientific Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA). The RT-PCR reaction was carried out at 65 °C for 5 min, 42 °C for 50 min and 85 °C for 5 min in a thermal cycler (Bio-Rad, USA). Synthesized cDNA was kept at −80 °C till further use. Quantitative real-time PCR (qPCR) (Roche's Lightcycler 480) was performed using Thermo Scientific Maxima SYBR Green qPCR Master Mix kit (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, the reaction mix prepared was: 1 µl template; 5 µl (2×) SYBR green mix, 0.5 µl each of reverse and forward primer, and 3 µl nuclease-free PCR grade water. Details of primers used for specific bovine IDO1, IL-10, IL-4, TGFβ1, IFNγ, TNFα, MX1, MX2, ISG15 and OAS1 genes have been presented in Table 1 (Sigma Chemicals Co., St. Louis, Missouri, USA). The following protocol and program was used: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s and then 72 °C for 5 min and final holding temperature was 4 °C. The expression of each gene was analyzed in triplicate and normalized with β-actin and GAPDH which were used as housekeeping genes. The relative gene expression level was evaluated by the 2−ΔΔCt method.

2.9. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software, version 5.01. All the data obtained from the study were expressed as mean ± standard error of the mean (S.E.M.) and were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A minimum of three independent experiments were performed for each experimental condition tested. The value of $P < 0.05$ was considered to be statistically significant.

3. Results

Plasma IFNτ and progesterone levels have been presented in Fig. 1. The plasma IFNτ level was non-detectable during all the days in non-pregnant cows whereas, in pregnant cows the plasma IFNτ level was significantly ($P < 0.05$) high on day 10^{th} and 18^{th} post-AI and reached to undetectable levels by day 36^{th} post-AI. Plasma progesterone level increased significantly ($P < 0.05$) upto day 18^{th} post-AI in pregnant cows and then remained constant afterwards. In non-pregnant cows, the progesterone level was low and further decreased significantly ($P < 0.05$) on day 18^{th} post-AI.

The relative mRNA expression of IDO1 gene in neutrophils and PBMCs of pregnant and non-pregnant cows has been portrayed in Fig. 2. The expression of IDO1 gene was significantly ($P < 0.05$) higher in both neutrophils and PBMCs of pregnant cows on day 10^{th} and 18^{th} post-AI as compared to the non-pregnant cows. The real time expression of IDO1 gene in neutrophils of pregnant cows was found to be 2–7 folds more as compared to non-pregnant ones. Whereas, in case of PBMCs, the mRNA

Table 1
Details of primers used in the study.

Genes	Sequence (5′→3′)	Acc. No.	Annealing Temp (°C)
IDO1	F: ACTTGTCTGGTTGGAAAGGCA R: GGTGAGCTGGTGGCATGTAT	NM_001101866.2	58
IL-10	F: AGGACCAACTGCACAGCTTA R: TGGCAACCCAGGTAACCCTTA	NM_174088.1	58
IL-4	F: CTGCCCCAAAGAACACAACTG R: TCCGCCCAGGAATTTGTTCA	NM_173921.2	58
TGFβ1	F: GGTGGAATACGGCAACAAAATC R: GAGAGAGCAACACAGGTTCG	NM_001166068.1	58
TNFα	F: GGTGGGACTCGTATGCCAAT R: TGGTGTGGGTGAGGAACAAG	NM_173966.3	58
IFNγ	F: CAAATTCCGGTGGATGATCTGC R: TCTCCGGCCTCGAAAGAGAT	NM_174086.1	58
MX1	F: GCTGTTATCGGGGACCAGAG R: GATGGCAATCTGGGCTTCAC	NM_173940.2	58
MX2	F: CTTCAGAGACGCCTCAGTCG R: TGAAGCAGCCAGGAATAGTG	AC_000158.1	58
ISG15	F: CCATGACGGTATCCGAGCTG R: GGGCCTCCCTTCAAAAGACA	NM_174366.1	58
OAS1	F: TTGTGAAGGGTGGCTCCTCA R: TCCTCTTTCGCTGCTTCAGG	NM_001029846.2	58
ß-Actin	F: CATCGCGGACAGGATGCAGAAAGC R: GCGCGATGATCTTGATCTTCATTG	NM_173979.3	58
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	NM_001034034	58

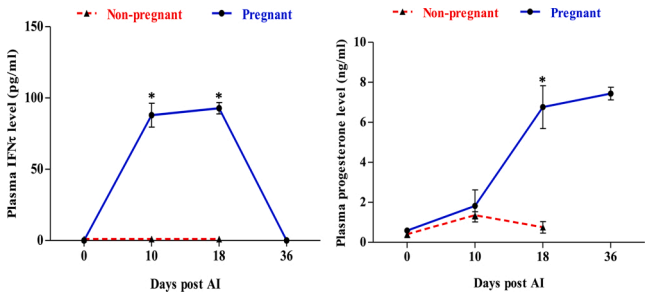

Fig. 1. Plasma IFNτ level (pg/mL) and progesterone level (ng/mL) in non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

transcript abundance of IDO1 gene was found to be 10–15 folds more in the pregnant cows as compared to the non-pregnant ones.

The relative mRNA expressions of anti-inflammatory cytokine genes (IL-4, IL-10 and TGFβ1) in neutrophils and PBMCs have been shown in Figs. 3 and 4 respectively. The mRNA expression pattern of IL-4, IL-10 and TGFβ1 genes in both neutrophils and PBMCs showed almost similar trends in all the sampling days when compared between pregnant and non-pregnant groups. The real time expression of IL-4 in both neutrophils and PBMCs increased significantly ($P < 0.05$) from day 10th to day 18th post-AI in pregnant cows as compared to non-pregnant ones. In case of IL-10, the mRNA transcript abundance was significantly ($P < 0.05$) high on day 10th and day 18th post-AI in neutrophils of pregnant cows in comparison to non-pregnant cows. However, in case of PBMCs the relative mRNA expression of IL-10 started to increase from day 10th onwards and was significantly ($P < 0.05$) more on day 18th post-AI in pregnant cows compared to non-pregnant cows. The gene expression analysis of TGFβ1 gene in neutrophils was found to be significantly ($P < 0.05$) high only on day 18th post-AI in pregnant cows compared to non-pregnant cows. However, in case of PBMCs, the real-time expressions of TGFβ1 gene significantly ($P < 0.05$) increased on day 10th and 18th post-AI in pregnant cows as compared to non-pregnant ones. The qPCR analysis of IL-4, IL-10 and TGFβ1 genes revealed further increase in their gene expression on day 36th post-AI in both PBMCs and neutrophils of pregnant cows. On comparison between neutrophils and PBMCs, the relative mRNA expression of anti-inflammatory genes (IL-4, IL-10 and TGFβ1) in neutrophils of pregnant cows was found to be 6, 3 and 2.5 fold changes more compared to non-pregnant cows. Whereas, in case of PBMCs, the mRNA transcript abundance of IL-4, IL-10 and TGFβ1 genes was found to be 12, 6 and 2.6 fold changes more in pregnant cows as compared to the non-pregnant ones.

The relative mRNA expressions of pro-inflammatory cytokines (IFNγ and TNFα) in neutrophils and PBMCs of pregnant and non-pregnant cows have been illustrated in Figs. 5 and 6 respectively. In case of neutrophils, the real time expression of IFNγ started to increase from day of AI (day 0) and was significantly ($P < 0.05$) more on 10th and 18th day

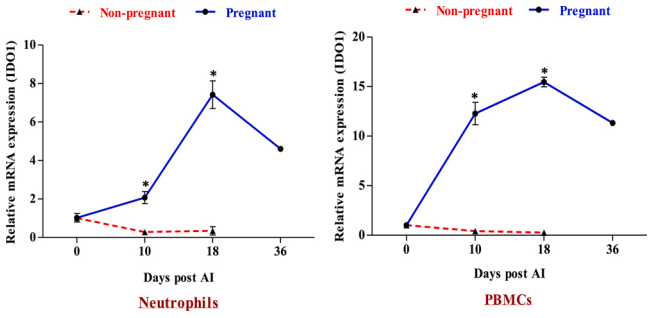

Fig. 2. Relative mRNA expression of IDO1 in neutrophils and PBMCs of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

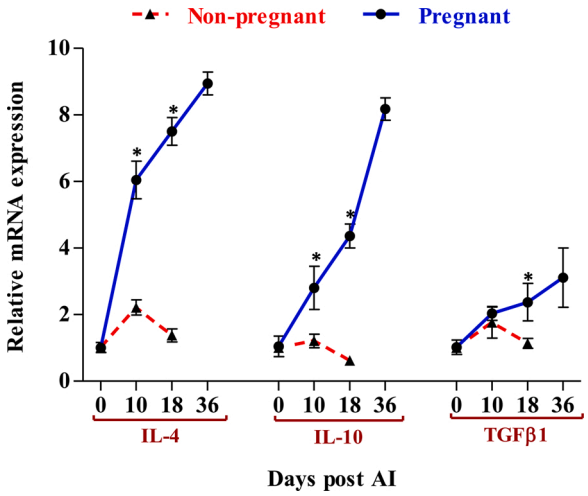

Fig. 3. Relative mRNA expression of anti-inflammatory cytokines (IL-4, IL-10 and TGFβ1) in neutrophils of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

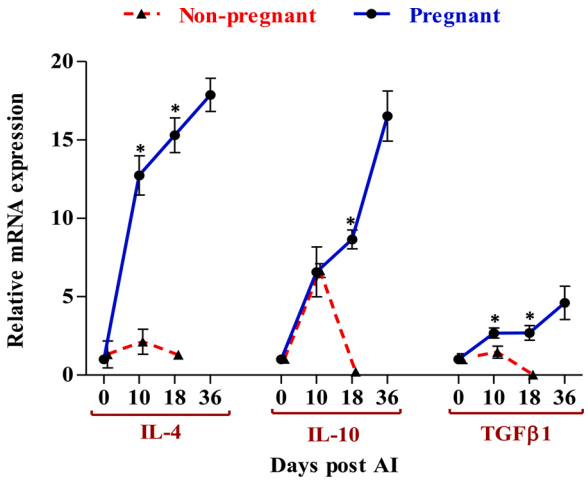

Fig. 4. Relative mRNA expression of anti-inflammatory cytokines (IL-4, IL-10 and TGFβ1) in PBMCs of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

post-AI in pregnant cows compared to non-pregnant ones. However, after 18th day post-AI in pregnant group and 10th day post-AI in non-pregnant group, a decreasing trend in the expression pattern of IFNγ was observed and the mRNA levels fell back to its basal levels. The relative mRNA expression of IFNγ in PBMCs started to rise from day 10th onwards in both pregnant and non-pregnant groups and was significantly ($P < 0.05$) more on day 18th post-AI in pregnant cows as compared to non-pregnant cows. However, after 18th day post-AI a decreasing trend in the expression pattern of IFNγ was observed in pregnant group and the mRNA levels fell back to its basal levels. Similar mRNA expression pattern was observed for TNFα gene in both neutrophils and PBMCs, which showed a significant ($P < 0.05$) increase in its expression on day 18th post-AI in pregnant cows compared to non-pregnant cows followed by a reduction in its mRNA transcript to the basal level after day 18th post-AI.

The relative mRNA expression levels of ISGs (ISG15, MX1, MX2 and OAS1) in neutrophils and PBMCs of pregnant and non-pregnant cows have been depicted in Figs. 7 and 8 respectively. The mRNA levels for ISG15, MX1, MX2 and OAS1 genes in both neutrophils and PBMCs started to increase from day 10th onwards and were significantly higher ($P < 0.05$) on day 10th and 18th post-AI only in pregnant cows. On 36th

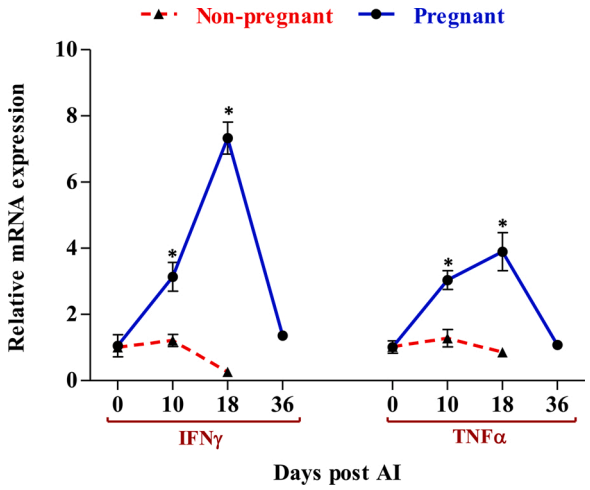

Fig. 5. Relative mRNA expression of pro-inflammatory cytokines (IFNγ and TNFα) in neutrophils of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

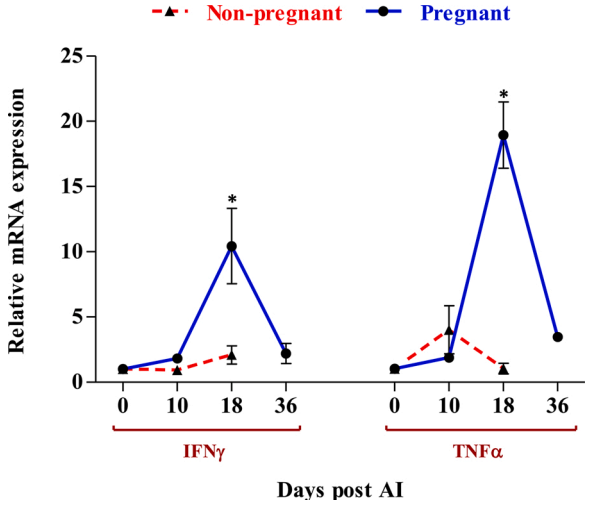

Fig. 6. Relative mRNA expression of pro-inflammatory cytokines (IFNγ and TNFα) in PBMCs of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

day post-AI, mRNA levels of all the four genes fell back to their basal levels in both neutrophils and PBMCs of pregnant cows.

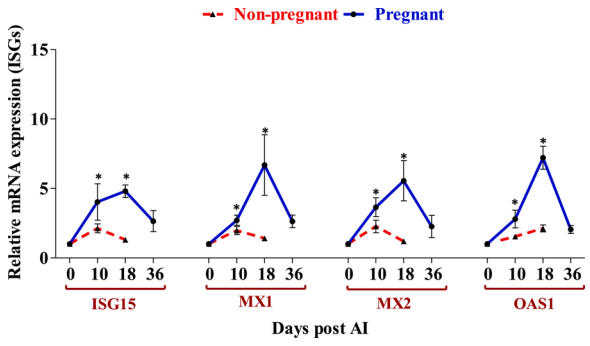

Fig. 7. Relative mRNA expression of interferon-stimulated genes (ISG15, MX1, MX2 and OAS1) in neutrophils of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

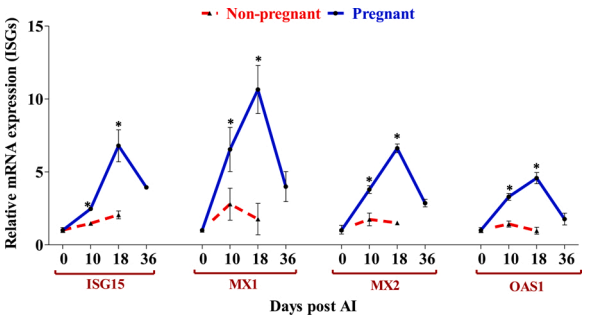

Fig. 8. Relative mRNA expression of interferon-stimulated genes (ISG15, MX1, MX2 and OAS1) in PBMCs of non-pregnant and pregnant cows. Asterisk (*) represents a significant difference ($P < 0.05$) between the groups.

4. Discussion

The exact mechanism behind maternal immune-modulation during early pregnancy establishment in cattle is still unexplored. Many researchers have discovered various molecules which directly or indirectly comfort the relationship between the semi-allogeneic fetus and the maternal immune-system. One of such molecule is IFNτ, which is known as the primary pregnancy recognition signal in cattle and is solely produced by the trophoblast cells of the conceptus (Flint et al., 1994). IFNτ is responsible for the successful implantation in bovines and its synthesis is initiated during the blastocoel formation (around 8 days) and goes up to 21–25 days with a peak around 14–18 days post fertilization (Mor et al., 2015). In accordance with this, we also found significantly ($P < 0.05$) higher IFNτ levels on 10^{th} and 18^{th} days post-AI in pregnant cows, however, in case of non-pregnant cows, the IFNτ levels remained non-detectable during all the days of sampling. The mechanism behind embryo recognition and successful implantation by IFNτ is mediated through the prevention of corpus luteum (CL) lysis. IFNτ acts locally on endometrial cells in a paracrine manner and inhibits the expression of the estrogen receptor which in turn abates the expression of oxytocin receptor in endometrium. This results in attenuation of the synthesis of luteolytic agent PGF2α and ultimately prevents the lysis of CL (Spencer et al., 1995). IFNτ mediated maintenance of CL enables sustained progesterone production which is essential for the establishment and maintenance of pregnancy. Progesterone plays a crucial role in the regulation of endometrial secretions along with the growth and differentiation of the conceptus, during early pregnancy of the cows (Lonergan et al., 2016). A positive correlation between the rise in plasma progesterone concentrations post-AI and embryonic development has been delineated in cattle (Carter et al., 2008). In our study we also observed significantly ($P < 0.05$) higher plasma progesterone level in pregnant cows on days 18 post-AI which resulted in successful maintenance of the pregnancy. Apart from the anti-luteolytic role of IFNτ, it is also known to have potential antiviral, immunomodulatory, and anti-proliferative properties (Roberts, 2007). It not only interacts locally at the maternal-fetal interface but also escapes into the systemic circulation and modulates various maternal immune cells like neutrophils and PBMCs in the peripheral blood circulation (Yang et al., 2014). Thus, we investigated the expression levels of IDO1 gene in maternal peripheral immune cells (neutrophils and PBMCs) and associated Th1-Th2 regulation along with ISGs expression under the influence of systemic IFNτ levels during early pregnancy in bovines.

IDO1 is the first and rate-limiting enzyme in tryptophan catabolism and acts via the kynurenine pathway to convert tryptophan into N-formyl-kynurenine and other biologically active compounds (Munn et al., 1998). IDO1 causes immunosuppression by consuming the available tryptophan present in the microenvironment and thereby causing starvation of the neighbouring maternal T-cell for this essential amino acid. Subsequently, the deficiency of tryptophan blocks the progression of cell cycle in activated alloreactive T-cells which leads to

T-cell arrest at mid-G1 phase of the cell cycle and the cells undergo apoptosis (Lee et al., 2002). This process leads to immunosuppression due to the inhibition of locally activated T-cell proliferation. However, in the context of bovine pregnancy, the mechanism behind it is largely unknown. In order to explore the mechanism of immune tolerance in bovine pregnancy during peri-implantation period, Groebner et al. (2011) revealed around 18 fold increase in the IDO1 expression in the endometrium of pregnant heifers on day 18 as compared to non-pregnant Simmental heifers. According to their report, this increase in IDO1 mRNA expression may be due to the trophoblast-derived IFNτ secretion. In our study, we observed significantly ($P < 0.05$) higher relative mRNA expression of IDO1 gene on day 10^{th} and 18^{th} post-AI in both neutrophils and PBMCs of pregnant cows. On comparison of the relative mRNA expression of IDO1 gene between neutrophils and PBMCs, we found that, there was around 2–7 fold more IDO1 expression in neutrophils and about 10–15 fold more IDO1 expression in PBMCs of pregnant cows. This systemic up-regulation of IDO1 in peripheral immune cells might be essential to prevent semi-allogeneic fetus from undergoing rejection and thus might be crucial for achieving successful implantation. Moreover, increased IDO1 expression might be a signal for the immune cells to synthesize and secrete cytokines in favour of the developing embryo so as to establish relationship between the embryo and the mother.

During early pregnancy establishment a delicate harmony between pro and anti-inflammatory cytokines is needed to increase the maternal tolerance towards the semi-allogeneic embryo (Wegmann et al., 1993). Any disturbance in the Th1:Th2 cytokine balance will lead to fetal death and failure in pregnancy (Sykes et al., 2012). During the time of implantation the immunosuppressive action of IDO1 abrogates the production of pro-inflammatory cytokines by suppressing the Th1 cell population and promotes Th2 cells like T-reg cells to produce more anti-inflammatory cytokines for the embryo survival (Xu et al., 2008). Our observations delineated significantly ($P < 0.05$) higher relative mRNA expression levels of various anti-inflammatory cytokines (IL-4, IL-10 and TGFβ1) on days 10 and 18 post-AI in pregnant cows compared to non-pregnant cows. IL-4 cytokine induces differentiation of naive helper T cells (Th0 cells) to Th2 cells, and subsequently these Th2 cells produce additional IL-4 in a positive feedback loop (Gool, 1999). IL-4 decreases the differentiation of Th1 cells and is a key regulator in humoral and adaptive immunity (Sokol et al., 2008). In our study we observed significantly ($P < 0.05$) increased real-time expressions of IL-4 gene in both neutrophils and PBMCs from day 10^{th} to day 18^{th} post-AI in only pregnant cows. Interleukin-10 cytokine is a potent anti-inflammatory agent primarily produced by lymphocytes, monocytes and Th2 cells under the influence of systemic IFNτ (Shirasuna et al., 2011). In our study, we also observed a significant ($P < 0.05$) increase in the IL-10 mRNA expression in neutrophils on day 10^{th} and 18^{th} post-AI in pregnant cows compared to non-pregnant ones. However, in case of PBMCs, the IL-10 expression in pregnant cows increased after day 10^{th} post-AI and was significantly ($P < 0.05$) higher on day 18^{th} post-AI as compared to non-pregnant cows. Transforming growth factor beta 1 (TGF-β1) is a multifunctional cytokine that exhibits potential immuno-regulatory and anti-inflammatory properties for the survival and growth of the allograft. The level of TGF-β1 has been reported to be higher in pregnant women and is suggested for its potential regulatory function in fetal allograft survival in successful pregnancies (Ayatollahi et al., 2007). In our study we also observed significant ($P < 0.05$) increase in the real-time expression of TGFβ1 gene in neutrophils after day 18^{th} post-AI whereas, in case of PBMCs its expression increased from day 10^{th} to day 18^{th} post-AI in pregnant cows as compared to non-pregnant ones. This up-regulation in the expression of various anti-inflammatory cytokine genes i.e., IL-4, IL-10 and TGFβ1 in pregnant cows might be implicated towards prevention of semi-allogeneic fetal rejection which ultimately leads to successful pregnancy establishment in cattle.

Up-regulation in the pro-inflammatory cytokines like IFNγ and TNFα (Th1 cytokines) are believed to be deleterious for the fetal survival during pregnancy establishment (Robertson et al., 2018). However, the exact mechanism behind the immuno-regulatory property of IFNγ and TNFα during pregnancy establishment is poorly understood. Therefore, extensive research has to be conducted to explore the hidden aspects of these pro-inflammatory cytokines in the context of immune-modulation during early pregnancy establishment in bovines. In our study we observed that the mRNA expression of IFNγ and TNFα genes were significantly ($P < 0.05$) higher in both neutrophils and PBMCs of pregnant cows on day 18^{th} post-AI compared to non-pregnant ones. However, the mRNA levels of both the genes fell back to their basal level after 18^{th} day post-AI. The possible reason behind the increased expression of IFNγ around day 18^{th} post-AI in pregnant cows might be implicated towards the stimulation of IDO1 expression as IFNγ which is a type-II interferon is also a potent stimulator of IDO1 expression along with IFNτ (Miwa et al., 2005). Our results are in contrast to Yang et al. (2016) in which they reported significantly higher expression of IFNγ in non-pregnant PBMCs as compared to pregnant PBMCs on similar days. However, according to Tuo et al. (1999) the expression of IFNγ could be up-regulated by IFNτ in bovines. In a positive note, firstly higher albeit controlled expression of IFNγ around 18 days of pregnancy (known as implantation window) might be responsible for the elimination of the pathogens if any in order to give emphasis to conservation of species through pregnancy as IFNγ is known to be an effector cytokines for the recruiting of immune cells (Nagaoka et al., 2003). Secondly it might affect the Th1 responses in a negative feedback manner (Feuerer et al., 2006) as higher Th1 response is detrimental for the developing embryo. The up-regulation in TNFα mRNA expression might be implicated towards increased PGE2 production (luteotrophic) and reduced PGF2α secretion (luteolytic) as reported by Szostek et al. (2014). Sakumoto et al. (2014) reported higher levels of TNFα mRNA and protein in the CL of pregnant cows compared to cyclic cows. From these studies it can be inferred that the higher level of TNFα mRNA in neutrophils and PBMCs of pregnant cows around day 18^{th} post-AI might be involved in the luteotrophic role of PGE2 on CL through the blood circulation and thus might be playing a crucial role in the establishment and maintenance of pregnancy.

Beside the role of systemic IFNτ in IDO1 expression, it also induces the synthesis and secretion of various ISGs like ISG15, MX1, MX2 and OAS1 in peripheral blood leucocytes in dairy cows (Gifford et al., 2007). These four genes have been studied extensively in the context of early pregnancy diagnosis in cattle because of their marked up-regulation during the early phase of the pregnancy establishment i.e., around day 18 post-AI (Kizaki et al., 2013; Shirasuna et al., 2011). Researchers have reported an up-regulation in the ISGs mRNA and protein levels in bovine peripheral blood leucocytes on days 18 and 20 post insemination in pregnant cows compared to non-pregnant ones (Gifford et al., 2007; Panda et al., 2020). In our study, we also observed similar results in relation to the qPCR analysis of four ISGs. Our findings delineated significantly ($P < 0.05$) higher relative mRNA expression of ISG15, MX1, MX2 and OAS1 genes in both neutrophils and PBMCs of pregnant cows on day 10^{th} and 18^{th} post-AI compared to non-pregnant cows. The expression profiles of all four ISGs in both neutrophils and PBMCs were found to be maximum on day 18^{th} post-AI in pregnant cows. Our results are in agreement with the above researchers. High expression levels of ISGs during early pregnancy particularly around day 18 post insemination may be subjected to IFNτ stimulation because of its maximum secretion at that time. ISG15 covalently conjugates with different proteins through ISGylation which is considered as pivotal for maintenance of pregnancy across all mammalian species (Hansen and Pru, 2014). MX1 and MX2 proteins have anti-viral properties and have a role in regulation of endometrial secretion, uterine remodeling and anti-luteolytic activities in pregnant animals (Hicks et al., 2003). OAS1 gene affects the PGF2α secretion in the endometrial epithelium by altering the arachidonic acid metabolism (Schmitt et al., 1993), thereby playing a crucial role in the maintenance of CL to achieve successful pregnancy. Altogether, increased expression of ISGs may be involved in

the maintenance of CL, protection of mother from the pathogenic insult and at the same time providing a protective microenvironment to the semi-allogeneic fetus to undergo successful implantation in cows.

5. Conclusions

The present study provides for the first time an insight into the possible role of IDO1 in systemic cytokine balance under the influence of circulating IFNτ during early pregnancy establishment in bovines. The distinct increased in the transcript of immunosuppressive IDO1 in circulating maternal immune cells (neutrophils and PBMCs) at the beginning of implantation may be necessary to modulate the cytokine balance towards anti-inflammatory side so as to protect the semi-allogeneic fetus from undergoing rejection. Higher expression of anti-inflammatory cytokines was observed in both neutrophils and PBMCs of pregnant cows which may be due to a shift towards Th2 cells during early pregnancy. Higher expression of pro-inflammatory cytokines was also seen around day 18th post insemination in both neutrophils and PBMCs of pregnant cows. Higher albeit controlled expression of IFNγ may be necessary to augment the expression of immunosuppressive IDO1 enzyme during early pregnancy establishment in bovines. On the other hand up-regulation of TNFα may be necessary for maintenance of CL via increased luteotrophic PGE2 production and reduced luteolytic PGF2α secretion in pregnancy. This study also confirms the role played by various ISGs on the blood neutrophils and PBMCs of pregnant cows during the implantation window as indicated by various researches. Higher expression of ISGs around day 18th post-AI in both neutrophils and PBMCs of pregnant cows might be playing a crucial role in endometrial secretion, uterine remodeling, anti-luteolytic activities and protection from pathogens for successful maintenance of pregnancy in bovines. We believe that extensive research on the mechanism of immune regulation by IDO1 and ISGs during the implantation window may contribute towards the identification of potential new pregnancy diagnosis markers in bovines.

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Declaration of Competing Interest

The authors reported no declarations of interest.

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