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Investigation of conceptus stimulated gene expression in buffalo peripheral blood mononuclear cells as potential diagnostic markers of early pregnancy

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Research Article

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Abstract

Exploration of novel strategies for early pregnancy diagnosis is pivotal in enhancing the reproductive potential and monetary gains from dairy herds. In buffalo, the trophectoderm cells of the elongating conceptus secrete interferon-tau that stimulates the transcription of various genes in peripheral blood mononuclear cells (PBMC) during the peri-implantation period. We explored the differential expression of classical (*ISG15*) and novel (*LGALS3BP* and *CD9*) early pregnancy markers in PBMC of buffaloes during various stages of pregnancy. Natural heat was detected in buffaloes by assessing the vaginal fluid, and artificial insemination (AI) was done. Whole blood was collected from the jugular vein in EDTA-containing vacutainers for PBMC isolation before AI (0-day) and 20, 25 and 40 d post-AI. On day 40, transrectal ultrasonography examination was performed to confirm pregnancy. The inseminated non-pregnant animals served as control. Total RNA was extracted using the TRIzol method. The temporal abundance of *ISG15, LGALS3BP* and *CD9* genes in PBMC was compared between pregnant and non-pregnant groups ($n = 9$ per group) using real time-qPCR. Results showed transcripts of *ISG15* and *LGALS3BP* were more abundant at 20 d in the pregnant group compared to the 0 d and 20 d values of the non-pregnant group. However, due to variability in expression, threshold (Ct) cycle of RT-qPCR alone could not distinguish pregnant and non-pregnant animals. In conclusion, *ISG15* and *LGALS3BP* transcripts abundance in PBMCs are potential candidate biomarkers for early prediction of buffalo pregnancy 20-days post-AI, but further work is required to allow the development of a reliable new methodology.

India is enormously privileged in terms of buffalo germplasm, with 109.85 million in number, contributing 20.45% of the total livestock population (Livestock Census, 2019) and responsible for 45% of total milk contribution in the year 2021 (statista.com). Buffalo also contribute significantly towards draught power, meat production, manure, and fuel. Buffalo milk has desirably high fat content, however, buffalo experience issues related to anestrous, delayed puberty and long calving intervals that ultimately decrease milk production efficiency. The possible solution to enhance milk production is to reduce the post-partum interval to conception. Such a reduction in time interval could be enabled by early pregnancy diagnosis following timed artificial insemination (AI) or natural service. Efficient reproductive management is a must for any productive and profitable dairy farm.

The potential of interferon-tau (IFNT)- stimulated genes (ISGs) in circulating peripheral blood mononuclear cells (PBMC) as pregnancy markers is worth investigating. In the cow, transcription of ISGs was increased from day 15 post-AI, reached a peak on day 20 and reduced from 22-day onwards (Pugliesi *et al*., 2014). Likewise, novel transcripts such as interleukin 6 (*IFI6),* radical S-adenosyl methionine domain containing 2 *(RSAD2),* interferon induced protein 44 *(IFI44),* 2′-5′-oligoadenylate synthetase 2 *(OAS2*), galectin 3 binding protein *(LGALS3BP),* interferon induced transmembrane protein 2 *(IFITM2),* TNF superfamily member 13B *(TNFSF13B),* C-type lectin domain family 3 member B *(CLEC3B),* and dermokine (*DMKN)* were expressed in bovine PBMCs during early pregnancy (Rocha *et al*., 2020). Other studies showed increased protein expression of myxovirus resistance protein 2 (MX2: Buragohain *et al*., 2016) and recombinant ISG15 (the product of interferon-stimulated gene 15: Batra *et al*., 2018) as well as mRNA expression of *ISG15, MX2,* 2′-5′-oligoadenylate synthetase 1 (*OAS1*) and *IFNT* in PBMC of early pregnant buffalo (Casano *et al*., 2022).

Here, we investigated the utility of two ISG stimulated genes, *ISG15* (a classical marker) and a novel marker galectin three binding protein (*LGALS3BP),* along with a receptor for

pregnancy-specific glycoprotein (*CD9*) as indicators of early pregnancy in buffalo. We hypothesized that buffalo conceptus would induce greater mRNA abundance of *ISG15, LGALS3BP* and *CD9* in PBMC and these would be useful either alone or in combination, as markers of early pregnancy in buffalo. Our study supports earlier research of *ISG15* as early pregnancy markers in cows and buffalo and identifies *LGALS3BP* as a novel pregnancy marker in buffalo.

Materials and methods

Experimental animals

Lactating Murrah buffaloes ($n = 18$) with a body condition score of 4.3 ± 0.5 maintained at the Directorate of Livestock Farms, Guru Angad Dev and Animal Sciences University, were used. The Institutional Animal Ethical Committee (IAEC) approved the use (GADVASU/2021/IAEC/61/05 dated 13/10/21). Only mature, cycling buffaloes without any reproductive issues were selected for this study. All animals were fed a high-quality buffalo diet, mineral salts and water *ad libitum* and were milked twice daily by hand. Veterinary assistance and good husbandry practices were assured throughout the study to maintain the good health of the animals. Natural heat was detected by assessing the vaginal fluid (Selvam and Archunan, 2017). Animals were artificially inseminated using frozen-thawed semen and day of insemination considered as day 0. After confirmation of pregnancy using ultrasonography and rectal palpation by veterinarians, buffaloes were categorized as pregnant ($n = 9$) and non-pregnant ($n = 9$) (online Supplementary Figs S1 and S2).

Sample collection and PBMC isolation

Blood samples were collected on day 0 and 20, 25 and 40 d post-insemination from the jugular vein in 10-ml EDTA-containing vacutainers (Tarsons, WB, India). Briefly, blood was diluted with isotonic PBS 1X (Sigma-Aldrich, CA, USA) in 1:1 and gently layered over a density gradient medium, HiSep™ LSM 1077 (HiMedia, CA, USA), and centrifuged at 400 × ***g*** for 30 min. The buffy coat layer containing PBMC was collected. The PBMC pellet was mixed with 1 ml Trizol (Life Technologies, CA, USA) and kept at −80°C until total RNA isolation.

RNA isolation, cDNA synthesis and RT-qPCR

RNA was isolated from PBMC cells using Trizol, following the manufacturer's guidelines with slight modifications. Briefly, PBMC were homogenized in 1 mL of Trizol using a handheld motor with stylet at room temperature (RT) until complete homogenization. After a brief incubation of homogenate (RT; 10 min), a 300 µl of chloroform (Himedia, CA, USA) was added. After incubation (RT; 15 min) the mixture was centrifuged (15 min, 12 000 rpm, 4°C). The aqueous phase was collected gently, and an equal volume of chilled isopropanol was added to the RNA pellet. Pelleted RNA was washed twice with 75% molecular grade ethanol (Himedia, CA, USA), centrifuged (5 min, 12 000 rpm, 4°C) and air-dried (RT; 20 min). The dried pellet was resuspended in 20 µl of nuclease-free water and kept at – 80°C. The quality and quantity of all RNA was estimated using NanoDrop Spectrophotometer (Thermo Fisher Scientific™, MA, USA).

A total of 2000 ng RNA was subjected to reverse transcription using AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, CA, USA) as per the manufacturer's protocol. The cDNA was kept at −20°C before RT-qPCR reactions were set up. Quantifying particular transcripts was done using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, MA, USA) and the reaction was performed in Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany). The targets were classic *ISG15*, novel *LGALS3BP* and *CD9,* and cyclophilin A (*PPIA*) was selected as reference gene. Primers were designed in-house and custom synthesized (Sahagene, TL, India) (online Supplementary Table S1). Reaction conditions of thermocycler included hold-1 at 50°C for 2 min, hold-2 at 95°C for 2 min, and a two-step cycling (40 cycles) with denaturation at 95°C for 3 s and annealing/extension at 60°C for 20 s. Specificity of PCR amplification was analysed using melt curve ramping by 1°C/min from 60°C to 95°C and agarose gel electrophoresis. Threshold cycles were exported out in excel and data were analysed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). The abundance of transcripts (fold change) was assessed by the mixed model using SAS software (SAS Institute, 2011). The Shapiro–Wilk test accessed the normal distribution of the data, and non-normalized data were transformed into a natural logarithmic scale to achieve normal distribution before the analysis. Graphical data representation used GraphPad (ver. 9) (GraphPad Software, CA, USA).

Statistical analyses

The data were analysed using the mixed linear mixed model in SAS. The Shapiro–Wilk test tested the data's normality, and the analysis did not consider outliers with values beyond mean ± 3 standard deviations. Animals and days were included as subjects and repeated factors, respectively. The main effects of the fold-change expression of *ISG15* and *LGALS3BP* genes were evaluated at four time points (4 levels: 0, 20, 25 and 40 d post-insemination), pregnancy status (2 levels: pregnant and non-pregnant) and the interaction between pregnancy status and time. Differences between the two groups were tested using a paired test assuming equal variances.

Ultrasound scanning

On 20, 25 and around 40 d post insemination, animals were checked for pregnancy by B-mode and colour Doppler mode transrectal ultrasonography using a duplex ultrasound instrument with linear multi-frequency transducer (Sonosite m turbo, 5–7.5 MHz). B-mode evaluation was performed at around day 40 post-insemination to detect the heartbeat of a viable embryo. Colour Doppler was used to assess luteal blood perfusion on days 20 and 25 to check the non-pregnant buffaloes undergoing luteolysis. The buffaloes with non-functional CL and absence of foetal mass on day 40 post-insemination were categorized as non-pregnant.

Results

The RNA extracted from 5 ml of whole blood ranged between 434.2 and 8159.4 ng/µl (mean concentration 1903.838 ± 236.2279 ng/µl) in 20 µl volume (online Supplementary Table S2). The method yielded RNA samples with OD ratio near 2.0 ($OD_{260/280} = 2.003 \pm 0.008$ and $OD_{260/230} = 1.630 \pm 0.085$), showing acceptable purity. The efficiency of PCR amplification of all the genes was between 95 and105%.

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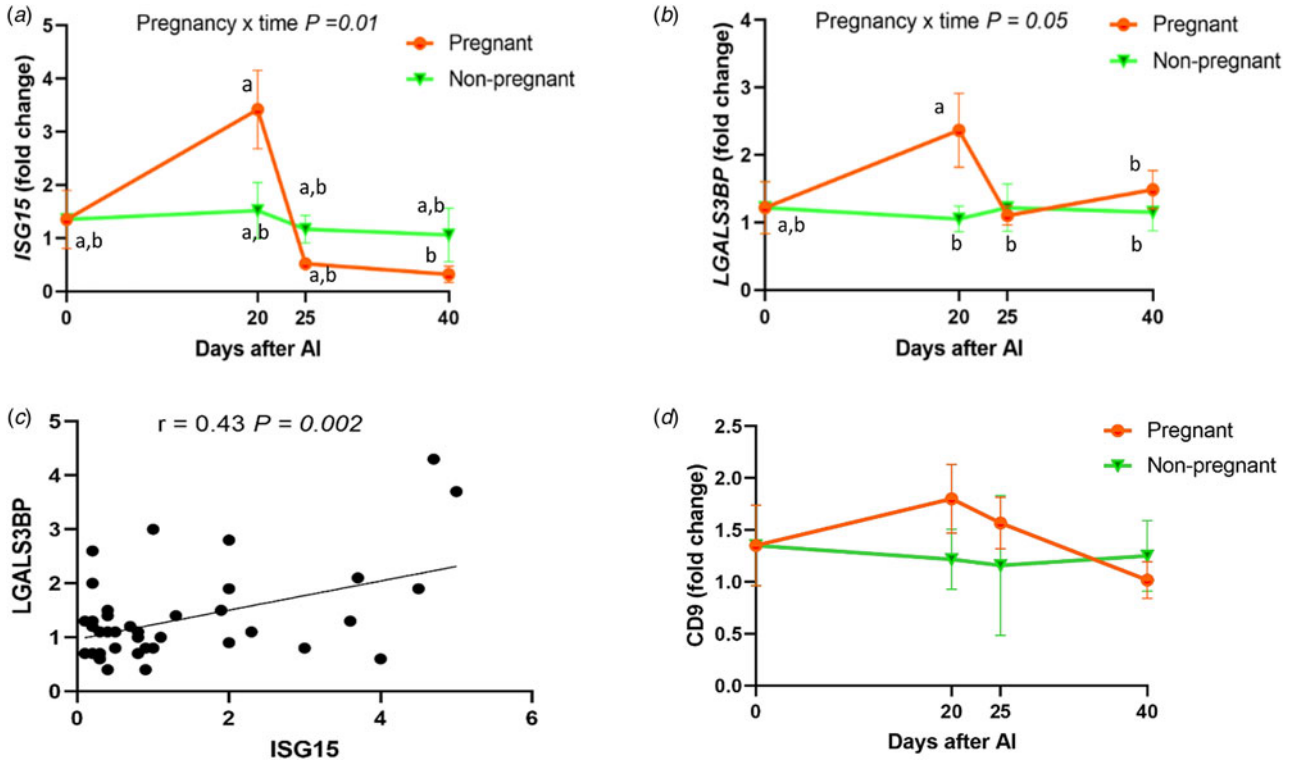

Figure 1. Differential gene expression of early pregnancy markers in PBMC of water buffalo. Fold change expression of *ISG15* (A) and *LGALS3BP* (B) in pregnant and non-pregnant animals depended on the time (0, 20, 25 and around 40 d post-insemination). Correlation analysis of expression of both marker genes was moderate and positive (*ISG15* is shown at C). Fold change of CD9 did not show any significance difference between pregnant and non-pregnant animals across various time points (D).

Ultrasound examination was done at 20, 25 and around 40 d post-insemination. Pregnancy was confirmed at the final examination, and other echoic features of the uterine environment of inseminated animals were examined. In early pregnant animals, luteal blood perfusion was observed at 20 and 25 d, and at around 40 d a viable foetus was observed (online Supplementary Fig. S2).

Expression of *ISG15* (as fold change) was affected by time ($P < 0.001$) and interaction between time and pregnancy status ($P = 0.01$) (Fig. 1A). Likewise, *LGALS3BP* expression was affected by pregnancy status ($P < 0.01$) and interaction between time and pregnancy status ($P = 0.05$) (Fig. 1B). The estimated marginal means of *ISG15* expression in PBMC on day 20 (vs. day 0) was higher in pregnant than non-pregnant group (3.4 ± 0.54 and 1.4 ± 0.54, respectively, $P < 0.05$). The expression of *LGALS3BP* was numerically (non-significantly) higher in pregnant than non-pregnant group (2.4 ± 0.55 and 1.2 ± 0.38, respectively, $P = 0.06$). A moderate positive correlation ($r = 0.43$; $P < 0.01$) was observed in the expression of *ISG15* and *LGALS3BP* (Fig. 1C). No changes in the expression of *CD9* were observed between the pregnant and non-pregnant group, although the expression values remained numerically higher at day 20 and 25 in pregnant animals (Fig. 1D).

Discussion

IFNT prevents the luteolytic mechanism of the endometrium and maintains the corpus luteum (CL) and progesterone secretion. IFNT also upregulates different genes coding for uterine-derived growth factors. These factors are vital in setting the uterus for placenta attachment and maintaining the uterine immune microenvironment to support early fetal development with functional CL throughout the pregnancy.

We evaluated the expression of classic (*ISG15*) and novel pregnancy markers (*LGALS3BP* and *CD9*) in PBMC of water buffalo before (day 0) and at 20, 25 and around 40 d post- insemination. Increased abundance of two genes, *ISG15* and *LGALS3BP,* at day 20 of early pregnancy was consistent with similar findings in cows (Ferraz *et al.*, 2021). *ISG15* expression was upregulated in many other bovine studies (Soumya *et al.*, 2017). In our non-pregnant buffalo group, no upregulated expression of *ISG15* was observed at day 20, suggesting the absence of a viable conceptus. As a result, the pregnant group had a higher expression *ISG15* than the non-pregnant group on day 20 of pregnancy. The significant upregulation of *ISG15* transcript and ultrasound examination of CL, evidenced by increased blood flow rate 20 d after insemination, were suggestive of pregnant animals. The ultrasonography confirmed the pregnancy on days 25 and around 40 d.

LGALS3BP gene is a member of the galectin family, and its role is in cell-cell and cell–matrix interactions. The enhanced expression of the *LGALS3BP* gene in PBMCs indicated its role in the adhesion of the conceptus to the endometrium (Bauersachs *et al.*, 2006). The expression of *LGALS3BP* was upregulated in early (day 20) pregnant buffaloes compared to the same animals at day 0 d and to the non-pregnant group at day 20, indicating its potential role in establishing a pregnancy (Baba *et al.*, 2019). Our results agree with the high expression of *LGALS3BP* observed in pregnant heifers (Rocha *et al.*, 2020), who showed that PBMC expression of *LGALS3BP* and other INFT-stimulated genes (*IFI6, IFI44, RSAD2, OAS2, IFITM2* and *CLEC3B*) was affected by pregnancy status at day 18 post

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insemination. In an earlier study, the abundance of *LGALS3BP* transcript in PBMC and polymorphonuclear cells in bovine heifers indicated differential expression patterns of *LGALS3BP* in two different cell types in the blood (Rocha *et al*., 2020). The study also suggested an earlier response of the galectin gene in PBMC than in polymorphonuclear cells.

We found no difference in the expression level of the *CD9* gene. CD9 regulates mouse embryo implantation and is differentially expressed in the uterus at the very initial stages of pregnancy (Weimin *et al*., 2007). Failure to detect the expression difference might be due to species differences (buffalo vs. mice) and tissue differences (PBMC vs. uterine tissue).

Determining differential gene expression of different immune cell types of early pregnant buffaloes will enhance understanding of the mechanistic regulation of immune cells on the conceptus-stimulated gene expression during the peri-implantation period. This effort could lead to identifying novel pregnancy markers in buffalo for early and indirect detection methods of pregnancy in buffaloes. The development of a novel pregnancy diagnosis method as early as day 20, compared to rectal palpation (after 40 d) and ultrasonography (after 35 d) could significantly reduce calving interval and the time of repeat insemination and estrus synchronization in buffaloes.

In conclusion, we have shown increased expression of *LGALS3BP* in PBMC to be a potential biomarker of early pregnancy in buffalo. A duplex probe-based method for precise determination of the transcripts of *ISG15* and *LGALS3BP* could serve as a diagnostic assay. In future research, identifying protein expression of these markers in blood or in milk, might also be of interest.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000304

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