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Dose- and time-dependent effects of interferon tau on bovine endometrial gene expression

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ABSTRACT

Failure by the developing conceptus to secrete sufficient interferon tau (IFNT), required for maternal recognition of pregnancy (MRP), at the appropriate time is related to early pregnancy loss in cattle. We aimed to test the hypothesis that there is a dose- and time-dependent relationship between IFNT and the endometrial expression of key interferon-stimulated genes (ISGs) involved in the signalling cascade leading to MRP in cattle. Candidate genes were identified first through a bioinformatic approach, where integrated transcriptomic data from two previous studies were analyzed to identify endometrial genes induced by IFNT. Next, expression of selected candidate genes was investigated in vitro in endometrial explants. Endometrial explants collected from cows (n = 8) in the late luteal phase of the estrous cycle were cultured in medium without (control) or with recombinant ovine IFNT (1, 10, 100 ng/mL) for 6 h. Simultaneously, endometrial explants were cultured in medium containing 100 ng/mL IFNT for different time periods (15 min, 30 min, 1 h, 3 h, 6 h). Gene expression was analyzed by RT-qPCR. We identified 54 endometrial genes responding to IFNT and to some degree to the conceptus, from which five ISGs (*CMPK2*, *BPNT1*, *IFI35*, *TNFSF10* and *TRIM38)* were further selected for the dose- and time-dependent experiments. Classical ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*) were up-regulated ($P < 0.05$) in endometrium by 1 ng/mL IFNT. However, other selected ISGs (*CMPK2*, *BPNT1*, *IFI35*, *TNFSF10* and *TRIM38)* were induced only by higher concentrations (10 and 100 ng/mL) of IFNT ($P < 0.05$). In terms of duration of exposure, IFNT at 100 ng/mL induced a significant ($P < 0.05$) increase in *ISG15* and *CMPK2* expression after 1 h incubation, while all other studied ISGs in the endometrium were upregulated when cultured for 3 or 6 h, but did not affect expression when the duration of culture was for 1 h or less. These results suggest that IFNT acts on the uterus in both a dose- and time-dependent manner in cattle and that timely exposure of the endometrium to sufficient IFNT is essential for appropriate signalling to ensure successful pregnancy establishment.

1. Introduction

In high-yielding dairy cattle, a significant proportion (up to 50% in some scenarios) of embryo loss occurs in the two weeks following natural breeding or artificial insemination [1–4]. Such early embryonic death results in significant economic loss in dairy farms by decreasing pregnancy rates and subsequent impacts on milk production. Most losses occur during the pre-implantation stage of pregnancy likely due to poor oocyte quality and developmental competence of the early embryo, sometimes accompanied by uterine dysfunction, resulting in failure of the conceptus to develop properly, signal maternal pregnancy recognition and/or undergo implantation and placentation [3]. It has been estimated recently that approximately 50% of pregnancy loss is due to luteal regression and about 50% is due to conceptus failure [5].

Using in vitro fertilization, bovine blastocysts can be efficiently produced in the laboratory in the absence of any interaction with the female reproductive tract; however, they must be transferred to a receptive uterus for growth and development into an elongated filamentous conceptus to occur. The elongation process does not occur in vitro or in vivo in the absence of uterine glands [6,7] indicating that the process is maternally-driven. Thus, appropriate communication between the developing embryo/conceptus and the mother in the first 2–3 weeks after fertilization is essential for pregnancy establishment.

Establishment of pregnancy in cattle begins at the conceptus stage

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and includes various steps including maternal pregnancy recognition, implantation, and placentation [8,9]. Following natural breeding or artificial insemination (Day 0), the oocyte is fertilized by sperm in the oviduct and the resultant embryo enters the uterus around Day 4, forming a blastocyst by Day 6–7, containing an inner cell mass and an outer layer of trophectoderm cells surrounding the blastocoel cavity. Next, the blastocyst hatches from the zona pellucida on Day 8–9 and develops into an ovoid, then tubular form which begins to elongate on Day 12–14, forming a filamentous conceptus [10]. Recent studies reported that interferon tau (IFNT) from Day 7–8 blastocysts activates interferon-stimulated genes (ISGs) locally in the uterus [11–13]. As the elongation process progresses, the trophectoderm secretes increasing amounts of IFNT [14], which is responsible for MRP around Day 16–17 of pregnancy. IFNT silences transcription of estradiol receptor alpha (ESR1) and, in turn, oxytocin receptors, thereby inhibiting oxytocin-induced luteolytic pulses of prostaglandin F2alpha from the uterus. Luteolysis is thus prevented and progesterone (P4) output by the corpus luteum (CL) is maintained [15].

Recent studies from our group and others have made significant progress in clarifying the role of the maternal environment, in particular the role of conceptus-derived IFNT in regulating endometrial function for the successful establishment of pregnancy in cattle [16,17]. Using an endometrial explant culture system, we have demonstrated a local IFNT-dependent effect of blastocysts on endometrial gene expression [13,18], identified IFNT-dependent and independent transcripts in the endometrium induced by the conceptus which may play a role in signalling to the endometrium around pregnancy recognition [19], and demonstrated that the endometrial transcriptome responds differently to long vs short (i.e., developmentally compromised) age-matched conceptuses which may be indicative of conceptus developmental competence [20]. These data highlight the importance of IFNT in signalling to the maternal endometrium and thereby maintaining P4 output from the CL, ensuring continued development of the conceptus. However, the threshold concentration of IFNT required to establish pregnancy in cattle remains unknown [16]. We have demonstrated that short conceptuses fail to induce a large number of ISGs in the bovine endometrium that are altered by both IFNT and age-matched long conceptuses, suggesting insufficient IFNT production, and consequent compromised maternal signalling, is a major contributing factor for lower survival of short conceptuses [20]. Thus, it appears that a threshold amount of IFNT is required for MRP and pregnancy establishment, and thus may be related to fertility in cattle. Interestingly, the transfer of conceptuses up to Day 16 of the oestrous cycle, but not on Day 17, can establish pregnancy in cattle [21] suggesting that the IFNT-signalling effect required to prevent luteolysis is quite acute. We hypothesized that there is a dose- and time-dependent relationship between IFNT and the endometrial expression of key ISGs involved in the signalling cascade leading to MRP, which may be associated with successful pregnancy establishment in cattle. Thus, we aimed to investigate the dose- and time-dependent effects of IFNT on the expression of key ISGs in bovine endometrium. In addition to well-known classical ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*), we screened other potential ISGs through a bioinformatics approach, involving the integration of data from previous studies from our group and the application of machine learning (ML) tools. Next, the selected ISGs were investigated in vitro by measuring their expression in endometrial explants treated with different concentrations of IFNT for various time periods.

2. Materials and methods

2.1. Ethics statement

All experimental procedures involving animals were approved by the Animal Research Ethics Committee of University College Dublin and were licensed by the Health Products Regulatory Authority, Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes).

2.2. Identification of candidate genes through a bioinformatics approach

Transcriptomic data from two independent studies [20,22], both of which measured the endometrial response through RNAseq in endometrial explants collected on Day 15 and co-cultured for 6 h with: (i) nothing (control, n = 10), (ii) 100 ng/mL of IFNT (n = 10), or (iii) a single Day 15 conceptus (n = 33), were employed to determine the candidate genes through R packages. First, data were integrated by removing the study effect with the sva package [23] (Supplementary Fig. 1). Next, data were pre-filtered by selecting the up-regulated differentially expressed genes (DEGs; false discovery rate<0.05) when contrasting between groups with the DESeq2 package [24]. Endometrial genes responding to IFNT were identified as those up-regulated DEGs overlapping between IFNT vs conceptus and IFNT vs control. These overlapping genes were further explored through the Boruta algorithm, to identify endometrial genes mainly responding to IFNT rather than to the conceptus. The Boruta method is a feature selection wrapper built around the Random Forest classification algorithm, which compares the importance of each gene to classify the groups with genes selected at random [25]. This process was repeated up to 10,000 until all the top genes were confirmed with a confidence of 99.9%.

The DAVID Bioinformatics Resource 6.8 [26] was used to carry out a Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the endometrial genes responding to IFNT identified by the Boruta algorithm. GO and KEGG categories were considered enriched when $P \leq 0.05$ and considered to be approaching significance when $0.05 \leq P \leq 0.1$. In addition, protein-protein interaction (PPI) network analysis was performed using the Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org/) to elucidate the association between two or more products.

2.3. Dose- and time-dependent effects of IFNT on endometrial ISG expression

Five endometrial genes exhibiting greater differences between the IFNT and control groups were selected from the genes identified by the Boruta algorithm for in vitro assessments using endometrial explants, in addition to four classical ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*).

2.3.1. Endometrial explant culture

Bovine reproductive tracts (n = 8) were collected from a local abattoir immediately after slaughter in the late luteal phase of the estrous cycle based on the ovarian status [27] and a previously described protocol [19]. Tracts were placed on ice and transported to the laboratory within 2 h. In the laboratory, the endometrial explants were prepared according to a previously described protocol Borges, Healey [28]. Briefly, the uterine horn ipsilateral to the CL was dissected from the rest of the reproductive tract, sprayed with 70% ethanol and washed with 1× PBS (Gibco, ThermoFisher Scientific) containing 1% (v/v) 100 × antibiotic-antimycotic (ABAM; Gibco). Next, the endometrium was incised longitudinally on the *anti*-mesometrial side and the endometrial luminal surface was washed with PBS containing 1% ABAM. At this stage, an 8 mm biopsy punch (Covetrus BV) was applied to obtain intercaruncular endometrial tissue from the middle third of the uterine horn. Sterile scissors were then used to separate the endometrium from the underlying tissue. The collected endometrial explants (50–80 mg) were washed in conical tubes containing 25 mL of Hank balanced salt solution (HBSS; Gibco, ThermoFisher Scientific) supplemented with 1% ABAM. The medium was removed, and explants were washed twice more in 25 mL of HBSS without ABAM. In a sterile safety cabinet, the explants were individually placed epithelial side up in culture wells of 4-well plate (Nunc™, ThermoFisher Scientific) containing 1 mL of Roswell Park Memorial Institute (RPMI) medium (Gibco) with 1% ABAM. The explants were cultured in 5% CO_2 and humidified air at

Table 1
List of the primers used in RT-qPCR.

Gene	Accession No.	Primer (Sequence 5′–3′)	Product length (bp)
ISG15	NM_174366.1	F: GTCTTTTGAAGGGAGGCCCA R: TACCCACCCCGAAGACGTAG	102
OAS1	NM_001029846.2	F: GACGTGCTTCCAAGAGTCCA R: GGAAGACGACAAGGTCAGCA	116
MX1	NM_173940.2	F: AGAGCAACCTGTACAGCCAAT R: CTCTGGTCCCCGATAACAGC	127
MX2	NM_173941.2	F: CTACAAGTGCACAGGTGACAA R: CGGGCACAGAACACAAAAGG	127
CMPK2	XM_002691489.5	F: TCTTGCATCAGCCAGTGGAG R: CGACAACCACAGGCGATTTG	133
BPNT1	NM_001034539.2	F: CTGGTGCCTGATAACGCTCT R: GTGGGACTGGATGCCATGAT	97
IFI35	NM_001075462.2	F: GGGTGAGACCAAAGCTCCTC R: AGAGCCGACATAGTCTGGGA	120
TNFSF10	NM_001319901.1	F: AAGGGTCCTAAGAGGGTAGC R: TTCTTGGAGCCTGGAACTGG	80
TRIM38	XM_010818483.3	F: CCCAAAGGGTCACACTGAACT R: AGCTCTGAACCCATTGAAGAGG	90
GAPDH	NM_001034034.2	F: TTCTACTGGCGCTGCCAAGG R: GATCCACAACAGACACGTTGGG	107
ACTB	NM_173979.3	F: CAGCAGATGTGGATCAGCAAGC R: AACGCAGCTAACAGTCCGCC	91
RPL19	NM_001040516.1	F: GAAAGGCAGGCATATGGGTA R: TCATCCTCCTCATCCAGGTT	86
PPIA	NM_178320.2	F: CATACAGGTCCTGGCATCTTGTCC R: CACGTGCTTGCCATCCAACC	108
YWHAZ	NM_174814.2	F: TGAAGCCATTGCTGAACTTG R: TCTCCTTGGGTATCCGATGT	114
RNF11	NM_001077953.1	F: TCCGGGAGTGTGTGATCTGTATGAT R: GCAGGAGGGGCACGTGAAGG	131
H3F3A	NM_001014389.2	F: CATGGCTCGTACAAAGCAGA R: ACCAGGCCTGTAACGATGAG	136
SDHA (new)	NM_174178.2	F: ACTTCACCGTTGATGGCAATAA R: CGCAGAAATCGCATCTGAAA	59

F = Forward, R = Reverse.

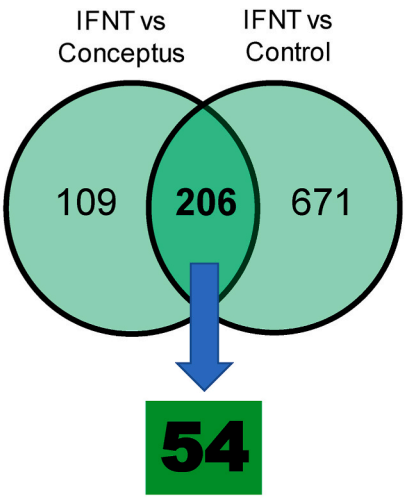
38.8 °C for 2 h before treatment with recombinant ovine IFNT (produced by G. Charpigny, INRAE, France).

2.3.2. Treatment of endometrial explants with IFNT

After 2 h culture of endometrial explants, the medium was fully aspirated and replaced with 1 mL of pre-warmed RPMI medium supplemented with 1% ABAM. Then, the explants were cultured in medium without (control) or with IFNT (1, 10, 100 ng/mL) for 6 h. In parallel, endometrial explants were cultured in medium containing 100 ng/mL IFNT for different time periods (15 min, 30 min, 1 h, 3 h, 6 h). Eight endometrial explants taken from the same region of each of the eight uteri were used for both dose- and time-dependent experiments in order to minimise variation. The 6 h culture duration was selected in the dose-dependent experiment based on previous studies from our group [13, 19]. For the time-dependent experiment, 100 ng/mL of IFNT was chosen on the basis of findings of an earlier study [20] in which 100 ng/mL of IFNT and a Day 15 long conceptus induced similar response in the uterine endometrium. In addition, Rizos et al. [14] reported that a conceptus of 20 mm length (long) produced about 10,000 IU/ml (equivalent to 100 ng/mL) IFNT in 24 h. At the end of the respective experiments, explants were snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.3.3. Explant RNA extraction, cDNA synthesis and RT-qPCR

Total RNA extraction from endometrial explants was carried out according to a protocol described by Mathew et al. [19]. Briefly, total RNA was extracted from ~50 mg of endometrial explant tissue by homogenization using 1 mL of Trizol reagent (Invitrogen) and a Qiagen



DYNC1H1	*MGAT1*	*TAF3*	*ACP2*	*HOMEZ*
SPTAN1	*MFN2*	*GOSR2*	*SDK2*	*POU6F1*
CMPK2	*TRIM21*	*IFI30*	*MITD1*	*TRIM14*
LRP10	*CTC1*	*RNASEL*	*KIF5C*	*TIMD4*
LOC781741	*ZFYVE26*	*SHROOM4*	*AGPAT3*	*ABI3*
EPSTI1	*TBC1D14*	*RUBCN*	*FAM110D*	*WDFY4*
LOC112441507	*TTC4*	*TENT5A*	*ZUP1*	*WDCP*
BPNT1	*IFI35*	*BLOC1S5*	*NOD1*	*C3AR1*
PFKP	*TNFSF10*	*RAPGEF5*	*KCTD2*	*CD180*
SCO1	*ATL3*	*SLC25A19*	*ADCK2*	*CLECL1P*
LGALS3BP	*TRIM38*	*MGAT2*	*PCK2*	

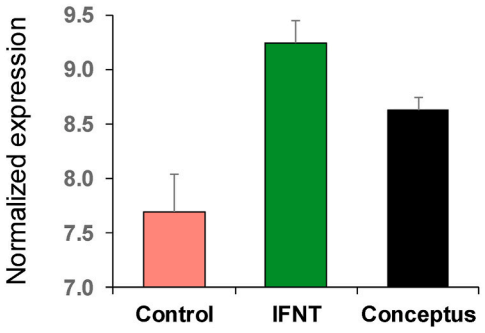


Fig. 1. Comparison of differentially expressed genes (DEGs) upregulated in bovine endometrium between IFNT vs conceptus and IFNT vs control. The overlapping genes were subjected to a Boruta algorithm to select the top genes. The average expression for the selected top genes is shown in the bar graphs. Control (n = 10), 100 ng/mL IFNT (n = 10), conceptus (n = 33).

RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. The RNA quantity and purity (verified through A_{260}/A_{280} ratio) were determined using the Nano Drop 1000 (Thermo Fisher Scientific) and RNA quality was checked by the Agilent Bioanalyzer (Agilent Technologies). The mean A_{260}/A_{280} ratio was 2.0 (range: 1.9–2.1), and the mean RNA integrity number (RIN) was 7.3 (range: 6.6–8.4).

Then, 1 μg of explant mRNA was reverse transcribed into cDNA using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific) in a 20 μL reaction following the manufacturer's instruction, the cDNA was diluted 1:10 with distilled water and 5.0 μl was used in each RT-qPCR. The primers of the target genes used in RT-qPCR are listed in Table 1. The RT-qPCR reactions were carried out using the Roche SYBR Green RT-qPCR master mix kit (Roche Diagnostics Ltd) and the Applied Biosystems 7500 Real-Time PCR system

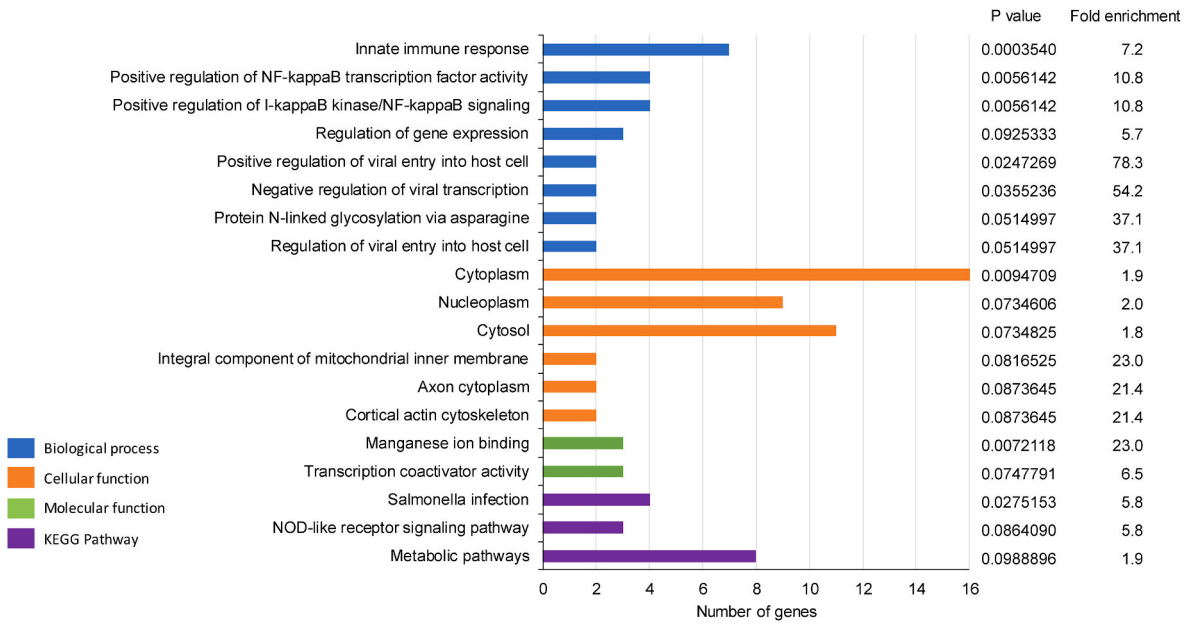


Fig. 2. Classification of 54 interferon tau-induced genes into functional categories based on the analysis of GO terms and KEGG pathways.

(ThermoFisher Scientific) following the manufacturer's recommendations, in a 20ul reaction containing 300pM final concentration of the forward and reverse primers. The RT-qPCR thermal cycler settings for all reactions consisted of an initial temperature of 95 °C for 10 min, followed by 40 PCR cycles consisting of melting at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. A dissociation analysis was included for each primer pair to evaluate primer specificity for the target sequence. The PCR primer amplification efficiencies (E) for each primer pair was calculated using a serial dilution of cDNA (1:4 over 7 points) and shown to lie between 90% and 110%.

The stability of eight potential reference genes was assessed using the geNorm [29] function within the qBASE + analysis package (Biogazelle, Zwijnaarde, Belgium). The reference genes H3 histone family 3A (*H3F3A*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) were shown to be the most stably expressed and were subsequently used to normalise the data. Calibrated normalized relative gene expression values (CNRQ) were calculated for all samples using the qBASE + software.

2.4. Statistical analysis

The qBASE + Stat wizard () was used for statistical analysis of the data. All values are presented as mean ± standard error mean of variance (SEM). One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was applied to compare the mean differences among the treatment groups. The treatment effect was considered statistically significant at $P < 0.05$.

3. Results

3.1. Selection of candidate genes

Transcriptome analysis results revealed that IFNT upregulated 877 and 315 transcripts compared to control and conceptus, respectively (Fig. 1 and Supplementary Tables S1–2). Among the 877 genes upregulated by IFNT compared to control, well-known classical genes (*ISG15*, *OAS1*, *MX1* and *MX2*) were not differentially expressed between IFNT and conceptus group (Supplementary Fig. 2). A total of 206 transcripts were commonly regulated by IFNT. From these overlapped 206 differentially expressed genes (DEGs), 54 genes were identified by the Boruta algorithm as the main endometrial genes upregulated by IFNT.

Gene ontology (GO) analysis of these 54 ISGs revealed eight enriched biological processes among which the top 3 categories concerned innate immune response (7 genes), positive regulation of NF-kappaB transcription factor activity (4 genes) and positive regulation of I -kappa B kinase/NF-kappaB signalling (4 genes) (Fig. 2 and Supplementary Table S3). The enriched cellular components (CC) and molecular functions (MF) associated with these selected genes were cytoplasm (16 genes) and nucleoplasm (9 genes), and manganese ion binding (3 genes) and transcription coactivator activity (3 genes), respectively. Furthermore, KEGG pathway analysis identified three enriched pathways including salmonella infection (4 genes), NOD-like receptor signalling pathway (3 genes) and metabolic pathways (8 genes). Furthermore, we obtained a protein-protein interaction (PPI) network (Fig. 3) composed of 52 nodes and 102 edges (expected number of edges: 55) with average node degree 3.92, and local clustering co-efficient 0.386. The network analysis identified significantly more interactions than expected (PPI enrichment P value: 7.75e-09).

From 54 ISGs, we further selected five genes (*CMPK2*, *BPNT1*, *IFI35*, *TNFSF10* and *TRIM38*), based on their increased expression in the endometrium in response to IFNT compared to the control group ($P < 0.05$; Fig. 4; Supplementary Table S4).

3.2. Dose- and time-dependant response of the endometrium to IFNT

The well-known classical ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*) were up-regulated ($P < 0.05$) in the endometrial explants by 1 ng/mL of IFNT, and the degree of the up-regulation was increased at higher concentrations (10 and 100 ng/mL) ($P < 0.05$, Fig. 5). On the other side, IFNT at 100 ng/mL stimulated ($P < 0.05$) *ISG15* after 1 h incubation, while other classical ISGs (*OAS1*, *MX1* and *MX2*) were upregulated ($P < 0.05$) in endometrial explants when cultured for 3 or 6 h, but not for shorter periods (15 min, 30 min, and 1 h, $P > 0.05$).

Unlike the well-known classical ISGs, the selected ISGs (*CMPK2*, *BPNT1*, *IFI35*, *TNFSF10* and *TRIM38*) were upregulated only at higher concentrations of IFNT (10 and 100 ng/mL), but not by 1 ng/mL of IFNT (Fig. 6). In terms of time dependence, IFNT at 100 ng/mL stimulated ($P < 0.05$) *CMPK2* after 1 h incubation; however, other selected ISGs (*BPNT1*, *IFI35*, *TNFSF10* and *TRIM38*) were only increased in endometrium when cultured for 3 or 6 h, but not shorter periods (15 min, 30 min, and 1 h ($P > 0.05$, Fig. 6).

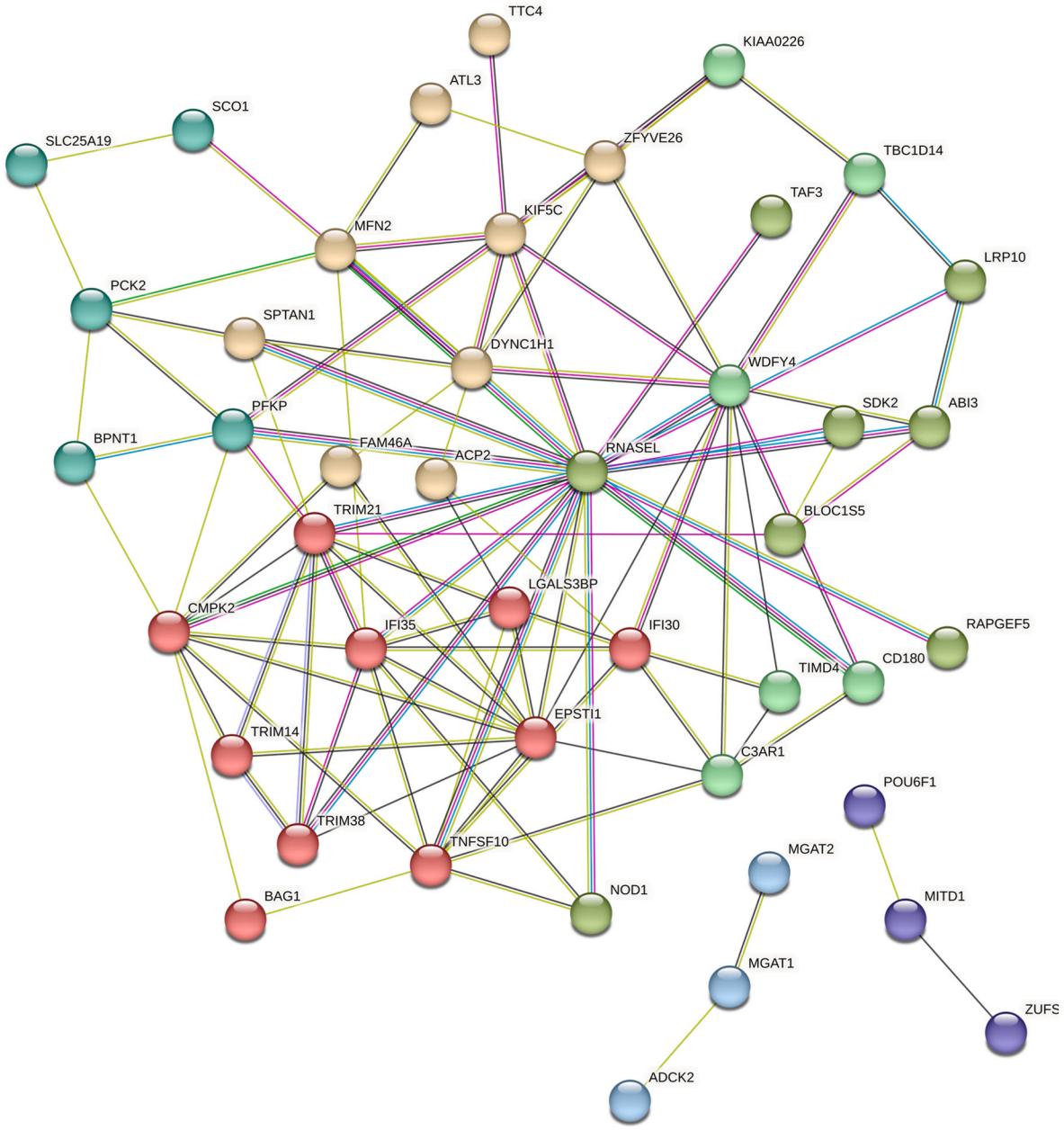


Fig. 3. The protein-protein interaction network of 54 ISGs. Nodes represent proteins and lines represent protein-protein interactions. The minimum required interaction score was set at 0.150.
Nodes with same colour represent same cluster with shared functions. Nodes joined by lines indicate interactions. The source of interactions is curated databases (——), experimentally determined (——), gene neighbourhood (——), gene co-occurrence (——), text-mining (——), and co-expression (——). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Improved understanding of the fundamentals of pregnancy recognition during the pre-implantation period, when a significant proportion of embryo loss occurs, could help to minimise early embryonic death and thereby improve reproductive efficiency in dairy cattle. The timely secretion of sufficient IFNT from the developing conceptus is crucial for MRP to occur. In the present study, we investigated the dose- and time-dependent effects of IFNT on the expression of key ISGs in bovine endometrium using an endometrial explant culture model. The major findings of this study are: (i) 54 ISGs responding to IFNT were identified in the endometrium through a bioinformatic approach (ii) the well-known classical ISGs (*ISG15*, *OAS1*, *MX1*, and *MX2*) transcripts were induced in the endometrium by lower concentrations of IFNT (1 ng/mL) and the intensity of the endometrial response to IFNT was increased at higher concentrations, (iii) higher concentrations (10 and 100 ng/mL) of IFNT were required to induce up-regulation of other selected ISGs (*CMPK2*, *BPNT1*, *IFI35*, *TNFSF10* and *TRIM38*) in the endometrium, and (iv) both well-known and other selected ISGs were up-regulated when endometrial explants were treated with IFNT for 3 or 6 h.

Endometrial explants have been successfully used to study the endometrial response to different stimuli under various patho-physiological conditions. For example, endometrial explants were used to investigate uterine responses to bacterial lipopolysaccharides [28,30], bacterial peptidoglycan [31], blastocyst stage embryos [13], IFNT [19,20], conceptus [19,20], and bovine conceptus secretory proteins (bCSP) [32]. In the present study, we employed an endometrial explant model to elucidate dose- and time-dependent effects of IFNT on

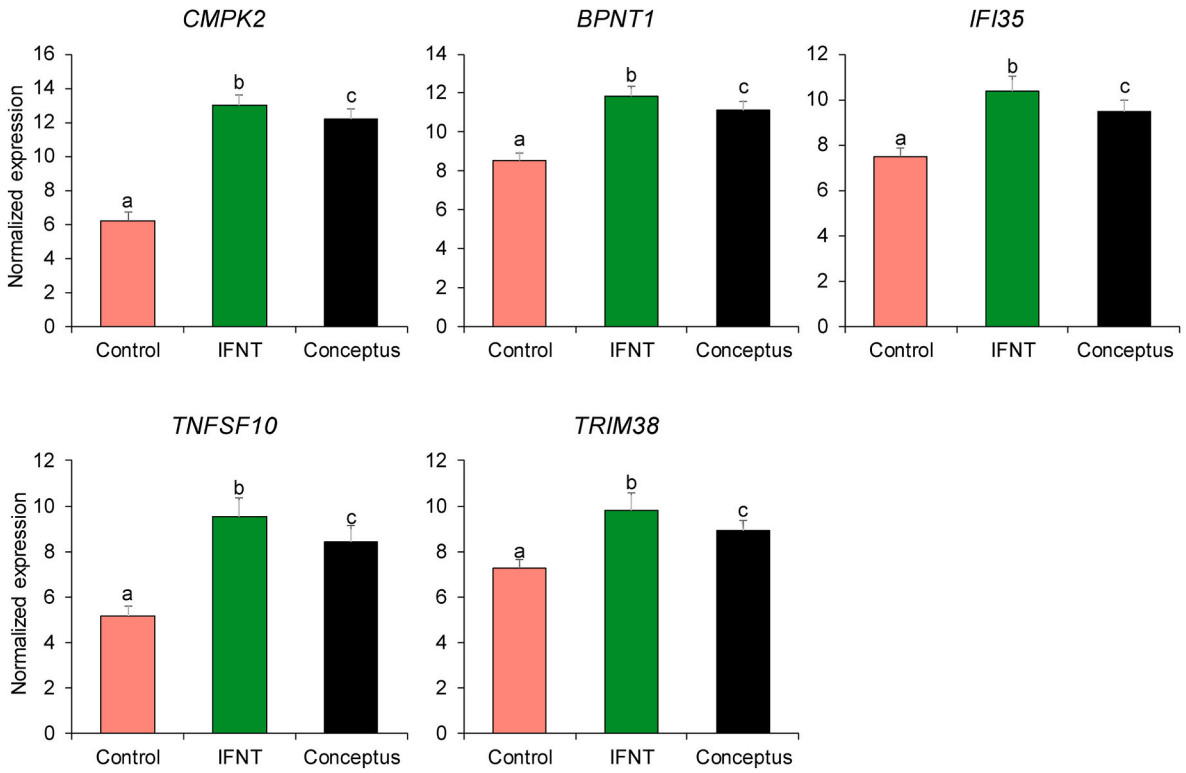


Fig. 4. Normalized expression of five selected genes from the 54 genes in the bovine endometrium responding to IFNT and also to some degree to the conceptus (see Fig. 1). Different letters (a–c) above the bar in the same graph indicate significant difference at $P < 0.05$. Control (n = 10), 100 ng/mL IFNT (n = 10), conceptus (n = 33).

endometrial expression of key ISGs. Endometrial explants collected from the late luteal phase can mimic the uterine milieu of early pregnancy and thus overcome limitations of studying conceptus (IFNT)-maternal interactions using an in vivo model. This is because explants maintain normal cellular and extracellular architecture which represents resident populations of endometrial cells that cannot be achieved with current 2D and 3D cell culture techniques.

Secretion of IFNT by the developing bovine embryo has been reported as early as Day 7 from blastocyst-stage embryos [33–36]. More recently, it has been demonstrated that the endometrium responds to this blastocyst-derived IFNT by upregulating ISG expression as early as Day 7 [11–13]. Such endometrial response to IFNT is amplified during the MRP period around Day 16 resulting in a pregnancy establishment in cattle. Indeed, IFNT secreted from the developing embryo/conceptus binds with type 1 IFN alpha receptors (IFNAR1 and IFNAR2) of endometrial cells [37] and induces both 'classical' and 'non-classical' ISGs via activation of JAK-STAT signal transduction pathway [38], and mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathway [39,40], respectively. These ISGs play pivotal roles in regulating uterine functions important for conceptus elongation, implantation and establishment of pregnancy [41–44]. In the last two decades, the well-known classical ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*) have been extensively identified and reported in the endometrium during early pregnancy in cattle [11,13,18,45,46]. However, other ISGs associated with conceptus-derived IFNT during early pregnancy in cattle have not received significant attention.

In the present study, we applied a Boruta algorithm to determine genes capable of discriminating one group from the other according to their expression. This method identified 54 candidate genes induced by IFNT and also to some degree by the conceptus, but with a distinct expression between one group and the other. The GO term analysis showed that the majority of the genes were associated with the biological processes involved in regulation of innate immunity (Fig. 2). Apart from its *anti*-luteolytic function, IFNT is also regarded as a potent immune regulator during pregnancy establishment in cattle [47] which involves both regulation of innate immune response and generation of adaptive/tolerogenic environment in the uterus [48,49]. Thus, activation of genes related to immune-related biological processes by IFNT might reflect the immune regulatory functions in the uterus during pregnancy establishment in cattle.

From these genes induced by IFNT, we further selected five genes (*CMPK2*, *BPNT1*, *IFI35*, *TFSF10*, and *TRIM38*) based on their expression level compared to the control for examining the effect of IFNT on their expressions in endometrial explants in vitro. The PPI network analysis done with the 54 genes showed that our selected fives genes are directly and/or indirectly associated with each other in which IFI35, CMPK2, TRIM38 and TNFSF10 formed more connections than BPNT1 in the whole network (Fig. 3). Among these five genes, *CMPK2*, *IFI35* and *TRIM38* are recognized members of ISG family [50–53], while *BPNT1* is a member of the magnesium-dependant phosphomonoesterase family mostly involved in metabolic pathway [54], and *TNFSF10* is a member of tumour necrosis factor (TNF) ligand family involved in immune response [55]. Both, *BPNT1* and *TNFSF10* have been reported to be upregulated in the endometrium by IFNT and Day 15 conceptus during early pregnancy in cattle [20,22]. The specific role of these selected ISGs during early pregnancy in cattle is unknown and warrants further investigations.

Well-known ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*) were induced in the endometrial explants by the lowest concentration of IFNT tested (1 ng/mL). Interestingly, higher concentrations of IFNT (10 and 100 ng/mL) were required to induce other selected ISGs (*CMPK2*, *BPNT1*, *IFI35*, *TNFSF10* and *TRIM38*), suggesting a dose-dependent effect of IFNT in modulating ISG expression in the endometrial explants. The dose-dependent effect of IFNT between well-known ISGs and selected ISGs could be due to their variations in the expression level (Figs. 5 and 6, Supplementary Table S5), where well-known classical ISGs were highly

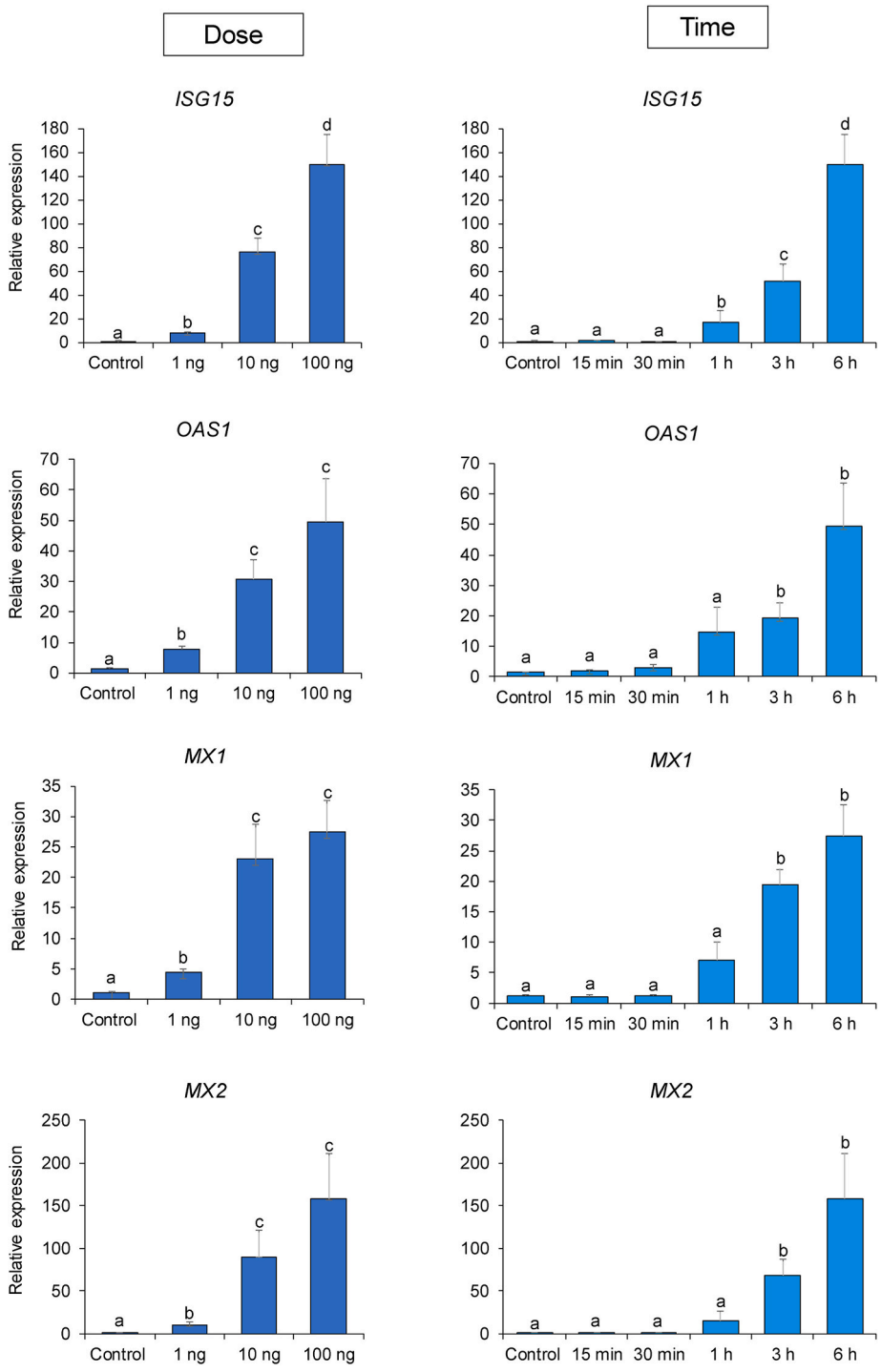


Fig. 5. Relative expression of well-known classical ISGs in endometrium induced by IFNT. Different letters (a–d) above the bar in the same graph indicate significant difference at $P < 0.05$. For time-dependent experiments, 100 ng/mL of IFNT was used. No. of replications = 8.

expressed. Indeed, *ISG15* expression can be induced in cultured luteal cells with as little as 0.1 ng/mL IFNT in sheep [56] and cattle [57]. Moreover, well-known classical ISGs (*ISG15*, *OAS1*, *MX1* and *MX2*) have been demonstrated to be induced in cultured bovine uterine epithelial cells in the presence of very low concentrations (0.1 ng/mL) of IFNT [12]. These results together with our findings support the concept that lower quantities of IFNT can induce well-known classical ISGs in the uterus, but an optimum amount of IFNT is required to induce a large scale of ISG expression in the uterus, which is likely associated with the amount of IFNT secreted from the developing conceptus. Consistent with this, our group previously demonstrated that short conceptuses were unable to induce a response similar in the bovine endometrium to that induced by age-matched long conceptuses and IFNT (100 ng/mL) [20]. In addition, a strong positive relationship between conceptus length and IFNT secretion has been reported [14,58]; conceptuses should therefore reach an adequate size in order to produce sufficient IFNT to prevent luteolysis.

In this study, we showed that IFNT begins to act in the uterus by stimulating all studied ISGs in the first 3 h which supports the notion of an acute action of IFNT on the uterus. Unlike the dose-dependent effect

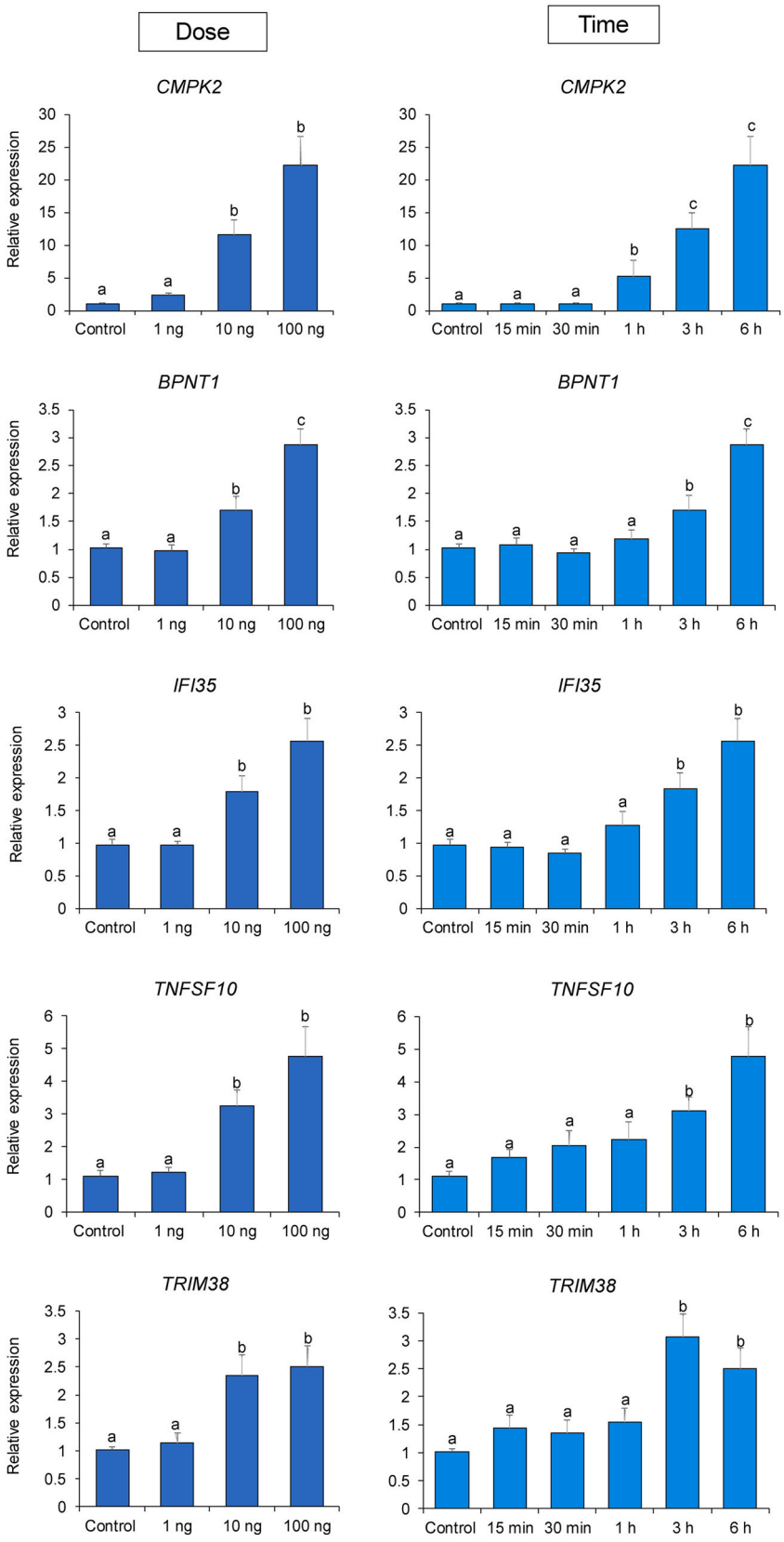


Fig. 6. Relative expression of five selected ISGs genes in endometrium induced by IFNT. Different letters (a–c) above the bar in the same graph indicate significant difference at $P < 0.05$. For time-dependent experiments, 100 ng/mL of IFNT was used. No. of replications = 8.

of IFNT, both well-known classical ISGs and selected ISGs found to be upregulated at the same time, suggesting that the action of IFNT is not only acute but it also induces broader changes in the uterus within this short period. Indeed, IFNT concentrations peak around Day 15–16 of pregnancy for MRP and continues thereafter over a period of week. While a sufficient IFNT level is required for pregnancy establishment [16], onset of its function in the uterus at the appropriate time is also important to prevent luteolysis. Interestingly, it is reported that conceptuses are capable of preventing luteolysis within a day or so of transfer in both cattle [21] and sheep [59]. The transfer of conceptuses from Day 10 to Day 16 of the oestrous cycle, but not on Day 17, can establish pregnancy in cattle [21]. Even transfer of 2 embryos (to maximise the luteotrophic or antiluteolytic effects of the conceptus) as late as Day 16 after estrus led to normal pregnancy but not thereafter. Similarly, it was also shown that removal of conceptuses at Day 17, 18 or 19 prolonged luteal lifespan compared to nonbred controls or when conceptuses were removed on Day 13 or 15 of pregnancy in cattle [60]. These findings clearly indicate an acute action of IFNT around Day 16 of pregnancy in cattle. In support of this concept of an acute action of IFNT, transcriptomic studies have shown that there is a dynamic temporal pattern to the endometrial gene expressions in cattle up to the time of maternal pregnancy recognition, however, such differences in the gene expression pattern of the endometrium of pregnant and cyclic heifers are little [45,61]. In contrast, from Day 15 [62] to Day 16 [45] onwards, significant endometrial changes occur, predominantly but not exclusively driven by IFNT. Similar data are also available in sheep [63].

5. Conclusion

In conclusion, through a bioinformatic approach we identified 54 ISGs potentially induced by IFNT in the endometrium around the period of maternal pregnancy recognition in cattle. Our findings from dose- and time-dependent experiments suggest that IFNT acts on the uterus in both a dose- and time-dependent manner in cattle and that timely exposure of the endometrium to sufficient IFNT is essential for appropriate signalling to ensure successful pregnancy establishment.

CRediT authorship contribution statement

A.K. Talukder: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, preparation. **M.B. Rabaglino:** Methodology, Investigation, Formal analysis, Writing – review & editing. **J.A. Browne:** Methodology, Writing – review & editing. **G. Charpigny:** Methodology. **P. Lonergan:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts 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.2023.07.033.

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