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SÃO PAULO STATE UNIVERSITY
INSTITUTE OF BIOSCIENCES
POSTGRADUATE PROGRAM IN PHARMACOLOGY AND BIO-TECHNOLOGY

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ISABELLA RIO FELTRIN

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ISABELLA RIO FELTRIN

Thesis submitted to the Postgraduate Program in Pharmacology and Biotechnology of the Institute of Biosciences at the São Paulo State University to obtain the PhD degree in Pharmacology and Biotechnology.

Advisor: Claudia Maria Bertan Membrive, PhD.
Co-Advisor: Guilherme Pugliesi, PhD.

Botucatu – SP
2024

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: MARIA CAROLINA A. CRUZ E SANTOS-CRB 8/10188

Feltrin, Isabella Rio.
Study of genes stimulated by interferon-T in immune cells as candidates for pregnancy markers in bovine females / Isabella Rio Feltrin. - Botucatu, 2024

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Claudia Maria Bertan Membrive
Coorientador: Guilherme Pugliesi
Capes: 50504002

1. Interferon. 2. Immunity, Cellular. 3. Genes Expression. 4. Animal Pregnancy. 5. Cattle Reproduction.

Palavras-chave: ISG; Immune cells; Interferon-T; Pregnancy diagnosis; Uterine flush.

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Date: 08/02/2024

CERTIFICADO

Certificamos que a proposta intitulada "Inovações no diagnóstico precoce da gestação em bovinos", protocolada sob o CEUA nº 8192280317, sob a responsabilidade de **Guilherme Pugliesi** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 10/05/2017.

We certify that the proposal "Innovations in the early pregnancy diagnosis in cattle", utilizing 90 Bovines (90 females), protocol number CEUA 8192280317, under the responsibility of **Guilherme Pugliesi** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 05/10/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 05/2017 a 04/2018 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 9 anos	N:	30
Linhagem:	Nelore			Peso:	400 a 700 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	12 a 24 anos	N:	30
Linhagem:	Nelore			Peso:	300 a 400 kg		
Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	2 a 7 anos	N:	30
Linhagem:	Holandesa			Peso:	450 a 700 kg		

Resumo: A identificação do estado gestacional mais precocemente aos métodos atuais realizados por volta do 30o dia pós-inseminação permite uma redução no intervalo de inseminações, o que pode resultar em menor período para concepção e maior rentabilidade ao sistema de produção pecuário. A tese central do projeto é que biomarcadores no sangue estimulados pelo concepto são detectáveis no início da gestação. Isto pode permitir o desenvolvimento de um método precoce e inovador de diagnóstico da gestação em bovinos. Assim, tem-se como objetivo principal desenvolver um método de diagnóstico através da quantificação da abundância de genes estimulados por IFN-tau (ISGs) já conhecidos ou por novos marcadores aliado a uma identificação precoce dos animais não-gestantes pela avaliação da função luteal. Para isso, no Estudo 1 novos marcadores do estado gestacional poderão ser identificados através do estudo transcriptômico das células imunes do sangue no dia 14 e 18 pós-inseminação. Pretende-se caracterizar a abundância de ISGs já conhecidos e de novos transcritos estimulados pela presença do concepto, no sangue total, nas células imunes polimorfonucleares (PMNs) e em células imunes do leite entre os dias 12 e 20 de gestação em vacas de corte e leite. A luteólise será detectada por ultrassonografia Doppler através das mudanças no tamanho e vascularização do corpo lúteo (CL) e pelas concentrações circulantes de progesterona (P4) para detecção de vacas com CL não-funcional. Para mensuração das concentrações de P4 objetiva-se desenvolver e validar um ensaio imunoenzimático com alta sensibilidade e especificidade para evitar o uso de radioisótopos. No Estudo 2 será determinado a acurácia de métodos de diagnóstico da gestação realizados antes do dia 20 pós-IA, e baseados no uso isolado ou combinado da expressão de ISGs e da funcionalidade luteal (pela concentração de P4 ou ultrassonografia Doppler). Os novos conhecimentos e métodos inovadores aqui propostos embasarão o desenvolvimento de tecnologias para a pecuária de leite e corte. A exequibilidade será garantida pelo trabalho integrado de especialistas em cada etapa analítica e pela infraestrutura das instituições envolvidas.

Local do experimento: CBRA/VRA/FMVZ

São Paulo, 12 de maio de 2017

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

I dedicate this thesis to my parents, Ana and Feltrin, who are the basis of my life and my greatest supporters. Everything I am today is thanks to you!

All my love for you!

ACKNOWLEDGMENTS

Firstly, my sincere thanks to my parents, ***Ana and Feltrin***, for all their unconditional support dedicated to my dreams and me. You are the foundation of my life and my greatest example of person's and character. Without you, none of this would be possible. All my love to you!

To my life partner, ***Vinicius***, for all the love, affection, and patience dedicated to my dreams and me. Thank you for being by my side every moment of my life. All my love for you!

To the ***Postgraduate program and the professors*** of the Pharmacology and Biotechnology program at the Institute of Biosciences of the São Paulo State University, I extend my gratitude for the opportunity provided to pursue my PhD and for the courses taught that significantly contributed to my professional and academic qualification.

To the ***São Paulo State Research Support Foundation (FAPESP)***, for the financial support granted for the project (grant number 2015/10606-9).

To the ***Coordination for the Improvement of Higher Education Personnel (CAPES)*** for the PhD scholarship that facilitated my scientific qualification.

To the ***Center for Biotechnology in Animal Reproduction (CBRA)*** of the Fernando Costa Campus of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo (FMVZ, USP – Pirassununga), for providing the animals used in this study, as well as for the infrastructure and logistics that enabled the successful completion of this project.

To the ***members of the evaluation committee***, Prof. Fernando Silveira Mesquita, Prof. Alfredo Quites Antoniazzi, Prof. Mateus José Sudano, and Prof. Luciano Andrade Silva, for their valuable scientific contributions to this thesis.

My sincere thanks to my advisor, ***Prof. Claudia M. Bertan Membrive***, for granting me the opportunity to pursue my master's and PhD degrees. Your support, teachings, and knowledge shared over the years have significantly contributed to my personal and intellectual growth. My eternal gratitude and admiration for you and for what you represent for science!

My sincere thanks to my co-advisor, ***Prof. Guilherme Pugliesi***, for being part of my academic journey. For all the knowledge, teachings, and scientific contributions that have made me the professional I am today; and for the opportunity to be part of the LFEM family, which has certainly changed my life forever. My eternal gratitude. You are an inspiration to us all!

To my dear friends from the Postgraduate - ***Amanda Guimarães, Ana Clara Degan, Karine Morelli, Thais Sayuri, and Cecília Rocha***; I am thankful for your unconditional support, companionship, and affection throughout these years. Your presence was indispensable in my journey. Thank you for everything, and I love you all!

Special thanks to ***my friends and members of LFEM*** - Amanda, Ana Clara, Karine, Thiago, Danilo, Igor, Diego, Ana Laura, Lucas, Samuel, and the scientific initiation students and trainees; for their assistance and time dedicated to the execution and logistics of this project.

Finally, I extend my gratitude to everyone who directly or indirectly contributed to the planning and execution of this study, leading to the development of my PhD. Thank you very much.

RESUMO

FELTRIN, I.R. **Estudo de genes estimulados por interferon-τ em células imunes como candidatos à marcadores de prenhez em fêmeas bovinas.** [Study of genes stimulated by interferon-τ in immune cells as candidates for pregnancy markers in bovine females]. 2024. 99 f. Tese (Doutorado em Farmacologia e Biotecnologia) – Instituto de Biociências, Universidade Estadual Paulista, Botucatu, 2024.

Nas fêmeas bovinas, as perdas embrionárias ocorridas entre o 14º e o 19º dia após o estro afetam significativamente a eficiência reprodutiva dos rebanhos. Portanto, o desenvolvimento de métodos diagnósticos capazes de detectar precocemente a gestação ou não gestação é um ponto importante para melhorar o desempenho reprodutivo dos bovinos. O interferon-τ (IFN-τ) produzido pelo concepto desempenha um papel importante durante o reconhecimento materno da prenhez (MPR) em ruminantes, que envolve mais do que a inibição dos pulsos luteolíticos da prostaglandina F2 alfa ($PGF_{2\alpha}$) para manter a funcionalidade do corpo lúteo (CL). Para uma prenhez bem-sucedida, o concepto semi-alogênico deve evitar a sua rejeição pelo sistema imunológico materno, destacando a importância de elucidar os mecanismos envolvidos durante o início da prenhez. Além disso, neste momento, o IFN-τ é liberado na circulação sanguínea e estimula a expressão de genes (ISGs). O estudo da expressão gênica dos ISGs clássicos (*ISG15*, *OAS1*, *MX1* e *MX2*) em células imunes do sangue periférico, como células mono (PBMC) e polimorfonucleares (PMN) já vem sendo utilizado como ferramenta de diagnóstico precoce da prenhez, porém apresentou baixa acurácia. Neste contexto, nossos objetivos neste estudo foram: 1) caracterizar a magnitude da resposta dos ISGs clássicos (*ISG15*) e não clássicos (*RSAD2* e *IFI44*); bem como o perfil das citocinas pró- (*IL1β*) e anti-inflamatórias (*IL10*) em PBMC e PMN estimuladas com IFN-τ (***Experimento 1***) ou lavados uterinos (UF) de vacas gestantes (***Experimento 2***); e 2) avaliar a acurácia desses biomarcadores recentemente identificados (*RSAD2* e IFI44) como preditores da prenhez precoce em fêmeas bovinas (***Experimento 3***). Nos Experimentos 1 e 2, PBMC e PMN foram isoladas do sangue de vacas Nelore não gestantes (N=9) entre 10-12 dias pós-ovulação (D0 = dia da ovulação), e estimuladas com 100 ng/mL de roIFNT ou UF de vacas no dia 18 de gestação. A expressão gênica foi determinada por qPCR. Para o Experimento 3, fêmeas Nelore (nulíparas, N=103; primíparas, N=53; pluríparas, N=91) foram submetidas à coleta de

sangue para isolamento das PMN, e foi realizada ultrassonografia Doppler para avaliar a funcionalidade do CL no D20 após inseminação artificial em tempo fixo (D0 = dia da IATF). A expressão gênica também foi determinada por qPCR. Além disso, foram realizadas curvas ROC para determinar a acurácia dos preditores da prenhez no D20 (ISGs e Doppler). Nos Experimentos 1 e 2, a expressão de todos os ISGs foi maior (P<0,05) em ambas as células tratadas com roIFNT e UF-Conceptus do que nos grupos Controle. O fold change indicou que o *ISG15* e *RSAD2* foram os genes mais estimulados (P<0,05) em PBMC e PMN. A expressão de *IL1β* foi menor (P<0,05) em PBMC e PMN tratadas com UF-Conceptus; no entanto, nenhuma diferença (P>0,1) foi observada entre os outros grupos de tratamento. Para o Experimento 3, todas as combinações genéticas foram testadas, e a melhor associação para aumento da acurácia (92,7%) e redução de resultados falsos negativos (0,9%, 2/233) foi obtida quando os animais foram considerados prenhes se um dos os quatro ISGs (*ISG15*, *OAS1*, *RSAD2* e *IFI44*) foram estimulados em fêmeas com CL ativo (> 25% de perfusão sanguínea) no D20. Concluindo, o *ISG15* e *RSAD2* foram os ISGs mais estimulados em PBMC e PMN, indicando que uma associação entre ISGs clássicos e não clássicos pode ser utilizada como uma ferramenta precoce para melhorar a predição da prenhez no D20 em fêmeas bovinas com CL ativo determinado através da US-Doppler.

Palavras-chave: ISG, células imunes, interferon-τ, lavado uterino, diagnóstico de prenhez.

ABSTRACT

FELTRIN, I.R. **Study of genes stimulated by interferon-τ in immune cells as candidates for pregnancy markers in bovine females.** [Estudo de genes estimulados por interferon-τ em células imunes como candidatos à marcadores de prenhez em fêmeas bovinas]. 2024. 99 f. Tese (Doutorado em Farmacologia e Biotecnologia) – Instituto de Biociências, Universidade Estadual Paulista, Botucatu, 2024.

In bovine females, embryonic losses occurring between the 14th and 19th day after estrus significantly affect the reproductive efficiency of herds. Therefore, the development of diagnostic methods capable of early detection of pregnancy or non-pregnancy is an important point for improving the reproductive performance of cattle. Interferon-τ (IFN-τ) produced by the conceptus plays an important role during maternal recognition of pregnancy (MPR) in ruminants, which involves more than the inhibition of luteolytic pulses of prostaglandin F2 alpha ($PGF_{2\alpha}$) to maintain the function of corpus luteum (CL). For a successful pregnancy, the semi-allogeneic conceptus must avoid rejection by the maternal immune system, highlighting the importance of elucidating the mechanisms involved during early pregnancy. Furthermore, at this moment, IFN-τ is released into the blood circulation and stimulates the expression of genes (ISGs). The study of gene expression of the classic ISGs (*ISG15, OAS1, MX1,* and *MX2*) in peripheral blood immune cells, such as mono (PBMC) and polymorphonuclear (PMN) cells has already been used as a form of early diagnosis of pregnancy, but showed low accuracy. In this context, our objective were: 1) characterize the response magnitude of classic (*ISG15*) and non-classic (*RSAD2* and *IFI44*) ISGs; as well as pro- (*IL1β*) and anti-inflammatory (*IL10*) cytokines in PBMC and PMN stimulated by IFN-τ (***Experiment 1***) or uterine flush (UF) from pregnant cows (***Experiment 2***); and 2) evaluate the accuracy of these recently identified biomarkers (*RSAD2* and *IFI44*) as early pregnancy predictors in bovine females (***Experiment 3***). In the *Experiment 1 and 2,* PBMC and PMN were isolated from the blood of non-pregnant Nelore cows (N=9) between 10-12 days post-ovulation (D0 = day of ovulation) and stimulated with 100 ng/mL of roIFNT or UF from day 18 of pregnancy cows. Gene expression was determined by qPCR. For the *Experiment 3,* Nelore females (nulliparous, N=103;

primiparous, N=53; pluriparous, N=91) were submitted to blood collection for PMN isolation, and Doppler ultrasonography was performed to assess the functionality of the corpus luteum (CL) on D20 after timed-artificial insemination (D0 = TAI day). Gene expression was also determined by qPCR. In addition, ROC curves were performed to determine the accuracy of pregnancy predictors on D20 (ISGs and Doppler). In *Experiments 1 and 2*, expression of all ISGs was greater (P<0.05) in both cells treated with roIFNT and UF-Conceptus than its controls. The fold change indicated that *ISG15* and *RSAD2* were the most stimulated genes (P<0.05) in PBMC and PMN. Expression of *IL1β* was lesser (P<0.05) in PBMC and PMN treated with UF-Conceptus; however, no difference (P>0.1) was observed between the other treatment groups. For the *Experiment 3*, all gene combinations were tested, and the best association for an increase in accuracy (92.7%) and reduction of false negative results (0.9%, 2/233) was obtained when pregnant animals were considered if one of the four ISGs (*ISG15, OAS1, RSAD2*, and *IFI44*) were stimulated in females with an active CL (> 25% blood perfusion) on D20. In conclusion, *ISG15* and *RSAD2* were the most stimulated ISGs in PBMC and PMN, indicating that an association between classic and non-classic ISGs can be used as an early tool to improve pregnancy prediction at D20 in bovine females with active CL determined by US-Doppler.

Keywords: ISG, immune cells, interferon-τ, uterine flush, pregnancy diagnosis.

SUMMARY

1. GENERAL INTRODUCTION

Brazil has the second-largest cattle herd in the world, totaling around 202 million animals, representing 12.18% of the global herd (ABIEC, 2023). Although the Brazilian beef industry is on the rise, the reproductive efficiency of herds is still low and deserves attention. For example, the calving interval in Brazilian beef cattle operations is around 16.3 months, far from the ideal calving period of 12-13 months (BARUSELLI et al., 2016). Therefore, developing and improving technologies that help increase the productivity of properties and optimize breeding systems and herd profitability are essential.

To achieve enhanced genetic and production gains, reproductive strategies must prioritize improving service rates and reducing the interval between inseminations, without compromising the viability of the already-established pregnancy (SÁ FILHO et al., 2014). Based on this concept, protocols for resynchronizing ovulation in females who did not become pregnant were developed. Thus, the success in reducing prolonged calving intervals is associated with an early pregnancy diagnosis combined with an efficient resynchronization protocol. In this context, early detection of embryonic loss is essential - which occurs between days 8 and 17 of pregnancy in 40% of cases (THATCHER et al., 2001) - or early detection of conception failure.

Embryonic losses significantly affect reproductive efficiency and result from failure in maternal pregnancy recognition (MPR). MPR occurs between days 15 and 19 post-ovulation in bovine females, through the release of the interferon-τ (IFN-τ), which is produced and secreted by the trophoblastic cells of the conceptus in the uterine environment, which blocks the mechanisms involved in pulsatile secretion of prostaglandin F2 alpha ($PGF_{2\alpha}$) by the endometrium (MARQUES et al., 2007). IFN-τ increases on day 15, peaks on day 20, and decreases to basal concentrations near day 25 (BAZER et al., 2009). In addition, IFN-τ acts on endometrial cells downregulating the estradiol (ER) and oxytocin

(OXTR) receptors (SPENCER et al., 2007). As a consequence, the pulsatile release of $PGF_{2\alpha}$, induced by the binding of oxytocin (OT) to its endometrial receptor, does not occur, and the corpus luteum (CL) remains active producing high concentrations of progesterone (P4) to maintain pregnancy.

Among the pregnancy diagnosis methods applied to cattle, the most used technique is B-mode ultrasonography between days 28 and 32 after timed artificial insemination (TAI) (MARQUES et al, 2012; STEVENSON et al., 2003). However, this method makes it impossible to identify non-pregnant cows immediately after MRP and before returning to estrus (FORDE et al., 2011). Actually, other techniques have become available for the diagnosis of pregnancy, one of which is the detection of structural luteolysis in non-pregnant females using Doppler ultrasonography (Doppler-US). The detection of structural luteolysis has been used in beef cows between 20 and 22 days after TAI (PUGLIESI et al., 2014) with accuracy and sensitivity exceeding 90%. However, this technique has a limiting factor: the high number of false positives.

In the last years, advances have been achieved, generating the possibility of performing an indirect pregnancy diagnosis through the expression of genes stimulated by IFN-τ (ISGs) on days 18 and 20 of pregnancy in peripheral blood immune cells. The most traditional ISGs used to detect pregnancy in immune cells are Ubiquitin-like Modifier 15 (*ISG15*), 2'-5'-Oligoadenylate Synthetase 1 (*OAS1*), MX dynamin as GTPase 1 (*MX1*), and MX dynamin as GTPase 2 (*MX2*) (KIZAKI et al. al., 2013; PUGLIESI et al., 2014). The increase of ISGs abundance in peripheral blood mononuclear cells (PBMC) has been reported in ewes (ANTONIAZZI et al., 2013), dairy cows (SHIRASUNA et al., 2012, FERRAZ et al., 2021), and beef cattle (PUGLIESI et al., 2014; DALMASO DE MELO et al., 2020). The expression of ISGs follows the IFN-τ release pattern, which may indi-

cate pregnancy earlier than other conventional diagnostic methods. Furthermore, expression of classic ISGs has also been reported in peripheral blood polymorphonuclear cells (PMN) in cattle (KIZAKI et al., 2013; TOJI et al., 2017; MELO et al., 2020), and there is evidence that PMNs are more sensitive to IFN-τ stimulation than PBMCs (KIZAKI et al., 2013).

The expression of classic ISGs in PBMC and PMN has been used in a few studies for prospective diagnosis of pregnancy on the 20th day post-TAI in beef cows; however, the maximum accuracy achieved was 87% (PUGLIESI et al., 2014; DALMASO de MELO et al., 2020). Thefore, in a previous study conducted by Rocha et al. (2020) using RNA sequencing, novel early-pregnancy-induced genes were identified in PBMC *(IFI6,* ***RSAD2****,* ***IFI44****, IFITM2, CLEC3B, OAS2, TNFSF13B, DMKN* and *LGALS3BP)* and PMN (***IFI44***, ***RSAD2,*** *OAS2, LGALS3BP, IFI6* and *C1R****)*** on the 18th day after TAI. These genes have the potential to be used as pregnancy biomarkers, offering an alternative for greater accuracy in the early diagnosis of pregnancy compared to the classic ISGs already in used. Thus, a better understanding of the transcriptional stimulus of each biomarker in immune system cells is a necessary aspect to be explored to deepen the selection criteria. Moreover, in the aforementioned study (Rocha et al., 2020), a limited sample size was used (N=6 animals/group), and as pregnancy assessment is a binary test, a larger number of animals must be explored to correctly select a pregnancy biomarker.

The herein study aims to determine novel pregnancy markers in peripheral blood immune cells that can serve as a basis for developing and improvement of molecular methods for pregnancy diagnosis in cattle. Our central hypothesis is that biomarkers in peripheral blood, stimulated by the presence of the conceptus, may be detectable at the early stages of pregnancy in circulating immune cells. This condition would enable the development of a method for early pregnancy diagnosis in bovine females 20 days after

mating. To test our hypothesis, the specific objectives of the current study were: 1) characterize the response magnitude of classic (*ISG15*) and non-classic (*RSAD2* and *IFI44*) ISGs; as well as pro- (*IL1β*) and anti-inflammatory (*IL10*) cytokines in PBMC and PMN stimulated by IFN-τ (***Experiment 1***) or uterine flush (UF) from pregnant cows (***Experiment 2***); and 2) evaluate the accuracy of these recently identified biomarkers (*RSAD2* and *IFI44*) as early pregnancy predictors in bovine females (***Experiment 3***).

This thesis is divided into two chapters. Chapter 1 reviews relevant topics related to the experiments conducted in the subsequent chapter. Chapter 2 describes the magnitude of the transcriptional response of each ISG and cytokine due to challenge by direct stimulation of IFN-τ or UF in PBMC and PMN, using an *in vitro* study model consisting of two experiments. Based on the results obtained in these studies, we conducted the third experiment. In the third experiment, we tested the accuracy of ISGs expression in PMN as early pregnancy predictors in a large number of animals. Chapter 2 was written in accordance with the standards of the journal *Biology of Reproduction*, to which the manuscript will be submitted. The final considerations at the end of this thesis summarize the most important findings of each experiment and discuss future perspectives for further studies.

We believe that the present study introduces an innovative aspect as it corresponds to the development of new techniques to diagnose pregnancy earlier than current methods, in addition to generating the possibility of identifying new molecular markers in peripheral blood immune cells, in addition to classic ISGs. Such advances can directly affect the efficiency of reproductive programs in cattle, especially those that use TAI.

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3.6 FINAL CONSIDERATIONS

The development of methods for early detection of pregnancy or embryonic/fetal mortality can contribute to improving the efficiency of reproductive programs in cattle worldwide. In this context, modulating the maternal immune system during early pregnancy has been reported as one of the main factors for gestational success and a significant cause of embryonic loss.

In the first studies (*Experiments 1 and 2*) described in Chapter 2, we initially sought to understand the response of immune cells from *Bos taurus indicus* heifers, stimulated by treatments with exogenous IFN-τ, or conditioned medium by the conceptus, through an *in vitro* cell culture system. In this model, we identified that the presence of a viable bovine conceptus modulates maternal immunological patterns in the uterus in favor of a TH2 anti-inflammatory response, through the downregulation of the cytokine *IL1β*, favoring the establishment of pregnancy. However, further studies are needed to elucidate which molecules secreted or induced by the conceptus, in addition to IFN-τ, may be inducing these maternal immune responses. Furthermore, we also observed that both treatments induced the expression of classic and novel ISGs, with *ISG15* and *RSAD2* being the most stimulated genes in both cell types, indicating that these markers have the potential to be evaluated for their accuracy as early pregnancy predictors on day 20 post-TAI. These findings may influence the improvement of molecular-based tests using ISGs to detect pregnancy in cattle, in addition to generating improvements in production systems, especially those that use fixed-time programs.

Although several reports describe a more pronounced response of PMNs to stimulation with IFN-τ, in the present study, we found no significant difference in the expression of ISGs between cell groups. Therefore, the response of PMNs was similar to that described in PBMCs, leading us to believe that both groups of cells can be used with

similar efficacy for the diagnosis of pregnancy. Therefore, based on the results obtained in the *in vitro* studies, we conducted an accuracy experiment (*Experiment 3*) using only PMNs.

To better select circulating transcripts for use as potential markers, larger numbers of animals under field conditions are necessary. For this reason, we conducted an *in vivo* study, where we analyzed the accuracy of the ISGs selected in the *Experiments 1 and 2*, as early pregnancy predictors in bovine females of different parity categories. Although all ISGs evaluated were classified as accurate predictors of pregnancy on D20 post-TAI, the use of these genes for pregnancy diagnosis still needs to overcome some barriers for practical application in the area. Firstly, although the expression of ISGs when associated with Doppler-US has generated satisfactory accuracy, the considerable number of false positive results, and mainly false negatives, significantly reduces the precision of the technique. Secondly, the technique for isolating immune cells and subsequent analysis of transcripts by qPCR is expensive and time-consuming, which would not justify the choice of this technique over others, using current methodologies. Furthermore, it is known that there is a difference in the expression patterns of ISGs between younger animals (heifers) compared to older (cows), making the use of this method even more limited.

Despite the obstacles raised, we believe that research using ISGs as pregnancy markers should be encouraged, as these genes respond early to a direct stimulus from the conceptus during early pregnancy. Furthermore, technology is constantly evolving, and today more sensitive and faster techniques are available, such as gene expression analysis by digital PCR, and more efficient forms of cell isolation. Moreover, the abundance of ISGs in the whole blood fraction could be explored, as we conclude that the cellular fraction does not affect the expression of these genes. This opens the way for the development

of rapid commercial kits, as well as metabolite analysis techniques through metabolomics, which can facilitate the application of this technique in the field.