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BALANÇO ENERGÉTICO NEGATIVO MODULA A EXPRESSÃO DE *ISG15* NO ENDOMÉTRIO E CORPO LÚTEO DURANTE O RECONHECIMENTO MATERNO DA GESTAÇÃO EM OVINOS

Santa Maria, RS

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Sanidade e Reprodução Animal da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Medicina Veterinária**.

Orientador: Prof. Alfredo Quites Antoniazzi

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Aprovado em 20 de fevereiro de 2020.

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Santa Maria, RS
2020

RESUMO

BALANÇO ENERGÉTICO NEGATIVO MODULA A EXPRESSÃO DE *ISG15* NO ENDOMÉTRIO E CORPO LÚTEO DURANTE O RECONHECIMENTO MATERNO DA GESTAÇÃO EM OVINOS

AUTOR: Igor Gabriel Zappe
ORIENTADOR: Alfredo Quites Antoniazzi

A mortalidade embrionária durante o reconhecimento materno da gestação é uma das principais causas de falha reprodutiva em ruminantes. Uma das causas de redução de fertilidade em ruminantes é a ocorrência de balanço energético negativo (BEN). A hipótese deste estudo é que o BEN pode estar relacionado à ocorrência de pior comunicação materno-embrionária, prejudicando o reconhecimento materno da gestação. O objetivo do nosso estudo foi avaliar os efeitos do BEN na expressão do *ISG15* no endométrio e no corpo lúteo no dia 17 de gestação. Para isso, 21 ovelhas mestiças da Texel-Corriedale tiveram estro e ovulação sincronizados e 18 foram acasaladas. As ovelhas foram divididas em quatro grupos experimentais: grupo controle não-prenhe, não acasaladas (CNP, n = 3); grupo controle prenhe (CP, n = 6); grupo de BEN durante a primeira semana de desenvolvimento embrionário (FW; n = 6); e grupo de BEN durante a segunda semana de desenvolvimento embrionário (SW; n = 6). As ovelhas do grupo FW jejuaram dos dias 0 a 7 e as ovelhas do grupo SW jejuaram dos dias 9 a 16 para a indução de BEN com hipercetonemia. O BEN foi induzido em dois estágios distintos de desenvolvimento embrionário: primeira semana de desenvolvimento embrionário e segunda semana de desenvolvimento embrionário. Durante o período experimental foram mensurados os níveis de β-hidroxibutirato (BHBA) no sangue e a glicemia. No dia 17 do ciclo estral ou da gestação, as ovelhas foram eutanasiadas e foram coletadas amostras de endométrio e corpo lúteo (CL). A expressão relativa de *ISG15* foi avaliada por q-PCR. Os animais que tiveram BEN induzido tiveram uma redução na glicemia e aumento na cetonemia. As ovelhas prenhes tiveram maior expressão de *ISG15* do que ovelhas não prenhes no endométrio do corno ipsilateral à ovulação e no CL. O grupo CP teve maior expressão de *ISG15* que o grupo CNP no endométrio do corno ipsilateral à ovulação e no CL. Os grupos FW e SW não mostraram diferença na expressão relativa do *ISG15* dos grupos CNP e CP e no endométrio dos cornos contralateral e ipsilateral à ovulação. O grupo FW teve maior expressão relativa de *ISG15* do que o grupo CNP no corpo lúteo. Como conclusão, ovelhas que tiveram períodos de BEN durante o desenvolvimento embrionário, especialmente durante a segunda semana de desenvolvimento embrionário, tiveram uma menor expressão relativa de *ISG15*, não apresentando tiveram diferença na expressão relativa de *ISG15* no endométrio do corno contralateral e ipsilateral à ovulação quando comparadas com os grupos CNP e CP.

Palavras-chave: *ISG15*, ovelhas, β-hidroxibutirato, gestação, endométrio, corpo lúteo

ABSTRACT

NEGATIVE ENERGY BALANCE MODULATES *ISG15* EXPRESSION IN THE ENDOMETRIUM AND THE CORPUS LUTEUM DURING MATERNAL RECOGNITION OF PREGNANCY IN EWES

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Embryonic mortality during maternal recognition of pregnancy is a major cause of reproductive failure in ruminants. One of the causes of reduced fertility in ruminants is the occurrence of a negative energy balance (NEB). The hypothesis of this study is that NEB may influence the occurrence of poor maternal-embryonic communication, impairing maternal recognition of pregnancy. The aim of our study was to evaluate the effects of NEB on the expression of *ISG15* in the endometrium and corpus luteum on day 17 of gestation. For this, 21 crossbred Texel-Corriedale ewes had estrus and ovulation synchronized and 18 mated. The ewes were allocated into four experimental groups: non-pregnant, non-bred control group (CNP, n = 3); pregnant control group (CP, n = 6); NEB group during the first week of embryonic development (FW; n = 6); and NEB group during the second week of embryonic development (SW; n = 6). The ewes in the FW group fasted from days 0 to 7 and the ewes in the SW group fasted from days 9 to 16 for the induction of NEB with hyperketonemia. BEN was induced in two distinct stages of embryonic development: first week of embryonic development and second week of embryonic development. During the experimental period, the blood levels of β-hydroxybutyrate (BHBA) and glycemic levels were measured. On day 17 of the estrous cycle or gestation, the sheep were euthanized and samples of the endometrium and corpus luteum (CL) were collected. The relative expression of *ISG15* was assessed by q-PCR. Animals that had NEB induced had a reduction in glycemic levels and an increase in BHBA blood levels. Pregnant ewes had higher *ISG15* relative expression of than non-pregnant ewes in endometrium from ipsilateral horn at ovulation and in the CL. The CP group had greater expression of *ISG15* than the CNP group in the endometrium from ipsilateral horn at ovulation and in the CL. The FW and SW groups showed no difference in the *ISG15* relative expression of the CNP and CP groups in the endometrium from contralateral and ipsilateral horn at ovulation. The FW group had a higher *ISG15* relative expression than the CNP group in the CL. In conclusion, ewes that had periods of NEB during embryonic development, especially during the second week of embryonic development, had a lower *ISG15* relative expression, with no difference in the *ISG15* relative expression in the endometrium from contralateral and ipsilateral horn at ovulation when compared with the CNP.

Keywords: *ISG15*, sheep, β-hydroxybutyrate, pregnancy, endometrium, corpus luteum

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1. INTRODUÇÃO

A reprodução em ruminantes, como em todos os mamíferos, é um processo biológico altamente complexo que requer um diálogo entre o concepto em desenvolvimento e o útero materno (FORDE, N.; LONERGAN, P., 2017). E o estabelecimento de uma gestação bem-sucedida em ruminantes domésticos precisa passar por três estágios distintos: sinalização e reconhecimento materno da gestação, implantação e placentação (GUILLOMOT, 1995; HANSEN, T. R; SINEDINO; SPENCER, T. E., 2017; SPENCER, T. E. *et al.*, 2007). Sendo que a mortalidade embrionária em estágio inicial de desenvolvimento, principalmente por falhas na sinalização durante o reconhecimento materno da gestação, é uma das maiores causas de falhas reprodutivas em ruminantes (DISKIN; MORRIS, 2008) e pode envolver de 20 a 30% das perdas totais (DIXON *et al.*, 2007; KAULFUSS *et al.*, 1997; WILTBANK *et al.*, 2016).

O reconhecimento materno da gestação é o período em que o concepto sinaliza sua presença para a mãe (FARIN, C. E.; IMAKAWA; ROBERTS, R. M., 1989; NISWENDER, G D *et al.*, 2000). Nos ruminantes, o período de sinalização coincide com o elongamento do embrião e com a produção máxima de interferon tau (IFNT) (ANTONIAZZI, A. Q. *et al.*, 2011), que ocorre com maior intensidade entre os dias 12 e 26 da gestação (FARIN, C. E. *et al.*, 1990; ROBERTS, R. Michael, 1993). A principal função do IFNT é evitar o retorno ao estro, preservando o funcionamento do corpo lúteo durante a gestação (NISWENDER, G D *et al.*, 2000). A ação direta do IFNT eleva a expressão de genes estimulados por interferon (*ISGs*), dentre os *ISGs* que aumentam a expressão durante o início da gestação estão os genes 2',5' oligoadenilato sintetase (OAS1) (MIRANDO *et al.*, 1991; SCHMITT, R. A. M. *et al.*, 1993), o gene de resistência ao myxovirus 1 (MX1) (OTT *et al.*, 1998) e o gene estimulado por interferon 15 (*ISG15*) (AUSTIN, Kathy J *et al.*, 1996).

Com a proximidade do parto e no início da lactação, durante o período de transição, ocorre o balanço energético negativo (BEN) em ovinos (LACETERA *et al.*, 2001; SAUN, 2000) e bovinos (DRACKLEY, 1999; HERDT, 2000). Durante o BEN, ocorrem alterações na regulação endócrina que levam à mobilização extensiva do tecido corporal, principalmente do tecido adiposo, a fim de atender aos requerimentos nutricionais para mantença e para a manutenção da gestação ou da produção de leite. Como consequência do BEN ocorre hipoglicemia, hipercetonemia e aumento da concentração circulante de ácidos graxos não esterificados (NEFA), que são liberados principalmente do tecido adiposo (DRACKLEY, 1999). Estudos sugerem que o BEN diminui a fertilidade em ruminantes como demora ao retorno à ciclididade (RIVERA *et al.*, 2010), menores taxas de gestação em vacas leiteiras

(CAIXETA *et al.*, 2017; OSPINA *et al.*, 2010; SANTOS, J E P *et al.*, 2010; WALSH, R. B. *et al.*, 2007) e menores taxas na primeira inseminação artificial (RIBEIRO *et al.*, 2013; WALSH, R. B. *et al.*, 2007), redução na qualidade do oocitária (DESMET *et al.*, 2016; HOECK, V. V. *et al.*, 2013; HOECK, Veerle VAN *et al.*, 2011) e embrionária (DESMET *et al.*, 2016).

Este trabalho utilizou a espécie ovina como um modelo experimental para estudar o efeito do BEN no reconhecimento materno da gestação. Para isso, 21 ovelhas com ovulação sincronizada foram distribuídas aleatoriamente em quatro grupos experimentais (grupo controle não-prenhe, CNP = 3; grupo controle prenhe, CP = 6; grupo com BEN na primeira semana de desenvolvimento embrionário, FW = 6; grupo com BEN na segunda semana de desenvolvimento embrionário, SW = 6). As ovelhas dos grupos CP, FW e SW foram submetidas a monta dirigida conforme identificação de cio por rufião. As ovelhas dos grupos FW e SW tiveram BEN induzido através de jejum alimentar por 7 dias na primeira e segunda semana de desenvolvimento embrionário, respectivamente. As ovelhas tiveram glicemia e β-hydroxibutirato (BHBA) monitorados e plasma e soro coletados durante o período experimental. Ao final do experimento as ovelhas foram eutanasiadas e foram coletadas amostras teciduais do endométrio dos cornos ipsi e contralaterais à ovulação, corpo lúteo, embrião e fígado.

Desta maneira, a nossa hipótese é de que o BEN interfere na comunicação materno-embrionária, prejudicando o reconhecimento materno da gestação. Assim, nosso trabalho teve como objetivo avaliar os efeitos do BEN, induzida em dois momentos distintos de desenvolvimento embrionário, na expressão de *ISG15* no endométrio e no corpo lúteo de ovelhas no dia 17 de gestação.

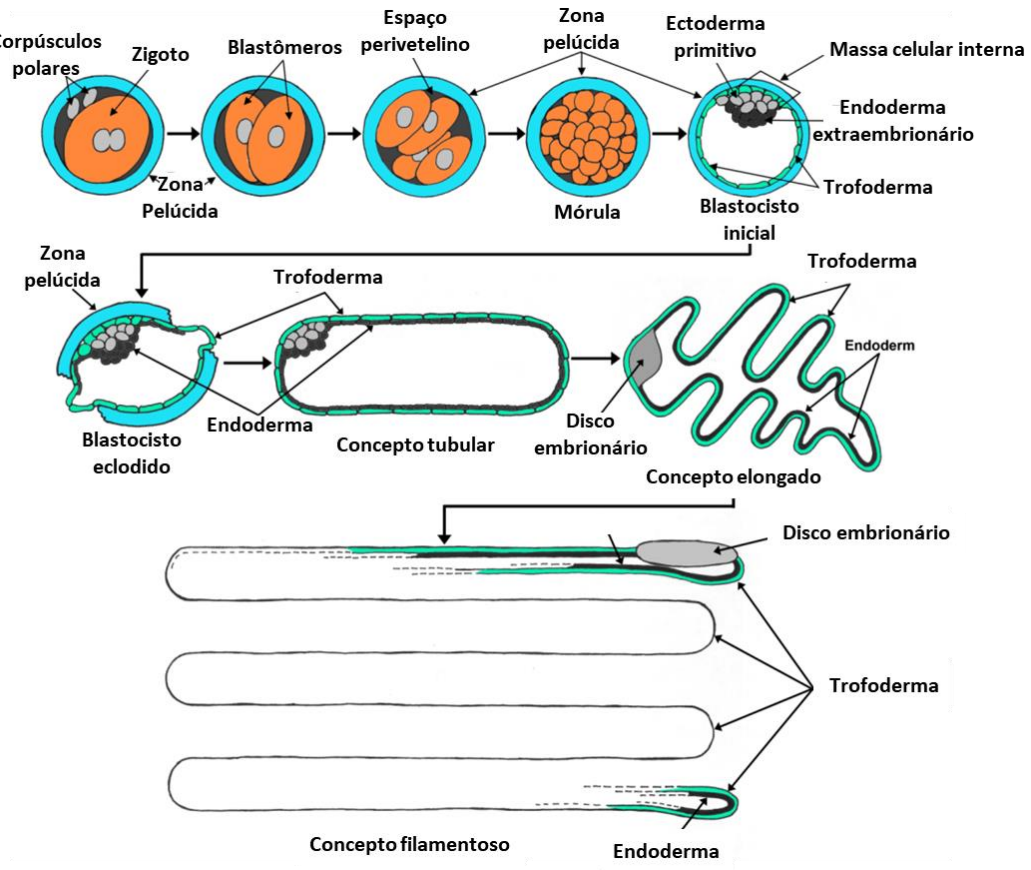
2. REVISÃO DE LITERATURA

2.1. RECONHECIMENTO MATERNO DA GESTAÇÃO EM RUMINANTES

O reconhecimento materno da gestação é um processo fisiológico em que o concepto sinaliza sua presença para a mãe e prolonga a vida útil do corpo lúteo (FARIN, C. E.; IMAKAWA; ROBERTS, R. M., 1989; NISWENDER, G D *et al.*, 2000). Em ruminantes, o período de sinalização coincide com a elongação do embrião e a máxima produção de IFNT (ANTONIAZZI, A. Q. *et al.*, 2011), acontecendo com maior intensidade entre os dias 12 e 26 de gestação (FARIN, C. E. *et al.*, 1990; ROBERTS, R. M., 1993). Esse processo em ruminantes requer que o concepto se elongue para produzir IFNT suficiente para sinalizar a gestação e suprimir o mecanismo luteolítico endometrial (ROBERTS, R. M. *et al.*, 1999; ROBERTS, R. M. *et al.*, 2008; SPENCER, T E; BAZER, F W, 1996; SPENCER, T. E; BAZER, F. W, 2002).

Após a fertilização, inicia-se o processo de desenvolvimento embrionário, conforme representado na Figura 1. O embrião sofre sucessivas mitoses que culminam na formação de uma massa celular sólida, denominada mórula (16-32 células), que permanece dentro da zona pelúcida do oócito. A mórula permanece no oviduto até entrar no útero por volta de 3 a 4 dias após fertilização em ovelhas. No dia 6, o embrião em desenvolvimento evolui para blastocisto. Nesse momento, os blastômeros pluripotentes começam a se diferenciar em massa celular interna e em trofectoderma. O blastocisto eclode da zona pelúcida entre os dias 8 e 9 (200 µm de diâmetro e contendo cerca de 300 células) e aumenta de tamanho (400-900 µm de diâmetro e contendo cerca de 400-900 células). O pequeno embrião esférico cresce de forma tubular até o dia 11, seguido de uma fase de rápido crescimento e elongamento entre os dias 12 e 16 (10-22 mm no dia 12, 10 cm no dia 14 e 25 cm no dia 17), assumindo uma forma filamentosa. Durante o período de elongamento precoce, o embrião permanece desprendido do endométrio uterino e dependente de nutrientes no lúmen uterino (JOHNSON, G. A. *et al.*, 2018). A expressão de IFNT aumenta à medida que ocorre o processo de elongação (HIRAYAMA *et al.*, 2014) e em ovinos o pico de produção de IFNT ocorre entre os dias 14 e 16 (SPENCER, T. E *et al.*, 2004), enquanto que em bovinos, o pico de produção de IFNT ocorre entre os dias 17 e 18 após a fertilização (FARIN, C. E. *et al.*, 1990).

Figura 1- Desenvolvimento embrionário inicial em ruminantes.



Fonte: adaptado de JOHNSON et al. (2018).

O IFNT é a principal citocina secretada pelas células do trofoblasto embrionário, sendo responsável pela sinalização durante o período de reconhecimento materno da gestação (ROBERTS, R. M. *et al.*, 1999). O mecanismo clássico de ação do IFNT consiste no controle da transcrição de receptores de estrógenos (ESR1) e, consequentemente receptores de ocitocina (OXTR) no epitélio luminal endometrial. Esse controle inibe os pulsos luteolíticos de prostaglandina F2 alfa (PGF), evitando o retorno à ciclicidade (SPENCER, T E; BAZER, F W, 1996).

2.1.1 O interferon tau (IFNT)

O IFNT é classificado como interferon do tipo I e sua principal função é evitar o retorno ao estro em ruminantes, preservando o funcionamento do corpo lúteo durante a gestação

(NISWENDER, G D *et al.*, 2000). Seu RNAm começa a ser expresso a partir do quarto dia do desenvolvimento embrionário *in vitro* (YAO *et al.*, 2009) e sua proteína é detectada a partir do sétimo dia do desenvolvimento embrionário, iniciando sua sinalização nas células do endométrio localizadas na região uterotubárica ipsilateral ao corpo lúteo (SPONCHIADO *et al.*, 2017). A expressão de IFNT termina com a implantação (DEMMERS; DERECKA; FLINT, 2001).

O IFNT liga-se a receptores de interferon tipo I (IFNAR1 e IFNAR2) e induz sua resposta por meio da sinalização via janusquinase (JAK) e proteínas transdutoras de sinais e ativadoras da transcrição (STAT), formando complexos multiméricos que agem como fatores de transcrição (BINELLI *et al.*, 2001). IFNAR1 e IFNAR2 são expressos em todos os tecidos corporais e têm como função principal mediar respostas antivirais (SADLER; WILLIAMS, 2008). Esses complexos se ligam a regiões definidas no DNA, chamadas de elementos responsivos à estimulação por interferons (ISREs), que regulam a expressão de genes estimulados por interferon (*ISGs*) (ANTONIAZZI, A. Q. *et al.*, 2011; HANSEN, T R *et al.*, 1999). Dentre os *ISGs* que aumentam a expressão de RNAm durante o início da gestação em resposta ao IFNT, estão os genes 2',5' oligoadenilato sintetase (OAS1) (MIRANDO *et al.*, 1991; SCHMITT, R. A. M. *et al.*, 1993), o gene de resistência ao myxovirus 1 (MX1) (OTT *et al.*, 1998) e o gene estimulado por interferon 15 (*ISG15*) (AUSTIN, K. J. *et al.*, 1996).

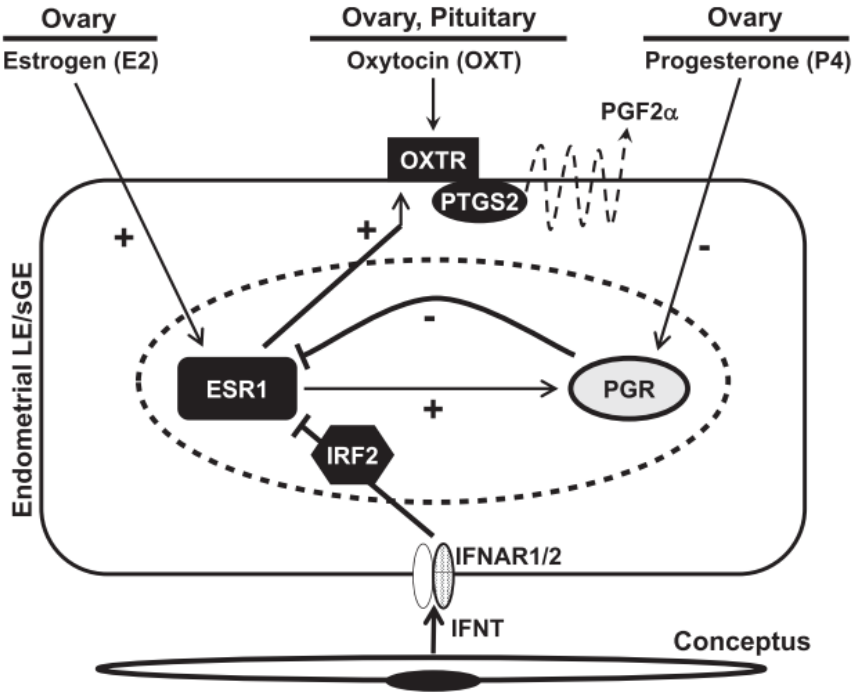
2.1.1.1 Ações do interferon tau

Atualmente, sabe-se que o IFNT atua mediante 3 vias de sinalização: parácrina, endócrina e autócrina. O IFNT também age pela via autócrina através de IFNAR1 (BROOKS; SPENCER, Thomas E., 2015; IMAKAWA *et al.*, 2002) aumentando a proliferação das células do trofoblasto e aumentando a expressão de *ISGs* (BROOKS; SPENCER, T. E., 2015; WANG, X. L. *et al.*, 2013).

Atua por via parácrina no útero, inibindo a expressão de ESR1 e de OXTR no epitélio luminal (SPENCER, T E; BAZER, F W, 1996). A supressão desses receptores evita a liberação de pulsos luteolíticos de PGF (SPENCER, T E; BAZER, F W, 1996), hormônio responsável pelo início da luteólise (MCCRACKEN; CUSTER; LAMSA, 1999), como demostrado na Figura 2. Ainda pela via parácrina, em ovelhas, verificou-se que o IFNT estimula a expressão de vários genes (*CST3, CST6, CTSL, GRP, HSD11B1, IGFBP1, LGALS15, SLC2A1, SLC2A5, SLC5A11, SLC7A2*) no epitélio luminal endometrial e (ou) no epitélio glandular endometrial,

que possuem atividades biológicas potencialmente importantes para o elongamento e implantação do embrião (SPENCER, T. E.; FORDE, N.; LONERGAN, P., 2016).

Figura 2 - Esquema ilustrativo da regulação hormonal do mecanismo luteolítico endometrial e dos efeitos parácrinos antiluteolíticos do interferon tau (IFNT) no endométrio uterino ovino.



Durante o estro e o metaestro, a expressão dos receptores de ocitocina (OXTR) pelos epitélios glandulares (sGE) e lúmen uterino (LE) aumenta em resposta aos estrógenos dos folículos ovarianos que primeiro estimulam a expressão do receptor alfa de estrógeno (ESR1) e os estrogénos agem via ESR1 para aumentar OXTR. Os receptores de progesterona (PGR) são expressos pelo LE durante o metaestro e o diestro, mas baixos níveis sistêmicos de progesterona são insuficientes para atuar via PGR para suprimir a expressão do gene ESR1 e OXTR. Durante o diestro inicial, a ESR1 endometrial e o estrogéno são baixos, mas os níveis de progesterona começam a aumentar com a formação do corpo lúteo (CL). A progesterona atua através do PGR para suprimir a síntese de ESR1 e OXTR por 8 a 10 dias. A exposição contínua do endométrio à progesterona acaba diminuindo a expressão do gene PGR no LE nos dias 11 a 12 do ciclo estral. A perda de PGR termina com o bloqueio de progesterona para a formação de ESR1 e OXTR. Assim, a ESR1 aparece entre os dias 11 e 12 após o estro, e na sequência ocorre aumento do OXTR nos dias 13 e 14. O aumento na expressão do OXTR é facilitado pelo aumento da secreção de estrógeno pelos folículos ovarianos. Nas ovelhas cíclicas e gestantes, a ocitocina é liberada do CL do ovário e da hipófise posterior a partir do dia 9. Nas ovelhas cíclicas, o OXT se liga ao OXTR no LE e aumenta a liberação dos pulsos luteolíticos da prostaglandina F2α (PGF2α) para regredir o CL através de uma via dependente de PTGS2. Nas ovelhas prenhes, o IFNT é sintetizado e secretado pelo concepto elongado a partir do dia 10 da gestação. O IFNT se liga aos receptores de IFN do tipo I (IFNAR) no LE endometrial e inibe a transcrição do gene ESR1 através de uma via de sinalização envolvendo o fator regulador de interferon 2 (IRF2). Essas ações antiluteolíticas do IFNT no gene ESR1 impedem a formação de OXTR, mantendo assim a produção de progesterona do CL, necessária para o estabelecimento e manutenção da gestação. E2, estradiol; ESR1, receptor alfa de estrogéno; IFNAR, receptor de IFN tipo I; IFNT, interferon-tau; IRF2, fator regulador de interferon 2; OXT, ocitocina; OXTR, receptor de ocitocina; P4, progesterona; PGF, prostaglandina F2α; PGR, receptor de progesterona; PTGS2, prostaglandina-endoperóxido-sintase 2 (prostaglandina G / H sintase e ciclo-oxigenase). Fonte: (HANSEN, T. R.; SINEDINO; SPENCER, T. E., 2017)

O primeiro indício de ação endócrina do IFNT foi descoberto por SCHALUE-FRANCIS *et al.* (1991), que detectaram atividade antiviral de IFN na veia uterina no dia 15 de gestação de ovelhas prenhes.. Em um outro estudo, observou-se atividade antiviral significativa no sangue da veia uterina de ovelhas prenhes no dia 15 de gestação (OLIVEIRA *et al.*, 2008). A atividade antiviral foi observada na veia uterina, mas não na artéria uterina, no dia 15 de gestação, quando comparada com ovelhas cíclicas (BOTT, R. C. *et al.*, 2010). Apesar do embrião de 14–15 dias produzir 20 milhões de unidades antivirais ou cerca de 200 µg de IFNT por dia, foi determinado que a quantidade de IFNT no sangue da veia uterina era de apenas 5–10 ng/mL (ROMERO, J. J *et al.*, 2015).

A expressão de *ISG15* pode ser induzida em culturas de células luteais pequenas, grandes e mistas com apenas 100 pg/mL de IFNT em ovinos (ANTONIAZZI, A. Q. *et al.*, 2013) e bovinos (HANSEN, T. R.; SINEDINO; SPENCER, T. E., 2017). Uma vez que a maioria dos ensaios têm sensibilidade de 1 ng/mL, os ensaios falhariam em detectar o IFNT, pois circula abaixo dessa concentração. Assim, a dificuldade em detectar o IFNT no soro periférico pode ser causada por níveis circulantes muito baixos de IFNT, que estão abaixo dos limites de detecção das tecnologias disponíveis atualmente (HANSEN, T. R; SINEDINO; SPENCER, T. E, 2017).

Evidências indiretas e correlativas em ovinos e bovinos apoiam o conceito de que o IFNT é liberado pelo embrião, percorre o endométrio, entra no sangue das veias uterinas e é entregue em quantidades suficientes para induzir *ISGs* nos tecidos periféricos (HANSEN, T. R; SINEDINO; SPENCER, T. E, 2017). A ação direta do IFNT em tecidos extrauterinos eleva a expressão de *ISGs* que, no corpo lúteo, estão envolvidos com a resistência luteal à ação luteolítica da PGF (ANTONIAZZI, A. Q. *et al.*, 2013).

2.1.2 Gene estimulado por interferon 15 (*ISG15*)

O *ISG15*, também conhecido por proteína de reação cruzada de ubiquitina, foi identificado logo após a descoberta da ubiquitina, e imediatamente reconhecido como um homólogo de ubiquitina (LOEB; HAAS, 1992). O *ISG15* é uma das principais proteínas induzidas durante a infecção viral via resposta por interferon tipo I (SADLER; WILLIAMS, 2008) e tem sido pesquisado também como marcador de gestação em bovinos e ovinos (HAN, H. *et al.*, 2006; OLIVEIRA, J. F. *et al.*, 2008; YOSHINO *et al.*, 2018). Ubiquitinas são pequenos polipeptídios e a ligação de uma ou várias resulta no processo denominado de ubiquitinação. A ubiquitinação frequentemente serve para marcar proteínas para degradação

proteolítica pelos lisossomas ou proteossomas, este último sendo o passo crítico na via de complexo principal de histocompatibilidade (MHC) de classe I do processamento e apresentação de antígeno (HERSHKO; CIECHANOVER; VARSHAVSKY, 2000). A ubiquitinação de proteínas regula muitos aspectos da resposta imune inata, incluindo a transdução de sinal intracelular, como a ativação do fator nuclear κB e funções do sistema imune adaptativo (LIU, Y. C.; PENNINGER; KARIN, 2005).

Além da sua função intracelular, o *ISG15* é secretado em grandes quantidades no ambiente extracelular e foi demonstrado como uma citocina moduladora das respostas imunes (D'CUNHA *et al.*, 1996). O mecanismo pelo qual as funções extracelulares de *ISG15* ocorrem não está claro. As ubiquitinas também são secretadas de células e têm efeitos imunomoduladores que não estão esclarecidos (MAJETSCHAK *et al.*, 2003). Em estudos realizados com ratos, a deleção de *ISG15* promoveu uma alta taxa de mortalidade embrionária (70%), principalmente quando os animais eram submetidos a situações de estresse (HENKES, L. E *et al.*, 2015).

Durante o início da gestação, a primeira resposta à liberação de IFNT pelo concepto é a indução de *ISGs*, principalmente o (*ISG15*) no endométrio (ROMERO, J. J *et al.*, 2015). No dia 11 de gestação em ovelhas, as níveis de mRNA do *ISG15* são baixas, iniciando a expressão no dia 13 com aumento significativo até o dia 15 (JOYCE *et al.*, 2005). Nos tecidos extrauterinos, como fígado e CL, o *ISG15* é expresso nos dias 14 e 15 de gestação em ovelhas (ANTONIAZZI, A. Q. *et al.*, 2013; BOTT, R. C *et al.*, 2010; OLIVEIRA, J. F. *et al.*, 2008).

O *ISG15* foi identificado no endométrio bovino em resposta à gestação, sendo observado coincidentemente com a liberação de IFNT pelo embrião (AUSTIN, K. J *et al.*, 1996; JOHNSON, G. A *et al.*, 1998). *ISG15* é detectada em quantidades significativas em lavados uterinos no dia 18 de gestação (AUSTIN, K. J *et al.*, 1996; JOHNSON, G. A *et al.*, 1998), e aos 20 dias de gestação em células mononucleares do sangue periférico (HAQ *et al.*, 2016). Portanto, o *ISG15* pode estar envolvido na regulação de proteínas essenciais para o estabelecimento da gestação em ruminantes (HAQ *et al.*, 2016).

Os *ISGs*, especialmente o *ISG15*, vem sendo investigados como possíveis métodos de diagnóstico de gestação em ruminantes (GREEN *et al.*, 2010; HAN, H. *et al.*, 2006; MAUFFRÉ *et al.*, 2016; PUGLIESI *et al.*, 2014; YOSHINO *et al.*, 2018) e, também, como preditores de mortalidade embrionária em bovinos (SHEIKH *et al.*, 2018) e ovinos (KOSE *et al.*, 2016), em que o decréscimo na expressão de *ISGs* está relacionado com a mortalidade embrionária.

2.2. BALANÇO ENERGÉTICO NEGATIVO EM RUMINANTES

O BEN durante o período de transição próximo ao parto é considerado a principal causa do desenvolvimento da hipercetonemia em ovinos (LACETERA *et al.*, 2001; SAUN, 2000) e em vacas leiteiras (DRACKLEY, 1999; HERDT, 2000). Durante o BEN, devido à redução da glicemia, ocorrem mudanças na regulação endócrina que causam extensiva mobilização de tecido corporal, principalmente tecido adiposo, a fim de atender as necessidades nutricionais para mantença e produção de leite (DRACKLEY, 1999). Resultado da lipólise do tecido adiposo, os NEFA circulantes entram no fígado e tem três destinos: 1) podem ser completamente oxidados para energia através do ciclo de Krebs; 2) convertidos em BHBA ou 3) podem ser re-sintetizados como triglicerídeos (TG), podendo ser liberados através de lipoproteínas de densidade muito baixa (VLDL) ou armazenados no fígado (INGVARTSEN; MOYES, 2013). Durante o BEN, ocorre um aumento as concentrações circulantes de NEFA e um aumento as concentrações de corpos cetônicos, reflexo da incompleta oxidação de ácidos graxos no fígado (DRACKLEY, 1999). A hipercetonemia não ocorre por causa do BEN e sim por falhas dos mecanismos adaptativos ao BEN, como falhas do fígado em atender as necessidades glicogênicas, controle deficiente da liberação de ácidos graxos não esterificados do tecido adiposo(HERDT, 2000). Assim, a severidade do BEN reduz os níveis de glicose e eleva os níveis de NEFA e corpos cetônicos (acetoacetato, acetona e BHBA) (DAVID BAIRD, 1982; DRACKLEY, 1999). O aumento nos níveis de NEFA e BHBA na circulação levam à ocorrência de cetose clínica ou subclínica (DRACKLEY, 1999). O teste “padrão ouro” para diagnóstico de cetose é o BHBA sanguíneo, pois esse corpo cetônico é mais estável no sangue que a acetona ou o acetoacetato (OETZEL, 2007).

Os corpos cetônicos servem como fonte alternativa de energia para muitos tecidos, mas não contribuem ou contribuem apenas em pequena escala para o suprimento de energia ao feto. A glicose continua sendo o metabólito mais importante para o crescimento fetal e placentário. A capacidade da ovelha para fornecer uma quantidade suficiente de glicose ao feto a partir de fontes dietéticas é limitada, pois cerca de 70 a 75% do carboidrato da dieta é convertido no rúmen em produtos não-glicogênicos. A fração restante fornece de 40 a 60% da glicose circulante através do propionato. Durante o BEN e demanda aumentada de glicose, até 23% da glicose pode ser sintetizada a partir do glicerol liberado do tecido adiposo (SCHLUMBOHM, C; HARMEYER, 2004).

Em bovinos, a hipercetonemia ocorre com maior frequência nos primeiros meses de lactação e estima-se que a prevalência de cetose clínica seja de 3,4% e que a prevalência de

cetose subclínica seja de em média 24,1% nas primeiras três semanas de lactação (BRUNNER *et al.*, 2019). Em ovinos, a toxemia da gestação (cetose clínica) ocorre com maior frequência nas últimas três a seis semanas de gestação (SAUN, 2000; SCHLUMBOHM, C.; HARMEYER, 2008), enquanto que a cetose subclínica ocorre com maior frequência das primeiras duas semanas após o parto até seis semanas após o parto (FEIJÓ *et al.*, 2015), com prevalência de 32,2% e 18%, respectivamente (PANOUSIS *et al.*, 2012).

2.2.1 Impacto do balanço energético negativo no desempenho reprodutivo

O sucesso do desempenho reprodutivo é consequência de uma cadeia de eventos, que consiste na retomada à ciclicidade após o parto, desenvolvimento e ovulação de um oócito viável, concepção, desenvolvimento embrionário, implantação, manutenção da prenhez e parto (GARNSWORTHY *et al.*, 2008). As falhas na comunicação entre o concepto e a mãe frequentemente resultam no término da gestação (SPENCER, T. E *et al.*, 2004).

Apesar da alta taxa de sucesso da fertilização em ruminantes, baixas taxas de nascimento indicam claramente a ocorrência de morte embrionária e perdas fetais durante a gestação (DISKIN; MURPHY, J. J.; SREENAN, 2006). A maioria das mortes embrionárias ocorre durante o estágio de peri-implantação e afeta diretamente a fertilidade, estendendo o intervalo entre partos e reduzindo o número de produtos em muitas espécies, incluindo ruminantes (DISKIN; MORRIS, 2008). A maioria das perdas na gestação (20% a 30%) ocorre durante o estágio embrionário da gestação em ovelhas (DIXON *et al.*, 2007; KAULFUSS *et al.*, 1997).

A severidade e duração do BEN e a perda de condição corporal durante os primeiros meses de lactação estão associados a ausência de ciclicidade ao fim do período de espera voluntária (RIVERA *et al.*, 2010). O efeito da cetose subclínica foi associado a menores taxas de prenhez em vacas leiteiras (CAIXETA *et al.*, 2017; OSPINA *et al.*, 2010; SANTOS, J E P *et al.*, 2010; WALSH, R. B. *et al.*, 2007) e menores taxas de concepção na primeira inseminação artificial (RIBEIRO *et al.*, 2013; WALSH, R. B. *et al.*, 2007).

A nutrição tem um importante papel na manutenção e estabelecimento da prenhez em ruminantes. A subnutrição reduz o número de folículos que emergem e, portanto, afeta o número de folículos capazes de ovular e também afeta o número de folículos com maior diâmetro (ABECIA, J. *et al.*, 2006). A qualidade oocitária é drasticamente reduzida após a exposição de NEFA durante a maturação final do oócito, afetando significativamente a qualidade do embrião e o metabolismo energético (HOECK, V. V. *et al.*, 2013; HOECK, V. V. *et al.*, 2011). Os embriões que foram expostos a NEFA tem um número reduzido de células

blastocitárias, aumento na taxa de células apoptóticas e padrões alternados de expressão gênica (DESMET *et al.*, 2016; HOECK, V. V. *et al.*, 2011). Um estudo realizado com um pequeno número de animais sugeriu um efeito da nutrição materna na secreção de IFNT do embrião, onde embriões coletados no dia 15 de gestação de ovelhas em subnutrição tiveram atividade antiviral reduzida (ABECIA, J. A.; FORCADA, F.; LOZANO, 1999).

2.2.2 Indução do balanço energético negativo

A elevada produção de corpos cetônicos que ocorre no BEN é desencadeada primariamente por uma carência de oxaloacetato ou dos seus precursores que limita a oxidação das grandes quantidades de acetil-CoA, provenientes do metabolismo dos NEFA, via ciclo tricarboxílico, desviando este composto para a cetogênese (BERGMAN, 1971; CALDEIRA, 2005). Entre os denominados corpos cetônicos (BHBA, acetona e aceto-acetato) o BHBA é mais analisado devido à sua estabilidade no soro (CALDEIRA, 2005; DUFFIELD, T., 2000; OETZEL, 2007).

O BHBA nos ruminantes tem duas origens que condicionam a sua interpretação: 1) em uma dieta equilibrada, o butirato produzido na fermentação ruminal é metabolizado na sua maior parte em BHBA na passagem através dos epitélios retículo-ruminal e omasal. A fração restante passa para o sangue portal e é captada pelo fígado onde é metabolizado em BHBA e aceto-acetato (HEITMANN; DAWES; SENSENIG, 1987; LOMAX, M A; BAIRD, 1983); 2) já em restrição alimentar, parcial ou total, o butirato deixa de ser o principal precursor de corpos cetônicos, passando o metabolismo de NEFA proveniente da mobilização do tecido adiposo ser o precursor responsável pela formação desses compostos no fígado (BAIRD, 1982; DRACKLEY, 1999; ZAMMIT, 1990).

Durante o jejum prolongado, 80-90% do requerimento energético é atingido pela oxidação da gordura corporal. No entanto, a necessidade de energia dos tecidos corporais não pode ser atendida apenas pelo metabolismo lipídico, sendo a glicose essencial por pelo menos cinco tecidos: sistema nervoso, músculo, síntese e renovação de gordura, feto e glândula mamária. Durante o jejum, os aminoácidos desaminados contribuem com 70% da necessidade de glicose através da gliconeogênese (CHOWDHURY; ØRSKOV, 1994). Durante jejum prolongado, os animais ruminantes não podem mais absorver nutrientes exógenos e devem utilizar energia e proteínas endógenas para manter a atividade vital. Dependendo da duração da privação alimentar, o jejum induz a adaptação fisiológica caracterizada por hipoglicemia, hiperlipidemia, hipercetonemia e hipoinsulinemia (CHOWDHURY; ØRSKOV, 1994).

O estado metabólico e fisiológico do animal também é determinante para a magnitude da cetogênese (CALDEIRA, 2005). As concentrações de NEFA aumentaram igualmente em vacas lactantes e não-lactantes em resposta a vários dias de jejum; porém, a resposta cetogênica é superior nos animais lactantes (BAIRD *et al.*, 1979). Em vacas lactantes os corpos cetônicos foram sete vezes maiores do que em vacas não-lactantes após seis dias de jejum (BAIRD; HEITZMAN, R. J.; HIBBITT, 1972).

Em contrapartida, CALDEIRA *et al.*, (2007) observaram que ovelhas não-lactantes e não-prenhes submetidas à restrição alimentar por várias semanas tiveram um decréscimo nos níveis de BHBA nas primeiras semanas. Em ovelhas prenhes, o aumento nas concentrações de BHBA é 5 vezes maior do que em ovelhas não-prenhes, quando submetidas ao jejum alimentar por seis dias (HERRIMAN; HEITZMAN, R J, 1978).

Em ovelhas não gestantes, o jejum continuado de 3 e 5 dias fez os níveis sanguíneos de NEFA atingirem níveis de 1,5 mmol/L e 2,5 mmol/L, respectivamente, e foram mantidos em altos níveis quando as ovelhas estavam em jejum por até 10 dias. Já ovelhas prenhes em jejum aumentaram mais rapidamente os níveis de NEFA durante 5 dias de jejum (ANNISON, 1960). REID & HINKS (1962), utilizando ovelhas não prenhes em jejum por 7 dias, observaram que os níveis de glicose decresceram para o mínimo após um período de 2-4 dias e nesse mesmo período os níveis de NEFA alcançaram seu valor máximo e os corpos cetônicos aumentaram constantemente. BOUCHAT *et al.* (1981) observaram que as concentrações de corpos cetônicos reduziram no primeiro dia e depois tiveram um aumento até o sexto dia seguido de estabilização e novo aumento próximo aos dias 10 e 11 de jejum. De forma semelhante, STEWART *et al.* (2018) observaram que as concentrações sanguíneas de BHBA reduziram até passadas 12 horas de jejum e aumentaram constantemente a partir de então. Já as concentrações de NEFA mantiveram-se constantes até 6 horas de jejum aumentando a partir de então.

Assim, em ruminantes machos ou nas fêmeas não-prenhes e não-lactantes, o BEN resultante da restrição alimentar não provoca geralmente um aumento da cetonemia, sendo apenas o jejum capaz de induzir um aumento significativo das concentrações sanguíneas de corpos cetônicos, tanto em bovinos (BAIRD *et al.*, 1979), como em ovinos (CAMERON; CIENFUEGOS-RIVA, 1994; FILSELL *et al.*, 1969; KATZ; BERGMAN, 1969).

3. ARTIGO 1 - NEGATIVE ENERGY BALANCE MODULATES *ISG15* EXPRESSION IN THE ENDOMETRIUM AND THE CORPUS LUTEUM DURING MATERNAL RECOGNITION OF PREGNANCY IN EWES

Negative energy balance modulates *ISG15* expression in the endometrium and the corpus luteum during maternal recognition of pregnancy in ewes

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Negative energy balance modulates *ISG15* expression in the endometrium and the corpus luteum during maternal recognition of pregnancy in ewes

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ABSTRACT

Embryonic mortality during maternal recognition of pregnancy is a major cause of reproductive failure in ruminants. One of the causes of reduced fertility in ruminants is the occurrence of a negative energy balance (NEB). The hypothesis of this study is that NEB influence the occurrence of poor maternal-embryonic communication, impairing maternal recognition of pregnancy. The aim of our study was to evaluate the effects of NEB on the expression of *ISG15* in the endometrium and corpus luteum on day 17 of early gestation. For this, 21 crossbred Texel-Corriedale ewes had estrus and ovulation synchronized and 18 were mated. The ewes were allocated into four experimental groups: non-pregnant, non-bred control group (CNP, n = 3); pregnant control group (CP, n = 6); NEB group during the first week of embryonic development (FW; n = 6); and NEB group during the second week of embryonic development (SW; n = 6). The ewes in the FW group fasted from days 0 to 7 and the ewes in the SW group fasted from days 9 to 16 for the induction of NEB with hyperketonemia. BEN was induced in two distinct stages of embryonic development: first week of embryonic development and second week of embryonic development. During the experimental period, the blood levels of β-hydroxybutyrate (BHBA) and glycemic levels were measured. On day 17 of the estrous cycle or gestation, the sheep were euthanized and samples of endometrium and corpus luteum (CL) were collected. The relative expression of *ISG15* was assessed by q-PCR. Animals that had NEB induced had a reduction in glycemic levels and an increase in BHBA blood levels. Pregnant ewes had higher *ISG15* relative expression of than non-pregnant ewes in endometrium from ipsilateral horn and in the CL. The CP group had greater expression of *ISG15* than the CNP group in the endometrium from ipsilateral horn and in the CL. The FW and SW groups showed no difference in the *ISG15* relative expression of the CNP and CP groups in the endometrium from contralateral and ipsilateral horn. The FW group had a higher *ISG15* relative expression than the CNP group in the CL. In conclusion, ewes that had periods of NEB during embryonic development, especially during the second week of embryonic development, had a lower *ISG15* relative expression, with no difference in the *ISG15* relative expression in the endometrium from contralateral and ipsilateral horn at ovulation when compared with the CNP.

Keywords: *ISG15*, sheep, β-hydroxybutyrate, pregnancy, endometrium, corpus luteum

1. INTRODUCTION

Reproduction in ruminants is a highly complex biological process that requires communication between the developing conceptus and the uterus. This communication must be established during the peri-implantation period to signal and stablish the pregnancy, and also for the regulation of uterine cell gene expression [1]. The establishment of a successful gestation in ruminants needs to go through three distinct stages: signaling and maternal recognition of pregnancy, implantation and placentation [2–4]. Embryonic mortality is recognized as one of the major causes of reproductive failure in ruminants during the early stage of embryonic development, mainly due to signaling failures during maternal recognition of pregnancy [5] and may involve 20-30% total losses [6–8].

The maternal recognition of pregnancy is the period in which the concept signals its presence to the mother [9]. In ruminants, the signaling period coincides with embryo elongation and maximum interferon tau (IFNT) production [10] and occurs with greater intensity between Days 12 and 26 of pregnancy [11,12]. IFNT is a protein secreted in large quantities by ruminant embryo trophoblast cells prior to implantation [13]; its mRNA begins to be expressed from the

Day 4 of embryonic development *in vitro* [14] and the protein is detected from the Day 7 of embryonic development, initiating its signaling in the cells of the endometrium located in the ipsilateral uterotubal region to corpus luteum (CL) [15]. IFNT expression ends with full implantation around Day 22 [16,17].

IFNT signals via paracrine, endocrine and autocrine manner. It acts paracrine in the uterus, inhibiting the expression of estrogen receptors (ESR1) and oxytocin (OXTR) in the luminal epithelium [18]. The suppression of these receptors prevents the release of prostaglandin F2α (PGF) luteolytic pulses [18], which is responsible for initiating luteolysis [19]. In the paracrine manner in sheep, IFNT has been found to stimulate the expression of several genes (*CST3, CST6, CTSL, GRP, HSD11B1, IGFBP1, LGALS15, SLC2A1, SLC2A5, SLC5A11, SLC7A2*) in the luminal and epithelial gland endometrium that have potentially important biological activities for embryo elongation and implantation [20]. The endocrine manner, IFNT acts in extrauterine tissues increasing the expression of interferon stimulated genes (ISGs), as in the CL, is involved to prevent apoptosis [21].

During early pregnancy, the first response to IFNT release by the concept is the induction of ISGs, especially Interferon Stimulated Gene 15 (*ISG15*) in the endometrium [22]. On Day 11 of pregnancy in ewes, *ISG15* concentrations are low, increasing the expression on Day 13 with significant raise up to Day 15 [23]. In the extrauterine tissues, such as liver and CL, *ISG15* is expressed on Days 14 and 15 of pregnancy in ewes [21,24,25]. The *ISG15* was identified in the bovine endometrium in response to pregnancy, being observed coincidentally with the release of IFNT by the embryo [26,27]. *ISG15* is detected in significant amounts in uterine flushing on Days 18 [26,27] and 20 of pregnancy in peripheral blood mononuclear cells [28]. Therefore, *ISG15* may be involved in the regulation of proteins essential for establishing pregnancy in ruminants [28]. The *ISG15* is one of the ISGs that has been studied as a possible method of pregnancy diagnosis in ruminants [29–33] and, have also been studied as indicators of embryonic mortality in cattle [34] and sheep [35].

In the last weeks of gestation and early lactation, during the transition period, a negative energy balance (NEB) period occurs, and this is the main cause of hyperketonemia in dairy cows [36,37] and sheep [38,39]. During the NEB there are changes in endocrine regulation that lead to extensive mobilization of body tissue, especially adipose tissue, in order to achieve the nutritional requirements for maintenance and for pregnancy or milk production. This endocrine regulation leads to: 1) decreased blood glucose concentrations; 2) increased circulating concentration of non-esterified fatty acids (NEFA), which are released mainly from adipose tissue; 3) and increased concentrations of β-hydroxybutyrate (BHBA) as a reflection of incomplete oxidation of NEFA in the liver [36]. Hyperketonemia does not occur because of NEB, but because of failures of the adaptive mechanisms to NEB [37]. The “gold standard” test for NEB and ketosis is BHBA because it is more stable in blood than acetone or acetoacetate [40].

NEB and the occurrence of hyperketonemia have been pointed as one of the causes of reduced fertility in ruminants. The severity and duration of NEB and that the loss of body condition during the first months of lactation are associated with the lack of cyclicity at the end of the voluntary waiting period in dairy cows [41]. The effect of subclinical ketosis was associated with lower gestation rates in dairy cows [42–45] and lower rates at the first artificial insemination [45,46]. Undernutrition decreases the number of follicles emerging and the number of follicles capable of ovulating in sheep [47]. Oocyte quality is dramatically reduced after exposure to NEFA during final maturation, affecting embryo quality [48–50]. Embryos exposed to NEFA have a reduced number of blastocyte cells, a larger number of apoptotic cells, and alternate patterns in gene expression [48]. Results from a small number of animals provide the first suggestion of an effect of maternal nutrition on IFNT secretion from the conceptus, the

embryos were collected on Day 15 of pregnancy from ewes underfed and presented lower antiviral activity [51].

Consequently, the hypothesis of this study is that NEB may reduce embryo signaling, weakening maternal recognition of pregnancy. The objective of our study was to evaluate the effects of NEB, induced in the first or the second week of embryonic development, on the expression of *ISG15* mRNA in the endometrium and CL of ewes on Day 17 of gestation. The two embryonic developmental stages studied in this paper were: 1) NEB during embryonic developmental stage from zygote to blastocyst; and 2) NEB during embryonic developmental stage from hatching of the blastocyst from the pellucid zone to mature filamentous conceptus.

2. MATERIALS AND METHODS

2.1. ANIMALS AND TREATMENTS

All procedures using animals were approved by the Institutional Committee for Ethics in Animal Experiments (protocol #5133030519). In early breeding season, 21 Texel-Corriedale crossbreed ewes were kept in open yards receiving a diet formulated to their maintenance requirements (Agricultural and Food Research Council, 1993), for the period of 30 Days before the onset of the experimental procedure, to allow adaptation to experimental conditions. Total diet comprised 1.5 kg of tifton85 hay (*Cynodon* spp. cv. Tifton 85) and 0.25 kg of cracked corn (*Zea mays)* per day, providing 2.2Mcal of metabolizable energy per ewe. The animals had unrestricted access to water and mineral supplement.

All ewes were synchronized using intravaginal sponges containing 62 mg medroxyprogesterone acetate, inserted for 14 Days. To induce ovulation, ewes received 400 IU eCG (Folligon®) in a single i.m. and administration and 0,132mg cloprostenol sodic (Cioprostinn®) in a single i.m administration at intravaginal sponge removal to induce luteolysis. Ovulation time (Day 0) was estimated to be 56 hours after intravaginal sponge removal [52]. Fertile rams (one ram per six ewes) mated 18 out of 21 ewes 4 h after intravaginal sponge withdrawal and ewes were examined for evidence of estrus every 1 h. Ewes were allocated into one of 4 experimental groups after breeding the: Open control group, not bred and not pregnant (CNP; n = 3); pregnant control group (CP; n=6); NEB during first week of embryonic development group (FW; n=6) and NEB during second week of embryonic development group (SW; n=6). Three ewes were excluded from the experiment because the absence of an embryo on the day of tissue collection, thus, the number of sheep in the experimental groups was CNP = 3, CP = 6, FW = 5 and SW = 4.

The control groups received the same balanced diet from the adaptation period throughout the experimental period (16 Days). the sheep were kept in collective pens, one pen for each experimental group, with free access to water and mineral supplement. The FW fasted from Days 0 to 7 and SW fasted from Days 9 to 16. In the remaining days from groups FW (Days 8 to 16) and SW (Days 0 to 8) received the same balanced diet as the controls. Body condition score (BCS) (scale of 1–5, 1 = lean and 5 = obese) were determined on Days 0, 7 and 16.

2.2. INDUCTION OF BEN AND HYPERKETONEMIA

Hyperketonemia due to BEN is primarily triggered by a lack of oxaloacetate or its precursors which limits the oxidation of large amounts of acetyl-CoA from NEFA metabolism via the tricarboxylic cycle, diverting this compound to ketogenesis [53,54]. BHBA is the most analyzed ketone body because of its stability in serum [40,54,55]. In nonpregnant and non-lactating females, dietary restriction generally does not cause an increase in ketonemia, only

fasting can induce a significant increase in blood concentrations of ketone bodies in cattle [56] and in sheep [57–61].

The ewes had BEN induced by fasting for 7 days to be able to cause an increase in ketonemia, similarly to what happens with high production dairy cows in the first months of lactation. The ewes from FW and SW groups fasted for 7 days were monitored four times a day during the fasting period by the same veterinarian to evaluate their health status. In addition, blood BHBA levels was monitored daily and used to identify possible risks of clinical ketosis.

2.3. BLOOD SAMPLING

Blood was collected to determine blood BHBA level and glycemic level on Days 0, 1, 4, 7, 10, 13 and 16 of all groups; on Days 2, 3, 5 and 6 of FW; and on Days 9, 11, 12, 14 and 15 of SW. A 10-hour fast was performed in the groups fed before each blood collection, so that there was no interference from BHBA from rumen fermentation. Prior to the beginning of the sampling all the animals had the neck region trimmed/shaved. Animals were physical restrained, and 2 mL of blood was collected from the jugular vein through vacutainer tubes with and without EDTA. Blood samples were centrifuged, and plasma or serum stored at -20C for further analysis.

2.4. SAMPLE COLLECTION AND DETECTION OF PREGNANCY OR STAGE OF ESTROUS CYCLE

On Day 17 of the estrous cycle or pregnancy, all ewes were euthanized to allow collection of endometrium from the ipsilateral horn to ovulation and endometrium from the contralateral horn to ovulation; and CL. Samples were snap-frozen in liquid nitrogen. Ewes were considered pregnant after visualization of one or more embryos in the uterus.

2.5. BLOOD METABOLITES ASSAY

Evaluations of blood β-hydroxybutyrate (BHBA) level and glycemic level were performed using a FreeStyle Precision Ketone Glucose and Blood Glucose Monitor (Abbott Diabetes Care) from blood drop obtained during blood collection [62].

2.6. RNA ISOLATION AND REVERSE TRANSCRIPTION

Extraction of RNA from samples of endometrium and CL were performed using TRIzol® according to manufacturer protocol. Briefly, the extraction used 1000µl TRIzol® reagent (Thermo Fisher, Waltham, MA, USA) and 200µl chloroform, followed by purification of the aqueous phase with 20mg 1.33µl Glycoblue (Thermo Fisher, Waltham, MA, USA) and 700µl isopropyl alcohol. Quantification and estimation of RNA purity was performed using a Nano-Drop spectrophotometer (Thermo Scientific – Waltham USA; Absorbance 260/280 nm ratio). RNA was treated with 0.1 U DNase Amplification Grade (Invitrogen) for 15 min at 27ºC, to digest any contaminating DNA. After, DNase was inactivated by adding 1 ul of EDTA at 65ºC for 10 min. Single-stranded cDNA was synthesized from 1000 ng of total RNA with final volume of 20 ul using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to the manufacturer's instructions.

2.7. QUANTITATIVE REAL TIME PCR

Quantitative polymerase chain reactions (qPCR) were conducted in a CFX384 thermocycler (BioRad) using a final volume of 10 ul per well using BRYT Green® dye and Taq DNA polymerase from GoTaq® qPCR Master Mix (Promega Corporation), with cDNA (2 ul) and the primers for *ISG15* (forward, 5'-TACAGCCAACCAGTGTCTGC-3'; reverse, 5'-ACTGCTTCAGCTCGGATACC-3'), GAPDH (forward, 5'-GATTGTCAGCAATGCCTCCT-3'; reverse, 5'-GGTCATAAGTCCCTCCACGA-3') were based on Oliveira et al. (2008) [25]. RPL19 primer is (forward, 5'–AGGTGGCGGAATATGAAAGG-3'; reverse, 5'–GCTCCTCCCATTATCTGTGAAG–3') based on Santos *et al.* (2018) [63]. Amplification was performed with initial denaturation at 95ºC for 5 min followed by 40 cycles of denaturation at 95ºC for 15 sec and annealing/extension at 60ºC for 30 sec. To optimize the qPCR assay, serial dilutions of cDNA templates were used to generate a standard curve. The standard curve was constructed by plotting the log of the starting quantity of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R^2) higher than 0.98 and efficiency between 85 to 110% were considered optimized. The relative standard curve method was used to assess the amount of a particular transcript in each sample. Samples were run in duplicate and results are expressed relative to *GAPDH* and *RPL19*. The calculation of the relative expression was be performed as recommended by Pfaffl (2001) [64].

2.8. STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad, Inc., San Diego, CA, USA). The data was tested for normality by the Shapiro-Wilk test. Two groups were compared using Student's *t*-test (if a normal distribution was expected) or Mann–Whitney *U* test (if an asymmetrical distribution of data was expected). Three or more groups were compared using ordinary one-way ANOVA followed by Tukey test. The effect of fasting time on blood BHBA levels or glycemic levels in hyperketonemia groups or the effect of experimental time on blood BHBA levels or glycemic levels in control groups were compared using nonparametric repeated measures one-way ANOVA (Friedman's test) and parametric repeated measures one-way ANOVA, respectively.

Pearson's coefficient of correlation was calculated by CORR procedure. All data are expressed as means ± standard error of the mean (±SEM). Treatment differences with $P<0.05$ were considered significant and $P<0.10$ were considered as a trend.

3. RESULTS

3.1. DESCRIPTIVE AND STATISTICAL ANALYSES

There were no statistic differences between groups regarding age, body condition score on Days 0, 7 and 16, and number of CL on Day 17. Among the pregnant control group, FW group and SW group there was no difference regarding the presence of embryo at euthanasia. Data on age, body condition score on Days 0, 7 and 16, number of CL, presence of embryo, number of embryos and size of embryos are shown in Table 1.

	Experimental groups			
	CNP (n=3)	**CP (n=6)**	**FW (n=5)**	**SW (n=4)**
Age (months)	24 ± 10.39	24 ± 14.70	21.6 ± 9.10	25.5 ± 15.78
BCS Day 0	3.50 ± 0.25	3.46 ± 0.56	3.60 ± 0.52	3.12 ± 0.32
BCS Day 7	3.50 ± 0.25	3.46 ± 0.56	3.10 ± 0.42	3.25 ± 0.35
BCS Day 16	3.25 ±0.25	3.63 ± 0.54	3.15 ± 0.38	2.87 ± 0.32
Number of CL	1.00 ± 0	1.33 ± 0.52	1.80 ± 0.45	1.50 ± 0.58
Presence of embryo	0%	100%	100%	100%
Number of embryo	-	1.16 ± 0.41	1.00 ± 0.00	1.00 ± 0.00
Embryo length (cm)	-	33.67 ± 14.22	31.60 ± 7.70	21.75 ± 9.32

Table 1- Descriptive and statistical analyses of non-pregnant control group (CNP), pregnant control group (CP), NEB during early embryonic development group (FW) and NEB during late embryonic development group (SW), about on age, body condition score on Days 0, 7 and 16; number of corpus luteum on Day 17; presence of embryo, number of embryos and size of embryos on Day 17. Values are represented as mean ± SEM. NEB groups = Groups that had negative energy balance induced.

3.2. GLYCEMIC LEVELS AND BLOOD BHBA LEVELS

The ewes that were fasted (FW group and SW group) were grouped for analysis of the effect of fasting on glycemic and BHBA levels. Thus day 0 is the day of beginning of fasting (zero hour) and day 7 is the day of ending of fasting (168^{th} hour). Fasted ewes had strong negative correlation between glycemic levels and fasting time in the first two days of fasting (Day 0 to Day 2) (-0.72; $p < 0.001$) and also a moderate positive correlation between blood BHBA levels and fasting time on the seven days of fasting (Day 0 to Day 7) (0.60; $p < 0.001$). On Day 2 of fasting, ewes had a reduction in glycemic levels and an increase in blood BHBA levels. In the control groups, there was no correlation between blood BHBA levels or glycemic levels at any experimental Day. The variation in glycemic levels and blood BHBA levels in fasted animals is shown in Figure 1.

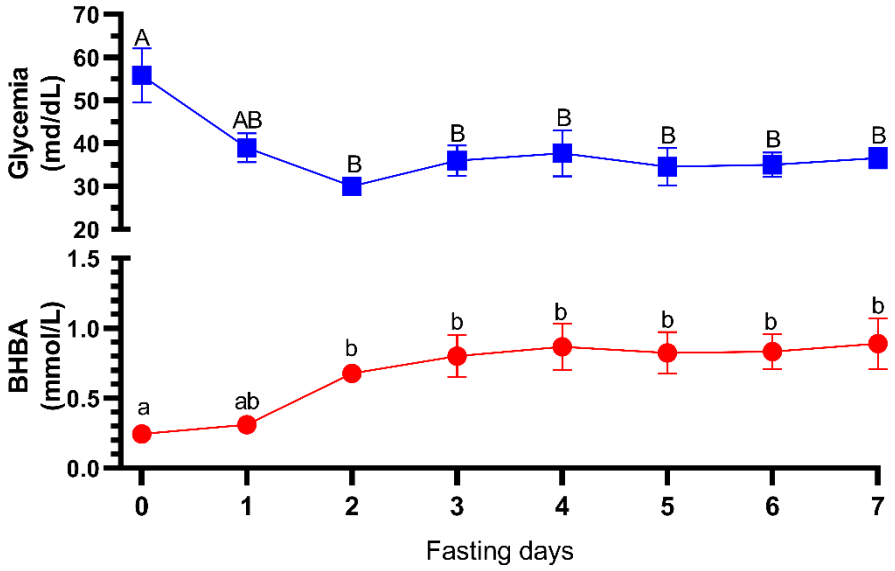

Figure 1 - Effect of fasting on glycemic and blood BHBA levels in ewes submitted to NEB during early embryonic development group (FW) and NEB during late embryonic development group (SW). Day 0 is the beginning day of fasting and Day 7 is the end day of fasting. Values are represented as mean ± SEM. Uppercase letters show differences among days within glycemic levels ($p < 0.05$). Lowercase letters show differences among Days within blood BHBA levels ($p < 0.05$).

The FW group between Days 0 and 7 showed a weak negative correlation (-0.32; $p<0.05$) between glycemic levels and experimental days and also showed a moderate positive correlation (0.51; $p<0.001$) between blood BHBA levels and experimental Days. The FW group between Days 9 and 16 did not show any correlation between glycemic levels or blood BHBA levels and experimental days. The SW group between Days 0 and 7 showed a moderate correlation (-0.52; $p<0.05$) between glycemic levels and experimental days and also showed a moderate negative correlation (-0.67; $p<0.01$) between blood BHBA levels and experimental days. The SW group between Days 9 and 16 showed no correlation between glycemic levels and experimental time and also showed a strong positive correlation (0.76; $p<0.001$) between blood BHBA levels and experimental days.

The glycemic levels and BHBA blood levels of experimental groups are show in Figure 2A. On Day 0, the control groups had lower glycemic level than the FW group ($p <0.001$) and the SW group ($p<0.05$). On Day 10, the SW group had a lower glycemic level than the control group ($p<0.05$) and the FW group ($p<0.01$).

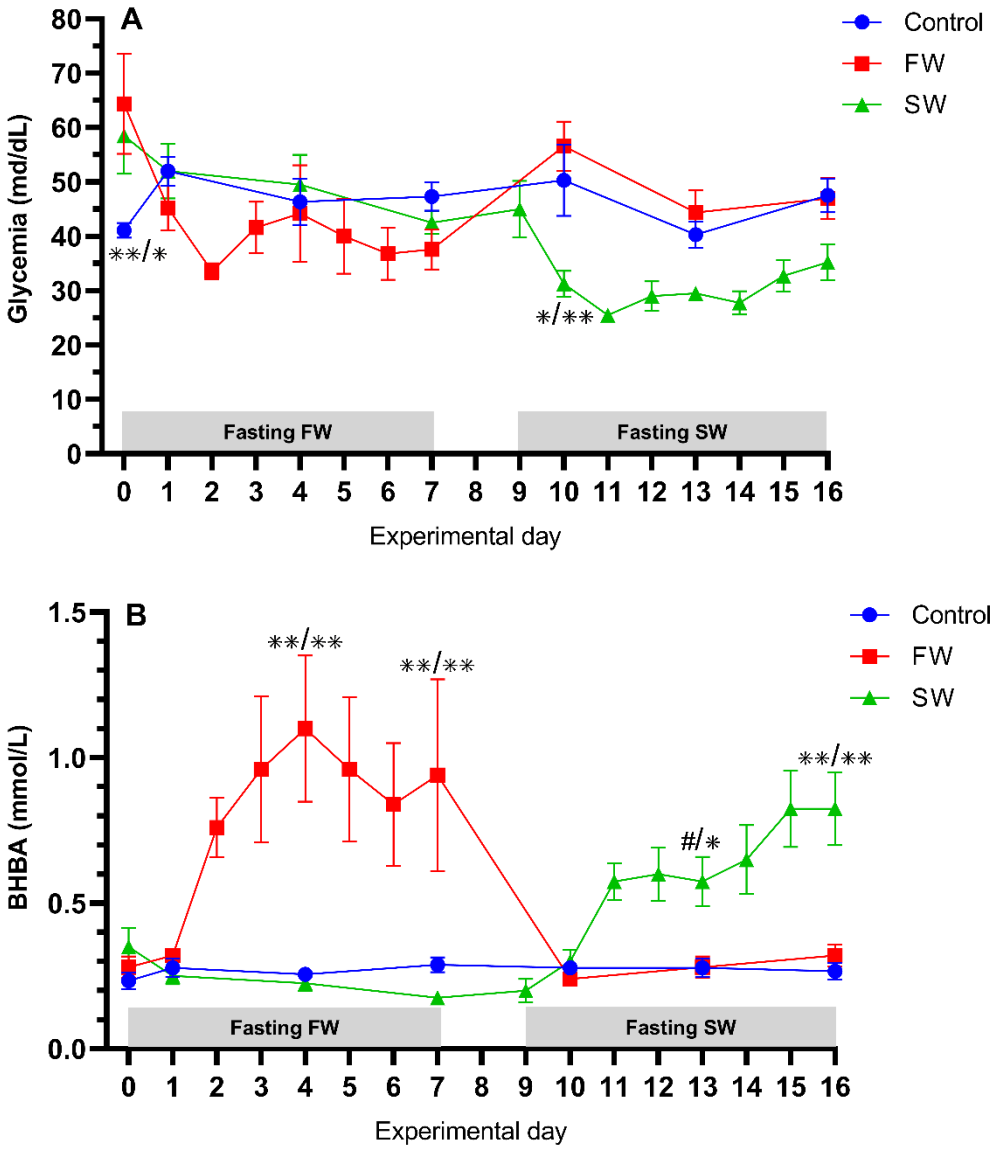

Figure 2 - Effect of experimental time on glycemic (A) and blood BHBA levels (B) in experimental groups. Control groups (non-pregnant control group and pregnant control group), NEB during early embryonic development group (FW) and NEB during late embryonic development group (SW). Values are represented as mean ± SEM. (*) indicates differences at $p < 0.05$, (**) indicates differences at $p < 0.01$ and (#) indicates probable differences at $p < 0.1$.

The blood BHBA levels of experimental groups are show in Figure 2B. FW group had a higher blood BHBA level than the control groups ($p<0.001$) and the SW group ($p<0.001$) on Days 4 and 7. On the other hand, the SW group on Day 13 had a higher blood BHBA level than the control groups ($p<0.05$) and tended to have a higher blood BHBA level than the FW group ($p<0.10$); on Day 16, the SW group had a higher blood BHBA level than the control groups ($p<0.001$) and the FW group ($p<0.001$).

3.3. *ISG15* RELATIVE EXPRESSION IN ENDOMETRIAL TISSUE

The pregnant ewes from the CP, FW and SW groups were grouped and compared with non-pregnant ewes from the CNP group to verify the effect of pregnancy on the relative

expression of *ISG15*. There was a difference in *ISG15* relative expression in the endometrium from the ipsilateral horn at ovulation ($p<0.05$) and there was a tendency in *ISG15* relative expression in the endometrium from the contralateral horn to ovulation ($p<0.10$). The *ISG15* relative expression on ipsilateral endometrium from the ipsilateral and contralateral horn to ovulation in pregnant and non-pregnant ewes are shown in Figure 3.

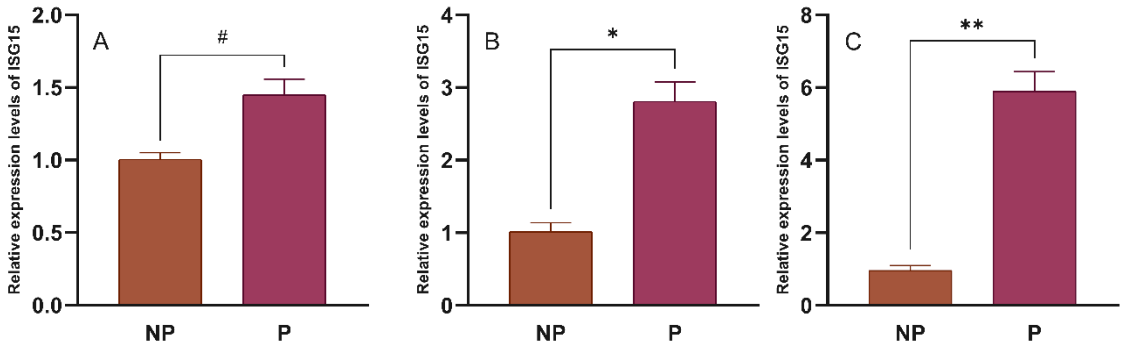

Figure 3 - *ISG15* relative expression in the non-pregnant (NP) and pregnant (P) ewes on endometrium from the contralateral horn at ovulation (A), endometrium from the ipsilateral horn at ovulation (B) and corpus luteum (C). Values are represented as mean ± SEM. (*) indicates differences at $p < 0.05$, (**) indicates differences at $p < 0.01$ and (#) indicates probable differences at $p < 0.1$.

The endometrium from contralateral horn at ovulation of CP tended to have a higher *ISG15* relative expression than the CNP ($p<0.10$). The groups with periods of NEB during embryonic development (FW and SW) showed no difference in the *ISG15* relative expression from the CNP and CP on endometrium from the contralateral horn to ovulation. The *ISG15* relative expression on endometrium from the contralateral horn to ovulation in experimental groups are show in Figure 4A.

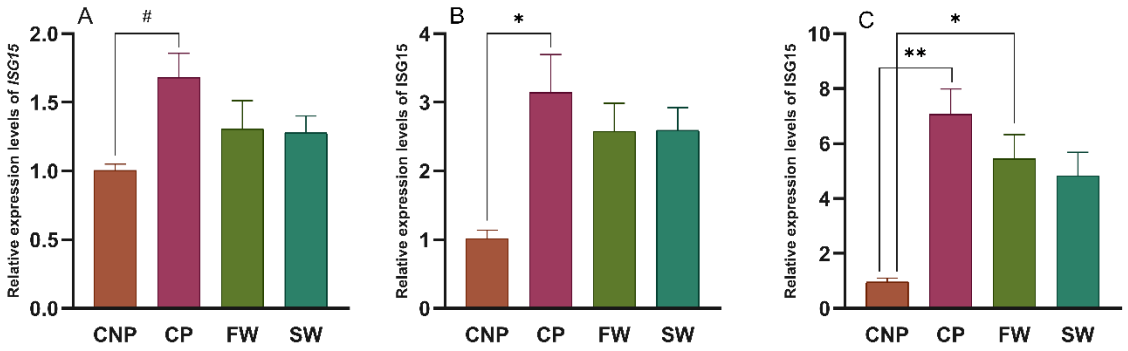

Figure 4 - *ISG15* relative expression in the non-pregnant control group (CNP), pregnant control group (CP), NEB during early embryonic development group (FW) and NEB during late embryonic development group (SW) on endometrium from the contralateral horn at ovulation (A), endometrium from the ipsilateral horn at ovulation (B) and corpus luteum (C). Values are represented as mean ± SEM. (*) indicates differences at $p < 0.05$, (**) indicates differences at $p < 0.01$ and (#) indicates probable differences at $p <0.1$.

The endometrium from ipsilateral horn at ovulation of pregnant control group had higher *ISG15* relative expression than the non-pregnant control group ($p<0.05$). The groups with periods of NEB during embryonic development (FW and SW) showed no difference in the *ISG15* relative expression from the CNP and CP on endometrium from the ipsilateral horn to ovulation different. The *ISG15* relative expression on endometrium from the ipsilateral horn to ovulation in experimental groups are show in Figure 4B.

3.4. *ISG15* RELATIVE EXPRESSION IN CORPUS LUTEUM

Comparing pregnant ewes from the CP, FW and SW groups were grouped and compared with non-pregnant ewes from the CNP group, there was a difference in *ISG15* relative expression in the CL ($p<0.01$). The *ISG15* relative expression on CL in pregnant and non-pregnant ewes are shown in Figure 3C.

The CP had higher *ISG15* relative expression than the CNP ($p<0.01$)). The FW had higher *ISG15* relative expression than the CNP ($p<0.05$). The SW showed no difference in the *ISG15* relative expression from the CNP and CP. The *ISG15* relative expression on corpus luteum in experimental groups are show in Figure 4C.

3.5. EMBRYO LENGTH VERSUS *ISG15* RELATIVE EXPRESSION

There was no difference between experimental groups regarding embryo size collected on Day 17. However, embryo size had a strong positive correlation with the *ISG15* relative expression in the endometrium from the ipsilateral horn at ovulation (0.78; $p<0.001$). There was also a moderate positive correlation between embryo size and *ISG15* relative expression in the corpus luteum (0.59; $p<0.05$).

4. DISCUSSION

This study investigated the effects of fasting-induced state of NEB on the *ISG15* relative expression in the endometrium and corpus luteum in ewes. In our experiment, we evaluated maternal recognition of pregnancy signaling through the *ISG15* expression. Our results indicate that ewes that had periods of NEB during embryonic development had a lower *ISG15* expression in the endometrium ipsilateral and corpus luteum. To our knowledge, this is the first study on NEB consequences on early on embryonic paracrine and endocrine signaling in ewes.

During early pregnancy, the first response to IFNT released by the concept is the induction of ISGs, especially *ISG15* in the endometrium [22]. On Day 11 of pregnancy in ewes, *ISG15* concentrations are low, initiating expression on Day 13 with significant increase up to Day 15 [23]. In our study, only the pregnant control group had a higher relative expression of *ISG15* than the non-pregnant control group on endometrium from ipsilateral horn at ovulation. In the extrauterine tissues, such CL, *ISG15* is expressed on Days 14 and 15 of pregnancy in ewes [21,24,25]. In our study, only the pregnant control group and FW group had a higher relative expression of *ISG15* than the non-pregnant control group on CL. In this way, the NEB may be interfering with the embryo-maternal signaling during the period of maternal recognition of pregnancy since *ISG15* may be involved in the regulation of proteins essential for establishing pregnancy in ruminants [28].

The *ISG15* has been investigated as possible methods of pregnancy diagnosis in ruminants [29–32,65]. More recently, *ISG15* has been investigated as a predictor of embryonic mortality in cattle [34] and ewes [35], as the decrease in *ISG15* relative expression is related to embryonic mortality. These changes in *ISG15* relative expression correspond to the plasma IFNT concentration in pregnant and late embryonic mortality cows [34]. Factors such postpartum metabolic issues, ill-time elongation and histotroph deficiencies may contribute to early embryonic mortality (WILTBANK *et al.*, 2016). Embryonic mortality during the early stage of embryonic development, mainly due to signaling failures during maternal recognition of pregnancy, is recognized as one of the major causes of reproductive failure in ruminants [5] and may involve 20-30% of total losses [6–8]. In this study, the decrease in the *ISG15* relative expression in the endometrium and CL observed in ewes with periods of NEB may be related to a possible embryonic death or reproductive failure in the near future, as NEB is related to

worse reproductive performance. NEB, NEFA exposure, hyperketonemia and poor nutrition have been linked to decreased reproductive performance, decreasing the number of follicles capable of ovulating [47], reducing oocyte [49,50] and embryonic quality [48,49], reducing IFNT production by the embryo [51] and reducing conception rates [45,46] and pregnancy rates [42–45].

In ovine, morula embryos enter the uterus around Days 4 and 5 post-conception, evolving into blastocysts by Day 6. After hatching of the zona pellucida on Day 8, blastocysts begin to elongate on Day 10 when they started to secrete IFNT, with a peak of production between Days 14 and 16, and the synthesis ceases by Day 25 of pregnancy [67]. IFNT is a protein secreted in large quantities by ruminant embryo trophoblast cells prior to implantation [13]. Its mRNA begins to be expressed from the Day 4 of embryonic development *in vitro* [68] and the protein is detected from the Day 7 of embryonic development, initiating its signaling in the cells of the endometrium located in the ipsilateral uterotubal region to the corpus luteum [15]. In the results presented here, although there was no difference in embryo size between the experimental groups, there was a positive correlation between embryonic size and *ISG15* relative expression in the endometrium from the ipsilateral horn at ovulation and CL. This suggests that the larger the size of the embryo, the greater the *ISG15* expression, even in ewes that had periods of NEB.

Results from 2 ewes provide the first suggestion of an effect of maternal nutrition on IFNT secretion from the conceptus. The embryos were collected on Day 15 of pregnancy from ewes underfed and presented lower antiviral activity [51]. It is important to note that before and during embryo elongation, the embryo is living free in the uterine lumen and is completely dependent on uterine secretions for all its metabolic needs [47]. Thus, the limiting factor of early embryonic mortality may be the mother's ability to promote embryo growth. If around the time of maternal recognition of pregnancy in ewes (Day 14), the embryo fails to deliver its signal (IFNT) in an appropriate pattern, luteolysis follows and pregnancy is not maintained [47]. Our results indicate that distinct periods of NEB during embryonic development negatively affect the *ISG15* relative expression. Our hypothesis for this reduction in *ISG15* relative expression is that NEB may affect directly or indirectly embryonic quality and, consequently, IFNT production.

Our results showed that fasting time influenced glycemic levels and blood BHBA levels. The longer the fasting time, the lower the glycemic levels and the higher the blood BHBA levels, leading the ewes to a condition of hyperketonemia without clinical signs. Hyperketonemia is characteristic of NEB in ruminants, especially dairy cows at the beginning of lactation (clinical ketosis and subclinical ketosis) [36,37] and in ewes at the end of pregnancy (pregnancy toxemia) or early lactation [38,39]. Our experimental design could be used to study NEB or hyperketonemia and its effects on reproductive performance, since it simulated the events of NEB and allowed the collection of material for analysis of indicators related to reproduction.

5. CONCLUSIONS

Ewes that had periods of NEB during embryonic development, especially during the second week of embryonic development, had a lower ISG15 relative expression, with no difference in the ISG15 relative expression in the endometrium from contralateral and ipsilateral horn at ovulation when compared with the CNP. This lower of *ISG15* relative expression caused by some effect of the negative energy balance may be related to some effect on embryonic quality, on the production of IFNT, on maternal-embryonic communication during the period of maternal recognition of pregnancy or on uterine quality and receptivity.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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4. CONCLUSÃO

As ovelhas que tiveram períodos de BEN durante o desenvolvimento embrionário, especialmente durante a segunda semana de desenvolvimento embrionário, tiveram uma menor expressão relativa de *ISG15*, não apresentando tiveram diferença na expressão relativa de *ISG15* no endométrio do corno contralateral e ipsilateral à ovulação quando comparadas com os grupos CNP e CP. Essa menor expressão relativa de *ISG15* causada pelo BEN pode estar relacionada a algum efeito na qualidade embrionária, na produção de interferon tau, na comunicação materno-embrionária durante o período de reconhecimento materno da gestação ou na qualidade e receptividade uterina. Mais estudos devem ser realizados para verificar o efeito do BEN na qualidade uterina, qualidade embrionária e outros marcadores da sinalização materno-embrionária durante o reconhecimento materno da gestação.

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