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Ana Paula da Silva

RESPOSTA ENDOMETRIAL E LUTEAL NO BALANÇO ENERGÉTICO NEGATIVO INDUZIDO 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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Santa Maria, RS
2021

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O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.

-José de Alencar-

RESUMO

RESPOSTA ENDOMETRIAL E LUTEAL NO BALANÇO ENERGÉTICO NEGATIVO INDUZIDO DURANTE O RECONHECIMENTO MATERNO DA GESTAÇÃO EM OVINOS

AUTORA: Ana Paula da Silva
ORIENTADOR: Alfredo Quites Antoniazzi

O balanço energético negativo (BEN) é uma das principais causas da redução da fertilidade em ruminantes. Nossa hipótese de estudo é que o BEN induzido influencia a comunicação materno-embrionária impactando negativamente no reconhecimento materno da gestação. Por esse motivo, o objetivo do nosso estudo foi avaliar os efeitos do BEN induzido, na primeira ou segunda semana de desenvolvimento embrionário, sobre o reconhecimento materno da gestação e do estresse oxidativo no endometrio e corpo lúteo em ovelhas prenhes no dia 17 de gestação. Para isso, 21 ovelhas foram alocadas em quatro grupos: grupo controle, não acasaladas e não gestantes (n = 3); grupo controle de gestantes (n = 6); BEN durante a primeira semana de desenvolvimento embrionário (FW; n = 6); e BEN na segunda semana (SW; n = 6). Nossos resultados demonstram diminuição moderada da expressão de *MX2* no CL no grupo em jejum na primeira semana e aumento na expressão de *MX1* em FW e SW no endométrio e aumento da concentração de progesterona em ovelhas submetidas ao jejum na segunda semana. Possivelmente, o jejum foi eficaz em causar hipercetonemia, mas não foi o suficiente para causar alterações significativas no endometrio e no corpo lúteo. Ovelhas prenhes submetidas a BEN induzido apresentaram o mesmo padrão de expressão de genes do estresse oxidativo e IFNs quando comparadas às ovelhas P controle. Em conclusão, nosso estudo expressa um efeito subclínico, normalmente animais que sofrem BEN apresentam níveis de BHB mais elevados do que os demonstrados aqui. Embora o BEN seja inevitável em certas fases do sistema de produção, minimizá-lo resultará em perdas mínimas.

Palavras-chave: Reconhecimento Materno da Gestação. Ovelhas. Balanço Energético Negativo.

ABSTRACT

ENDOMETRIAL AND LUTEAL RESPONSE IN THE NEGATIVE ENERGY BALANCE INDUCED DURING THE MATERNAL RECOGNITION OF PREGNANCY IN EWES

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Negative energy balance (NEB) is one of the main causes of reduced fertility in ruminants. Our study hypothesis is that induced NEB influences maternal-embryonic communication, negatively impacting maternal recognition of pregnancy. For this reason, the aim of our study was to evaluate the effects of induced-NEB, in the first or second week of embryonic development, on maternal recognition of pregnancy and oxidative stress in the endometria and corpora lutea on Day 17 pregnant ewes. For this, 21 sheep were placed into four groups: control group, non-bred and non-pregnant (n=3); control group of pregnant women (n=6); NEB during the first week of embryonic development (FW; n=6); and NEB in the second week (SW; n=6). Our results demonstrate a moderate decrease in the expression of *MX2* in the CL in the fasting group in the first week and an increase in the expression of *MX1* in FW and SW in the endometrium and increased progesterone concentration in ewes submitted to fasting in the second week. Possibly, fasting was effective in causing hyperketonemia, but it was not enough to cause significant changes in the endometria and corpora lutea. Pregnant ewes under induced NEB showed the same pattern of oxidative stress gene expression and IFNs when compared with P control sheep. In conclusion, our study expresses a subclinical effect, normally animals that suffer NEB have higher BHB levels than those demonstrated here. Although NEB is inevitable in certain phases of the production system, minimizing it will result in minimal loss.

Keywords: Maternal Recognition of Pregnancy. Ewes. Negative Energy Balance.

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1 INTRODUÇÃO

O reconhecimento materno da gestação é caracterizado pelo período em que o concepto sinaliza sua presença para a mãe (BAZER *et al.*, 1986). Em ruminantes, o período de sinalização coincide com o alongamento do embrião e a produção máxima de interferon tau (IFNT) (ANTONIAZZI, Alfredo Quites *et al.*, 2011). O IFNT é a principal citocina responsável pela interação materno-embrionária para evitar a luteólise, estabelecer e manter a gestação (MCCRACKEN; CUSTER; LAMSA, 1999; NISWENDER *et al.*, 2000). O IFNT é produzido pelo trofoblasto embrionário no período pré-implantação e atua no útero de forma parácrina, inibindo a expressão de receptores de estrogênio e ocitocina endometrial no epitélio luminal, evitando pulsos luteolíticos de prostaglandina F2 alfa (PGF) (SPENCER, T E; BAZER, 1996).

Além de seus efeitos antiluteolíticos, o IFNT aumenta a expressão de vários genes estimulados por IFN (ISG) no útero, como 2 ', 5'-oligoadenilato sintetase (*OAS1*), resistência ao mixovírus (vírus da gripe) 2 (*MX2*) e proteína 15-kDa do gene estimulado por IFN tipo ubiquitina (*ISG15*) (JOHNSON, Greg A. *et al.*, 1999; OTT, Troy L. *et al.*, 1998). A expressão de proteínas induzidas por IFNT durante o início da gestação, como *MX* e *ISG15*, foi recentemente demonstrado que ocorre em outros tecidos extra-uterinos como o corpo lúteo (CL). A expressão do gene *MX* aumenta de 4 a 5 vezes nas células mononucleares do sangue periférico (PBMC) dentro de 24-48 h da sinalização inicial de IFNT em ovelhas prenhes, bem como a expressão de *ISG15* no CL após infusão intrauterina ou injeção IM com roIFNT (CHEN *et al.*, 2006; YANKEY *et al.*, 2001). A bioatividade antiviral do IFN no sangue foi significativamente maior no sangue da veia uterina quando comparada ao sangue da artéria uterina e veia jugular no dia 15 da gestação, fornecendo a primeira evidência de que um IFN tipo I é realmente liberado do útero e tem ação endócrina. A detecção de proteínas mRNA e ISG no CL significa que o CL pode ser regulado durante a gestação não apenas por fatores endócrinos liberados pelo útero, como o IFNT, mas também pela expressão de ISG específico no ovário (OLIVEIRA *et al.*, 2008).

A mortalidade embrionária durante a fase inicial do desenvolvimento embrionário, principalmente devido a falhas de sinalização durante o reconhecimento materno da gestação, é reconhecida como uma das principais causas de falha reprodutiva em ruminantes (DISKIN; MORRIS, 2008), bem como balanço energético negativo (BEN) e a ocorrência de hipercetonemia tem sido apontadas como causas da redução da fertilidade em ruminantes. Durante o período de transição, ocorre um período de BEN, que é a principal causa de hipercetonemia em vacas leiteiras (DRACKLEY, 1999; HERDT, 2000) e ovelhas

(LACETERA *et al.*, 2001). A toxemia da gestação (cetose clínica) ocorre frequentemente nas últimas três a seis semanas de gestação (SCHLUMBOHM, Christina; HARMEYER, 2008), enquanto a cetose subclínica ocorre frequentemente nas primeiras duas semanas após o parto até seis semanas após o parto (FEIJÓ *et al.*, 2015).

A gravidade e a duração do BEN e a perda da condição corporal durante os primeiros meses de lactação estão associadas à falta de ciclicidade, assim como a cetose subclínica foi associada a taxas de prenhez mais baixas em vacas leiteiras (CAIXETA *et al.*, 2017; RIVERA *et al.*, 2010; WALSH *et al.*, 2007a). A desnutrição diminui o número de folículos emergentes e o número de folículos capazes de ovular em ovelhas (ABECIA, José Alfonso *et al.*, 2006). A qualidade do oócito é drasticamente reduzida após a exposição ao NEFA durante a maturação final, afetando a qualidade do embrião (DESMET *et al.*, 2016; VAN HOECK, V. *et al.*, 2013). Embriões expostos a NEFA têm um número reduzido de células de blastocisto, um número maior de células apoptóticas e padrões alternativos na expressão gênica (DESMET *et al.*, 2016). 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 desnutridas tiveram redução da atividade antiviral (ABECIA, J.A.; FORCADA; LOZANO, 1999). No entanto, este experimento não abordou ISGs de maneira parácrina e endócrina.

A excessiva mobilização lipídica que ocorre durante o período de BEN também desempenha um papel importante entre o metabolismo energético, estresse oxidativo e eficiência do sistema imunológico (SORDILLO, L. M., 2016; SORDILLO, Lorraine M.; RAPHAEL, 2013). Assim, durante o período de BEN, os processos intensificados de oxidação de NEFA resultam no aumento da produção de ROS (do inglês, *Reactive Oxygen Species*) e no desenvolvimento de estresse oxidativo (BIONAZ *et al.*, 2007). Conforme Van Hoeck et al. (2013), o estresse oxidativo é uma das principais vias pelas quais as quantidades elevadas de NEFA afetam oócitos e embriões bovinos. Além dos NEFA, muitos estudos têm demonstrado que BHB está associado com metabolismo energético e isso pode causar estresse oxidativo, resposta inflamatória e apoptose celular (BERNABUCCI *et al.*, 2005; LI *et al.*, 2016; SHI *et al.*, 2014). Processos patológicos podem ocorrer quando um desequilíbrio entre a produção de ROS e a capacidade antioxidante ocorre nas células (SLIMEN *et al.*, 2014; SOYSAL *et al.*, 2017), que é controlado por um sistema antioxidante complexo constituído de antioxidantes enzimáticos e não enzimáticos, incluindo três enzimas importantes: superóxido dismutase (*SOD*), catalase (*CAT*) e glutationa peroxidase (*GPX*) (SOYSAL *et al.*, 2017). ROS intracelulares aumentados podem causar danos ao DNA e mitocondrial (SLIMEN *et al.*, 2014),

levando à modificação não específica de lipídios e proteínas (WANG *et al.*, 2013), ativando os mecanismos de autofagia celular e apoptose. Além das ROS, as (HSPs) representam um grupo heterogêneo de chaperonas moleculares, protegendo as células de danos letais (SLIMEN *et al.*, 2014). Um estudo do nosso grupo apresenta a interação do estresse oxidativo e térmico, e a qualidade do embrião em relação à síntese de IFNT (AMARAL *et al.*, 2020).

Portanto, nossa hipótese de estudo é que o BEN induzido influencia a comunicação materno-embrionária, impactando negativamente no reconhecimento materno da gestação. O objetivo do nosso estudo foi avaliar os efeitos do BEN induzido, na primeira ou segunda semana de desenvolvimento embrionário, no reconhecimento materno da gestação e no estresse oxidativo no endométrio e CL de ovelhas no dia 17 de gestação. Os estágios de desenvolvimento embrionário avaliados foram: 1) estágio de desenvolvimento embrionário do zigoto ao blastocisto; 2) estágio de desenvolvimento embrionário do blastocisto eclodido ao concepto filamentoso.

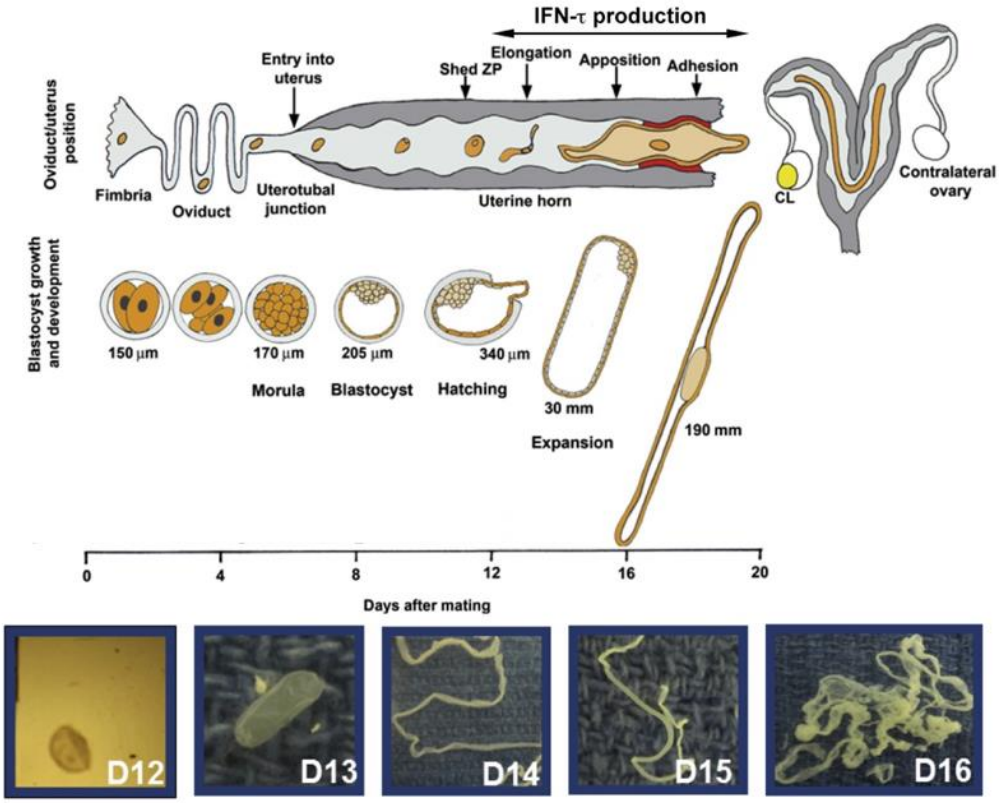
2 REVISÃO DE LITERATURA

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; IMAKAWA; ROBERTS, 1989; NISWENDER *et al.*, 2000). Em ruminantes, o período de sinalização coincide com a elongação do embrião e a máxima produção de interferon tau (IFNT) (ANTONIAZZI, Alfredo Quites *et al.*, 2011) acontecendo com maior intensidade entre os dias 12 e 26 de gestação (FARIN *et al.*, 1990; ROBERTS, R. Michael, 1993). Esse processo em ruminantes requer que o concepto elongue-se para produzir IFNT suficiente para sinalizar a gestação e suprimir o mecanismo luteolítico endometrial (ROBERTS, R. M. *et al.*, 1999; ROBERTS, R. Michael *et al.*, 2008; SPENCER, T E; BAZER, 1996; SPENCER, Thomas E; BAZER, 2002).

Após a fertilização, inicia-se o processo de desenvolvimento embrionário. 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 dos dias 3 ou 4 em ovelhas. Em seguida, o embrião em desenvolvimento evolui para blastocisto no dia 6. Nesse momento, os blastômeros pluripotentes começam a se diferenciar em massa celular interna (ICM) 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 inicial, o embrião permanece desprendido do endométrio uterino e dependente de nutrientes no lúmen uterino (JOHNSON, Greg 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, Thomas 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 *et al.*, 1990).

Figura 1- Desenvolvimento embrionário inicial em ruminantes.



Fonte: (BAZER, Fuller W. et al., 2015).

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, 1996).

2.1.1 O interferon tau

O IFNT é classificado como interferon do tipo I e sua principal função é evitar o retorno ao estro, preservando o funcionamento inicial do corpo lúteo durante a gestação (NISWENDER *et al.*, 2000). Seu RNAm começa a ser expresso a partir do quarto dia do desenvolvimento

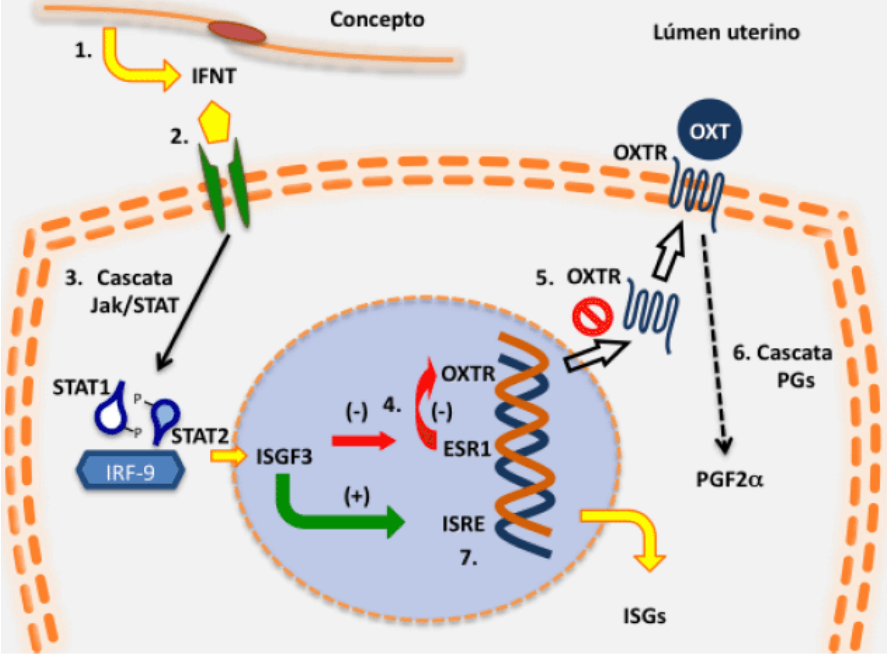
embrionário *in vitro* (YAO *et al.*, 2009) e sua proteína é detectada a partir do sétimo dia do desenvolvimento embrionário, iniciando sua sinalização nas células do endométrio localizadas na região uterotubárica ipsilateral ao CL (SPONCHIADO *et al.*, 2017). A expressão de IFNT termina com a implantação (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 janus quinase (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 ISGs (ANTONIAZZI, Alfredo Quites *et al.*, 2011; HANSEN, T R *et al.*, 1999).

2.1.2 Ações do interferon tau

Atualmente, sabe-se que o IFNT age de formas parácrina, endócrina e autócrina. Atua por via parácrina no útero, inibindo a expressão dos receptores de estrógenos (ESR1) e de ocitocina (OXTR) no epitélio luminal (SPENCER, T E; BAZER, 1996). A supressão desses receptores evita a liberação de pulsos luteolíticos de prostaglandina F2 alfa (PGF) (SPENCER, T E; BAZER, 1996), que é o hormônio responsável pelo início da luteólise (MCCRACKEN; CUSTER; LAMSA, 1999). Ainda pela via parácrina (Figura 2), em ovelhas, verificou-se que o IFNT estimula a expressão de vários genes no epitélio luminal endometrial e (ou) no epitélio glandular endometrial que possuem atividades biológicas potencialmente importantes para o elongamento e implantação do embrião (SPENCER, Thomas E.; FORDE; LONERGAN, 2016).

Figura 2- Via de sinalização parácrina do reconhecimento materno da gestação em ruminantes.

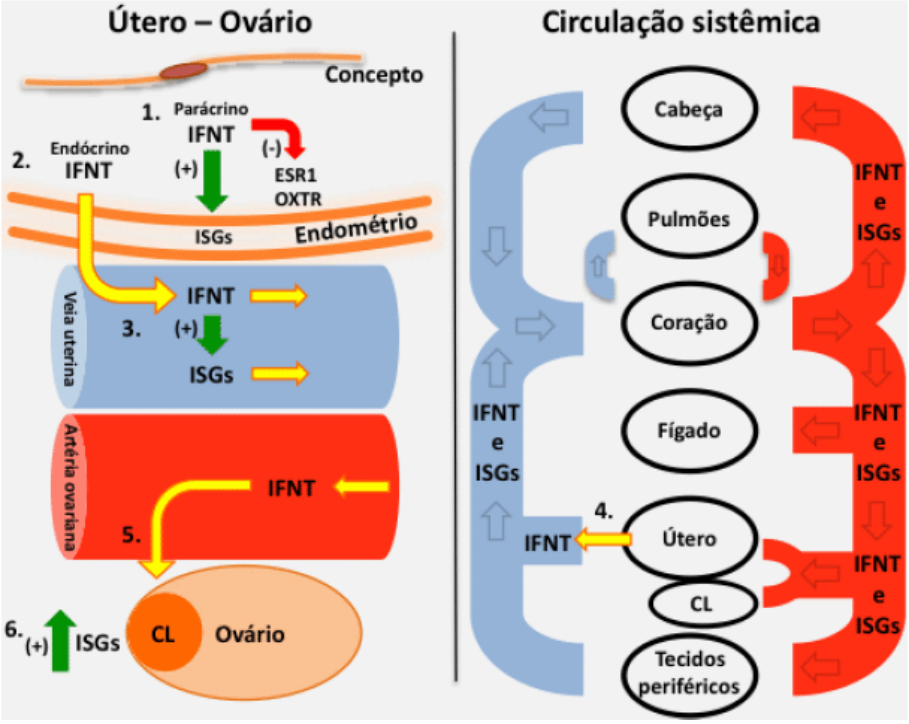


Fonte: (ANTONIAZZI, Alfredo Quites et al., 2011).

O IFNT também tem ação autócrina através de IFNAR1 (BROOKS; SPENCER, 2015; IMAKAWA *et al.*, 2002) aumentando a proliferação das células do trofoblasto e aumentando a expressão de ISGs (BROOKS; SPENCER, 2015; WANG *et al.*, 2013). O primeiro indício de ação endócrina do IFNT (figura 3), foi descoberto por SCHALUE-FRANCIS *et al.* (1991) que detectaram atividade antiviral de IFN na veia uterina no dia 15 de gestação de ovelhas prenhas mas não detectaram atividade antiviral na artéria ovariana ou veia jugular, indicando que o IFNT pode ser rapidamente removido da circulação sistêmica. Semelhantemente OLIVEIRA *et al.* (2008) encontrou atividade antiviral significativa no sangue da veia uterina de ovelhas prenhes no dia 15 de gestação. Atividade antiviral foi observada na veia uterina, mas não na artéria uterina, do dia 15 de gestação quando comparada com ovelhas cíclicas (BOTT *et al.*, 2010).

Evidências indiretas e correlativas em ovinos e bovinos apoiam o conceito de que o IFNT é secretado pelo embrião, sinaliza o endométrio e tecidos periféricos (HANSEN, Thomas R; SINEDINO; SPENCER, 2017). A ação direta do IFNT em tecidos extrauterinos eleva a expressão de genes estimulados por interferon (*ISGs*) que, no CL, estão envolvidos com a resistência luteal à ação luteolítica da PGF (ANTONIAZZI, A. Q. *et al.*, 2013).

Figura 3- Mecanismo de ação endócrino do reconhecimento materno da gestação em ruminantes.



Fonte: (ANTONIAZZI, Alfredo Quites et al., 2011).

2.2.3 Receptores de interferon tipo I e genes estimulados por interferon (*ISGs*)

O IFNT liga-se a receptores de interferon tipo I (IFNAR1 e IFNAR2) e induz sua resposta por meio da sinalização via JAK/STAT (BINELLI *et al.*, 2001). Os receptores IFNAR1 e IFNAR2 são expressos em todos os tecidos corporais e têm como principal função mediar respostas antivirais. Também estão localizados no endométrio para as respostas maternas ao IFNT produzido pelo embrião (JOHNSON, Gregory A *et al.*, 1998).

O IFNT se liga a esses receptores para exercer sua ação pela via de transdução de sinais JAK/STAT, onde as proteínas tirosino-quinases fosforilam proteínas STAT formando complexos multiméricos que agem como fatores de transcrição (BINELLI *et al.*, 2001). Esses complexos atuam basicamente em 2 vias: a primeira consiste no controle da transcrição de ESR1 e consequentemente OXTR no epitélio luminal endometrial (SPENCER, Thomas E.; BAZER, 1996); e a segunda ocorre quando os complexos se ligam a regiões definidas no DNA,

chamadas de elementos responsivos à estimulação por interferons, que regulam a expressão de ISGs (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 (*OAS*1) (MIRANDO *et al.*, 1991; SCHMITT *et al.*, 1993), o gene de resistência ao myxovirus 1 (*MX1*) (OTT, T L *et al.*, 1998) e o gene estimulado por interferon 15 (*ISG15*) (AUSTIN *et al.*, 1996). Estudos demonstraram a expressão de ISGs em células do sangue e células luteais (OLIVEIRA *et al.*, 2008) logo após a sinalização por IFNT no início da gestação em ruminantes.

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; R.J., 2000) e em vacas leiteiras (DRACKLEY, 1999; HERDT, 2000). Durante o BEN, devido a 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 BHB ou 3) podem ser re-sintetizados como triglicerídeos (TG), podendo serem 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 (HERDT, 2000). Assim, a severidade do BEN reduz os níveis de glicose e eleva os níveis de NEFA e corpos cetônicos (acetoacetato, acetona e BHB) (DAVID BAIRD, 1982; DRACKLEY, 1999). O aumento nos níveis de NEFA e BHB na circulação levam a ocorrência de cetose clínica ou subclínica (DRACKLEY, 1999). O teste "padrão ouro" para diagnóstico de cetose é o BHB sanguíneo, 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 (R.J., 2000; SCHLUMBOHM, Christina; 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 18,0%, respectivamente (PANOUSIS *et al.*, 2012).

2.2.1 Impacto do BEN no desempenho reprodutivo

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; 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 *et al.*, 2010; WALSH *et al.*, 2007b) e menores taxas de concepção na primeira inseminação artificial (RIBEIRO *et al.*, 2013; WALSH *et al.*, 2007b).

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, José Alfonso *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; VAN HOECK, Veerle *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; VAN HOECK, Veerle *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; LOZANO, 1999).

2.2.2 Indução do Balanço Energético Negativo (BEN)

A elevada produção de corpos cetônicos que ocorre no BEN é desencadeada primariamente por uma diminuição 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 (BHB, acetona e aceto-acetato) o BHB é mais analisado devido a sua estabilidade no soro (CALDEIRA, 2005; DUFFIELD, 2000; OETZEL, 2007). BHB 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 BHB 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 BHB e aceto-acetato (HEITMANN; DAWES; SENSENIG, 1987; LOMAX; BAIRD, 1983); 2) já em restrição alimentar, parcial ou total, o butirato deixa de ser o principal precursor de corpos cetonicos passando o metabolismo de NEFA proveniente da mobilização de lipídios ser o primeiro responsável pela formação desses compostos (DAVID 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). Após 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). Em vacas lactantes e não-lactantes em resposta à vários dias de jejum as concentrações de NEFA aumentaram igualmente 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; HIBBITT, 1972). Em contrapartida, CALDEIRA *et al.*, (2007) observaram que ovelhas não-lactantes e não-prenhes submetidas a restrição alimentar por várias semanas tiveram um decréscimo nos níveis de BHB nas primeiras semanas. Em ovelhas prenhes o aumento nas concentrações de BHB é 5 vezes maior do que em ovelhas não-prenhes, quando submetidas ao jejum alimentar por seis dias (HERRIMAN; HEITZMAN, 1978). Taghipour et al. (2010) definiram que valores entre 0,8 e 1,6 mmol/L de BHB são indicativos de cetose subclínica e presença de balanço energético negativo (BEN), enquanto que níveis superiores a 1,6 mmol/L podem ser considerados indicativos de cetose clínica (TAGIPOUR *et al.*, 2010).

Em ovelhas não gestantes, o jejum continuado fez os níveis sanguíneos de NEFA atingirem níveis de 1,5 mmol/L e 2,5 mmol/L em 3 e 5 dias de jejum 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). Em 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 REID & HINKS (1962). 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 BHB 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). Deste modo, para a indução do balanço energético negativo com hipercetonemia no nosso trabalho as ovelhas foram submetidas a jejum alimentar por um período de 7 dias, avaliando as concentrações de glicose e BHB.

2.3 ESPÉCIES REATIVAS DE OXIGÊNIO E ESTRESSE OXIDATIVO

Os radicais livres mais abundantes nos sistemas biológicos são os centrados no oxigênio e seus metabólitos, geralmente chamados de ROS (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993). As ROS são formadas continuamente como subprodutos normais do metabolismo celular e se caracterizam por moléculas que contém um ou mais elétrons desemparelhados na última camada, se tornando instáveis. Em baixas concentrações, ROS participam de processos fisiológicos no organismo (DRÖGE, 2002; SUGINO, 2006), incluindo fosforilação de proteínas, ativação de fatores de transcrição, diferenciação celular, apoptose, esteroidogênese, maturação oocitária, ovulação, formação do corpo lúteo, luteólise, manutenção da gestação, inicio do parto, defesa e imunidade celular (AGARWAL; GUPTA; SHARMA, 2005; DRÖGE, 2002; MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993; RIZZO, A. *et al.*, 2012) Portanto, apesar de certo nível de ROS ser desejável, não se sabe o nível ideal para cada processo fisiológico em ruminantes (CELI, 2010).

Fisiologicamente, a produção de ROS e antioxidantes permanecem em equilíbrio. No entanto, quando em concentrações elevadas, ROS possui efeitos deletérios sobre as células, uma vez que essas não são neutralizadas pelas defesas antioxidantes (LYKKESFELDT; SVENDSEN, 2007). Nesse caso, devido a superprodução de ROS ou depleção de antioxidantes ocorre um processo denominado de estresse oxidativo (AGARWAL; GUPTA; SHARMA, 2005; CELI, 2010). O estresse oxidativo pode danificar todas as moléculas biológicas, como DNA, RNA, colesterol, lipídios, carboidratos e proteínas. Por sua vez, a oxidação dessas macromoléculas produz vários produtos finais que podem ser medidos para avaliar o estresse oxidativo *in vivo*. Conforme Dalle-Donne et al. (2005), as proteínas são as moléculas mais suscetíveis ao dano oxidativo nas células porque muitas vezes são catalisadores. Ainda, a excessiva concentração de ROS induz uma cascata de reações em cadeia que pode desencadear a peroxidação lipídica nos fosfolipídios de membrana, afetando a função e a permeabilidade das membranas celulares, gerando danos ao DNA, atraso meiótico e disfunção mitocondrial, que ativam a cascata das caspases e culminam com a morte irreversível das células (COMBELLES; GUPTA; AGARWAL, 2009; TRIPATHI *et al.*, 2016). Conforme Celi e Gabai

(2015), as principais causas de estresse oxidativo em animais são oriundas de eventos metabólicos, inflamatórios e fatores ambientais, como estresse calórico e nutrição. Ainda, em ruminantes, fatores como alta produção de leite (LÖHRKE *et al.*, 2005), escore de condição corporal ao parto (BERNABUCCI *et al.*, 2005), BEN (PEDERNERA *et al.*, 2010) e dieta (CELI *et al.*, 2012; CELI; GABAI, 2015) são fatores que contribuem para o aumento do estresse oxidativo. Assim, o estresse oxidativo pode estar envolvido em várias condições patológicas, incluindo as que são relevantes para a produção e o bem-estar animal (CELI, 2010).

Em vacas leiteiras, o estresse oxidativo tem sido associado tanto a doenças (LYKKESFELDT; SVENDSEN, 2007), incluindo mastite (RANJAN *et al.*, 2005), acidose, cetose, enterite, pneumonia, doenças respiratórias (CELI, 2011) e retenção de placenta (KANKOFER *et al.*, 2010) quanto a problemas reprodutivos (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993), como perdas embrionárias (CELI, 2011; CELI *et al.*, 2012) e cistos foliculares (RIZZO, Annalisa *et al.*, 2009), alterando vários eventos fisiológicos que culminam com a diminuição das taxas de prenhez (AGARWAL; GUPTA; SHARMA, 2005; AL-GUBORY; FOWLER; GARREL, 2010).

A manutenção de um equilíbrio entre ROS e antioxidantes no período de transição de vacas leiteiras é crucial para impedir o prolongamento do estro pós-parto e intervalos de concepção entre partos e, consequentemente, perdas embrionárias (KANKOFER *et al.*, 2010; RIZZO, A. *et al.*, 2007; RIZZO, Annalisa *et al.*, 2009). Assim, uma suplementação adequada de antioxidantes é aconselhável para manter o estresse oxidativo sob controle, melhorar as funções imunológicas e reduzir a incidência de doenças pós-parto (BALDI *et al.*, 2004).

3 ARTIGO – INDUCED-NEGATIVE ENERGY BALANCE DO NOT ALTER PARACRINE AND ENDOCRINE EMBRYONIC SIGNALING ON EARLY PREGNANT EWES

Artigo submetido para revista

Induced-negative energy balance do not alter paracrine and endocrine embryonic signaling on early pregnant ewes

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Induced-negative energy balance do not alter paracrine and endocrine embryonic signaling on early pregnant ewes

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Short title: negative energy balance on embryonic signaling

Abstract

Negative energy balance (NEB) is one of the main causes of reduced fertility in ruminants. Our study hypothesis is that induced NEB influences maternal-embryonic communication. For this reason, the aim of our study was to evaluate the effects of induced-NEB, in the first or second week of embryonic development, on maternal recognition of pregnancy and oxidative stress in the endometria and corpora lutea on Day 17 pregnant ewes. For this, 21 sheep were placed into four groups: control group, non-bred and non-pregnant (n=3); control group of pregnant women (n=6); NEB during the first week of embryonic development (FW; n=6); and NEB in the second week (SW; n=6). Our results demonstrate a moderate decrease in the expression of *MX2* in the CL in the fasting group in the first week and an increase in the expression of *MX1* in FW and SW in the endometrium and increased progesterone concentration in ewes submitted to fasting in the second week. Possibly, fasting was effective in causing hyperketonemia, but it was not enough to cause significant changes in the endometria and corpora lutea. Pregnant ewes under induced NEB showed the same pattern of oxidative stress gene expression and IFNs when compared with P control sheep. In conclusion, our study expresses a subclinical effect, normally animals that suffer NEB have higher BHB levels than those demonstrated here. Although NEB is inevitable in certain phases of the production system, minimizing it will result in minimal loss.

Keywords: Maternal recognition of pregnancy, ewes, negative energy balance.

Introduction

Maternal recognition of pregnancy is the period when the conceptus signals to the mother its presence (Bazer, Vallet, Roberts, Sharp, & Thatcher, 1986). In ruminants, the signaling period coincides with embryo elongation and maximum production of interferon tau (IFNT) (Alfredo Quites Antoniazzi, Henkes, Oliveira, & Hansen, 2011). IFNT is the major cytokine responsible for the embryo-maternal interaction to avoid luteolysis and establish and maintain pregnancy (Mccracken, Custer, & Lamsa, 1999; Niswender, Juengel, Silva, Rollyson, & McIntush, 2000). IFNT is produced by embryonic trophoblast at pre-implantation period and acts in the uterus in a paracrine manner, inhibiting endometrial estrogen and oxytocin receptors expression in the luminal epithelium, avoiding prostaglandin F2 alpha (PGF) luteolytic pulses (Thomas E. Spencer & Bazer, 1996). In the paracrine manner, IFNT has been found to stimulate the expression of several genes in the luminal and glandular epithelial endometrium that have potentially important biological activities for embryo elongation and implantation (Thomas E. Spencer, Forde, & Lonergan, 2016).

In addition to its antiluteolytic effects, IFNT increases the expression of several genes stimulated by IFN (*ISG*) in the uterus, such as 2 ', 5'-oligoadenylate synthase (*OAS1*), resistance to mixovirus (influenza virus) 2 (*MX2*) and protein 15-kDa of the ubiquitin-like IFN-stimulated gene (*ISG15*) (Johnson et al., 1999; Troy L. Ott et al., 1998). IFNT-induced protein expression during early pregnancy, such as *MX* and *ISG15*, has recently been shown to occur in other extra-uterine tissues such as the corpus luteum (CL). The antiviral bioactivity of IFN in the blood was significantly greater in the blood of the uterine vein when compared to the blood of the uterine artery and jugular vein on day 15 of pregnancy, providing the first evidence that a type I IFN is actually released from the uterus and has endocrine action. The detection of mRNA and ISG proteins in the CL means that the CL can be rescued during pregnancy not only by endocrine

factors released by the uterus, such as IFNT, but also by the expression of specific ISG in the ovary (Oliveira et al., 2008).

Embryonic mortality during the early stage of embryonic development, mainly due to signaling failures during maternal recognition of pregnancy, is recognized as a major cause of reproductive failure in ruminants (Diskin & Morris, 2008), as well as negative energy balance (NEB) and the occurrence of hyperketonemia has been identified as a cause of reduced fertility in ruminants. During the transition period, there is a period of NEB, which is the main cause of hyperketonemia in dairy cows (Drackley, 1999; Herdt, 2000) and sheep (Lacetera, Bernabucci, Ronchi, & Nardone, 2001).

The severity and duration of NEB and loss of body condition during the first months of lactation are associated with a lack of cyclicality, as subclinical ketosis has been associated with lower pregnancy rates in dairy cows (Caixeta, Ospina, Capel, & Nydam, 2017; Rivera et al., 2010; Walsh et al., 2007). Embryos exposed to NEFA have a reduced number of blastocyst cells, a greater number of apoptotic cells and alternative patterns in gene expression (Desmet et al., 2016). A study conducted with a small number of animals suggested an effect of maternal nutrition on the IFNT secretion of the embryo, where embryos collected on the 15th day of gestation from malnourished sheep had reduced antiviral activity (J.A. Abecia, Forcada, & Lozano, 1999). However, the experiment did not evaluated ISGs in a paracrine and endocrine manner.

The excessive occurrence of lipid mobilization during the NEB, also plays an important role between energy metabolism, oxidative stress and efficiency of the immune system (L. M. Sordillo, 2016; Lorraine M. Sordillo & Raphael, 2013). As a consequence of BEN and the intensified NEFA oxidation processes there is in an increase in production of ROS (Reactive Oxygen Species) and the development of oxidative stress (Bionaz et al., 2007). According to Van Hoeck et al. (2013), oxidative stress is one of the main ways in which the high amounts of

NEFA affect bovine oocytes and embryos. In addition to NEFA, many studies have shown that BHB is associated with energy metabolism and this can cause oxidative stress, inflammatory response and cell apoptosis (Bernabucci, Ronchi, Lacetera, & Nardone, 2005; Li et al., 2016; Shi et al., 2014). Increased intracellular ROS can cause DNA and mitochondrial damage (Slimen et al., 2014), leading to a non-specific modification of lipids and proteins (Wang et al., 2013), activating the mechanisms of cellular autophagy and apoptosis (Brunet et al., 2004). One study by our group, presents the interaction of heat and oxidative stresses, and the embryo quality regarding to *IFNT* Synthesis (Amaral et al., 2020).

Therefore, our study hypothesis is that induced NEB influences maternal-embryonic communication, negatively impacting maternal recognition of pregnancy. The aim of our study was to evaluate the effects of induced NEB, in the first or second week of embryonic development, on maternal recognition of pregnancy and oxidative stress in the endometrium and CL of sheep on Day 17 of pregnancy. The stages of embryonic development were: 1) stage of embryonic development from zygote to blastocyst; 2) stage of embryonic development of the blastocyst hatched to the filamentous conceptus.

Material and methods

Animals

All procedures using animals were approved by the Institutional Committee for Ethics in Animal Experiments (protocol #5133030519). During early breeding season, 21 Texel-Corriedale crossbreed ewes were kept in open paddocks receiving a maintenance diet for 30 days previous the experimental period, to adapt to experimental conditions. Ewes were fed total daily diet containing 1.5 kg of tifton85 hay and 0.25 kg of crushed corn, providing 2.2Mcal of metabolizable energy per animal. All the animals had unrestricted access to water and mineral supplement.

116

117 ***Experimental design***

118 All ewes were synchronized using intravaginal devices containing 62 mg
119 medroxyprogesterone acetate, inserted for 14 days. To induce ovulation, ewes received 400 IU
120 equine chorionic gonadotropin (eCG) (Folligon®, MSD Animal Health, Kenilworth, N.J.,
121 USA) and 0,132mg cloprostenol sodium (Cioprostinn®, Boehringer Ingelheim Animal Health
122 do Brasil Ltda, Paulínia, S.P., Brazil) in a single IM administration at the time of the removal
123 intravaginal device. Ovulation time (Day 0) was estimated to be 56 hours after intravaginal
124 device removal (Martinez-Ros, Rios-Abellan, & Gonzalez-Bulnes, 2019). After removal the
125 vaginal device, ewes were examined every hour for evidence of estrus. Four hours later, ewes
126 were placed to fertile rams (1 to 6) that mated 18 out 21 females. After breeding, the ewes were
127 allocated into one of four experimental groups: Open control group, not bred and not pregnant
128 (NP; n=3); pregnant control group (P; n=6); NEB on the first week of embryonic development
129 group (FW; n=6) and NEB on the second week of embryonic development group (SW; n=6).
130 The control groups (NP and P) received the same diet from the adaptation period during the
131 whole experimental period (16 days). The FW fasted from Days 0 to 7 and SW fasted from
132 Days 9 to 16. In the remaining days the FW (Days 8 to 16) and SW (Days 0 to 8) received the
133 similar diet as the control groups. All the animals had unrestricted access to water and mineral
134 supplement. Body condition score (BCS) (scale of 1–5, 1 = lean and 5 = obese) were determined
135 on Days 0, 7 and 16.

136

137 ***Induction of NEB and hyperketonemia***

138 Ewes from FW and SW groups fasted for 7 days and were examined four times a day
139 during fasting period by the same person to evaluate health status. In addition, blood BHB levels
140 were monitored daily to identify possible risks of clinical ketosis.

Blood sampling

Blood was collected to determine BHB and glycemic levels on Days 0, 1, 4, 7, 10, 13 and 16 of all groups; additional Days 2, 3, 5 and 6 on FW group; and Days 9, 11, 12, 14 and 15 on SW group. A 10-hour fast eas done before each blood collection. Animals were physically restrained, and 2 mL of blood was collected from the jugular vein using vacutainer tubes with or without EDTA. Blood samples were centrifuged at 2000 x g and samples stored at -20ºC for further analysis.

Tissue sample collection

All ewes were euthanized on Day 17 of the estrous cycle or pregnancy. Samples of endometrium ipsilateral and contralateral to the CL, and CL were collected. The samples were snap-frozen in liquid nitrogen for mRNA and protein studies. Tissues were placed in 4%PFA for 24h and after in 70% alcohol for histology studies. The ewes were considered pregnant through the visualization of one or more normally developed embryos in the uterus.

Blood metabolites assay

Blood assay of β-hydroxybutyrate (BHB) and glycemic levels were obtained using FreeStyle Precision Ketone and Glucose Blood Monitor (Abbott, Diabetes Care Ltd., Oxon, UK) (Panousis et al., 2012).

Progesterone concentration

Concentration of progesterone was measured on Day 16 serum. The progesterone concentration was determined by the chemiluminescence kit (ADVIA Centaur, Siemens) following the manufacturer's recommendations. The sensitivity of the assay was 0.15 ng/mL.

RNA extraction, reverse transcription and real time PCR

Total RNA was extracted from endometrium and luteal samples using TRIzol® according to manufacturer instructions. Briefly, extraction used 1000µl TRIzol® reagent (Thermo Fisher, Waltham, MA, USA) and 200µl chloroform, followed by purification of the aqueous phase with 400µl isopropyl alcohol. Quantification and estimation of RNA purity were performed using a NanoDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA; Absorbance 260/280nm ratio). RNA was treated with 0.1 U DNAse Amplification Grade (Thermo Fisher, Waltham, MA, USA) for 15 minutes at 27ºC to degrade DNA molecules. DNAse was inactivated with 1µl EDTA for 10 minutes at 65ºC. Reverse transcription was performed by adding 1U iScript cDNA synthesis Kit (BioRad, Hercules, CA, USA) for 5 minutes at 25ºC followed by 30 minutes at 42ºC and 5 minutes at 85ºC. Quantitative polymerase chain reaction (qPCR) was conducted in a thermocycler (BioRad, Hercules, CA, USA) using 2µl of cDNA and 8µl of MIX containing forward and reverse bovine specific primers (Table 1), nucleases free water, and GoTaq® Master Mix (Promega Corporation, Madison, USA). Amplification was performed with an initial denaturation at 95ºC for 5 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds and annealing/extension at 60ºC for 30 seconds. To optimize the RT-qPCR assays, serial dilutions of cDNA templates were used to generate a standard curve. Reactions with a coefficient of determination (R^2) higher than 0.98 and efficiency between 85 to 110% were considered optimized. Samples were run in duplicate, and the results of all genes were expressed relative to the *GAPDH* reference gene, according to (Pfaffl, 2001).

Statistical analysis

The analysis of variance was performed with software JMP (SAS Institute) with the treatment as main effect and replicates as a random variable. Differences between the means

were tested with the Tukey multiple comparison test. The homogeneity of variance was tested with O'Brien test. Data that did not follow a normal distribution (Shapiro-Wilk test) were transformed into logarithms. Results are presented as mean ± standard error of the mean (SEM), and $P < 0.05$ was considered significant.

Results

Descriptive analyses

There were no differences among groups regarding age, body condition score on Days 0, 7 and 16, and number of CL on Day 17. Among the P, FW and SW groups there was no difference regarding to the presence of embryo on the Day 17.

Glycemic levels and blood BHB levels

Two days after fasting beginning all the ewes declined glycemic and increased BHB levels. On Day 0, control group had a 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 ($P < 0.01$). Blood BHB the FW group on Day 4 had a greater blood BHB level than the control groups ($P < 0.001$) and the SW group ($P < 0.001$); on Day 7, the FW group had a higher blood BHB level than the control groups ($P < 0.001$) and the SW group ($P < 0.001$). During the fasting period, ewes from FW group and SW group presented a strong negative correlation for glycemic levels in the first two days of fasting (Day 0 to Day 2) (-0.72; $P<0.001$) and a moderate positive correlation for blood BHB levels on the seven days of fasting (Day 0 to Day 7) (0.60; $P < 0.001$) (data not shown).

mRNA relative expression of interferon-stimulated genes in the endometrium

The mRNA expression for *ISG15*, *MX1* and MX2 were evaluated to determine the endometrial response (paracrine) to IFNT action. NP group had lower expression of *ISG15* m RNA than all pregnant groups (P, FW and SW), in the ipsilateral horn to the CL ($P < 0.05$; Fig. 1A). In the contralateral horn, the NP group also showed lower relative expression of *ISG15* when compared to the P group ($P < 0.05$; Fig. 1B). No difference was identified for the relative expression of *MX1* between the experimental groups in the contralateral horn ($P > 0.05$; Fig. 1D). In the ipsilateral horn, there was a difference between the experimental groups of the relative expression of *MX1* ($P < 0.05$, Fig. 1C). The relative expression of the *MX2* mRNA was greater in all pregnant groups (P, FW and SW) compared to the NP ($P < 0.05$) in the ipsilateral horn (Fig. 1E). In the contralateral horn, a greater expression in the pregnant control group after contrast ($P < 0.05$; Fig. 1F).

mRNA relative expression of interferon-stimulated genes in CL

The levels of mRNA expression for *ISG15*, *MX1* and *MX2* were evaluated in the CL in order to evaluate endocrine action of IFNT. The *ISG15* and *MX2* mRNA expressions were different when comparing the NP and P groups ($P < 0.05$) (Fig. 2A and 2C). The NP group had a lower relative expression for *ISG15* than the P, FW and SW groups ($P < 0.05$) (Fig. 2A). The relative expression for *MX2* was lower for the NP and FW groups than for the P and SW groups ($P < 0.05$) (Fig. 2C). There was no difference in the relative expression of *MX1* between the experimental groups ($P > 0.05$) (Fig. 2B). All pregnant groups (P, FW and SW) had no difference for the expression of the *ISG15* mRNA ($P > 0.05$), while the group (FW) showed less relative expression for *MX2* than other pregnant groups ($P < 0, 05$) (Fig. 2A and 2C).

Endometrial and luteal mRNA relative expression of IFN receptors (IFNAR1 and 2) and IFNT pathway

The endometrial levels of mRNA for *IFNAR1* were not different between the experimental groups (P> 0.05), in the ipsilateral and contralateral horns to CL (Fig. 3A and 3B). The *IFNAR2* mRNA had no difference between the groups in the ipsilateral horn (P > 0.05; fig. 3C). However, in the contralateral horn, *IFNAR2* was greater in the SW group when compared to the other groups (NP, P and FW) (P <0.05, fig. 3D). In the CL, all groups showed relative mRNA expression for both *IFNAR1* and *IFNAR2* mRNA. The levels of mRNA for *IFANR2* (P > 0.05; Fig. 3F) were not different between experimental groups, while the *IFNAR1* mRNA was greater in NP sheep (P <0.05; Fig. 3E).

The functionality of the IFNT signaling pathway was assessed through the relative mRNA expression of *JAK1*, *STAT1* and *2* and *IRF9*. In the ipsilateral horn, there was no difference for *JAK1* (P > 0.05; Fig. 5A). While for *STAT1* and *2*, and *IRF9* there was a difference (P <0.05; fig. 4A; 4B and 5B,), the NP group had a lower expression of these genes, and a greater expression in the SW group when compared with other pregnant groups. In the contralateral horn there was no difference for the *JAK1* and *IRF9* genes (P > 0.05; Fig. 5C and 5D); There was a difference for *STAT1* and *2* in the contralateral horn (P <0.05; fig. 4C and 4D), with a greater expression in the FW group. When evaluating these genes in the CL we found no difference for *STAT1* and *2*, and *IRF9* (P> 0.05; Fig. 4E; 4F and 5F; respectively), for *JAK1* there was a greater expression in the NP group compared with the P group and the SW group (P <0.05; fig. 5E).

Endometrial and luteal mRNA relative expression of oxidative stress genes

The relative expression of *SOD1* and *SOD2*, *GPX1* and *GPX4*, and catalase (*CAT*) were evaluate identify oxidative stress control. Endometria mRNA levels for *SOD1* (P> 0.05; fig. 6 A, C and E), *GPX1* and *GPX4* (P> 0.05; fig. 7) were not different when comparing the experimental groups. While SOD2 (P <0.05) had greater expression in the pregnant groups

267 when compared to NP in the ipsilateral horn (fig. 6 B) and greater expression in SW group
268 compared to NP in the contralateral horn (Fig. 6 D). On the contrary, *CAT* had greater ($P < 0.05$)
269 expression in NP group compared to the other groups in the CL (Fig. 8 C).
270
271 ***Endometrial and luteal mRNA relative expression of cell stress genes***
272 Cellular stress was assessed by mRNA of the heat shock protein 90 members of the
273 family A class B (*HSP90B1*) and of the heat shock protein member of the family A 1A (*HSP70*).
274 No differences for *HSP90B1* and *HSP70* ($P > 0.05$; fig. 9) in ipsilateral and contralateral
275 endometria and CL.
276
277 ***Progesterone concentrations and mRNA relative expression of 3βHSD in CL***
278 There was no difference in progesterone concentrations (ng/mL) for NP, P and FW
279 groups ($P > 0.05$; Fig. 10 A). Induced NEB ewes in the second week of gestation (SW group)
280 presented a greater concentration of progesterone when compared to the other groups ($P < 0.05$).
281 On the other hand, when evaluating the relative expression of *3βHSD* in the CL, there was a
282 significant difference ($P < 0.05$; fig. 10B), where a lower expression is observed in the NP group
283 (NP) compared to the groups of pregnant (P, FW and SW).
284
285 **Discussion**
286 The present study propose a model of induced-NEB at the beginning of pregnancy in
287 sheep to assess its influence during maternal recognition of pregnancy on endometria and CL
288 and oxidative stress responses. Initially, our induced-NEB model was validated in ewes in the
289 first or the second week of early embryonic development. On Day 0 to 7 in the FW and from
290 Day 8 to 16 in the SW group, there was a decline in glycemic and an increase in BHB levels,
291 confirming the NEB status. Not even one ewe had BHB above the threshold of subclinical

292 ketosis. To confirm the pregnancy, it was observed normally developed embryos at Day 17,
293 and to assess embryonic health status it was verified ISGs induction in all pregnant groups in
294 the endometria and CL. Following these validations, it was studied the IFN receptors and
295 signaling pathways and oxidative stress targets.

296 The classic mechanism of action of IFNT controls the transcription of estrogen receptor
297 (ESR1) and, consequently, oxytocin receptor (OXTR) in the endometrial tluminal epithelium.
298 This control inhibits the luteolytic pulses of prostaglandin F2 alpha (PGF), preventing return to
299 cyclicity (T E Spencer & Bazer, 1996). *IFNT* mRNA begins to be expressed on the fourth day
300 of embryonic development *in vitro* (Yao et al., 2009), and its protein is detected on Day 7 of
301 embryonic development, starting its signaling in the endometrial cells located at the ipsilateral
302 uterotubal junction to the CL (Sponchiado et al., 2017). There are many genes positively
303 regulated by IFNT in early pregnancy and, among all ISGs, we can highlight *ISG15*, *MX1*, *MX2*
304 and *OAS* (Green, Okamura, Poock, & Lucy, 2010; Haq et al., 2016). The first response to the
305 conceptus IFNT release is the induction of ISGs in the endometrium, especially *ISG15* (Romero
306 et al., 2015). On Day 11 of pregnancy in sheep, *ISG15* concentrations are low, increasing the
307 expression on Day 13 and 15 (Joyce et al., 2005). In our study, only the NP had lower expression
308 of *ISG15* than all the pregnant groups (P; FW and SW). This confirm the presence of a viable
309 embryo signaling in the uterus. The induced-NEB groups had no difference on *ISG15*
310 expression when compared to P group. This indicates NEB did not interfere in embryo-maternal
311 ISGs signaling during the period of maternal recognition of pregnancy. *ISG15* may be involved
312 in the regulation of essential proteins for the establishment of pregnancy in ruminants (Haq et
313 al., 2016).

314 Several IFNT-stimulated genes (ISGs), including *MX* genes, are expressed in the uterus.
315 These ISGs can regulate endometrial receptivity to implantation, as well as fetal survival,
316 growth and development. The relative expression of *MX1* and *MX2* in the endometrium was

317 greater in P than NP ewes, which is expected since MX is stimulated in response to IFNT
318 (Charleston & Stewart, 1993; Johnson et al., 1999; T L Ott et al., 1998). *MX2* showed no
319 difference between the P group and the NEB groups (FW and SW), while for *MX1* there was a
320 difference between the groups showing greater expression in the NEB groups in the ipsilateral
321 horn (Fig. 3C). However, in the contralateral horn there was no difference in the expression of
322 *MX1*, which may be related to the fact that there is lower expression of *MX* in the contralateral
323 horn (Charleston & Stewart, 1993).

324 IFNT also acts in an endocrine manner in the CL to signal maternal recognition in sheep
325 (Oliveira et al., 2008). The maintenance of CL in ruminants occurs in response to IFNT secreted
326 by the conceptus (Thatcher et al., 2001). In the CL, ISGs profile was similar, where P and NEB
327 groups, have a greater expression, but the FW group had lower expression for *MX2* mRNA, this
328 may be related to metabolic stress of these animals. Moreover, these animals had a greater
329 concentration of blood ketone bodies (BHB) than the SW group.

330 One study, suggested an effect of maternal undernutrition on IFNT secretion on Day 15,
331 these sheep had reduced antiviral activity. The results indicate that malnutrition can reduce the
332 ability of embryos to secrete IFNT and, therefore, an increase in the production of endometrial
333 PGF, which can initiate luteolysis (J.A. Abecia et al., 1999). In our study, we evaluated the
334 expression of the ISGs, however, differently from Abecia et al. (1999), NEB ewes had increased
335 expression of *MX1* when compared to P ewes. In a study, Wathes et al. (2009), when evaluating
336 NEB in gene expression and immune responses in the uterus of postpartum dairy cows showed
337 that several genes associated with interferons were regulated positively in cows with severe
338 NEB grade, including *MX1* and *MX2* (Wathes et al., 2009). Type 1 interferons increase in
339 response to many viral infections, have potent antiviral activity, and can also promote an
340 increased capacity for cellular response to other stimulus, including LPS, in addition to potent
341 antiangiogenic activity in endothelial cells (Naschberger et al., 2004).

IFNT acts binding to *IFNAR1* and *IFNAR2* receptors, which have greater expression and colocalization in the cells of the luminal and glandular epithelium of the endometrium, as well as in the CL during pregnancy. In addition, the expression of endometrial subunits are present during pregnancy and the estrous cycle, although regulated positively in the presence of the conceptus (A. Q. Antoniazzi et al., 2013; Han, Mathialagan, Klemann, & Roberts, 1997; Rosenfeld et al., 2002). In the present study, we found no difference for *IFNAR1* mRNA in the ipsilateral and contralateral horns, while the *IFNAR2* in the contralateral horn was greater when compared to SW group. *IFNAR2* has an extracellular region of only two domains, but is considered the subunit that contributes most to interferon binding, its long intracellular region of amino acids links *JAK1* and *STAT2* and possibly other signal transduction components (Rosenfeld et al., 2002). We verified the presence of *IFNAR1* and *IFNAR2* in the CL on Day 17 NP and P ewes and our results demonstrate that IFNT seems to regulated *IFNAR1* unit during pregnancy in the CL.

We investigated IFN type I signaling pathway in endometrial and luteal tissue in order to verify whether NEB may impair IFN signaling pathway. The cascade *JAK1*, *STAT1* and *STAT2* and the regulatory factor IRF9 did not present any difference between the groups in the CL. In the endometria, the ipsilateral horn, had a positive regulation for *STAT1*, *STAT2* and *IRF9* in the P groups, as expected, we also found levels of expression similar to NP in FW group. The contralateral horn, also had a positive regulation in the P groups, however, contrary to the ipsilateral, we observed a greater expression in the FW group. Thus, no differences were observed in all the IFN pathway genes of sheep submitted to NEB. P ewes under induced NEB showed the same pattern of gene expression as the ISGs and IFN pathways when compared with P control ewes. Although, in FW group, there was a tendency to decrease the expression of genes from the ISGs pathway (*MX2*) in the CL and IFN (*STAT1* and *STAT2*) in the uterus,

366 which can also be associated with the fact that this group of sheep presented a greater
367 concentration of BHB.

368 NEB also modulated the progesterone concentration. Progesterone is the main hormone
369 of early pregnancy (Garcia-Ispierto & López-Gatius, 2017) and its low concentration has been
370 associated to negative effects on embryonic development and elongation (Carvalho et al.,
371 2017). The effects of NEB may result in inadequate CL function, leading to suboptimal
372 progesterone concentrations, which may be responsible for a suboptimal microenvironment in
373 the uterus, unable to sustain early embryonic life (Thatcher et al., 2001). In contrast, our study
374 shows that pregnant sheep in NEB with greater BHB in the second week of embryonic
375 development (SW), have a greater concentration of progesterone than P control ewes. In
376 addition to serve as energy to extra hepatic tissues such as brain, heart or skeletal muscle, ketone
377 bodies play essential roles as signaling mediators, conductors of post-translational protein
378 modification (PTM) and modulators of inflammation and oxidative stress (Puchalska &
379 Crawford, 2019). During NEB and especially in the transition period, the changes that occur in
380 metabolism are a reflection of metabolic changes in the liver (Grum, Drackley, Younker,
381 LaCount, & Veenhuizen, 1996). Studies in ovine have shown that increased food intake causes
382 an increase in hepatic blood flow and a decrease in circulating progesterone concentrations
383 (Parr, Davis, Miles, & Squires, 1993). The increase in progesterone hormone metabolism may
384 also be related to decreased fertility in overfed females (Sartori & Guardieiro, 2010). In these
385 results, due to NEB and greater BHB on the SW group, we may consider the reduction of
386 hepatic metabolism as the cause of the greater serum concentration of progesterone.

387 During NEB, intensified NEFA oxidation processes result in increased ROS production
388 and the development of oxidative stress (Bionaz et al., 2007). According to Van Hoeck et al.
389 (2013), oxidative stress is one of the main ways in which the high amounts of NEFA affect
390 bovine oocytes and embryos. In addition to NEFA, many studies have shown that BHB is

associated with energy metabolism and this can cause oxidative stress, inflammatory response and cell apoptosis (Bernabucci et al., 2005; Li et al., 2016; Shi et al., 2014). The main causes of oxidative stress in animals come from metabolic, inflammatory events and environmental factors, such as heat stress and nutrition (Celi & Gabai, 2015). Physiologically, the production of ROS and antioxidants remains in balance. However, when in high concentrations, ROS has deleterious effects on cells, since they are not neutralized by antioxidant defenses (Lykkesfeldt & Svendsen, 2007). In this case, due to overproduction of ROS or depletion of antioxidants, an oxidative stress process occurs (Agarwal, Gupta, & Sharma, 2005; Celi, 2010).

Antioxidant enzymes superoxide dismutase 1 and 2 (*SOD1* and *SOD2*), glutathione peroxidase 1 and 4 (*GPX1* and *GPX4*) and catalase (*CAT*) were investigated to understand whether the antioxidant system is effective in balancing the increase in ROS. *SOD* belongs to the class of enzymes that catalyze the hydrolysis of the superoxide anion in oxygen and hydrogen peroxide; *CAT* and *GPX* catalyze the reduction of hydrogen peroxide in water and, together, stabilize the injured cell (Slimen et al., 2014). All antioxidant enzymes prevent the accumulation of ROS in cells. The mRNA expressions of *SOD1*, *GPX1* and *GPX4* did not differ in the evaluated tissues. Our data showed lower expression of CAT in luteal cells in P ewes compared to NP ewes and higher expression of *SOD2* in P ewes in the endometrium. The activities of antioxidant enzymes in the CL of sheep are subject to major changes during the estrous cycle, maintained levels of antioxidant enzymes in the CL may be linked to ROS generated continuously in the steroidogenic active luteal cells and may be involved in the maintenance of the luteal steroidogenic activity and integrity (Al-Gubory, Bolifraud, Germain, Nicole, & Ceballos-Bicot, 2004). The increase in *SOD2* in the endometrium may be related to the advancement in embryonic development. In the sheep species, the blastocyst loses the pellucid zone approximately 7 days before implantation, this is accompanied by an increase in the generation of ROS, related to an increase in the cell for cellular contact and for activation

of NADPH oxidase (Aurousseau, Gruffat, & Durand, 2006). In addition, in newly formed cells, physiological lipid peroxidation occurs that triggers the expression of *SOD2*, this leads to an attenuation of ROS mediated effects (Silva, Marques, & Chaveiro, 2014).

Lower expression of antioxidant enzymes, can lead to low antioxidant action, and consequently we investigate whether HSPs would be modulated in tissues exposed to NEB. HSPs belong to the class of proteins called chaperones and are induced as a defense mechanism in any type of cellular stress (Lindquist, Biology, & Craig, 1988). We evaluated the expression of the *HSP90B1* and *HSP70* genes. In this study, groups and treatments did not modify the expression of these genes. Members of the HSP family are also constitutively expressed in cells. HSPs families induced and expressed constitutively assist in the normal folding of various polypeptides (Hartl & Hayer-Hartl, 2002).

In conclusion, the NEB induced in the first or second week of embryonic development did not negatively affect maternal recognition of pregnancy. Our results demonstrate a moderate decrease in the expression of *MX2* in the CL in the fasting group in the first week and an increase in the expression of *MX1* in FW and SW in the endometrium and increased progesterone concentration in ewes submitted to fasting in the second week. Possibly, fasting was effective in causing hyperketonemia, but it was not enough to cause significant changes in the endometrium and CL. Thus, no differences were observed in all genes of the IFN pathway of sheep submitted to NEB. Pregnant sheep under induced NEB showed the same pattern of oxidative stress gene expression and IFNs when compared with P control sheep. Our study expresses a subclinical effect, normally animals that suffer NEB have higher BHB levels than those demonstrated here. Although NEB is inevitable in certain phases of the production system, minimizing it will result in minimal loss. Therefore, additional analyzes should be done to assess other possible effects on paracrine and endocrine signaling in early pregnancy in sheep during negative energy balance.

441

442 **Declaration of interest**

443 The authors have nothing to declare.

444

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448

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699 **Figure legends**

700

701 **Figure 1:** Relative mRNA expression of Interferon-stimulated genes on ovine endometrium
702 ipsilateral horn (A: I*SG15*; C: *MX*1; E: *MX2*), and contralateral horn (B: *ISG15*; D: *MX1*; F:
703 *MX2*) in all groups (NP; P; FW; SW). Bars represent the group mean ± SEM. Asterisk
704 represents contrast between groups (P <0,05). Different letters indicate statistical significance
705 among groups (P <0.05).

706

707 **Figure 2:** Relative mRNA expression of Interferon-stimulated genes on ovine luteal cells. (A:
708 *ISG15*; B: *MX1* and C: *MX2*) in all groups (NP; P; FW; SW). Bars represent the group mean ±
709 SEM. Different letters indicate significance among groups (P < 0.05).

710

711 **Figure 3:** Relative mRNA expression of type I interferon receptor genes on ovine endometrium
712 and ovine luteal cells. Ipsilateral horn (A: *IFNAR1*; C: *IFNAR2*); contralateral horn (B: *IFNAR1*;
713 D: *IFNAR2*) and ovine luteal cells (E: *IFNAR1*; F: *IFNAR2*) in all groups (NP; P; FW; SW).
714 Bars represent the group mean ± SEM. Different letters indicate significance among groups (P
715 < 0.05).

716

717 **Figure 4:** Relative mRNA expression of Interferon-pathway genes on ovine endometrium and
718 ovine luteal cells. Ipsilateral horn (A: *STAT1*; B: *STAT2*); contralateral horn (C: *STAT1*; D:
719 *STAT2*) and ovine luteal cells (E: *STAT1*; F: *STAT2*) in all groups (NP; P; FW; SW). Bars
720 represent the group mean ± SEM. Different letters indicate significance among groups ($P < 0.05$).

721

722

723 **Figure 5:** Relative mRNA expression of Interferon-pathway genes on ovine endometrium and
724 ovine luteal cells. Ipsilateral horn (A: *JAK1*; B: *IRF9*); contralateral horn (C: *JAK1*; D: *IRF9*)
725 and ovine luteal cells (E: *JAK1*; F: *IRF9*) in all groups (NP; P; FW; SW). Bars represent the
726 group mean ± SEM. Different letters indicate significance among groups ($P < 0.05$).

727

728 **Figure 6:** Relative mRNA expression of Oxidative stress genes on ovine endometrium and
729 ovine luteal cells. Ipsilateral horn (A: *SOD1*; B: *SOD2*); contralateral horn (C: *SOD1*; D: *SOD2*)
730 and ovine luteal cells (E: *SOD1*; F: *SOD2*) in all groups (NP; P; FW; SW). Bars represent the
731 group mean ± SEM. Different letters indicate significance among groups ($P < 0.05$).

732

733 **Figure 7:** Relative mRNA expression of Oxidative stress genes on ovine endometrium and
734 ovine luteal cells. Ipsilateral horn (A: *GPX1*; B: *GPX4*); contralateral horn (C: *GPX1*; D: *GPX4*)
735 and ovine luteal cells (E: *GPX1*; F: *GPX4*) in all groups (NP; P; FW; SW). Bars represent the
736 group mean ± SEM. Different letters indicate significance among groups ($P < 0.05$).

737

738 **Figure 8:** Relative mRNA expression of Oxidative stress genes on ovine endometrium:
739 Ipsilateral horn (A: *CAT*); contralateral horn (B: *CAT*) and ovine luteal cells (C: *CAT*) in all

groups (NP; P; FW; SW). Bars represent the group mean ± SEM. Different letters indicate significance among groups ($P < 0.05$).

Figure 9: Relative mRNA expression of Oxidative stress genes on ovine endometrium. Ipsilateral horn (A: *HSP70*; B: *HSP90B1*); contralateral horn (C: *HSP70*; D: *HSP90B1*) and ovine luteal cells (E: *HSP70*; F: *HSP90B1*) in all groups (NP; P; FW; SW). Bars represent the group mean ± SEM. Different letters indicate significance among groups ($P < 0.05$).

Figure 10: Progesterone concentration (ng/mL) on blood serum on Day 16 (A) and relative mRNA expression of *3BHSD* gene on ovine luteal cells (B). Bars represented the group mean ± SEM. Different letters indicate statistical significance among groups ($P < 0,05$).

Tables

Table 1. Primers designed for real-time PCR analysis.

Target	**Accession number**	**Primer sequence**
GAPDH	NM_001034034.2	F: TGACCCCTTCATTGACCTTC
		R: CGTTCTCTGCCTTGACTGTG
ISG15	NM_001009735.1	F: GGTATCCGAGCTGAAGCAGTT
		R: ACCTCCGTGCTGTCAAGGT
MX1	NM_173940.2	F: GTACGAGCCGAGTTCTCCAA
		R: ATGTCCACAGCAGGCTCTTC
MX2	NM_173941.2	F: CTTCAGAGACGCCTCAGTCG
		R: TGAAGCAGCCAGGAATAGT
IFNAR1	NM_174552.2	F: GAATCAGCTCTACCCGCTAAT
		R: GCTCTGGCTTTGACACAATAC
IFNAR2	NM_174553.2	F: AGCCAGAATGTGTCAGCGAT
		R: AGAACAGGCGCAACATACGA

STAT1	NM_001077900.1	F: CAAAGGAAGCCCCAGAGCCTA
		R: ACATGCCACTCTTCTGTGTTCA
STAT2	NM_001205689.1	F: CAGCCCGTTTCAGGATCAGC
		R: CAGTGCAGCTTTCTGCCAGT
JAK1	XM_024989564.1	F: GGGGTTAGCCGCTTAGGGAG
		R: CCATTCAGAGCTGAGCACTTCC
IRF9	NM_001024506.1	F: GGTTCCTGAGATCGGCCACA
		R: CCTGATTGAGCGGGGACAGT
SOD1	NM_174615.2	F: AAGGCCGTCTGCGTGCGAA
		R: CAGGTCACCAACATGCCTCT
SOD2	NM_201527.2	F: CCCATGAAGCCTTTCTAATCCTG
		R: TTCAGAGGCGCTACTATTTCCTTC
GPX1	NM_174076.3	F: TTGGGCATCAGGAAAACGCC
		R: TTCTCGCCATTCACCTCGCA
GPX4	NM_001346430.1	F: CGCCGAGTGTGGTTTAC
		R: AGGTCCTTCTCTATCACCAG
CAT	NM_001035386.2	F: GTTCGCTTCTCCACTGTT
		R: GGCCATAGTCAGGATCTT
HSP90B1	NM_001012670.2	F: AGTCCTTCAGCCAAGATGCC
		R: GACAGCCAAGTGATCCTCCC
HSP70	NM_203322.3	F: CTTCAACATGAAGAGCGCCG
		R: TGATGGGGTTACACACCTGC
3βHSD	NC_040252.1	R: TCTCTGCAGTACTGGCTTGC
		F: GTCACTAGGTGGCGGTTGAA

Abbreviations: F: Forward; R: Reverse.

Figures

Figure 1:

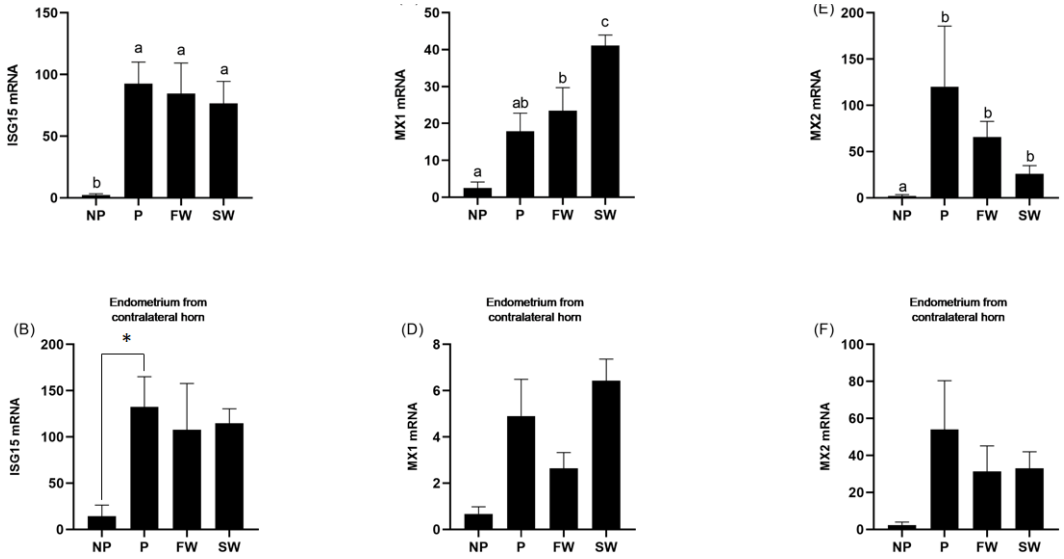


Figure 2:

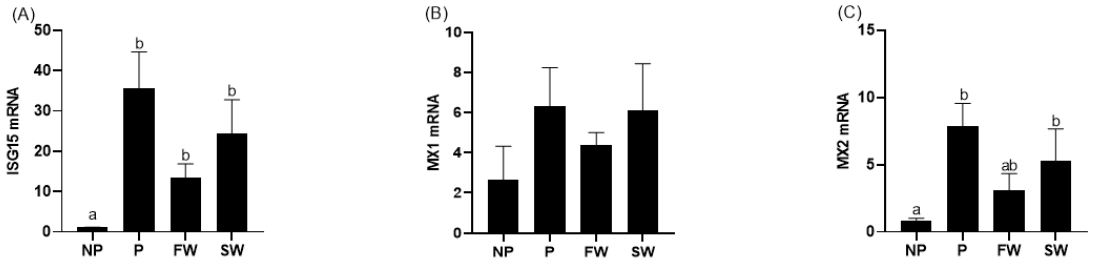


Figure 3:

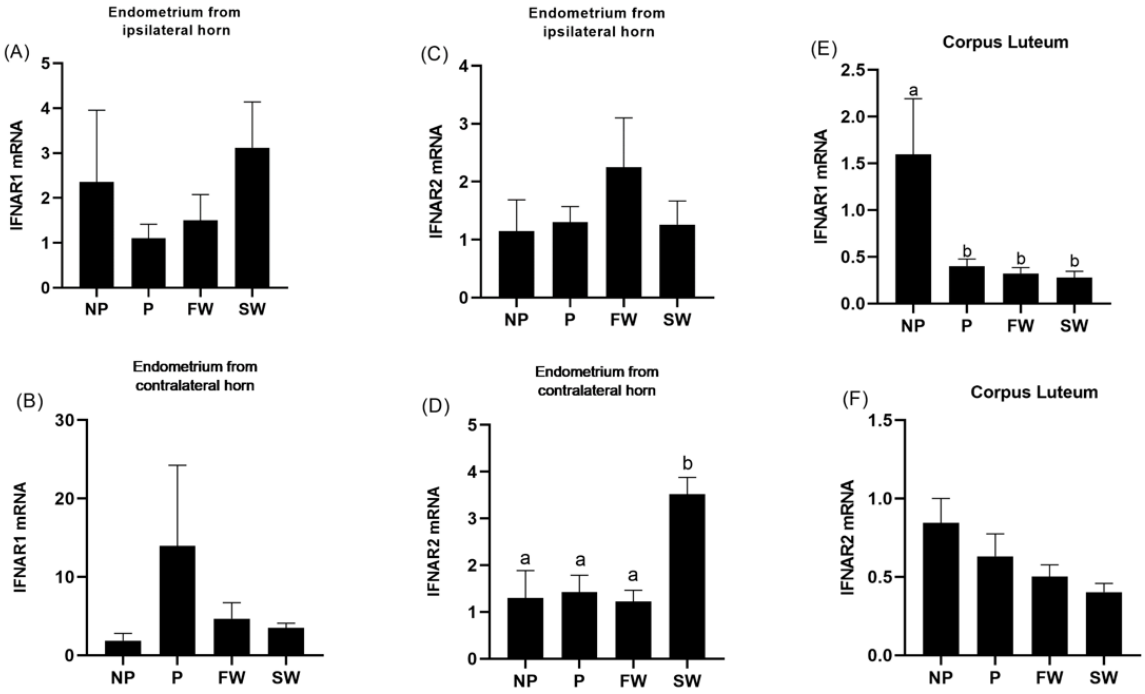
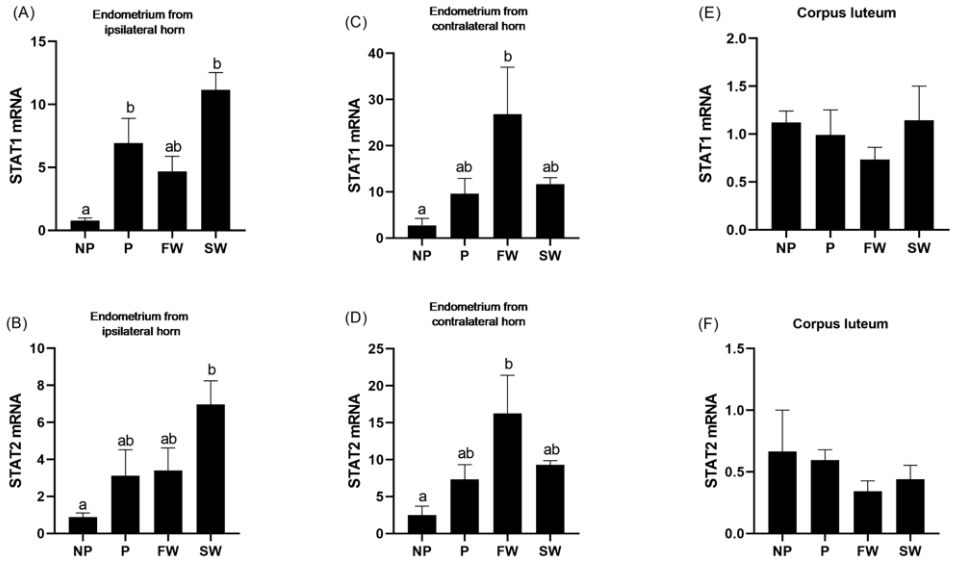


Figure 4:



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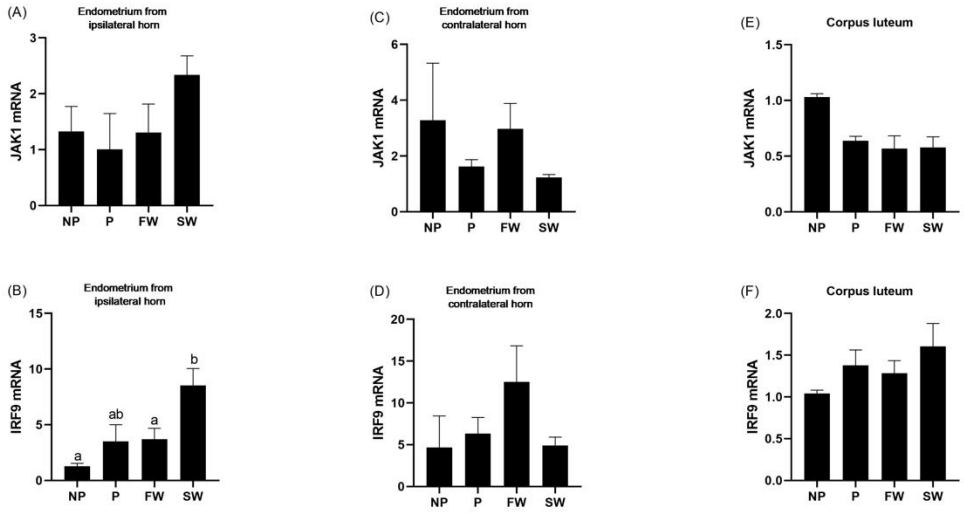

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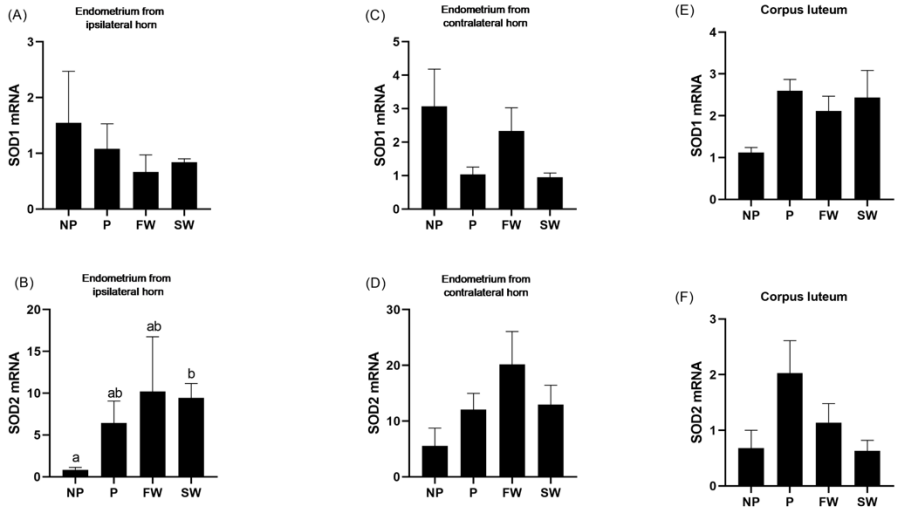

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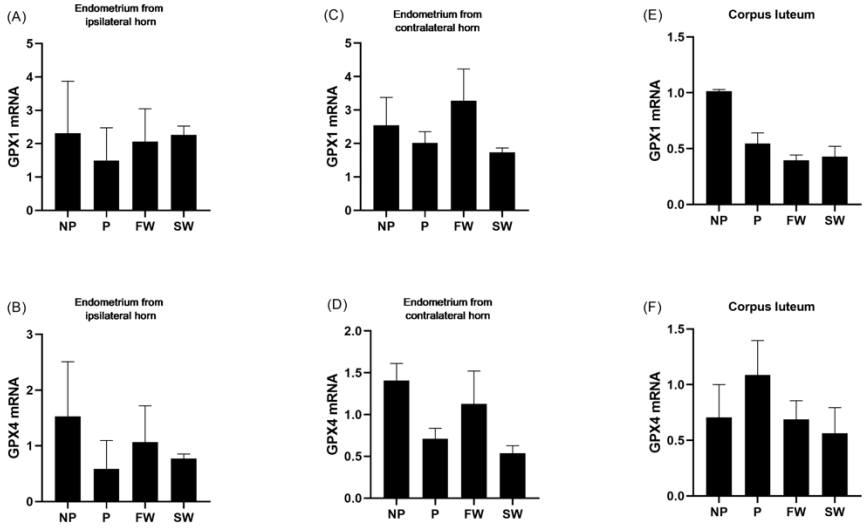


Figure 8:

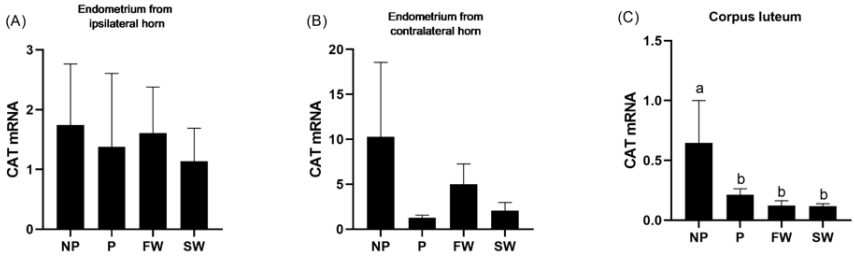


Figure 9:

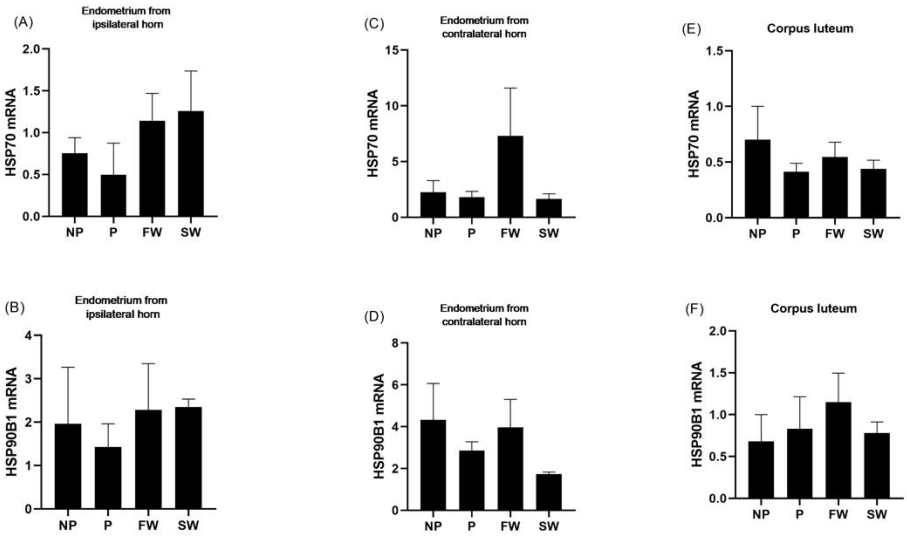
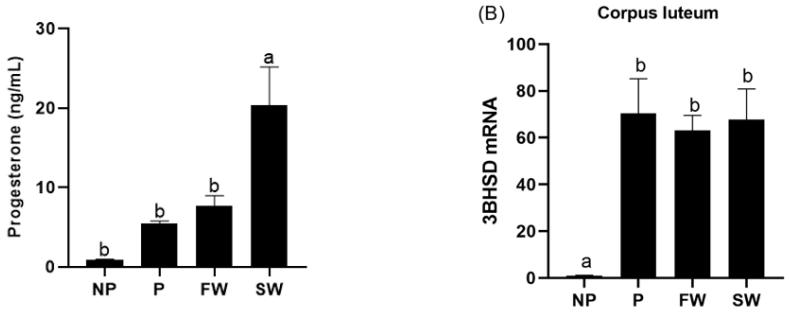


Figure 10:



4 CONCLUSÃO

O balanço energético negativo induzido na primeira ou segunda semana de desenvolvimento embrionário não afetou negativamente o reconhecimento materno da gestação. Nossos resultados demonstram diminuição moderada da expressão de *MX2* no CL no grupo em jejum na primeira semana e aumento na expressão de *MX1* no FW e SW no endométrio e aumento da concentração de progesterona em ovelhas submetidas ao jejum na segunda semana. Possivelmente, o jejum foi eficaz em causar hipercetonemia, mas não foi o suficiente para causar alterações significativas no endométrio e CL. Assim, não foram observadas diferenças em todos os genes da via do IFN de ovelhas submetidas ao BEN. Ovelhas prenhes sob BEN induzido apresentaram o mesmo padrão de expressão do gene do estresse oxidativo e IFNs quando comparadas às ovelhas P controle. Nosso estudo expressa um efeito subclínico, normalmente animais que sofrem de BEN apresentam níveis de BHB mais elevados do que os demonstrados aqui. Embora o BEN seja inevitável em certas fases do sistema de produção, minimizá-lo resultará em perdas mínimas. Portanto, análises adicionais devem ser feitas para avaliar outros possíveis efeitos na sinalização parácrina e endócrina no início da gestação em ovelhas durante o balanço energético negativo.

5 REFERÊNCIAS

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