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ESTRESSE METABÓLICO EM CÉLULAS ENDOMETRIAIS E ESPERMATOZOIDES BOVINOS: EFEITO DE ÁCIDOS GRAXOS NÃO ESTERIFICADOS E HIPOCALCEMIA

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutora em Medicina Veterinária**.

Orientador: Prof. Dr. Paulo Bayard Dias Gonçalves

Coorientador: Prof. Dr. Rogério Ferreira

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RESUMO

IMPACTO DO ESTRESSE METABÓLICO E HIPOCALCEMIA SUBCLÍNICA NA FERTILIDADE DE VACAS LEITEIRAS: ANÁLISE DOS EFEITOS NO MICROAMBIENTE UTERINO

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No período de transição, o qual compreende três semanas antes e três semanas após o parto, as fêmeas bovinas desenvolvem balanço energético negativo (BEN). O BEN é o período em que as demandas de energia são maiores do que o aporte nutricional, levando à intensa mobilização de reversas corporais, o que se reflete no aumento dos níveis sanguíneos de ácidos graxos não esterificados (NEFA) e β-hidroxibutirato (BHBA), e diminuição das concentrações de insulina e glicose. Estudos demonstraram que essas alterações sanguíneas têm reflexo no microambiente ovidutal e uterino de bovinos. Por isso, é importante avaliar como essas alterações podem afetam os espermatozoides quando eles entram em contato com o trato reprodutivo da fêmea. Portanto, o objetivo do primeiro trabalho desta tese é avaliar os efeitos dos NEFA na morfologia, cinética e status oxidativo dos espermatozoides bovinos *in vitro*. Os resultados demonstram que diferentes concentrações de ácido palmítico (PA), ácido esteárico (SA) e ácido oleico (OA) não alteram a cinética e morfologia espermática, nem o status oxidativo dos espermatozoides. O objetivo do segundo trabalho desta tese é avaliar os efeitos da hipocalcemia subclínica transitória sobre a via de sinalização de insulina uterina em vacas leiteiras. Sabe-se que as vacas leiteiras no pós-parto apresentam hipocalcemia, e que esta condição é um fator de risco para infecções uterinas e podem culminar em alterações na sinalização da insulina. Os resultados primeiramente demonstraram que o nosso protocolo de indução de hipocalcemia com solução de EDTA a 5% foi eficaz reduzindo as concentrações de cálcio ionizado circulante. Observamos que os níveis sanguíneos de glicose, insulina e NEFA não foram alterados pela hipocalcemia, enquanto os níveis de BHBA foram reduzidos. Com relação à expressão de genes relacionados a via de sinalização de insulina e glicose, INSR, IRS1, IGF, GLUT1 e GLUT3, a hipocalcemia não afetou negativamente nenhum dos genes avaliados. A partir dos dados obtidos com esta tese foi possível determinar que os NEFA nas condições testadas não afetam negativamente a viabilidade de espermatozoides bovinos e que a hipocalcemia subclínica transitória não altera a via de sinalização da insulina no útero de vacas leiteiras.

Palavras-chave: Balanço energético negativo. Ácidos graxos não esterificados. Resistência à insulina. Hipocalcemia. Endométrio.

ABSTRACT

IMPACT OF METABOLIC STRESS AND SUBCLINICAL HYPOCALCEMIA ON FERTILITY OF DAIRY COWS: AN ANALYSIS OF THE EFFECTS ON THE UTERINE MICROENVIRONMENT

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In the transition period, which comprises three weeks before and three weeks after calving, bovine females develop a negative energy balance (BEN). The BEN is the period in which the energy demands are greater than the nutritional intake, leading to the intense mobilization of body reverses, which is reflected in the increase in blood levels of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB), and decreased insulin and glucose concentrations. Studies have shown that these blood alterations are reflected in the oviductal and uterine microenvironment of cattle. Therefore, it is important to evaluate how these changes can affect sperm when they meet the female's reproductive tract. Therefore, the objective of the first work of this thesis is to evaluate the effects of NEFA on the morphology, kinetics, and oxidative status of bovine spermatozoa *in vitro*. The results demonstrate that different concentrations of palmitic acid (PA), stearic acid (SA), and oleic acid (OA) do not alter sperm kinetics, morphology, or sperm oxidative status. The objective of the second work of this thesis is to evaluate the effects of transient subclinical hypocalcemia on the uterine insulin signaling pathway in dairy cows. It is known that postpartum dairy cows have hypocalcemia and that this condition is a risk factor for uterine infections and can culminate in changes in insulin signaling. The results first demonstrated that our protocol for inducing hypocalcemia with 5% EDTA solution was effective in reducing circulating ionized calcium concentrations. We observed that blood levels of glucose, insulin, and NEFA were not altered by hypocalcemia, while BHB levels were reduced. Regarding the expression of genes related to the insulin and glucose signaling pathway, INSR, IRS1, IGF, GLUT1, and GLUT3, hypocalcemia did not negatively affect any of the genes evaluated. From the data obtained with this thesis, it was possible to determine that NEFA in the tested conditions does not negatively affect the viability of bovine spermatozoa and that transient subclinical hypocalcemia does not alter the insulin signaling pathway in the uterus of dairy cows.

Keywords: Negative energy balance. Non-esterified fatty acids. Insulin resistance. Hypocalcemia. Endometrium.

LISTA DE FIGURAS

ARTIGO 1

ARTIGO 2

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

SUMÁRIO

1. INTRODUÇÃO

A redução da fertilidade de vacas leiteiras no pós-parto tem sido associada a ocorrência de doenças metabólicas (LEROY et al., 2015). Nesse sentido, o estresse metabólico em vacas leiteiras leva ao balanço energético negativo (BEN), uma questão multifatorial com implicações negativas para vários sistemas fisiológicos, incluindo a reprodução. Na reprodução, BEN influencia o microambiente folicular (LEROY et al., 2005), ovidutal (FENWICK et al., 2008) e uterino (WATHES et al., 2009), diminuindo a eficiência reprodutiva. Durante o período de BEN, há um aumento nas concentrações plasmáticas de ácidos graxos não esterificados (NEFA) e corpos cetônicos, enquanto os níveis de fator de crescimento semelhante à insulina 1 (IGF1), glicose e insulina diminuem (KAWASHIMA et al., 2012). Em vacas leiteiras em BEN, os principais NEFA que aumentam são o oleico (OA, C18:1), palmítico (PA, C16:0) e esteárico (SA, C18:0; VAN HOECK et al., 2014).

Assim como outros metabólitos, concentrações elevadas de NEFA no plasma resultam em concentrações elevadas em outros fluidos, como fluido folicular (LEROY et al., 2005) e secreções ovidutais em vacas (JORDAENS et al., 2017). Considerando que os microambientes do útero e do oviduto desempenham papéis fundamentais nos processos de seleção, armazenamento, motilidade espermática e fecundação (COY et al., 2012; GHERSEVICH; MASSA; ZUMOFFEN, 2015; HOLT; FAZELI, 2010), alterações na composição desses ambientes durante o transporte espermático pode afetar a viabilidade espermática e, consequentemente, a fertilização. Estudo anterior demonstrou que a exposição *in vitro* de espermatozoides bovinos a altas concentrações combinadas dos principais NEFAs (PA, SA e OA), reduz a motilidade total e progressiva, velocidade média da trajetória, velocidade em linha reta, e velocidade curvilínea dos espermatozoides (DESMET et al., 2018). Sabe-se então que os NEFAs combinados prejudicam os espermatozoides quando expostos *in vitro*, porém não se sabe os efeitos individuais dos principais NEFAs. Uma vez que foi observado em outras células, como as células endometriais, que o PA é responsável por alterar parâmetros genéticos e os ácidos OA e SA alteram o perfil oxidativo das células (FERST et al., 2021). É importante analisar os efeitos individuais de PA, SA, e OA na motilidade, perfil oxidativo e morfologia espermatozoides bovinos *in* vitro.

Além do aumento de NEFA, outra alteração metabólica do pós-parto de vacas é a resistência à insulina (RI). A RI é definida como um estado em que determinada concentração fisiológica de insulina induz uma resposta biológica diminuída em tecidos sensíveis à insulina (RONALD KAHN, 1978). Assim, a RI tem duas apresentações distintas, redução na responsividade e na sensibilidade à insulina. Num primeiro momento há redução da sensibilidade à insulina no qual uma maior concentração de insulina é necessária para atingir a resposta máxima nos tecidos sensíveis, logo após se o quadro de RI se manter há redução da responsividade à insulina no qual o tecido produz uma resposta menor ao hormônio (MUNIYAPPA et al., 2008; RONALD KAHN, 1978). Em vacas leiteiras ocorre resistência periférica à insulina no final da gestação e início da lactação (BARUSELLI et al., 2016; DE KOSTER; OPSOMER, 2013; KERESTES et al., 2009b), sendo essa condição necessária para garantir suprimento suficiente de glicose para o útero gestante e para a glândula mamária lactante (BELL; BAUMAN, 1997).

O mecanismo da RI em tecidos reprodutivos, como o útero, ainda não foi totalmente elucidado. Sabe-se que a RI uterina ocorre por meio de alterações nas vias de sinalização PI3K e MAPK (ZHANG et al., 2016), o que culmina em menor expressão gênica do receptor de insulina (INSR), substrato do receptor de insulina tipo 1 (IRS1) e IGF1. Estudos avaliando alterações na via de sinalização da insulina no endométrio de vacas têm demonstrado que essas alterações prejudicam a involução uterina (LLEWELLYN et al., 2008; WATHES et al., 2011) e a resposta inflamatória pós-parto (WATHES et al., 2009). Diversas condições e estímulos que aumentem o risco de inflamação podem causar RI (HIRABARA et al., 2012; KHODABANDEHLOO et al., 2016), entre elas a hipocalcemia.

A hipocalcemia ocorre no início da lactação, pois as vacas não conseguem se adaptar à alta demanda de cálcio necessária para a produção leiteira e manter a normocalcemia (GOFF, 2008). A diminuição das concentrações de cálcio circulantes nesse período é um fator de risco para distúrbios uterinos como a involução uterina tardia (HEPPELMANN et al., 2015), prolapso uterino (RISCO; REYNOLDS; HIRD, 1984), retenção de placenta (MELENDEZ et al., 2004), metrite e endometrite (MARTINEZ et al., 2012; RIBEIRO et al., 2013). Portanto, a hipocalcemia pode contribuir para a RI uterina, uma vez que vacas com hipocalcemia subclínica apresentam maior predisposição para desenvolverem alterações inflamatórias uterinas (MARTINEZ et al., 2012). E por

sua vez essa inflamação uterina pode alterar a via de sinalização a insulina (HIRABARA et al., 2012; KHODABANDEHLOO et al., 2016). Por isso, nossa hipótese é que as alterações inflamatórias provenientes da hipocalcemia, causa alterações na via de sinalização à insulina uterina no pós-parto de vacas leiteiras.

Portanto, os objetivos dessa tese foram: (1) avaliar os efeitos dos NEFA na morfologia, cinética e status oxidativo dos espermatozoides bovinos *in vitro*; e (2) avaliar os efeitos das alterações inflamatórias provenientes da hipocalcemia subclínica transitória sobre a via de sinalização à insulina uterina em vacas leiteiras.

2. REVISÃO BIBLIOGRÁFICA

2.1. Período de transição de balanço energético negativo em vacas

O período mais crítico na vida produtiva de vacas leiteiras de alto rendimento é conhecido como o período de transição, que compreende o período entre três semanas pré-parto e três semanas pós-parto (RINGSEIS; GESSNER; EDER, 2015). Esse período é marcado por intensas mudanças metabólicas, endócrinas e imunológicas, as quais refletem na alta incidência de doenças (DRACKLEY, 1999). No final da gestação, o crescimento fetal associado à produção de leite impõe demandas nutricionais elevadas (BELL, 1995). Na mesma proporção em que a demanda nutricional aumenta há uma diminuição do consumo de matéria seca. Esse declínio do consumo de matéria seca induz a mobilização de reservas corporais antes do parto, tendo como consequência o aumento dos níveis de NEFA e β-hidroxibutirato (BHBA; BELL, 1995). O aumento dos níveis sanguíneos de NEFA e BHBA caracteriza o BEN, período em que as demandas de energia são maiores que o aporte nutricional e levam a mobilização de reservas corporais (DRACKLEY; CARDOSO, 2014). Nessa fase, o aumento de NEFA e BHBA tanto no sangue quanto no fluido do oviduto, pode afetar o oócito e os espermatozoides.

Os NEFA estão presentes nos fluidos biológicos na sua forma livre e, são definidos como ácidos graxos que não estão ligados a qualquer fração lipídica (VAN HOECK et al., 2014). Eles podem ser classificados como saturados ou insaturados, sendo que os ácidos graxos insaturados podem ser monoinsaturados (MUFA) ou poliinsaturados (PUFA), dependendo do número de ligações duplas que possuem entre os pares de carbono. Os principais ácidos graxos aumentados no período de transição em vacas leiteiras em BEN são o ácido monoinsaturado oleico (OA, C18:1), e os ácidos saturados palmítico (PA, C16:0) e esteárico (SA, C18:0; VAN HOECK et al., 2014).

Durante o BEN, os níveis de NEFA dependem da intensidade da deficiência energética (BELL, 1995), causando efeitos no sistema imunológico e predispondo a doenças metabólicas. Níveis elevados de NEFA induzem o acúmulo de lipídios nos hepatócitos que, associado à baixa disponibilidade de glicose e redução do oxalacetato, impedem a oxidação completa das moléculas de acetil-CoA, oriundas da β-oxidação

(BELL, 1995). O acúmulo de acetil-CoA é convertido em corpos cetônicos (acetoacetato, β-hidroxibutirato e acetona) ou pode ser substrato para a síntese de acetato (MULLIGAN; DOHERTY, 2008). O BHBA é a cetona mais abundante e sua concentração sanguínea é um indicador de cetose em vacas (LEBLANC, 2010). As condições metabólicas das vacas no BEN passam para um estado de catabolismo, causando aumento plasmático do hormônio do crescimento (GH), das concentrações de NEFA, diminuição das concentrações do IGF1, insulina e glicose (KAWASHIMA et al., 2012). Além da queda nos níveis de insulina, a sua sensibilidade também é afetada, gerando a resistência à insulina. A RI é a condição em que as células falham em responder às ações normais do hormônio insulina (VAN HOECK et al., 2014).

O aumento nas concentrações de NEFA é um fator chave para o desequilíbrio metabólico, disfunção celular e apresentação de patologias com a RI (JORRITSMA et al., 2004; LEROY et al., 2004, 2005; VANHOLDER et al., 2005). Por isso, as concentrações sanguíneas de BHBA e NEFA podem ser utilizadas como ferramentas para avaliação da intensidade do BEN e da predisposição à ocorrência de doenças. Sendo que a mensuração de NEFA demonstra a intensidade de mobilização de reservas corporais, e a mensuração do BHBA demonstra o grau de oxidação dos ácidos graxos mobilizados (BELL, 1995; LEBLANC, 2010). Assim, essas ferramentas podem ser utilizadas para monitorar o *status* metabólico das vacas leiteiras durante o período crítico do pós-parto e, acompanhar as alterações reprodutivas que podem aparecer nesse período.

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, 2016; SORDILLO; RAPHAEL, 2013). Assim, durante o período de BEN, os processos intensificados de oxidação de NEFA resultam no aumento da produção de espécies reativas a oxigênio (ROS - do inglês, *Reactive Oxygen Species*) e no desenvolvimento de estresse oxidativo (BIONAZ et al., 2007). Análise de expressão gênica e ensaios funcionais de oócitos, células do cumulus e blastocistos expostos aos NEFA apontaram a importância de vias relacionadas ao metabolismo oxidativo, *status* redox e estresse oxidativo (VAN HOECK et al., 2013), havendo um crescente reconhecimento que o estresse oxidativo pode estar implicado em muitas condições fisiopatológicas de vacas leiteiras que incluem distúrbios reprodutivos (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993). O estresse oxidativo é

uma das principais vias pelas quais as quantidades elevadas de NEFA afetam oócitos e embriões bovinos (VAN HOECK et al., 2013). Uma vez que os espermatozoides expostos a elevadas concentrações de NEFA apresentam motilidade reduzida (DESMET et al., 2018), nossa hipótese é que essa alteração pode estar ligada a alterações no *status* redox dos espermatozoides.

2.2. Resistência à insulina

A RI é definida como um estado em que uma concentração normal de insulina induz uma resposta biológica diminuída nos tecidos sensíveis à insulina (RONALD KAHN, 1978). Além disso, a RI pode ser subdividida com base em duas características distintas: diminuição da sensibilidade à insulina e diminuição da responsividade à insulina. Sendo que, o efeito máximo da insulina determina a resposta à insulina dos tecidos. Num primeiro momento há redução da sensibilidade à insulina no qual uma maior concentração de insulina é necessária para atingir a resposta máxima nos tecidos sensíveis, logo após se o quadro de RI se manter há redução da responsividade à insulina no qual o tecido produz uma resposta menor ao hormônio (MUNIYAPPA et al., 2008; RONALD KAHN, 1978). As vacas leiteiras apresentam RI periférica no final da gestação e início da lactação (BARUSELLI et al., 2016; DE KOSTER; OPSOMER, 2013; KERESTES et al., 2009a). Essa condição de resistência à insulina é necessária para garantir um suprimento de glicose suficiente para o útero gestante e a glândula mamária em lactação em apoio ao crescimento do terneiro, tanto no pré-natal quanto no pós-natal (BELL; BAUMAN, 1997).

As concentrações de insulina e a resposta dos tecidos ao seu estímulo podem prejudicar a fertilidade das vacas leiteiras. Condições adversas, como balanço energético negativo que leva ao estado de RI, podem afetar a foliculogênese levando a problemas subsequentes na competência dos oócitos no momento da ovulação (BARUSELLI et al., 2016). Há uma gama de estudos avaliando os efeitos da RI em diversos tecidos, como: tecido adiposo (KARIS et al., 2020; SAKODA et al., 2000), hepatócitos (LU et al., 2017), células da granulosa (XU et al., 2019; YUAN et al., 2014), células da teca (ZHAO et al., 2011) e oviduto (FENWICK et al., 2008), e em processos como o desenvolvimento

folicular (LLEWELLYN et al., 2007), ovulação (HACKBART et al., 2013) e no embrião pré-implantação (SINCLAIR, 2010). Contudo, carecemos de estudos voltados para os efeitos da RI no tecido uterino.

A insulina é um dos hormônios que promove metabolismo adequado, equilíbrio energético e manutenção do peso corporal normal (KANZAKI; PESSIN, 2001). A ligação da insulina a subunidade α do seu receptor (INSR) ativa a atividade quinase da subunidade β do receptor (PATTI; KAHN, 1998). Que fosforila 10 substratos proteicos em tirosina, sendo o mais importante o substrato do receptor de insulina 1 (IRS1; WHITE, 1998). As proteínas IRS, os principais substratos do receptor de insulina, funcionam como andaimes de sinalização que propagam a ação da insulina através da criação de sítios de reconhecimento de moléculas homólogas a Src 2 (SH2; KHAN; PESSIN, 2002; LE ROITH; ZICK, 2001). Onde destaca-se as três principais vias de sinalização que são propagadas em resposta à ativação da INSR: fosfatidilinositol 3-quinase (PI3K), MAP quinase e a via Cbl/CAP (SALTIEL; KAHN, 2001). A cascata MAP quinase leva a um aumento da proliferação celular, enquanto a cascata Cbl/CAP media o transporte de glicose pela membrana plasmática (LODHI et al., 2007).

E a cascata PI3K é ativada para desencadear as funções metabólicas da insulina, como por exemplo enviar um sinal para o transportador de glicose sensível à insulina (GLUT4; WHITE, 1998). A cascata PI3K é um dos efetores downstream mais bem caracterizados de proteínas IRS (CANTLEY, 2002). Ele se associa a proteínas IRS fosforiladas por Tyr após a estimulação da insulina e catalisa a formação de fosfatidilinositol-3,4,5-trifosfato, que estimula a atividade da quinase dependente de fosfoinositídeo (PDK-1) e inicia a ativação de seus efetores downstream da proteína quinase B (PKB, Akt), alvo mamífero de rapamicina (mTOR), e p70 S6 quinase (S6K1), levando ao transporte de glicose e síntese de proteínas e glicogênio (BANDYOPADHYAY et al., 1997; WANG et al., 1999).

Há uma variedade de agentes e condições que induzem RI nos tecidos, como TNFα, ácidos graxos livres e estresse celular, que ativam várias proteínas quinases que têm como alvo elementos ao longo da via de sinalização da insulina (WELLEN; HOTAMISLIGIL, 2005). O IRS1 é importante para que ocorra a sinalização de insulina corretamente, uma vez que foi observado de camundongos knockout para IRS1 apresentam resistência à

insulina e retardo de crescimento (SAAD et al., 1994). Alterações na fosforilação das proteínas IRS inibem a sua função e interferem com a sinalização da insulina, levando assim ao desenvolvimento de um estado de RI (BOURA-HALFON; ZICK, 2009). Embora o útero possa não ser um tecido alvo clássico para a ação da insulina, existem evidências que sugerem que o INSR e seus alvos downstream contribuem para a regulação da função reprodutiva (ZHANG et al., 2016).

2.4. Hipocalcemia

O cálcio é um mineral importante para diversos processos biológicos. O papel do cálcio na contração muscular, inflamação e função imune, tornam este mineral um componente chave na ocorrência de alguns transtornos metabólicos e infecciosos no pós-parto (KIMURA; REINHARDT; GOFF, 2006). Os diversos papéis fisiológicos do cálcio, como na mineralização óssea, coagulação, potenciais de ação cardíaca, sinalização celular como segundo mensageiro e contratilidade muscular, requerem uma regulação extremamente precisa do cálcio nos fluidos intracelulares e extracelulares (WILKENS et al., 2020). A hipocalcemia grave pode levar à paresia ou morte. Mesmo pequenos desequilíbrios na homeostase do cálcio podem ter efeitos prejudiciais nas funções muscular, endócrina e imunológica, o que pode explicar os efeitos negativos da hipocalcemia na saúde e produção de vacas leiteiras.

A função do cálcio na musculatura desempenha um papel crucial no parto e na função gastrointestinal de vacas periparturientes. Sua presença extracelular é fundamental para a excitabilidade de neurônios e células musculares, especialmente no músculo liso, onde é essencial para o acoplamento excitação-contração (HAN; TRINIDAD; SHI, 2015). A hipocalcemia pode ter efeitos negativos na contratilidade e motilidade dos tratos reprodutivo e gastrointestinal devido ao papel do cálcio nessas funções (AL-EKNAH; NOAKES, 1989; DANIEL, 1983; HEPPELMANN et al., 2015; JORGENSEN et al., 1998). Além disso, o cálcio atua como segundo mensageiro em diversas funções endócrinas e imunológicas de vacas periparturientes. Em células imunes e nas células β pancreáticas, sua função como segundo mensageiro pode explicar a redução nas

concentrações de insulina e a ativação de neutrófilos observadas em vacas leiteiras com subclínica hipocalcemia (MARTINEZ et al., 2014).

No início da lactação, a homeostase do cálcio é perturbada por um redirecionamento maciço de cálcio para a produção do colostro e de leite (GOFF; EL-SAMAD; KHAMMASH, 2000), gerando hipocalcemia pós-parto. A hipocalcemia pode ser clínica, conhecida como a febre do leite, ou subclínica. A febre do leite é um problema reconhecido há décadas, sendo sabido quase a um século que é causada por hipocalcemia. Surpreendentemente, apesar dos aumentos significativos na produção de leite por vaca nas últimas décadas, a incidência da febre do leite não aumentou (REINHARDT et al., 2011). No entanto, a hipocalcemia subclínica ainda é uma ocorrência persistente. Dado que diversos processos fisiológicos vitais dependem do cálcio, os efeitos da hipocalcemia subclínica têm um impacto negativo significativo na saúde e na produtividade das vacas leiteiras.

A hipocalcemia subclínica é definida como concentração sanguínea de Ca abaixo do limite desejado, porém as vacas não apresentam sinais clínicos (COUTO SERRENHO et al., 2021). Assim, testes para medir a concentração de cálcio no sangue são necessários para o diagnóstico. A hipocalcemia subclínica é uma condição prevalente associada ao comprometimento da saúde e do desempenho pós-parto do gado leiteiro, uma vez que os baixos níveis séricos de cálcio persistem por vários dias (COUTO SERRENHO et al., 2021). Isso muitas vezes dificulta avaliar o impacto da hipocalcemia no desenvolvimento de doenças, pois é necessário diferenciar se a redução da calcemia é a causa da maior incidência de enfermidades ou, se a enfermidade é a responsável pela redução dos níveis séricos de cálcio (COUTO SERRENHO et al., 2021).

A hipocalcemia subclínica, embora menos óbvia, é muito mais comum e pode ter efeitos significativos na saúde e na produção das vacas em nível de rebanho. Estudos recentes que utilizam diferentes limiares diagnósticos para o cálcio total sérico ou plasmático, variando de 2,0 a 2,15 mmol/L nas 24 e 48 horas pós-parto, indicam que esse distúrbio pode afetar até 50% das vacas nesse período (MARTINEZ et al., 2012; REINHARDT et al., 2011). Essas pesquisas, juntamente com outras, evidenciam que vacas com hipocalcemia subclínica têm de 3 a 5 vezes mais chances de desenvolver doenças pós-parto e 50% mais probabilidade de serem removidas do rebanho no início da

lactação, em comparação com vacas com níveis normais de cálcio sérico (CHAPINAL et al., 2011a; RODRÍGUEZ; ARÍS; BACH, 2017; VENJAKOB et al., 2018).

A hipocalcemia é frequentemente referida como uma condição de porta de entrada para resultados indesejáveis. Concentrações sanguíneas de cálcio abaixo de vários limites são relatadas como um fator de risco para cetose (RODRÍGUEZ; ARÍS; BACH, 2017), abomaso deslocado (CHAPINAL et al., 2011b; MARTINEZ et al., 2018; RODRÍGUEZ; ARÍS; BACH, 2017) e distúrbios uterinos (MARTINEZ et al., 2012, 2018; RODRÍGUEZ; ARÍS; BACH, 2017). Embora essas associações sejam descritas na literatura, o emaranhado de interações entre alterações metabólicas periparto e inflamação torna um desafio estudar a relação entre hipocalcemia e saúde, produção de leite e desempenho reprodutivo.

A diminuição das concentrações de cálcio circulantes no pós-parto é um fator de risco para distúrbios uterinos como a involução uterina tardia (HEPPELMANN et al., 2015), prolapso uterino (RISCO; REYNOLDS; HIRD, 1984), retenção de placenta (MELENDEZ et al., 2004), metrite e endometrite (MARTINEZ et al., 2012; RIBEIRO et al., 2013). Portanto, a hipocalcemia pode contribuir para a RI uterina, uma vez que vacas com hipocalcemia subclínica apresentam maior predisposição para desenvolverem alterações inflamatórias uterinas (MARTINEZ et al., 2012). E por sua vez essa inflamação uterina pode alterar a via de sinalização a insulina (HIRABARA et al., 2012; KHODABANDEHLOO et al., 2016). Por isso, nossa hipótese é que as alterações inflamatórias provenientes da hipocalcemia, causa alterações na via de sinalização à insulina uterina no pós-parto de vacas leiteiras.

ARTIGO 1

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Motility, oxidative status, and morphology of frozen-thawed bovine semen are not impacted by fatty acid exposure in vitro.

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Motility, oxidative status, and morphology of frozen-thawed bovine semen are not impacted by fatty acid exposure in vitro.

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Abstract

While sperm migrate within the reproductive tract of cows experiencing negative energy balance, they come into contact with elevated concentrations of non-esterified fatty acids (NEFA). For this reason, this study aimed to investigate the effects of three different NEFA - palmitic acid (PA), stearic acid (SA), and oleic acid (OA) - on bovine sperm motility, kinetic parameters, oxidative status, and morphology. Frozen thawed semen samples from Bos taurus bulls were incubated with varying concentrations of each fatty acid, and the sperm's characteristics were analyzed at different time points. Computer-Assisted Sperm Analysis (CASA) was employed to assess sperm motility and kinetic parameters. Concurrently, the production of reactive oxygen species (ROS) and total antioxidant capacity were measured to determine the oxidative status. Additionally, sperm morphology was evaluated. In Experiment 1, different concentrations of PA did not show significant effects on total motility, progressive motility, or any kinetic parameters analyzed. Similarly, PA did not have a significant impact on oxidative status or sperm morphology. In Experiment 2, SA at various concentrations did not lead to significant changes in total motility, progressive motility, or any kinetic parameters evaluated. Furthermore, SA did not affect oxidative status or sperm morphology. In Experiment 3, the concentrations of OA used did not result in significant changes in total motility, progressive motility, or any kinetic parameters studied. Likewise, OA did not induce any alterations in oxidative status or sperm morphology. Overall, the results from all three experiments indicate that PA, SA, and OA, at the in vitro conditions and tested concentrations, do not exert detrimental effects on bovine sperm function and morphology. These results provide insights that contribute to our understanding of how fatty acids can impact the reduction of fertility rates in cows facing negative energy balance. This, in turn, lays the foundation for additional critical investigations in this area. Further studies are necessary to validate these findings in vivo.

1. INTRODUCTION

Maternal metabolic diseases have been associated with reduced fertility, particularly in postpartum dairy cows (Leroy et al., 2015). Metabolic stress in dairy cows can lead to negative energy balance (NEB), a multifactorial issue with implications for various physiological systems, including reproduction. NEB can impact reproductive efficiency by influencing the microenvironment of the follicular (Leroy et al., 2005), oviductal (Fenwick et al., 2008), and uterine systems (Wathes et al., 2009). During the NEB period, there is an increase in plasma concentrations of non-esterified fatty acids (NEFA) and ketone bodies, while levels of insulin-like growth factor 1 (IGF-1), glucose, and insulin in the blood decrease (Kawashima et al., 2012). NEFA refers to fatty acids present in biological fluids in their free form, not associated with lipid fractions (Van Hoeck et al., 2014). In dairy cows during the transition period, the main NEFA that increase are monounsaturated oleic acid (OA, C18:1), saturated palmitic acid (PA, C16:0), and saturated stearic acid (SA, C18:0) (Van Hoeck et al., 2014).

Elevated NEFA concentrations in plasma also result in similar changes in other fluids, such as follicular fluid (Leroy et al., 2005) and oviductal fluid in cows (Jordaens et al., 2017). The uterine and oviductal microenvironments play pivotal roles in sperm selection, storage, motility, and fertilization processes (Coy et al., 2012; Ghersevich et al., 2015; Holt & Fazeli, 2010), and any alterations in these environments can impact sperm viability. Research on boar sperm by Zhu et al., (2020) indicates that NEFA (OA and PA) serve as an energy source via β-oxidation, promoting increased motility, mitochondrial activity, and reduced apoptosis. Similar observations have been made in bovine spermatozoa when testing PA and SA, showing increased linear sperm motility due to NEFA serving as an energy source (Islam et al., 2021).

Despite these promising findings, the exact mechanism through which NEFA protects progressive motility and sperm viability remains unclear. Conflicting reports in the literature regarding the effects of NEFA on sperm function may be attributed to variations in the type of NEFA used, their concentrations, and the exposure time. For instance, exposure to OA for 4 hours has been shown to increase the motility, viability, and acrosome reaction of boar sperm (Hossain et al., 2007). Conversely, exposure to OA and PA did not affect motility but reduced mouse sperm fertility (Quinn & Whitfingham,

1982). Additionally, exposure to SA did not affect motility or stimulate the acrosome reaction in hamster spermatozoa (Meizel & Turner, 1983).

Considering the diverse outcomes reported in various species, the question arises as to how NEFA may influence the function and fertility of bovine spermatozoa. In this study, we hypothesize that exposure to NEFA may affect sperm physiology and oxidative state. To investigate this hypothesis, we examined the influence of three NEFA - oleic, palmitic, and stearic acids - on sperm kinetics, oxidative state, and bovine sperm morphology. The aim of this research is to shed light on the potential impact of NEFA on bovine reproductive outcomes, contributing to a deeper understanding of fertility-related challenges in dairy cows with negative energy balance.

2. MATERIAL AND METHODS

2.1. Experimental design

This study employed semen samples from four Bos taurus bulls with confirmed fertility, which were housed at Renascer Biotecnologia, a breeding center. These semen samples were generously provided by the breeding center, having undergone full processing. Authors had no direct involvement with the animals or the sample processing procedures until after the samples had been frozen. It should be mentioned that, in accordance with Brazilian regulations, the ethics committee does not evaluate the procedures for such cases. It is essential to note that Renascer Biotecnologia adhered to all ethical requirements for international semen marketing.

Spermatozoa were selected and divided into NEFA treatment groups using the Percoll gradient, following the experimental setup. The samples were incubated at 37ºC with a concentration of 4 x 106 spermatozoa/mL for 3 hours. The effect of NEFA on spermatozoa was analyzed by evaluating sperm kinetics at 0, 30, 60, 90, 120, 150, and 180 minutes. Oxidative status was evaluated at 90 and 180 minutes by assessing the production of reactive oxygen species (ROS) and total antioxidant capacity. Sperm morphology analysis was performed at 180 minutes. The literature still lacks data on the exact concentrations of each NEFA present in the uterine and oviductal environment. For the study, we considered the concentrations of NEFA described by Leroy et al., (2005) in

serum, in which the authors describe the variation from 22.3 μM to 200 μM depending on the type of NEFA (PA, SA, or OA). Based on these data, we tested a concentration-response curve of 0, 50, 100, and 200 μM for each NEFA.

In experiment 1, the effects of PA on sperm were evaluated, using four treatment groups: control (0 μM), 50 μM, 100 μM, and 200 μM PA. In experiment 2, the effects of SA were evaluated at the following concentrations: control (0 μM), 50 μM, 100 μM, and 200 μM of SA. Experiment 3 evaluated the effects of OA at concentrations of control (0 μM), 50 μM, 100 μM, and 200 μM of OA. Each experiment was repeated three times.

2.2. Chemicals

The PA (ref. 10006627) and SA (ref. 10011298) used in this study were acquired from Cayman Chemical, and OA (O1008) was obtained from Sigma-Aldrich Chemical Company. NEFA was diluted in pure ethyl alcohol (Sigma E7023) at concentrations of 80 mM of PA, 70 mM of SA, and 70 mM of OA. The dilutions were performed in sp-TALP medium to produce concentrations of 50, 100, and 200 μM of PA in experiment 1, SA in experiment 2, and OA in experiment 3.

2.3. Sperm preparation

For this study, we used frozen semen, because artificial insemination has been widely used in dairy farming and we need to understand how cow-related factors can interfere with frozen semen. For this study, four semen straws were used per replicate, one from each bull. Therefore, we used three semen straws per bull totaling twelve straws per experiment. We chose to make a semen pool to reduce a possible individual effect on the experiment since our goal was to evaluate the effect of NEFA and we would not be able to isolate the effect of the individual.

For the experiment, a semen straw from each bull was thawed at 37°C for 30 seconds, and the samples were mixed to create a pooled sperm sample. The samples were homogenized, spermatozoa were selected, and the diluent from semen was removed, using a discontinuous Percoll density gradient. A Percoll solution was prepared for the formation of 90% and 45% solutions with a modified Talp-Fert medium. Briefly, 300 μL

of thawed semen was placed at the top of the gradient, and the tubes were centrifuged for 5 minutes at 2,200 x g. The pellet was then resuspended in sp-TALP and centrifuged for 1 minute at 2,200 x g. Finally, the pellet was collected to determine the final sperm concentration and exposed to the treatment groups in sp-TALP with the addition of NEFA, with a dose of 4 x 106 sperm/mL.

To assess the effects of NEFA on sperm kinetics, sperm were distributed into treatment groups according to the experiment and incubated for 30, 60, 90, 120, 150, and 180 minutes at 37°C. At 90 and 180 minutes, oxidative status was determined. Additionally, at 180 minutes, sperm morphology analysis was performed.

2.4. Sperm kinetics

Sperm kinetic evaluations were conducted using the SCA 6.6.15 system (Sperm Class Analyser, Microptic). The CASA settings were followed in accordance with the manufacturer's instructions. Briefly, an aliquot of 5 µL of the semen sample was placed on a preheated microscope slide (37°C) and covered with a preheated 15 x 15 mm slide. The parameters for sperm kinetics were analyzed in 5 different fields using an objective of 10x positive phase contrast. The fields were analyzed by capturing 25 frames/field at a rate of 25 frames/s. The specific parameters assessed were as follows: head area: 25-70 µm²; velocity limit for slow spermatozoa: 10 µm/s, velocity limit for medium spermatozoa: 25 µm/s, velocity limit for fast spermatozoa: 50 µm/s, minimal straightness for progressive spermatozoa: 70%, and the maximal percentage of linearity: 50%. The following parameters were analyzed: total motility (%); progressive motility (%); curvilinear velocity (VCL, µm/s); straight line velocity (VSL, µm/s); average path velocity (VAP, µm/s); linearity (LIN, %); straightness index (STR, %); the amplitude of the lateral displacement of the head (ALH, µm); cross beat frequency (BCF, Hz); Wobble (WOB, %); and hyperactivity (%, spermatozoa with VCL >35µm/s, ALH >2.5 µm and STR >85%), as proposed by Mortimer, (2000).

2.5. Semen oxidative status

To evaluate oxidative status, the samples underwent two assessments: ROS production and total antioxidant capacity. The ROS production was determined using a spectrofluorimetric method (Loetchutinat et al., 2005). In brief, the samples were incubated in the dark with 5 ml of 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA). Upon oxidation, DCHF-DA is converted into fluorescent 2',7'- dichlorofluorescein (DCF). The oxidation of DCHF-DA to DCF allowed the detection and measurement of intracellular ROS concentrations. The fluorescence intensity emitted at 520 nm (excitation at 488 nm) was monitored 60 minutes after adding DCF-DA. The results are expressed in units of fluorescence (UF).

For the assessment of total antioxidant capacity, the ferric reducing antioxidant potential (FRAP) technique was employed, based on the iron ion's ability to be reduced to the ferrous state at low pH (Benzie & Strain, 1996). The formation of an intense, blue-colored product was quantified using spectrophotometry at 593 nm, employing a standard curve with ascorbic acid as an antioxidant. The results are expressed as micrograms of ascorbic acid equivalents.

2.6. Sperm morphology

A semen aliquot was fixed in 4% formaldehyde, and the morphology of two hundred spermatozoa per slide was determined using differential interference contrast microscopy with an oil immersion objective at 1,000x magnification. Morphological defects were classified according to the region of the spermatozoa. The classification system identified five general categories: morphologically normal, head defects, midpiece defects, tail defects, and cytoplasmic droplets, including the subcategories proximal and distal (Burns et al., 2013; Fordyce et al., 2006).

2.7. Statistical analysis

The effect of treatment, time, and their interaction on sperm kinetics was evaluated using mixed models with a repeated measurement statement followed by Tukey HSD post-hoc test to compare all possible pairs of treatment groups at each specific time-point. Different covariance structures were tested for each model, and the one with the smallest

Akaike Information Criteria (AIC) was selected as the best fit. The differences in oxidative status variables were analyzed by ANOVA followed by Tukey HSD post-hoc test. The percentage of sperm defects and normal sperm cells was evaluated using the Kruskal-Wallis test. To assess the normal distribution of continuous data and model residuals, the Shapiro-Wilk test was used. All statistical analyses were performed using JMP Statistical software (SAS Institute Inc.), and a significance level of $P < 0.05$ was used to determine statistical significance.

3. RESULTS

3.1. Experiment 1: palmitic acid

The incubation of semen with different concentrations of PA did not lead to significant changes in total motility and progressive motility (Figure 1, $P > 0.05$). Furthermore, none of the kinetic parameters assessed by CASA, including VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF, showed any significant alterations in response to PA concentrations at any of the analyzed time points (Figure 2a-h; $P > 0.05$).

To investigate the effects of PA on the oxidative status of bovine spermatozoa, we evaluated the production of reactive oxygen species and total antioxidant capacity. Two measurements were conducted, one at 90 minutes of incubation (Figure 3a-b) and the other at the end of incubation (180 minutes, Figure 3c-d). However, no statistically significant differences were observed in the evaluated parameters at either time point. Similarly, PA did not have any significant impact on normal sperm morphology, total defects, head defects, intermediate part defects, tail defects, or cytoplasmic droplets (Table 1).

The findings from Experiment 1 indicate that the different concentrations of PA tested did not exert significant effects on sperm motility, kinetic parameters, oxidative status, or sperm morphology in bovine semen.

3.2. Experiment 2: stearic acid

In this experiment, none of the concentrations of SA used showed any significant effects on total motility and progressive motility at any of the analyzed time points (Figure 4). Similarly, the concentrations of SA did not lead to any significant alterations in the kinetic parameters evaluated, including VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF (Figure 5 a-h; $P > 0.05$).

Moreover, SA did not induce changes in the oxidative status of bovine spermatozoa. The production of ROS and FRAP were analyzed at 90 minutes and 180 minutes of incubation, respectively (Figure 6 a-b and c-d). However, there were no statistically significant differences in the evaluated parameters at either time point.

Furthermore, the different concentrations of SA used in the experiment did not affect normal sperm morphology, total defects, head defects, midpiece defects, tail defects, or cytoplasmic droplets (Table 2).

The results from Experiment 2 indicate that the various concentrations of SA tested did not have significant effects on sperm motility, kinetic parameters, oxidative status, or sperm morphology in bovine semen.

3.3. Experiment 3: oleic acid

In this experiment, the concentrations of OA used did not result in significant changes in total motility or progressive motility of the spermatozoa (Figure 7, $P > 0.05$). Furthermore, there were no alterations in any of the kinetic parameters evaluated, including VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF, at the analyzed time points (Figure 8 a-h; $P > 0.05$).

Regarding the oxidative state, none of the OA concentrations significantly affected the production of ROS or the FRAP when evaluated after 90 minutes (Figure 9 a-b) and 180 minutes of incubation (Figure 9 c-d). Similarly, OA did not induce any changes in normal sperm morphology, total defects, head defects, intermediate part defects, tail defects, or cytoplasmic droplets (Table 3).

Overall, the results from Experiment 3 demonstrate that the various concentrations of OA tested did not have significant effects on sperm motility, kinetic parameters, oxidative status, or sperm morphology in bovine semen.

4. DISCUSSION

In this study, we set out to explore the potential effects of three distinct fatty acids - PA, SA, and OA - on the motility, kinetic parameters, oxidative status, and sperm morphology of bovine spermatozoa. Understanding the impact of these fatty acids on sperm function is crucial, especially considering the question regarding whether the oviduct of cows in a NEB, with increased levels of fatty acids (Jordaens et al., 2017), could influence sperm function. Addressing the implications of NEB in cows, we have found that none of the three fatty acids studied exhibited interference with the characteristics assessed through CASA or oxidative status. These findings provide significant reassurance for reproductive processes and future research in cattle under these specific conditions.

There are reports in the literature of NEFA causing changes in the oxidative stress of other cells, hence the importance of evaluating the effects on spermatozoa. In bovine endometrial cells, OA and SA were responsible for lipid accumulation in these cells, and the association of three NEFA (PA, SA, and OA) increased ROS production when compared to the control group (Ferst et al., 2021). In human hepatoma HepG2 cells with high glucose in culture, PA caused severe oxidative stress, increasing ROS production, lipids, proteins, and DNA damage to the cells (Alnahdi et al., 2019). However, these effects on the oxidative state were not observed in our study with bovine spermatozoa. The mechanism by which NEFA can cause oxidative stress in cells is not yet well defined, so more studies are needed in this area.

The effects of PA, in the first experiment, were assessed. Interestingly, none of the concentrations of PA used in this study showed any significant alterations in total motility, progressive motility, or any of the kinetic parameters evaluated. These findings suggest that PA, at the tested concentrations, does not influence sperm movement or velocity. A recent study demonstrated that rapid incubation (30 minutes) with PA increases the progressive motility of bovine spermatozoa (Islam et al., 2021) and after incubation for 3 hours in boar spermatozoa (Zhu et al., 2020). Both studies demonstrate that PA is used as an energy source by β-oxidation (Islam et al., 2021; Zhu et al., 2020). On the other hand, PA does not alter sperm motility in long incubations from 1 to 4 hours,

in boar (Am-in et al., 2011; Hossain et al., 2007), human (Aksoy et al., 2006; Siegel et al., 1986), and mouse spermatozoa (Quinn & Whitfingham, 1982). Moreover, the oxidative status of the spermatozoa, measured by ROS production and total antioxidant capacity, remained unchanged in the presence of PA for 90 and 180 minutes. When evaluating the generation of ROS in bull spermatozoa stored for up to 7 days, it is observed that the PA reduces ROS on days 1 and 3 compared to the control (Kiernan et al., 2013). Furthermore, there were no observable effects on normal sperm morphology or any specific defects in the sperm structure. These results collectively indicate that PA, within the concentrations tested, does not have a detrimental impact on bovine sperm function and morphology.

The focus on SA was studied in the second experiment. Similarly, to PA, SA did not cause any significant alterations in total motility, progressive motility, or the various kinetic parameters evaluated. These findings align with those observed in other species, including boar (Am-in et al., 2011; Hossain et al., 2007), hamster (Meizel & Turner, 1983), and human (Aksoy et al., 2006; Siegel et al., 1986). In these studies, incubation was carried out for 1 to 4 hours using similar methodologies, and no significant alterations in sperm motility were noted compared to the control group. The sperm's oxidative status was also unaffected, as there were no changes in ROS production or total antioxidant capacity. Furthermore, SA did not lead to any abnormal sperm morphology or defects in the head, midpiece, tail, or cytoplasmic droplets. These findings demonstrate that SA, like PA, does not negatively affect bovine sperm functionality and morphology.

The effects of OA on bovine spermatozoa were investigated in the third experiment. Once again, no significant changes were observed in total motility, progressive motility, or any of the kinetic parameters studied. A study involving boar spermatozoa revealed that incubation for 3 hours with OA results in increased total and progressive motility as well as VSL (Zhu et al., 2020). The authors attributed these changes in parameters to the utilization of OA as an energy source through β-oxidation (Zhu et al., 2020). Furthermore, Hossain et al. (2007) reported that in addition to enhancing motility, OA also triggers the acrosome reaction in boar sperm. Contrarily, studies in humans have demonstrated that OA either has no impact (Aksoy et al., 2006) or may even decrease total motility (Siegel et al., 1986). The oxidative status of the spermatozoa, as indicated by ROS production and FRAP, remained unaltered in the presence of OA. When assessing the generation of

ROS in bull spermatozoa stored for a duration of up to 7 days, it becomes evident that OA postpones the peak of ROS production to day 3, in contrast to the control group where the peak is observed on day 1 (Kiernan et al., 2013). Our study marks the initial assessment of the oxidative condition of bovine spermatozoa after exposure to OA. Additionally, there were no adverse effects on sperm morphology, including no changes in normal sperm structure or defects in any specific regions of the sperm.

Overall, the findings from all three experiments suggest that PA, SA, and OA, at the concentrations used in this study, do not have any detrimental effects on the motility, kinetic parameters, oxidative status, or morphology of bovine spermatozoa. It's important to emphasize that this study primarily concentrated on the immediate effects of these fatty acids. The assessments were carried out after 90 minutes and 180 minutes of incubation, which is roughly around half the time it takes for a sperm cell to penetrate an oocyte in physiological terms (Wilmut & Hunter, 1984).

However, it is crucial to acknowledge certain limitations in our study. Firstly, the investigation was confined to a specific set of fatty acids, and there may be other compositions worth exploring, such as linoleic, palmitoleic, and myristic acids, which are also elevated in NEB (Leroy et al., 2005). Additionally, our experiments were carried out in vitro, and in vivo studies are imperative to validate these findings in a real-life setting, specifically to assess the impact of the cow's uterine and oviduct conditions in a NEB on spermatozoa for fertilization. This study represents one of the initial efforts focusing on the effects of fatty acids on bovine spermatozoa (Desmet et al., 2018; Islam et al., 2021; Kiernan et al., 2013).

5. CONCLUSION

In conclusion, our comprehensive study provides evidence that PA, SA, and OA, when tested at the specified concentrations under in vitro conditions, exert no detrimental impact on bovine sperm activity and morphology as assessed by CASA or oxidative status. These findings contribute valuable insights to the growing comprehension of how fatty acids impact low fertility rates in cows with NEB, thus paving the way for future research in this critical area.

AUTHOR CONTRIBUTIONS

Camila Cupper Vieira: Conceptualization, Methodology, Investigation, Writing - Original Draft.

Daniele Missio: Conceptualization, Writing - Review & Editing, Project administration.

Daniela dos Santos Brum: Methodology, Investigation, Resources.

Rafaela Dalmolin Menezes: Methodology, Investigation.

Francielli Weber Santos Cibin: Methodology, Investigation, Resources.

Fernando Silveira Mesquita: Investigation, Writing - Review & Editing.

Paulo Bayard Dias Gonçalves: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition.

Rogério Ferreira: Conceptualization, Methodology, Formal analysis; Investigation, Resources; Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author, Ferreira, R. upon reasonable request.

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Legends:

Figure 1 - Total motility (a) and progressive motility (b) of bovine spermatozoa at 0, 30, 60, 90, 120, 150, and 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of palmitic acid (PA). The data are expressed as a percentage means ± SEM of three repetitions.

Figure 2 - Curvilinear velocity (VCL; a), straight-line velocity (VSL; b), mean path velocity (VAP; c), linearity (LIN; d), straightness (STR; e), wobble (f), the amplitude of lateral head displacement (ALH, g), and beat cross frequency (BCF, h) of bovine spermatozoa at 0, 30, 60, 90, 120, 150, and 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of palmitic acid (PA). The data are expressed as means ±SEM of three replications. $P > 0.05$

Figure 3 – Production of reactive oxygen species (ROS) and ferric reducing potential (FRAP) of bovine spermatozoa after 90 minutes (a, b) and 180 minutes (c, d) of in vitro exposure without or with 50, 100, or 200 μM of palmitic acid (PA). The data are expressed as means ± SEM of three repetitions. $P > 0.05$

Figure 4 - Total motility (a) and progressive motility (b) of bovine spermatozoa at 0, 30, 60, 90, 120, 150, and 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of stearic acid (SA). The data are expressed as a percentage means ± SEM of three repetitions.

Figure 5 - Curvilinear velocity (VCL; a), straight-line velocity (VSL; b), mean path velocity (VAP; c), linearity (LIN; d), straightness (STR; e), wobble (f), the amplitude of lateral head displacement (ALH, g), and beat cross frequency (BCF, h) of bovine spermatozoa at 0, 30, 60, 90, 120, 150, and 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of stearic acid (SA). The data are expressed as means ±SEM of three replications. $P > 0.05$

Figure 6 – Production of reactive oxygen species (ROS) and ferric reducing potential (FRAP) of bovine spermatozoa after 90 minutes (a, b) and 180 minutes (c, d) of in vitro exposure without or with 50, 100, or 200 μM of stearic acid (SA). The data are expressed as means ± SEM of three repetitions. $P > 0.05$

Figure 7 - Total motility (a) and progressive motility (b) of bovine spermatozoa at 0, 30, 60, 90, 120, 150, and 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of oleic acid (OA). The data are expressed as a percentage means ± SEM of three repetitions.

Figure 8 - Curvilinear velocity (VCL; a), straight-line velocity (VSL; b), mean path velocity (VAP; c), linearity (LIN; d), straightness (STR; e), wobble (f), the amplitude of lateral head displacement (ALH, g), and beat cross frequency (BCF, h) of bovine spermatozoa at 0, 30, 60, 90, 120, 150, and 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of stearic acid (SA). The data are expressed as means ±SEM of three replications. $P > 0.05$

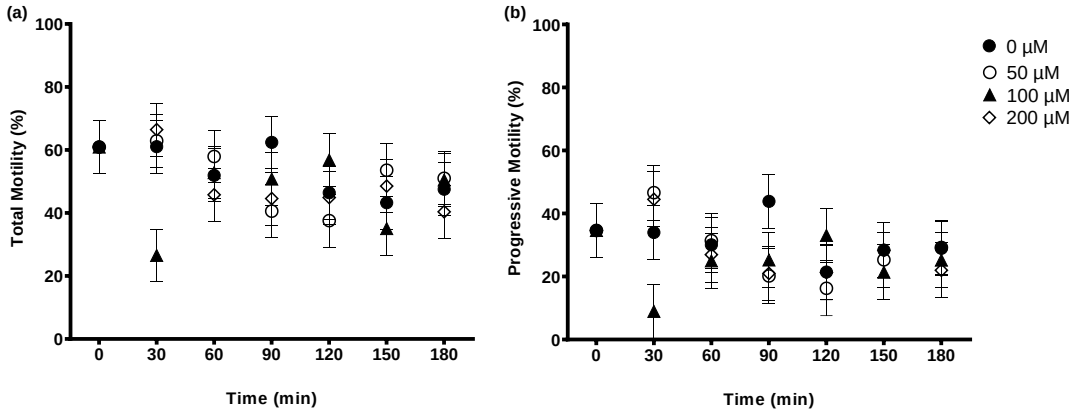
Figure 9 – Production of reactive oxygen species (ROS) and ferric reducing potential (FRAP) of bovine spermatozoa after 90 minutes (a, b) and 180 minutes (c, d) of in vitro exposure without or with 50, 100, or 200 μM of stearic acid (SA). The data are expressed as means ± SEM of three repetitions. $P > 0.05$.

Table 1 - Morphology of bovine spermatozoa after 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of palmitic acid (PA). The data are expressed as the median (lower and upper confidence limit of 95%).

Table 2 - Morphology of bovine spermatozoa after 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of stearic acid (SA). The data are expressed as the median (lower and upper confidence limit of 95%).

Table 3 - Morphology of bovine spermatozoa after 180 minutes of in vitro exposure without or with 50, 100, or 200 μM of oleic acid (OA). The data are expressed as the median (lower and upper confidence limit of 95%).

558 Figure 1.



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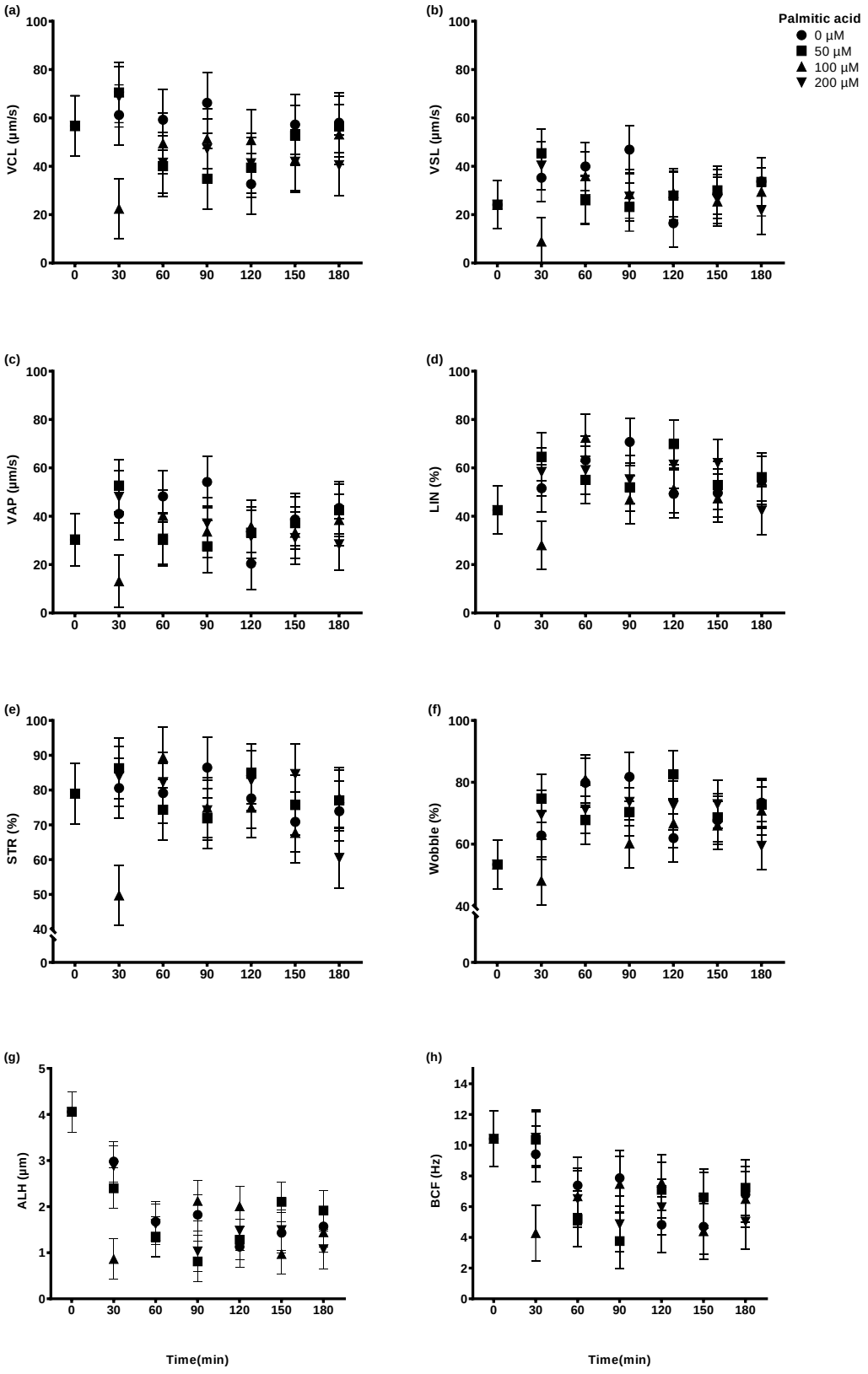

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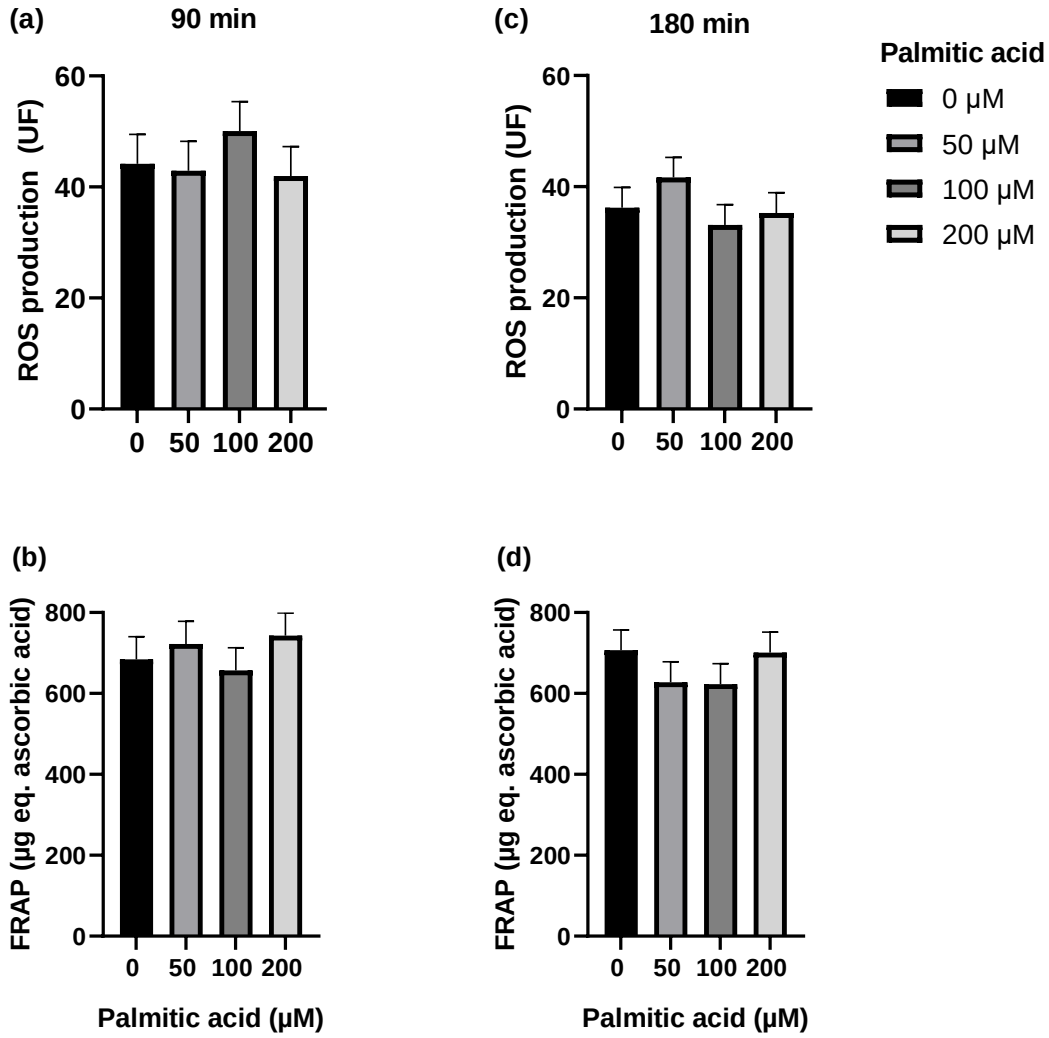

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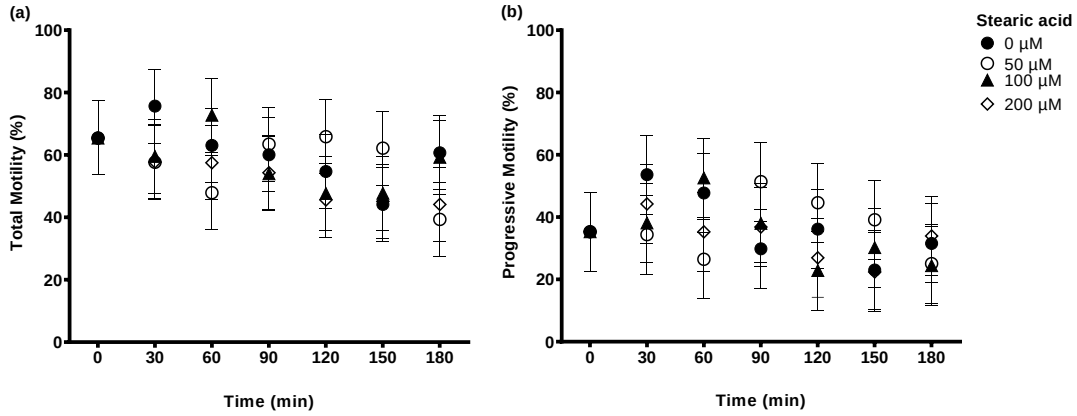

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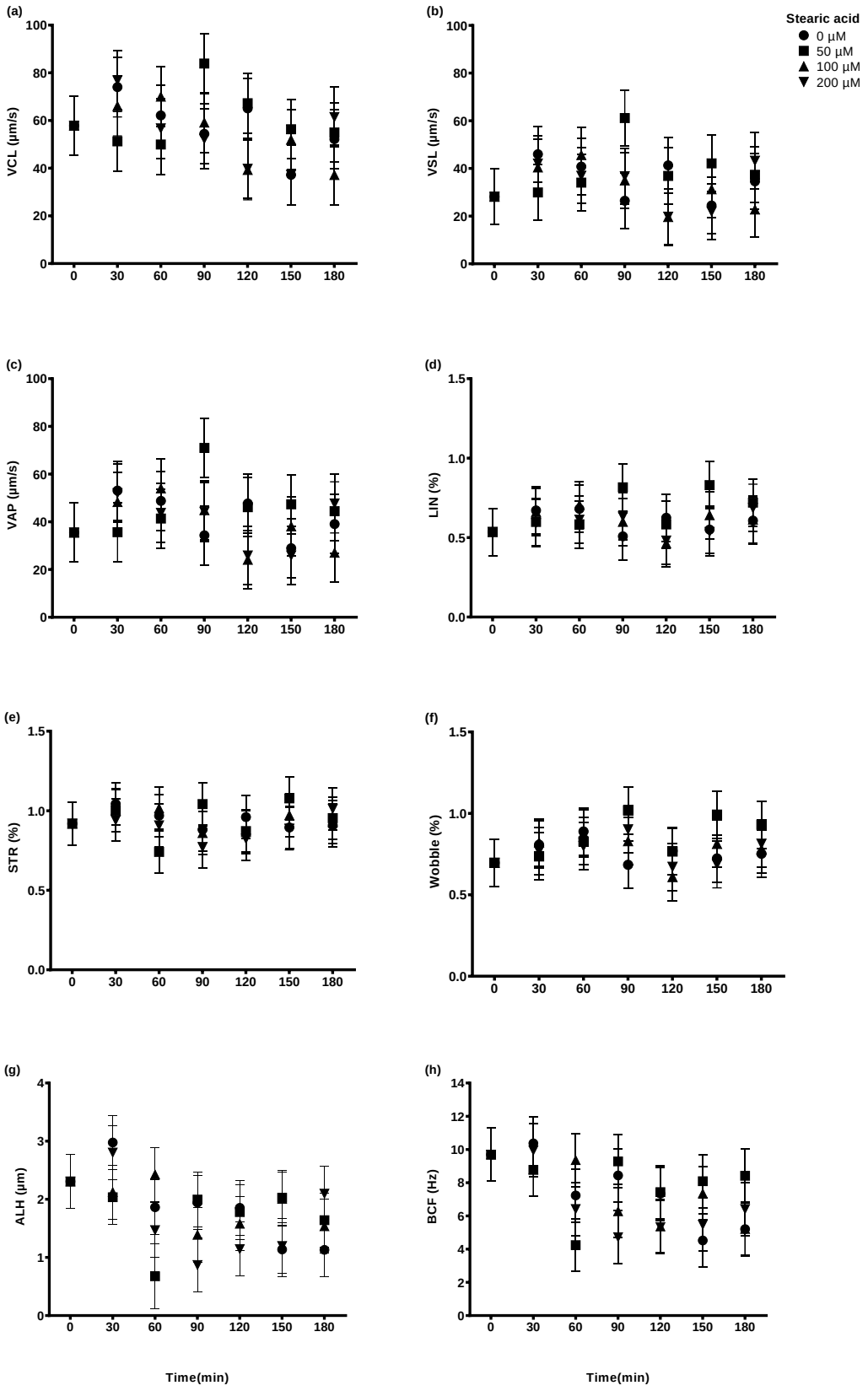

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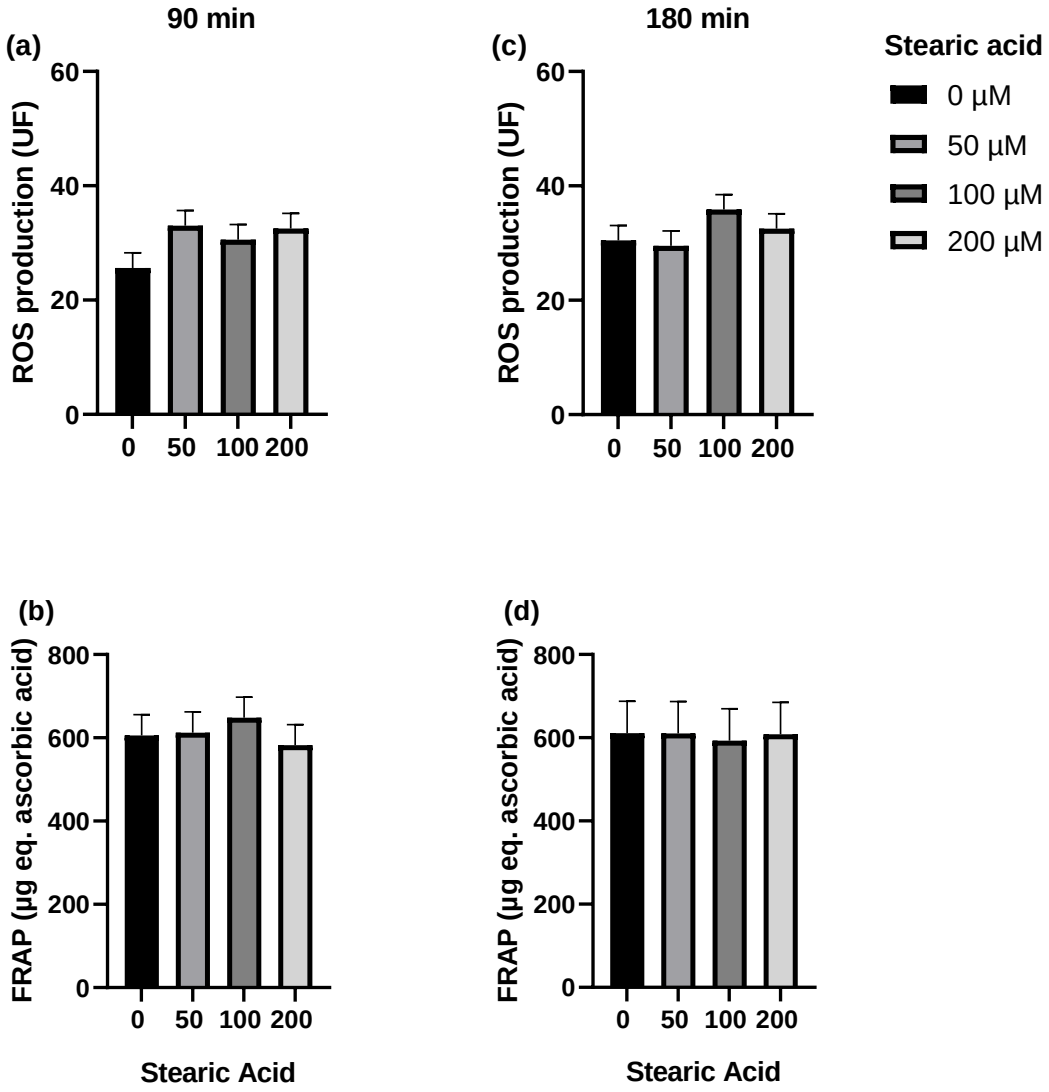

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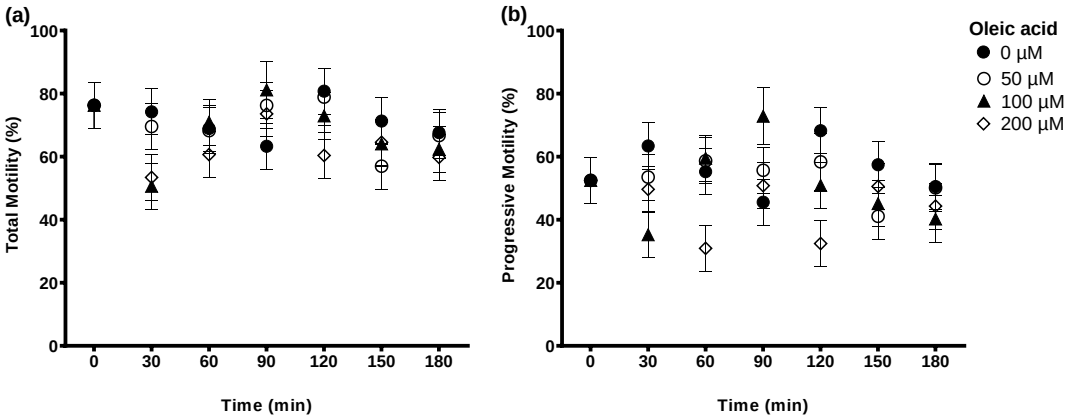

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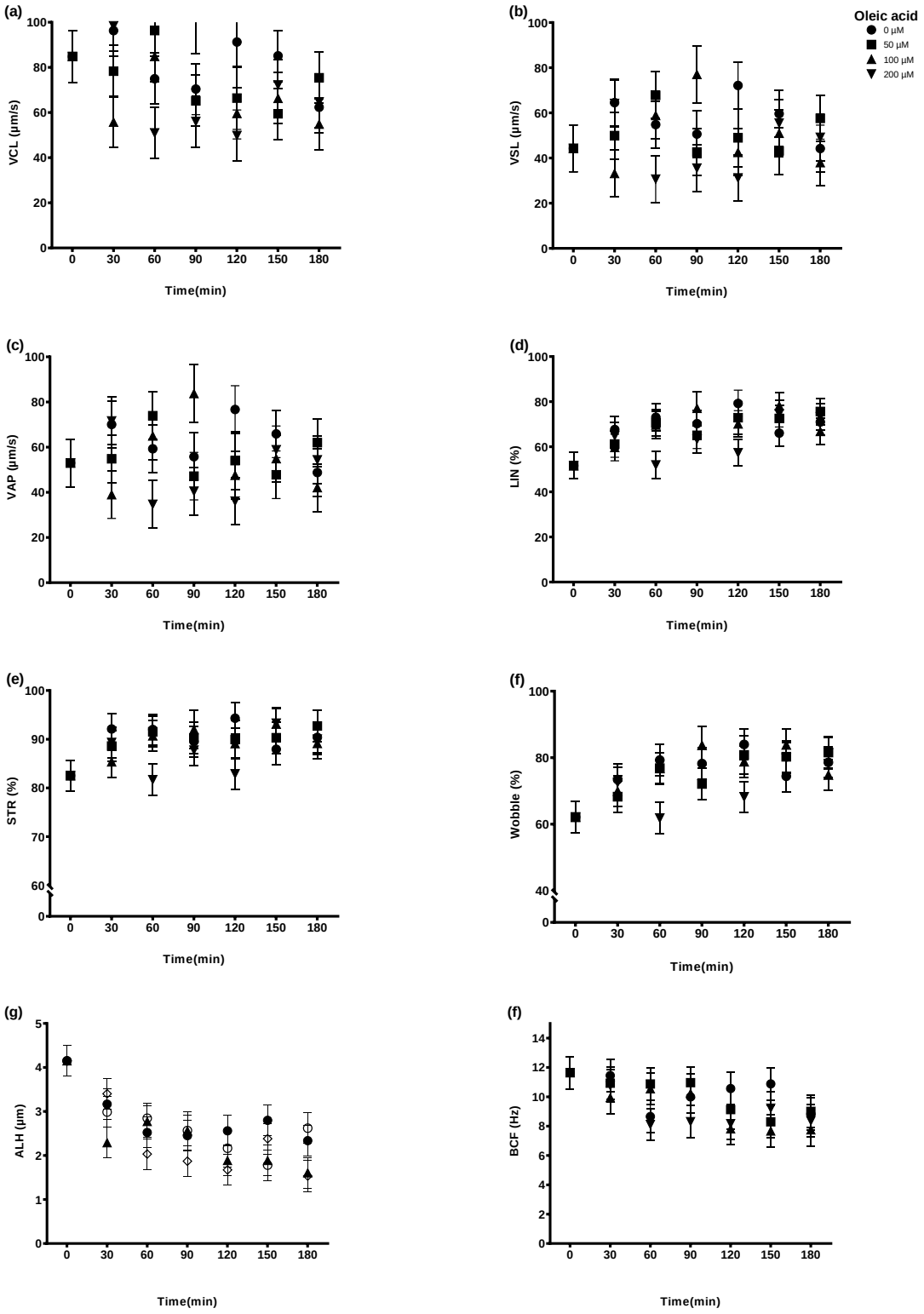

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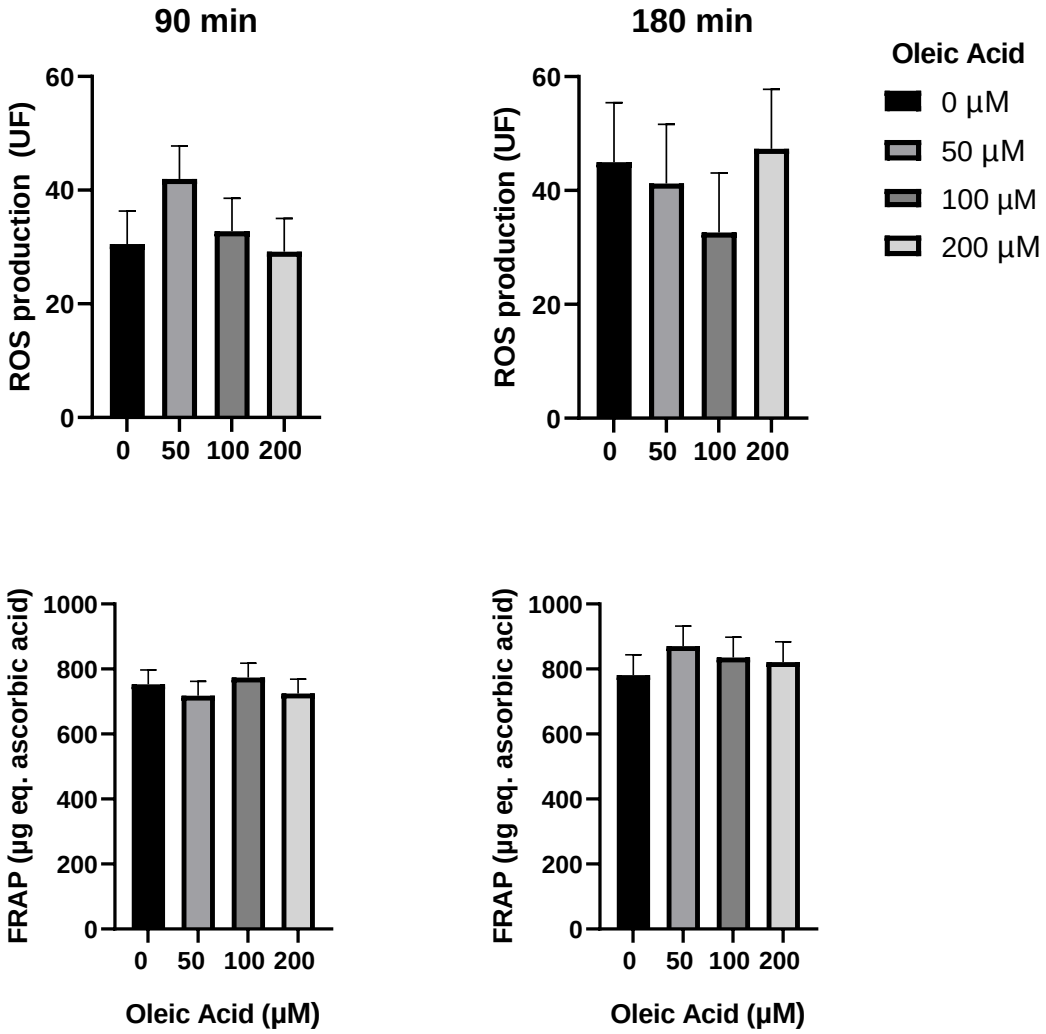

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583 Table 1.

	PA (µM)				
Variable (%)	**0**	**50**	**100**	**200**	***p-value***
Normal spermatozoa	92.5 (92-96)	93.5 (85-94.5)	90.5 (89.5-91.5)	94 (91.5-94.5)	0.3062
Total defects	7.5 (4-8)	6.5 (5.5-15)	9.5 (8.5-10.5)	6 (5.5-8.5)	0.3062
Head defects	5.5 (3.5-5.5)	5 (3.5-10)	6 (5.5-9.5)	5.5 (3.5-7)	0.6423
Midpiece defects	0 (0-0)	0.5 (0-0.5)	0.5 (0-1)	0 (0-0)	0.1818
Tail defects	2 (0.5-2)	1 (1-4.5)	2.5 (0.5-3)	1.5 (0-2)	0.8252
Cytoplasmic droplets	0 (0-0.5)	0 (0-1)	0 (0-0)	0 (0-0.5)	0.9999
Proximal droplets	0 (0-0.5)	0 (0-1)	0 (0-0)	0 (0-0.5)	0.9999
Distal droplets	-	-	-	-	-

584

585 Table 2.

	SA (µM)				
Variable (%)	**0**	**50**	**100**	**200**	***p-value***
Normal spermatozoa	89 (84.5-92)	88.5 (87-93.5)	93 (88.5-93)	87 (86.5-88)	0.2858
Total defects	11 (8-15.5)	11.5 (6.5-13)	7 (7-11.5)	13 (12-13.5)	0.2858
Head defects	6.5 (2-14)	11 (4.5-11.5)	5.5 (5-6)	11 (9.5-12)	0.4736
Midpiece defects	0 (0-1)	0 (0-0.5)	0 (0-0.5)	0 (0-0.5)	0.9999
Tail defects	1.5 (1.5-6)	0.5 (0-1.5)	1.5 (0.5-2)	1 (0.5-3)	0.3844
Cytoplasmic droplets	0 (0-2)	0.5 (0-1)	0 (0-0)	0.5 (0-0.5)	0.5909
Proximal droplets	0 (0-2)	0 (0-0.5)	0 (0-0)	0 (0-0.5)	0.9999
Distal droplets	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-0.5)	0.9999

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587 Table 3.

	OA (µM)				
Variable (%)	**0**	**50**	**100**	**200**	***p-value***
Normal spermatozoa	90.5 (90-91.5)	88.5 (88-95)	92 (91.5-94.5)	91 (86-92)	0.5256
Total defects	9.5 (8.5-10)	11.5 (5-12)	8 (5.5-8.5)	9 (8-14)	0.5256
Head defects	7.5 (5.5-10)	6.5 (4-7)	4 (3.5-6.5)	7.5 (4.5-13)	0.2451
Midpiece defects	0.5 (0-0.5)	0 (0-0)	0 (0-0)	0 (0-0)	0.1818
Tail defects	1.5 (0-1.5)	3 (1-4.5)	2 (1-2.5)	1.5 (1-2.5)	0.5625
Cytoplasmic droplets	0 (0-1)	0.5 (0-2)	0.5 (0-2)	0 (0-1)	0.8714
Proximal droplets	0 (0-1)	0.5 (0-2)	0 (0-1.5)	0 (0-0)	0.6182
Distal droplets	0 (0-0)	0 (0-0)	0.5 (0-0.5)	0 (0-1)	0.5091

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ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

Transient-induced subclinical hypocalcemia does not alter uterine insulin sensitivity in dairy cows.

Camila Cupper Vieira, Jerbeson Hoffmann da Silva, Monique Tomazele Rovani, Daniele Missio, Fernando Silveira Mesquita, Paulo Bayard Dias Gonçalves, Rogério Ferreira

Reproduction in Domestic Animals, 2024

Transient-induced subclinical hypocalcemia does not alter uterine insulin sensitivity in dairy cows.

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Abstract

The cause of insulin resistance (IR) in uterus has not yet been fully elucidated. In the postpartum period, dairy cows go through several changes and stimuli that can cause IR in the uterus, such as hypocalcemia. Knowing that hypocalcemia causes several reproductive disorders, we hypothesized that hypocalcemia causes changes in uterine insulin sensitivity in the postpartum period of dairy cows. To test this hypothesis, we used an induced subclinical hypocalcemia model to isolate other postpartum factors and evaluate exclusively the effect of hypocalcemia on uterine insulin sensitivity. The study was carried out with eight dairy cows, in the 2x2 crossover model, for the control groups (normocalcemia; C-NOR) and EDTA (induced subclinical hypocalcemia; T-ISH). All animals were evaluated 7 days before each round (M7) by clinical examination and endometrial cytology. On the day of treatment (M0), blood was collected, and the mammary vein was cannulated for infusion of the treatments. Cows in the C-NOR group were infused for 45 min with saline solution and those in the T-ISH group were infused with a calcium chelator, 5% EDTA. After 15 min infusions (M1), blood samples were collected. After 45 min infusions (M2), blood samples were collected, and all animals received an intrauterine challenge with LPS to generate an endometrial immune response. After 3 hours of the challenge with LPS (M3), blood collection and uterine biopsy were performed. Total and ionized calcium levels were measured in blood samples from all time points (M0, M1, M2, and M3), and glucose, insulin, nonesterified fatty acids, and beta hydroxybutyrate (BHBA) levels were also measured in samples from M3. Gene expression analysis of glucose and insulin receptors and genes of the insulin signaling pathway was performed in the uterine biopsies. The significant reduction in tCa and iCa levels after EDTA infusion confirmed the successful induction of subclinical hypocalcemia. Despite the decrease in calcium levels, none of the cows showed clinical symptoms of hypocalcemia, underscoring the subclinical nature of the condition induced in the study. The T-ISH group exhibited a significant reduction in BHBA levels compared to the control group. However, no significant differences were observed in glucose, insulin, and non-esterified fatty acid concentrations between the two groups. Our comparative analysis between the C-NOR and T-ISH groups did not reveal significant differences in the expression levels of the genes of interest, INSR, IRS1, IGF1, GLUT1, and GLUT3. This study provides valuable information on the metabolic effects of transient induced subclinical hypocalcemia, as it does not adversely affect insulin sensitivity and responsiveness in utero.

Keywords: induced hypocalcemia, blood metabolites, insulin responsiveness

1. Introduction

Insulin resistance (IR) is defined as a state in which a physiological concentration of insulin induces a decreased biological response in insulin-sensitive tissues (Ronald Kahn, 1978). In addition, IR has two distinct presentations, reduced insulin sensitivity and reduced insulin responsiveness. At first, there is a reduction in insulin sensitivity, in which a higher concentration of insulin is necessary to achieve the maximum response in sensitive tissues, and soon after, if the IR condition is maintained, there is a reduction in insulin responsiveness, in which the tissue produces a lower response to the hormone (Muniyappa et al., 2008; Ronald Kahn, 1978). Dairy cows show peripheral IR in late pregnancy and early lactation (Baruselli et al., 2016; De Koster & Opsomer, 2013; Kerestes et al., 2009). This insulin-resistant condition is necessary to ensure a sufficient supply of glucose to the pregnant uterus at the end of pregnancy and to the lactating mammary gland (Bell & Bauman, 1997).

The mechanism of IR in reproductive tissues such as the uterus has not yet been fully elucidated. Uterine IR is known to occur through alterations in the PI3K and MAPK signaling pathways (Zhang et al., 2016), which culminates in lower gene expression of insulin receptor (INSR), insulin receptor type 1 substrate (IRS1), and insulin-like growth factor type 1 (IGF1). Studies evaluating changes in endometrial insulin sensitivity have demonstrated that these alterations impair uterine involution (Llewellyn et al., 2008; Wathes et al., 2011), and the postpartum immune response (Wathes et al., 2009).

Several conditions and stimuli that increase the risk of inflammation can cause IR (Hirabara et al., 2012; Khodabandehloo et al., 2016), including hypocalcemia. Hypocalcemia occurs in early lactation because cows cannot adapt to high calcium demand and maintain normocalcemia (Goff, 2008). The decrease in calcium concentrations during this period is a risk factor for uterine disorders such as delayed uterine involution (Heppelmann et al., 2015), uterine prolapse (Risco et al., 1984), retained placenta (Melendez et al., 2004), metritis and endometritis (Martinez et al., 2012; Ribeiro et al., 2013). Therefore, hypocalcemia may contribute to uterine IR, since cows with subclinical hypocalcemia are more predisposed to develop uterine inflammatory changes (Martinez et al., 2012). And in turn, this uterine inflammation can alter the insulin signaling pathway (Hirabara et al., 2012; Khodabandehloo et al., 2016). Therefore, our

hypothesis is that the inflammatory changes resulting from hypocalcemia cause changes in the uterine insulin signaling pathway in the postpartum period of dairy cows. The objective of this study was to evaluate the effects of inflammatory changes resulting from transient subclinical hypocalcemia on the uterine insulin signaling pathway in dairy cows.

2. Materials and Methods

2.1 Animals

The experiment was conducted on a dairy farm located in South Brazil, from April to May 2023. The study was carried out with 8 multiparous Holstein dairy cows, non-pregnant and non-lactating cows. The study was approved by the Committee for Ethics in Animal Use of the University of Passo Fundo (protocol nº 027/2022).

2.2 Preparation of 5% EDTA solution

A 5% (w/v) ethylenediaminetetraacetic acid (EDTA) solution was used to induce subclinical hypocalcemia. The solution was prepared using 900 mL of 0.9% (w/v) sodium chloride saline solution, to which 50 g of EDTA was added. The pH was corrected to 7.4 using about 50 mL of 5 M sodium hydroxide solution. The solution was filtered using a 0.45 μm filter, stored at 4ºC, and used within 10 days of preparation.

2.3 Experimental design

The study was carried out in the 2x2 crossover model, for the control groups (normocalcemia; C-NOR) and EDTA (induced subclinical hypocalcemia; T-ISH) as depicted in Figure 1. Each animal underwent both treatments, with a 3-week interval between treatments, to minimize the risks of interference with the results due to the previous treatment. All animals were evaluated 7 days before each round (M7) through clinical examination and endometrial cytology, to exclude cows with previous endometrial inflammation.

On the day of treatment (M0), the mammary vein was cannulated with a 14G catheter for the infusion of the treatments. Immediately before the start of the infusion with the treatments, blood was collected in tubes with coagulum activator. After blood collection, the cows in the C-NOR group received an infusion of saline solution and those in the T-ISH group received an infusion with a previously prepared Ca chelator. The initial infusion rate was 500 mL/h, which was adjusted according to the levels of iCa and tCa, and the infusion was performed for approximately 45 min (Martinez et al., 2014). Every 15 minutes of infusion, blood samples were collected to measure serum levels of total calcium (tCa) and ionized calcium (iCa) in loco using portable analyzers (Ca+ Vet TD-5220 from Eco Diagnóstico Veterinário; and HI98190 and HI4104, from Hanna Instruments). The infusion was maintained until the levels of tCa were between 5.5 and 8 mg/dl and iCa 2.8 and 3.6 mg/dl, thus inducing a subclinical hypocalcemia state. None of the animals presented clinical hypocalcemia (tCa <5.5 mg/dl) during the experiment.

After 15 min of infusions (M1), blood samples were collected for subsequent Ca analysis. After the infusions (M2), blood samples were collected, and all animals received an intrauterine challenge with LPS to generate an endometrial immune response. A uterine infusion containing 20 mL of 0.9% NaCl and 300 μg of LPS (Escherichia coli O26:B6, SIGMA) was performed with a rigid probe and sanitary sleeve, and the vulva was properly cleaned before the procedure. After 3 hours of the LPS challenge (M3), blood collection and uterine biopsy were performed, which were stored in liquid nitrogen until processing.

2.4 Processing of blood samples

Blood samples were collected from the coccygeal vein with the aid of the vacutainer system and tubes with coagulation accelerator gel, at the following moments: M0, M1, M2, and M3. The samples were centrifuged at 1,000 g for 10 minutes, the serum was collected and stored at -20ºC. The serum samples were sent to a commercial laboratory for animal clinical analysis. At all-time points (M0, M1, M2, and M3) the levels of total and ionized calcium were measured, and in the samples at the last moment (M3), the levels of glucose, insulin, NEFA, and BHBA were also measured.

2.5 Gene expression

Total RNA was extracted from uterine biopsies for gene expression analysis. The RNA was extracted using AllPrep® DNA/RNA/Protein Mini Kit (Qiagen, Ref. 80004), according to the manufacturer's instructions. During RNA purification, it was treated with an RNase-Free DNase Set (Qiagen, Ref. 79254) for 15 min, following the manufacturer's instructions. RNA was quantified using a Nano-Vue Plus spectrophotometer (Healthcare Bio-Sciences). The samples were then incubated with iScript cDNA Synthesis Kit (BioRad) for complementary DNA (cDNA) synthesis, according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR) was conducted in the Bio-Rad CFX Opus 384 equipment, using the BRYT Green® fluorophore and Taq DNA Polymerase present in the GoTaq® qPCR Master Mix (Promega), specific primers for cattle and according to pre-established criteria (Taylor & Mrkusich, 2014). All samples were analyzed in duplicate. The variability in the amount of messenger RNA (mRNA) was corrected by the geometric mean of the expression of constitutive genes. To ensure the accuracy of our findings, we initially examined the expression of five reference genes and identified GAPDH and PPIA as the most suitable representatives of our dataset. The reference and interest genes are listed in Table 1.

2.7 Statistical analysis

Serum ionized calcium and total calcium concentrations were analyzed using mixed models. Treatment, time, and their interaction were included as fixed effects, with the cow as the subject. Different covariance structures were tested for each model, and the one with the smallest AIC (Akaike Information Criteria) value was chosen. Other continuous variables were analyzed by ANOVA. The data and residuals of each model were tested for normal distribution using the Shapiro-Wilk test. Homoscedasticity was assessed using Levene's test. Variables without a Gaussian distribution were transformed according to each distribution. Data are presented as mean ± SEM, and mean differences were considered significant when $P < 0.05$. All analyses were performed using JMP software (JMP Statistical Discovery LLC, Cary, NC).

3. Results

3.1 Induction of subclinical hypocalcemia

All cows in the T-ISH group underwent induction of subclinical hypocalcemia via infusion of 5% EDTA solution (Figure 2). Before the initiation of the infusion, the mean tCa levels were 9.13±0.66 and 7.94±0.66 mg/dl in the C-NOR and T-ISH groups, respectively. Following a 45 min infusion with 5% EDTA, the T-ISH group exhibited a reduction in tCa levels to 6.14±0.94 mg/dl. This reduction was similarly observed for iCa, which decreased from 2.98±0.53 mg/dl before infusion to 1.84±0.53 mg/dl afterward. It is noteworthy that none of the animals presented clinical hypocalcemia symptoms.

3.2 Blood metabolites

In this experiment, no statistical differences were observed in the blood concentrations of glucose, insulin, and NEFA between the C-NOR and T-ISH groups, three hours after exposure to LPS (Figure 3). However, an intriguing finding emerged with a notable reduction in BHBA levels within the T-ISH group (0.16±0.04 mmol/l) compared to the C-NOR group (0.38±0.09 mmol/l; $P < 0.05$).

3.3 Insulin signaling pathway

To assess the potential impact of subclinical hypocalcemia on insulin sensitivity, we analyzed gene transcription related to insulin receptors and glucose transponders (INSR, GLUT1, and GLUT3), as well as genes implicated in insulin signaling pathways (IRS1 and IGF1). Our comparative analysis between the C-NOR and T-ISH groups revealed no significant differences in the expression levels of the genes of interest (Figure 4).

4. Discussion

Pregnant and lactating dairy cows undergo intense hormonal and metabolic adaptations typical of this period. To isolate the effects of these adaptations, we used non-pregnant or lactating dairy cows as a model for the induction of subclinical hypocalcemia. The significant reduction in both tCa and iCa levels following the EDTA infusion confirmed the successful induction of subclinical hypocalcemia (Ro et al., 2020). Despite the decrease in calcium levels, none of the cows exhibited clinical symptoms of hypocalcemia, highlighting the subclinical nature of the condition induced in the study.

Blood metabolite analysis revealed intriguing findings regarding the levels of BHBA, a ketone body associated with metabolic stress and negative energy balance (NEB) in dairy cattle (Bell, 1995; Drackley & Cardoso, 2014; Kawashima et al., 2012). The T-ISH group exhibited a significant reduction in BHBA levels compared to the control group, suggesting potential alterations in energy metabolism or hepatic ketogenesis pathways associated with subclinical hypocalcemia. However, no significant differences were observed in the concentrations of glucose, insulin, and non-esterified fatty acids (NEFA) between the two groups.

Towards the end of pregnancy, bovine females experience significant nutritional demands due to fetal growth associated with milk production (Bell, 1995). This period often entails NEB, wherein energy demands surpass nutritional intake, leading to the mobilization of body reserves (Drackley & Cardoso, 2014). Consequently, there is a rise in NEFA and BHBA levels, accompanied by a decline in IGF1, insulin, and glucose concentrations (Bell, 1995; Kawashima et al., 2012). These physiological changes also trigger the secretion of large quantities of calcium in the colostrum, inducing a state of hypocalcemia in the animals. Cows inherently struggle to mobilize calcium from their bones to restore normocalcemia (Goff, 2008). Such alterations serve as primary risk factors for reproductive changes, underlining the importance of studying them to comprehend their occurrence and interconnections. Exploring these changes is crucial for identifying potential strategies to mitigate their adverse effects.

We anticipated that inducing subclinical hypocalcemia would lead to an increase in NEFA and BHBA levels, given previous findings suggesting elevated plasma levels of these metabolites in cows with subclinical hypocalcemia (Martinez et al., 2012; Ribeiro et al., 2013). To facilitate this increase, the body needs to mobilize reserves, resulting in

heightened NEFA levels. Elevated NEFA prompts lipid accumulation in hepatocytes, which coupled with limited glucose availability and reduced oxaloacetate, hinders the complete oxidation of acetyl-CoA (Bell, 1995). Consequently, acetyl-CoA is converted into ketone bodies, leading to increased BHBA levels (Mulligan & Doherty, 2008). However, contrary to our expectations, our data did not exhibit this pattern; NEFA levels remained unchanged, and BHBA levels decreased. This discrepancy likely arose because the cows were not experiencing NEB.

The study on pancreatic β cells emphasizes the essential role of calcium influx into the cytosol for insulin granule release and responsiveness to insulin during glucose tolerance tests (Rorsman et al., 2012). Additionally, dairy cows afflicted with milk fever exhibit impaired insulin release linked to spontaneous hypocalcemia (Littledike et al., 1968). Moreover, inducing subclinical hypocalcemia in non-pregnant, non-lactating dairy cows has been shown to elevate glucose levels while decreasing blood insulin levels (Martinez et al., 2014). These findings underscore the influence of calcium levels on insulin sensitivity and responsiveness. Nevertheless, despite these observed effects, the absence of significant differences in glucose and insulin levels, as well as in the expression of insulin sensitivity-related genes, suggests that the impact of subclinical hypocalcemia on insulin sensitivity may be intricate and multifaceted, necessitating further investigation.

For an effective insulin response to occur within tissues, insulin must bind to the α subunit of the insulin receptor (INSR), triggering kinase activity in the β subunit of the receptor (Patti & Kahn, 1998). Following activation, ten protein substrates undergo phosphorylation, primarily IRS1 (insulin receptor substrate 1). The phosphorylation of IRS1 activates three main pathways: PI3K, CAP/Cbl, and MAPK, which play pivotal roles in increasing glucose uptake and promoting cell growth (White, 1998). Disruption in IRS1 function results in insulin resistance and growth retardation, as observed in rats lacking IRS1 (Saad et al., 1994). While the uterus may not conventionally be regarded as a primary target tissue for insulin action, emerging evidence suggests that the insulin receptor and its downstream targets contribute to the regulation of reproductive function (Zhang et al., 2016).

It is crucial to acknowledge the limitations of the study, foremost among them being the short-term nature of the experimental induction of subclinical hypocalcemia. This short duration may not adequately reflect the long-term metabolic adaptations that dairy cows might undergo in response to chronic hypocalcemia. Future research endeavors should aim to elucidate the longitudinal effects of subclinical hypocalcemia on metabolic parameters and insulin sensitivity in dairy cattle across varying management and nutritional contexts.

5. Conclusion

In conclusion, this study provides valuable insights into the metabolic effects of transient-induced subclinical hypocalcemia. Particularly, it reveals that such inflammation arising from hypocalcemia and challenge with LPS does not adversely affect the insulin signaling pathway in the uterus. These findings underscore the necessity for additional research to elucidate the intricate interplay among calcium homeostasis, energy metabolism, and insulin signaling pathway in the context of dairy cattle health and production.

AUTHOR CONTRIBUTIONS

Camila Cupper Vieira: Conceptualization, Methodology, Investigation, Writing - Original Draft.

Jerbeson Hoffmann da Silva: Conceptualization, Methodology, Investigation.

Monique Tomazele Rovani: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Project administration.

Daniele Missio: Conceptualization, Writing - Review & Editing, Project administration.

Fernando Silveira Mesquita: Investigation, Writing - Review & Editing.

Paulo Bayard Dias Gonçalves: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition.

Rogério Ferreira: Conceptualization, Methodology, Formal analysis; Investigation, Resources; Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition.

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CONFLICTS OF INTEREST STATEMENT

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author, Ferreira, R. upon reasonable request.

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LEGENDS

FIGURE 1 – Experimental Design. Eight Holstein multiparous dairy cows were submitted to the 2x2 crossover model. Seven days before treatment (M7) the cows were evaluated by clinical examination and endometrial cytology. Immediately before the infusions with treatment (M0), blood was collected from the animals. The cows in the C-NOR group received an infusion of saline solution and those in the T-ISH group received an infusion with a previously prepared 5% EDTA solution. After fifteen minutes of infusion (M1), blood was collected from the animals. After 45 min of infusion (M2), blood samples were collected, and all animals received an intrauterine challenge with LPS. Three hours after the LPS challenge (M3), blood collection and uterine biopsy were performed. After 21 days, the design was repeated, inverting the cows in the groups, so that they underwent each treatment once.

FIGURE 2 – Serum ionized calcium (iCa; a) and total calcium (tCa; b) concentrations in cows submitted to normocalcemia (C-NOR) or induced subclinical hypocalcemia (T-ISH). Immediately before the infusion (M0), after 15 min of infusion (M1), at the end of the 45 min of infusion (M2), and three hours after the LPS challenge (M3). For iCa, statistical analysis revealed the effects of time ($P=0.002$) and treatment-time interaction ($P=0.004$), but not of treatment ($P=0.163$). For tCa there were no effects of treatment ($P=0.051$), time ($P=0.183$), and treatment-time interaction ($P=0.067$).

FIGURE 3 – Blood metabolite concentrations in cows submitted to normocalcemia (C-NOR) or induced subclinical hypocalcemia (T-ISH) three hours after the LPS challenge (M3). Blood concentrations of (a) glucose, (b) insulin, (c) BHBA, and (d) NEFA. The data are expressed as means ± SEM and statistical differences ($P < 0.05$) were identified with different letters.

FIGURE 4 – Relative mRNA abundances in uterine biopsies of cows submitted to normocalcemia (C-NOR) or induced subclinical hypocalcemia (T-ISH) three hours after

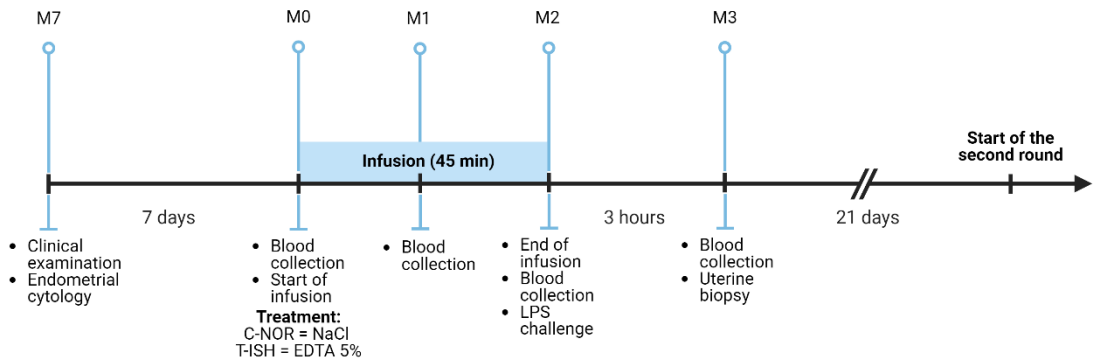
453 the LPS challenge (M3). Relative mRNA abundances for genes of insulin and glucose
454 receptors (INSR, GLUT1, and GLUT3) and those involved in insulin signaling pathways
455 (IRS1, and IGF1). Results are presented as means ± SEM, there was no statistical
456 difference ($P > 0.05$).

457

458 **TABLE 1 -** List of primers used as reference genes and genes of interest in qPCR.

459

460 Figure 1.



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465 Figure 2.

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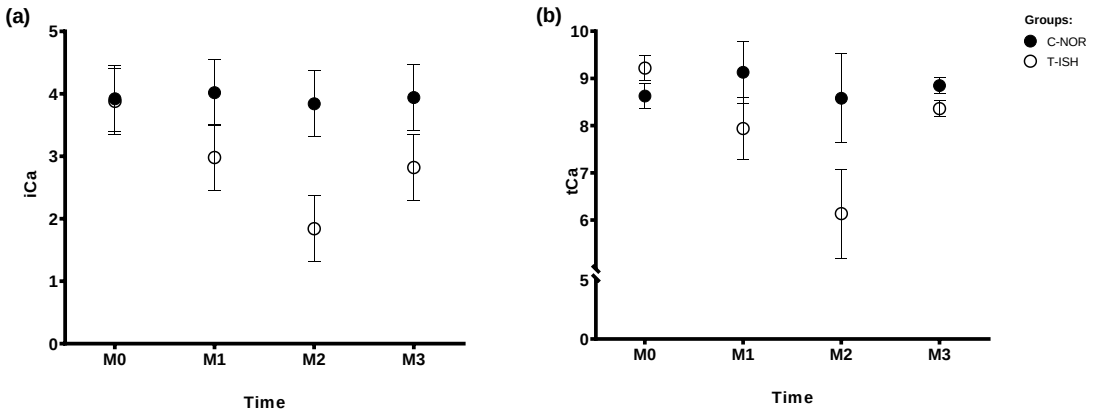

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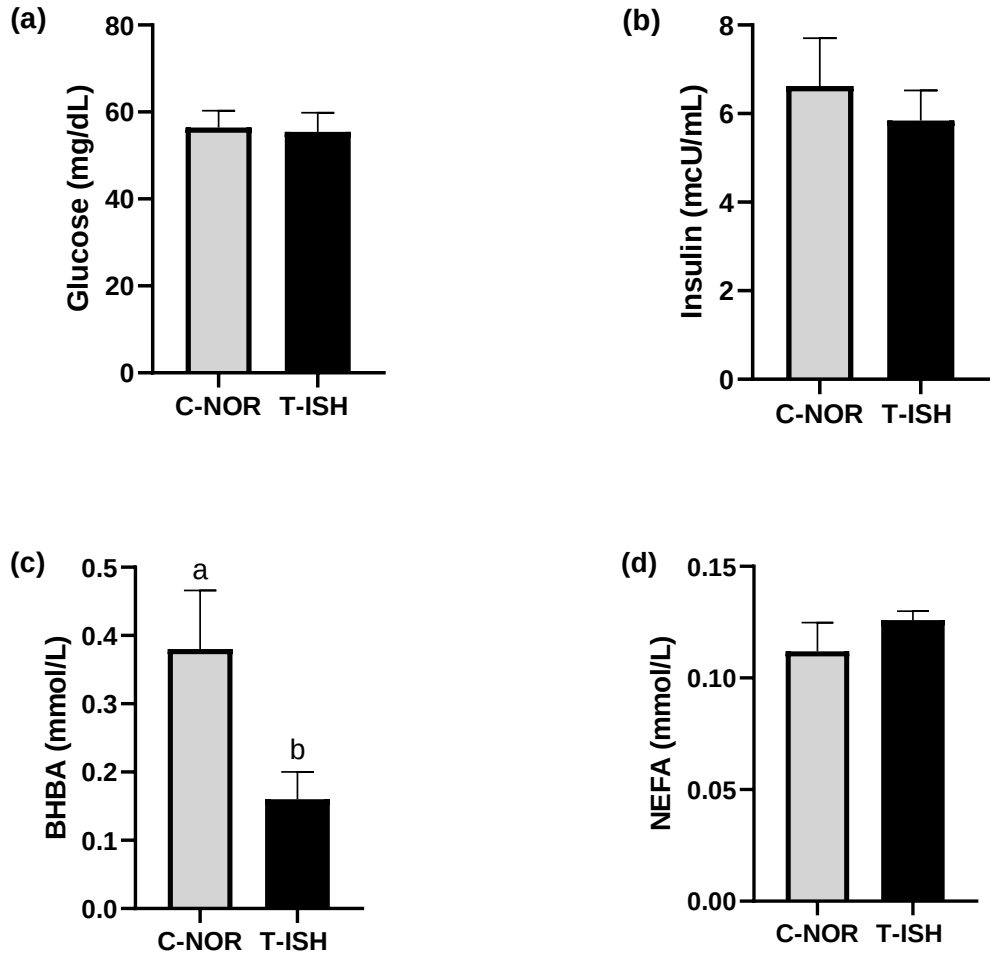
469 Figure 3.



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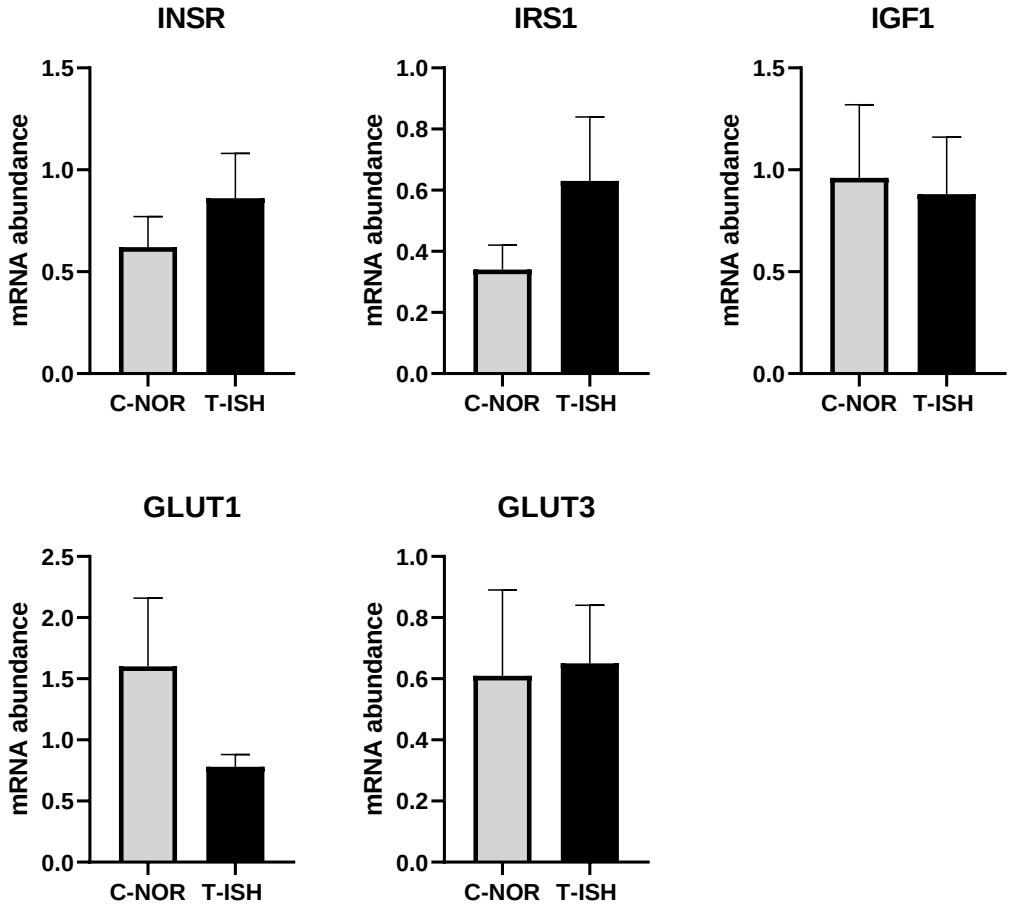
474 Figure 4.



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478 Table 1.

Gene name	Sequence (5’ to 3’)	Accession number
GAPDH	F: GCCATCAATGACCCCTTCAT R: TGCCGTGGGTGGAATCA	NM_001034034.2
PPIA	F: GGTCATCGGTCTCTTTGGAA R: TCCTTGATCACACGATGGAA	NM_174152.2
INSR	F: TCCTCAAGGAGCTGGAGGAGT R: TTTCCTCGAAGGCCTGGGGAT	XM_590552.4
IRS1	F: TCCGCCTTTCCTCAAGTTCC R: AGCTGTGTCCACCTTTCGAG	XM_003585773.5
GLUT1	F: CCTTCACTGTCGTGTCGCTA R: GCCACAATGCTCAGGTAGGA	NM_174602.2
GLUT3	F: GCCGCCGATAGAGGACATTT R: ATGGCGAAGATCAGAGGTGC	NM_174603.3
IGF1	F: AGTTGGTGGATGCTCTCCAGT R: CTGATTCTCCCTGGGCAAAG	NM_001077828.1

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3. DISCUSSÃO

No período de transição, as vacas leiteiras passam por diversas mudanças metabólicas, endócrinas e imunológicas, as quais refletem em alta incidência de doenças (DRACKLEY, 1999), o que compromete a fertilidade das fêmeas. O BEN, que ocorre nesse pós-parto, é um dos principais fatores de risco determinantes de alterações reprodutivas e é caracterizado pelo aumento dos níveis circulantes de NEFA, BHBA e diminuição das concentrações do IGF1, insulina e glicose (KAWASHIMA et al., 2012). Nesse sentido, Jordaens et al., (2017) demonstraram que ocorre um aumento de NEFA no fluido ovidutal refletindo os níveis sanguíneos. Além disso, ocorre queda nos níveis de insulina, sendo a sua sensibilidade afetada e gerando RI. Essa condição de RI é necessária para garantir um suprimento suficiente de glicose para o útero gestante no final da gestação e para a glândula mamária lactante (BELL; BAUMAN, 1997). O mecanismo da RI em tecidos reprodutivos, como o útero, ainda não foi totalmente elucidado. No pós-parto, as vacas leiteiras passam por diversas alterações e estímulos que podem causar RI no útero, como a hipocalcemia. Nesse sentido, nosso grupo de pesquisa vem tentando entender quais são os efeitos de NEFA nos gametas e como os metabólitos e as alterações do BEN interferem ambiente uterino de bovinos. A partir dos resultados obtidos nesta tese, foi possível observar que as concentrações específicas de PA, SA e OA quando testadas em espermatozoides bovinos *in vitro*, não exercem impacto negativo na cinética e morfologia espermática nem no status oxidativo das células. Ainda, foi possível demonstrar que a indução de hipocalcemia subclínica transitória, não prejudica a sensibilidade uterina à insulina.

Esta tese é constituída por dois trabalhos. No primeiro, nos utilizamos uma metodologia *in vitro* para avaliar o impacto de NEFA (PA, SA e OA) na cinética, status oxidativo e morfologia de espermatozoides bovinos. Os resultados revelaram que diferentes concentrações de PA, SA ou OA isoladamente, não alteram a motilidade total e progressiva dos espermatozoides bovinos. Além disso, nenhum dos parâmetros cinéticos avaliados pelo CASA, incluindo VCL, VSL, VAP, LIN, STR, WOB, ALH e BCF, apresentou alterações significativas em resposta às concentrações de PA, SA ou OA em qualquer um dos momentos analisados. Para investigar os efeitos de PA, SA e OA, no status oxidativo das células espermáticas, avaliamos a produção de ROS e a

capacidade antioxidante total em duas mensurações. No entanto, nenhuma diferença foi observada. Da mesma forma, os NEFA não tiveram qualquer impacto significativo na morfologia normal dos espermatozoides, defeitos totais, defeitos de cabeça, defeitos de peça intermediária, defeitos de cauda ou gotas citoplasmáticas. Existem estudos demonstrando que NEFA causam estresse oxidativo em diferentes tipos celulares, como nas células endometriais bovinas (FERST et al., 2021) e em hepatoma humano (células HepG2; ALNAHDI; JOHN; RAZA, 2019), por isso a importância de avaliar o status oxidativo nos espermatozoides. O mecanismo pelo qual os NEFA podem causar estresse oxidativo nas células ainda não está bem definido, por isso são necessários mais estudos nesta área. Portanto, os resultados do primeiro trabalho indicam que diferentes concentrações de PA, SA e OA não exerceram efeitos significativos sobre a cinética, status oxidativo ou morfologia de espermatozoides bovinos. Este estudo representa um dos esforços iniciais focados nos efeitos dos ácidos graxos nos espermatozoides bovinos (DESMET et al., 2018; ISLAM et al., 2021; KIERNAN; FAHEY; FAIR, 2013).

No segundo trabalho, buscamos compreender o que ocorre com a sensibilidade à insulina em células uterinas de vacas com hipocalcemia subclínica. Assim, primeiramente observamos que o nosso protocolo de indução de hipocalcemia subclínica transitória utilizando solução de EDTA a 5% foi eficaz reduzindo as concentrações de cálcio ionizado circulantes. Observamos que os níveis sanguíneos de glicose, insulina e NEFA não foram alterados pela hipocalcemia, enquanto os níveis de BHBA foram reduzidos. Com relação a expressão de genes relacionados a sensibilidade à insulina como INSR, IRS1, IGF, GLUT1 e GLUT3, a hipocalcemia não afetou nenhum deles. A indução de hipocalcemia subclínica em vacas leiteiras não prenhes e não lactantes demonstrou elevar os níveis de glicose enquanto diminui os níveis de insulina no sangue (MARTINEZ et al., 2014). Esses achados reforçam a influência dos níveis de cálcio na sensibilidade e responsividade à insulina. No entanto, apesar dos efeitos observados, a ausência de diferenças nos níveis de glicose e insulina, bem como na expressão de genes relacionados à sensibilidade à insulina, sugere que o impacto da hipocalcemia subclínica sobre a sensibilidade à insulina pode ser intrincado e multifatorial, necessitando mais investigações.

A partir dos dados obtidos com esta tese foi possível fornecer evidências de que PA, SA e OA, quando testados em concentrações específicas sob condições in vitro, não

exercem impacto prejudicial sobre a viabilidade espermática em bovinos. Esses resultados contribuem com insights valiosos para a crescente compreensão de como os ácidos graxos afetam a fertilidade em vacas com BEN, abrindo caminho para pesquisas futuras nesta área. Ainda, fornece informações importantes sobre os efeitos metabólicos da indução de hipocalcemia subclínica transitória, revelando que a hipocalcemia não afeta negativamente a sensibilidade à insulina no útero bovino. Esses achados ressaltam a necessidade de pesquisas adicionais para elucidar a intrincada interação entre a homeostase do cálcio, o metabolismo energético e a sensibilidade à insulina no contexto da saúde e produção de bovinos leiteiros.

4. CONCLUSÃO

Com os resultados obtidos no conjunto de experimentos que compõem esta tese, foi possível demonstrar os efeitos de NEFA nos espermatozoides. Foi observado que PA, SA e OA, quando testados em concentrações específicas sob condições *in vitro*, não exercem impacto prejudicial sobre a cinética, morfologia e status oxidativo de espermatozoides bovinos. Ainda, foi observado que a hipocalcemia transitória subclínica e o desafio com o LPS, não prejudica a via de sinalização a insulina no útero de vacas. Esses resultados fornecem informações importantes para a compreensão do efeito de NEFA na fertilidade de vacas em BEN, e sobre os efeitos metabólitos da hipocalcemia subclínica induzida transitoriamente. Esses achados abrem caminho para pesquisas adicionais sobre os efeitos de NEFA e a interação entre a homeostase do cálcio, o metabolismo energético e a via de sinalização a insulina no contexto da saúde e produção de bovinos leiteiros.

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