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Daniele Missio

ESTRESSE METABÓLICO EM BOVINOS: EFEITO EM GAMETAS E CÉLULAS FOLICULARES

Santa Maria, RS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-graduação em Medicina Veterinária, Área de concentração de Sanidade e Reprodução Animal da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do grau de **Doutor em Medicina Veterinária**.

Orientador: Prof. Dr. Paulo Bayard Dias Gonçalves
Coorientador: Prof. Dr. Rogério Ferreira

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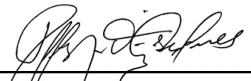

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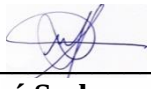

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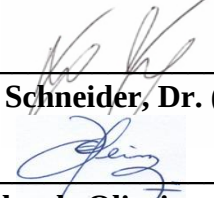

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Aos meus pais Valdecir e Rosane, com todo meu amor e gratidão, por tudo que fizeram por mim ao longo da minha vida.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

(Madre Teresa de Calcutá)

RESUMO

ESTRESSE METABÓLICO EM BOVINOS: EFEITO EM GAMETAS E CÉLULAS FOLICULARES

AUTORA: Daniele Missio
ORIENTADOR: Paulo Bayard Dias Gonçalves

A fertilidade de vacas leiteiras tem diminuído nos últimos anos com a intensificação para a produção de leite e os distúrbios metabólicos que ocorrem durante o período de transição têm contribuído para a performance reprodutiva inferior. Durante o período de balanço energético negativo (BEN), há um aumento das concentrações plasmáticas de ácidos graxos não esterificados (NEFA) e corpos cetônicos, como o β-hidroxibutirato (BHBA). Estudos demonstraram que essas alterações têm reflexo no microambiente folicular, ovidutal e uterino bovino. No entanto, os efeitos dos NEFA e do BHBA em células foliculares e gametas não está totalmente elucidado. Portanto, os objetivos desta tese foram avaliar o efeito de concentrações elevadas de BHBA sobre gametas, crescimento folicular e ovulação de bovinos e analisar o efeito do uso de Vitamina E para impedir o estresse oxidativo no sangue e fluido folicular de vacas em BEN. Primeiramente, se investigou os efeitos do BHBA na viabilidade de espermatozoides bovinos. Os resultados demonstraram que altas concentrações de BHBA prejudicam a cinética espermática, sem alterar o status oxidativo e a morfologia das células. Posteriormente, avaliamos se a injeção intrafolicular (IIF) de BHBA compromete o crescimento folicular e a cascata ovulatória, bem como a produção de esteroides e a abundância de genes relacionados a esteroidogênese, estresse oxidativo e de retículo endoplasmático (RE) e de apoptose em células da granulosa. O BHBA diminui o diâmetro do folículo dominante e a taxa de crescimento folicular. No entanto, nesse trabalho não foi observado nenhum efeito prejudicial do BHBA na cascata ovulatória ou na funcionalidade das células da granulosa. Com o objetivo de compreender os efeitos do BHBA na expansão, no status oxidativo e no estresse do RE em células do cumulus de bovinos e na taxa de maturação nuclear e clivagem do oócito foi desenvolvido o terceiro experimento. Os resultados desse estudo indicam que o BHBA induz estresse de RE em células do cumulus até as 12 h de MIV, sem comprometer as demais variáveis analisadas. Para avaliar o efeito do uso parenteral de vitamina E para prevenir o estresse oxidativo no sangue e fluido folicular de vacas em BEN, nós realizamos o último trabalho incluído nessa tese. Os resultados demonstraram que a concentração de vitamina E no sangue e no fluido folicular foi maior nas vacas que receberam o antioxidante em comparação com as que não receberam Vitamina E. Os resultados demonstraram que os animais que não receberam antioxidante tiveram maior produção de ROS no fluido folicular comparado aos animais tratados com 1000 UI de Vitamina E, demonstrando a eficácia do antioxidante. A partir dos dados obtidos com essa tese foi possível determinar que o BHBA possui efeitos deletérios sobre os gametas e células foliculares e que a utilização de alternativas antioxidantes pode minimizar esses efeitos.

Palavras-chave: β-hidroxibutirato. Ácidos graxos não esterificados. Período de transição. Vacas leiteiras.

ABSTRACT

METABOLIC STRESS IN CATTLE: EFFECT ON GAMETES AND FOLLICULAR CELLS

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ADVISER: Paulo Bayard Dias Gonçalves

The fertility of dairy cows has declined in recent years and the metabolic disturbances that occur during the transition period have contributed to lower reproductive performance. During the period of negative energy balance (NEB), there is an increase in plasma concentrations of non-esterified fatty acids (NEFA) and ketone bodies, such as β-hydroxybutyrate (BHBA). Studies have shown that these changes are reflected in the bovine follicular, oviductal and uterine microenvironment. However, the effects of NEFA and BHBA in follicular cells and gametes is not fully elucidated. Therefore, the aim of this thesis was to evaluate the effect of high concentrations of BHBA on gametes, follicular growth, and ovulation in cattle and the effect of using Vitamin E to prevent oxidative stress in blood and follicular fluid of cows in NEB. First, the effects of BHBA on the viability of bovine spermatozoa were investigated. The results showed that high concentrations of BHBA impair the sperm kinetics, without altering the oxidative status and morphology of the cells. Subsequently, we evaluated whether intrafollicular injection (IIF) of BHBA compromises follicular growth and ovulatory cascade as well as steroid production and the abundance of genes related to steroidogenesis, oxidative and endoplasmic reticulum (ER) stress, and apoptosis in granulosa cells. BHBA decreases the diameter of the dominant follicle and the follicular growth rate. However, in this study no harmful effect of BHBA was observed on the ovulatory cascade or on the functionality of granulosa cells. To understand the effects of BHBA on the expansion, oxidative status, and ER stress in bovine cumulus cells and on the rate of nuclear maturation and oocyte cleavage, the third experiment was performed. The results of this study showed that BHBA induces ER stress in cumulus cells up to 12 h of IVM, without compromising the other variables analyzed. To evaluate the effect of parenteral use of vitamin E to prevent oxidative stress in blood and follicular fluid of cows in NEB we performed the last study included in this thesis. The results showed that the concentration of vitamin E in the blood and follicular fluid were higher in cows that received the antioxidant in comparison without Vitamin E. Furthermore, in this study, we can observe that animals that did not receive antioxidant had higher ROS production in the follicular fluid compared to animals treated with 1,000 IU of Vitamin E, demonstrating the effectiveness of the antioxidant. From these data, it was possible to determine that BHBA and NEFA have deleterious effects on gametes and follicular cells and vitamin E, as antioxidant, can minimize these negative effects.

Keywords: β-hydroxybutyrate. Non-esterified fatty acids. Transition period. Dairy cows**.**

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ARTIGO 2

fasting for four days and treated intramuscularly with 1,000 IU vitamin E (Fasting + VitE). Values are means ± SEM. $P < 0.05$…….

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LISTA DE ABREVIATURAS E SIGLAS

3BHSD	*3 Beta hydroxysteroid dehydrogenase*
ACTB	*Actin beta*
ADAM17	*ADAM metallopeptidase domain 17*
AI	*Artificial insemination*
ALH	*Amplitude of lateral head*
AREG	*Amphiregulin*
ATF4	*Activating transcription factor 4*
ATF6	*Activating transcription factor 6*
BCF	*Beat cross frequency*
BEN/NEB	Balanço energético negative/*negative energy balance*
BHBA	*Beta-hydroxybutyrate/* Beta hidroxibutirato
BSA	Albumina sérica bovina
C16:0	*Palmitic acid*
C16:1	*Palmitoleic acid*
C17:1	*Methylpalmitoleic acid*
C18:0	*Stearic acid*
C18:1n9	*Oleic acid*
CASA	*Computer-assisted sperm analysis*
CAT	Catalase
cDNA	DNA complementar
CHOP	*DNA damage inducible transcript 3*
COC	*Cumulus-oocyte complexes*
CYP19A1	Citocromo P450, familia 19, subfamília A, polipeptídeo 1
DCF	2′,7′-dichlorofluorescein
DCHF-DA	2′,7′-dichloro dihydrofluorescein diacetate
E2	Estradiol
EB	*Estradiol benzoate*
ER/RE	*Endoplasmic reticulum/*retículo endoplasmático
EREG	*Epiregulin*
FA	*Fatty acids*
FF	*Follicular fluid/*fluido follicular

FRAP	*Total antioxidant potential*
FSH	Hormônio folículo estimulante
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
GnRH	*Gonadotropin-releasing hormone*
GPX	Glutationa peroxidase
GSR	Glutationa reductase
H2AFZ	*H2A.Z variant histone 1*
HAS2	*Hyaluronan synthase 2*
HSPA5	*Heat shock 70 kDa protein 5*
IGF	Fator de crescimento semelhante a insulina
IM	Intramuscular
IVD	*Intravaginal device*/dispositivo intravaginal
IVM/MIV	*In vitro maturation*/maturação *in vitro*
LC3	*Microtubule-associated protein 1A/1B-light chain 3*
LH	Hormônio luteinizante
LIN	*Linearity*
MDA	*Malondialdehyde*
mRNA	RNA mensageiro
MUFA	Ácido graxo monoinsaturado
NEFA	*Non-esterified fatty acids*/ácidos graxos não esterificados
OPU	*Ovum pickup*
P4	*Progesterone*/progesterona
PBS	*Phosphate- buffered saline*
PGF2α	*Prostaglandin F2α analogue*
PPIA	*Peptidylprolyl isomerase A*
PTGS2	*Prostaglandin-endoperoxide synthase 2*
PUFA	Ácido graxo poliinsaturado
RNA	Ácido ribonucleico
ROS	*Reactive oxygen species*/espécies reativas de oxigênio
RPL19	*Ribosomal protein L19*
RPS18	*Ribosomal protein S18*
SCA	*Sperm class analyzer*
SLC16A1	*Solute carrier family 16 member 1*

SLC16A7	*Solute carrier family 16 member 7*
SOD	*Superoxide dismutase*
SOFaaci	*Synthetic oviduct fluid*
STAR	*Steroidogenic acute regulatory protein*
STR	*Straightness index*
TBA	*Thiobarbituric acid*
TBARS	*Thiobarbituric acid reactive species*
TNFAIP6	*Tumor necrosis factor alpha-induced protein 6 -*
TPTZ	*2,4,6-tri(2-pyridyl)-striazine*
UF	Unidade de fluorescência
UPR	*Unfolded protein response*
VAP	*Mean path velocity*
VCL	*Curvilinear velocity*
VSL	*Straight line velocity*
WOB	*Wobble*
XBP1s	*X-box binding protein 1 spliced*
XBP1u	*X-box binding protein 1 unspliced*
XIAP	*X-Linked inhibitor of apoptosis*

SUMÁRIO

1. INTRODUÇÃO

O estresse metabólico em vacas leiteiras é um problema multifatorial, que afeta a eficiência reprodutiva direta ou indiretamente, causando alterações no microambiente folicular e uterino (TRIPATHI et al., 2016). O balanço energético negativo (BEN), que ocorre no período de transição das fêmeas leiteiras, é um dos principais fatores de risco determinantes de alterações reprodutivas. Nesse sentido, a cetose é considerada uma das principais causas de perdas econômicas por diminuir diretamente a produção de leite e ser uma condição que desencadeia outras enfermidades durante esse período em vacas leiteiras (LYKKESFELDT; SVENDSEN, 2007).

Durante o BEN, a mobilização de reservas corporais tem como consequência o aumento dos níveis de ácidos graxos não esterificados (NEFA) séricos, oriundos do catabolismo de triglicerídeos nos adipócitos em resposta aos estímulos lipolíticos. No fígado, os NEFA são oxidados gerando acetil coenzima A (Acetil CoA) que pode ser usado para produção de energia via ciclo de Krebs. Porém, como durante o período de BEN, o ciclo de Krebs está com sua atividade diminuída e sem disponibilidade de oxaloacetato, devido a baixas concentrações de glicose, a quantidade excessiva de acetil-CoA é desviada para a síntese de novo colesterol ou via cetogênese para a produção dos corpos cetônicos (LASSEN; FETTMAN, 2004). Portanto, a elevação dos níveis de NEFA, juntamente com hipoglicemia durante o BEN, predispõe a esteatose hepática e a cetose (DRACKLEY et al., 2005)

Ao final da gestação, ocorre redução da responsividade e da sensibilidade tecidual à insulina, o que favorece a cetogênese e subsequente elevação plasmática de corpos cetônicos, como β-hidroxibutirato (BHBA) e acetoacetato (RHOADS et al., 2004) . Altas concentrações plasmáticas dos NEFA e BHBA também refletem no microambiente folicular e uterino bovino (LEROY et al., 2005; TRIPATHI et al., 2016; WATHES et al., 2007). Como resultado dessas alterações, têm-se a diminuição da qualidade do oócito (LEROY et al., 2004, 2005), viabilidade celular da granulosa (SHARMA et al., 2019; VANHOLDER et al., 2005; YENUGANTI; VIERGUTZ; VANSELOW, 2016), competência oocitária (AARDEMA et al., 2011; JORRITSMA et al., 2003; SUTTON-MCDOWALL et al., 2016; VAN HOECK et al., 2013) e subsequente qualidade embrionária e, portanto, a fertilidade de vacas leiteiras (LEROY et al., 2005; VAN HOECK et al., 2011). Ainda, NEFA reduz a proliferação, migração e capacidade de ligação espermática em células epiteliais do oviduto *in vitro* (JORDAENS et al., 2015). Recentemente nosso grupo de pesquisa revelou que NEFA prejudica o crescimento do folículo

dominante (FERST et al., 2020) e que em células uterinas NEFA e BHBA são responsáveis por induzir o acúmulo de lipídios, causar estresse oxidativo e alterar a morfologia de células durante cultivo *in vitro* (FERST et al., 2021).

Durante o período de BEN ocorre um aumento da produção de espécies reativas de oxigênio (ROS) e, consequentemente, de estresse oxidativo (BIONAZ et al., 2007). Assim, o estresse oxidativo pode ser implicado em condições fisiopatológicas de vacas leiteiras que culmina com distúrbios reprodutivos (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993). Conforme VAN HOECK et al. (2013), o estresse oxidativo é uma das principais vias pelas quais as quantidades elevadas de NEFA afetam oócitos e embriões bovinos. Além dos NEFA, estudos têm apresentado que BHBA pode causar estresse oxidativo e estresse de retículo endoplasmático (RE; BERNABUCCI et al., 2005; ISLAM et al., 2022; LI et al., 2016; SHI et al., 2021; SONG et al., 2012). Ainda, devemos considerar que o aumento da produção de ROS e estresse oxidativo durante o período de BEN pode estar relacionado com o esgotamento das defesas antioxidantes, como relatado para a vitamina E (LEBLANC et al., 2004), cuja concentração sérica diminui rapidamente próximo ao parto, principalmente devido ao transporte deficiente de vitamina E no plasma, aumento do armazenamento de lipídios no fígado (HERDT; SMITH, 1996) e transferência para o colostro (GOFF; KIMURA; HORST, 2002).

Considerando os resultados encontrados até o momento, nosso grupo investigou se o aumento de BHBA no ambiente uterino e ovidutal compromete a viabilidade espermática durante a passagem pelo trato reprodutivo, pois apesar da exposição aos NEFA não influenciar na capacidade fecundante de espermatozoides (DESMET et al., 2017), não há estudos avaliando o efeito de corpos cetônicos nesses gametas. Além disso, nosso grupo buscou compreender quais as consequências de níveis elevados de BHBA no folículo sobre o crescimento folicular, ovulação e maturação do oócito. Portanto, o entendimento do mecanismo pelo qual os NEFA e o BHBA afetam a reprodução de vacas durante o período de transição é de suma importância para desenvolver tecnologias para melhorar a fertilidade das vacas em BEN. Nesse sentido, nós sugerimos que a utilização do antioxidante, Vitamina E, de forma parenteral pode impedir o aumento de ROS no fluido folicular e prevenir os efeitos deletérios causados por NEFA no folículo de vacas durante o período de transição.

Os objetivos do presente trabalho foram: 1) avaliar o efeito de concentrações elevadas de BHBA na morfologia, cinética e status oxidativo dos espermatozoides *in vitro*; 2) verificar se o corpo cetônico, BHBA, compromete o crescimento e a ovulação do folículo pré-ovulatório de bovinos através do estresse celular; 3) determinar os efeitos do BHBA na expansão dos

complexos cumulus-oócito, status oxidativo, estresse de RE e autofagia em células do cumulus e taxas de maturação nuclear e clivagem de oócitos bovinos durante a maturação *in vitro*; e, 4) analisar o efeito do uso de Vitamina E (α-tocoferol) intramuscular para prevenir o estresse oxidativo no sangue e fluido folicular (FF) de vacas em BEN.

2. REVISÃO BIBLIOGRÁFICA

2.1 PERIODO DE TRANSIÇÃO, BALANÇO ENERGÉTICO NEGATIVO E CETOSE EM VACAS

A infertilidade em bovinos leiteiros é um problema multifatorial, que pode estar envolvido com alterações do desenvolvimento folicular, detecção inadequada de estro, qualidade inferior do oócito, transporte alterado de espermatozoides, problemas na fecundação e ambiente sub ótimo do trato reprodutivo feminino ou uma combinação desses fatores (MATOBA et al., 2012). Um dos momentos mais críticos na saúde das vacas leiteiras é o período de transição (GOFF; HORST, 1997).

O período de transição compreende o período entre três semanas pré-parto e três semanas pós-parto e representa o período mais crítico na vida produtiva de vacas leiteiras de alta produção (RINGSEIS; GESSNER; EDER, 2015). Durante esse intervalo de tempo, a necessidade energética das vacas aumenta drasticamente, excedendo a quantidade de matéria seca que a vaca consegue ingerir (TURK et al., 2013). Assim, as vacas entram em BEN, o que desencadeia vias catabólicas, aumentando a produção celular de ROS (CELI, 2011a; CELI, 2011b; PEDERNERA et al., 2010) e podendo comprometer a saúde, produtividade e fertilidade dos animais.

Durante o período de BEN, as vacas usam fontes alternativas de energia para suprir a diminuição da glicose disponível proveniente da gliconeogênese, sendo que a incapacidade das vacas de lidar com o BEN e a queda de glicose leva a uma mobilização excessiva das reservas adiposas, liberando concentrações anormais de NEFA e corpos cetônicos (acetona, acetoacetato e β-hidroxibutirato; BENEDET et al., 2019) no sangue. Fisiologicamente, a intensa mobilização de triglicerídeos do tecido adiposo ocasiona o aumento das concentrações sanguíneas de NEFA, que são metabolizados pelos hepatócitos via β-oxidação para acetil-CoA. Porém, como durante o período de BEN, o ciclo de Krebs está com sua atividade diminuída em razão da baixa disponibilidade de oxaloacetato, a quantidade excessiva de acetil-CoA é desviada para a síntese de triglicerídeos ou via cetogênese para a produção dos corpos cetônicos (LASSEN; FETTMAN, 2004). Dessa forma, NEFA e BHBA são parâmetros usados como indicadores da mobilização de gordura (BELL, 1995; RUKKWAMSUK; WENSING; KRUIP, 1999), sendo

que os corpos cetônicos fornecem uma importante fonte de energia para os tecidos periféricos nos casos em que os níveis de carboidratos são reduzidos.

Os NEFA são definidos como ácidos graxos que não estão ligados a qualquer fração lipídica presente nos fluídos biológicos (VAN HOECK et al., 2013), ou seja, estão na sua forma livre. Eles podem ser classificados como saturados ou insaturados, sendo que os ácidos graxos insaturados podem ser mono (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 palmítico (C16:0), esteárico (C18:0) e o oleico (C18:1n9).

O BHBA é usado para o diagnóstico de cetose (OETZEL, 2015), pois é o corpo cetônico predominante e mais estável, sendo que vacas leiteiras com níveis séricos de BHBA acima de 1,4 mM são consideradas em cetose (DUFFIELD et al., 2009). No caso de desequilíbrio nutricional persistente, as vacas desenvolvem cetose clínica grave (BHBA > 3 mM; FOSTER, 1988). No entanto, alguns autores observaram que a ocorrência de cetose clínica pode estar associada com concentrações de BHBA $\geqslant$1,1 mmol/L (SEIFI et al., 2011; SONG et al., 2012). A cetose ocorre principalmente entre 8 e 60 dias pós-parto, juntamente com a retomada da ciclicidade das fêmeas bovinas. Assim, tanto a cetose subclínica como a cetose clínica afetam a produção de leite, performance reprodutiva e saúde de vacas leiteiras(RABOISSON; MOUNIÉ; MAIGNÉ, 2014), e assim, aumentam as taxas de descartes nas propriedades e os custos de produção (MOSTERT et al., 2017; SEIFI et al., 2011).

O efeito negativo do estresse metabólico devido ao BEN é um importante fator identificado na patogênese de subfertilidade durante o período de transição (BERNABUCCI et al., 2005), podendo interferir sobre o microambiente folicular, ovidutal e uterino (BEAM; BUTLER, 1997; DUPONT; SCARAMUZZI; REVERCHON, 2014). Nesse sentido, há uma forte correlação entre os níveis de NEFA e BHBA no soro e no FF (LEROY et al., 2005) sendo que concentrações aumentadas desses metabólitos no FF comprometem a qualidade do oócito, viabilidade das células da granulosa (SHARMA et al., 2019; VANHOLDER et al., 2005; YENUGANTI; VIERGUTZ; VANSELOW, 2016) competência oocitária (AARDEMA et al., 2011; JORRITSMA et al., 2003; SUTTON-MCDOWALL et al., 2016a; VAN HOECK et al., 2013), e subsequente qualidade embrionária. Portanto, a fertilidade de vacas leiteiras é afetada (VAN HOECK et al., 2011; VANHOLDER et al., 2005). Além disso, os NEFA diminuem a esteroidogênese e proliferação das células da teca (VANHOLDER et al., 2005) e afetam a fisiologia das células epiteliais do oviduto *in vitro*, diminuindo a proliferação, capacidade de migração celular e de ligação espermática (JORDAENS et al., 2015). Ainda, estudos tem

mostrado os efeitos prejudiciais de NEFA e BHBA em células endometriais (FERST et al., 2021; LI et al, 2019). *In vivo*, BUTLER & SMITH (1989) relatam que a duração e a severidade do BEN estão relacionadas com o intervalo para retomada da atividade ovulatória após o parto, uma vez que as concentrações circulantes elevadas de NEFA e BHBA e diminuídas de insulina, fator de crescimento semelhante a insulina 1(IGF1) e glicose estão envolvidas no desempenho reprodutivo. Ainda, recentemente, nosso grupo de pesquisa também demonstrou que os NEFA comprometem o crescimento folicular (FERST et al., 2020).

O ambiente intrafolicular durante o desenvolvimento do folículo dominante e a maturação do oócito, pode ser um dos principais fatores que determinam a fertilidade subsequente (LEROY et al., 2004). Estudos revelaram que as concentrações de NEFA e BHBA no FF também estão aumentadas durante o BEN (Leroy et al., 2004). Nesse sentido, LEROY et al. (2005), após determinarem a composição e as concentrações dos componentes do FF de vacas leiteiras no período pós-parto, adicionaram NEFA no FF durante a maturação oocitária *in vitro* (MIV). Com esse experimento, esses autores observaram que C16:0 e C18:0 tiveram um efeito negativo na maturação meiótica, fecundação e produção embrionária. Além disso, eles relataram que a exposição a altas concentrações de NEFA causa apoptose e necrose nas células do cumulus cultivadas *in vitro* (LEROY et al., 2005). Ainda, SUTTON-MCDOWALL et al. (2016) demonstraram que quantidades elevadas de NEFA durante a MIV prejudicam a viabilidade e o metabolismo dos oócitos e causam estresse de RE, especialmente nas células do cumulus.

2.2 ESPÉCIES REATIVAS DE OXIGÊNIO E ESTRESSE OXIDATIVO EM VACAS LEITEIRAS

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

Fisiologicamente, a produção de ROS e antioxidantes permanecem em equilíbrio. No entanto, quando em concentrações elevadas, ROS possuem efeitos deletérios sobre as células, uma vez que essas não são neutralizadas pelas defesas antioxidantes (LYKKESFELDT; SVENDSEN, 2007). Nesse caso, devido a superprodução de ROS ou depleção de antioxidantes ocorre um processo denominado de estresse oxidativo (AGARWAL; GUPTA; SHARMA, 2005; CELI, 2011b). O estresse oxidativo pode danificar todas as moléculas biológicas, como DNA, RNA, colesterol, lipídios, carboidratos e proteínas. Por sua vez, a oxidação dessas macromoléculas produz vários produtos que podem ser medidos para avaliar o estresse oxidativo *in vivo e in vitro*.

As principais causas de estresse oxidativo em animais são oriundas de eventos metabólicos, inflamatórios e fatores ambientais, como estresse térmico e nutrição (CELI; GABAI, 2015). Ainda, em ruminantes, fatores como alta produção de leite (LÖHRKE et al., 2005), escore de condição corporal ao parto (BERNABUCCI et al., 2005), BEN (PEDERNERA et al., 2010) e dieta (CELI et al., 2012; CELI; GABAI, 2015; GABAI et al., 2004) são fatores que contribuem para o aumento do estresse oxidativo. Assim, o estresse oxidativo pode estar envolvido em várias condições patológicas, incluindo as que são relevantes para a produção e o bem-estar animal (CELI, 2010). Em vacas leiteiras, o estresse oxidativo tem sido associado tanto a doenças (LYKKESFELDT; SVENDSEN, 2007), incluindo mastite (RANJAN et al., 2005), acidose, cetose, enterite, pneumonia, doenças respiratórias (CELI, 2011b) e retenção de placenta (KANKOFER et al., 2010), quanto a problemas reprodutivos (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993a), como perdas embrionárias (CELI, 2011b; CELI et al., 2012) e cistos foliculares (RIZZO et al., 2009), alterando vários eventos fisiológicos que culminam com a diminuição das taxas de prenhez (AGARWAL; GUPTA; SHARMA, 2005; AL-GUBORY; FOWLER; GARREL, 2010).

A 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 ROS 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, 1993a). Nesse sentido, conforme VAN HOECK et al. (2013), o estresse oxidativo é uma das principais vias pelas quais as quantidades elevadas de NEFA afetam oócitos e embriões bovinos. Além dos NEFA, muitos estudos têm demonstrado que BHBA está associado com estresse metabólico e isso pode causar estresse oxidativo, resposta inflamatória e apoptose celular em hepatócitos (BERNABUCCI et al., 2005; LI et al., 2016; SONG et al., 2012). Deve-se considerar que o aumento da produção de ROS e, consequente, estresse oxidativo durante o período de BEN pode estar relacionado com o esgotamento das defesas antioxidantes, como relatado para a vitamina E (LEBLANC et al., 2004). A vitamina E, é um conhecido captador de radicais livres (PRZYBYLSKA; ALBERA; KANKOFER, 2007) cuja concentração sérica diminui rapidamente próximo ao parto, devido ao transporte deficiente no plasma, aumento da estocagem no fígado (HERDT; SMITH, 1996) e alta acumulação no colostro (GOFF; KIMURA; HORST, 2002).

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

2.3 DEFESAS ANTIOXIDANTES

Os antioxidantes podem ser divididos em dois grandes grupos: antioxidantes enzimáticos e antioxidantes não enzimáticos (SUGINO, 2006). Os antioxidantes enzimáticos são as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GSR) e representam a principal forma de defesa intracelular. A SOD é o primeiro antioxidante enzimático no ovário que desempenha um papel protetor vital ao catalisar a conversão de radicais superóxido em peróxido de hidrogênio (H_2O_2), enquanto a catalase e a glutationa peroxidase GPx convertem H_2O_2 em água (AL-GUBORY; FOWLER; GARREL, 2010). Além disso, sugere-se que esses antioxidantes atuam dentro dos folículos em crescimento, células da granulosa do folículo dominante, endométrio e corpo lúteo para regular importantes funções reprodutivas como ovulação, fecundação e desenvolvimento embrionário (RIZZO et al., 2012). Os antioxidantes não enzimáticos podem ser divididos em hidrofílicos como a vitamina C e lipofílicos como as vitaminas A e E (PIERCE; CACKLER; ARNETT, 2004).

A capacidade antioxidante total é variável no período de lactação. Conforme CASTILLO et al. (2005) a capacidade antioxidante total no final da lactação em vacas prenhas é menor que a observada em vacas leiteiras no pico de lactação. Nesse sentido, pode se inferir que a produção de ROS, que acompanha a esteroidogênese (SUGINO, 2006a), é menor do que ocorre em intensas mudanças metabólicas, como o BEN (ALBERA; KANKOFER, 2011). De forma geral, o equilíbrio adequado entre oxidantes e antioxidantes é essencial para manter a saúde e o desempenho das vacas leiteiras (TURK et al., 2013). Entre os antioxidantes, o α-tocoferol mostra a atividade da vitamina E como um removedor de radicais livres, protegendo todas as membranas contendo fosfolipídios da peroxidação lipídica causada pelo estresse oxidativo (PRZYBYLSKA; ALBERA; KANKOFER, 2007).

A vitamina E é uma descrição genérica para duas famílias de compostos lipossolúveis, os tocoferóis e os tocotrienóis. Existem quatro compostos diferentes de tocoferol e tocotrienóis designados como α, β, γ e δ (POLITIS, 2012), sendo que a forma mais abundante e biologicamente ativa na natureza é o α-tocoferol (BRIGELIUS-FLOHÉ; TRABER, 1999). Todas as formas de vitamina E consistem em um núcleo hidroquinol com uma cadeia isoprenóide (quatro átomos de carbono em uma cadeia reta e uma cadeia lateral de um único carbono), que é repetido três vezes em sucessão. Os tocoferóis têm sua cadeia lateral completamente saturada (POLITIS, 2012). A vitamina E é um antioxidante lipossolúvel primário, importante para a defesa contra o estresse oxidativo (IBRAHIM et al., 1997), que impede a peroxidação de lipídios através da neutralização dos radicais livres por meio da formação do radical α-tocoferoxil (KAMAL-ELDIN; APPELQVIST, 1996). Por sua vez, o radical α-tocoferoxil é regenerado pela atividade da glutationa reduzida, vitamina C e coenzima Q10. Entretanto, durante o estresse oxidativo, a quantidade desses compostos disponíveis para a regeneração do α-tocoferol não é suficiente para reduzir os níveis de α-tocoferoxil, o que pode levar a danos relacionados ao estresse oxidativo. A vitamina E é transportada no plasma, principalmente, pela lipoproteína de densidade muito baixa (DUTTA-ROY, 1999) e, portanto, o risco de baixos níveis plasmáticos de vitamina E é mais pronunciado durante o período periparto das vacas.

A ingestão de vitamina E recomendada pelo Conselho Nacional de Pesquisa dos EUA (NRC, 2001) é de 80 a 20 mg/Kg de matéria seca, o que corresponde a cerca de 1000-1400 e 400-500 mg por dia para vacas secas e lactantes, respectivamente. Essas quantidades representam um requisito mínimo para evitar sintomas de deficiência e garantir um desempenho razoável dos animais. Existem evidências na literatura sobre a importância de concentrações adequadas de α-tocoferol no sangue e o papel da suplementação de vitamina E durante o período

de transição na saúde periparto de vacas leiteiras (PONTES et al., 2015). Nesse sentido, caso a ingestão dietética de vitamina E seja inadequada, as concentrações de α-tocoferol diminuem no sangue e aumentam os riscos de enfermidades (LEBLANC et al., 2004; MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993). É importante considerar que os níveis de α-tocoferol são fisiologicamente reduzidos desde cerca de dez dias antes do parto até cerca de duas semanas após o parto (GOFF; KIMURA; HORST, 2002b; HERDT; SMITH, 1996). Dada a sua importância como antioxidante, pode-se esperar que as concentrações de vitamina E nos tecidos sofram grandes mudanças, influenciando na ocorrência de estresse oxidativo (BOUWSTRA et al., 2008). Por exemplo, DE BIE et al. (2016) demonstraram que as concentrações de α-tocoferol no FF em vacas é 3 a 4 vezes inferior aos níveis plasmáticos, o que pode comprometer o ambiente folicular e a fertilidade dessas fêmeas.

Vários mecanismos têm sido propostos pelos quais os antioxidantes, incluindo a vitamina E, podem melhorar a reprodução em bovinos (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993a). Tem sido proposto que a vitamina E pode ter efeitos sobre os folículos que se desenvolvem durante o período pré-parto tardio ou no pós-parto e, potencialmente, alterar a capacidade fecundante futura desses oócitos (LEROY et al., 2008). Além disso, estudos têm sugerido que elevadas concentrações de BHBA antes do parto coincide com baixos níveis de α-tocoferol (LI et al., 2016b; PILOTTO et al., 2016; QU et al., 2014), demonstrando a relação entre BEN e antioxidantes, uma vez que a atividade metabólica mais alta é acompanhada pelo aumento da produção de ROS (LÖHRKE et al., 2005).

No ovário mamífero, o α-tocoferol é o antioxidante mais importante que protege as células das ROS (ARIAS-ÁLVAREZ et al., 2018). O α-tocoferol está presente no FF tanto em animais como em humanos (CASSANO et al., 1999; PALINI et al., 2014), e tem um papel importante no desenvolvimento folicular e maturação do oócito (SCHWEIGERT et al., 2003). Estudos *in vitro* têm demonstrado que a suplementação do meio de cultivo com α-tocoferol aumenta a viabilidade de células da granulosa (MCCLUSKEY; HALL; STANTON, 1999), foliculogênese, qualidade oocitária e desenvolvimento embrionário de bovinos (OLSON; SEIDEL, 2000), porcas (TAREQ et al., 2012) e ovelhas (NATARAJAN; SHANKAR, 2010). Ainda, o uso de α-tocoferol durante a maturação *in vitro* de oócitos de coelhos aumentou a competência oocitária através da diminuição do estresse oxidativo, de danos as células e apoptose tanto em oócitos como em células do cumulus (ARIAS-ÁLVAREZ et al., 2018). Considerando que a vitamina E é um poderoso antioxidante, que influencia a saúde de vacas leiteiras no período de transição, é possível que a administração de α-tocoferol impeça o estresse oxidativo no folículo ovariano e assim melhore os índices reprodutivos dessas fêmeas.

Estudos relatam que durante o período pré-parto é crucial a suplementação de vitamina E em vacas leiteiras, pois pode impedir parcialmente o declínio nas concentrações plasmáticas de α-tocoferol (MILLER; BRZEZINSKA-SLEBODZINSKA; MADSEN, 1993a; POLITIS, 2012). Existem duas maneiras de suplementar vacas leiteiras com vitamina E: (a) suplementação dietética diária de 1000 a 3000 UI / vaca por dia e/ou (b) injeções parenterais durante as últimas 2 semanas antes do parto. Estudos relataram que a administração de 1.000 UI de vitamina E melhora alguns índices reprodutivos (BALDI et al., 2000; CAMPBELL; MILLER, 1998; PONTES et al., 2015). PONTES e colaboradores (2015) observaram que a administração de 1000 UI de α-tocoferol em intervalos semanais durante três semanas antes do parto diminui a incidência de retenção de placenta e melhora as taxas de prenhez/inseminação artificial com diminuição do intervalo entre partos. Resultados similares, foram encontrados por ARÉCHIGA et al. (1998) que relataram que a suplementação com 500 mg de vitamina E melhora a fertilidade, com redução no intervalo entre partos e gestação e aumento da prenhez/IA no segundo serviço. Esses resultados podem estar associados com a redução do estresse oxidativo, pois a suplementação de vacas com antioxidantes exógenos aumenta os níveis séricos das vitaminas e reduz o acúmulo de ROS (RIZZO et al., 2013).

2.4 ESTRESSE DE RETÍCULO ENDOPLASMÁTICO EM VACAS LEITEIRAS

O RE desempenha diferentes funções na célula, que incluem síntese, alterações conformacionais e transporte de proteínas secretoras e de membrana, biossíntese de lipídios, armazenamento e sinalização de Ca^{2+} e requer uma alta área de superfície e uma distribuição por todo o citoplasma (RINGSEIS; GESSNER; EDER, 2015). O estresse do RE é definido como um desequilíbrio entre a capacidade de modificações conformacionais das proteínas no RE e a carga proteica, assim ocorre, consequente, alterações conformacionais ou proteínas malformadas, que acabam se acumulando no lúmen do RE, perturbando, desse modo, a homeostase da organela (CNOP; FOUFELLE; VELLOSO, 2012). Em situações de estresse, o ambiente do RE é comprometido e a maturação das proteínas é prejudicada, levando ao acúmulo de proteínas mal-formadas e uma resposta ao estresse característica chamada de *unfolded protein response* (UPR; RUTKOWSKI; KAUFMAN, 2004)**,** que visa impedir o estresse e restaurar a homeostase (CNOP; FOUFELLE; VELLOSO, 2012; RINGSEIS; GESSNER; EDER, 2015). Tem sido sugerido que a proteína-78 regulada pela glicose (GRP78/Bip), também conhecida como HSPA5, é um importante marcador de estresse de RE, uma vez que é uma chaperona residente no RE e desempenha um papel vital na regulação da

homeostase do RE (LIU et al., 2011). Além da GRP78, a GRP94 também contribui para o dobramento de proteínas, armazena o cálcio no RE e auxilia no direcionamento de proteínas mal dobradas para degradação (MARZEC; ELETTO; ARGON, 2012).

Níveis sanguíneos elevados de NEFA e BHBA são considerados importantes indutores do estresse de RE de células hepáticas de vacas leiteiras em BEN (ISLAM et al., 2022; SHI et al., 2021), sendo que em outras espécies, condições metabólicas como obesidade ou diabetes, levam ao estresse de RE dessas células (CNOP; FOUFELLE; VELLOSO, 2012; KAWASAKI et al., 2012). Estudo avaliando o efeito dos NEFA no estresse de RE durante a maturação *in vitro* de oócitos bovinos demonstrou que houve um aumento nos níveis de expressão dos genes marcadores de estresse de RE, ATF4 e HSPA5 (SUTTON-MCDOWALL et al., 2016). ALEMU et al. (2018) observaram que o estresse térmico aumenta acentuadamente o acúmulo de HSPA5 nas células da granulosa cultivadas *in vitro* após 24 h de exposição. Em células da granulosa de ratos, CHEN et al. (2019) relataram que o ácido palmítico induz apoptose através de estresse de RE. NIVALA et al. (2013) propuseram que o efeito indutor de estresse de RE pelos NEFA séricos é mediado, principalmente, pelos níveis de ácidos graxos saturados livres, uma vez que esses ácidos graxos são importantes determinantes para a homeostase do RE no fígado. Nesse sentido, sabe-se que o estresse de RE, associado aos ácidos graxos saturados, ocorre porque esses ácidos, comparados aos insaturados, são pouco convertidos em triacilgliceróis e permanecem na forma livre, onde podem perturbar a morfologia e função do RE (GENTILE; FRYE; PAGLIASSOTTI, 2011).

Além dos NEFA, o estresse oxidativo tem se mostrado um iniciador e um dos principais contribuintes para o estresse de RE (HOTAMISLIGIL, 2010). Níveis elevados de ROS são considerados como mensageiros locais entre estresse de RE e a mitocôndria (CSORDÁS; HAJNÓCZKY, 2009). Considerando a relação entre NEFA, ROS e estresse de RE, tem sido relatado que o ácido palmítico induz estresse de RE através do dano ao DNA mitocondrial devido ao estresse oxidativo (YUZEFOVYCH et al., 2013). Ainda, estresse oxidativo e estresse de RE podem induzir a autofagia (YORIMITSU et al., 2006). A autofagia, bem como a UPR, são mecanismos desencadeados para citoproteção principalmente em casos de estresse celular (FERNANDEZ, 2015), como é o caso dos transtornos metabólicos. No entanto, não há estudos avaliando o efeito dos corpos cetônicos, como o BHBA, no estresse oxidativo e estresse de RE em células foliculares de bovinos.

ARTIGO 1

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High concentrations of β-hydroxybutyrate alter the kinetics of bovine spermatozoa

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Running head: β-hydroxybutyrate on kinetics of bovine sperm

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ABSTRACT

Postpartum cows, mainly with metabolic diseases, such as ketosis, usually experience an increased number of services per conception. During ketosis, high concentrations of β-hydroxybutyrate (BHBA) in follicular, uterine and oviductal fluid have been considered to cause subfertility in cows. However, the effect of sperm exposure to an environment with high BHBA concentration is not known. This study investigated the influence of high levels of BHBA on kinetics, oxidative status and morphology of bovine spermatozoa. To assess the effect of BHBA after sperm selection, bovine spermatozoa were incubated (180 min) with different BHBA concentrations: 0 (Control),

0.8, 2.4 or 5 mM. Sperm kinetics was evaluated after 30, 60, 120 and 180 min, and oxidative status and morphology were analyzed at 180 min. Oxidative status was evaluated through the production of reactive oxidative species (ROS), total antioxidant capacity and lipid peroxidation. High concentrations of BHBA decreased the curvilinear velocity, straight line velocity, mean path velocity, linearity, straightness and hyperactivity of spermatozoa. However, there was no effect of BHBA on oxidative and antioxidant capacity as well as on sperm morphology. In conclusion, exposure of bovine spermatozoa to high levels of BHBA impairs sperm kinetics without altering oxidative and antioxidant mechanisms.

1. INTRODUCTION

Resumption of ovarian cyclicity and uterine recovery during the postpartum period is critical for subsequent fertility in dairy cows (Sheldon, 2004). Metabolic stress conditions, leading to negative energy balance (NEB), have been associated with reduced fertility in dairy cows (Raboisson et al., 2014; Roche et al., 2017). Negative energy balance can affect many physiological systems, including the reproductive system and, as a consequence, resulting in a low pregnancy rate. During periods of NEB, the blood plasma concentration of non esterified fatty acids and ketone bodies is elevated, whereas blood glucose levels are reduced, which affect the follicular, uterine and oviduct fluid (Leroy et al., 2005; Tripathi et al., 2016; Wathes et al., 2007). The uterus and oviduct play a vital role in early embryo development and are also involved in sperm selection, storage, motility, transport and fertilization (Coy et al., 2012; Ghersevich et al., 2015; Holt & Fazeli, 2010). Therefore, changes in the female genital tract milieu can affect sperm viability.

The main and most stable ketone body circulating in dairy cows is β-hydroxybutyrate (BHBA; Duffield et al., 2009) and its concentration is an index of fatty acid oxidation. The BHBA increases considerably during fasting, prolonged exercise or diabetic ketoacidosis in humans and ketosis in cattle (Newman & Verdin, 2014). The BHBA serum levels above 1.4 mM indicate ketosis in cows (Duffield et al., 2009) and can reach levels of 6–8 mM (Duffield et al., 2009; Foster, 1988). As the maternal metabolic state is reflected in blood and follicular fluid, a recent study demonstrated that BHBA is present in high concentrations in oviductal and uterine fluids during postpartum in ewes (Tripathi et al., 2016). In sheep, BHBA concentrations in the oviductal fluid were 0.33 ± 0.02 and 0.58 ± 0.11 mM in ewes in the control group and 17–25 days postpartum respectively. In the uterine fluid, BHBA concentrations were 0.38 ± 0.09 and 0.72 ± 0.10 for ewes in the control and postpartum group respectively (Tripathi et al., 2016). Moreover, BHBA has been related to oxidative stress in different cells like hepatocytes

and endometrial cells (Cheng et al., 2019; Li et al., 2019; Shi et al., 2014). Consequently, high levels of BHBA present in the fertilization milieu may affect the oxidative stress levels and the sperm viability.

The effect of high levels of BHBA, which is found in the uterus and oviduct during bovine ketosis, on sperm function is not yet defined. Therefore, we hypothesized that semen exposure to high concentrations of BHBA affect directly sperm kinetics and morphology by increasing oxidative stress. To test this hypothesis, we aimed to assess the effect of elevated BHBA concentrations on sperm morphology, kinetics and oxidative status *in vitro*.

2. MATERIAL AND METHODS

2.1 Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Company. The BHBA was dissolved in phosphate-buffered saline (PBS) at the concentration of 50 mM. Dilutions were performed in sp-TALP medium to yield the concentrations of 0.8, 2.4 and 5.0 mM of BHBA.

2.2 Sperm preparation

Frozen bovine semen from one ejaculate of four *Bos taurus* bulls of proven fertility were used in the experiment. For the experiment, a semen straw of each bull was thawed at 37°C for 30 s and mixed in sperm pools. Samples were homogenized and the spermatozoa were selected by discontinuous Percoll gradient. To verify the effects of BHBA in sperm kinetics, the spermatozoa were distributed and incubated for 30, 60, 120 and 180 min at 37°C in sp-TALP medium without BHBA (Control) or with 0.8 mM, 2.4 mM or 5.0 mM of BHBA. At 180 min, the oxidative status and sperm morphology were determined. Since no data are available regarding the concentrations of BHBA in the uterus or oviductal fluid in cows under metabolic

stress conditions, the concentrations of BHBA used here are based on those established in bovine endometrial cells by Li et al. (2019). Each treatment was replicated six times.

2.3 Sperm selection

Sperm cells were selected by discontinuous Percoll density gradient. A Percoll solution was used for the preparation of 90, 60 and 30 % solutions with modified Talp-Fert media (Parrish et al., 1986). The Percoll density gradient was created according to Gonçalves et al. (2018). Briefly, 300 µl thawed semen was layered on the top of the gradient and the tubes were centrifuged twice for 5 min and 1 min at 2.200 × g. Finally, the pellet was resuspended in sp-TALP to spermatozoa final concentration of 4×10^6 /mL and exposed in different concentrations of BHBA (0.8; 2.4 or 5 mM) or Control for 30, 60, 120 or 180 min. The sperm kinetics was analyzed at 30, 60, 120 and 180 min. The oxidative status and sperm morphology were analyzed only at 180 min.

2.4 Sperm kinetics

Sperm kinetics evaluations were performed with the SCA 5.0 system (Sperm Class Analyser, Microptic). The CASA settings were followed according to the manufacturer's instructions. In brief, a 5 µL aliquot of semen sample was placed on a pre-warmed (37°C) microscope slide and covered it with a 15 × 15 mm pre-warmed coverslip. Sperm kinetics parameters were analyzed in different fields (varying between 15 and 20 fields per treatment per replicate) using positive phase contrast 10× objective. The fields were analyzed by capturing 25 frames/field at a rate of 25 frames/s; 25–70 μm^2 for head area, 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); mean path velocity (VAP, μm/s); linearity (LIN, %); straightness index (STR, %); amplitude of lateral head displacement (ALH, μm); beat cross frequency (BCF, Hz); wobble (WOB, %); and hyperactivity (%, spermatozoa with VCL >35 μm/s, ALH >2.5 μm and STR >85%), as proposed by Mortimer (2000).

2.5 Semen oxidative status

To assess the oxidative status, the samples were submitted to three evaluations: production of reactive oxygen species (ROS), total antioxidant capacity and lipid peroxidation. The ROS production was determined with a spectrofluorimetric method according Loetchutinat et al. (2005). Briefly, the samples were incubated in the dark with 5 μL of 2′,7′-dichloro dihydrofluorescein diacetate (DCHF-DA). The DCHF-DA, upon oxidation, is converts to the fluorescent 2′,7′-dichlorofluorescein (DCF). The oxidation of DCHF-DA to DCF was used to detect and measure intracellular ROS concentrations. The fluorescence intensity emitted at 520 nm (488 nm excitation) was monitored 60 minutes after the addition of DCF-DA. The total antioxidant potential (FRAP) in the sample was determined by ability of antioxidants to reduce Fe^{+3} to Fe^{+2}, which is chelated by 2,4,6-tri(2-pyridyl)-striazine (TPTZ) to form Fe^{+2} -TPTZ with maximum absorption at 593 nm (Benzie & Strain, 1996). Lipid peroxidation was determined by the formation of thiobarbituric acid reactive species (TBARS). Malondialdehyde (MDA) was reacted with thiobarbituric acid (TBA) to form a color complex, which was determined spectrophotometrically at 532 nm as described by Ohkawa et al. (1979).

2.6 Sperm morphology

An aliquot of semen was fixed in 4 % formaldehyde, and then the morphology of minimum of 200 spermatozoa per slide was determined by examining a thin cover-slip

preparation of semen using differential interference contrast microscopy (oil immersion objective at 1,000× magnification). Morphologic defects were classified according to the region of the spermatozoa. The classification system identified five overall categories as previously described by Burns et al. (2013) and Fordyce et al. (2006): morphologically normal, head defects, midpiece defects, tail defects and cytoplasmic droplets (including the subcategories proximal, distal).

2.7 Statistical analysis

The effect of treatment, time and their interaction on sperm kinetics was assessed using mixed models with a repeated measure statement. Different covariance structures were tested for each model and it was accepted the one with smaller Akaike Information Criteria (AIC). Differences between sperm kinetics at a specific time point were determined after establishing the final model by Student's t-test using least squares corrected means (lsmeans). Differences in the oxidative status variables were analyzed using a one-way ANOVA. The percentage of sperm defects and normal sperm cells was accessed by the Kruskal–Wallis test. Shapiro–Wilk test was used to test the normal distribution of continuous data e model residuals. Continuous data are presented as lsmeans ± SEM. All the analyses were performed using the SAS Statistical Package (SAS Institute Inc.), and $P < 0.05$ was considered statistically significant.

3. RESULTS

None of the BHBA concentrations used in this study altered the total motility ($P = 0.866$) and progressive motility ($P = 0.853$) in any of the analyzed times (Figure 1). However, the kinetics parameters (VCL, VSL and VAP) were reduced when sperm cells were exposed to 2.4 or 5.0 mM of BHBA for 60 minutes (Figure 2, $P < 0.05$). When sperm cells were incubated in the presence of 2.4 (72.07 ± 8.48 μm/s) or 5.0 mM (73.93 ± 5.38 μm/s) of BHBA, VCL was

reduced significantly in comparison to control group (92.51 ± 9.51 µm/s; Figure 2A). Also, reduction in VSL was observed when sperm cells were incubated in concentrations of 5.0 mM (44.69 ± 8.76 µm/s) compared to control group (67.41 ± 8.13 µm/s, Figure 2B). The VAP at 60 minutes in. 2.4 mM (56.13 ± 6.35 µm/s) of BHBA was also reduced when compared to the control (73.18 ± 7.93 µm/s, $P<0.03$, Figure 2C). Sperm linearity (LIN) after 120 and 180 minutes and straightness (STR) after 60 minutes were reduced when incubated with 5.0 mM BHBA in comparison to sperm cells from the control group ($P < 0.05$; Figure 3A and 3B), which revealed the effect of BHBA on sperm swimming pattern and progression. At 60 minutes of exposure to 5.0 mM of BHBA (8.22 ± 3.17%), the sperm hyperactivity (Figure 3C) was lower than those observed in the control medium (16.12 ± 3.66 %, $P= 0.02$). Sperm cells incubated with or without BHBA, at different times, did not differ statistically in regard to ALH, BCF and wobble. Independently of the presence of BHBA, the incubation time had a significant effect on all kinetics sperm parameters ($P < 0.05$).

To analyze the effects of BHBA on oxidative status of bovine sperm cells, we evaluated the reactive oxygen species, total antioxidant capacity and lipid peroxidation in sperm samples at 180 minutes (Figure 4A, B, and C, respectively). After three hours of incubation, the production of ROS was similar for the groups control (25.53 ± 2.1 UF), 0.8 mM (22.03 ± 1.6 UF), 2.4 mM (18.79 ± 2.4 UF), and 5 mM of BHBA (25.96 ± 2.8 UF). The total antioxidant capacity and lipid peroxidation did not differ between sperm cells that were incubated without BHBA (55.5 ± 9.9 mcg and 0.03 ± 0.005 nmol MDA/mL) or with 0.8 mM (57.3 ± 8.2 mcg and 0.04 ± 0.006 nmol MDA/mL), 2.4 mM (51.8 ± 12.8 mcg and 0.03 ± 0.003 nmol MDA/mL), and 5 mM (37.1 ± 8.4 mcg and 0.03 ± 0.004 nmol MDA/mL). In the same way, BHBA did not affect the normal sperm morphology, total defects, head defects, midpiece defects, tail defects, and cytoplasmic droplets (Table 1).

4. DISCUSSION

In this study, we observed that the kinetic parameters (VCL, VSL and VAP) were reduced when spermatozoa were incubated in the presence of 2.4 and 5.0 mM BHBA for 60 min. There was a decrease of hyperactivity and sperm STR at 60 min of incubation when samples were exposed to 5 mM of BHBA, and linearity reduced after 120 min when samples were exposed to the same concentration of BHBA. Moreover, we found that sperm morphology and oxidative status were not affected by BHBA. These results demonstrate that high concentrations of BHBA negatively affected the kinetics of bovine spermatozoa, without inducing oxidative stress, a classical cellular deleterious BHBA effect.

The VCL, VSL, VAP, LIN and STR parameters that were affected by high concentrations of BHBA are reliable predictors of bull semen fertility *in vivo* (Farrell et al., 1998; Gliozzi et al., 2017; Kathiravan et al., 2011; Michos et al., 2017; Nagy et al., 2015). The sperm kinetics variables have been considered strong indicators of sperm functionality and fertility (Budworth et al., 1988). The VAP, VSL, STR and LIN variables are markers of sperm progression and STR and LIN are indicators of sperm swimming pattern (Duty et al., 2013). In addition, these are among the most accurate prediction parameters of abnormal sperm motion, which were associated with reduced fertility or embryonic implantation losses (Kawaguchi et al., 2004). *In vitro*, embryonic development rate was reduced when VAP, VSL, BCF, STR and LIN were decreased (Campanholi et al., 2017). In our study, hyperactivity was another parameter that was impaired by high concentrations of BHBA, which should be related to sperm capacitation, and should affect fertilization *in vitro* and *in vivo* (Mortimer, 2000). Therefore, our results suggest that high doses of BHBA can impair sperm progression, hyperactivity and, consequently, the fertility of bovine spermatozoa in cows under ketosis conditions. Although high concentrations of BHBA altered the parameters of sperm kinetics, the rates of total and progressive sperm motility did not differ between control and treatment groups. These results

demonstrated that BHBA had no toxic effect in spermatozoa. Unfortunately, our study does not allow us to answer the question of what factors were responsible for the changes in speed patterns. Therefore, further studies are needed to understand the mechanisms involved in the effect of BHBA on sperm kinetics.

In view of these findings, we decided to assess the effect of BHBA on sperm oxidative status, which was not altered after 3 h of exposure to this ketone body. Bovine spermatozoa are characterized by the abundance of polyunsaturated fatty acids and low concentration of intracellular antioxidant enzymes, becoming highly vulnerable to oxidative stress, which can alter the sperm kinetics (Kang et al., 2008). The relationship between oxidative stress and sperm motility is quite complex. On the one hand, the decrease in motility increases the production of ROS. On the other hand, the increase in ROS ends up decreasing motility. In our study, although BHBA caused changes in sperm kinetics parameters, there was no difference in oxidative status, this may have occurred due to the antioxidant defenses present in spermatozoa being able to neutralize ROS. In other cell types, antioxidant defenses were unable to neutralize ROS, which caused oxidative stress mainly when high concentrations of BHBA were used (Li et al., 2019; Shi et al., 2014; Tian et al., 2014).

The ketone body BHBA did not affect the sperm morphology, which was expected because the main sperm defects are associated with spermatogenesis (Blom, 1973). In this study, commercial semen was used, with a total of at least 70% of normal morphology and, in addition, sperm cells were selected by a discontinuous Percoll density gradient. However, morphology changes caused by incubation medium can be observed in sperm acrosome, intermediate piece and tail, affecting fertilization and sperm kinetics (Ahmed et al., 2019; Ashrafi et al., 2013). Moreover, we must consider that BHBA is a metabolic intermediate, mainly produced in the liver, and serve as an energy source during the transition period in cows.

Therefore, under the conditions of this study, BHBA may have been partially metabolized by spermatozoa (Tanaka et al., 2004), not impairing sperm morphology.

The time period in which the spermatozoa are exposed to a particular factor is crucial to cause cell damage. In this study, selected sperm cells were incubated for 3 h with BHBA, which was enough time to reduce sperm kinetics. It is well known that bovine spermatozoa are transported to the oviducts in two phases; a rapid phase, in which the sperm cells are transported to the oviducts by the female reproductive tract contractions in a few minutes, and a gradual increase phase, when the sperm cells reach the fertilization site after being in the oviduct for several hours (Hawk, 1983). The period of time during which the spermatozoa remains in the female reproductive tract from ejaculation to fertilization is approximately 22 to 24 h (Hawk, 1983). Therefore, the spermatozoa are exposed to high concentrations of BHBA when the cow is in ketosis for several hours.

The negative effects in sperm kinetics observed in this study occurred when doses greater than 2.4 mM for at least 60 min of incubation. In low concentrations or for a short period of time, BHBA supplies energy to the cells (Newman & Verdin, 2014). This fact may explain the absence of effects on the spermatozoa when the BHBA concentration was of 0.8 mM or spermatozoa were exposed for 30 min. Bovine spermatozoa are characterized by a high energy demand and BHBA could be used as energy supply and ATP production (Laeger et al., 2010). Therefore, we suggest that BHBA may have been partially metabolized by the sperm cells (Tanaka et al., 2004). In murine, the motility was recovered when immotile spermatozoa from the epididymis were incubated with high concentrations of BHBA (Tanaka et al., 2004). Therefore, the cells might be using BHBA as an energy source (Tanaka et al., 2004), decreasing the concentration of this ketone body in the incubation medium, which was not investigated in this study. *In vivo*, the ketone bodies do not decrease and this effect is not observed because BHBA levels are kept high in cows with ketosis (Mahrt et al., 2014).

The direct effect of BHBA on the sperm cells is difficult to be examined *in vivo*. Therefore, the present experiment carried out *in vitro* becomes relevant for us to understand how sperm cells behave in the face of high concentrations of BHBA. A computer-assisted sperm analysis (CASA) system provides an objective and quantitative evaluation to study the direct effect of this ketone body on cell kinetics. Therefore, we can conclude that the parameters of bovine sperm kinetics (VCL, VSL, VAP, STR and LIN) are affected by concentrations greater than 2.4 mM of BHBA after of 60 min of exposure *in vitro*. The negative action of BHBA in the sperm kinetics cannot be explained by oxidative stress as observed in other cell types.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, Ferreira, R. upon reasonable request.

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416

Legends:

Figure 1. Total motility (A) and progressive motility (B) of bovine sperm at 0, 30, 60, 120, and 180 minutes of *in vitro* exposure without or with 0.8, 2.4 or 5.0 mM of β-hydroxybutyrate (BHBA). The data are expressed as percentage means ± SEM of six replications.

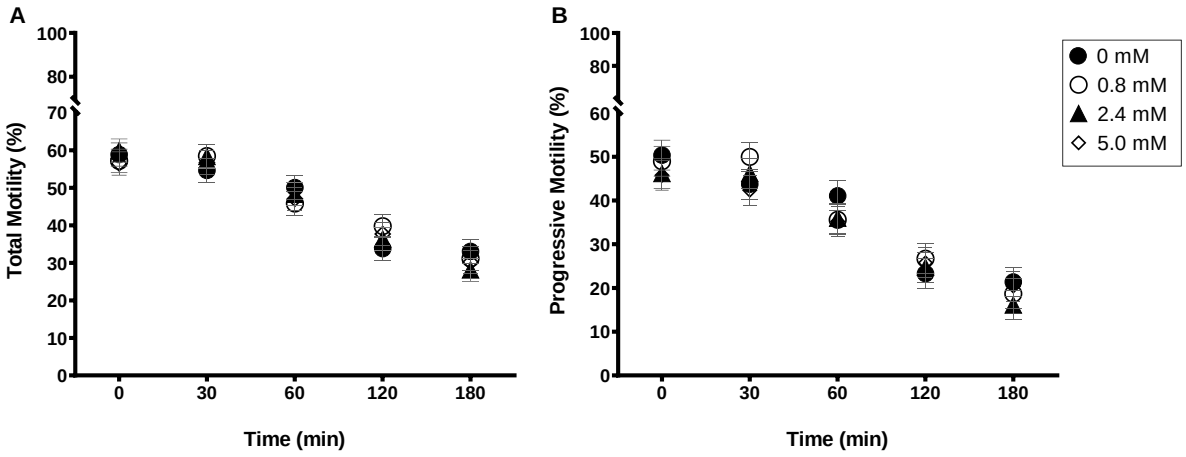
Figure 2. Curvilinear velocity (VCL; A); Straight line velocity (VSL; B), and mean path velocity (VAP; C) of bovine sperm at 0, 30, 60, 120, and 180 minutes of *in vitro* exposure without or with 0.8, 2.4 or 5.0 mM of β-hydroxybutyrate (BHBA). The data are expressed as means ± SEM of six replications. Asterisk (*) indicates difference from control group ($P < 0.05$).

Figure 3. Linearity (LIN; A), straightness (STR; B), and hyperactivity (C) of bovine sperm at 0, 30, 60, 120, and 180 minutes of *in vitro* exposure without or with 0.8, 2.4 or 5.0 mM of β-hydroxybutyrate (BHBA). The data are expressed as percentage means ± SEM of six replications. Asterisk (*) indicates difference from control group ($P < 0.05$).

Figure 4. Reactive oxygen species (ROS; A), Ferric reducing potential (FRAP; B), and Lipid peroxidation (TBARS; C) of bovine sperm after of 180 minutes of *in vitro* exposure without or with 0.8, 2.4 or 5.0 mM of β-hydroxybutyrate (BHBA). The data are expressed as means ± SEM of six replications. $P < 0.05$.

Table 1. Morphology of bovine sperm after of 180 minutes of in vitro exposure without or with 0.8, 2.4 or 5.0 mM of β-hydroxybutyrate (BHBA) The data are expressed as median (lower and upper 95% confidence limit) of six replications.

443 **Figure 1:**



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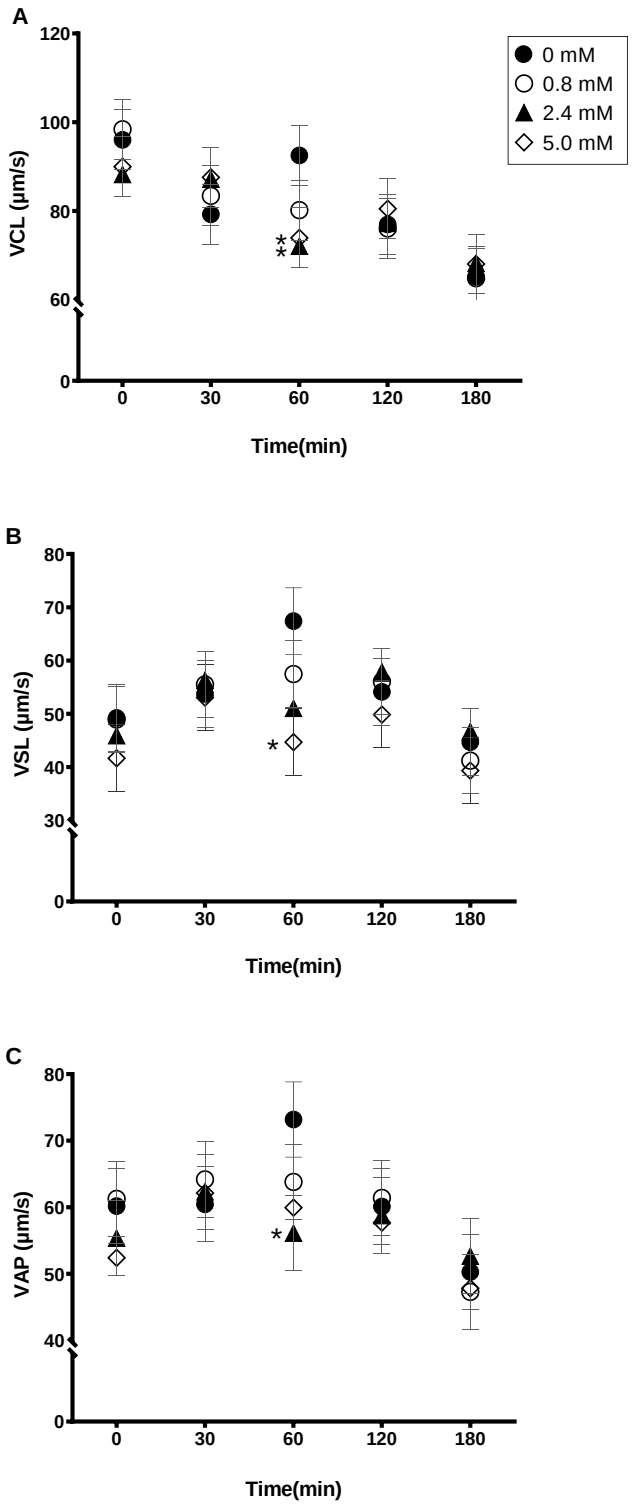
445 **Figure 2:**



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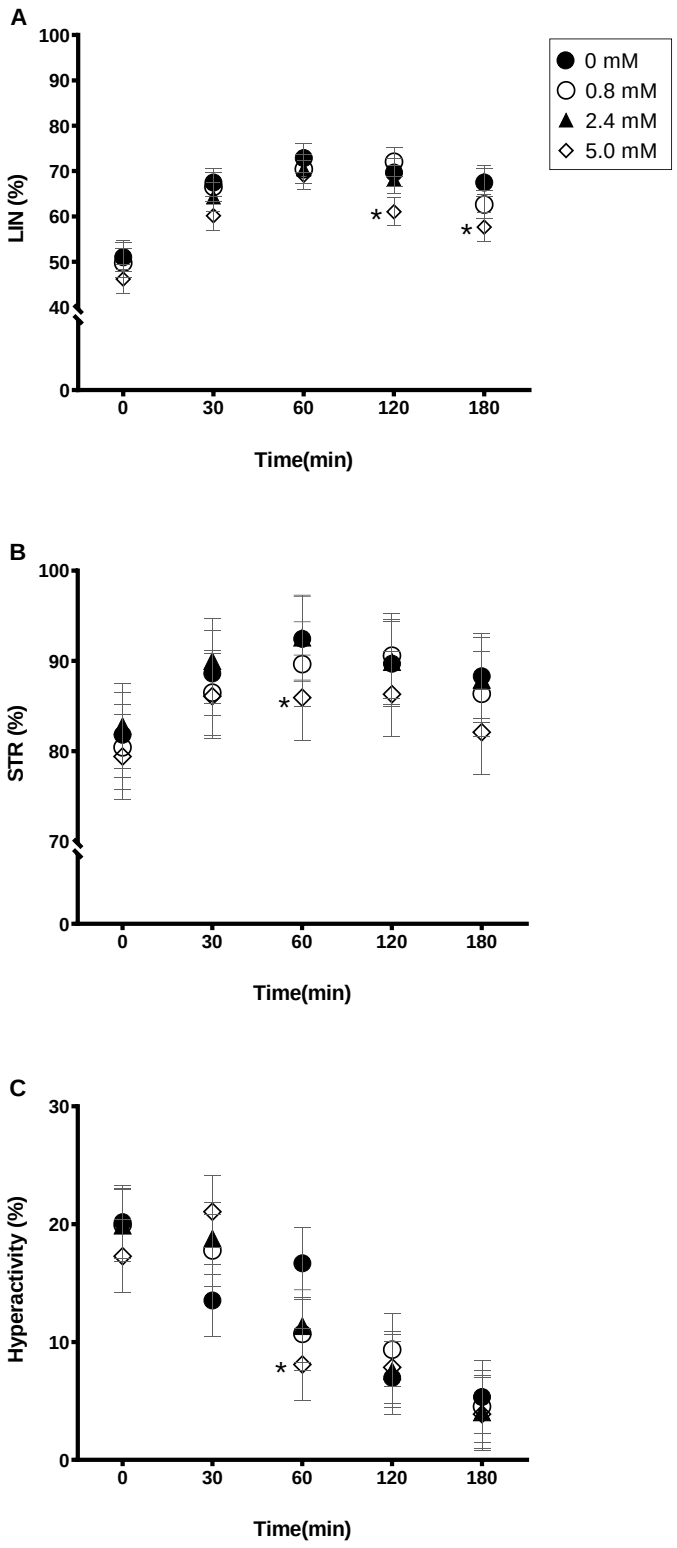
448 **Figure 3:**



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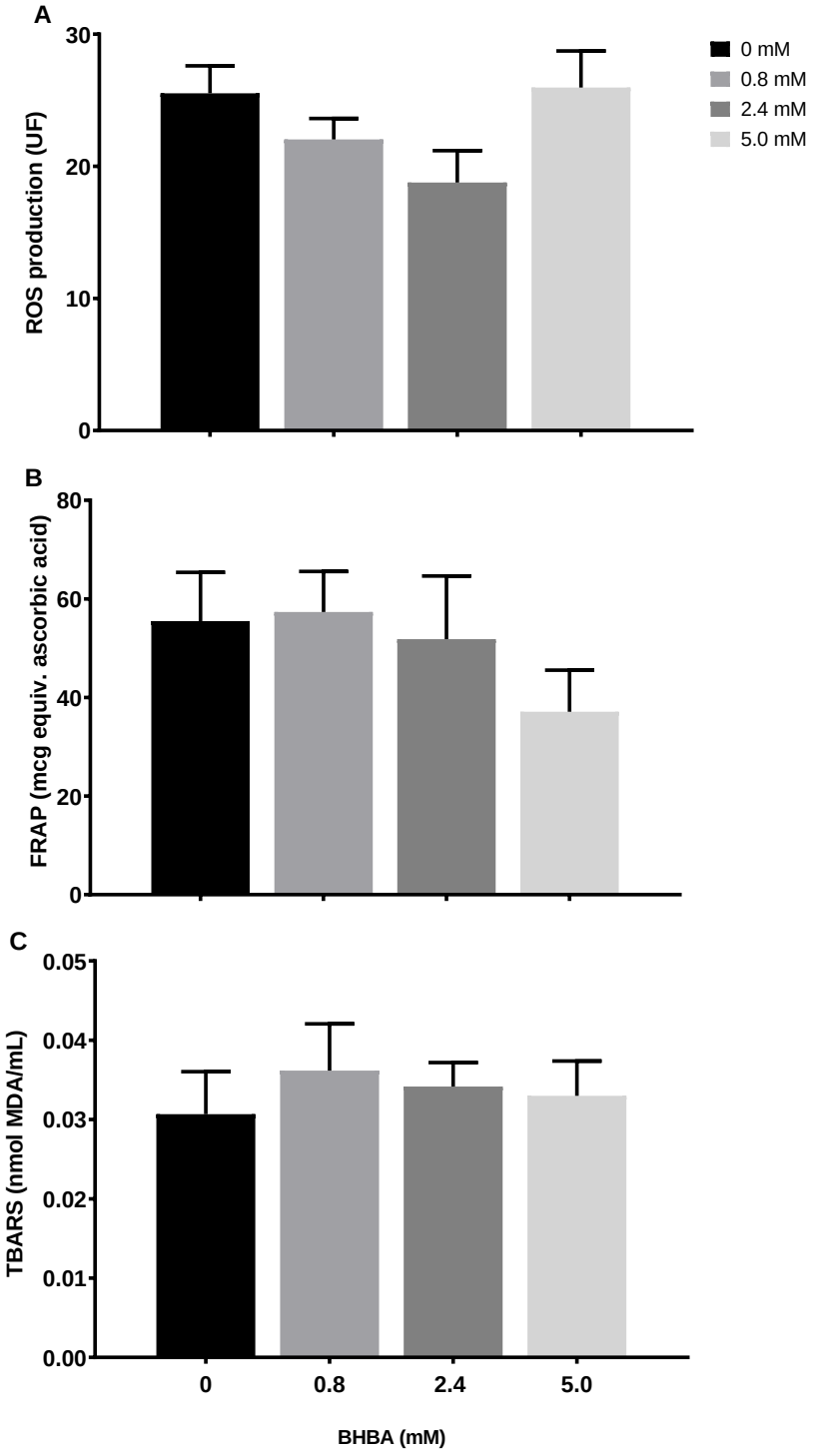
450 **Figure 4:**



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452 **Table 1:**

Variable (%)	BHBA (mM)				*P-value*
	0	**0.8**	**2.4**	**5**	
Normal spermatozoa	85.5 (81-88)	86 (83-88)	86.5 (84-88)	85.5 (82-88)	0.8302
Total defects	14.5 (12-19)	14 (12-17)	13.5(12-16)	14.5(12-18)	0.8302
Head defects	3 (0-6)	4 (0-6)	2.5 (1-5)	3 (1-7)	0.9773
Midpiece defects	1 (0-3)	0 (0-2)	2 (0-3)	2 (0-3)	0.4059
Tail defects	5.5 (3-10)	4.5 (1-8)	4.5 (1-8)	6(4-11)	0.4878
Cytoplasmic droplets	3.5 (2-7)	5 (4-9)	5 (2-6)	3.5(1-5)	0.1753
Proximal droplets	1(0-3)	1(0-3)	1(0-2)	0.5 (0-2)	0.8316
Distal droplets	3(1-6)	4(3-6)	4.5(0-5)	2 (1-4)	0.2271

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ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

High β-hydroxybutyrate levels reduces follicular growth in cattle

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Animal Reproduction Science, 2022

High β-hydroxybutyrate levels reduces follicular growth in cattle

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ABSTRACT

Metabolic stress conditions caused by negative energy balance (NEB) have been associated with reduced fertility in cows. β-hydroxybutyrate (BHBA) is the main circulating ketone body, which accumulate within follicular fluid. The aim of this study was to evaluate the effects of BHBA on follicle growth and on ovulatory mechanism in cattle. At 72 h after intrafollicular BHBA injection, there was a decrease in follicular diameter compared to control ($P = 0.02$). Furthermore, follicle growth rate was reduced post-treatment with BHBA in comparison to the control group ($P < 0.03$). However, the BHBA intrafollicular injection in follicles ≥ 12 mm did not affect E2 and P4 concentrations in the follicular fluid. In addition, the relative abundance of genes involved in ovulatory cascade (*ADAM 17, AREG, EREG, PTGS2*), steroidogenesis (*CYP19A1, 3BHSD, STAR*), cellular stress (*SOD1, CAT, GPX1, HSPA5, XBP1s, XBP1u, ATF4, ATF6*), monocarboxylic acid transporters (*SLC16A1, SLC16A7*) and apoptosis (*XIAP*) was similar between groups. In conclusion, the results of this study indicate that the increase in intrafollicular concentrations of BHBA affects the follicular growth, but it does not compromise the ovulatory cascade and cellular homeostasis in bovine granulosa cells.

Keywords: Ketosis; Ovulation; Granulosa cells; Follicle; Negative energy balance.

1. Introduction

The harmful effect of metabolic stress due to negative energy balance (NEB) is an important factor identified in the pathogenesis of subfertility during the postpartum period of dairy cattle (Bernabucci et al., 2005). During NEB there is an increase in circulating levels of non-esterified fatty acids (NEFA) and ketone bodies. It is well reported that there is a strong correlation between NEFA and β-hydroxybutyrate (BHBA) levels in serum and follicular fluid (FF; Leroy et al., 2004). In this sense, high levels of BHBA may interfere with the follicular, oviductal and uterine microenvironment (Beam and Butler, 1997; Dupont et al., 2014). The increased concentrations of these metabolites in FF impair oocyte quality, granulosa cell viability (Sharma et al., 2019; Vanholder et al., 2005; Yenuganti et al., 2016), oocyte competence (Aardema et al., 2011; Jorritsma et al., 2004; Sutton-Mcdowall et al., 2016; Van Hoeck et al., 2013) and subsequent embryonic quality, affecting the fertility in postpartum dairy cows (Van Hoeck et al., 2011; Vanholder et al., 2005).

Ketone bodies are small molecules synthesized primarily in the liver from fats that circulate through the bloodstream during fasting, prolonged exercise, and when carbohydrates are restricted (Newman and Verdin, 2014). Beta-hydroxybutyrate is the most abundant ketone body in mammals and emerging evidence suggest that BHBA not only is a passive carrier of energy but also has a variety of signaling functions (Newman and Verdin, 2014). In cultured granulosa cells, BHBA caused a reduction in estradiol and progesterone production (Vanholder et al., 2006). In cattle, it is well established that animals with elevated blood BHBA after calving have lower pregnancy success at first artificial insemination (AI), greater number of inseminations per pregnancy, shorter activity at estrus, and longer interval from calving to onset of first estrus than healthy cows (Rutherford et al., 2016; Walsh et al., 2007). In addition, ovulation occurs earlier in cows that have lower serum BHBA concentration ten days before AI (Hill et al., 2018). Despite these results, little is known about the direct effects of high

concentrations of BHBA on follicular growth and ovulation in cattle. Thus, our hypothesis is that high levels of BHBA in dominant follicle impair the follicle growth and ovulation through cell stress mechanisms in cattle. To test this hypothesis, intrafollicular injections of BHBA were performed, followed by evaluation of follicular development, ovulation and mRNA abundance of genes involved in steroidogenesis, ovulation, oxidative stress, endoplasmic reticulum (ER) stress and apoptosis in granulosa cells.

2. Material and Methods

2.1. Animals

The procedure for Experiment 1 was approved by the Ethics Committee on Animal Use of the University of Santa Catarina State (protocol number 2841210316). The procedure for Experiment 2 was approved by the Federal University of Pampa Animal Care and Use Committee (046/2018). In all experiments, non-lactating, estrous cycling, multiparous (4–6 years old) *Bos taurus* cows with a body condition score of 3-4 (on a scale of 1-thin to 5-fat) were used in the study (Moraes et al., 2007). All animals were maintained in large paddocks for grazing of pastures and received water *ad libitum*.

2.2. Hormonal protocol

Emergence of a new follicular wave was induced by using progesterone-releasing intravaginal device (IVD; Primer, Tecnopec, São Paulo, Brazil; 1 g progesterone) and an intramuscular (IM) injection of 2 mg estradiol benzoate (EB; Gonadiol, Zoetis, São Paulo, Brazil). In experiment 1, the IVD was removed 4 days after the time of its insertion. Prostaglandin F2α analogue (PGF2α; 500 μg cloprostenol, Estron, Agener União Saúde Animal, São Paulo, Brazil) was administered intramuscularly at the time of IVD removal, and the ovaries were monitored daily using transrectal ultrasonography procedures. In experiment

2, after intravaginal device removal (day 9), ovaries were examined by transrectal ultrasonography, using an 8 MHz linear-array transducer (AquilaVet scanner, Pie Medical, Netherlands) and cows that had gonadotropin-releasing hormone (GnRH)-responsive preovulatory follicles (≥12 mm) were subjected to intrafollicular treatment described below and with 630 µg of buserelin acetate (Sincroforte, Ouro Fino, Brazil) intramuscular. GnRH-treated cows were then ovariectomized 6 h post-GnRH.

2.3. BHBA preparation

The BHBA (Sigma-Aldrich, Missouri, USA, catalog number 54965) was dissolved in phosphate buffered saline (PBS) at the concentration of 100 mM. The final concentration of BHBA, inside the follicle, was 15 mM. In the control cows, the follicles were injected with PBS.

2.4. Intrafollicular injection

Intrafollicular injections were performed using a system with two sterile needles guided using ultrasonic procedures with a 7.5 MHz vaginal probe (Ferreira et al., 2007). Before injection, the inner needle system was washed consecutively with 70 % alcohol and sterile saline solution (0.9 % NaCl), and then filled with treatment solutions. After epidural anesthesia, intrafollicular injection was performed by pushing the outer needle until close to target follicle. The inner needle was pushed until the bevel appear inside de follicle on ultrasound image and the treatments were immediately applied. The swirling of the fluid entering the follicle was monitored as an indicator that the injection was successful. The intrafollicular injection volume was adjusted based on follicle size to obtain the desired BHBA concentration inside the follicle. The follicular fluid volume was estimated using the linear regression equation V = - 685.1 + 120.7D, where V corresponds to the estimated follicular volume, and D to the diameter of the

follicle to be injected (Ferreira et al., 2007). When the diameter of an injected follicle reduced more than 1 mm within 2 h after injection, the cow was excluded from the experiment. In Experiment 1, the ovaries were examined once a day using transrectal ultrasonography equipped with an 8 MHz linear array transducer (Aquila Vet scanner, Pie Medical, Netherlands). All follicles larger than 5 mm were plotted using three to five virtual slices of the ovary, allowing for three-dimensional localization of the follicles, and monitoring of individual follicles during the follicular wave. In Experiment 2, intrafollicular injection was performed in follicles equal or greater than 12 mm in diameter to analyze the effect of BHBA on ovulatory mechanism and cell stress.

2.5. Isolation of follicular fluid and granulosa cells

GnRH-treated cows were then ovariectomized 6 h post-GnRH via colpotomy in the standing position, as described by Drost et al. (1992). Immediately after ovariectomy, follicular fluid was recovered by aspiration and granulosa cells were collected from the injected follicles by repeated flushing with PBS. The samples were immediately frozen in liquid nitrogen where they remained until processing for analysis (hormonal dosage in FF or mRNA extraction from cells).

2.6. RNA extraction and real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, EUA) according to the manufacturer's instructions and was quantified at 260 nm wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific, Wilmington, DE, USA). RNA was reverse transcribed (RT) using the iScript™ cDNA Synthesis Kit (Bio-Rad, Des Plaines, IL, USA) at 25 °C for 5 min and 46 °C for 30 min. The reaction was ended by incubation at 95 °C for 5 min. The cross-contamination with theca cells and oocyte, in each

sample was checked by qRT-PCR detection of mRNA encoding *CYP17A1* and *GDF9* genes, respectively (data not shown). All qRT-PCR analyses were conducted in a CFX384 thermocycler (BioRad), by using the GoTaq®qPCR Master Mix (Promega,Wisconsin, USA) and bovine-specific primers (Table 1). Standard two-step reactions were performed with initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. To optimize the qRT-PCR assay, serial dilutions of cDNA templates were used to generate a standard curve. The standard curve was constructed by plotting the log of the starting quantity of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R^2) greater than 0.98 and efficiency between 90 % and 105 % were considered optimized. The relative standard curve method was used to assess the amount of a particular mRNA transcript in each sample, and all samples were evaluated in duplicate. The PCR machine was set to Cq CV's up to 7 % between duplicates. When the CV between a duplicate was larger than 7 %, the sample and a calibrator were re-evaluated (Pfaffl, 2001). Results are expressed relative to the geometric average of H2A.Z variant histone 1 (*H2AFZ)*, actin beta (*ACTB*), ribosomal protein L19 (*RPL19*), and peptidylprolyl isomerase A (*PPIA*) as reference genes (Pfaffl, 2001; Vandesompele et al., 2002).

2.7. Hormone assays

Follicular fluid samples were collected and individually stored at – 196 ºC. Follicular fluid concentrations of E2 and P4 were determined using the Estradiol ELISA kit (501890, Cayman Chemical, Ann Arbor, USA) and Progesterone Elisa Kit (562601, Cayman Chemical, Ann Arbor, USA) respectively. For E2 analysis, the dilution of FF samples ranged between 1:500 to 1:1000. The intra-assay coefficient of variation was 3.6 %. For P4 analysis, the dilution of FF samples was 1:500. The intra-assay coefficient of variation was 7.5 %. One sample from

the BHBA group was excluded from further analysis since intrafollicular E2:P4 ratio was lower than 1, indicating to be an atretic follicle (Cheong et al., 2016; Ireland and Roche, 1982).

2.8. Experimental design

In Experiment 1, to assess the effect of increased BHBA concentration on follicular development, sixteen adult cyclic cows had the emergence of a new follicular wave induced. When the follicles reached a diameter of 8-9 mm, which represents the size to predict future dominant follicle (Ferreira et al., 2011), the animals were intrafollicularly injected with BHBA (15 mM; n = 8) or PBS (control; n = 8). Follicular growth was monitored daily until ovulation or for 72 h. For ovulation rate, the preovulatory follicle was monitored for 120 h after treatment.

In Experiment 2, the effect of high levels of BHBA on preovulatory follicle was assessed to study the ovulatory mechanism. Nine adult cyclic cows had the emergence of a new follicular wave induced. In D9, the follicles with diameter ≥ 12 mm, which represents the size of predicted future ovulatory follicle (Ferreira et al. 2011), were injected intrafollicularly with BHBA (15 mM; n = 4) or PBS (n = 5). The cows were ovariectomized after 6 h of BHBA injection as described above. After ovariectomy, follicular fluid and granulosa cell samples were collected from the follicles for steroid assay and evaluation of relative abundance of mRNA transcripts, respectively.

2.9. Statistical analysis

The effect of intrafollicular injection of BHBA or PBS on follicular development was assessed by mixed models for repeated data. The effect of group, time and group time interaction were included in statistical model. Cow was included as subject. Differences between follicular sizes at a specific time point were compared between groups using Students' pair-wise test. Different covariance structures were tested for each model and the one with the

189 least Akaike Information Criteria (AIC) was used. The effect of treatments on ovulation rate
190 was analyzed by chi-square test. Differences in the relative abundance of mRNA transcripts,
191 estradiol and progesterone concentrations were analyzed using a one-way ANOVA. All
192 continuous data and residuals were tested for normal distribution using the Shapiro–Wilk test
193 and normalized when necessary. Data are presented as least square corrected means ± SEM. All
194 the analyses were performed using the SAS Statistical Package (SAS Institute Inc., Cary, NC),
195 and significant differences were considered when $P < 0.05$.
196
197 **3. Results**
198 *3.1. Experiment 1: Effect of intrafollicular injection of BHBA on follicular growth and*
199 *ovulation*
200 The dominant follicle had a size reduction 72 h (Fig. 1A) after intrafollicular injection
201 of BHBA (7.7 ± 1.6 mm) compared to control follicles that received PBS (11.5 ± 0.6 mm; $P =$
202 0.02). In all times that preceded 72 h, the diameter of follicles that received BHBA did not
203 differ from those observed in the control group (0 h: 8.5 ± 0.3 mm and 8.0 ± 0.2 mm; 24 h: 8.4
204 ± 0.6 mm and 7.8 ± 0.3 mm; 48 h: 9.5 ± 0.5 mm and 8.7 ± 0.9 mm; respectively for the follicular
205 sizes of the control and treatment follicles; $P > 0.05$). Furthermore, in the BHBA-treated group,
206 follicle growth rate was reduced post-treatment (0.17 ± 0.2, Fig. 1B) when compared to the
207 control group (0.9 ± 0.2, $P < 0.03$). Although the number of animals was small to analyze the
208 ovulation rate, there were 25 percentage point reduction in the ovulation rate of follicles that
209 received BHBA (6/8; 75%) in comparison to those in control group (8/8; 100%).
210
211 *3.2. Experiment 2: Effect of BHBA in follicular hormone concentration and relative abundance*
212 *of mRNA transcripts in granulosa cells*

To study the effect of BHBA in genes related to ovulation, steroidogenesis, oxidative and ER stress, and apoptosis in granulosa cells, dominant follicles were collected 6 h after the injections with BHBA or PBS. Twelve cows were excluded from the experiment because they presented a dominant follicle smaller than 12 mm on the day of intrafollicular injection. The average diameter of dominant follicle at the time of injection was similar between control (12.6 ± 0.58 mm) and BHBA groups (12.03 ± 0.78 mm; $P = 0.26$).

The BHBA intrafollicular injection did not affect estradiol ($P = 0.89$), progesterone concentration ($P = 0.15$) and E2:P4 ratio ($P = 0.37$) in the FF (Fig. 2). In addition, the relative abundance of *ADAM17, AREG, EREG, PTGS2* (proteins regulating ovulation, Fig. 3), *CYP19A1, 3BHSD, STAR* (proteins regulating steroidogenesis; Fig. 4), *SOD1, CAT, GPX1, HSPA5, XBP1s, XBP1u, ATF4, ATF6* (proteins regulating oxidative stress and ER stress; Fig. 5), *SLC16A1, SLC16A7* (proteins regulating monocarboxylic acid transporters, Fig. 6) and *XIAP* (protein regulating apoptosis; Fig. 7) mRNA transcripts did not differ in granulosa cells from follicles injected with PBS or BHBA.

4. Discussion

In cattle, it is well established that animals with elevated blood BHBA after calving have impaired fertility (Rutherford et al., 2016; Walsh et al., 2007). The present study provides the first evidence that BHBA affects follicular growth. In addition, in the present study the BHBA was not associated with changes in estradiol and progesterone concentration in FF, as well as with the relative abundances of mRNA transcripts for proteins involved in ovulatory cascade, steroidogenesis, oxidative stress, ER stress, monocarboxylic acid transporters and apoptosis in granulosa cells 6 h after the injection of BHBA. To our knowledge, the effect of isolated BHBA on the preovulatory follicle had not yet been demonstrated. It is important to note that during the period of NEB, other metabolites are increased such as NEFA and may compromise

ovulation in cows. NEFA are known to have harmful effects on several cell types as oocyte, granulosa cells, and hepatocytes (Aardema et al., 2011; Shi et al., 2015; Vanholder et al., 2005).

At 72 h after BHBA injection, follicular size was smaller in the BHBA treated follicles than the control follicles. Our results demonstrate that BHBA affected follicular growth rate, but it did not cause atresia in the injected follicles as most of them ovulated. *In vivo*, ovarian cells use ketones as energy substrate (Rabiee et al., 1997) as an alternative at low glucose levels (Veech, 2004) in NEB conditions. The BHBA are used as energy in different cellular types. Therefore, as the animals used in this study were not in NEB, BHBA may have been used by the cells, decreasing their follicular concentration, and allowing granulosa cells to restore its functionality and the follicle to ovulate.

In this sense, to verify whether BHBA affects ovulation marker genes on granulosa cells, we performed the second experiment. We analyzed key genes involved in ovulation such as *ADAM17*, *AREG*, *EREG*, and *PTGS2*. The ovulation process depends on the luteinizing hormone (LH) peak to initiate the signaling cascade necessary for the release of an oocyte and the formation of a corpus luteum (Thatcher, 2017). In the present study, no difference was observed in the expression of genes of the ovulatory cascade between groups, with no harmful effect of BHBA when the follicles are responsive to LH (≥ 12 mm). Studies have shown that postpartum ovulation rates are lower in cows with higher serum concentrations of BHBA (Bossaert et al., 2008; Hill et al., 2018; Stevenson et al., 2020). This lower ovulation rate found must be related to the lower follicular growth and consequently lower ovulatory capacity of the follicle as demonstrated in our results. In addition, we decided to verify steroidogenesis by evaluating key genes such as *STAR, 3BHSD,* and *CYP19A1*. According to our data, the BHBA intrafollicular injection did not change the mRNA levels of *STAR, HSD3B1* and *CYP19A1*. In addition, no changes were observed in the levels of estrogen and progesterone in the FF of follicles injected with BHBA. These results appear to be contradictory to previously published

data (Vanholder et al., 2006), which demonstrated that BHBA reduced progesterone and estradiol production in *in vitro* cultured granulosa cells for 48 h. However, our study and the study by Vanholder et al. (2006) are based on very different experimental approaches. Furthermore, the absence of alterations in gene expression and hormone production may be related to the short interval between injection and ovariectomy (6 h). It is important to emphasize that *in vivo*, during NEB, BHBA levels are increased for several days which can compromise cell viability and steroidogenesis.

Importantly, oxidative stress is observed in the ketotic cows and is positively related to high BHBA and NEFA levels (Li et al., 2016). Moreover, BHBA has been associated with oxidative stress in different cells like hepatocytes and endometrial cells (Cheng et al., 2019; Ferst et al., 2021; Li et al., 2019; Shi et al., 2015). Oxidative stress has proved to be an initiator and one of the main contributors to ER stress (Hotamiligil, 2010). In the present study, there was also evaluation of the relative abundance of mRNA for proteins involved in the regulation of oxidative stress (*SOD1, CAT* and *GPX1*) and ER stress (*HSPA5, ATF4, ATF6, XBP1s* and *XBP1u*) to investigate whether high BHBA concentrations causes stress in granulosa cells. We must also consider that the absence of effects on oxidative stress in this study may have occurred due to the short exposure time of the cells to BHBA and its use by cells. Previously, our group demonstrated that the ROS production did not increase in endometrial cells cultured for 3 days with BHBA; however, it caused an increase in ROS production when cells were cultured for 7 days (Ferst et al., 2021). The ER is a subcellular organelle that ensures the smooth function of synthetic pathways, including lipogenesis. Any perturbation of its homeostasis causes ER stress, resulting in the accumulation of unfolded proteins on the organelle. NEB state affects the expression of ER stress-related genes and proteins in the liver of starved dairy cows and in bovine mammary epithelial cells cultured *in vitro* for 24 h (Zhang et al., 2020). Recently, Shi et al. (2021) and Islam et al. (2022) demonstrated that ER stress occurred in hepatocytes of

cows with ketosis. However, BHBA did not alter the expression of *HSPA5*, an ER stress marker, in endometrial cells cultivated *in vitro* (Ferst et al., 2021).

To assess if treatment with BHBA affected gene transcription, the relative mRNA abundance of candidate genes involved in the apoptosis (*XIAP*) was evaluated in granulosa cells after of intrafollicular injection with BHBA. The relative mRNA abundance of this gene did not differ from the control cells. It is known that the production of the protein encoded by this gene is induced by gonadotropins in granulosa cells during follicular development and that this protein has an important function as a cell survival factor in the control of follicular atresia (Phillipps and Hurst, 2012). In accordance with the non-observance of stress in these cells, *XIAP* levels did not differ in treated and control cells.

In this study, we used a well-established *in vivo* procedure based on the intrafollicular injection to evaluate the effect of BHBA on growing dominant follicles and ovulation in cows without metabolic stress. Our results demonstrated that a single intrafollicular injection of BHBA, despite of preovulatory follicle growth reduction, did not cause stress and did not impair ovulation and candidate gene levels in granulosa cells of preovulatory follicles.

5. Conclusion

Findings from this study revealed that high intrafollicular concentrations of BHBA are detrimental to follicle development in cows. In addition, BHBA did not alter steroids concentrations in follicular fluid and relative abundance of mRNA transcripts encoded by genes involved in the regulation of important functions in granulosa cells from preovulatory follicle.

CRediT authorship contribution statement

Daniele Missio: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Project administration. **Alexandro**

Fritzen: Conceptualization, Methodology, Investigation, Writing - Review & Editing. **Camila Cupper Vieira:** Investigation. **Juliana Germano Ferst:** Methodology, Investigation, Writing - Review & Editing. **Mariani Farias Fiorenza:** Investigation, Writing - Review & Editing. **Leonardo Guedes de Andrade:** Investigation, Writing - Review & Editing. **Bento Martins de Menezes Bisneto:** Investigation. **Monique Rovani:** Investigation, Writing - Review & Editing. **Bernardo Gasperin:** Investigation, Resources, 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.

Declaration of competing interests

The authors declare that they have no conflict of interests.

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Legends:

Table 1: List of primer used in the qRT-PCR reactions.

Fig. 1. Effect of intrafollicular injection with β-hydroxybutyrate (BHBA) on follicular diameter (A) and follicular growth rate (B). A new follicular wave was induced and when the follicles reached 8-9 mm an intrafollicular injection containing BHBA (15 mM; n = 8,) or PBS (control; n = 8) was performed. Follicular diameters were measured daily by transrectal ultrasonography. Asterisk (*) indicates statistical difference between groups ($P \leq 0.05$).

Fig. 2. Estradiol (A), Progesterone (B) concentrations, and E2:P4 ratio (C) in follicular fluid of BHBA-treated and control follicles. A new follicular wave was induced and there were intrafollicular injections of BHBA (15 mM; n = 4) or PBS (control; n = *5*) into the dominant follicle of each cow when it was ≥ 12 mm in diameter. Cows were ovariectomized and follicular fluid was collected 6 h after the BHBA injections.

Fig. 3. Effect of treatment with BHBA on the relative abundance of mRNA transcripts for ovulation related proteins in granulosa cells. A new follicular wave was induced and there were intrafollicular injections of BHBA (15 mM; n = 4) or PBS (control; n = *5*) into the dominant follicle of each cow when it was ≥ 12 mm in diameter. Cows were ovariectomized and follicular fluid was collected 6 h after the BHBA injections.

Fig. 4. Effect of treatment with BHBA on the relative abundance of mRNA transcripts for steroidogenesis related proteins in granulosa cells. A new follicular wave was induced and there were intrafollicular injections of BHBA (15 mM; n = 4) or PBS (control; n = *5*) into the dominant follicle of each cow when it was ≥ 12 mm in diameter. Cows were ovariectomized and follicular fluid was collected 6 h after the BHBA injections.

Fig. 5. Effect of treatment with BHBA on the relative abundance of mRNA transcripts for oxidative stress and endoplasmic reticulum stress related proteins in granulosa cells. A new follicular wave was induced and there were intrafollicular injections of (15 mM; n = 4) or PBS (control; n = *5*) into the dominant follicle of each cow when it was ≥ 12 mm in diameter. Cows were ovariectomized and follicular fluid was collected 6 h after the BHBA injections.

Fig. 6. Effect of treatment with BHBA on the relative abundance of mRNA transcripts for monocarboxylic acid transporters related proteins in granulosa cells. A new follicular wave was induced and there were intrafollicular injections of BHBA (15 mM; *n* = 4) or PBS (control; *n* = *5*) into the dominant follicle of each cow when it was ≥ 12 mm in diameter. Cows were ovariectomized and follicular fluid was collected 6 h after the BHBA injections.

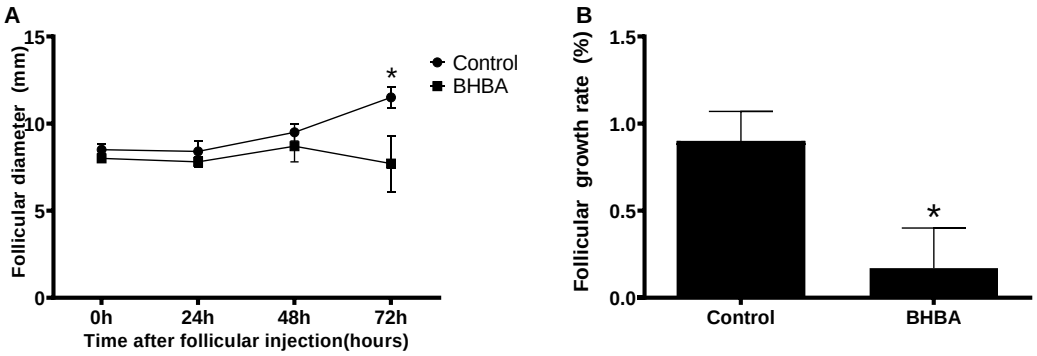
Fig. 7. Effect of treatment with BHBA on the relative abundance of mRNA transcripts for apoptosis related proteins in granulosa cells. A new follicular wave was induced and there were intrafollicular injections of BHBA (15 mM; *n* = 4) or PBS (control; *n* = *5*) into the dominant follicle of each cow when it was ≥ 12 mm in diameter. Cows were ovariectomized and follicular fluid was collected 6 h after the BHBA injections.

526 **Table 1**:

Gene name	Sequence (5′ to 3′)	Accession number
H2AFZ	F:GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	NM_174809.2
ACTB	F:GGATGAGGCTCAGAGCAAGAGA R: TCGTCCCAGTTGGTGACGAT	NM_173979.3
RPL19	F:CCGGCTGCTTAGACGATACC R:CCGCTTGTTTTTGAACACGTT	NM_001040516.1
PPIA	F:GGTCATCGGTCTCTTTGGAA R: TCCTTGATCACACGATGGAA	NM_178320.2
CYP19A1	F: GTGTCCGAAGTTGTGCCTATT R: GGAACCTGCAGTGGGAAATGA	NM_174305.1
3BHSD	F:GCCCAACTCCTACAGGGAGAT R:TTCAGAGCCCACCCATTAGCT	NM_174343.3
ADAM17	F:TTCATGGGACAATGCAGGTTT R: GAAGTGCCTTTCACCAGGTTTT	XM_002691486.2
EREG	F:ACTGCACAGCATTAGTTCAAACTGA R: TGTCCATGCAAACAGTAGCCATT	XM_002688367
AREG	F: CCATTTTCTTGTCGAAGTTTCTTTC R:TGTTTTTATTACAATCCTGCTTCGAA	XM582419
PTGS2	F: TTTGACCCAGAGCTGCTTTT R: GAAAGACGTCAGGCAGAAGG	NM_174445.2
STAR	F: CCCAGCAGAAGGGTGTCATC R: TGCGAGAGGACCTGGTTGAT	NM_174189.3
SOD1	F: ATACACAAGGCTGTACCAGTGC R: CACATTGCCCAGGTCTCCAA	NM_174615.2
CAT	F: AGAGGAAACGCCTGTGTGAG R: ATGCGGGAGCCATATTCAGG	NM_001035386.1
GPX1	F: GCATCAGGAAAACGCCAAGA R: CCATTCACCTCGCACTTTTCG	NM_174076.3
HSPA5	F: CGTGCGTTTGAGAGCTCAGT R: GACAGCTTCATCTTTCCAGCG	NM_001075148.1
ATF6	F: GAACTTCGAGGATGGGTTCATAGG R: CCAGAGCACCCTGAAGAATACG	XM_024989877.1
ATF4	F:AGTAGTGGTGGAGTCTGGCT R: GGTGCCCCCTTCACTTTCTT	NM_001034342.2
XBP1s	F: AGCAGAGACCAAGGGGAATG R: TCAGAGTCCATGGGGAGATGT	NM_001034727.3
XBP1u	F: GCAGAGACCAAGGGGAATGG R:GGGTCCAAGTTGAACAGAATGC	NM_001271737.1
XIAP	F: GAAGCACGGATCATTACATTTGG R:CCTTCACCTAAAGCATAAAATCCAG	XM_024987764.1
SLC16A1	F:TGGCATCTTGTCAGGCAGTGG R: CCAGCCACACAGCAGTTTAATAG	NM_001037319.1
SLC16A7	F: CTCATGGACCTTGTTGGTGC R: CACTGGGACTTTGAGATGTCTTC	NM_001076336.2

527

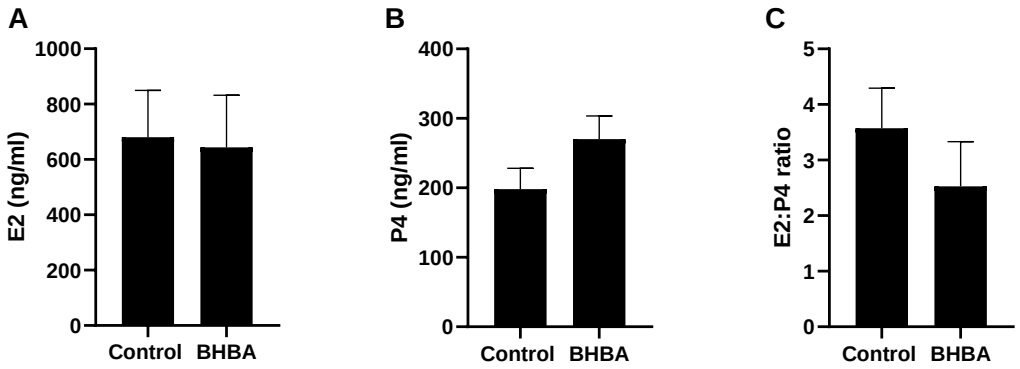
528 **Fig. 1.**



529

530

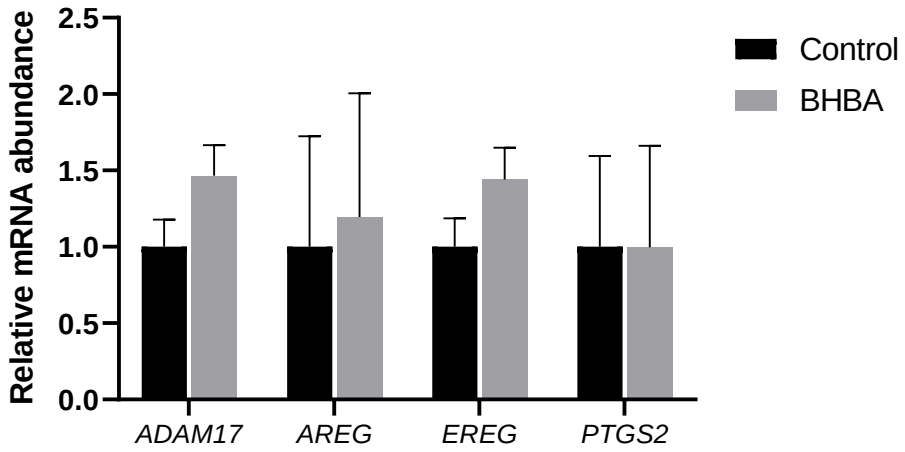
531 **Fig. 2.**



532

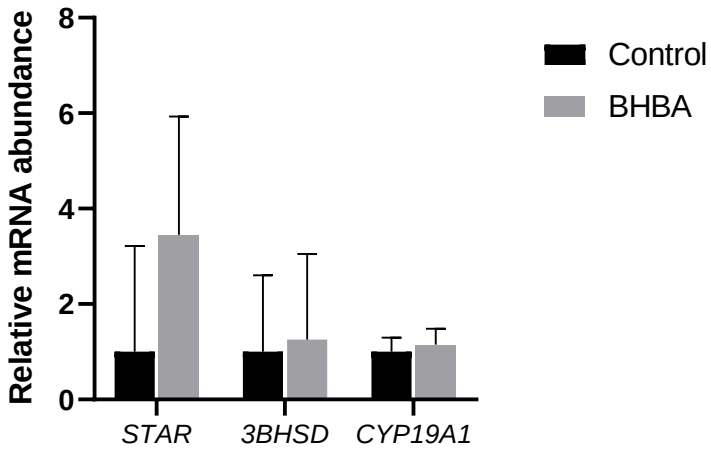
533

534 **Fig. 3.**


Relative mRNA abundance
2.5
2.0
1.5
1.0
0.5
0.0
ADAM17
AREG
EREG
PTGS2
Control
BHBA

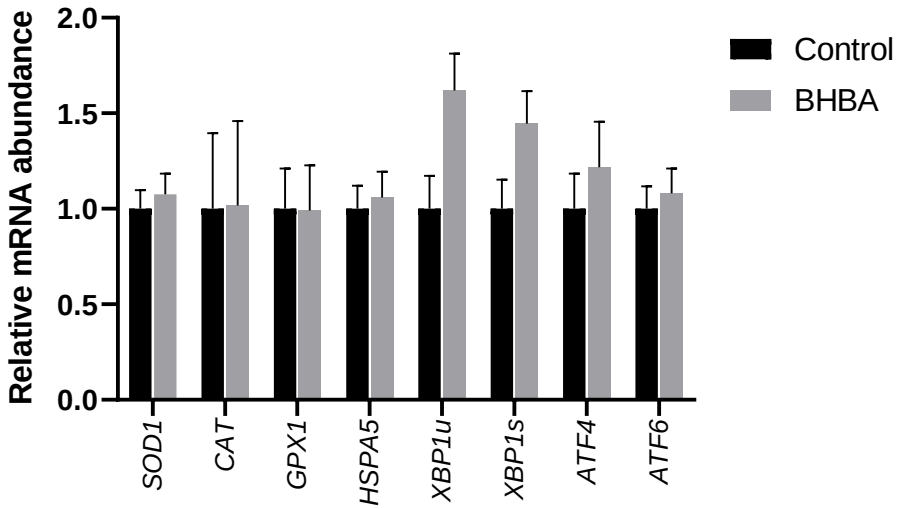
535

536 **Fig. 4.**


Relative mRNA abundance
8
6
4
2
0
STAR
3BHSD
CYP19A1
Control
BHBA

537

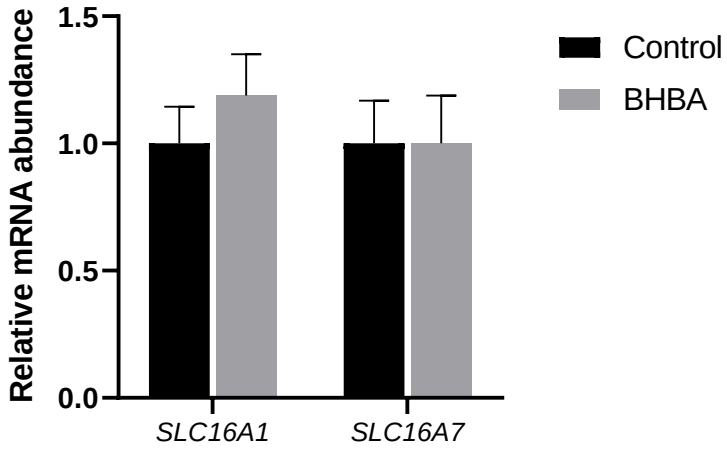
538 **Fig. 5.**


Relative mRNA abundance
2.0
1.5
1.0
0.5
0.0
Control
BHBA
SOD1
CAT
GPX1
HSPA5
XBP1u
XBP1s
ATF4
ATF6

539

540

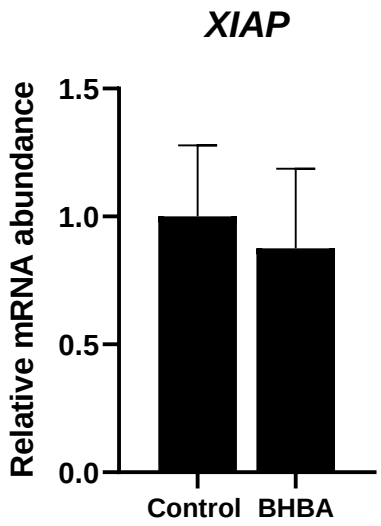
541 **Fig. 6.**



542

543 **Fig. 7.**

544



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ARTIGO 3

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

β-Hydroxybutyrate induces reticulum stress in bovine cumulus cells during oocyte maturation *in vitro*

Daniele Missio, Julia Koch, Zigomar da Silva, Valério Valdetar Marques Portela Junior, Francielli Weber Santos Cibin, Marcos Henrique Barreta, Vitor Braga Rissi, Fernando Silveira Mesquita, Paulo Bayard Dias Gonçalves, Rogério Ferreira

Theriogenology, 2022

β-Hydroxybutyrate induces reticulum stress in bovine cumulus cells during oocyte maturation *in vitro*

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ABSTRACT

Metabolic stress conditions, leading to negative energy balance (NEB), have been associated with reduced fertility in cows. Ketosis is a major metabolic disorder caused by negative energy balance (NEB). β-hydroxybutyrate (BHBA) is the most abundant circulating ketone body and is known to cause oxidative stress and accumulate within follicular fluid during NEB period. Thus, BHBA may compromise the viability and functionality of bovine cumulus cells and oocyte. The aim of this study was to determine the effects of BHBA on expansion, oxidative status, and endoplasmic reticulum (ER) stress in cumulus cells, oocyte nuclear maturation and cleavage rate in cattle. Cumulus-oocyte complexes were treated with 0 (control), 2 or 4 mM of BHBA for 6, 12, 18 or 24 h during oocyte *in vitro* maturation (IVM). Cumulus expansion and mRNA genes related to expansion, oxidative status, ER stress, and autophagy were evaluated. The oocytes were fixed for nuclear maturation analysis at 24 h of IVM. In addition, cleavage rate of embryos was examined. In conclusion, BHBA induces reticulum stress in bovine cumulus cells during *in vitro* maturation, without compromising the expansion, oocyte nuclear maturation, oxidative status, and cleavage rate.

Keywords: Cattle; Oocyte, β-hydroxybutyrate, Ketose.

1. Introduction

Metabolic stress conditions, leading to negative energy balance (NEB), have been associated with reduced fertility in cows [1,2]. The period of NEB coincides with the resumption of ovarian activity during the postpartum period [1]. In this period, the blood plasma concentrations of non-esterified fatty acids (NEFA) and ketone bodies are elevated whereas blood glucose levels are reduced, which are reflected in the follicular fluid [3–7].

β-hydroxybutyrate (BHBA) is the most abundant circulating ketone body and its concentration is a marker of fatty acid oxidation. The BHBA increases considerably during fasting, prolonged exercise or diabetic ketoacidosis in humans and ketosis in cattle [8]. Dairy cows with BHBA levels above 1.4 mM are in ketosis [9]. In case of persistent nutritional imbalance, cows develop severe clinical ketosis (BHBA > 3 mM) [10]. There is a strong correlation between levels of BHBA in the serum and follicular fluid [11]. The follicle is the microenvironment where cumulus-oocyte complexes (COC) develop and mature [12]. In this sense, cumulus cells are exposed to high levels of this endogenous metabolite in ketotic cows [13].

Throughout follicular development, the oocyte and cumulus cells communicate via a bi-directional pathway [14]. Cumulus cells play several critical roles during oocyte development and maturation, including the exchange of molecules that promote meiotic resumption, protection against oxidative stress, oocyte molecular and cytoplasmic maturation, and ovulation [15,16]. Thus, after the blood-follicle barrier, the cumulus cell layer forms the second barrier between blood and the oocyte [17]. Studies have shown that during the maturation stage, cumulus cells appear to effectively protect oocytes against stress caused by fatty acid in cattle [5] or environmental contaminants in rats [18].

Ketone bodies, including BHBA, increase the production of reactive oxygen species (ROS) [19] and promote oxidative stress [20–22]. Oxidative stress has proved to be an initiator

and one of the main contributors to endoplasmic reticulum (ER) stress [23]. ER stress is a cytoprotective mechanism activated under non physiological condition [24] as in cases of ketosis in dairy cows. However, to our knowledge, no studies are available on the effects of BHBA on bovine cumulus cells. The aim of our study was to determine the effects of BHBA on cumulus expansion, oxidative status, ER stress, and autophagy in cumulus cells, and oocyte nuclear maturation and cleavage rates in cattle.

2. Materials and Methods

All chemicals used were purchased from Sigma Chemicals Company (St. Louis, MO, USA), unless otherwise stated.

2.1.Oocyte recovery and in vitro maturation

Ovaries were obtained from cows at different estrous cycle stages in an abattoir and transported to the laboratory at 30°C in saline solution (0.9% NaCl), containing 100 IU/mL penicillin and 50 μg/mL streptomycin sulphate. The COC were aspirated from 3-8 mm ovarian follicles. Only COC grade 1 were recovered and selected under a stereomicroscope according to Leibfried and First [25]. Subsequently, the COC were transferred to four-well culture dishes (Nunc®, Roskilde, Denmark) with maturation medium and the components of each treatment. The basic maturation medium used was Medium 199 (1X) containing Earle's salts, L-glutamine, 2.2 mg/mL sodium bicarbonate and 25 mM Hepes (Gibco Labs, Grand Island, NY, USA), supplemented with 0.2 mM pyruvic acid, 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 μg/mL of FSH (Folltropin®-V, Bioniche, ON, CA), 0.4% (v/v) bovine serum albumin (BSA), 100 IU/mL penicillin and 50 μg/mL streptomycin sulphate. Then, the COC were cultured at 39 °C in an atmosphere containing 5% CO_2 in air, at 95% relative humidity, for 6, 12, 18 or 24 h.

2.2.Cumulus expansion evaluation

Cumulus-oocyte complexes expansion was measured at 0, 6, 12, 18 and 24 h of IVM. Digital images of the COC were captured through the Leica Application Suite (LAS, Version 3.8) software at 100× magnification. With the obtained images, the total COC area was analyzed using the ImageJ software (version 1.47, National Institutes of Health, Bethesda, MD, USA) at different times and treatments with BHBA.

2.3. Assessment of oocyte nuclear maturation

To analyze the effect of BHBA treatment in nuclear maturation, after 24 h of IVM, the cumulus cells were removed by repeated pipetting, and denuded oocytes were fixed in 4 % paraformaldehyde for 15 min., followed by permeabilization of the nuclear membranes with 0.5 % Triton X-100 until evaluation. For assessment of nuclear maturation phase, the oocytes were exposed to 10 µg/mL of bisbenzimide (Hoescht 33342) for 15 min. Stained oocytes were classified under UV light (wavelength of 340-380 nm) in a fluorescence microscope (Leica DMI4000B, Wetzlar, Germany) and considered mature if it displayed a chromatin configuration corresponding to metaphase II stage.

2.4. Evaluation of oxidative status in cumulus cells

To assess the oxidative status, the samples of cumulus cells were submitted to two evaluations: production of reactive oxygen species (ROS) and total antioxidant capacity at 0, 12 and 24 h of IVM. The ROS production was determined with a spectrofluorimetric method according by Loetchutinat et al. [26]. Briefly, the samples were incubated in the dark with 5 µL of 2′,7′-dichloro dihydrofluorescein diacetate (DCHF-DA). The DCHF-DA, upon oxidation, is

converted to the fluorescent 2′,7′-dichlorofluorescein (DCF). The oxidation of DCHF-DA to DCF was used to detect and measure intracellular ROS concentrations. The fluorescence intensity emitted at 520 nm (488 nm excitation) was monitored 60 min after the addition of DCHF-DA. The total antioxidant potential (FRAP) in the sample was determined by ability of antioxidants to reduce Fe^{+3} to Fe^{+2}, which is chelated by 2,4,6-tri(2-pyridyl)-striazine (TPTZ) to form Fe^{+2} -TPTZ with maximum absorption at 593 nm [27].

2.5.RNA isolation, reverse transcription and quantitative real-time PCR

The cumulus cells were removed by repeated pipetting and immediately stored in TRIzol®, with objective of evaluation BHBA treatment influence the cumulus expansion, oxidative stress, ER stress and autophagy. Total RNA was extracted using TRIzol® according to the manufacturer's instructions and was quantified at 260 nm wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific, Wilmington, DE, USA). Total RNA (200 ng) was first treated with 0.2 µL DNAse (Invitrogen, Carlsbad, CA, USA) at 27°C for 15 min. to digest any contaminating DNA, followed by 0.2 µL EDTA (Invitrogen, Carlsbad, CA, USA) addition and heating to 65°C for 10 min. RNA was reverse transcribed (RT) with 1 µL iScript™ cDNA Synthesis Kit (Bio-Rad, Des Plaines, IL, USA) at 25°C for 5 min. and 46°C for 30 min. The reaction was ended by incubation at 95 °C for 5 min.

Quantitative Real-Time PCR was performed using in a CFX384 thermocycler (BioRad) using BRYT Green® dye and Taq DNA polymerase from GoTaq® qPCR Master Mix (Promega Corporation) and specific primers (Table 1). Standard two-step RT-qPCR was performed with initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. The reaction was performed in duplicate, and the melting-curve was analyzed to determine the product's identity. The target mRNA concentration was normalized to the amplification of the reference genes

glyceraldehyde-3-phosphate dehydrogenase - *GAPDH*, ribosomal protein S18 - *RPS18*, and peptidylprolyl isomerase A -*PPIA*. Relative expression calculation was performed as described by Pfaffl [28].

2.6. In vitro production of embryos and cleavage rate evaluation

To evaluate the potential deleterious effect of BHBA on *in vitro* cleavage, after IVM, oocytes were placed into Fert-TALP medium and inseminated with tested frozen semen pool from two sires. The semen was thawed and fractionated on discontinuous Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradients [29] and diluted to a final concentration of 2×10^6 sperm/mL in Fert-TALP medium containing 10 μg/mL heparin, 20 μg/mL penicillamine, 10 μM hypotaurine, and 1 μM epinephrine. *In vitro* fertilization was carried out by co-culture of sperm and oocytes for 18 h in four-well plates in the same atmospheric conditions as the ones used for maturation. After gamete co-incubation period, the cumulus cells were removed by 2 min vortexing. Presumptive zygotes were cultured at 38.5 °C in 400 μL synthetic oviduct fluid (SOFaaci; [30]) medium in four-well plates (Nunc, Roskilde, Denmark) under a saturated humidity atmosphere containing 5 % CO_2, 5 % O_2, and 90 % N_2. The cleavage rates (cleaved/oocyte) were determined 48 h after *in vitro* insemination. *In vitro* embryo production was performed in 3 replicates.

2.7. Experimental design

To understand the effect of BHBA in bovine cumulus cell, grade 1 COC were randomly divided into groups (n = 5 COC/group/repeated 4 times) and cultured in 200 μL of maturation medium. COC were treated with 0 (control group), 2 or 4 mM of BHBA (Sigma-Aldrich, St. Louis, MO, USA). After 6, 12, 18 and 24 h of *in vitro* maturation, cumulus expansion was evaluated and subsequently the cumulus cells were removed by successive pipetting. Cumulus

cells were used for evaluation of gene expression and oxidative status and oocyte was used for nuclear maturation assessment. To understand the effect of BHBA in status oxidative of cumulus cells (n = 45-50 COC/group/repeated 4 times) and cleavage rate (n = 45-50 presumptive zygotes/group/repeated 3 times), grade 1 COC were randomly divided into groups and cultured in 400 μL of maturation medium for up to 24 h.

2.8.Statistical analysis

All analysis were performed with JMP software (SAS Institute, Inc., Cary, NC, USA). Continuous data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary, according to data distribution and residuals of each statistical model. The effect of treatments on COC area over time was analyzed by mixed models for repeated data including each COC as subject. Different covariance structures were tested for each model and the one with the least Akaike Information Criteria (AIC) was used. Differences on COC diameter at specific time point were compared by Tukey HSD. Other continuous dependent variables were submitted to one-way ANOVA and Tukey HSD as *post-hoc* test. $P < 0.05$ was considered statistically significant. Data are presented as least square corrected means + SEM (standard error of mean).

3. Results

3.1.Effects of BHBA in COC expansion and oocyte nuclear maturation

The total area of COC was evaluated at 0, 6, 12, 18, and 24 h of IVM. The treatment with BHBA did not alter COC area during the time of IVM (Fig. 1). Concerning the effect of BHBA on the stage of meiotic progression, the number of oocytes incubated in the presence of

188 2 mM or 4 mM of BHBA that reached metaphase II (81.9 ± 2.11 %; 82.6 ± 2.11 %, respectively)
189 in 24 h did not differ those in control group (84 ± 1.79 %; $P = 0.72$).
190
191 *3.2.Effects of BHBA on expression of cumulus cell expansion genes*
192 In this study, the expression of COC expansion-related genes (hyaluronan synthase 2 -
193 *HAS2* and tumor necrosis factor alpha-induced protein 6 - *TNFAIP6*) were examined at 6, 12,
194 18, and 24 h of IVM. BHBA at 2 or 4 mM concentrations did not affect the expression of *HAS2*
195 or *TNFAIP6* genes at the different times examined, except for *HAS2* at 6 h of COC maturation
196 ($P < 0.01$; Fig. 2A and B).
197
198 *3.3.Effects of BHBA on mRNA levels of antioxidant enzymes and oxidative status in cumulus*
199 *cells*
200 To analyze the antioxidant status of cumulus cells matured for 6, 12, 18 or 24 h in the
201 presence or absence of BHBA, Cu/Zn superoxide dismutase (*SOD1*), catalase (*CAT*), and
202 glutathione peroxidase (*GPX1*) mRNA levels were evaluated (Fig. 3). The *SOD1*, *CAT* and
203 *GPX1* mRNA abundance in cumulus cells was not affected by 2 or 4 mM of BHBA during
204 oocyte maturation at different times examined (Fig. 3A, B and C). To confirm these results, we
205 evaluated ROS production and total antioxidant capacity in cumulus cells at 12 and 24 h of
206 IVM and none of these parameters were affected by the BHBA treatment (2 or 4 mM; Fig. 4;
207 $P > 0.05$).
208
209 *3.4.Effects of BHBA on abundance of reticulum stress and autophagic genes in cumulus cells*
210 The mRNA abundances of five ER stress marker genes (heat shock 70 kDa protein 5 -
211 *HSPA5*, activating transcription factor 6 - *ATF6*, X-box binding protein 1 spliced - *XBP1s*, X-
212 box binding protein 1 unspliced - *XBP1u*, and DNA damage inducible transcript 3 -*CHOP)*

213 were evaluated (Fig. 5). *HSPA5, XBP1s,* and *XBP1u* mRNA levels increased in cumulus cells
214 when 2 mM BHBA was present in the maturation medium for 6 and/or 12 h, but this increase
215 was not observed at any other time points. (Fig. 5, $P < 0.05$). The abundance of the other ER
216 stress marker genes examined did not differ from control cumulus cells (Fig. 5, $P > 0.05$). In
217 addition, in response to oxidative and ER stress, cells can trigger an autophagic response to
218 reduce their damage. Thus, we examined microtubule-associated protein 1A/1B-light chain 3
219 (*LC3,* main autophagic marker) gene expression in bovine cumulus cells exposed to different
220 BHBA concentrations. BHBA did not alter *LC3* mRNA levels when compared to those cultured
221 in the control group in none of IVM times (Fig. 5F).
222
223 *3.5.Effects of BHBA in cleavage rate*

224 BHBA had no effect on the embryo cleavage rate at concentrations of 2 mM of BHBA
225 (52.1 ± 6.4 %) or 4 mM of BHBA (55.1 ± 6.4%) when compared to control group (66.0 ± 6.4%;
226 $P > 0.05$; Fig. 6).
227
228 **4. Discussion**

229 In the present study, the effect of high levels of BHBA during *in vitro* maturation on the
230 cumulus cells and oocyte were investigated. High concentrations of BHBA cause increased ER
231 stress at 6 and 12 h of IVM in bovine cumulus cells. These results suggest that this ketone body
232 affects temporarily bovine cumulus cells, and they respond to stress by altering their gene
233 expression and activating cytoprotective pathways to maintain a cellular homeostasis.
234 The BHBA did not alter COC expansion, but 2 mM of BHBA increased *HAS2* mRNA
235 expression at 6 h of IVM , which may be not so important at this point of maturation because
236 *TNFAIP6* expression and COC area was not altered. *HAS2* and *TNFAIP6* are necessary for the
237 synthesis of hyaluronic acid [31] and *HAS2* expression is correlated with oocyte development

238 [32]. In this study, no changes were observed regarding oocyte nuclear maturation and cleavage
239 rate during IVM with the presence of BHBA. Another hypothesis is that apoptosis of cumulus
240 cells can be prevented by increasing levels of *HAS2*. Recently, hyaluronic acid and *HAS2* were
241 reported to protects against apoptosis induced by environmental stress and to promote survival
242 in various types of cells, including fibroblasts [33], granulosa cells [34,35], articular
243 chondrocytes [36], and human aortic smooth muscle cells [37]. At the same IVM time, the
244 expression of genes related to ER stress (*XBP1s* and *XBP1u*) were elevated in BHBA groups
245 and these pathways trigger cellular stress. However, we were unable to elucidate this in our
246 experiment and further studies should be performed to assess this mechanism.

247 BHBA in IVM medium of bovine oocytes has a detrimental effect in embryo
248 development, which is variable according to the glucose concentration used [38]. In this sense,
249 we evaluated whether this BHBA exposure would affect the capacity of the oocyte to reach
250 metaphase II stage and cleavage rate. BHBA did not change nuclear maturation and cleavage
251 rates comparing to control, probably because the cumulus cells protect the oocyte from the
252 effects of BHBA, as observed in the presence of NEFA in cattle [5]. In agreement with our data,
253 Sangalli et al. [39] also observed no difference in the rate of oocytes that reached metaphase II
254 stage and cleavage rate after being matured with 2 mM of BHBA. Leroy et al. [38] did not
255 observe any adverse effect of 4 mM of BHBA on cleavage and embryo production rates in
256 cattle. In swine, BHBA at various concentrations during IVM had no effect on the capacity of
257 the oocyte to reach metaphase II and on the cleavage rate [40]. It is important to point out that
258 BHBA is not present only during oocyte maturation *in vivo*, reaching the oocyte during
259 follicular wave or even in preantral follicles [41]. *In vivo*, COC from cows are exposed to
260 elevated BHBA for a prolonged time during the transition period and early stages of
261 folliculogenesis [5], which may compromise follicular cell functionality and impair
262 reproductive performance.

263 Cumulus cells play an important role in oocyte maturation and protect oocytes against
264 cell damage produced by oxidative stress during IVM [42,43]. Importantly, oxidative stress is
265 observed in the ketotic cows and is positively related to high BHBA and NEFA levels [44].
266 Therefore, this study examined the effect of BHBA on oxidative status in bovine cumulus cells
267 through the evaluation of *SOD1*, *CAT* and *GPX1* gene expression, ROS production and total
268 antioxidant capacity. According to our results, Sangalli et al. [13] observed that *SOD1, CAT*
269 and *GPX1* mRNA abundance in early embryo stages did not change when 6 mM of BHBA was
270 present during *in vitro* embryo development. However, BHBA was responsible for increasing
271 *SOD1* and catalase activity in other cell types, including renal tissue [45], spinal cord [46] and
272 cardiomiocytes [47]. In addition, antioxidant defenses were unable to neutralize ROS in other
273 cells than cumulus cells, which caused oxidative stress mainly when high concentrations of
274 BHBA were used [48–51]. However, there was no difference in oxidative status, this may have
275 occurred due to the antioxidant defenses being able to neutralize ROS. In according to Tatemoto
276 et al. [52] cumulus cells surround oocytes during folliculogenesis act as a mechanical barrier
277 that protects oocytes from entering apoptosis induced by oxidative stress.

278 Oxidative stress has proved to be an initiator and one of the main contributors to ER
279 stress [23]. In addition to the oxidative stress, we hypothesized that high BHBA levels during
280 IVM induce ER stress in cattle cumulus cells and assessed their effect on ER stress gene
281 responses. In the present study, we examined the levels of mRNA for proteins involved in the
282 regulation of ER stress (*HSPA5, ATF6, CHOP, XBP1s* and *XBP1u*). Our results demonstrate
283 that BHBA increased mRNA levels at 6 and/or 12 h of IVM for *HSPA5*, *XBP1u* and *XBP1s*
284 genes . Disturbances in ER homeostasis cause stress, resulting in accumulation of unfolded
285 proteins in the organelle. The NEB state has been demonstrated to affect the expression of ER
286 stress-related genes and proteins in the liver of starved dairy cows and in bovine mammary
287 epithelial cells cultured *in vitro* for 24 h [53]. Furthermore, recently, Shi et al.[54] and Islam et

al. [55] demonstrated that ER stress occurs in hepatocytes of cows with ketosis. Activation of unfolded protein response signal pathways demonstrates that BHBA increased misfolding protein in the ER lumen in cumulus cells, which may compromise cell viability. However, the detrimental effects of BHBA seemed to be prevented by activation of cytoprotective mechanisms. This assumption is based on our IVM results that was not observed effect of BHBA on ER stress marker genes in cumulus cells after 12 h. Studies have shown that the increase in NEFA levels, which are also elevated during the NEB period in cows, causes COC reticulum stress during IVM in different species [56–58]. Thus, the increase of NEFA and BHBA in the follicular microenvironment during transition period causes ER stress in COC and may compromise the cell viability.

Oxidative and ER stress also leads to the induction of autophagy [59]. In this study, we analyzed *LC3* gene expression in cumulus cell of COC matured in different BHBA concentrations. Our results showed that BHBA did not alter *LC3* gene abundance during IVM . Autophagy is recognized as an important regulatory mechanism for cell death, special in unfavorable conditions [60]. The level of intracellular autophagy is often related to oxidative stress, ER stress and cell death versus survival [61,62]. Therefore, in this study, we observed that BHBA did not activate the mechanisms of autophagy, probably because the effect on the cumulus cells was transitory.

5. Conclusion

In conclusion, β-hydroxybutyrate induces endoplasmic reticulum stress in bovine cumulus cells during *in vitro* maturation but does not affect the expansion, oocyte nuclear maturation, oxidative status, and cleavage rate.

CRediT authorship contribution statement

Daniele Missio: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Project administration. **Julia Koch:** Investigation, Methodology. **Valério Valdetar Marque Portela Junior**: Investigation, Resources **Francielli Cibin:** Investigation, Resources. **Marcos Henrique Barreta**: Investigation, Resources. **Vitor Rissi:** Investigation. **Fernando Silveira Mesquita:** Investigation, Writing - Original Draft. **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.

Declaration of competing interests

The authors declare that they have no conflict of interests.

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Legends:

Table 1: List of primer used in the qRT-PCR reactions.

Fig. 1. Total area of bovine COC at 0, 6, 12, 18, and 24 h of oocyte *in vitro* maturation (IVM) without (control) or with 2 or 4 mM of β-hydroxybutyrate (BHBA). Data are presented as mean ± SEM of four replications.

Fig. 2. Messenger RNA abundance of hyaluronan synthase 2 (*HAS2;* A) and tumor necrosis factor alpha-induced protein 6 (*TNFAIP6;* B) in cumulus cells after cumulus-oocyte complexes *in vitro* maturation without (control) or with 2 or 4 mM of β-hydroxybutyrate (BHBA). Data are presented as mean ± SEM of four replications. Different letters at the same time point indicates statistical difference ($P < 0.05$).

Fig. 3. Messenger RNA abundance of Cu/Zn superoxide dismutase (*SOD1*; A), Catalase (*CAT; B*) and glutathione peroxidase (*GPX1; C*) in cumulus cells after maturation of cumulus-oocyte complexes (COC) without (control) or with 2 or 4 mM of β-hydroxybutyrate (BHBA). Data are presented as mean ± SEM of four replications ($P < 0.05$).

Fig. 4. Reactive oxygen species (ROS; A) and Ferric reducing potential (FRAP; B) in cumulus cells after maturation of cumulus-oocyte complexes (COC) without (control) or with 2 or 4 mM of β-hydroxybutyrate (BHBA). Data are presented as mean ± SEM of four replications ($P < 0.05$).

Fig. 5. Messenger RNA abundance of heat shock 70 kDa protein 5 (*HSPA5;A*), X-box binding protein 1 unspliced (*XBP1u; B*), X-box binding protein 1 spliced (*XBP1s;C*), activating transcription factor 6 (*ATF6; D*), DNA damage inducible transcript 3 (*CHOP; E*), and microtubule-associated protein 1A/1B-light chain 3 (*LC3; F*) in cumulus cells after maturation of cumulus-oocyte complexes (COC) without (control) or with 2 or 4 mM of β-hydroxybutyrate (BHBA). Data are presented as mean ± SEM of four replications. Different letters represent statistical difference ($P < 0.05$).

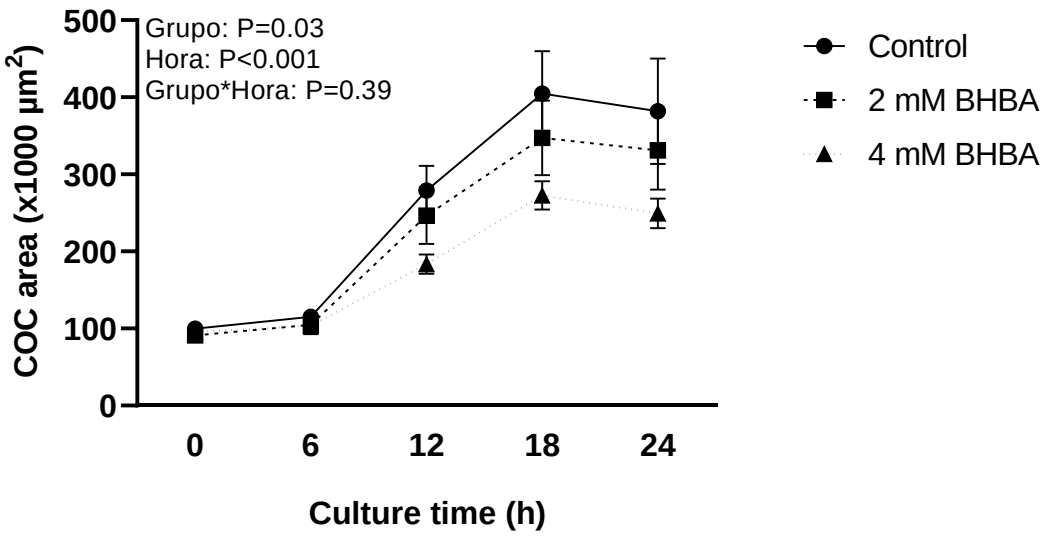
Fig. 6. Cleavage rate of oocytes *in vitro* matured without (control) or with 2 or 4 mM of β-hydroxybutyrate (BHBA). Data are presented as mean ± SEM of three replications ($P < 0.05$).

596 **Table 1.** List of primer used in the Quantitative Real-Time PCR in cumulus cells.

Gene name	Sequence (5' to 3')	Accession number
GAPDH	F: GATTGTCAGCAATGCCTCCT R: GTCATAAGTCCCTCCACGA	NM_001034034.2
PPIA	F: GGTCATCGGTCTCTTTGGAA R: TCCTTGATCACACGATGGAA	NM_178320.2
RPS18	F: CCTTCCGCGAGGATCCATTG R:CGCTCCCAAGATCCAACTAC	NC_037350.1
SOD1	F:ATACACAAGGCTGTACCAGTGC R:CACATTGCCCAGGTCTCCAA	NM_174615.2
CAT	F:AGAGGAAACGCCTGTGTGAG R:ATGCGGGAGCCATATTCAGG	NM_001035386.1
GPX1	F: GCATCAGGAAAACGCCAAGA R: CCATTCACCTCGCACTTTTCG	NM_174076.3
HSPA5	F:CGTGCGTTTGAGAGCTCAGT R:GACAGCTTCATCTTTCCAGCG	NM_001075148.1
XBP1u	F: GCAGAGACCAAGGGGAATGG R:GGGTCCAAGTTGAACAGAATGC	NM_001271737.1
XBP1s	F: AGCAGAGACCAAGGGGAATG R: TCAGAGTCCATGGGGAGATGT	NM_001034727.3
ATF6	F:GAACTTCGAGGATGGGTTCATAGG R:CCAGAGCACCCTGAAGAATACG	NC_037330.1
CHOP	F:GGTGCTGTCCTCAGATGAAAATCG R:GGTCCTGGCTCCTCAGTAAGC	NM_001078163.1
LC3	F:GCCGAACCTTCGAACAAAGAG R:TGAGCTGTAAGCGCCTTCTT	NC_037345.1
HAS2	F:GCATGTCACCCAGTTGGTCT R:TGGGTCAAGCATGGTGTCTG	NC_037341.1
TNFAIP6	F:GCTCACGGATGGGGATTCAA R:CGTGCTTCCCTGTGGTAGAC	NC_037329.1

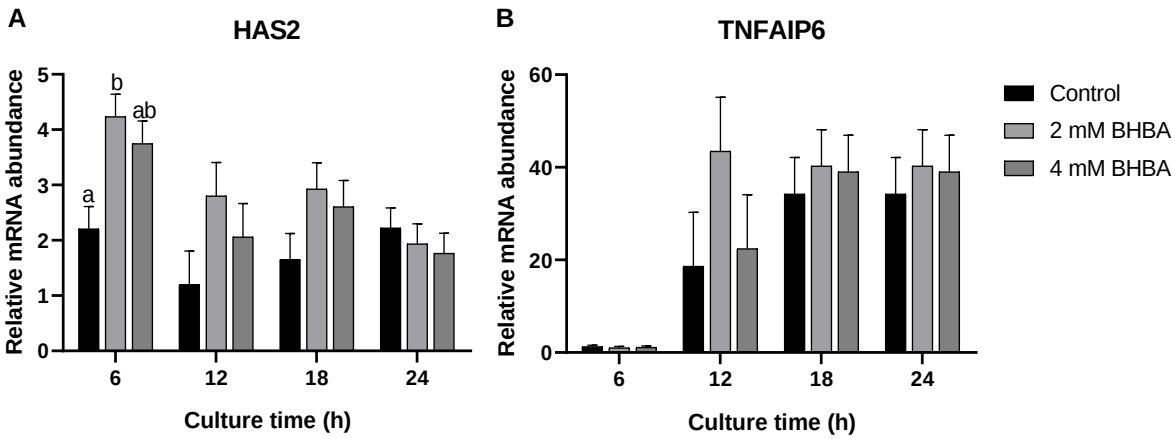
597 F: Forward primer; R: Reverse primer.
598

599 **Fig. 1:**


Grupo: P=0.03
Hora: P<0.001
Grupo*Hora: P=0.39
COC area (x1000 µm2)
500
400
300
200
100
0
0
6
12
18
24
Culture time (h)
Control
2 mM BHBA
4 mM BHBA

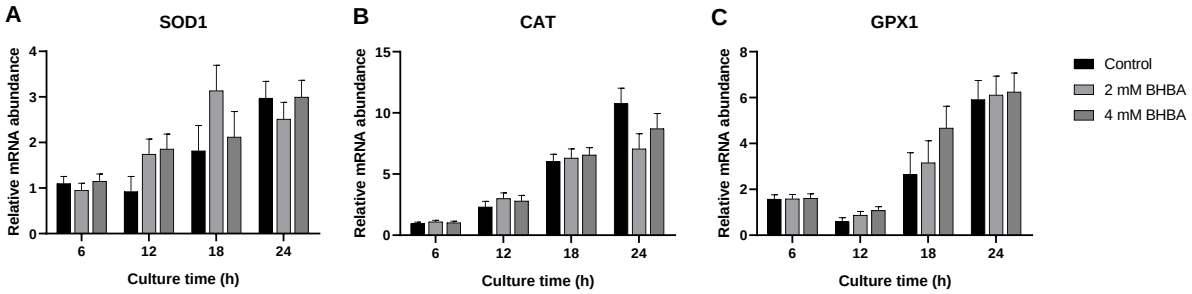
600

601 **Fig. 2:**


A
HAS2
Relative mRNA abundance
0
1
2
3
4
5
a
b
ab
6
12
18
24
Culture time (h)
B
TNFAIP6
Relative mRNA abundance
0
20
40
60
6
12
18
24
Culture time (h)
Control
2 mM BHBA
4 mM BHBA

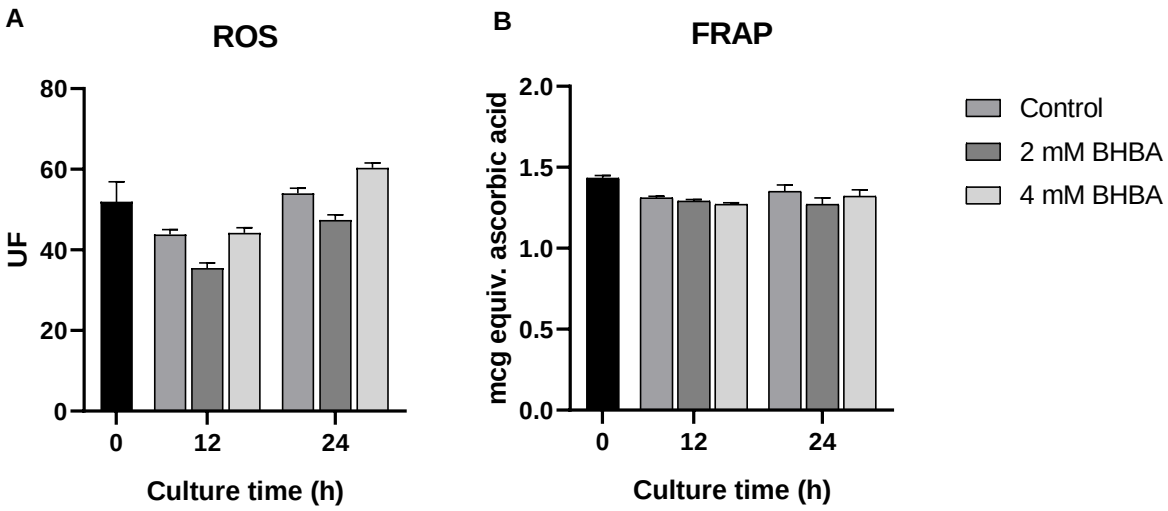
602

603 **Fig. 3:**


A
SOD1
Relative mRNA abundance
0
1
2
3
4
6
12
18
24
Culture time (h)
B
CAT
Relative mRNA abundance
0
5
10
15
6
12
18
24
Culture time (h)
C
GPX1
Relative mRNA abundance
0
2
4
6
8
6
12
18
24
Culture time (h)
Control
2 mM BHBA
4 mM BHBA

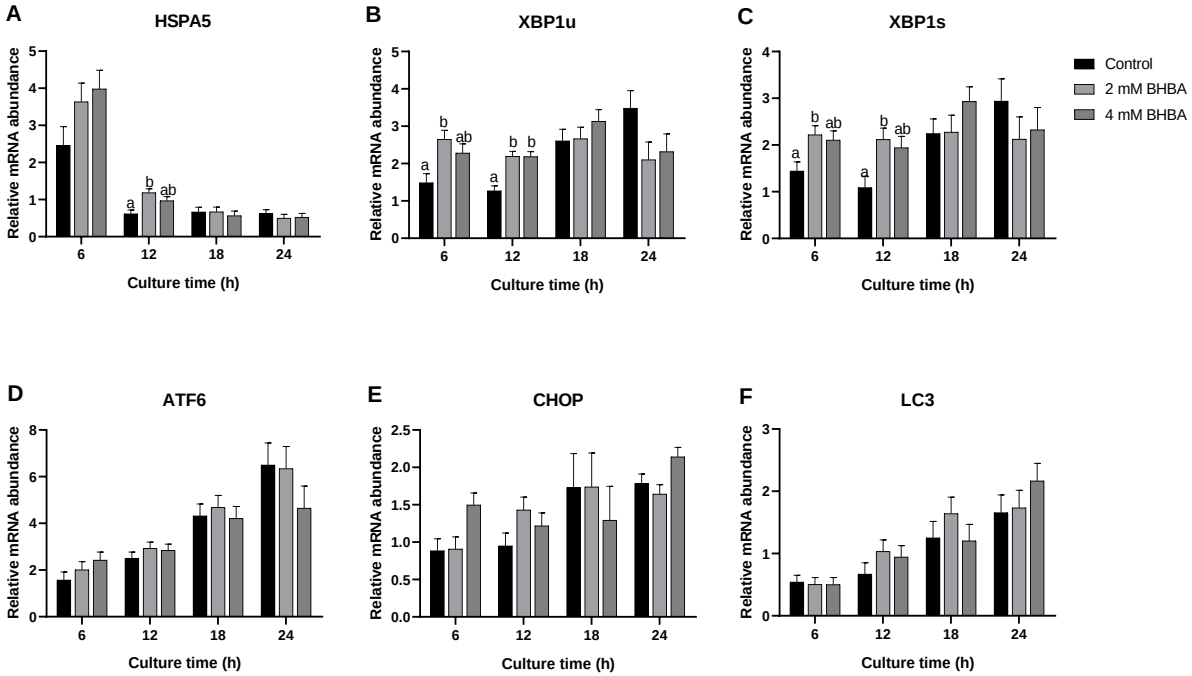
604

605 **Fig. 4:**

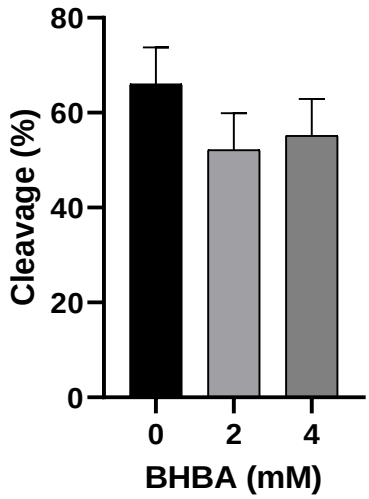

A
ROS
UF
80
60
40
20
0
0
12
24
Culture time (h)
B
FRAP
mcg equiv. ascorbic acid
2.0
1.5
1.0
0.5
0.0
0
12
24
Culture time (h)
Control
2 mM BHBA
4 mM BHBA

606

Fig. 5:


A
HSPA5
B
XBP1u
C
XBP1s
D
ATF6
E
CHOP
F
LC3
Relative mRNA abundance
Culture time (h)
6
12
18
24
a
b
ab
Control
2 mM BHBA
4 mM BHBA

610 **Fig. 6:**



611

612

ARTIGO 4

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

Vitamin E reduces the reactive oxygen species production in dominant follicle during the negative energy balance in cattle

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Animal Reproduction Science, 2022

Vitamin E reduces the reactive oxygen species production in dominant follicle during the negative energy balance in cattle

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ABSTRACT

The aim this study was to evaluate the effect of parenteral α-tocopherol to prevent oxidative stress in serum and follicular fluid of cows in NEB. Twenty-nine *Bos taurus* beef cows were divided into three groups: 1) control; 2) Fasting for four days; and 3) Fasting + VitE. The cows were submitted to a hormonal protocol to synchronize a new wave of follicular growth. Four days after the start of the hormonal protocol (D0), cows were randomly divided into three groups. Between D0 and D4 blood samples were taken daily to assess circulating levels of non esterified fatty acids, reactive oxygen species production, total antioxidant capacity, lipid peroxidation and α-tocopherol (Vitamin E). On D4 ultrasound-guided follicular aspiration was performed for analysis of follicular fluid (FF) from the dominant follicle. Our results demonstrate that fasting was effective in causing increased fat mobilization in animals. The increase in serum levels of C18:1n9 reflected in the FF of fasting cows. Serum a-tocopherol concentration was higher in the control and Fasting + VitE groups compared to the Fasting group. In FF, there was an increase of α-tocopherol in the Fasting + VitE in comparison to Fasting cows. There, there was an increase of ROS production in the serum of fasting cows. ROS production in FF was higher in the Fasting compared to Fasting + VitE group. Total antioxidant capacity and lipid peroxidation did not differ among groups in serum and FF. Vitamin E has beneficial effects in reducing production of ROS in preovulatory follicle of cows in NEB.

Keywords: Fatty acids; Follicular fluid; Antioxidants; Oxidative stress

1. Introduction

In the transition period of dairy cows, between 3 weeks before and 3 weeks after parturition, energy requirements increase dramatically, exceeding the amount of dry matter that a cow can ingest, undergoing negative energy balance (NEB; Turk et al., 2013). During the NEB period, cows use alternative energy sources to an excessive mobilization of adipose reserves, releasing abnormal concentrations of non esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) in blood (Bell, 1995; Benedet et al., 2019). In addition, there is a strong correlation between serum and follicular fluid concentration levels of NEFA and BHBA (FF; Leroy et al., 2005). The increase of these metabolites in the follicle compromises granulosa cell viability (Sharma et al., 2019; Vanholder et al., 2005; Yenuganti et al., 2016), oocyte competence (Aardema et al., 2011; Jorritsma et al., 2004; Sutton-mcdowall et al., 2016; Van Hoeck et al., 2013); embryonic quality; and subsequent fertility of dairy cows (Van Hoeck et al., 2011; Vanholder et al., 2005).

The increase in NEFA levels during NEB period results in increased production of reactive oxygen species (ROS) and oxidative stress (Abuelo et al., 2015; Bionaz et al., 2007; Sordillo and Raphael, 2013) in different cell types. The oxidative stress interferes with the follicular, oviductal and uterine microenvironment (Beam and Butler, 1997; Dupont et al., 2014), causing reproductive disorders and subfertility in dairy cows (Bernabucci et al., 2005; Miller et al., 1993). Furthermore, the increase in ROS production and oxidative stress has been related to the depletion of antioxidant defenses, as reported for vitamin E deficiency (LeBlanc et al., 2004).

Vitamin E is a primary fat-soluble antioxidant, important for defense against oxidative stress (Ibrahim et al., 1997). Vitamin E comprises four tocopherols and four tocotrienols. α-Tocopherol is considered to be the most important active compound to quantify vitamin E in serum or plasma. The vitamin E in serum rapidly decreases during transition period in cows,

mainly due to deficient transport in plasma, increase of lipid storage in the liver (Herdt and Smith, 1996), and transfer to colostrum (Goff et al., 2002). In addition, α-tocopherol concentrations in FF are 3 to 4 times lower than in plasma, which can compromise the follicular environment and fertility of cows (De Bie et al., 2016).

Supplementation with vitamin E increases incorporation of α-tocopherol into the cell membranes (Weiss et al., 1997), which enhances oxidative burst and increases the activity against pathogens (Hogan et al., 1993). In this sense, the understanding of the relationship between NEFA and oxidative stress in the follicular environment is essential to propose solutions to increase the fertility of postpartum cows under metabolic stress. However, studies evaluating the effect of NEFA and oxidative stress in the ovarian follicle are scarce and those published are mostly performed *in vitro.* Therefore, the aim this study was to evaluate the effect of parenteral α-tocopherol to prevent oxidative stress in serum and follicular fluid of cows in NEB. To this end, a short-term elevation of free fatty acid concentrations in blood and follicular fluid was induced by an acute period of fasting (Jorritsma et al., 2003).

2. Material and Methods

2.1. Animals

All experiment procedures using cattle were approved by the Federal University of Pampa Animal Care and Use Committee (protocol number 046/2018). Twenty-nine beef cows non-lactating, estrous cyclic, multiparous (4–6 years old) with a body condition score of 2.87 ± 0.05 (on a scale of 1-thin to 5-fat) were used in the study. The animal model of the fasting beef cows for four days was used to induce differences in serum NEFA concentrations (Jorritsma et al., 2003; Mohamed et al., 2004; Ono et al., 2011). All animals had free access to water throughout the experiment.

2.2.Experimental design

Estrous cycle and follicular wave synchronization were necessary to obtain the presence of a well-defined preovulatory follicle at the end of fasting (4 days of fasting, Figure 1). This was accomplished by using a progesterone-releasing intravaginal device (D-4; IVD; Primer, Tecnopec, São Paulo, Brazil; 1 g progesterone) and an intramuscular (IM) injection of 2 mg estradiol benzoate (EB; Gonadiol, Zoetis, São Paulo, Brazil). Four days after prostaglandin F2α analogue (D0, PGF2α; 500 μg cloprostenol, Estron, Agener União Saúde Animal, São Paulo, Brazil) was administered intramuscularly. At D0, saline solution or α-tocopherol (Monovin E, Bravet, Rio de Janeiro, Brazil) at single dose of 1,000 IU was administered intramuscularly in cows that made up the groups: 1) control (n = 9); 2) Fasting + VitE (n = 10); and 3) Fasting (n = 10). The IVD was removed 8 days after the time of its insertion. At the time of IVD removal, the ovaries were evaluated by ultrasonography and the dominant follicle was aspirated using transrectal ultrasonography procedures (Fig. 1).

2.3. Blood sampling and measurements

During the days 0 and 4 of experiment, blood samples were collected daily from coccygeal vein of all animals. Blood samples were taken for determination of NEFA (to validate the model), vitamin E (α-tocopherol), ROS production, total antioxidant capacity (FRAP) and lipid peroxidation. On D4, the ovaries were examined by transrectal ultrasonography to measure the diameter of the preovulatory follicle. Follicular fluid was collected from the preovulatory follicle to evaluate the same variables assessed in serum and estradiol concentration. The blood samples were collected into Vacuette tubes with heparin for ROS, FRAP and lipid peroxidation or without anti-coagulant for NEFA and α-tocopherol analyzes.

2.4. Follicular aspiration

Cows were subjected to perineal cleaning and epidural anesthesia with lidocaine 2 % before follicular aspiration. At day 4, the follicular fluid was collected from the dominant follicle with the aid of transvaginal ultrasound (AquilaVet, PieMedical Esaote, Maastricht, The Netherlands) equipped with a 5 MHz micro convex probe, using a conventional ovum pickup (OPU) system with 16 G catheters (Jelco; Smiths Medical, Southington, CT, USA) attached to a silicon hose and a 5 mL syringe. This technique enabled us to collect follicular fluid without or with only a very limited amount of contaminating blood. The obtained follicular fluid was centrifuged during 10 min at 2.000 x *g* and stored in liquid nitrogen until analyses for NEFA, α-tocopherol, ROS, FRAP, and estrogen concentration.

2.5. Oxidative status in blood and follicular fluid

To assess the oxidative status, the samples were submitted to three evaluations: ROS, total antioxidant capacity and lipid peroxidation. The ROS production was determined with a spectrofluorimetric method as described by Loetchutinat et al. (2005). Briefly, the samples were incubated in the dark with 5 mL of 2′,7′-dichloro dihydrofluorescein diacetate (DCHF-DA). The DCHF-DA, upon oxidation, is converts to the fluorescent 2′,7′-dichlorofluorescein (DCF). The oxidation of DCHF-DA to DCF was used to detect and measure intracellular ROS concentrations. The fluorescence intensity emitted at 520 nm (488 nm excitation) was monitored 60 min after the addition of DCF-DA. The total antioxidant potential (FRAP) in the sample was determined by ability of antioxidants to reduce Fe^{+3} to Fe^{+2}, which is chelated by 2,4,6-tri(2-pyridyl)-striazine (TPTZ) to form Fe^{+2} -TPTZ with maximum absorption at 593 nm (Benzie and Strain, 1996). Lipid peroxidation was determined by the formation of thiobarbituric acid reactive species (TBARS). Malondialdehyde (MDA) was reacted with thiobarbituric acid (TBA) to form a color complex, which was determined spectrophotometrically at 532 nm as described by Ohkawa et al. (1979).

141

142 *2.6. Alpha tocopherol analysis*

143 Alpha tocopherol was extracted from the samples (serum and follicular fluid) using the
144 methodology described by Charão et al. (2012). Chromatographic analyses were performed
145 using a CBM-20A Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with degasser
146 (DGU20A5 prominence, Shimadzu, Japan), column oven (CTO-20A prominence, Shimadzu,
147 Japan) and coupled to a UV/Vis detector (SPD-20AV prominence, Shimadzu, Japan) and a
148 fluorescent detector (RF-20A prominence, Shimadzu, Japan) in series. Separation was
149 performed in a reverse-phase C-18 Agilent Microsorb-MV 100-5 C column (5 µm particle size,
150 250 mm, 4.6 mm) at 30 °C. Injection volume was 20 µL and the mobile phase was a linear
151 gradient of an acetonitrile/methanol//MTBE mixture (65:25:10 v/v) at a flow rate of 0.8
152 mL/min.

153 The chromatograms for quantification purposes were obtained with fluorescence
154 detection for α-tocopherol (excitation wavelength of 295 nm and emission wavelength of 325
155 nm). Calibration curves were constructed using stock solutions α-tocopherol (Sigma T3251)
156 standards reference. Calibration curve α-tocopherol: y = 866019x - 319270 (r=0.999). The limit
157 of detection (LoD) and limited of quantification (LoQ) for α-tocopherol were 0.052 and 0.159
158 ppm, respectively.

159

160 *2.7. Non esterified fatty acids determination*

161 NEFA extracted from bovine follicular fluid and serum were determined by gas
162 chromatography according to the methodology described by Han et al. (2011), with some
163 modifications. Briefly, 35 µL of the sample was added to tubes already containing 15 µg of
164 internal standard (heptadecanoic acid, Sigma H3500). After, was added 175 µL of 0.4M

KOH/CH3OH; the mixture was vortexed for 30 s and then was held at room temperature for 10 min. Then, 1750 μL of n-hexane was added. After phase separation, the upper layer was removed. The lower phase, where the NEFA and the internal standard were dried under a stream of N_2 and solubilized with 1700 μL of 5 % (v/v) H2SO4/CH3OH. The tubes were sealed and incubated at 70 °C for 30 min with frequent agitation. After the tubes cooled down to room temperature, the reactions were quenched by adding 700 μL water. Extraction was conducted by adding 1750 μL n-hexane. Upper layers containing fatty acids (FA) methyl ester derivatives were removed to new vials for injection. Samples were injected in an Agilent Technologies gas chromatograph (HP6890 N) equipped with a capillary column Agilent DB-23 (60 m x 0.25 mm; 0.25 μm) and flame ionization detector (FID). The temperature of the injection port was maintained at 250 °C and the carrier gas was nitrogen (0.6 mL/min). After injection (2 μL, splitless), the oven temperature was held at 150 °C for 1 min, then it was increased to 240 °C at 4 °C.min−1 and maintained at this temperature for 12 min. A standard mix of FA methyl esters (37-Component FAME Mix and PUFA number 2 from Sigma, Saint Louis, MO, USA) were run under the same conditions, and the subsequent retention times were used to identify the FA. The internal standard was used to calculate the proportion of NEFA and the results were expressed as % of the sample.

2.8. Steroid assay

Follicular fluid samples from follicles were collected and individually stored at – 196 ºC. Follicular fluid concentration of E2 was determined using the Estradiol ELISA kit (501890, Cayman Chemical, Ann Arbor, USA). The dilution of follicular fluid samples ranged between 1:40 to 1:1,000. The intra-assay coefficient of variation was 11.5 %.

2.9. Statistical analysis

The effect of treatments over time of fasting was assessed by mixed models for repeated data. The effect of group, time and group by time interaction were included in the statistical model, and cow was included as subject. Differences at each time point were determined by Student's pair-wise test. Different covariance structures were tested for each model and the one with the least Akaike Information Criteria (AIC) was used. Other continuous non-repeated data were analyzed using mixed models followed by Student's pair-wise test. All continuous data and residuals were tested for normal distribution using Shapiro–Wilk test and normalized according to distribution of data. Data are presented as least square corrected means ± SEM. All the analyses were performed using the SAS Statistical Package (SAS Institute Inc., Cary, NC). $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Validation of the experimental model*

To validate the experimental model of fasting (Fig. 2), we performed the profile of the main NEFA present in the serum during fasting days and in follicular fluid after 4 days of fasting. In serum, it was possible to observe that there was an increase in the rate of palmitic acid (C16:0, Fig. 2A) in fasting cows on D1 when compared to those that received vitamin E (Fasting + VitE group). On Day 3, the C16:0 rate was higher in the fasting groups (Fasting and Fasting + VitE) compared to the control group. In addition, the NEFA proportion remained high in the Fasting + VitE compared to the control group ($P = 0.04$) on D4. Blood palmitoleic acid levels (C16:1, Fig. 2B)were increased in fasting when compared to the control cows ($P < 0.05$) on D1. Furthermore, the C16:1 ratio was higher in the Fasting group compared to the Fasting + VitE group ($P = 0.04$) in D1. There was no difference among the groups for the rates of C16:1 in D0, D2, D3 and D4 of fasting. For methylpalmitoleic acid (C17:1; Fig. 2C) there was no difference among groups the on any of the evaluated days ($P > 0.05$). The stearic acid (C18:0;

Fig. 2D) ratio did not differ among groups between D0-D3. However, in D4 the C18:0 ratio was higher in the control group compared to the Fasting + VitE group ($P = 0.04$), but it did not differ from the Fasting group ($P = 0.09$). The proportion of oleic acid (C18:1n9; Fig. 2E) in serum was higher in D2, D3 and D4 in the fasting groups (Fasting and Fasting + VitE) compared to the control group..

To assess the fat mobilization of fasting cows, we observed the proportion of the main NEFA (C16: 0 + C18: 0 + C18: 1n9; Fig. 3) in serum. On Day 1, fasting cows that received vitamin E had a reduction in NEFA levels ($P = 0.03$), which was not observed in fasting animals without vitamin E. However, NEFA levels in the animals of the three groups did not differ on D2 and, in fasting cows, regardless of whether they received VitE, the NEFA levels were higher than those in the control cows on days 3 and 4 (P £ 0.01). On Day 4, the increase of C18:1n9 levels in serum was also observed in follicular fluid ($P < 0.05$), which did not occur in relation to other NEFA (C16:0, C16:1; C:17:1 and C18:0; $P > 0.05$; Fig. 4). These data evidence that the NEFA in blood and follicular fluid are different and a short-term fasting period alters the NEFA composition in both blood and follicular fluid.

3.2. *Levels of α-tocopherol in serum and follicular fluid*

After validation of the experimental model, we evaluated the concentrations of vitamin E (α-tocopherol) from D0 to D4 in serum and in D4 on follicular fluid. Serum α-tocopherol concentrations decreased in Fasting animals, showing a significant difference in D2 and D4 ($P < 0.05$; Fig. 5A). With the application of vitamin E (Fasting + VitE), α-tocopherol levels did not differ from those observed in control cows in serum. When assessing serum and follicular fluid concentrations of α-tocopherol in D4 (Fig. 5B), the reduction in α-tocopherol levels observed in the serum of Fasting animals was not observed in the follicular fluid compared to control cows. However, an increase in α-tocopherol in FF was also detected in animals that

received vitamin E. These results show that the parenteral administration of vitamin E increased the α-tocopherol blood levels, reaching high levels in the dominant follicle.

3.3. *Status antioxidant*

To assess the effect of fasting on blood oxidative status, ROS, FRAP and lipid peroxidation levels were determined during the four days of fasting. Only on D4, ROS levels were increased in the serum of fasting animals ($P = 0.04$; Fig. 6A), which was not reduced by the administration of vitamin E ($P > 0.05$). On the other days, all cell oxidation parameters did not differ from control cows ($P > 0.05$; Fig. 6B, C). Contrary to what was observed in blood level, in follicular fluid there was decreased ROS production in fasting cows that received vitamin E compared to fasted animals without vitamin E ($P < 0.05$; Fig. 7A) but not FRAP ($P > 0.05$; Fig. 7B).

3.4. *Follicular diameter and estrogen concentration*

The mean diameter of the dominant follicle observed in fasting cows was 1.1 mm smaller than those in the control group, which was not statistically different ($P > 0.05$; Figure 8A). Similarly, the concentrations of estradiol in the follicular fluid did not differ from the control cows ($P > 0.05$; Fig. 8, B).

4. Discussion

We showed that α-tocopherol in a dose of 1,000 IU can reduce the production of ROS in the dominant follicle when cows are in negative energy balance. To simulate what normally occurs in cows during transition period, a model based on fasting was used (Mohamed et al., 2004; Ono et al., 2011; Jorritsma et al., 2003). In our study, using this model, we observed an increase in blood and follicular fluid NEFA levels during fasting. In validating the experimental

model, we observed that: 1) NEFA concentrations in follicular fluid reflect blood levels, as reported by other authors (Aardema et al., 2013; Leroy et al., 2004); 2) the increase in NEFA causes an increase in the production of ROS in the follicular fluid; and 3) intramuscular application of vitamin E reduces ROS levels in the dominant follicle.

In the present study, the four days fasting period resulted in an increase in NEFA concentrations with a chain length of 16–18 carbon atoms in serum, which denotes that the fatty acid are mobilized during energy deprivation (Rukkwamsuk et al., 2000). The proportion of fatty acid that differed between groups in the follicular fluid was oleic acid. The effects of oleic acid on different cell types are contradictory. In human lymphocytes, oleic acid promotes apoptosis and necrosis (Cury-Boaventura et al., 2006). In addition, oleic acid inhibits the proliferation and causes an excessive inflammatory reaction in fibroblasts (Jiang et al., 2012). In granulosa cells, oleic acid reduced proliferation, estradiol production (Vanholder et al., 2005), and morphological, physiological, and molecular transformations (Aardema et al., 2013). In endometrial cells, oleic acid was responsible for the intracellular accumulation of lipids (Ferst et al., 2021). *In vivo*, the intrafollicular injection of oleic acid reduced estradiol concentration and decrease ovulation rate (Sharma et al., 2019). Additionally, NEFA can alter or delay folliculogenesis and follicular growth (Ferst et al., 2020), with reduced ovulatory follicle size being associated with lower pregnancy rate and poor corpus luteum function (Jorritsma et al., 2003). Despite that, in the present study, no difference in follicular diameter and estradiol concentration among groups was observed. The follicular diameter and estrogen production were not affected probably due to the short period of cow exposure to high levels of NEFA. In our study, follicles were exposed to acute metabolic stress for 4 days during final follicular growth, whereas granulosa cells from transition period cows are exposed to elevated NEFA for weeks during early stages of folliculogenesis (Aardema et al., 2013), which may compromise the functionality follicular cells and impair reproductive performance.

290 During the transition period, the NEB leads to a disequilibrium in oxidant or antioxidant
291 capacity, resulting in oxidative stress, which impairs the reproductive functions such as
292 production of steroids and prostaglandins, and embryonic development (Aréchiga et al., 1998).
293 We hypothesized that a dose of vitamin E (1,000 UI) at the time of follicular divergence
294 decreases the risk of oxidative stress in the dominant follicle. Our results demonstrated that
295 intramuscular injection of a dose of vitamin E did not reduce the production of ROS in blood,
296 but it did in the follicular fluid. It is known that the circulating concentration of α-tocopherol is
297 highly correlated with the intake of vitamin E (Weiss et al., 1997), suggesting oral or parenteral
298 administration can maintain blood vitamin E concentration in transition cows. In the present
299 study, although fasting decreased the levels of α-tocopherol, Fasting + VitE prevented this
300 decline compared to the control group, which may be indicative for the prevention of oxidative
301 stress during NEB. In sheep, ROS-induced lipid peroxidation in mammary gland cells can be
302 prevented with α-tocopherol (Colitti et al., 2000). In erythrocytes, antioxidant activity was
303 higher when the animals were treated with 1,000 IU of vitamin E (Agarwal et al., 2012). These
304 findings in other cell types corroborate the antioxidant capacity of vitamin E. In our study, the
305 total antioxidant capacity in serum and follicular fluid was similar among groups. The possible
306 reasons for the absence of difference in total antioxidant capacity was the α-tocopherol levels
307 that were within acceptable levels. The levels of α-tocopherol capable of preventing oxidative
308 stress must be equal to or above 3.0 μg/mL (Weiss et al., 1997), which was observed in all
309 animals in the present study.

310 Another point is that vitamin E is a lipophilic molecule found mainly in cellular
311 membranes that inhibit lipid peroxidation by reacting and neutralizing lipid peroxyl radicals
312 (Wang, 1999). TBARS is indicative of lipid peroxidation and increased levels after calving
313 indicates the imbalance between oxidants and antioxidants (Bernabucci et al., 2005). However,
314 our results did not show difference among groups in lipid peroxidation. These results are

different from those obtained elsewhere (Bouwstra et al., 2010, 2008), which demonstrated that TBARS concentration was lower in vitamin E treatment groups. Unfortunately, in the present study we were unable to assess TBARS in follicular fluid due to the small sample volume. Therefore, the mechanisms by which supplemental vitamin E decrease the ROS production in ovarian follicle were not fully elucidated in this study.

There are considerable differences in the results of studies that investigate the effect of vitamin E on reproductive performance. At least part of these differences may be attributed to dose and administration frequency during transition period of cattle. In this study we used 1,000 IU concentration as suggested by Pontes et al. (2015) in a single application. We chose a single application because it could be used during fixed-time artificial insemination protocols to decrease the effects of NEFA on follicular fluid and improve reproductive performance. In some studies, it has been reported that the administration of 1,000 IU vitamin E improves the reproductive rates (Baldi et al., 2000; Campbell and Miller, 1998; Pontes et al., 2015). It was described that the administration of 1,000 IU α-tocopherol at weekly intervals decreases the incidence of postpartum illnesses and improves pregnancy/artificial insemination rates (Pontes et al., 2015). Similar results were described that supplementation with 500 mg vitamin E improves fertility, with reduction in pregnancy interval and increase in pregnancy/AI (Aréchiga et al., 1998).

5. Conclusion

The results of this study indicated that oxidative stress occurs during NEB in serum and follicular fluid in cows and supplementation of vitamin E has beneficial effects in reduce production of ROS in preovulatory follicle of animals. More studies are needed to assess whether vitamin E maintains follicular development and oocyte competence in cows during the period of metabolic stress.

CRediT authorship contribution statement

Daniele Missio: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Project administration **Fabio Gallas Leivas**: Investigation, Resources. **Francielli Cibin:** Investigation, Resources. **Tatiana Emanuelli**: Investigation, Resources. **Sabrina Somacal:** Investigation. **Vanessa Buss:** Investigation. **Bernardo Gasperin:** Investigation, Resources. **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.

Declaration of competing interests

The authors declare that they have no conflict of interests.

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Figures legends:

Fig. 1. Experimental design. The three bars at the bottom indicate the applied feeding regime. EB = 2 mg of estradiol benzoate; CIDR: 1 g progesterone-releasing intravaginal device; PG: injection with prostaglandin F2a; SS: saline solution; FF: collection of follicular fluid; B: blood sampling for analysis of NEFA, ROS production, TBARS, FRAP and α-tocopherol.

Fig. 2. Ratio of non-esterified fatty acids in the serum of cows fed *ad libitum* (control), fasting for four days (Fasting), and fasting for four days and treated with 1,000 IU parenterally of vitamin E (Fasting + VitE). C16:0 = Palmitic Acid (A); C16:1 = Palmitoleic acid (B); C17:1 = Methylpalmitoleic acid (C); C18:0 = Stearic acid (D); C18:1n9c = Oleic acid (E). Values are means ± SEM. * indicates statistical difference between control and Fasting group ($P < 0.05$); ┼ indicates statistical difference between Fasting and Fasting +VitE group ($P < 0.05$); # indicates statistical difference between control and Fasting + VitE group ($P < 0.05$).

Fig. 3. Ratio of Palmitic (C16:0), Stearic (C18:0) and Oleic (C18:1n9) acids in the serum of cows fed *ad libitum* (control), fasting for four days (Fasting) and fasting for four days and treated with 1,000 IU of parenteral vitamin E (Fasting + VitE). Values are means ± SEM. * indicates statistical difference between control and Fasting group ($P < 0.05$). # Indicates statistical difference between control and Fasting + VitE group ($P < 0.05$).

Fig. 4. Ratio of non-esterified fatty acids in the preovulatory follicle fluid of cows fed *ad libitum* (control), fasting for four days (Fasting), and fasting for four days and treated with 1,000 IU parenterally of vitamin E (Fasting + VitE). C16:0 = Palmitic Acid; C16:1 = Palmitoleic acid; C17:1 = Methylpalmitoleic acid; C18:0 = Stearic acid; C18:1n9c = Oleic acid. Values are means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

Fig. 5. Concentration of α-tocopherol (means and SEM) in serum (A) or serum and preovulatory follicle fluid (B) in D4 of cows fed *ad libitum* (control), fasting for four days (Fasting), and fasting for four days and treated with 1,000 IU parenterally of vitamin E (Fasting + VitE). A: *indicates statistical difference between control and Fasting group ($P < 0.05$); # indicates statistical difference between control and Fasting +VitE group ($P < 0.05$). B: Bars with different letters are significantly different ($P < 0.05$).

Fig. 6. Production of reactive oxygen species (ROS; A), total antioxidant capacity (FRAP, B) and lipid peroxidation (TBARS; C) in the serum of cows fed *ad libitum* (control), fasting for four days (Fasting), and fasting for four days and treated with 1,000 IU parenterally of vitamin E (Fasting + VitE). *indicates statistical difference between control and Fasting group ($P < 0.05$). # indicates statistical difference between control and Fasting +VitE group ($P < 0.05$).

Fig. 7. Production of reactive oxygen species (ROS; A) and total antioxidant capacity (FRAP, B) in the preovulatory follicle fluid of cows fed ad libitum (control), fasting for four days (Fasting), and fasting for four days and treated with 1,000 IU parenterally of vitamin E (Fasting + VitE). Values are means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

Fig. 8. Follicular diameter (A) and estradiol concentration in follicular fluid (B) in cows fed *ad libitum* (control), fasting for four days (Fasting), and fasting for four days and treated intramuscularly with 1,000 IU vitamin E (Fasting + VitE). Values are means ± SEM. $P < 0.05$.

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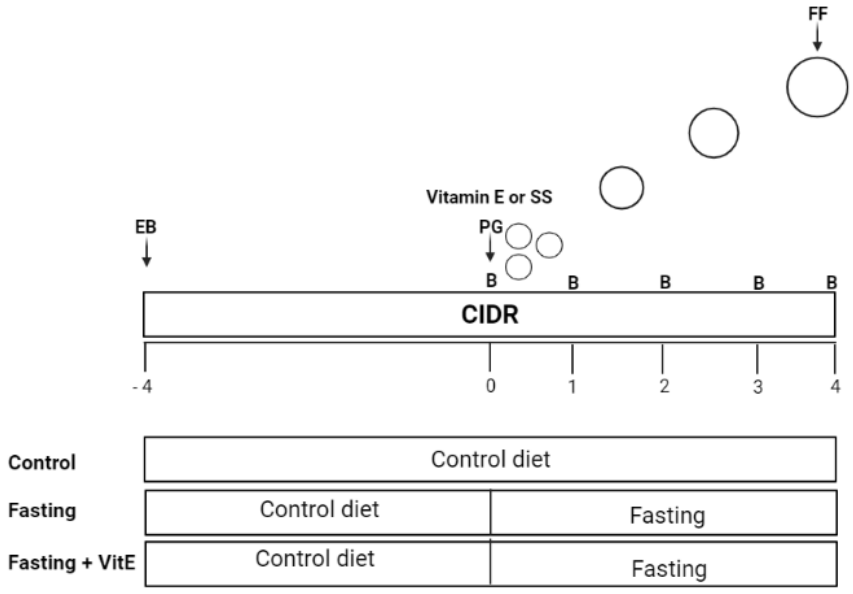

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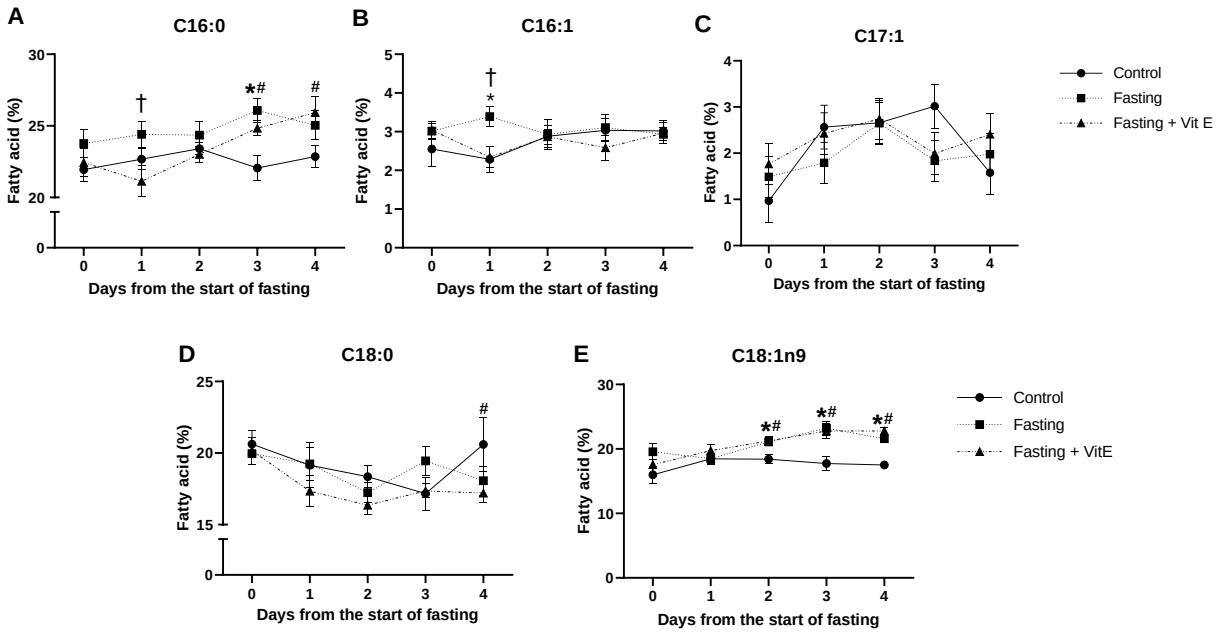

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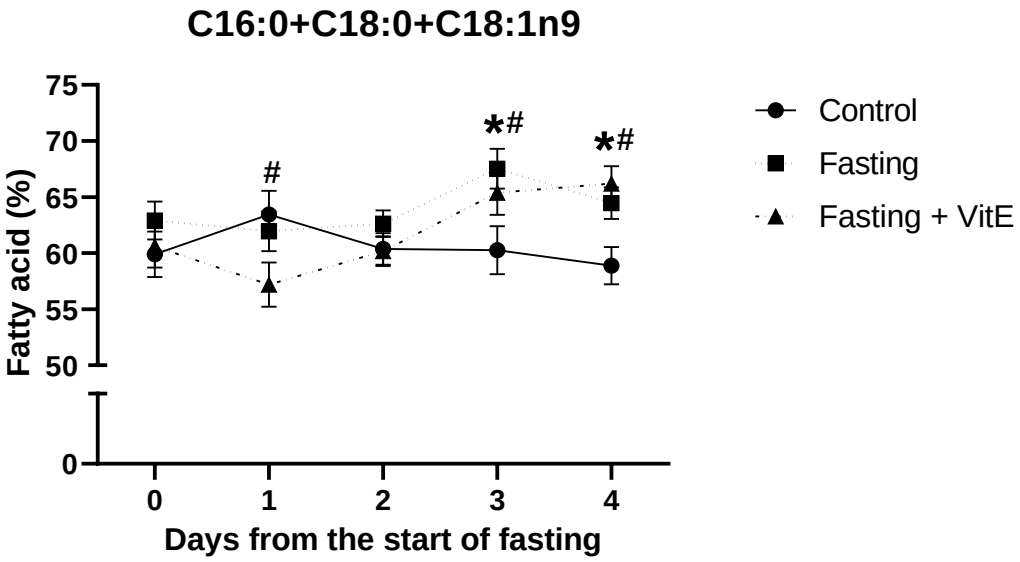

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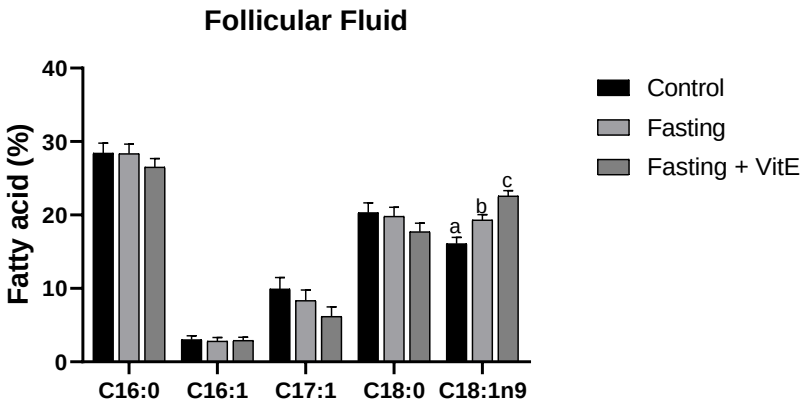
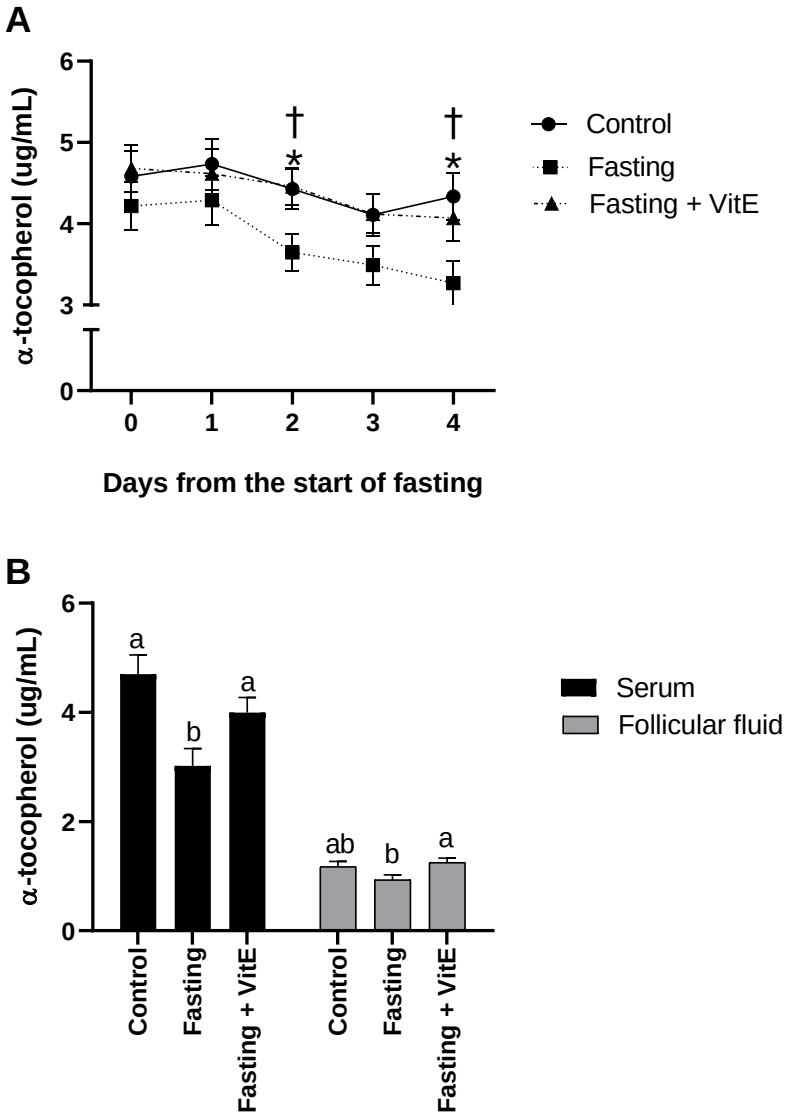

Follicular Fluid
Fatty acid (%)
40
30
20
10
0
a
b
c
C16:0
C16:1
C17:1
C18:0
C18:1n9
Control
Fasting
Fasting + VitE

592

593 **Fig. 5:**



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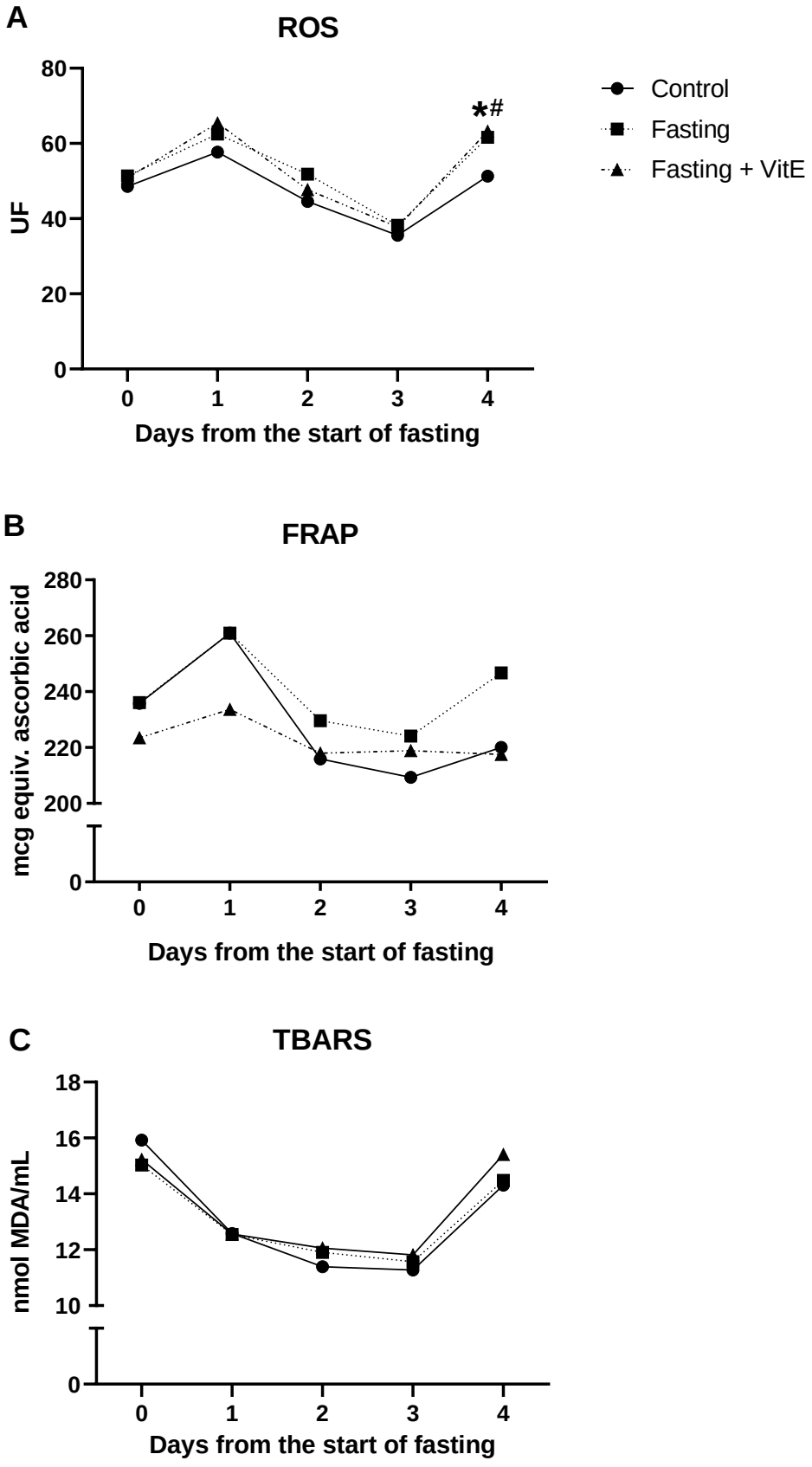
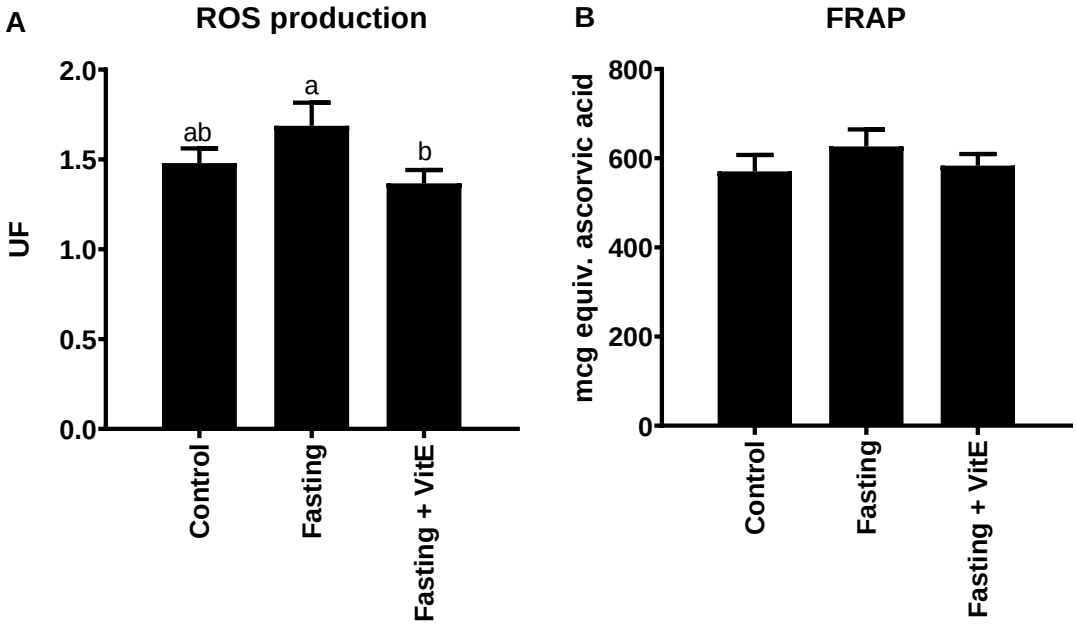

A
ROS
UF
80
60
40
20
0
*#
0
1
2
3
4
Days from the start of fasting
Control
Fasting
Fasting + VitE
B
FRAP
mcg equiv. ascorbic acid
280
260
240
220
200
0
Days from the start of fasting
C
TBARS
nmol MDA/mL
18
16
14
12
10
0
Days from the start of fasting

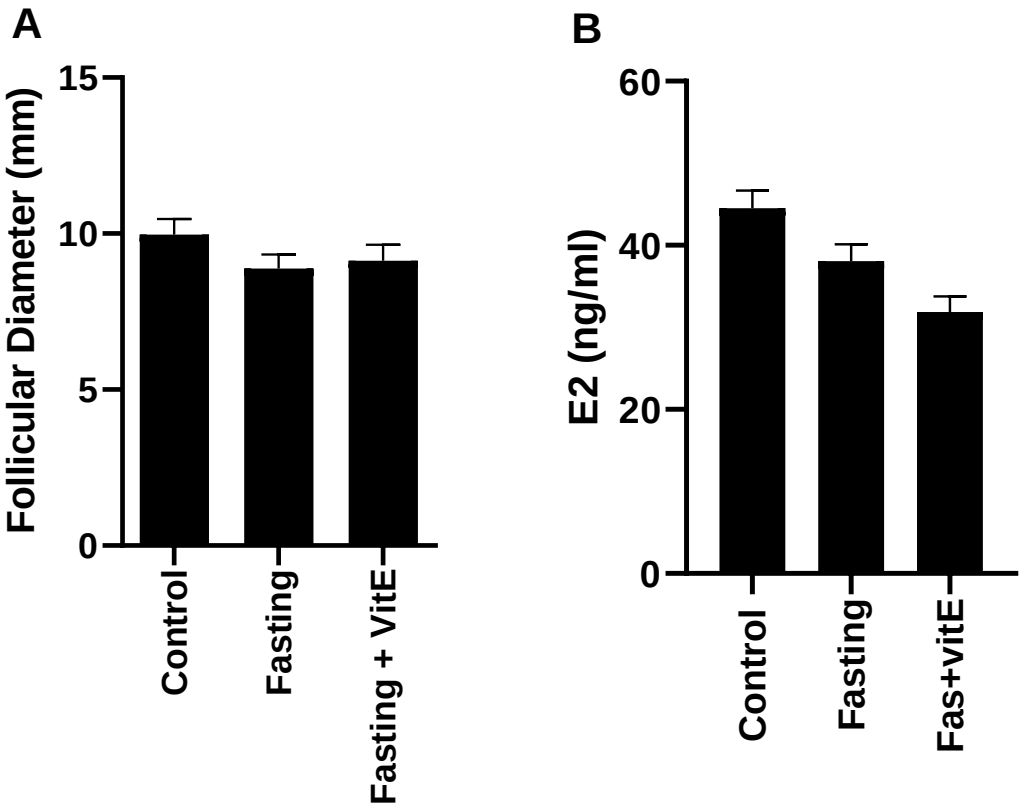
597

598 **Fig. 7:**


A
ROS production
UF
2.0
1.5
1.0
0.5
0.0
ab
a
b
Control
Fasting
Fasting + VitE
B
FRAP
mcg equiv. ascorvic acid
800
600
400
200
0
Control
Fasting
Fasting + VitE

599

600 **Fig. 8:**


A
Follicular Diameter (mm)
15
10
5
0
Control
Fasting
Fasting + VitE
B
E2 (ng/ml)
60
40
20
0
Control
Fasting
Fas+vitE

601

3. DISCUSSÃO

Nas últimas décadas tem ocorrido uma diminuição da fertilidade de vacas leiteiras. A redução nas taxas de prenhez tem sido associada ao estresse metabólico que ocorre durante o período de transição, sendo essa fase a mais crítica da vaca leiteira em função de todas as mudanças metabólicas e fisiológicas que ocorrem. Durante esse período há uma complexa mudança nas concentrações de diversas moléculas, como o aumento dos níveis circulantes de ácidos graxos não esterificados e de corpos cetônicos como BHBA, diminuição dos níveis séricos de glicose, IGF-1 e insulina. Essas alterações séricas também são refletidas no ambiente folicular, ovidutal e uterino. Nesse sentido, nosso grupo de pesquisa tem tentado elucidar como essas moléculas atuam sobre as células foliculares e os gametas para traçar alternativas que diminuam os efeitos do estresse metabólico no ambiente reprodutivo e consequentemente as perdas econômicas.

Através da realização de experimentos *in vivo* e *in vitro*, os resultados dessa tese demonstram que altas concentrações de BHBA prejudicam a cinética de espermatozoides bovinos, o crescimento folicular, causam estresse de RE em células do cumulus durante a maturação *in vitro*, sem, no entanto, causar estresse oxidativo em qualquer um destes tipos celulares, comprometer a cascata de genes ovulatórios em células da granulosa ou diminuir a viabilidade oocitária. Ainda, nossos resultados demonstram que o jejum aumenta NEFA e a produção de ROS no folículo pré-ovulatório e a administração intramuscular de Vitamina E, como antioxidante, é capaz de reduzir essa produção no folículo dominante de vacas em NEB.

Essa tese é constituída por quatro trabalhos. No primeiro trabalho nos utilizamos uma metodologia *in vitro* para investigar se o transporte dos espermatozoides bovinos pelo trato reprodutivo da fêmea em cetose afeta a sua viabilidade, o que poderia comprometer a fertilidade desses animais. Os resultados revelaram que a exposição de células espermáticas após uma hora em concentrações de BHBA superiores a 2,4 mM prejudica padrões de cinética espermática como velocidade curvilínea (VCL), velocidade retilínea (VSL), velocidade média da trajetória (VAP), retilinearidade (STR) e linearidade (LIN), sendo que essas alterações não foram causadas por estresse oxidativo. As variáveis VAP, VSL, STR e LIN são marcadores de progressão espermática, já STR e LIN são indicadores do padrão de movimento espermática (DUTY et al., 2004). Diversos estudos têm demonstrado que essas variáveis são preditores confiáveis da fertilidade do sêmen de touros *in vivo* (FARRELL et al., 1998; GLIOZZI et al., 2017; KATHIRAVAN et al., 2011; MICHOS et al., 2017). Além disso, *in vitro*, a taxa de

desenvolvimento embrionário foi reduzida quando VAP, VSL, BCF, STR e LIN foram diminuídos (CAMPANHOLI et al., 2016). Apesar de ser o primeiro estudo avaliando a cinética de espermatozoides bovinos expostos ao BHBA, DESMET et al., (2017) incubou espermatozoides com NEFA, que também estão em níveis elevados em vacas em BEN, e observou que os parâmetros de velocidade foram reduzidos quando altas concentrações de ácidos graxos foram utilizadas. Assim podemos sugerir que o movimento espermático é prejudicado durante a passagem pelo fluido uterino e ovidutal de vacas em BEN. Nesse primeiro trabalho, embora BHBA causou mudanças nos parâmetros de cinética espermática, nenhuma diferença foi observada nos status oxidativo avaliado através da produção de ROS, capacidade antioxidante total e peroxidação lipídica. Podemos sugerir que as defesas antioxidantes presentes nas células espermáticas foram eficazes em neutralizar ROS. É bem relatado na literatura que BHBA pode causar aumento de ROS em diferentes tipos de células (FERST et al., 2021; SHI et al., 2014; TIAN et al., 2014). Portanto, os resultados desse primeiro artigo apresentaram que o BHBA altera a cinética espermática e isso não ocorre por estresse oxidativo, sendo necessárias mais investigações para elucidar o mecanismo de ação do BHBA nas células espermáticas.

A partir do segundo trabalho, buscamos compreender o que ocorre nas células foliculares e em oócitos de vacas que estão em estresse metabólico. Assim, através da injeção intrafolicular (IIF) de BHBA nós apresentamos que o BHBA diminui o diâmetro e a taxa de crescimento folicular após a injeção com o corpo cetônico. No entanto, apesar do crescimento do folículo dominante ser prejudicado não houve diferença na taxa de vacas em que os folículos ovularam. Isso pode ter ocorrido pois as células ovarianas utilizam corpos cetônicos como fonte de energia (RABIEE et al., 1997), diminuindo a sua concentração no folículo e permitindo restabelecimento da homeostase e ovulação. Além do BHBA, nosso grupo de pesquisa mostrou recentemente que a IIF de NEFA durante a divergência diminui o crescimento folicular (FERST et al., 2020). Considerando os resultados obtidos, ainda no segundo trabalho nos decidimos elucidar o que ocorre quando há altos níveis de BHBA no folículo pré-ovulatório (≥ 12mm) sobre o mecanismo ovulatório e em vias de estresse em células da granulosa. Os resultados obtidos através da expressão gênica demonstram que a cascata de genes ovulatórios (ADAM17, AREG, EREG e PTGS2) não é alterada por altas quantidades de BHBA, bem como a produção de esteroides e a abundância relativa de genes associados a esteroidogênese (3BHSD, CYP19A1 e STAR), estresse oxidativo (SOD1, CAT e GPX1), estresse de RE (HSPA5, ATF4, ATF6, XBP1u e XBP1s) e apoptose (XIAP) 6 h após a administração de BHBA no folículo. Esses resultados diferem de VANHOLDER et al. (2006) que apresentaram que BHBA diminui

a concentração de estrógeno e progesterona em células da granulosa cultivadas *in vitro*. Além disso, apesar do o estresse oxidativo ser positivamente correlacionado com o aumento de NEFA e BHBA em vacas (LI et al., 2016a), nenhuma alteração foi observada no nosso trabalho. Diferentes estudos demonstraram que o BHBA causa estresse oxidativo e estresse de RE em hepatócitos (ISLAM et al., 2022; SHI et al., 2015), epitélio da glândula mamária (ZHANG et al., 2020), células uterinas (FERST et al., 2021), e células do abomaso (TIAN et al., 2014). É importante salientar que para a realização desse trabalho nós utilizamos vacas que não estavam em NEB para ver o efeito direto do BHBA no folículo, portanto, as células podem ter utilizado o corpo cetônico como uma fonte de energia. Outro ponto, é que durante o período de transição os corpos cetônicos ficam com níveis elevados por vários dias da foliculogênese, o que pode comprometer a homeostase das células da granulosa e consequentemente a ovulação. Apesar de HILL et al. (2018) sugerir que a concentração de BHBA sérica dez dias antes da inseminação artificial é menor em vacas que ovulam, durante o BEN uma complexa alteração de moléculas ocorre e podem corroborar com esses resultados, como os próprios NEFA. Nesse sentido, SHARMA et al. (2019) apresentou que a IIF com ácido oleico reduziu a concentração de estradiol e tendeu a inibir a ovulação. Portanto, com a realização do segundo trabalho podemos concluir que o BHBA isolado apesar do prejudicar o desenvolvimento folicular não causa estresse e compromete a funcionalidade celular nas células da granulosa no folículo pré-ovulatório na ovulação.

Posteriormente, nós desenvolvemos o terceiro trabalho para avaliar o que ocorre nas células do cumulus e no oócito de vacas em cetose após o pico de LH. Para isso, nós utilizamos o modelo da maturação *in vitro* e ao longo desse período podemos investigar o que ocorre nos complexos cumulus-oócito as 6, 12, 18 e 24 h de MIV. Nenhuma diferença foi observada na expansão do cumulus, percentagem de oócitos que atingiram a metáfase II às 24 horas, taxa de clivagem às 48 horas, bem como o status oxidativo e a abundância relativa de genes associados a enzimas antioxidantes (SOD1, CAT e GPX1) e autofagia (LC3). No entanto, pela primeira vez, nos apresentamos que 2 mM de BHBA ativa vias UPR através do aumento da expressão de genes como HSPA5 (marcador de estresse de RE), XBP1u e XBP1s, demonstrando que BHBA causa estresse nas células do cumulus e vias de citoproteção são ativadas. Corroborando com nossos resultados, alguns estudos demonstram que BHBA não altera a taxa de maturação e clivagem de oócitos bovinos (SANGALLI et al., 2018) e suínos (TSUZUKI et al., 2009). Ainda, estudos demonstram que BHBA ativa o via UPR em diferentes tipos celulares como citado acima. A ativação das vias UPR demonstram que BHBA aumenta a quantidade de proteínas malformadas, o que pode comprometer a viabilidade celular, no entanto, em nosso

trabalho esse mecanismo de citoproteção foi efetivo uma vez que as 18 h nenhuma alteração foi constatada e as taxas de maturação e clivagem não foram afetadas. No entanto, mais estudos são necessários para avaliamos se os embriões apresentam um desenvolvimento inicial normal ou há comprometimento molecular. Portanto, o terceiro trabalho dessa tese mostrou que BHBA causa estresse de RE em células do cumulus.

Os três primeiros trabalhos dessa tese trabalharam especificamente com o corpo cetônico BHBA, o principal e mais estável corpo cetônico de vacas em cetose. Já o último trabalho dessa tese foi realizado para avaliar o efeito do uso intramuscular de Vitamina E (α-tocoferol) para impedir o estresse oxidativo no sangue e no fluido folicular de vacas em NEB. Nesse trabalho, nos utilizamos um produto comercial citado por outros autores (PONTES et al., 2015), pois poderia ser adquirido facilmente e com um custo acessível para os produtores rurais. Para realizar esse estudo nos induzimos NEB em vacas de corte não lactantes através de um jejum agudo de quatro dias. Para validação de nosso modelo, realizamos o perfil dos principais NEFA no sangue e no fluido folicular através da cromatografia gasosa e espectrofotometria de massa. O jejum agudo foi capaz de elevar os níveis circulatórios de NEFA. No fluido folicular, as concentrações de ácido oleico foram superiores nas vacas em jejum comparado ao grupo controle, comprovando a validação do nosso modelo experimental. Como utilizamos um produto comercial, nós também realizamos o perfil de α-tocoferol no sangue e no fluido folicular dos animais após a administração. Os resultados demonstraram que a concentração de vitamina E no sangue e no fluido folicular foi superior nas vacas que receberam o antioxidante comparado ao grupo que nas mesmas condições alimentares não recebeu o produto. Ainda, nesse estudo podemos observar que após quatro dias de jejum os animais que não receberam antioxidante tiveram maior produção de ROS no fluido folicular comparado aos animais tratados com 1.000 UI de Vitamina E, demonstrando a eficácia do antioxidante. Há uma grande variação nos resultados dos estudos que investigam o efeito da vitamina E no desempenho reprodutivo, pelo menos parte disso é atribuível as diferentes concentrações e frequência de administração em que é utilizada durante o período de transição. Neste estudo usamos a concentração de 1.000 UI conforme PONTES et al. (2015) em uma única aplicação, portanto, sugerimos que esse método pode ser utilizado durante protocolos de inseminação artificial em tempo fixo para diminuir os efeitos do NEFA no fluido folicular e melhorar o desempenho reprodutivo. Porém, mais estudos são necessários para avaliarmos a dose e os períodos de aplicação. Estudos relataram que a administração de 1.000 UI de vitamina E melhora algumas taxas reprodutivas (BALDI et al., 2000; CAMPBELL; MILLER, 1998; PONTES et al., 2015). PONTES et al., (2015) descobriram que a administração de 1.000 UI de α-tocoferol em

intervalos semanais diminui a incidência de doenças pós-parto e melhora as taxas de prenhez por inseminação artificial. Resultados semelhantes foram encontrados por ARÉCHIGA et al. (1998) que relataram que a suplementação com 500 mg de vitamina E melhora a fertilidade, com redução do intervalo entre partos. Em conclusão os dados do quarto estudo indicaram que o estresse oxidativo acontece durante a NEB no soro e fluido folicular em vacas e a suplementação de antioxidantes como a vitamina E tem efeitos benéficos na redução da produção de ROS no folículo pré-ovulatório dos animais, o que pode melhorar o desempenho reprodutivo das vacas durante o período de transição. Nossos resultados não mostraram nenhuma alteração na concentração de estradiol e no diâmetro folicular. Portanto, mais estudos são necessários para avaliar se a vitamina E mantém a competência oocitária em vacas durante o período de estresse metabólico.

A partir dos dados obtidos com essa tese foi possível determinar que o BHBA prejudica a cinética de espermatozoides bovinos, o crescimento folicular e causa estresse em células do cumulus. Ainda, que a utilização de antioxidante é capaz de prevenir o estresse oxidativo no folículo de vacas em estresse metabólico. Os resultados dessa tese abrem caminho para novos estudos que abordem os mecanismos de ação dos NEFA e do BHBA em gametas e células foliculares.

4. CONCLUSÃO

Com os resultados obtidos no conjunto de experimentos que compõe essa tese foi possível demonstrar o efeito que os metabólitos do balanço energético negativo causam em gametas e células foliculares. Foi observado que o BHBA causa diminuição na cinética de espermatozoides bovinos, altera o desenvolvimento folicular, mas não altera a cascata ovulatória em folículos dominantes e causa estresse de retículo endoplasmático em células do cumulus durante a maturação *in vitro*. Ainda, foi demonstrado que a utilização de vitamina E é eficiente em impedir o aumento da produção de ROS no folículo pré-ovulatório de vacas em BEN. Portanto, os resultados mostrados nessa tese fornecem novas informações sobre os efeitos de NEFA e BHBA na reprodução de vacas em estresse metabólico e trazem uma alternativa para tentar reduzir esses efeitos.

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