UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Veterinária
Programa de Pós-Graduação em Veterinária

Tese

Estratégias nutracêuticas no período de transição em bovinos leiteiros e seus efeitos sobre parâmetros metabólicos e fertilidade

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Pelotas, 2016

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Tese apresentada ao Programa de Pós-Graduação em Veterinária da Faculdade de Veterinária da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de concentração: Sanidade Animal).

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Pelotas, 2016

Dados Internacionais de Catalogação na Publicação:
Bibliotecária Daiane Schramm – CRB-10/1881

A185e Acosta, Diego Andres Velasco

Estratégias nutracêuticas no período de transição em bovinos leiteiros e seus efeitos sobre parâmetros metabólicos e fertilidade. / Diego Andres Velasco Acosta. Orientador: Prof. Dr. Marcio Nunes Corrêa. – Pelotas, 2016.

132f.

Tese (Doutorado em Ciências) – Faculdade de Veterinária. Programa de Pós-Graduação em Veterinária. Universidade Federal de pelotas.

1. Cholina. 2. Fertilidade. 3. Metionina. 4. Somatotropina. I. Corrêa, Marcio Nunes; orient. II. Título.

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Tese aprovada como requisito parcial para obtenção do grau de Doutor em Ciências, Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas.

Data da Defesa: 12/01/2016

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Dedicatória

Dedico está tese a meus pais que foram fundamentais para que eu pudesse chegar até este ponto de minha carreira professional

Agradecimentos

À minha família, quero agradecer pelo incentivo, pelo amor que têm comigo, por tudo que me ensinaram e continuam ensinando para que eu possa ser um ótimo professional e especialmente uma ótima pessoa.

À minha namorada, quero agradecer por tudo o amor, a companhia, e pelo incentivo nos momentos difíceis.

A meu orientador, o Professor Marcio Nunes Corrêa pela confiança, apoio e pelos ensinamentos.

À meus co-orientadores, Dr. Felipe Cardoso, pela oportunidade de trabalhar junto e fazer parte de um excelente time (Dairy Focos Team), pelos ensinamentos e pela amizade, ao Dr. Augusto Schneider por todos ensinamentos, pelas orientações, e pela amizade.

Ao NUPEEC, que tournou-se uma segunda família, onde conheci pessoas maravilhosa e obrigado pela ajuda durante todo este processo.

À meus amigos, prefiro não mencionar nenhum pois posso ser injusto, quero agradecer por todos os bom momentos que fizeram parte fundamental de minha vida aqui no Brasil.

Resumo

ACOSTA, Diego Andres Velasco. **Estratégias nutracêuticas no período de transição em bovinos leiteiros e seus efeitos sobre parâmetros metabólicos e fertilidade.** 2016. 132f. Tese (Doutorado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2016.

O balanço energético negativo (BEN) está associado com alterações na fertilidade de vacas leiteiras, portanto diferentes estratégias nutricionais e farmacológicas vêm sendo utilizadas para minimizar os efeitos negativos do BEN, visando aumentar a fertilidade. A aplicação pré-parto de somatotropina recombinante bovina (rbST) pode levar a uma redução da concentração sérica dos ácidos graxos não esterificados (AGNE) e β-hidroxibutirato (BHBA) no período pós-parto. Além disso têm sido demonstrado efeito significativo no desenvolvimento folicular ovariano pós-parto. Da mesma forma, estratégias nutricionais e o manejo da alimentação durante o período pré e pós-parto têm um impacto na saúde, produtividade e fertilidade da vaca leiteira. Estudos recentes mostram que a adição de aminoácidos na dieta durante o período de transição de vacas leiteiras pode ter efeitos benéficos no metabolismo, contribuindo para melhorar a performance reprodutiva. Diante desta problemática, esta tese apresenta três artigos, cujos objetivos foram: 1) determinar o efeito da administração pré-parto de somatotropina em novilhas leiteiras sobre marcadores do BEN, retomada da atividade ovariana e produção de leite. 2) Determinar o efeito da suplementação de metionina e colina protegidas da degradação ruminal, isoladas e em combinação, sobre o potencial esteroidogenico do folículo dominante da primeira onda folicular pós-parto de vacas leiteiras. 3) Determinar o efeito da suplementação de metionina e colina protegidas da degradação ruminal, isoladas e em combinação, sobre o desenvolvimento embrionário e nível de metilação global de DNA do embrião. O tratamento com somatotropina pré-parto em novilhas leiteiras aumentou a concentração de IGF-I intrafolicular e a expressão do receptor de LH e da proteína reguladora aguda da esteroidegênese em células foliculares do folículo dominante da primeira onda pós-parto. Essas mudanças foram associadas a um aumento da concentração de estradiol sérica e intrafolicular, o que pode, potencialmente, aumentar a chance de ovulação da primeira onda folicular pós-parto. Entretanto, somatotropina não afetou outros parâmetros metabólicos e produtivos no período pós-parto recente. A suplementação com metionina, colina ou ambas durante o período de transição parece não ter efeito sobre o desenvolvimento folicular pós-parto da primeira onda em vacas da raça Holandês. No entanto, a suplementação apenas com metionina durante o período de transição aumenta a expressão de *3β-HSD* nas células foliculares. Além disso, a suplementação com metionina e colina induziu a uma *down regulation* dos genes pró-inflamatórios, indicando um menor processo inflamatório nas células foliculares. Em relação ao desenvolvimento embrionário, a suplementação com metionina durante o período de transição reduziu a metilação

global do DNA e aumentou o acúmulo de lipídios nos embriões. A utilização de estratégias nutracêuticas durante o período de transição aumentaram o potencial esteroidogenico do folículo dominante da primeira onda folicular pós-parto e aumentou a expressão de genes relacionados a esteroidogenese, assim como uma redução na expressão de genes pró inflamatórios, indicando efeitos benéficos na fertilidade de bovinos leiteiros. Além disso, a suplementação de metionina parece aumentar a capacidade de sobrevivência dos embriões, desde que há fortes indícios de que as reservas lipídicas endógenas servem como um substrato energético.

Palavras-chave: Colina; fertilidade; metionina; somatotropina

Abstract

ACOSTA, Diego Angres Velasco. **Nutraceutical strategies during the transition period in dairy cattle and the effects on metabolic parameters and fertility.** 2016. 132f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2016.

The negative energy balance (NEB) is associated with changes in the fertility of dairy cows, therefore different nutritional and pharmacological strategies are being used to minimize the negative effects of the NEB in order to increase fertility. Pre-partum recombinant bovine somatotropin (rbST) administration can lead to a reduction in serum non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) in the postpartum period, in addition to improving postpartum ovarian follicular development. Similarly nutritional strategies during the pre and postpartum period have a positive impact on health, productivity and fertility of dairy cows. Recent studies show that the use of amino acids in the diet during the transition period may have beneficial effects in metabolism. Faced with this problem, this thesis presents three papers whosethe objectives were: 1) Determine the effect of pre-partum administration of somatotropin in dairy heifers on the markers of NEB, resumption of ovarian activity and milk production. 2) Determine the effect of methionine, choline supplementation isolated and in combination on esteroidogenic potential of the fist postpartum dominant follicle. 3) Determine the effect of methionine, choline supplementation isolated and in combination on embryo development and global DNA methylation. Pre-partum somatotropin treatment in dairy heifers increased intrafollicular IGF-I and mRNA expression of LH receptor and steroidogenic acute regulatory protein in follicular cells of the first postpartum dominant follicle. These changes were associated to increased intrafollicular and serum estradiol concentration, which can potentially increase the chance of ovulation of the first postpartum follicular wave. Despite that, somatotropin did not affect other metabolic and productive parameters in the early postpartum period. Supplementing methionine, choline or both during the transition period seemed to have no effect on postpartum follicular development of the first postpartum wave in Holstein cows. However, supplementing methionine, during the transition period increased follicular cells *3β-HSD* expression, methionine and choline supplementation also induced a down regulation of pro-inflammatory genes, indicating a lower inflammatory processes in follicular cells. Regarding to embryo development, supplementation of methionine, only during the transition period reduce global DNA methylation, moreover, supplemented methionine increase lipid accumulation in preimplantation embryos. The use of nutraceutical strategies during the transition period increased the steroidogenic potential of the first postpartum dominant follicle and increase the expression of genes related steroidogenesis also a down regulation of pro-

inflammatory genes indicating beneficial effects on fertility in dairy cattle. Moreover, the methionine supplementation seems to impact the preimplantation embryo in a way that enhances its capacity for survival since there is strong evidence that endogenous lipid reserves serve as an energy substrate.

Keywords: Choline; fertility; methionine; somatotropin

Lista de Abreviaturas e Siglas

AA	Aminoácidos
AGNE	Ácidos graxos não esterificados
BEN	Balanço Energético Negativo
BHBA	B-hydroxibutirato
CL	Corpo lúteo
CPDR	Colina protegida da degradação ruminal
FC	Fosfatidilcolina
GH	Hormônio do crescimento
GHR	Receptor do hormônio de crescimento
GHR 1A	Receptor do hormônio de crescimento 1A
IGF	Fator de crescimento semelhante a insulina
IGF-I	Fator de crescimento semelhante a insulina tipo I
IGF-II	Fator de crescimento semelhante a insulina tipo II
IGFBPs	Proteínas de ligação do fator de crescimento semelhante a insulina
IMS	Ingestão de matéria seca
MPDR	Metionina protegida da degradação ruminal
PAPP-A	Proteína plasmática A associada a prenhez
rbST	Somatotropina recombinante bovina
VLDL	Lipoproteína de baixa densidade

Sumário

1 Introdução

O período do final da gestação e início da lactação, definido como período de transição, compreende as três semanas que antecedem ao parto até três semanas após o parto (Grummer, 1995). Em vacas de alta produção, durante o período de transição as demandas energéticas aumentam muito, e ultrapassam a capacidade de ingestão, fazendo com que entrem em balanço energético negativo (BEN) (Bauman and Currie, 1980). O BEN mais intenso ocorre normalmente dentro de uma a duas semanas após o parto (Grummer, 2007). A intensidade e duração do BEN durante as primeiras semanas pós-parto têm sido correlacionadas com o intervalo entre parto e a primeira ovulação (Lucy et al., 1991, Beam and Butler, 1998). É geralmente aceito que a vaca que retoma a função ovariana rapidamente tem maior fertilidade (Staples et al., 1990, Darwash et al., 1997). No entanto, os fatores que controlam a primeira ovulação pós-parto não foram completamente elucidados (Kawashima et al., 2007).

Durante o período de transição ocorrem alterações nas concentrações circulantes de glicose e hormônios reguladores do metabolismo, incluindo hormônio do crescimento (GH), insulina e fator d\e crescimento semelhante à insulina (IGF-I) (Butler, 2000, Roche et al., 2000, Jorritsma et al., 2003). Logo, diversos fatores metabólicos podem afetar o crescimento folicular e, portanto, o momento da primeira ovulação pós-parto. O desempenho reprodutivo de vacas leiteiras, principalmente a probabilidade de concepção, tem sido associado negativamente com a magnitude e severidade do BEN no inicio da lactação (Butler and Smith, 1989). Isto se torna muito importante quando consideramos que, no período pós-parto recente a vaca encontra-se em BEN e diversos hormônios metabólicos estão alterados na circulação (Butler,2000).

Assim, diferentes estratégias vêm sendo utilizadas para minimizar os efeitos negativos do período de transição sobre o metabolismo e consequentemente sobre

a reprodução. O uso de somatotropina exógena durante o período pré-parto aumenta as concentrações plasmáticas de glicose e insulina no período pós-parto, concomitante com redução da concentração de ácidos graxos não esterificados (AGNE) (Putnam et al., 1999), em função do aumento da ingestão de matéria seca (IMS), estimulada no período pós-parto (Gulay et al., 2004). A aplicação de somatotropina exógena aumenta a produção de leite e altera a relação entre lipogênese e lipólise, através de seus efeitos no tecido adiposo e metabolismo de lipídeos (Etherton and Bauman, 1998). Além disso, sabe-se que tratamentos com somatotropina exógena tiveram um efeito significativo no desenvolvimento folicular ovariano (Gong et al., 1991, Gong et al., 1993) e na função do corpo lúteo (CL) (Lucy et al., 1999).

De mesma forma, estratégias nutricionais e o manejo da alimentação durante o período pré e pós-parto têm um impacto na saúde, produtividade e fertilidade da vaca leiteira. Estudos recentes mostram que a utilização de aminoácidos na dieta durante o período de transição podem ter efeitos benéficos sobre o metabolismo (Osorio et al., 2013, 2014).

A suplementação de metionina protegida da degradação ruminal tem um efeito positivo sobre a síntese de proteína do leite em vacas leiteiras (Pisulewski et al., 1996, Ordway et al., 2009, Osorio et al., 2013). Embora o papel de metionina no desenvolvimento embrionário de bovinos ainda é desconhecido, há evidências de que a disponibilidade de metionina altera o transcriptoma de embriões bovinos *in vivo*(Penagaricano et al., 2013). Além do mais, as reações para metilação do DNA dependem da disponibilidade de grupos metil, que podem ser fornecidos por aminoácidos, tal como a metionina, e por outros compostos de vias metabólicas do carbono, tal como a colina (Van den Veyver, 2002). A colina é um componente importante de fosfolipídios e esfingomielina, um componente de acetilcolina que participa diretamente no processo de neurotransmissão (Zeisel, 1997), e também atua na manutenção da integridade das membranas e nos processos de metilação (Niculescu and Zeisel, 2002, Niculescu et al., 2006). Alguns estudos avaliaram os efeitos da suplementação de colina na forma protegida da degradação ruminal sobre a reprodução e a saúde de vacas leiteiras, indicando resultados promissores (Ardalan et al., 2010, Lima et al., 2012).

Hipótese

A administração de somatotropina durante o período pré-parto ou a suplementação com metionina e colina durante o período pré e pós-parto aumentam a fertilidade de bovinos leiteiros.

Objetivo Geral

Determinar os efeitos da administração de somatotropina durante o período pré-parto ou a suplementação com metionina e colina durante o período pré e pós-parto sobre a fertilidade de bovinos leiteiros.

Objetivos específicos

- Determinar o efeito da administração pré-parto de somatotropina sobre marcadores do BEN, retomada da atividade ovariana e produção leiteira em novilhas da raça Holandês.

- Determinar o efeito da suplementação com metionina e colina durante o período pré e pós-parto sobre o potencial esteroidogênico do folículo dominante da primeira onda folicular pós-parto em vacas da raça Holandês.

- Determinar o efeito da suplementação com metionina e colina durante o período pré e pós-parto sobre o desenvolvimento embrionário e nível de metilação global do DNA dos embriões de vacas da raça Holandês.

2 Revisão da Literatura

2.1 Período de transição e fertilidade

Os mecanismos através dos quais o BEN durante o período de transição tem um efeito sobre o desempenho reprodutivo ainda não são totalmente compreendidos (Chapinal et al., 2012). Sabe-se que envolve a redução nos hormônios metabólicos, como a insulina e IGF-I (Leon et al., 2004) e aumento nos níveis de AGNE e β–hydroxibutirato (BHBA). Além do mais, Leroy et al. (2006) mostraram um efeito direto da toxicidade dos AGNE e BHBA na maturação *in vitro* de oócitos.

Durante o pós-parto recente existe uma grande demanda de glicose pela glândula mamária, o que pode diminuir sua disponibilidade para outros tecidos, incluindo aqueles que estão envolvidos na reprodução (Wathes et al., 2011, Green et al., 2012). Sabe-se que as alterações nas concentrações sanguíneas de glicose são diretamente refletidas no fluido folicular do folículo dominante (Leroy et al., 2004b). Landau et al. (2000) mostraram que uma baixa concentração de glicose intra-folicular coincide com baixa concentração de insulina no fluido folicular, e que as concentrações de ambos parâmetros são influenciadas pela dieta. Os resultados de Stewart et al. (1995) indicam que a disponibilidade da glicose é extremamente importante para a proliferação das células da teca e esteroidogênese, bem como para as células da granulosa para a produção de progesterona em suínos (Veldhuis et al., 1983), e para a produção de estradiol pelo folículo de ratos (Boland et al., 1994).

Logo após o parto, devido ao intenso BEN, as vacas leiteiras têm uma redução drástica na concentração sanguínea de insulina, devido ao direcionamento de glicose pela glândula mamária para a síntese de lactose (Butler, 2003). No ovário, receptores de insulina estão amplamente distribuídos pelos diferentes tipos de células, incluindo células da granulosa, teca e estroma (Poretsky and Kalin, 1987) e luteínicas (Mamluk et al., 1999). A insulina, *in vitro*, aumenta a esteroidogênese em resposta as gonadotrofinas (Stewart et al., 1995, Silva and Price, 2002).

Adicionalmente, baixas concentrações de insulina no sangue induzem a lipólise e elevadas concentrações de AGNE, o que, por sua vez, têm um efeito prejudicial sobre a função das células da granulosa (Vanholder et al., 2005).

Leroy et al. (2004a) observaram que as concentrações de BHBA no soro e no fluido folicular foram similares, concluindo que elevados concentrações de BHBA no soro (cetonemia) refletem na composição do fluido folicular. Além do mais, as concentrações de AGNE no fluido folicular também aumentaram em resposta a elevação dos níveis de AGNE no soro (Leroy et al., 2004a). Estes achados são similares a estudos anteriores com vacas submetidas a uma restrição alimentar aguda simulando um período de BEN (Comin et al., 2002, Jorritsma et al., 2003). Vacas anovulatórias no pós-parto recente apresentaram concentrações plasmáticas elevadas de AGNE e corpos cetônicos (Kawashima et al., 2007b). Além disso, os AGNE podem ser considerados como relativamente tóxicos para o oócito, considerando os resultados de estudos efetuados em ovinos (Herdt et al., 1988).

O desempenho reprodutivo de vacas leiteiras, principalmente a probabilidade de concepção tem sido associado negativamente com a magnitude e severidade do BEN no inicio da lactação. Isto se torna muito importante quando consideramos que no período pós-parto recente a vaca encontra-se em BEN e diversos hormônios metabólicos estão alterados na circulação.

2.2 Somatotropina

A somatotropina bovina foi descoberta em 1920 por Evans e Simpson e, somente na década de 80, conseguiu-se produzir uma somatotropina bovina recombinante (rbST) pela técnica de DNA recombinante (Bauman, 1992). A somatotropina bovina, também conhecida como hormônio do crescimento (GH), é um hormônio peptídico produzido naturalmente pela glândula pituitária (Bauman, 1992), com ação homeorrética, atuando na regulação da partição de nutrientes, sendo administrado para aumentar a produção e melhorar a eficiência na síntese de leite (Bauman, 1999).

O início de lactação está associada a um aumento nas concentrações de GH (Snijders et al., 2000), o que impulsiona o direcionamento de nutrientes, principalmente no fígado e tecido adiposo, para suportar as necessidades da vaca leiteira para a produção de leite (Lucy, 2008). A somatotropina estimula a produção hepática de IGF-I, e a exposição prolongada a concentrações elevadas de somatotropina circulante pode induzir a resistência à insulina (Scaramuzzi et al., 2011). A maior parte do IGF-I sérico é sintetizado no fígado, em resposta ao GH agindo através de seu receptor (Jiang and Lucy, 2001). Sabe-se que um mecanismo semelhante existe no ovário de vacas, assim o GH pode atuar diretamente sobre os folículos ovarianos, estimulando a produção local de IGF-I (Lucy, 2008). O GH controla o desenvolvimento de células da granulosa e teca, além de estimular o início do crescimento de folículos primordiais e auxiliar o desenvolvimento de folículos primários e secundários (Sluczanowska-Glabowska et al., 2012). Silva et al. (2009) sugerem que o GH é um fator de sobrevivência para os folículos primários e regula a diferenciação de células da granulosa. Entretanto, as ações do GH podem ser indiretamente mediadas por ambos, IGF-I e IGF-II, que são sintetizados no ovário (Silva et al., 2009), sendo que o IGF-I é o controlador primário do metabolismo.

As concentrações plasmáticas de IGF-I diminuem a partir de 3 semanas pré-parto até 3 semanas após o parto, apesar das concentrações de GH estarem aumentadas (Meikle et al., 2004, Taylor et al., 2004). Logo após o parto, a drástica redução das concentrações sanguíneas de insulina, devido ao intenso BEN, leva a uma redução na expressão hepática do receptor do GH (GHR), especialmente do GHR-1A (Butler, 2003), que compreende 50% do GHR hepático (Jiang and Lucy, 2001). Como o IGF-I é produzido em resposta a ativação do GHR pelo GH (Jones e Clemmons, 1995), nesta situação há uma dissociação do eixo GH/IGF-I, pois com a redução do GHR há uma síntese hepática e nível circulante de IGF-I (Fenwick et al., 2008).

De fato, o IGF-I derivado do fígado é um fator de regulação da maturação final do folículo dominante durante a primeira onda folicular pós-parto (Beam and Butler, 1998), pois sabe-se que o IGF-I circulante em vacas que ovulam a primeira onda folicular pós-parto é maior do que em vacas anovulatórias (Beam and Butler, 1998, Kawashima et al., 2007a, Kawashima et al., 2007b). Além disso, um estudo

mostrou que o IGF-I parece desempenhar um papel chave no aumento da sensibilidade de pequenos folículos antrais (5 mm de diâmetro) à ação de gonadotrofinas (Scaramuzzi et al., 2011). O IGF-I também desempenha um papel importante na transição dos folículos para a fase folicular dependente de gonadotrofinas (Mazerbourg et al., 2003).

A ação do IGF-I no folículo é modulada por interações complexas com o seu receptor e as várias proteínas de ligação (Scaramuzzi et al., 2011). Em bovinos, as proteínas de ligação do IGF (IGFBPs) têm quatro funções essenciais na regulação das atividades do IGF: (1) atuam como proteínas de transporte no plasma, (2) prolongam a vida media dos IGFs, regulando sua depuração metabólica (3) proporcionam que tecidos e células de tipos específicos controlem a ação do IGF livre e seus efeitos no crescimento tecidual, e (4) diretamente modulam a interação dos IGFs com os seus receptores e, assim, controlam indiretamente a sua bioreatividade (Silva et al., 2009).

Sabe-se que as baixas concentrações das IGBPs em folículos saudáveis na fase de crescimento são causadas pelo aumento das taxas de degradação proteolítica de IGFBP-2, -4 e -5 pela PAPP-A (do inglês pregnancy-associated plasma protein-A) (Mazerbourg et al., 2003). Em novilhas a atividade da PAPP-A foi maior em folículos dominantes do que em folículos subordinados nos dias 2 e 3 da primeira onda folicular (Rivera et al., 2001). Uma redução inicial nos níveis de IGFBPs, principalmente devido à proteólise da IGFBPs pela PAPP-A resultou no aumento da disponibilidade de IGF livre no fluido folicular (Rivera and Fortune, 2003). Embora tenha sido relatado que as concentrações dos IGFs para folículos dominantes e subordinados em bovinos sejam as mesmas (de la Sota et al., 1996, Stewart et al., 1996) outros estudos com experimentos *in vivo* sugerem uma associação positiva entre IGF livre e a seleção para dominância folicular (Beg et al., 2002).

O fornecimento de IGF-I total para o folículo está fora do controle do eixo reprodutivo, portanto, a atividade do IGF intra-folicular é regulada localmente por fatores intra-ovarianos, principalmente pelas IGFBPs (Scaramuzzi et al., 2011). Os hormônios metabólicos exercem diferentes sinais metabólicos que atingem o folículo. Assim, é improvável que exista um único mecanismo ou um mediador

metabólico único que influencie a atividade ovariana no pós-parto recente, provavelmente diversos mecanismos convirjam afetando o folículo.

Hormônios metabólicos, especialmente GH e IGF-I, afetam o eixo reprodutivo através de efeitos diretos nas células ovarianas e também através de seus efeitos sobre as gonadotrofinas, assim o entendimento dos mecanismos de controle entre os hormônios metabólicos e a função ovariana podem levar a uma melhora na atividade ovariana pós-parto.

2.3 Metionina

Alguns aminoácidos (AA) são limitantes para a uma ótima produção leiteira (Socha et al., 2005, Cho et al., 2007, Patton, 2010). Geralmente os primeiros três AA limitantes para a produção leiteira são a metionina, lisina e histidina (Socha et al., 2005). O interesse em proteger a metionina da degradação ruminal remonta a década de 1960 e início dos anos 1970, quando se tornou evidente a partir de ensaios de infusão abomasal, intestinal e intravenosa que o perfil da metionina absorvida não era sempre ótimo em ruminantes (Chalupa, 1975). A metionina protegida da degradação ruminal (MPDR) foi promovida como um produto que aumenta significativamente a porcentagem de proteína do leite (Rulquin and Delaby, 1997, Socha et al., 2005), entretanto, há alguns resultados contraditórios (Papas et al., 1984, Davidson et al., 2008).

Um aumento da porcentagem de proteína do leite como resposta a alimentação com MPDR resultou em um aumento significativo no rendimento da proteína do leite em alguns estudos (Armentano et al., 1997, Rulquin and Delaby, 1997, Samuelson et al., 2001), sendo negativo em outro estudo (Benefield et al., 2009). Além disso, alguns estudos têm relatado o aumento da porcentagem de gordura no leite (Overton et al., 1998, Samuelson et al., 2001), bem como aumento na produção de gordura do leite (Overton et al., 1996, Schmidt et al., 1999, Krober et al., 2000). Porém, outros estudos não encontraram um aumento significativo na produção de gordura do leite (Chilliard and Doreau, 1997, Socha et al., 2005). Samuelson et al. (2001) relataram um aumento da produção de leite, enquanto Chilliard e Doreau, (1997), relataram que a produção de leite diminuiu significativamente em resposta a suplementação com MPDR.

A metionina desempenha um papel direto na síntese de lipoproteína de baixa densidade (VLDL) em bovinos (Auboiron et al., 1995), diminuindo os efeitos negativos da acumulação de ácidos graxos no fígado (Drackley, 1999). Além disso, o aumento da biodisponibilidade de metionina em vacas suplementadas com MPDR (Graulet et al., 2005) tende a aumentar a entrada de metionina no ciclo do metabolismo de 1-carbono, onde inicialmente pode ser convertida a s-adenosilmetionina (SAM), a molécula biológica mais importante em termos de doação de grupamentos metil (Martinov et al., 2010). A disponibilidade de SAM é essencial para ao mecanismo de metilação do DNA, um processo biológico importante para a regulação da expressão gênica (Li and Zhang, 2014).

Numerosos estudos têm avaliado os efeitos de AA protegidos, particularmente metionina, na produção de leite. Infelizmente, existem poucos estudos na literatura científica que foram especificamente concebidos e adequadamente desenhados para avaliar os efeitos de aminoácidos específicos sobre a eficiência reprodutiva de vacas leiteiras em lactação. O maior estudo (Polan et al., 1991) foi um combinado dos resultados de 259 vacas, de 6 Universidades diferentes, avaliando a suplementação de metionina e lisina protegidas da degradação ruminal, onde não foi detectado efeito significativo sobre dias para o primeiro serviço, serviços por concepção, ou intervalo entre partos.

Souza et al., (2012), em um estudo *in vitro*, avaliaram um total de 570 embriões e não encontraram diferenças na fertilização, qualidade ou desenvolvimento do embrião precoce, pelo menos do ponto de vista morfológico. Entretanto, Ikeda et al., (2012) observaram que a peturbação do metabolismo da metionina induzida pelo tratamento com etionina prejudicou a transição de mórula para blastocisto em embriões bovinos. Além disso, é bem sabido que a metionina durante a maturação oocitária e desenvolvimento embrionário inicial pode ter efeitos drásticos sobre o epigenoma do embrião (Sinclair et al., 2007). Alguns dos efeitos sobre o desenvolvimento do embrião podem não se manifestar em aparência morfológica, mas resultam em diferenças drásticas na expressão de genes, tal como observado no estudo utilizando RNASeq em embriões produzidos em vacas que foram suplementadas com metionina (Penagaricano et al., 2013).

2.4 Colina

A colina é uma amina quaternária trimetilada que é crucial para o cérebro e sinalização neuromuscular (Hartwell et al., 2000). A colina foi classificada como um das vitaminas do complexo B, mas não satisfaz a definição padrão de uma vitamina pois é sintetizada endogenamente e não há provas de que é uma enzima. Além disso, ao contrário de outras vitaminas solúveis em água, é difícil identificar os efeitos de uma deficiência de colina em mamíferos saudáveis, devido a sua inter-relação com a metionina, ácido fólico, e vitamina B12 (Pinotti et al., 2005). É bem estabelecido que a colina é um nutriente essencial para mamíferos quando não há suficiente metionina e folato disponível na dieta (Zeisel et al., 1991, Zeisel, 2000).

A colina é um componente chave para a síntese de duas importantes moléculas, a fosfatidilcolina (FC) e a acetilcolina (Pinotti et al., 2002). FC é essencial para manter a membrana estrutural das células (Davidson et al., 2008), a síntese hepática e a secreção de lipoproteínas de baixa densidade (VLDL) (Cooke et al., 2007). Quando a FC é limitante, a suplementação com colina pode melhorar a taxa de síntese de VLDL e assim, evitar a acumulação excessiva de triacilglicerol (TAG) no fígado (Grummer, 2008).

Os primeiros estudos que avaliaram o efeito da suplementação dietética de colina sobre a produção de leite, avaliaram também o fluxo duodenal e indicaram rápida e extensa degradação ruminal (Atkins et al., 1988). Com isso, há necessidade de proteção contra a degradação no rúmen para poder estar disponível e ser absorvida no duodeno (Sharma and Erdman, 1988). Posteriormente, numerosos estudos avaliaram os efeitos da alimentação de colina protegida da degradação ruminal (CPDR) sobre a produção, reprodução e saúde das vacas leiteiras, e assim como os estudos com metionina, os resultados não são consistentes.

Alguns estudos demonstraram um efeito positivo da suplementação de CPDR aumentando a produção de leite (Pinotti et al., 2003), produção de proteína do leite (Zom et al., 2011), redução do acúmulo de gordura no fígado (Cooke et al., 2007, Zom et al., 2011) e aumento da ingestão de matéria seca (Zahra et al., 2006). Entretanto, Deuchler et al. (1998) não observaram efeitos positivos na produção e gordura do leite. Piepenbrink and Overton (2003) descobriram que a suplementação

com CPDR reduziu a esterificação de ácidos graxos, sem alterar oxidação, sugerindo assim um aumento da secreção de lipoproteínas, o que poderia reduzir os riscos relacionados a distúrbios metabólicos dos lipídios e melhorar a fertilidade (Walsh et al., 2007).

A maioria dos estudos de investigação têm centrado seu interesse em avaliar os efeitos da CPDR sobre os parâmetros de produtividade, estado metabólico (Guretzky et al., 2006, Zahra et al., 2006), e mobilização de gorduras (Cooke et al., 2007, Goselink et al., 2013). Poucos estudos têm dedicado o seu interesse nos possível efeitos da CPDR sobre a melhora de parâmetros reprodutivos e incidência de doenças metabólicas (Ardalan et al., 2010, Lima et al., 2012, Bisinotto et al ., 2012). Suplementar CPDR não afetou a retomada da ciclicidade ovariana pós-parto, prenhez por IA na primeira e segunda inseminações ou de manutenção da prenhez nos primeiros 60 dias de gestação (Bisinotto er al ., 2012).

3 Artigos

3.1 Artigo 1

Effect of somatotropin injection in late pregnant Holstein heifers on metabolic parameters and esteroidogenic potential of the first postpartum dominant follicle

Diego Andres Velasco Acosta, Augusto Schneider, Carolina Bespalhok Jacometo, Joao Alvarado Rincon, Felipe Cardoso, Marcio Nunes Corrêa

Submetido à revista Theriogenology

Effect of somatotropin injection in late pregnant Holstein heifers on metabolic parameters and esteroidogenic potential of the first postpartum dominant follicle

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ABSTRACT

The aim of this study was to determine the effect of pre-partum injections of somatotropin in dairy heifers on metabolic markers and esteroidogenic potential of the first postpartum dominant follicle. Fifty-nine late pregnant Holstein heifers from a commercial dairy herd in southern Brazil were used. Heifers were randomly assigned to two treatments: somatotropin treated (**ST**; n = 29), that received two doses of somatotropin (500 mg/dose) at -28 and -14 days relative to calving; and control (**CTL**; n = 30), that did not received somatotropin. Follicular development was monitored via ultrasound every 3 days starting at 8 days in milk (DIM) until the day the first dominant follicle reached a diameter of 16 mm. Follicular fluid from eighteen cows (ST; n = 8 and CTL; n = 10) was aspirated and the follicular cells were collected to evaluated gene expression of *LHCGR, STAR, 3β-HSD, P450scc, P450c17, IGFr* and *CYP19A1*, and intrafollicular concentrations of NEFA, IGF-I, progesterone (P4) and estradiol (E2). Blood samples were collected weekly since -28 from expected calving to 28 ± 2 DIM to analyze NEFA, IGF-I, BUN, P4 and E2. Follicular fluid IGF-I concentrations of the first postpartum dominant follicle was higher (P=0.05) in ST (87.09 ± 7.73 mg/mL) than CTL (64.26 ± 6.84 mg/mL) treated heifers. The expression of *LHCGR* and *STAR* mRNA in follicular cells was higher (P<0.05) in ST than CTL. Nonetheless, 3β-*HSD, P450scc, P450c17, IGFr* and *CYP19A1* mRNA expression did not change between treatments (P>0.05). Serum IGF-I concentration was higher in ST treated heifers during the pre-partum period (P<0.05) and no difference was observed in the postpartum period (P>0.05). Concentrations of NEFA, BHBA and BUN did not change between treatments (P>0.05). In conclusion, prepartum somatotropin treatment in dairy heifers increased intrafollicular IGF-I and expression of *LHCGR* and *STAR* mRNA in follicular cells of the first postpartum dominant follicle. These changes were associated to increased intrafollicular and serum E2 concentration, which can potentially increase the chance of ovulation of the first postpartum

follicular wave.

Key words: IGF-I, LH receptor, follicular cells.

1. INTRODUCTION

The transition from pregnancy to lactation is critical to define the reproductive performance of a dairy cow [1]. The resumption of ovarian activity and conception in a maximum of 100 days after calving are critical steps to determine the profitability of a dairy production system [2]. During the early lactation in dairy cows the energy demands increase and exceed the intake capacity resulting in a period of negative energy balance (NEB) [3, 4]. The extension and intensity of the NEB is negatively associated with reproductive performance. Cows that experience a more intense NEB have a delayed return of postpartum ovarian activity and, consequently, take longer to conceive [1]. In this sense, the more estrous cycles before the time of first insemination, the higher the probability of pregnancy [5-7]. Therefore, the use of strategies that anticipate the time to first ovulation may also benefit the moment of conception.

Several strategies have been used to improve the metabolic condition of cows in the transition period and thus the reproductive performance. Administration of low doses of somatotropin for cows in the periparturient period have been associated with beneficial effects on the physiological adaptation and liver function [8, 9]. Somatotropin regulates nutrient partitioning, and is traditionally used to increase milk production and improve the efficiency of milk synthesis in the postpartum period [10], as well as indirect effects expressed in the mammary gland and other tissues that are mediated by insulin-like growth factor I (IGF-I) [11]. In this regard, somatotropin injection during the peripartum period is able to increase serum IGF-I concentration, milk production [12], dry matter intake, glucose and reduce the concentration of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) at the

postpartum period [13]. This indicates that somatotropin can improve metabolic adaptation during the transition period in dairy cows.

Most positive effects of somatotropin are exerted by IGF-I [11]. Increased plasma concentration of IGF-I is beneficial for follicle development, since it acts as a modulator of gonadotropin action in the ovary, stimulating granulosa and theca cell proliferation and differentiation [14] and preventing follicular apoptosis [15]. Dairy cows that ovulated the first postpartum dominant follicle have higher serum IGF-I concentration in the pre and postpartum period [16]. The number of recruited follicles is increased in dry cows or heifers treated with somatrotopin [17-19] and this effect persisted for at least 21 days after termination of treatment [19]. Moreover prepartum somatotropin treatment increases the ovulation of the first postpartum dominant follicle [20], although the mechanism is not yet fully understood.

Based on these observations, the aim of this study was to determine the effect of pre-partum injections of somatotropin on serum metabolic markers, esteroidogenic potential of the first postpartum dominant follicle and milk production in Holstein dairy heifers.

2. MATERIALS AND METHODS

All procedures performed in this experiment were approved by the Committee for Ethics in Animal Experimentation from the Universidade Federal de Pelotas (Pelotas, RS, Brazil).

2.1 Location and experimental design

For this study 59 late pregnant Holstein heifers from a commercial dairy herd in southern Brazil (32º 16’ S, 52º 32’ W) were used. The heifers had a mean body condition score (BCS) of 2.9 ± 0.3 (ranging from 2.5 to 4.0) at the beginning of the experiment. All calving occurred in a 40 days interval during the winter season. Heifers were randomly

assigned to two treatments: Somatotropin treatment (ST; n = 29) that received two doses of somatotropin (500 mg/cow sc, of Lactotropin®, Elanco, Sao Paulo, Brazil) at −28 and −14 days relative to calving, and Control (CTL; n = 30) that did not received somatotropin application. All heifers were managed under the same conditions and nutritional regimen (pasture-based system, Table 1).

2.2 Reproductive management, ultrasonographic evaluations and follicular aspiration

Transrectal ultrasonography examination was performed using a 7.5-MHz linear array probe (Welld® Wed-3000V, Shenzhen, Guangdong, China) every 3 days from 8 days in milk (DIM) up to the day that the dominant follicle (DF) reached a diameter of 16 mm (which is the average diameter of the follicle to ovulate during the first postpartum wave in Holstein cows, [16, 21]. In that point follicular fluid from eighteen cows (ST; n = 8 and CTL; n = 10) was aspirated. The DF was aspirated by ultrasound-guided transvaginal follicular aspiration. The area above the first intercoccygeal space was clipped and disinfected with an iodine scrub solution and 70% ethanol. Lidocaine (5 mL, 2% lidocaine hydrochloride solution) was injected into the first intercoccygeal space and time was allowed for the anesthesia to take effect. The vulva and perianal area were cleaned and disinfected with iodine scrub solution.

An ultrasound scanner (Welld Wed-3000V) equipped with a 7.5-MHz probe was used for the follicle aspiration procedure. The ultrasound probe was enclosed within a custom-made handle. The handle enclosed the probe cord and fixed the head of the probe at a 30° angle relative to the needle guide. The needle guide ended at the base of the probe, just above the probe head. The probe and handle were lubricated with a sterile water-based lubricant and positioned in the vagina slightly posterior to the cervix. The ovary containing the DF was manipulated toward the ultrasound probe to be inspected and measured before aspiration. An 18-gauge aspiration needle was passed through the needle guide of the handle and then through the vagina. The aspiration needle was guided through the stroma of the ovary and into

the DF and the contents were aspirated with a 10 mL syringe. The follicular fluid was retrieved and frozen at −80°C for subsequent analysis. The follicular cells were immediately pelleted by centrifugation, and the follicular fluid was decanted. The cells were homogenized with Trizol reagent (Life Technologies, Inc., Grand Island, NY, USA), snap frozen in liquid nitrogen and stored at −80°C. Only estrogen active follicles (ST n=6 and CTL n=8), selected based on estradiol (E2):progesterone (P4) ratio higher than 1 in the follicular fluid [22], were used for gene expression and follicular fluid content analysis.

2.3 Hormones and metabolites analyses

Blood samples were collected weekly since 28 days before expected calving until 28 ± 2 DIM via venipuncture of the coccigeal vein in one tube with and without anticoagulant. Samples were collected in the interval after milking and before feeding. Samples for plasma were placed on ice immediately after collection. Samples for serum were allowed to clot at room temperature for at least 20 min and then placed on ice. All tubes were centrifuged, within 2 h of collection, at 4°C for 15 min at 1000 x g. After centrifugation, aliquots of plasma and serum were kept at −80°C until later analysis. NEFA concentration was determined with a commercially available enzymatic-colorimetric kit (NEFA, Wako Chemicals USA Inc., Richmond, VA, USA) by the micro-method described by Ballou, [23]. Plasma urea concentration (BUN) was measured with QuantiChrom Urea assay kit DIUR 500 (BioAssay Systems, Hayward, CA, USA). The intra and inter-assay CV for NEFA and BUN were less than 10%.

Serum and follicular fluid concentrations of IGF-I were determined using a competitive, liquid-liquid phase, double-antibody IGF-I radioimmunoassay procedure as described previously by Lalman, [24]. Serum samples were thawed, mixed thoroughly, and 10 µL of serum sample pipetted into individual wells of a 96 deep-well plate. Immediately after, 400 µL of 1M glycine (pH 3.2) was added to acidify each sample followed by the addition of

500 µL of PABET+P (consisting of 0.1% gelatin, 0.01 M EDTA, 0.9% NaCl, 0.01 M PO_4, 0.01% sodium azide, 0.05% Tween-20, 0.02% Protamine SO_4, pH = 7.5). The acidified-diluted aliquots were then individually sealed within each well and incubated at 37°C for 48 h. Thereafter, samples were neutralized by addition of 90 µL of 0.5M NaOH before being submitted to the IGF-I assay. IGF-I assay procedures were adapted from those described by Holland, [25]. Recombinant human IGF-I was used for iodination and standards (UBI-01-141, Amgen Corp., Thousand Oaks, CA, USA). Antiserum (UB3-189) was provided by the National Hormone and Pituitary Program and used at a final assay tube dilution of 1:10,000. Sample (40 µL of the acidified-diluted sample; in triplicate determinations), antisera, and PABET+P were combined (total volume balanced to 300 µL with PABET+P) and incubated at 4°C for 24 h. ^{125}I-IGF-I (^{125}I-IGF-I; 25,000 cpm) was then added and incubation continued at 4°C for an additional 16h. The antigen-antibody complex was then precipitated following a 15 min, 22°C incubation with 100 µL of a precipitated sheep-anti-rabbit second antiserum, by centrifugation at 3,000 × *g* for 30 min, and the supernatant discarded by aspiration. Assay tubes containing the precipitated antigen-1° antibody complex were counted for 1 min on a LKB1277 gamma counter (LKB Wallac, Turku, Finland). The inter- and intra-assay CV for IGF-I were < 6%.

The serum and intrafollicular concentration of P4 was determined using a commercial radio immuno assay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, USA) as previously described by Burke, [26]. All progesterone analyses were performed in a single batch and the intra-assay CV was less than 10%. Heifers with P4 concentrations in plasma above 1 ng/mL in two consecutive samples were considered as having resumed ovarian activity [27]. Concentrations of E2 in serum and follicular fluid were measured using the procedures reported by Rozell and Keisler [28] and later described by Kirby, [19] with the substitution of a second antibody precipitation procedure in place of the charcoal extraction

procedure. The E2 assay was sensitive to 0.5 pg/mL, and had an intra-assay CV of 9% and an inter-assay CV of 11%.

2.4 Milk production, body condition score and health

In the postpartum period heifers were milked twice daily at 0330 and 1530. Milk production was recorded daily (Alpro®, DeLaval, Kansas City, USA) and combined for weekly averages. The BCS was evaluated by the method described by Ferguson, [29]. Two observers independently assigned a body condition score (five-point scale, 0.5 increments) and described the appearance of seven body regions of heifers. Areas described were the thurl region, ischial and ileal tuberosities, ilio-sacral and ischio-coccygeal ligaments, transverse processes of the lumbar vertebrae, and spinous processes of the lumbar vertebrae. An absolute BCS was designated for each heifers based on the mean score of the two evaluators.

All heifers were observed daily during the first 28 DIM. Incidence of specific diseases (fetal membranes retention, metritis and mastitis) were recorded on the day of diagnosis and placed in the herd health records. From these records, the incidence rates (number of diseased heifers divided by the total number of heifers in each group) are reported for the two groups.

2.5 Real-time PCR

Total RNA was extracted using Trizol reagent (Life Technologies, Inc., Grand Island, NY, USA) according to the manufacturer's instructions. Integrity of the extracted RNA was determined by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Only RNA with intact 18S and 28S bands was used. Total RNA was treated with DNAse I (DNAse Amp Grade, Invitrogen®, Carlsbad, USA) to remove genomic DNA contamination and primed with oligo dT to synthesize single strand cDNA (SuperScript III First-Strand Synthesis Supermix, Invitrogen®, Carlsbad, USA). The PCR amplifications and fluorescence detection were performed in duplicate in the ECORealTimePCR System (Ilumina Inc., San Diego, CA USA), using the SYBR Green detection chemistry (Power SYBR Green, Life

Technologies, Carlsbad, USA), as recommended by the manufacturer. The PCR parameters were 5 min at 50ºC and 10 min at 95ºC followed by 40 cycles of 95ºC for 30 s and 60ºC and 72ºC for 1 min each. Primers are showed in Table 1 and *H2AFZ* expression was used as an internal control. For each sample, a mean cycle threshold of the duplicate PCR reactions was calculated. In addition, the expression of each target gene of interest was calculated relative to *H2AFZ* using the equation: relative target gene expression = (1/Etarget^CTtarget)/(1/E-*H2AFZ*^CT-*H2AFZ*), where E was the reaction efficiency and CT was the cycle threshold [30]. The mean coefficient of variation among sample CT's was 1.2%. The specificity of each primer was verified by the detection of only one fluorescence peak at the dissociation curve for each replicate at the end of the PCR.

2.6 Statistical analyses

The results are presented as means ± standard error of the mean. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Analyses involving repeated measures over time (e.g. NEFA, BUN, IGF-I BCS, milk production and serum E2 concentration relative to the moment of the aspiration) were compared between treatments by analysis of variance for repeated measures using the Proc MIXED, fixed effects were treatment (Trt) (somatotropin, control), time (day or week), and interaction (Trt*time). Two separated models were generated for the prepartum and postpartum periods. The random effects included heifer. The Shapiro–Wilk test was used to test for normal distribution of the data, and most of the parameters showed normal distribution. A logarithmic transformation was used for the NEFA, IGF-I and BUN concentration for better homogeneity of the residuals distribution. Means shown in table for these variables are back transformed. Percentage of cows ovulating before 30 days and percentage of heifers that had a disease were analyzed by chi-square. Gene expression results were log2-scale transformed if needed to comply with

normal distribution of residuals. Least squares means separation was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

3. RESULTS

There was observed a treatment by day interaction for serum NEFA concentrations during the pre-partum period (Trt*day, P<0.01) with an overall increasing closer to calving (P<0.01) but no effect of the treatment was observed (P=0.17). In the postpartum period there was only a reduction in serum NEFA over time (P<0.01). Pre-partum serum IGF-I concentration had an effect for interaction (Trt*day, P<0.01), day (P<0.01) and treatment (P=0.01), being higher for ST (112.78±3.95 mg/mL) than CTL (97.37±4.61mg/mL) heifers. In the postpartum period there was only a time effect, with lower serum IGF-I concentrations closer to calving (P<0.01; Table 3). A treatment by day interaction effect for serum BUN concentration during the pre-partum period was observed (Trt*day, P=0.01) with an overall decreasing closer to calving (P<0.01) but no difference between Trt (P=0.85). In the postpartum period there was only observed a serum BUN reduction with time (P=0.03; Table 3).

The IGF-I concentration in the follicular fluid of the first postpartum dominant follicle was higher (P=0.05) for ST (87.09 ± 7.73 mg/mL) than CTL (64.26 ± 6.84 mg/mL; Figure 1) treated heifers. Follicular fluid NEFA concentration was not different (P=0.69) between ST (0.71 ± 0.30 mEq/L) and CTL heifers (0.86 ± 0.33 mEq/L; Figure 1). Intrafollicular P4 concentration was also not different (P=0.35) between ST (24.9 ± 12.5 ng/mL) and CTL heifers (32.67± 11.9 ng/mL; Figure 1). However, E2 concentration in the follicular fluid was higher (P=0.02) for ST (199.74 ± 55.95 ng/mL) than CTL heifers (74.46 ± 37.73 ng/mL; Figure 1). Heifers were assigned to the presumed ovulatory group if circulating estradiol was ≥ 2 ng/mL at least one day before aspiration and these heifers had a dominant follicle > 10 mm in diameter (CTL n=8; ST n=6) Cheong et al. [21]. In addition, heifers that had follicles

that reached 16 mm in diameter, but did not have a rise in circulating estradiol were considered as non-ovulatory and were not used in subsequent gene expression analyses (CTL n=2; ST n=2; Figure 2). Serum E2 concentration relative to the moment of the aspiration was higher (P=0.02; Figure 2) for ST ovulatory group (3.68 ± 0.27 ng/mL) than CTL ovulatory group (2.57 ± 0.20 ng/mL). Follicular fluid E2:P4 ratio was higher (P=0.03) for ST (6.04 ± 1.24) than CTL heifers (2.18 ± 0.68). Despite the differences in serum and intrafollicular E2 concentrations, the resumption of ovarian activity in the first 3 weeks postpartum was not different (P=0.30) between ST (76.5%; 13/17) and CTL (66.6%; 10/15) heifers.

Expression of *LHCGR, STAR, 3β-HSD, P450scc, IGFr* and *CYP19A1* mRNA in follicular cells is presented in Figure 3. *LHCGR* and *STAR* mRNA expression was higher in ST (8.06 ± 0.22, 7.52 ± 0.40 respectively) than in CTL (1.00 ± 0.14, 1.00 ± 0.13 respectively) ovulatory groups (P<0.05). Expression of *3β-HSD, P450scc,* and *CYP19A1* mRNA was not different between groups (P>0.05).

Milk production was not different (Trt: P=0.50; Trt*week: P=0.97) between CTL (18.20 ± 0.55 kg/day) and ST treatments (17.68 ± 0.53 kg/day). There was only an effect of the week (P<0.01) for milk production, with increasing production over time. There was a pre-partum decrease (*P*<0.01) in BCS for all cows, being higher at week -4 when compared with -1 week and there was a postpartum increase (P = 0.01) in BCS for all cows, being lower at week 1 that week 4. However, no treatment effect (P>0.05) on BCS was observed between CTL and ST treatments for pre and postpartum period (Table 3).

4. DISCUSSION

Somatotropin injection during the peripartum period is able to increase serum IGF-I concentration as observed in the current study and previously by others [12, 20]. In the ovary, there is considerable evidence that GH, as well as systemic and locally produced IGF-I, can modulate the final stages of follicular development [31]. In addition to that, the earliest stages

of follicular development in cows are regulated, in part, by concentration of IGF in follicular microenvironment [32]. In the present study, we found that pre-partum somatotropin treatment increased the concentration of IGF-I and E2 in the follicular fluid and the expression of LH receptor and *STAR* mRNA in granulosa cells of the fist postpartum dominant follicle.

Studies *in vitro* have shown that IGF-I stimulates steroidogenesis by thecal cells and the proliferation and differentiation of granulosa cells [33, 34]. The secretion of E2 by granulosa cells in culture is also stimulated by IGF-I [35]. In our study we found a higher concentration of IGF-I and E2 in the follicular fluid, in addition to higher *LHCGR* and *STAR* mRNA expression on follicular cells of cows treated pre-partum with somatotropin. These changes persisted even so IGF-I concentration was higher in the pre-partum period, but not in the postpartum period. Britt, [36] hypothesized that the developmental competence of the oocyte and the steroidogenic capacity of the follicle, in high yielding dairy cows, is determined by their biochemical environment during the long period (up to 80 days) of follicular growth prior to ovulation. It was also previously demonstrated that somatotropin injection can have a residual effect in follicular development [19]. Therefore, there may be a connection between higher pre-partum levels of IGF-I and increased intrafollicular IGF-I at the first postpartum follicular wave. As mentioned before, intrafollicular IGF-I has a positive effect on steroidogenesis [37-40]. In agreement with that, in our study the higher levels of IGF-I were associated to increased intrafollicular E2. Furthermore, E2 can induce follicle-stimulating hormone/LH receptor expression in granulosa cells [41] as was also observed in our study for somatotropin treated cows. *STAR* expression is hormonally and developmentally regulated [42, 43] and its transcription can be up regulated by IGF-I [44, 45], which also confirms our observation and the feed-back loop between IGF-I, estradiol, LH receptor and *STAR mRNA* expression. The higher serum and intrafollicular concentration of E2 and the

presence of more LH receptors can be beneficial for improving the chance of ovulation of the first postpartum dominant follicle, as demonstrated before [46, 47] and can be related to the increased ovulation rate observed for prepartum somatotropin heifers described by Schneider, [20]. We did not observed improvement of ovulation rates in the present study as previously reported [20], which can be related to the small number of heifers used in the current study.

Although a significant increase in NEFA concentrations was observed in the first days after calving, no difference between groups was observed, similar to the previously reported [8, 48]. The effect of pre-partum somatotropin injection on NEFA and BHBA concentrations postpartum is controversial, and while some report a positive effect [13, 20], others found no relationships [48, 49]. In accordance to that no difference between treatments was observed for BCS, although previous studies had indicated that treated cows had a better postpartum BCS recovery [8, 20]. Cows with high milk production have more energy demand and mobilize more body fat depots to overcome the greater energy requirement, which exacerbates the changes in metabolic and hormonal factors during early lactation [50, 51]. The concentration of NEFA and BHBA are commonly used as indices of NEB and ketosis in transition dairy cows [52]. In this study there was no significant increase in NEFA, which indicate that these cows were not in a severe NEB, which could explain the lack of effect of the pre-partum somatotropin on milk production when compared to previous studies [13, 20]. However, it also indicates that the positive changes observed in the follicular development occurred independent of any changes at overall metabolic level (BUN, BHBA, NEFA, BCS, milk production) and can point to a direct effect of prepartum IGF-I concentration on follicular development. However, different research strategies are necessary to establish the effect of IGF-I without the interference of the transition cow metabolic adaptations.

In addition to these dramatic physiological changes, the periparturient transition period, is critical in determining welfare and profitability of individual cows during the subsequent

lactation [53]. Somatotropin orcheastrates many physiological processes that may benefit the transition dairy cow and indirect effects expressed in mammary gland and other tissues can mediated by IGF-I [11]. It also may have the potential to reduce mastitis incidence in ruminants, as indicated by the reduction in somatic cells count (SCC) [9, 20]. However, in the present study we did not observed an effect of treatment for retained fetal membranes or clinical mastitis. Other studies did not observed differences between treatments for retained fetal membranes [9, 20], in accordance with our study.

In conclusion, prepartum somatotropin treatment in dairy heifers increased intrafollicular IGF-I and expression of *LHCGR* and *STAR* mRNA in follicular cells of the first postpartum dominant follicle. These changes were associated to increased intrafollicular and serum E2 concentration, which can potentially increase the chance of ovulation of the first postpartum follicular wave. Despite that, somatotropin did not affect other metabolic and productive parameters in the early postpartum period.

ACKNOWLEDGEMENTS

The authors thank to Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho de Desenvolvimento Cientifico e Tegnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG) for the financial support. Appreciation is also extended to Granjas 4 Irmãos S/A, which provided animals and farm facilities.

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Table 1. Ingredient composition of pre-partum and postpartum diets.

Ingredients	Pre-partum		Postpartum
	-60 days to -30 days for expected calving	-30 to 0 days for expected calving	0 to 30 days in milk
Rice straw	*Ad libitum*	*Ad libitum*	-
Native pasture	*Ad libitum*	*Ad libitum*	-
Ryegrass pasture	-	-	*Ad libitum*
White clover pasture	-	-	*Ad libitum*
Sorghum Silage, kg	-	10.00	10.00
Items		3 kg	10 kg
Soybean meal, %	-	23.2	23.2
Rice Bran, %	-	9.27	9.27
Corn Bran, %	-	9.7	9.7
Soybean hulls, %	-	48.5	48.5
Mineral BCA[1], %	-	4.73	-
Mineral NNBI + MB[2], %	-	1.7	1.7
Calcitic limestone, %	-	2.85	2.85

[1]Mineral and vitamin mix (BCA PREPARTO, Tortuga, Mairinque, SP, Brazil) was formulated with Ca, 55g/kg, Cl, 89 g/kg, S, 160 g/kg, Mg, 45 g/kg, N, 50 g/kg, Cu, 400 g/kg, Cr, 16 g/kg, vitamin A, 50000 UI/kg, vitamin D3, 10000 UI/kg, vitamin E, 4000 UI/kg.

[2] Mineral and vitamin mix (NOVO BOVIGOLD, Tortuga, Mairinque, SP, Brazil) was formulated with Ca^{+}, 190g/kg, P, 60 g/kg, S, 20 g/kg, Mg, 20 g/kg, K, 35 g/kg, Na, 70 g/kg, Co, 15 mg/kg, Cu, 700 mg/kg, Cr, 10 mg/kg, Fe, 700 mg/kg, I, 40 mg/kg, Mn, 1600 mg/kg, Se, 19 mg/kg, Zn, 2500 mg/kg, vitamin A, 200000 UI/kg, vitamin D3, 50000 UI/kg, vitamin E, 1500 UI/kg, F, 600mg/kg.

Table 2. Primer pairs used in real time PCR reactions

Genes	Forward primer 5' to 3'	Reverse primer 5' to 3'	Reference
Gonadotropin receptor			
LHCGR	TGACTATGGTTTCTGCTTACCCAA	CCATAATGTCTTCACAGGGATTGA	*Spicer et al. [54]*
Progesterone production			
STAR	AGAACCCAAGGATCAGGTTGTC	TGGTAGAGGAGCAGAGGGTTGT	*Spicer et al.[54]*
P450scc	CTTCATCCCACTGCTGAATCC	GGTGATGGACTCAAAGGCAAA	*Tajima et al. [55]*
3B-HSD	CCAAGCAGAAAACCAAGGAG	ATGTCCACGTTCCCATCATT	*Nishimura et al. [56]*
Estrogen production			
CYP19A1	TGCCAAGAATGTTCCTTACAGGTA	CACCATGGCGATGTACTTTCC	*Spicer et al.[54]*
Growth-promoting			
IGFr	TGACTATGGTTTCTGCTTACCCAA	CCATAATGTCTTCACAGGGATTGA	*Spicer et al. [54]*
Internal control			
H2AFZ	GAGGAGCTGAACAAGCTGTTG	TTGTGGTGGCTCTCAGTCTTC	*Portela et al. [57]*

Table 3. Blood metabolic markers and body condition score (BCS) in control heifers (no treatment) and heifers treated with somatotropin during the prepartum period.

Variable		Treatment		SEM	P-value[1]		
		Control	Somatotropin		Trt*Time[2]	Time[2]	Trt
IGF-I (ng/ml)	Prepartum	97.38[a]	112.78[b]	4.62	<0.01	<0.01	0.01
	Postpartum	89.53	94.19	3.21	0.45	<0.01	0.19
NEFA (mEq/L)	Prepartum	0.49	0.6	0.02	<0.01	<0.01	0.17
	Postpartum	0.55	0.37	0.03	0.75	<0.01	0.61
BUN (mg/dL)	Prepartum	30.12	31.15	1.40	0.01	<0.01	0.85
	Postpartum	21.14	22.96	0.98	0.63	<0.01	0.40
BCS (1-5 scale)	Prepartum	2.80	2.77	0.10	0.30	<0.01	0.51
	Postpartum	2.52	2.37	0.11	0.21	<0.01	0.49

[a,b,] Differences (P<0.05) between treatment

[1]*P* value for treatment (Trt), time, or their interaction (Trt *Time) in the pre and postpartum separately

[2] The effect of time was measured in days for IGF-I, NEFA and BUN, and in weeks for BCS.

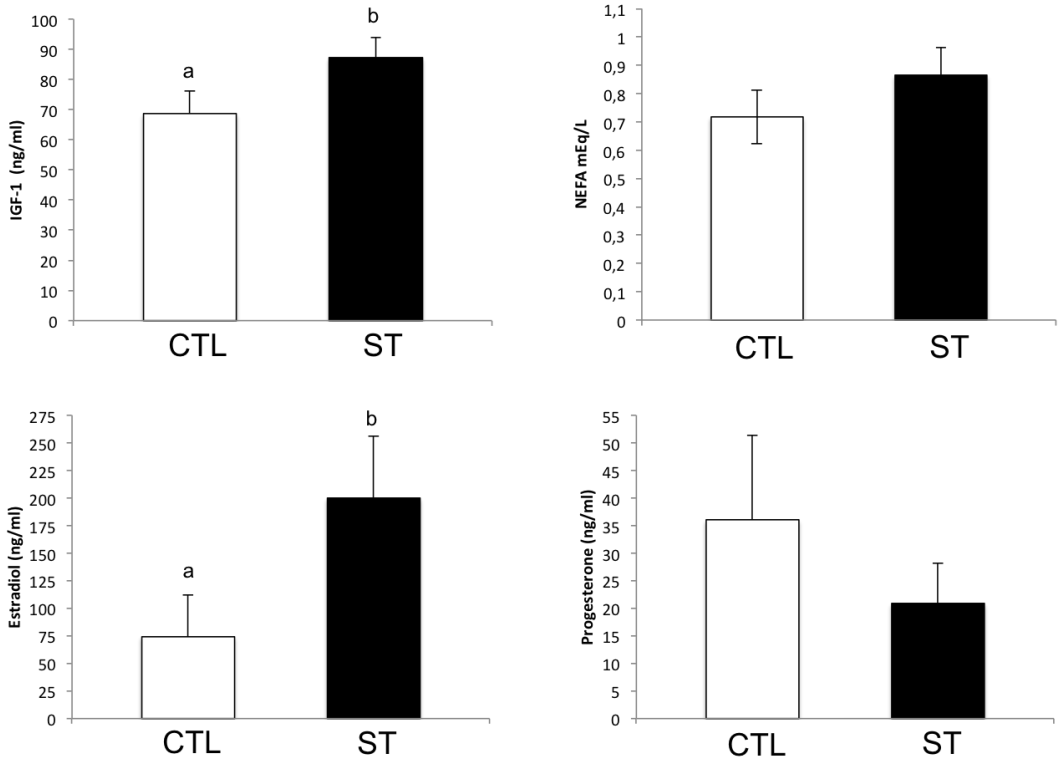

Figure 1. Insulin-like growth factor type I (IGF-I), non-esterified fatty acids (NEFA), estradiol and progesterone concentration in the follicular fluid of the first postpartum dominant follicle for control (white bar, n=8) and somatotropin (black bar, n=6) heifers. All values are shown as mean ± SEM. Values with different letters (a,b) indicate difference between groups ($P<0.05$).

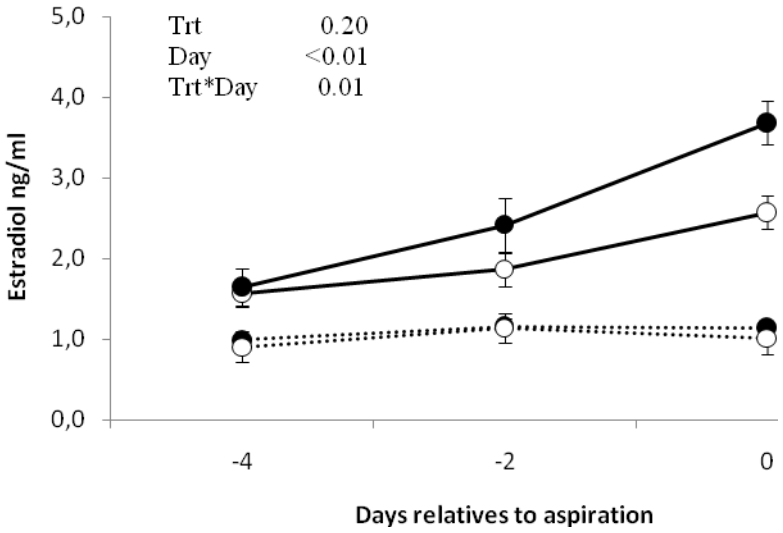

Figure 2. Serum estradiol concentrations from -4 to 0 days relative to the moment of the aspiration for somatrotopin treated (●) or control heifers (○) that had a crescent E2 (──) or not (----).

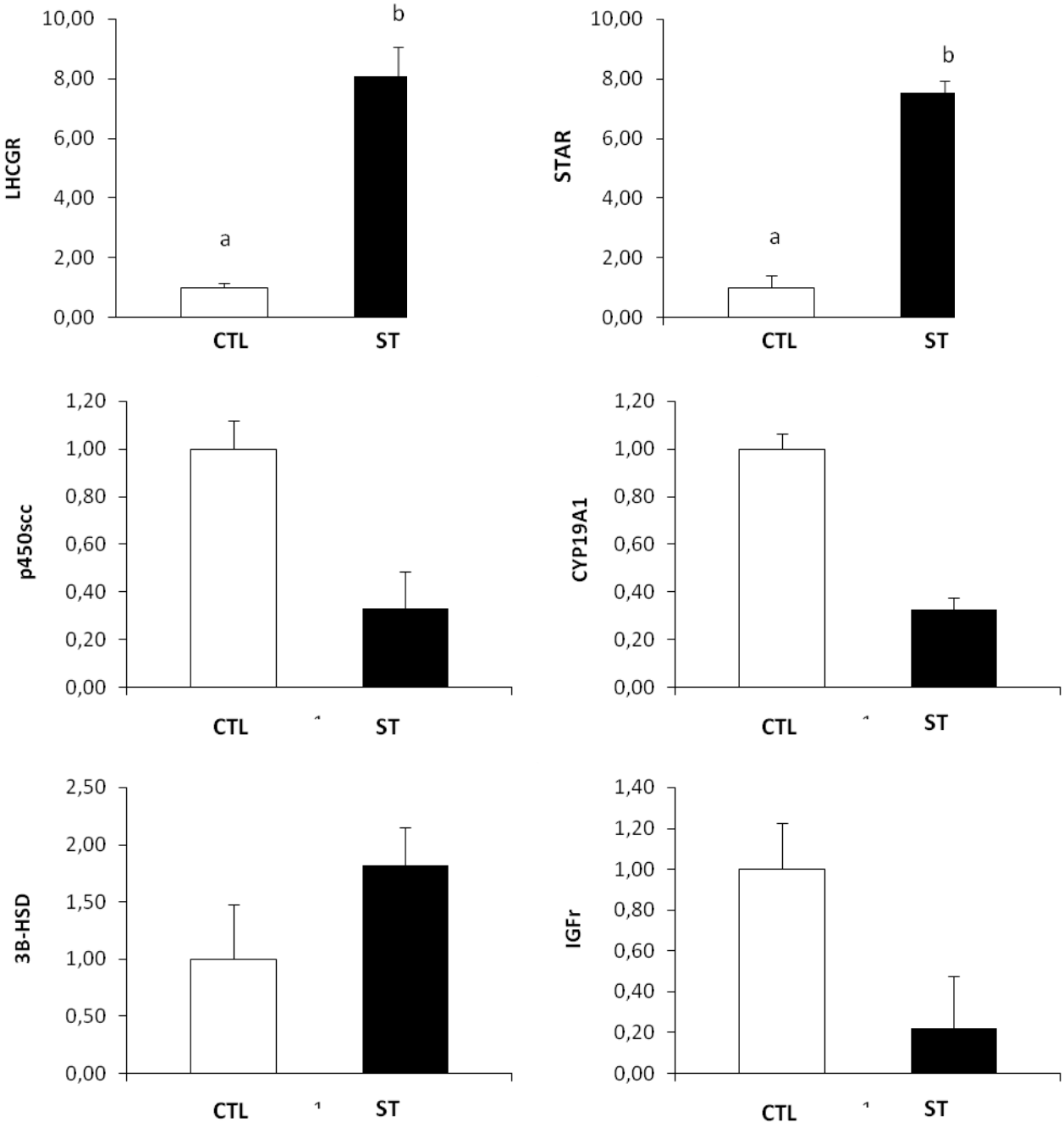

Figure 3. mRNA expression of *LHCGR*, *STAR*, *3β-HSD*, *P450scc*, *CYP19A* and *IGFr* in the follicular cells of the fist postpartum dominant follicle (estrogem activity follicles) for somatotropin (black bar) and control heifers (white bar). All values are shown as mean ± SEM. Values with different letters (a,b) indicate difference between treatments ($P<0.05$).

3.2 Artigo 2

Effects of rumen-protected methionine and choline supplementation on esteroidogenic potential of the first postpartum dominant follicle in dairy cows

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Será submetido à revista Journal of Dairy Science

Effects of rumen-protected methionine and choline supplementation on esteroidogenic potential of the first postpartum dominant follicle in dairy cows

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ABSTRACT

Our objective was to determine the effects of supplementing methionine and choline during the transition period on the esteroidogenic potential of the first postpartum dominant follicle (DF) in Holstein cows. Multiparous cows were assigned in a randomized complete block design into four treatments from 21 days before calving to 30 days in milk (DIM). Treatments were: **MET [**n = 19,fed the basal diet + rumen-protected methionine (RPM) at a rate of 0.08% (w/w) of the dry matter (DM), Smartamine® M], **CHO** (n = 17,fed the basal diet + choline 60 g/day, Reashure®), **MIX** (n = 21,fed the basal diet + Smartamine® M and 60 g/day Reashure®) and **CON** (n = 20, no supplementation, fed the close-up and fresh cow diets). Follicular development was monitored via ultrasound every 2 days starting at 7 DIM until ovulation or aspiration of the first postpartum DF. Follicular fluid from 40 cows was aspirated and cells were retrieved immediately by centrifugation and stored at -80 ^{o}C until analysis. Gene expression of *LHCGR, STAR, 3β-HSD, P450scc, P450c17, CYP19A1, IRS1, IGF, MAT1A, SAHH, TLR4, TNF, IL1-β, IL8* and *IL6* was measured. Supplementation of methionine, choline and both methionine and choline during the transition period did not affect days to first ovulation (CON 14.9 ± 1.1 d, MET 14.3 ± 1.1 d, CHOL 14.3 ± 1.2 d and MIX 13.4 ± 1.0 d; P = 0.74) and the number of cows that ovulated the first follicular wave (CON 5/10 cows, MET 5/10 cows, CHO 5/8 and MIX 6/9 cows; P = 0.18). Treatments did not affect mRNA expression of *LHCGR*, *STAR*, *P450scc*, *CYP19A*, *SAHH*, *MAT1A* and *IL6* (P>0.05) however, *3β-HSD* expression was higher (P<0.05) for MET (1.46±0.3) and MIX (1.25±0.3) than CON (0.17±0.04) and CHO (0.26±0.1) cows. For *TNF*, *TLR4* and *IL1-B* mRNA expression was higher (P<0.05) for CON (11.70±4.6, 21.29±10.4, 6.28±1.4) than CHO (2.77±0.9, 2.16±0.9, 2.29±0.7) and MIX (2.23±0.7, 1.46±0.6, 2.92±0.8). There was higher (P<0.05) *IL1*-β expression in CON (6.27±1.4,) than MET (3.28±0.6). Expression of

IL8 mRNA was lower (P<0.05) for CHO (0.98±0.3) than CON (4.90±0.7), MET (6.10±1.7) and MIX (5.05±1.8). Plasma estradiol ($E_{2)}$ and progesterone (P4) concentration was not different between treatments (P>0.05). Also E_2 and P_4 concentrations in the follicular fluid of the first follicular wave postpartum were not different between treatments (P>0.05). Methionine concentrations in the follicular fluid of the first postpartum DF was higher (P < 0.05) in MET cows than in CON cows. In conclusion, supplementing choline and methionine during the transition period changed mRNA expression in follicular cells and increased concentrations of methionine of the first postpartum dominant follicle in Holstein cows.

Key words: methionine, choline, gene expression

INTRODUCTION

The transition period for a dairy cow has traditionally been defined from three weeks pre-calving until three weeks post-calving (Grummer, 1995, Drackley, 1999). In this period high-producing dairy cows experience negative energy balance (NEB) that induces excessive tissue mobilization, primarily of fat but also of protein (Komaragiri and Erdman, 1997). This is important during the transition period when amino acids make up a large proportion of the precursors needed for gluconeogenesis (Drackley, 1999). Moreover, NEB have been shown to have effects on metabolic status and reproductive performance of dairy cows (Butler, 2003, Drackley and Cardoso, 2014). Perhaps the most detrimental impact of NEB on reproductive performance is due to a delayed return to cyclicity. In this sense, the more estrous cycles before the time of first insemination, the higher the probability of pregnancy (Thatcher and Wilcox, 1973, Walsh et al., 2007, Galvao et al., 2010). Therefore, the use of strategies that anticipate the time to first ovulation will also benefit the moment of conception.

In that circumstance, the main factors affected are decreased dominant follicle (DF) growth and estradiol (E2) production and probably related to the decrease in luteinizing hormone (LH) (Grainger et al., 1982) pulses as well as the decrease in circulating insulin and IGF-I(Canfield and Butler, 1990, Butler, 2003). Furthermore, immune function is also suppressed over the periparturient period (Mallard et al., 1998, Kehrli et al., 1999, Butler, 2003), and NEB and fatty liver can impair peripheral blood neutrophil function (Zerbe et al., 2000, Hammon et al., 2006). Circulating metabolites are reflected in the follicular fluid of DF (Leroy et al., 2004a, Leroy et al., 2004b) and consequently may have a direct influence on granulosa cell function (Vanholder et al., 2005). It has been proposed that expression of LH receptor (*LHCGR*) mRNA (Bao et al., 1997a) and 3beta-Hydroxysteroid dehydrogenase (*3β-HSD*) mRNA in granulosa cells can play a role in the establishment of follicular selection (Bao et al., 1997b); expression of *3β–HSD* mRNA was first detected in follicles >8.0 mm and

significantly increased as follicles grew to a mean diameter of 10.2 mm.

Some amino acids are limiting for optimal milk production as evidenced by an increase in milk and protein yields, and percentage of protein in milk after supplementation with specific, rumen-protected amino acids (Socha et al., 2005, Cho et al., 2007, Patton, 2010). Generally the three most important rate-limiting amino acids for milk production are considered to be methionine, lysine and histidine. In addition, many amino acids can have positive effects on physiological processes that are independent of their effects on synthesis of proteins.

A number of reviews have highlighted the importance of nutrition in regulating bovine reproductive efficiency (Wiltbank et al., 2006, Grummer et al., 2010, Santos et al., 2010, Cardoso et al., 2013). However, the precise effects of rumen protect methionine (RPM) supplementation on reproductive efficiency of dairy cattle have not yet been fully evaluated (Penagaricano et al., 2013) Furthermore, methionine is often a limiting amino acid in early lactation (Schwab et al., 1992), so the reduced availability of methionine at this time could decrease the synthesis of phosphatidylcholine and impair lipid metabolism. Increased methionine bioavailability is likely to increase entry of methionine into the 1-carbon metabolism cycle where it is initially converted into S-adenosylmethionine (SAM), the major biological methyl donor (Martinov et al., 2010). DNA methylation is an important mechanism for regulation of gene expression. DNA methylation depends on the availability of methyl donors supplied by amino acids such as methionine and by compounds of one-carbon metabolic pathways such as choline (Van den Veyver, 2002). It has been established that choline is an essential nutrient for mammals when sufficient methionine is not available in the diet (Zeisel et al., 1991, Zeisel, 2000). Increasing rumen-protected choline (RPC) reduced the esterification of fatty acids (NEFA) in the liver without altering fatty acid oxidation (Piepenbrink and Overton, 2003), thereby suggesting decreased liver TAG accumulation. In

addition, RPC supplementation reduced the plasma NEFA concentration and NEFA/cholesterol ratio around parturition(Pinotti et al., 2003), which could reduce the risk of lipid-related metabolic disorders and improve fertility (Walsh et al., 2007). Moreover, accumulation of triacylglycerol in the liver is positively correlated with the high number of days from calving to first ovulation (Rukkwamsuk et al., 1999)

We hypothesized that supplementing RPM, RPC and both RPM/RPC during the transition period reduce the interval between calving to first ovulation, increase expression of genes related to steroidogenes pathway and reduce expression of genes related to pro-inflammatory process that could improve fertility of dairy cows. The objective of the current study were to determine the effects of supplementing RPM, RPC and both RPM/RPC during the transition period on resumption of ovulation, expression of genes related to steroidogenic pathway and pro-inflammatory process of the follicular cells of the first postpartum dominant follicle in dairy cows.

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee. A total of 77 pregnant Holstein cows entering their second or greater lactation were used (parity 3.1 ± 1.2, BW 773.5 ± 16.4 kg). During the prepartum period, cows were housed in free stalls with individual Calan feed gates (American Calan Inc., Northwood, NH, USA). Approximately two days before expected parturition, cows were moved to individual maternity pens in the same barn until parturition. After parturition, cows were housed in tie stalls with mangers designed for measurement of feed intake. Cows were milked three times daily at 0600, 1400, and 2100 h.

During the experimental period, cows were fed for ad libitum intake. Diets (pre and postpartum) were formulated to meet or exceed cows requirements (NRC, 2001) and were delivered once daily (1400 h) as a TMR.

All cows received the same far-off diet [1.40 Mcal/kg of dry matter (DM), 10.2% rumen degradable protein (RDP), and 4.1% rumen undegradable protein (RUP)] from -50 to – 22 d before expected calving, close-up diet (1.52 Mcal/kg of DM, 9.1% RDP, and 5.4% RUP) from – 21d to expected calving, and lactation diet from calving (1.71 Mcal/kg of DM, 9.7% RDP, and 7.5% RUP) through 30 days in milk (DIM) (Table 2) and the nutrient composition (Table 3). Composite samples were analyzed for contents of DM, CP, ADF, NDF, lignin, starch, fat, ash, NFC, Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn, Mo, and S using wet chemistry methods (Dairy One, Ithaca, NY; http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures.pdf). Value for NEL was provided by the lab and calculated based on NRC (2001).

At – 21 ± 2 days before calving, cows were randomly assigned to one of four treatments (TRT), given as a top-dress on a TMR, the TMR DM for the close-up and lactation diets was measured weekly for estimation of daily TMR DM offered. Supplementation with RPM; [**MET;** n = 19; RPM at rate of 0.08% (w/w) of the DM, Smartamine® M (Adisseo, Alpharetta, GA, USA)], RPC; [**CHO;** n = 17; were received 60 g/day choline, Reassure® (Balchem Corporation, New Hampton, NY, USA)], both feed supplements [(**MIX;** n = 21; rumen-protected methionine at a rate of 0.08% (w/w) of the DM (Smartamine® M) and 60 g/day choline (Reashure®)] or no supplementation (**CON;** n = 20), Supplementation of Smartamine® M (0.08% DM) was calculated using the data of TMR offered on a DM basis. Smartamine® M was supplied as small pellets containing a minimum of 75% DL-methionine, physically protected by a pH-sensitive coating, which is considered to have a methionine bioavailability of 80% (Schwab, 2007); therefore, cows received 6 g metabolizable

methionine per 10 g of Smartamine® M fed. The Reashure® product contained 24% (w/w) choline so that 14.4 g/day choline was provided from the 60 g of the top-dressed supplement. Cows were blocked with regard to lactation number and previous lactation 305-dayz milk yield to ensure that these variables had minimal chance of influencing the outcome variables of the study.

Reproductive management, ultrasonography evaluations

The first postpartum follicular wave was monitored in 37 cows (MET, n = 10; CHO n= 8; MIX n = 9 and CON, n = 10) beginning at 7 DIM and then examinations were performed every 2 days until ovulation using transrectal ultrasonography (IBEX® E.I. Medical Imagin, Loveland, CO, USA) with 7.5-MHz linear array probe. At each examination, a sketch of each ovary was made, and the diameter and location of follicles > 3 mm in diameter were recorded (Ginther et al., 1989). Ovulation was defined as the disappearance (from one examination to the next) of a previously identified follicle > 8 mm in diameter (Martinez et al., 2005) along with the detection of a CL in the same ovary in a further examination.

Follicular Aspiration

Follicular aspiration was performed when the DF reached a diameter of 16 mm [average diameter of the follicle to ovulate during the first postpartum wave in Holstein cows, Butler et al. (2006)]. In that point follicular fluid from 40 cows (MET, n = 9; CHO, n= 9; MIX, n = 12; CON, n = 10) was aspirated. The DF was aspirated by ultrasound-guided transvaginal follicular aspiration. Briefly, the area above the first intercoccygeal space was clipped and disinfected with an iodine scrub solution and 70% ethanol. Lidocaine (5 mL, 2% lidocaine hydrochloride solution) was injected into the first intercoccygeal space and time was allowed for the anesthesia to take effect. The vulva and perianal area were cleaned and disinfected with iodine scrub solution.

An IBEX® ultrasound scanner equipped with a 7.5-MHz probe was used for the

follicle aspiration procedure. The ultrasound probe was enclosed within a custom-made handle. The handle enclosed the probe cord and fixed the head of the probe at a 30° angle relative to the needle guide. The needle guide ended at the base of the probe, just above the probe head. The probe and handle were lubricated with a sterile water-based lubricant and positioned in the vagina slightly posterior to the cervix. The ovary containing the DF was manipulated toward the ultrasound probe to be inspected and measured before aspiration. A 18-gauge aspiration needle was passed through the needle guide of the handle and then through the vagina. The aspiration needle (18 G, WTA, Cravinhos, SP, Brazil) was guided through the stroma of the ovary and into the DF. The follicular fluid was retrieved and frozen at −80°C for subsequent analysis of hormones. The follicular cells were immediately pelleted by centrifugation, and the follicular fluid was decanted. The cells were homogenized with Qiazol reagent (Qiagen, Hilden, Germany), snap frozen in liquid nitrogen and stored at −80°C.

RNA extraction, target gene cDNA synthesis and qPCR

For the follicular cells RNA extraction the miRNeasy kit (Quiagen, Hilden, Germany) was used following the manufacturer's protocols. Samples were treated on-column with DNaseI (Qiagen), quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE). Complementary DNA was synthesized using 100 ng RNA. Firstly random primers (10 mM) (Invitrogen Corp. CA) and DNase/RNase free water were mixed and incubated at 65 °C for 5 min and kept on ice for 3 min. Then a second mix containing DNase/RNase free water, first strand buffer (5X), oligo dT18 (Operon Biotechnologies, AL), dNTP mix (10 mM) (Invitrogen Corp. CA), RevertAid Reverse Transcriptase (200 U/μL) (Fermentas Inc., MD) and RNase Inhibitor (20 U/ μL) (Promega, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min.

cDNA was then diluted 1:3 with DNase/RNase free water.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL of SYBR Green master mix (Quanta Biosciences, Gaithersburg, MD), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmpTM Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 6 point relative standard curve plus the non-template control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA) using the following conditions: 5 min at 95 °C, 40 cycles of 1 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s, 65 °C for 15 s plus 95 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA).

Primers were designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible amplicons of 100-120 bp were chosen) and limited 3' G+C (Applied Biosystems, CA) (see table 3). When possible, primer sets were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (Bos taurus) Genome Browser Gateway. Prior to qPCR primers were tested in a 20 μL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from different bovine samples) to ensure identification of desired genes. Five μL of the PCR product were run in a 2% agarose gel stained with SYBR safe. Only those primers that did not present primer-dimers and a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for qPCR. The accuracy of a primer pairs also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. The extraction and qPCR analysis were

performed using previusly established protocols (Khan et al., 2013; Khan et al., 2014; Moyes et al., 2014). The final data were normalized using the geometric mean of *GAPHD, B-ACT*, and *H2AFZ*, which were validated as suitable internal control genes in bovine liver and adipose tissue.

Hormones and metabolites analyses

Blood samples were collected every two days from 7 DIM until the moment of the first ovulation or follicle aspiration, via venipuncture of the coccygeal vein in one tube with and without anticoagulant. Samples for plasma were placed on ice immediately after collection. Samples for serum were allowed to clot at room temperature for at least 20 min and then placed on ice. All tubes were centrifuged, within 2 h of collection, at 4°C for 30 min at 2000 x g. After centrifugation, aliquots of plasma and serum were kept at −20°C until later analysis.

The serum concentration of progesterone (P4) was determined using a commercial radio immune assay kit (Coat-A-Count®, Diagnostic Products Corporation, Los Angeles, USA) as previously described by Burke et al. (2003). Concentrations of estradiol were measured using the procedures reported by Rozell and Keisler (1990) and later described by Kirby et al. (1997) with the substitution of a second antibody precipitation procedure in place of the charcoal extraction procedure. The estradiol assay was sensitive to 0.5 pg/mL, and had an intra-assay CV of 9% and an inter-assay CV of 11%. Follicular fluid and serum analysis of methionine from nineteen cows (MET; n = 9 and CNT; n = 10) were evaluated and prepared for gas cromatography analysis by using a commercial kit (EZ: faast ^{TM} GC- FID Physiological, Phenomenex®).

Statistical analyses

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Cow was considered as the experimental unit. Analyses involving repeated measures

over time (e.g. Progesterone, Estradiol, and follicular growth) were compared among treatments by analysis of variance for repeated measures using the MIXED procedure for the fixed effects of treatment, day and interaction. Random effect included cow. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers (Littell et al., 2002). Variables were subjected to 5 covariance structures: compound symmetry, autoregressive order 1, autoregressive heterogeneous order 1, unstructured, and toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was compound symmetry and used in the model (Littell et al., 2002).

A logarithmic transformation was used for progesterone and estradiol concentrations for better homogeneity of the residuals distribution. Means shown for these variables are back transformed.Residual distribution was evaluated for normality and homoscedasticity. Gene expression results were log2-scale transformed if needed to comply with normal distribution of residuals, and subsequently back-trasformed. When the interaction between treatment and time was significant ($P< 0.05$), pair-wise comparison of individual means was conducted. The model used for each variable is indicated in the results section for each outcome of interest. Statistical significance was declared at $P< 0.05$ and trends at $0.05 <P< 0.10$.

RESULTS

Supplementation of methionine, choline and both methionine and choline during the transition period did not affect days to first ovulation (CON 14.9 ± 1.1 d, MET 14.3 ± 1.1 d, CHOL 14.3 ± 1.2 d and MIX 13.4 ± 1.0 d; $P = 0.74$), the number of cows that ovulated the first follicular wave (CON 5/10 cows, MET 5/10 cows, CHO 5/8 and MIX 6/9 cows; $P = 0.18$), the diameter of the first postpartum DF (CON 20.5 ± 3.5 mm, MET 22.6 ± 3.5 mm, CHOL 15.8 ± 3.9 mm, and MIX 17.3 ± 2.6 mm; $P = 0.54$), or the growing rate of the first postpartum DF (CON 1.1 ± 0.2 mm/d, MET 1.4 ± 0.2 mm/d, CHOL 1.2 ± 0.2 mm/d, and

MIX 1.0 ± 0.2 mm/d; *P* = 0.62). Also the diameter of the DF at -4 d, -2 d, and 0 d (the day relative to the moment of the first ovulation) were not different among treatments (*P* = 0.25) and interaction treatment by day (*P* = 0.35) only an effect of day (*P*< 0.01; Figure 1).

Plasma concentration of estradiol at days -4 d, -2 d, and day 0 relatives to the aspiration was not different (*P*> 0.05) among treatments (CON 1.2 ± 0.2 ng/ml, MET 2.0 ± 0.2 ng/ml, CHO 1.5 ± 0.2 ng/ml, MIX 1.8 ± 0.2 ng/ml) and the interaction of treatment by day, only an effect (*P*< 0.01) of day (-4d; 1.5 ± 0.1 ng/ml, -2d; 1.8 ± 0.1ng/ml, 0d; 2.2 ± 0.1 ng/ml). Similarly, plasma concentration of estradiol at -4 d, -2 d and day 0 relatives to the moment of the first ovulation postpartum were not different (*P*> 0.05) among treatments (CON 1.5 ± 0.3 ng/ml, MET 2.0 ± 0.3 ng/ml, CHO 1.7 ± 0.4 ng/ml, MIX 2.2 ± 0.3 ng/ml) or the effect of day and the interaction treatment by day. Plasma progesterone concentration at -4 d, -2 d and day 0 relatives to the moment of the first ovulation were not different (*P*> 0.05) among treatments (CON 0.15 ± 0.03 ng/ml, MET 0.15 ± 0.03 ng/ml, CHO 0.08 ± 0.04 ng/ml, MIX 0.12 ± 0.03 ng/ml), or the effect of day and the interaction treatment by day. Plasma progesterone concentrations at -4 d, -2 d, and day 0 relatives to the aspiration were not different (*P* > 0.05) among treatments (CON 0.10 ± 0.02 ng/ml, MET 0.14 ± 0.03 ng/ml, CHO 0.12 ± 0.02 ng/ml, MIX 0.10 ± 0.02 ng/ml), or the effect of day and the interaction treatment by day.

Follicular fluid estradiol concentration of the first postpartum DF was not different (*P* = 0.31) among treatments (CON 630.3 ± 229.8 ng/ml, MET 663.6 ± 229.8 ng/ml, CHO 501.4 ± 216.6 ng/ml, MIX 1029.3 ± 195.9 ng/ml). Also, follicular fluid progesterone concentration of the first postpartum DF was no different (*P* = 0.19) among treatments (CON 39.6 ± 7.9 ng/ml, MET 56.4 ± 8.0 ng/ml, CHO 51.4 ± 7.9 ng/ml, MIX 35.3 ± 7.2 ng/ml).

Plasma from MET cows at the day of follicular aspiration of the DF of the first follicular wave postpartum had higher (*P*< 0.01) concentrations of methionine (25.1 ± 1.7.

μM) when compared with CON cows (15.8 ± 1.1. μM). However, we did not see differences (P>0.05) for Lysine and Histidine between MET cows (93.2 ± 7.7 μM, 58.1 ± 4.1 μM respectively) and CON cows (95.1 ± 7.6 μM, 54.4 ± 3.3 μM respectively). Follicular fluid from cows of the dominant follicle of the first follicular wave postpartum from MET cows had high (P = 0.01) concentrations of methionine (18.2 ± 0.1 μM) when compared with CON cows (11.1 ± 0.9 μM). Also we found a tendency for higher (P = 0.07) concentrations of Histidine in MET cows (56.4 ± 3.4 μM) when compared with CON cows (47.4 ± 3.2 μM). No effect (P =0.88) for lysine concentrations in follicular fluid of the dominant follicle of cows from the first follicular wave postpartum Figure 2.

Treatments did not affect mRNA expression of *LHCGR*, *STAR*, *P450scc*, *CYP19A*, *SAHH*, *MAT1A* and *IL6* (P > 0.05) however, *3β-HSD* expression was higher (P < 0.05) for MET (1.46 ± 0.3) and MIX (1.25 ± 0.3) than CON (0.17±0.04) and CHO (0.26 ± 0.1). For *TNF*, *TLR4* and *IL1-B* mRNA expression was higher (P < 0.05) for CON (11.70 ± 4.6, 21.29 ± 10.4, 6.28 ± 1.4) than CHO (2.77 ± 0.9, 2.16 ± 0.9, 2.29 ± 0.7) and MIX (2.23 ± 0.7, 1.46 ± 0.6, 2.92 ± 0.8). There was higher (P <0.05) *IL1*-β expression and a tendency (P = 0.07) for higher TNF expression in CON (6.27 ± 1.4, 11.70 ± 4.6) than MET (3.28 ± 0.6, 3.06 ± 0.8). There was no difference (P = 0.43) between CON and MET for *TLR4*. Expression of IL8 mRNA was lower (P < 0.05) for CHO (0.98 ± 0.3) than CON (4.90 ± 0.7), MET (6.10 ± 1.7) and MIX (5.05 ± 1.8) Figure 3.

DISCUSSION

The aim of this study was to determine the effects of rumen-protected methionine and rumen-protected choline on esteroidogenic potential of the first postpartum dominant follicle in dairy cows. We postulated that methionine and choline supplementation would impact the lipid metabolism and improve fertility of dairy cows. In fact, however, neither treatment

affected days to first ovulation, the number of cows that ovulated the first follicular wave, the diameter of the first postpartum DF or the growing rate of the first postpartum DF.

Supplementation of periparturient dairy cows with RPM and RPC has been used as a strategy to improve lipid metabolism and alleviate hepatic lipidosis. The inclusion of supplemental choline in the diet from approximately 25 days before to 80 days after calving reduced loss of body condition postpartum and concentrations of BHBA in plasma, which resulted in lower incidence of clinical and subclinical ketosis despite the increase in fat-corrected milk (Lima et al., 2012). Although feeding RPC reduced morbidity, and improved metabolic health, no benefits were observed for reproduction. Supplemental rumen-protected choline did not affect the resumption of postpartum ovarian cyclicity, pregnancy per AI at the first and second inseminations, or maintenance of pregnancy in the first 60 days of gestation (Lima et al., 2012), in agreement with the present study that show not treatment effect on days to first ovulation, the number of cows that ovulated the first follicular wave, the diameter of the first postpartum DF. Moreover, supplementation of rate-limiting amino acids as methionine can have substantial effects on milk protein content and yield (Socha et al., 2005, Cho et al., 2007, Patton, 2010) however, effects on reproduction have not yet been adequately evaluated. Poland et al (1991) detected no significant effect on days to first service, services per conception, or calving interval. Futhermore, Souza et al (2012) did not found differences in fertilization or embryo quality.

Although no differences was observed between treatments for progesterone and estradiol concentrations in the follicular fluid and serum, a higher mRNA expression of *3β-HSD* gene in follicular cells of the first postpartum DF was observed when methionine was include in the diet. Also, a down regulation of genes involved in the proinflamatory response was observed when we suplemented RPM and RPC. Continued growth of dominant follicles is accompanied by an increase in expression of LH receptor and *3β-HSD* mRNA in granulosa

cells (Bao et al., 1997a). In addition, LH receptor mRNA expression in granulosa cells is limited to healthy follicles, which also express greater levels of mRNA for P450scc and P450arom in granulosal cells than do subordinate follicles (Bao et al., 1997b). Suggesting that not only LH receptor mRNA expression, but also *3β-HSD*, may be related to the selection of the dominant follicle in cattle (Bao and Garverick, 1998). A higher expression of *3β-HSD* can be beneficial, because *3β-HSD* is essential for the biosynthesis of all classes of steroid hormones, including progesterone, estrogens (Boerboom and Sirois, 2001).

Bacterial infections of the uterus or mammary gland perturb ovarian dominant follicle growth and endocrine function (Sheldon et al., 2002, Sheldon et al., 2009, Lavon et al., 2011). Although LPS has been found in follicular fluid from dominant follicles of animals with uterine disease (Herath et al., 2007), healthy ovarian follicles are devoid of immune cells (Spanel-Borowski et al., 1997, Bromfield and Sheldon, 2011). Granulosa cells collected from dominant follicles expressed mRNA TLRs and also had rapid responses to LPS, with increased abundance of IL6, IL1B, IL10, TNF, IL8 mRNA (Price et al., 2013). Probably these explain why the CNT cows had higher expression of genes involved in the innate immunity, maybe in the CNT group we have more cows with uterine disease and probably RPM and RPC supplementation cows had a lower incidence of disease, as demonstrated before (Lima et al., 2012) and can be related to the increased the proliferative response of peripheral lymphocytes in blood as described by Soder and Holden, (1999).

In cattle, the proportion of cultured embryos that develop to the blastocyst stage is affected by amino acid composition of the culture medium (6-8). One amino acid that might be important for embryonic development and survival is the essential amino acid methionine. Also, methionine in the follicular fluid was associated positive with morphological assessments of cumulus–oocyte complexes (COC) quality (Sinclair et al., 2008). However, little is known about the specific requirements of methionine in the follicular fluid of bovines.

In the present study MET had a higher concentration of methionine in plasma when compared with CNT in agreement with previous results that show increased methionine concentration in the plasma in response to feeding supplemental methionine (Koenig and Rode, 2001). Interesting, we found a high concentration of methionine in the follicular fluid in cows supplemented with methionine. It has been proposed and subsequently shown in postpartum cows that due to the close correlation between follicular fluid and serum levels of certain metabolites, metabolic changes in serum concentrations will be reflected in the follicular fluid and therefore may affect the quality of both the oocyte and the granulosa cells (Leroy et al., 2004a, Leroy et al., 2004b).

CONCLUSIONS

In conclusion, supplementing methionine, during the transition period increased *3β-HSD* expression also, methionine and choline supplementation induced a down regulation of pro-inflammatory genes indicating a lower inflammatory processes in follicular cells. It is important to highlight that methionine is higher in the follicle of supplemented cows and can potentially affect oocyte quality. The total understanding on how this may affect reproductive performance at the farm level is still do be studied.

ACKNOWLEDGEMENTS

This project was supported in part by a grant from Adisseo USA. Special appreciation is extended to the staff of the University of Illinois Dairy Research Unit and alsothe authors thank to Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG) for the financial support.

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Table 1. Ingredient composition of diets fed during far-off (-50 to -21 d relative to calving), close-up (-21 d to calving) and early lactation (calving to 30 d) periods.

	Diet		
Ingredient (% of DM)	Far-off	Close-up	Lactation
Alfalfa silage	12.00	8.34	5.07
Alfalfa hay	-	4.29	2.98
Corn silage	33.00	36.40	33.41
Wheat straw	36.00	15.63	2.98
Cottonseed	-	-	3.58
Wet Brewers grains	-	4.29	9.09
Ground shelled corn	4.00	12.86	23.87
Soy hulls	2.00	4.29	4.18
Soybean meal, 48% CP	7.92	2.57	2.39
Expeller Soybean meal[1]	-	2.57	5.97
Soychlor[2]	0.15	3.86	-
Blood meal, 85% CP	1.00	-	-
ProVAAl AADvantage[3]	-	0.86	1.50
Urea	0.45	0.30	0.18
Rumen-inert fat[4]	-	-	1.02
Limestone	1.30	1.29	1.31
Salt	0.32	0.30	0.30
Dicalcium phosphate	0.12	0.18	0.30
Magnesium oxide	0.21	0.08	0.12
Magnesium sulfate	0.91	0.99	-
Sodium Bicarbonate	-	-	0.79
Potassium carbonate	-	-	0.30
Calcium sulfate	-	-	0.12
Mineral vitamin mix[5]	0.20	0.17	0.18
Vitamin A[6]	0.015	-	-
Vitamin D[7]	0.025	-	-
Vitamin E[8]	0.38	0.39	-
Biotin	-	0.35	0.35

[1]SoyPLUS (West Central Soy, Ralston, IA)
[2]By West Central Soy
[3]Perdue AgSolutions LLC
[4]Energy Booster 100 (MSC, Carpentersville, IL)
[5]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2200 kIU of vitamin A/kg, 660 kIU of vitamin D3/kg, and 7,700 IU of vitamin E/kg.
[6]Contained 30,000 kIU/kg
[7]Contained 5,009 kIU/kg
[8]Contained 44,000 kIU/kg

Table 2. Nutrient composition and evaluation (NRC, 2001) of prepartal and postpartal diets fed to multiparous Holstein cows supplemented with rumen-protected MET (Smartamine M, Adisseo NA) or rumen-protected CHO (ReaShure; Balchem Inc.) during the peripartal period[1].

	Prepartum					Postpartum			
		Close-up[2]							
Chemical component	Far off	CNT	MET	CHO	MIX	CNT	MET	CHO	MIX
NE_L (Mcal/kg of DM)	1.40	1.52	1.52	1.51	1.51	1.72	1.70	1.72	1.70
CP (% of DM)	14.3	14.6	14.6	14.4	14.4	17.2	17.3	17.2	17.3
RDP (% of DM)	10.2	9.2	9.2	9.1	9.0	9.7	9.6	9.7	9.6
RUP (% of DM)	4.1	5.3	5.4	5.3	5.4	7.4	7.7	7.4	7.7
NDF (% of DM)	51.1	42.0	41.9	41.7	41.7	33.9	33.9	33.7	33.6
ADF (% of DM)	35.4	28.3	28.3	28.2	28.1	21.4	21.4	21.3	21.2
RDP supplied (g/d)	1248	1180	1289	1231	1332	1679	1862	1662	1849
RDP balance (g/d)	138	-51	-61	-63	-79	-120	-141	-121	-144
RUP supplied (g/d)	507	680	763	714	798	1280	1481	1268	1474
RUP required (g/d)	92	184	159	190	169	1787	2149	1814	2062
RUP balance (g/d)	415	496	605	525	629	-507	-669	-546	-587
MP supplied (g/d)	1058	1255	1390	1314	1445	2090	2374	2070	2361
MP balance (g/d)	324	404	493	426	513	-434	-573	-467	-504
Lys: Met	3.89:1	3.62:1	2.81:1	3.61:1	2.79:1	3.54:1	2.71:1	3.54:1	2.71:1
Lys (% of MP)	7.24	6.74	6.66	6.72	6.63	6.33	6.24	6.33	6.24
MP-Lys (g)	77	85	93	88	96	132	148	131	147
Met (% of MP)	1.86	1.86	2.37	1.86	2.38	1.79	2.30	1.79	2.30
MP-Met (g)	20	23	33	24	34	37	55	37	54

[1]The NRC (2001) evaluation of diets was based on final DMI and production data and feed analysis.

Table 3. Hybridization position, sequence, GeneBank accession number and amplicon size of primers for Bos taurus used to analyse gene expression

Gene	Primer squence (5'-3')	Accession number	bp
LHCGR	F:AGAACACTAAAAACCTGGTGCACAT	NM_174381.1	100
	R: GGAAGCTTGTGGATGCCTGTA		
STAR	F:TGGCATGGCCACACTCTATG	NM_174189.2	118
	R:TGAGAAGTGCTGATGTACCA		
3B-HSD	F:TGTCATTGACGTCAGGAATGC	NM_176644.2	100
	R:TACGCTGGCCTGGACACA		
P450scc	F:CGTCAGCCTCCTGCACAAG	NM_174093.1	100
	R:GGTGATGGACTCAAAGGCAAA		
CYP19A1	F:AGCATAGATTTCGCCACTGAGTT	NM_174305.1	100
	R:GCGCTGCGATCAGCATTT		
IL1β	F: ATTCTCTCCAGCCAACCTTCATT	NM_174093.1	100
	R: TTCTCGTCACTGTAGTAAGCCATCA		
IL6	F: CCAGAGAAAACCGAAGCTCTCAT	NM_173923.2	100
	R: CCTTGCTGCTTTCACACTCATC		
IL8	F: GACAGCAGAGCTCACAAGCATCT	NM_173925.2	105
	R: AAGCTGCCAAGAGAGCAACAG		
IGF1	F: CCAATTCATTTCCAGACTTTGCA	NM_001077828.1	103
	R: CACCTGCTTCAAGAAATCACAAAA		
IRS	F: TGTTGACTGAACTGCACGTTCT	XM_003585773.3	112
	R: CATGTGGCCAGCTAAGTCCTT		
TLR4	F:TGCGTACAGGTTGTTCCTAACATT	NM_174198.6	109
	R: TAGTTAAAGCTCAGGTCCAGCATCT		
TNFα	F: CCAGAGGGAAGAGCAGTCCC	NM_173966.3	114
	R: TCGGCTACAACGTGGGCTAC		
MAT1A	F: GGCACTGTCTATTTCCATCTTTACCTA	NM_001046497.1	110
	R: AGTCCAAGTCCCTGACGATAACA		
SAHH	F: TGTCAGGAGGGCAACATCTTT	NM_001034315.1	109
	R: AGTGCCCAATGTTACACACAATG		

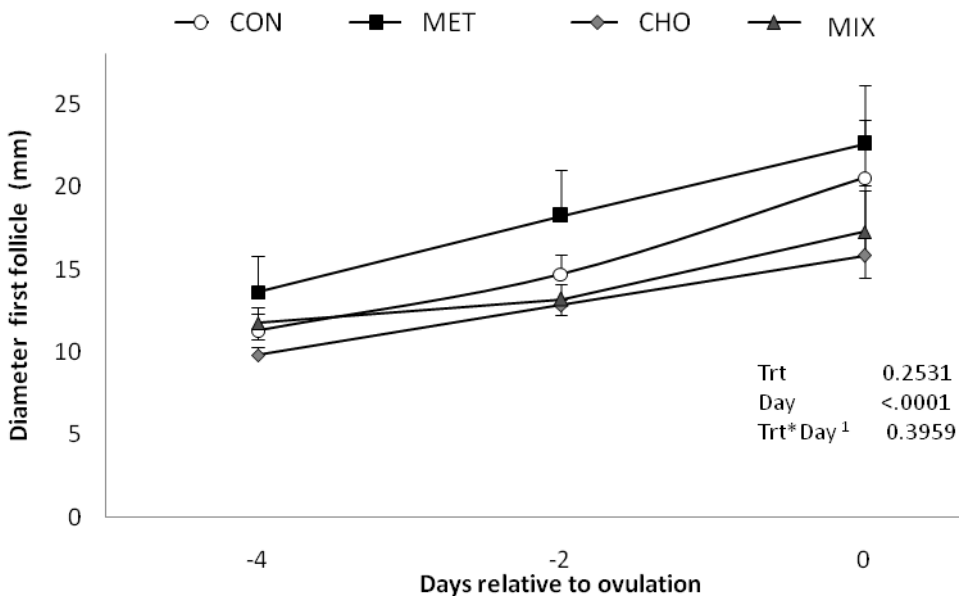

Figure 1. Diameter relative to the moment of the first ovulation of the first postpartum dominant follicle.Treatmens [**Trt**; fed the close-up and fresh cow diets supplemented with methionine (MET, n=5), choline (CHO, n=5) or both (MIX, n=7) and non-supplemented (CON, n=7)]. All values are shown as mean SEM.[1]Trt*Day: Treatment by day interaction.

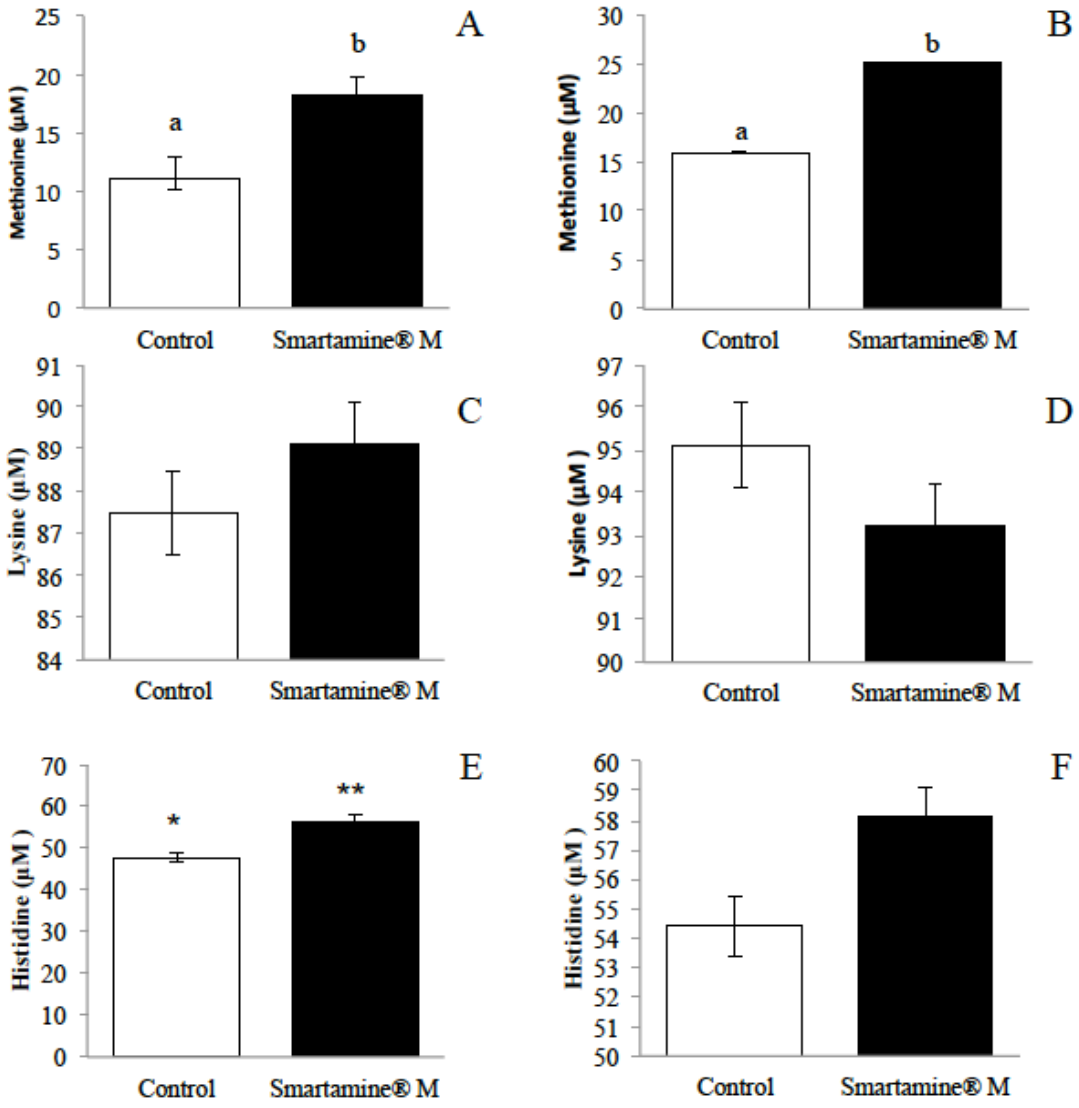

Figure 2. A) Methionine, C) Lysine, E) Histidine in the follicular fluid from the dominant follicle of cows from the first follicualr wave postpartum. B) Methionine, D) Lysinie, F) Histidine in plasma concentration from the day relative to the moment of the aspiration for control (non-supplemented; white bar, n=10) and Smartamine ® M (fed the close-up and fresh cow diets supplemented with methionine; black bar, n=9) cows. All values are shown as mean ± SEM. Values with different letters (a,b) indicate difference between groups ($P<0.05$). Values with asterisk (*) indicated a tendency ($P = 0.07$)

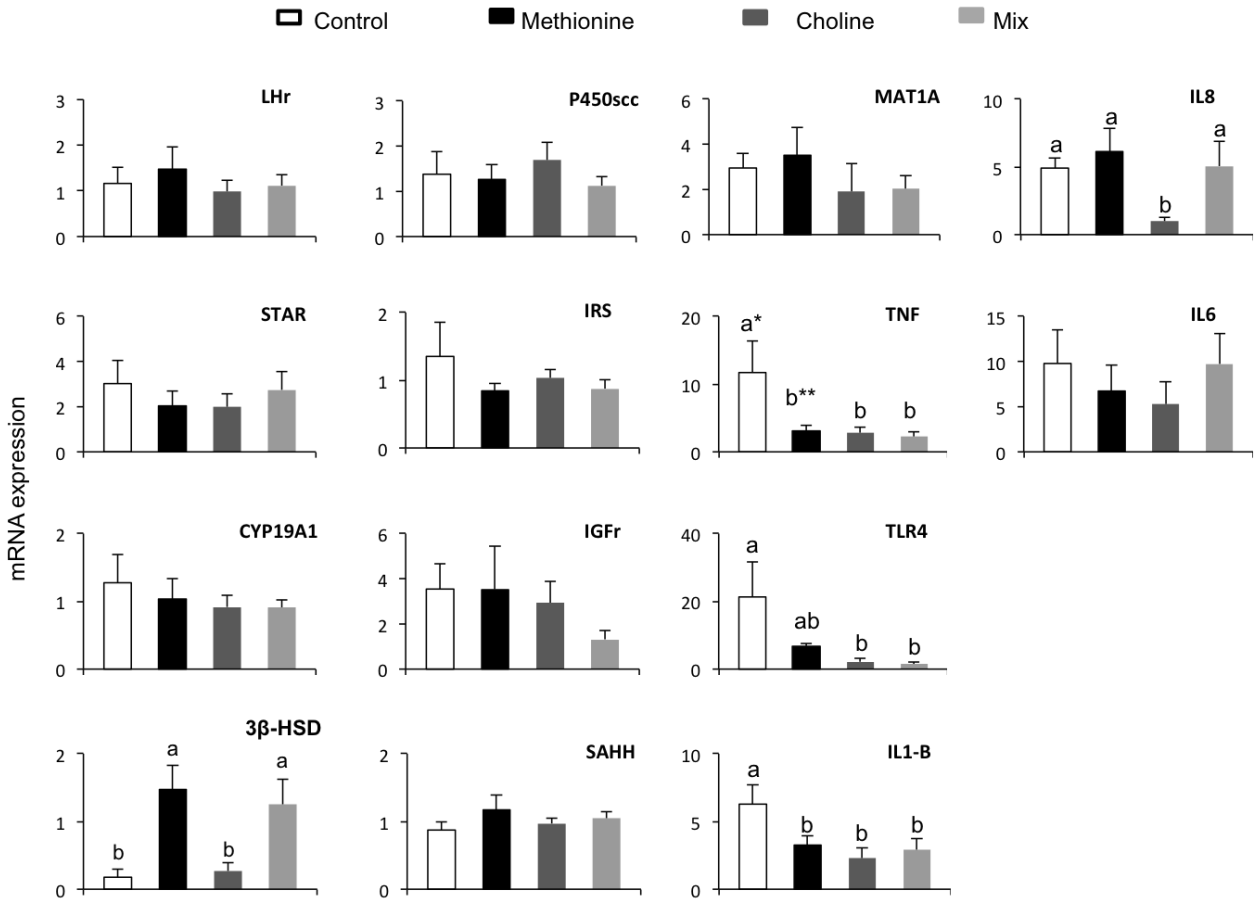

Figure 3. mRNA expression of *LHCGR*, *STAR*, *3β-HSD*, *P450scc*, *CYP19A*, IRS, *IGF1r*, *SAHH*, *MAT1A*, *TNF*, *TLR4*, *IL1-B*, *IL8* and *IL6* in the follicular cells of the fist postpartum dominant follicule, Treatmens [fed the close-up and fresh cow diets supplemented with methionine (MET, n=9), choline (CHO, n=9) or both (MIX, n=12) and non-supplemented (CON, n=10)]. All values are shown as mean SEM. Values with different letters (a,b) are different between treatments (P<0.05). Values with asterisk (*) indicated a tendency (P = 0.07).

3.3 Artigo 3

Effects of rumen-protected methionine and choline supplementation on the preimplantation embryo in Holstein cows

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Submetido à revista Theriogenology

Effects of rumen-protected methionine and choline supplementation on the preimplantation embryo in Holstein cows

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ABSTRACT

Our objective was to determine the effects of supplementing methionine and choline during the pre and postpartum periods on preimplantation embryos of Holstein cows. Multiparous cows were assigned in a randomized complete block design into four treatments from 21 days before calving to 30 days in milk (DIM). Treatments (**TRT**) were: **MET [**n = 9,fed the basal diet + rumen-protected methionine (RPM) at a rate of 0.08% (w/w) of the dry matter (DM), Smartamine® M], **CHO** (n = 8,fed the basal diet + choline 60 g/day, Reashure®), **MIX** (n = 11,fed the basal diet + Smartamine® M and 60 g/day Reashure®) and **CON** (n = 8, no supplementation, fed the close-up and fresh cow diets). Cows were randomly re-assigned to two new groups (**GRP**) to receive the following diets from 31 to 72 DIM; **CNT (**control, n = 16, fed a basal diet) and **SMT** [n = 20, fed the basal diet + 0.08% (w/w) of the DM intake as methionine]. A CIDR® device was inserted in all cows after follicular aspiration (60 DIM) and superovulation began at day 61.5 using FSH in 8 decreasing doses at 12 h intervals over a four days period. At day 63 and 64 all cows received two injections of PGF2α. CIDR® was removed at day 65. 24 h after CIDR® removed, ovulation was induced with GnRH. Cows received AI at 12 h and 24 h after GnRH. Embryos were flushed 6.5 days after AI. Global methylation of the embryos was assessed by immunofluorescent labeling of 5-methylcytosine, while lipid content was assessed by staining with Nile red. Nuclear staining was used to count the total number of cells per embryo.There was no difference between TRT, GRP or their interaction ($P > 0.05$) for embryo recovery, embryo quality, embryo stage or cells per embryo. Methylation of the DNA was not different among TRT but there was a TRT by GRP interaction ($P = 0.01$). Embryos from cows in CON-CNT had greater ($P = 0.04$) methylation [0.87 ± 0.09 arbitrary units (AU)] than embryos from cows in MET-CNT (0.44 ± 0.07 AU). The cytoplasmic lipid content was not affected (P > 0.05) by TRT or their interaction, but

lipid content was greater (P = 0.04) for SMT (7.02 ± 1.03 AU) than in CNT (3.61 ± 1.20 AU). In conclusion, cows in MET-CNT had embryos with lower methylation and SMT cows had a higher lipid content than non-supplemented cows. Methionine supplementation seems to impact the preimplantation embryo in a way that enhances its capacity for survival since there is strong evidence that endogenous lipid reserves serve as an energy substrate.

Key words: Methionine, choline, methylation, lipid, embryo.

1. INTRODUCTION

Studies over the last two decades clearly established the link between nutrition and fertility in ruminants [1-5]. Dietary changes can cause an immediate and rapid alteration in a range of humoral factors that can alter endocrine and metabolic signaling pathways crucial for reproductive function [6, 7]. Moreover, periconceptional nutritional environment in humans and other animals is critical for the long-term setting of postnatal phenotype [8]. Restricting the supply of B-vitamins and methionine during the periconceptional period in sheep, for example, results in adverse cardiometabolic health in postnatal offspring [9]. Feeding female mice a low protein diet during the preimplantation period of pregnancy resulted in reduction in amino acid (**AA**) concentration in uterine fluid and serum and attendant changes in the AA profile of the blastocyst [10].

Strategies have been used to improve the reproductive performance of dairy cows through alteration of nutritional status [11, 12]. In other species, dietary supplementation with specific amino acids (e.g., arginine, glutamine, leucine, glycine and methionine) had beneficial effects on embryonic and fetal survival and growth through regulation of key signaling and metabolic pathways [13, 14].

Methionine is the most limiting AA in lactating cows [15] but supplementation of diets with crystalline methionine has been excluded because free methionine is quickly and almost totally degraded by the microorganisms in the rumen [15]. In contrast, supplementing rumen-protected methionine has a positive effect on milk protein synthesis in dairy cows [16-18]. Although the role of methionine in bovine embryonic development is unknown there is evidence that methionine availability alters the transcriptome of bovine preimplantation embryos in vivo [19].

The DNA methylation is an important mechanism for regulation of gene expression. Among other roles, it is important for the irreversible and silencing of genes on the inactive X-chromosome and for the parent-of-origin dependent silencing of one allele of imprinted genes [20]. It has been proposed to be a repression mechanism used in complex organisms to reduce transcriptional noise [21]. DNA methylation in promoters is involved in gene silencing. However, DNA methylation in other regions may have a more complex role in regulation of transcription [20, 22, 23].

The DNA methylation depends on the availability of methyl donors supplied by amino acids such as methionine and by compounds of one-carbon metabolic pathways such as choline [22]. Increased methionine bioavailability is likely to increase entry of methionine into the 1-carbon metabolism cycle where it is initially converted into S-adenosylmethionine (SAM), the major biological methyl donor [24].

Oral administration of choline to rats increased plasma total cholesterol, phospholipid, high density lipoprotein, and low density lipoprotein [25]. Choline is a major component of phospholipids and sphingomyelin, a component of acetylcholine that participates directly in neurotransmission [26], membrane integrity and methylation pathways [27, 28]. Early studies evaluating the effect of dietary choline on milk yield and duodenal flow indicated its rapid and extensive rumen degradation for absorption in duodenum [29, 30]. Subsequently, numerous studies have evaluated the effects of feeding choline in rumen-protected form on reproduction and health of dairy cows [31, 32].

Animals fed diets deficient in methyl donors (e.g. choline and methionine) have hypomethylated DNA [33, 34]. These changes occur not only in global methylation [35], but also in the methylation of specific genes [36]. Preimplantation development of the bovine embryo is characterized by dynamic changes to DNA methylation, in particular, global

methylation declines to a nadir at the 6-8 cell stage and increases thereafter[37]. However, effects of methionine in preimplantation embryos are still controversial. Bonilla et al. [38] suggests that extracellular methionine is not required for DNA methylation in the cultured blastocyst. Nevertheless, gene expression changes caused by alteration of DNA methylation (i.e. absence of the methylase genes) can result in embryo lethality or developmental defects in preimplantation embryos [39].

The hypothesis of the present study was that dietary supplementation with rumen-protected methionine and rumen-protected choline increases DNA methylation in preimplantation embryos in dairy cows and is beneficial to embryonic development. The objective of this study was to determine the effects of methionine and choline on DNA methylation and lipid accumulation in preimplantation embryos of Holstein cows.

2. MATERIALS AND METHODOS

Institutional Animal Care and Use Committee (IACUC) from the University of Illinois (Urbana-Champaign, IL, USA) approved all procedures performed in this experiment.

2.1 Experimental design and sample collection

A total of 36 pregnant Holstein cows entering their second or greater lactation were utilized (parity 2.89 ± 0.25, Age 3.35 ± 0.41 y). During the prepartum period, cows were housed in free stalls with individual Calan feed gates (American Calan Inc., Northwood, NH, USA). Approximately two days before expected parturition, cows were moved to individual maternity pens in the same barn until parturition. After parturition, cows were housed in tie stalls with mangers designed for measurement of feed intake. Cows were milked three times daily. Values for milk yield were: wk 1 to 4 = 41.1 kg/day (range: 38.1 to 42.3 kg/day) and

wk 5 to 10 = 46.4 (44.4 to 48.3 kg/day). During the experimental period, cows were fed for *ad libitum* intake. Diets (pre and postpartum) were formulated to meet or exceed cows' requirements according to NRC [15] and were delivered once daily as a total mixed ration (TMR).

All cows received the same basal diet, close-up diet [1.52 Mcal/kg of dry matter (DM), 9.1% RDP, and 5.4% RUP] from 21days before the expected calving, fresh cow diet from calving (1.71 Mcal/kg of DM, 9.7% RDP, and 7.5% RUP) through 30 days in milk (DIM), and high cow diet (1.69 Mcal/kg of DM, 9.5 % RDP, and 7.3% RUP) from 31 to 72 DIM. At 21 days before calving, cows were randomly assigned to one of four treatments (**TRT**), given as a top-dress on a TMR: supplementation with methionine; [**MET;** n = 9; RPM at a rate of 0.08% (w/w) of the DM, Smartamine® M (Adisseo, Alpharetta, GA, USA)], choline; [**CHO;** n = 8; were received 60 g/day of RPC, Reashure® (Balchem Corporation, New Hampton, NY, USA)], both feed supplements [(**MIX;** n = 11; RPM at a rate of 0.08% (w/w) of the DM (Smartamine® M) and 60 g/day of RPC (Reashure®)] or no supplementation (**CON;** n = 8). Cows were randomly re-assigned to receive one of two new feed regimens from 30 ± 1 to 72 ± 1 DIM. The two new groups (**GRP**) were control (**CNT**; n = 16, fed a basal diet) and methionine [**SMT**; n = 20, fed the basal diet plus RPM at a rate of 0.08% (w/w) of the DM (Smartamine® M)]. Supplementation of Smartamine® M (0.08% of DM) was calculated using the data of TMR offered on a DM basis. Smartamine® M was supplied as small pellets containing a minimum of 75% DL-methinine, physically protected by a pH-sensitive coating, which is considered to have a methionine bioavailability of 80% [63]; therefore, cows received 6 g metabolizable methionine per 10 g of Smartamine® M fed. The Reashure® product contained 24% (w/w) choline so that 14.4 g/day choline was provided from the 60 g of the top-dressed supplement. A schematic design of the experimental groups can be found in Figure 1A. Cows were blocked with regard to lactation number and previous

lactation 305-d milk yield to ensure that these variables had minimal chance of influencing the outcome variables of the study. Dry matter intake (DMI) was determined daily throughout the dry period and first 72 days post-calving. Body weight (BW) and body condition score (BCS) (scale of 1 = emaciated to 5 = obese) were obtained weekly throughout the study. The BCS was assigned in quarter-unit increments by two individuals each time and the average of the score was used for that week.

2.2 Synchronization, superovulation, and flushing protocols

Synchronization protocol started (n = 36) on day 30 ± 1 relative to calving. Estrous cycles were presynchronized with 1 injection, im, of PGF2α (25 mg of dinoprost tromethamine; 5 mL of Lutalyse, Zoetis Animal Health, NJ, USA). On day 44, cows received the first injection of GnRH (100 μg of gonadorelin hydrochloride; 2 mL of Factrel, Zoetis Animal Health) and a controlled internal drug-release insert (CIDR; Eazi-Breed CIDR, Zoetis Animal Health) containing 1.38 g of progesterone. Seven days later (day 51), cows received an injection of PGF2α, im, concurrent with the removal of the CIDR insert. On day 53, cows received the second injection of GnRH. Seven days later (day 60), superstimulatory treatment was initiated.

Superstimulatory treatments with FSH (Folltropin-V; Bioniche Life Sciences, Belleville, ON, Canada) were initiated on days 8 to 10 of the an estrus cycle in order to stimulate follicles at the start of the second follicular wave when a dominant follicle is not present. On day 60, all dominant follicles greater than 5 mm were aspirated with an ultrasound-guided transvaginal approach using a 18-gauge X 55 cm aspiration needle. A CIDR device was inserted in all cows after follicular aspiration (day 60) and superovulation as started at day 61.5 using FSH treatment equivalent to 400 mg of NIHFSH- P1 (Folltropin-V). Eight decreasing FSH doses were administered at 12-h intervals over a period of four days

(80 mg a.m., 80 mg p.m., 60 mg a.m., 60 mg p.m., 40 mg a.m., 40 mg p.m., 20 mg a.m., 20 mg p.m.). During the superovulatory period, all cows received two PGF2α injections at days 63 and 64 (concomitant with the 5th and 7th FSH injections), and CIDR was removed at day 65 (concomitant with the last FSH injection). Twenty-four hours after the CIDR removed, ovulation was induced with GnRH injection. Cows were artificially inseminated at 12 h (day 66) and 24 h (day 67) after GnRH using a fertile sire (014HO05388; 2 doses per insemination). The same technician performed all artificial inseminations.

Embryos were flushed 6.5 days after the first timed artificial insemination using a transcervical non-surgical technique by the same technician. A schematic design of the superovulatory treatments can be found in Figure 1B. Embryos were evaluated according to the embryo grading system recommended by the manual of the International Embryo Transfer Society (IETS) [40]. The embryos were typically at the morula or blastocyst stage. Recovered embryos were evaluated for fertilization and scored for quality as, 1) perfect for the stage of the embryo, compact blastomeres, no debris (excellent), 2) having trivial imperfections such as oval zona, few small loose blastomeres (good), 3) having definite but not severe problems such as moderate numbers of loose blastomeres (fair), 4) partly degenerated, with vesiculated cells and greatly varying sizes of cells (poor), 5) severely degenerated (very poor), or 6) unfertilized. Embryos are collected 6.5 days after estrus (IETS Manual's standards) for the stages likely to be encountered at that time are described below. Unfertilized oocyte (Stage code 1). Unfertilized (Stage code 2): 2 to 15 cells. Morula (Stage code 3): A mass of at least 16 cells. Individual blastomeres are difficult to discern from one another. The cellular mass of the embryo occupies most of the perivitelline space. Compact morula (Stage code 4): Individual blastomeres have coalesced, forming a compact mass. The embryo mass occupies 60 to 70 % of the perivitelline space. Early blastocyst (Stage code 5): An embryo that has

formed a fluid-filled cavity or blastocele and gives a general appearance of a signet ring. The embryo occupies 70 to 80% of the perivitelline space. Early in this stage the embryo may appear of questionable quality because it is difficult to differentiate inner cell mass from trophoblast cells at this time. Blastocyst (Stage code 6): Pronounced differentiation of the outer trophoblast layer and of the darker, more compact inner cell mass is evident. The blastocele is highly prominent, with the embryo occupying most of the perivitelline space. Visual differentiation between the trophoblast and the inner cell mass is possible at this stage of development. Expanded blastocyst (Stage Code 7): The overall diameter of the embryo dramatically increases, with a concurrent thinning of the zona pellucida to approximately one-third of its original thickness. Hatched blastocyst (Stage code 8): Embryos recovered at this developmental stage can be undergoing the process of hatching or may have completely shed the zona pellucida. Hatched blastocysts may be spherical with a well defined blastocele or may be collapsed. Identification of hatched blastocysts can be difficult unless they reexpand when the signet ring appearance is again obvious. Superovulatory response was measured by counting number of CL in each ovary.

2.3 Processing for evaluation of global DNA methylation and lipid content in embryos

Upon collection, embryos at stage 4 (compact morula) of development or higher, when present, were randomly selected for analysis of global DNA methylation and cytoplasmic lipid content. Degenerated embryos were not included in this analysis. Embryos were washed three times in Dulbecco's phosphate buffered saline (DPBS) containing 0.2% (w/v) polyvinyl pyrrolidone (DPBS/PVP), fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature, and washed three more times in DPBS/PVP. Embryos were then placed in a 1.5 mL microcentrifuge tube containing 1 mL of cold DPBS/PVP and shipped cold

overnight to the Hansen laboratory (Department of Animal Sciences, University of Florida, Gainesville, FL, USA) for further processing as described below.

2.4 Immunolabeling with 5-methylcytosine antibody and analysis of global DNA methylation

All steps were performed at room temperature unless otherwise specified. Embryos were first washed three times in wash buffer [DPBS containing 0.1% (w/v) fraction V BSA (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (v/v) Tween 20 (Thermo Fisher Scientific, Waltham, MA, USA)], permeabilized for 30 min in DPBS/PVP containing 0.25% (v/v) of Triton-X 100 (Thermo Fisher Scientific), and washed again three times with wash buffer. Next, embryos were incubated with 50 μg/mL RNAse A for 1h at 37°C in the dark, washed three times in wash buffer, incubated with a solution of 3 M HCl/0.1% (w/v) PVP for 30 min at 37°C in the dark, followed by incubation with 100 mM Tris-HCl/1% (w/v) PVP, pH 8.5, for 10 min to neutralize the pH. This step was followed by three washes with wash buffer and incubation in blocking buffer [DPBS containing 5% (w/v) fraction V bovine serum albumin (BSA) and 0.1% (v/v) Tween 20] overnight at 4°C in the dark. The next morning, embryos were incubated for 1 h with 1 μg/mL primary antibody [affinity purified mouse monoclonal anti-5-methylcytosine antibody (Calbiochem, Darmstadt, Germany)] diluted in antibody buffer [DBPS containing 1% (w/v) fraction V BSA and 0.1% (v/v) Tween 20], washed three times in wash buffer and incubated for 1h with 1 μg/mL secondary antibody [anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Abcam, Cambridge, MA, USA). In embryos used as no primary control, the primary antibody was replaced by mouse IgG1 (Sigma-Aldrich), and the same incubation period and conditions were applied. After three washes in wash buffer, nuclear labeling was performed by a 15 min of incubation with 50 μg/mL propidium iodide on DPBS/PVP. Embryos were washed three more times with wash

buffer and mounted in slides containing a 10 µl drop of Slow Fade® Gold antifade reagent (Life Technologies, Carlsbad, CA, USA) and covered with a coverslip.

Image acquisition was preformed using a Zeiss Axioplan epifluorescence microscope with a 40X objective, using FITC and rhodamine filters. Exposure time was held constant for all embryos, including no primary control, in each replicate. Image analyses were performed using ImageJ software (version 1.60_41, National Institutes of Health, Washington DC, USA). All cell nuclei were selected using the free hand tool, and intensity of fluorescence was measured in the rhodamine (propidium iodide) and FITC (antibody) channels. Global methylation estimates were obtained by calculating the ratio of 5-methylcytosine to propidium iodide. Cell counting was performed using ImageJ, and was based on the number of cell nuclei labeled with propidium iodide.

2.5 Nile red labeling and analysis of lipid content

All steps were performed at room temperature. Fixed embryos were incubated for 30 min in a solution of 1 µg/mL Nile red (Invitrogen, Eugene, OR, USA) and 1 µg/mL Hoescht 33342 (Sigma-Aldrich) in DPBS/PVP in the dark. Following incubation, embryos were washed three times in DPBS/PVP and mounted in slides containing a 10 µl drop of Slow Fade® Gold antifade reagent (Life Technologies) and covered with a coverslip. Negative control embryos were incubated with 100% ethanol for 30 min before incubation with Nile red and Hoescht.

Images were acquired using Zeiss Axioplan epifluorescence microscope with a 40X objective, FITC and DAPI (UV) filters. Exposure time was held constant for all embryos, including no primary control, in each replicate. Image analyses were performed using ImageJ software, by selecting the entire embryo area enclosed by the zona pellucida and measuring

intensity of fluorescence using the FITC channel (Nile red labeling). Lipid accumulation was estimated by subtracting background fluorescence (obtained by averaging FITC fluorescence intensity in negative control embryos) from the fluorescence intensity obtained by FITC analysis of sample embryos. Cell counting was based on the number of nuclei labeled with Hoescht 33342.

2.6 Statistical analyses

A final dataset including all the variables was constructed in SAS (SAS v9.4 Institute Inc., Cary, NC, USA). A linear mixed model (MIXED procedure) was constructed to explore associations between treatments and variables related to embryo and embryo quality, total CL at the time of flush, total embryos recovered, total embryos that received a quality score (1 to 6), total embryos with a code stage score (1 to 8), percentage of embryos recovered (number of embryos/number of CL), methylation, lipid accumulation, number of cells, DMI, BW and BCS. The fixed effects of TRT (MET, CHO, MIX, CON), GRP (CNT, SMT), and their interaction were analyzed. Cow was treated as a random effect. Degrees of freedom were adjusted by using the Kenward-Roger method [41]. Residual distribution was evaluated for normality and homoscedasticity using the Univariate procedure in SAS. A logarithmic transformation was used for the methylation and lipid accumulation data to improve homogeneity of the distribution of residuals. Means shown in graphs for these variables are back transformed.When the interaction between TRT and GRP was not significant ($P > 0.15$) the interaction was excluded from the model and data reanalyzed with only TRT and GRP in the model. Statistical significance was declared as P values ≤ 0.05, and tendency declared for P values > 0.05 and ≤ 0.10.

3. RESULTS

The ingredient composition of the diets fed to cows is detailed in Table 1 and the analyzed chemical composition is shown in Table 2. Body weight, DMI, and were not affected by treatment ($P > 0.17$) at any time in the experiment. Values for body weight were as follows: wk –3 to 0 = 770.6 kg (range: 765.6 to 786.2), wk 1 to 4 = 675.8 kg (range: 660.5 to 702.5 kg) and wk 5 to 10 = 640.3 kg (range 612.7 to 655.7). Values for DMI were: –3 to 0 = 13.7 kg/day (range: 12.7 to 14.7), wk 1 to 4 = 18.2 kg/day (range: 17.1 to 19.2 kg/day) and wk 5 to 10 = 21.9 kg/day (range: 20.4 to 23.2 kg/day). Body condition score was not affected by TRT (P = 0.49) in wk 1 to 4, but there was a difference among treatments ($P = 0.004$) for wk 5 to 10. The average BCS were: wk 1 to 4 = 3.4 (range 3.36 to 3.44) and of wk 5 to 10 = 3.1 (range 2.80 to 3.20). In wk 5 to 10, CON-CNT had the lowest BCS of any TRT (mean = 2.80), and CON-SMT had the highest (mean = 3.20) ($P = 0.004$). There were no differences among the other TRT ($P > 0.05$). BCS from MET/SMT (mean = 3.01), MET/CNT (mean = 3.09), CHO/CNT (mean = 3.07), CHO/SMT (mean = 3.12), MIX/CNT (mean = 3.03) and MIX/SMT (mean = 3.16).

The methionine and choline effects on embryo recovery, embryo quality, embryo stage and number of CL are detailed in Table 3. Recovery rate per flushing was not affected by TRT, GRP or their interaction. The number of embryos recovered was not affected by TRT , GRP or their interaction. The number of CL was not affected by TRT or GRP. However, there was a TRT by GRP interaction. The number of CL for MET-SMT and MIX-SMT were greater (13.1 ± 1.6 and 13.1 ± 2.0 respectively) than MIX/CNT (3.0 ± 1.9). Embryo quality was not affected by TRT, GRP or their interaction. Similarly, embryo stage was not affected by TRT , GRP or their interaction.

Effects of methionine and choline on embryo cell number, methylation and lipid accumulation in the embryo are detailed in Table 4. The number of cells in embryos recovered was not affected by TRT, GRP or their interaction.

Representative images of labeling of embryos for DNA methylation are shown in Figure 2. There was no difference in DNA methylation among TRT or between GRP. There was, however, a TRT by GRP interaction (P = 0.01) where the cows that received control treatment during all periods of the experiment had greater methylation (CON-CNT; 0.87 ± 0.09) than cows that received methionine during the transition period and after 30 DIM switched to control with no methionine supplementation (MET-CNT; 0.44 ± 0.07).

Representative images of labeling of embryos for lipid using Nile red are shown in Figure 3. The cytoplasmic lipid content was not different between TRT and was not affected by the interaction of TRT by GRP. When we excluded the interaction of the model, lipid content was significantly greater (P = 0.04) in SMT (7.02 ± 1.03) than in CNT (3.61 ± 1.20; Figure 4).

4. DISCUSSION

The aim of this study was to determine the effects of rumen-protected methionine and rumen-protected choline on development, DNA methylation and lipid accumulation in preimplantation embryos of Holstein cows. We postulated that methionine and choline supplementation would impact the preimplantation embryo in a way that enhanced its capacity for survival so that reproductive function would be enhanced. In fact, however, neither treatment affected embryonic survival nor cell number and choline also had no effect on DNA methylation or lipid accumulation. Supplemental feeding of methionine increased lipid accumulation in embryos but, surprisingly, decreased DNA methylation.

The increase in lipid accumulation in preimplantation embryos from cows supplemented with methionine is in agreement with previous results that show increased fat synthesis in the mammary gland in response to feeding supplemental methionine [42] or methionine and lysine [43, 44]. It is possible that some AA are diverted to lipid because of excess of methionine or lysine or because of imbalances in other AA. This suggests that methionine could be a limiting AA, that its supplementation diverted nutrients from milk fat synthesis.

Interestingly enough, Penagaricano et al. [19] reported the top 30 most significant genes in preimplantation embryos that showed differential expression between non-supplemented (control) and methionine-supplemented cows. Only the expression of two genes showed higher expression in the methionine-supplemented cows than controls: LOC100849660 (uncharacterized) and LOC510193 (apolipoprotein L, 3-like). Apolipoproteins are involved in the transport and metabolism of lipids, including cholesterol, and allow the binding of lipids to organelles [65]. This data corroborate with our hypothesis that methionine is influencing lipid metabolism in the preimplantation embryo.

Lipid accumulation in embryos might also be caused by altered energy metabolism [45], which was proposed by Bavister [46] as being caused by an excess of glucose metabolism via glycolysis, with inhibition of oxidative phosphorylation. Lipid accumulation is one consequence of excess glycolysis caused by rising cellular concentrations of lipid synthesis precursors [47]. Lipid accumulation could also cause an imbalance in the cellular oxidation-reduction process, affecting mitochondrial metabolism and impairing metabolism of lipid complexes through β-oxidation [48, 49]. Whatever the mechanism(s), an increase in intracellular lipids could impair the survivability of the embryos by increasing sensitivity to oxidative stress and cryopreservation [50]. It has been reported that tolerance to

cryopreservation of bovine embryos developed from in vitro-fertilized oocytes cultured in a serum-supplemented medium could be increased after removal of cytoplasmic lipid droplets by centrifugation, suggesting that lipid droplets may directly affect the sensitivity of embryos to chilling and freezing [51]. Similarly, Nagashima et al. [52] demonstrated more successful cryopreservation after microsurgical delipidation of porcine embryos.

However, another major question still unresolved in the bovine embryo is the role of intracellular lipid in ATP production and other cellular functions. Lipid seems to be a source of energy for the early embryo. Triglycerides represent the major component of intracellular lipids in immature oocytes and may be metabolized during oocyte maturation, fertilization and first embryonic cleavage in cattle [53, 54]. There is strong evidence that endogenous lipid reserves serve as an energy substrate for the preimplantation embryo [64]. In addition to storing energy, lipid droplets provide reservoirs of lipids (such sterols, fatty acids and phospholipids) for membrane synthesis [55]. Given their diverse functions, lipid droplets are situated at the crossroads of membrane biology and energy metabolism and are important organelles in maintaining cell homeostasis [55] and for structure and function of membrane proteins[56].

DNA methylation patterns are altered by dietary and other environmental exposures throughout life [57, 58]. Previous studies have suggested that global DNA methylation is changed in models of developmental programming [59, 60]. Furthermore a number of studies have suggested that diet or metabolic defects that influence S-adenosyl methionine or homocysteine may alter total methylation [61, 62]. Surprisingly, cows that received methionine supplementation produced embryos with lower global DNA methylation than control cows. Penagaricano et al. [19] found that expression of several genes in preimplantation embryos (e.g. *VIM, IFI6, BCL2A1, TBX15*), were decreased by methionine supplementation, probably due to increased DNA methylation in CpG islands in the

promoters of these genes. In contrast, our data is more similar to that of that found by Bonilla et al. [38], working with in vitro produced embryos, where global DNA methylation was unaffected by local methionine availability. It remains a possibility that, while methionine concentration does not affect global DNA methylation, it does alter gene region-specific CpG islands. Bonilla et al. [38] also found that methionine concentration in embryo culture medium improved the development; however optimal development was achieved at methionine concentrations lower than what usually exists in the uterus of cows with mean plasma methionine concentrations of ~ 20µmol/L.

In conclusion, supplementation of methionine only during the periparturient period and 30 days after calving switched to control diet affect global DNA methylation. Also, when we analyze the effect of GRP, supplementation of methionine increase the lipid accumulation in preimplantation embryos. Methionine supplementation seems to impact the preimplantation embryo in a way that enhances its capacity for survival since there is strong evidence that endogenous lipid reserves serve as an energy substrate.

ACKNOWLEDGEMENTS

This project was supported in part by a grant from Adisseo USA. Special appreciation is extended to the staff of the University of Illinois Dairy Research Unit; Dr. Glaucio Lopes and Accelerated Genetics for providing semen; and Dr. Richard Wallace and Zoetis for providing CIDR, Factrel, and Lutalyse used in this projectand alsothe authors thank to Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Programa de Estudantes-Convênio de Pós-Graduação (PEC-PG) for the financial support.

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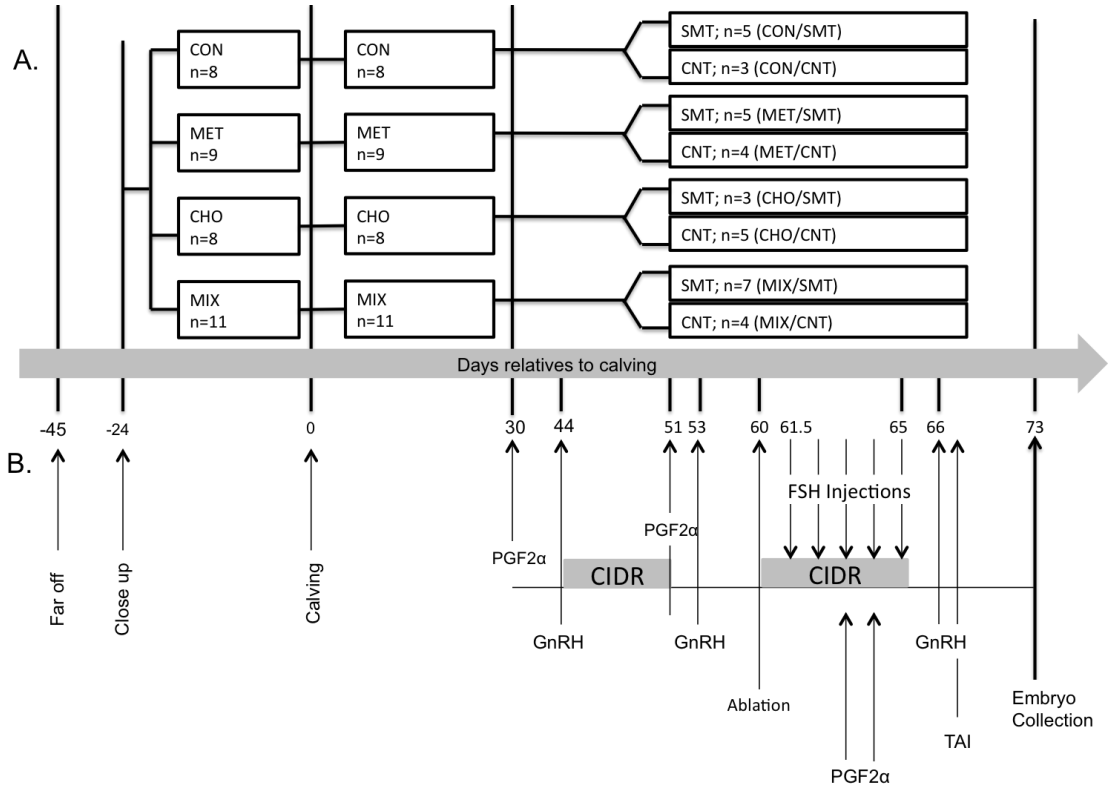

Figure 1. Schematic design of the experimental groups (A) and protocols for synchronization, superstimulation and embryo collection (B). Note that there were a total of 8 treatments after 30 DIM: **CON-CNT** (n = 3), **CON-MET** (n = 5), **MET-CNT** (n = 4), **MET-SMT** (n = 5), **CHO-CNT** (n = 5), **CHO-SMT** (n =3), **MIX-CNT** (n = 4), and **MIX-SMT** (n = 7).

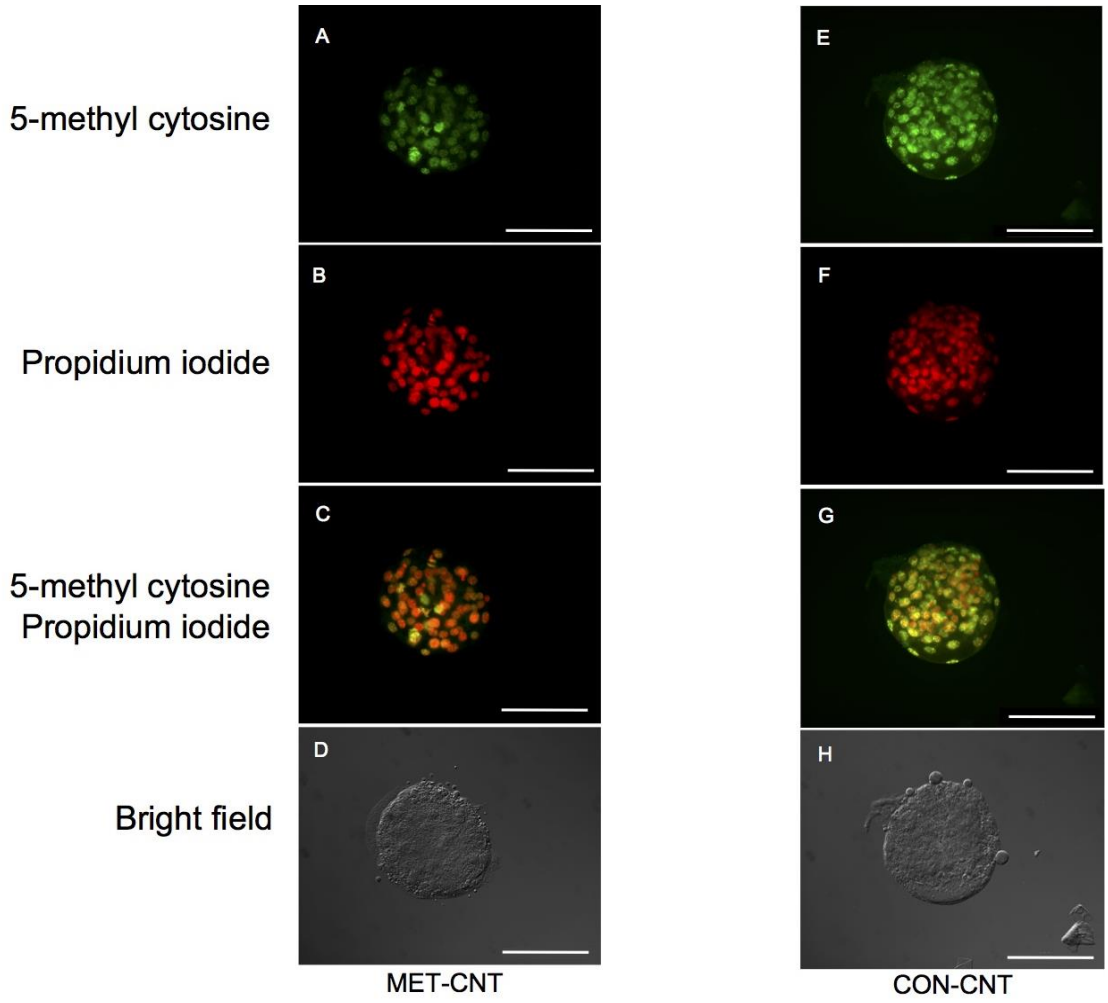

Figure 2. Immunolabeling of 5-methyl cytosine in embryos from cows fed a methionine supplemented diet (MET/CNT; panels A to D) or a control diet (CON/CNT, panels E to H) (scale bars = 100 μm). Note that the labeling intensity in E is higher than A. A, E: 5-methyl cytosine; B, F: propidium iodine; C, G: merged image of 5-methyl cystosine and propidium idoine; D, H: bright field image.

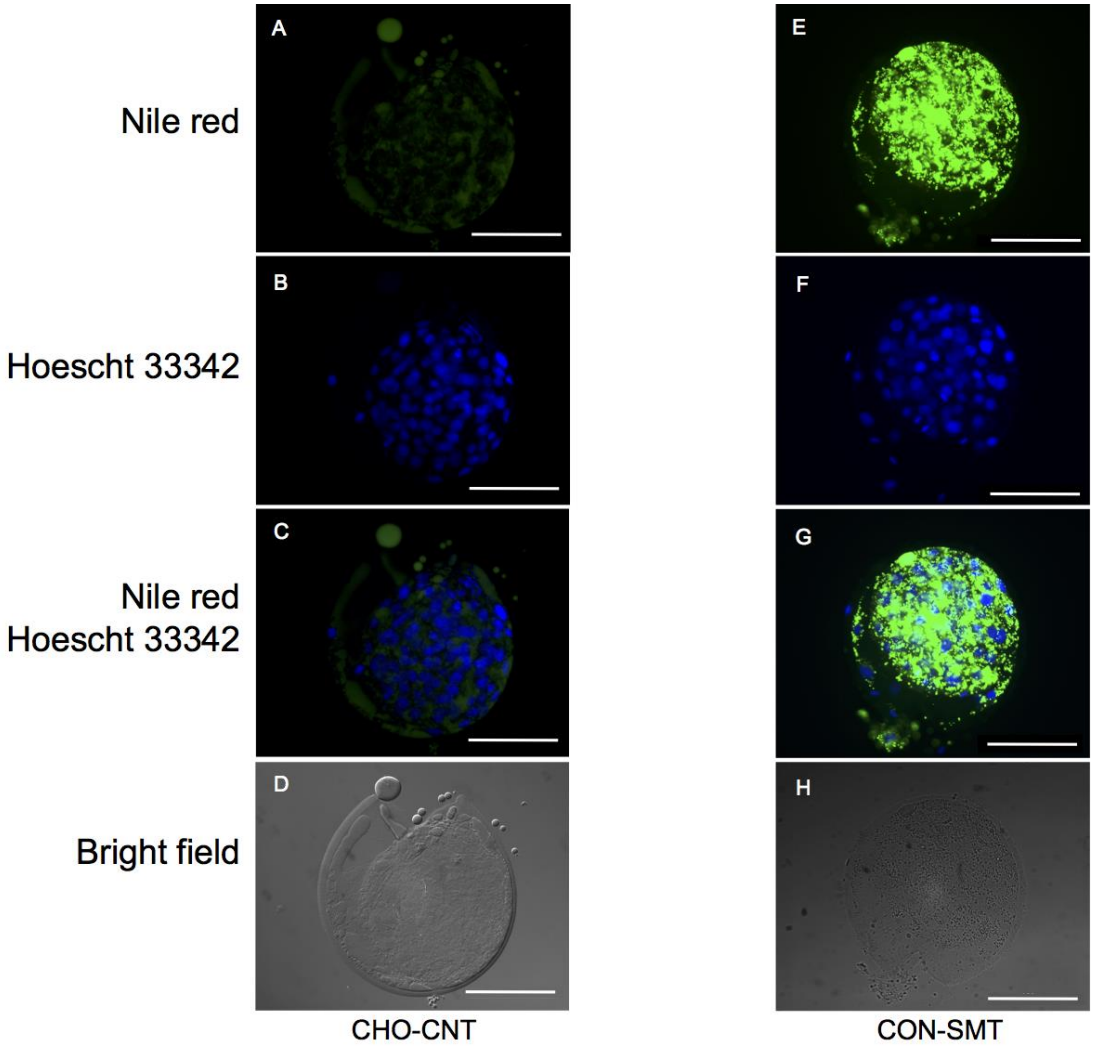

Figure 3. Nile red labeling for analysis of lipid content in embryos produced in vivo from cows fed methionine (**SMT**, fed the basal diet plus methionine) (panels A to D) or a control diet (**CNT**, fed a basal diet) after 30 days in milk (panels E to H) (scale bars = 100 μm). Note that the labeling intensity in A is higher than E. A, E: Nile red labeling; B, F: Hoescht 33342 labeling (nuclear stain); C, G: merged image of Nile red and nuclear labeling; D, H: bright field image.

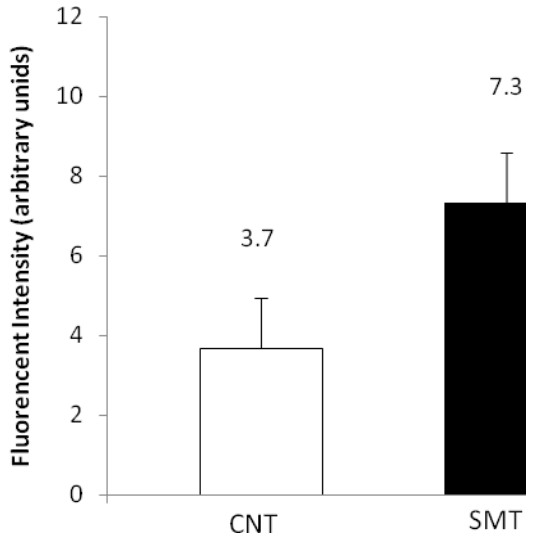

Figure 4. Lipid accumulation in embryos from cows supplemented with rumen-protected methionine (SMT) or non-supplemented (CNT). There was no treatment [**TRT**; fed the close-up and fresh cow diets supplemented with methionine (MET), choline (CHO) or both (MIX) and non-supplemented (CON) from -21 to 30 days in milk (DIM)] effect (P = 0.64) or interaction TRM by Group [**GRP**; fed the high cow diets supplemented with 0.08% (w/w) of the dry matter intake as methionine (SMT) and non-supplemented CNT from 31 to 72 DIM] (P = 0.58). There was an effect of GRP (P = 0.04).

Table 1. Ingredient composition of diet fed to cows from 21 days before calving until 30 days after calving and from 31 to 72 days after calving.

	Period		
Ingredient, % of DM	1[a]	2[b]	3[c]
Alfalfa silage	8.34	5.07	6.12
Alfalfa hay	4.29	2.98	6.94
Corn silage	36.40	33.41	35.09
Wheat straw	15.63	2.98	-
Cottonseed	-	3.58	3.26
Wet brewers grains	4.29	9.09	8.16
Ground shelled corn	12.86	23.87	25.09
Soy hulls	4.29	4.18	4.74
Soybean meal, 48% CP	2.57	2.39	2.45
Expeller Soybean meal[2]	2.57	5.97	1.22
Blood meal	0.86	1.50	1.43
Urea	0.30	0.18	0.33
Rumen-inert fat[1]	-	1.02	1.43
Limestone	1.29	1.31	1.14
Salt	0.30	0.30	0.30
Dicalcium phosphate	0.18	0.30	0.30
Magnesium oxide	0.08	0.12	0.12
Sodium bicarbonate	-	0.79	0.78
Potassium carbonate	-	0.30	0.30
Calcium sulfate	-	0.12	0.12
Mineral and vitamin mix[3]	0.17	0.18	0.53

[1]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN)
[2]SoyPLUS (West Central Cooperative, Ralston IA)
[3]Mineral and vitamin mix was formulated with 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg/kg of Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg of Se, 2,200 kIU/kg of vitamin A, 660 kIU/kg of vitamin D_3, and 7,700 IU/kg of vitamin E
[a]Period 1; a close-up cow diet from 21 days before calving until parturition.
[b]Period 2; a fresh cow diet from 0 to 30 days in milk (DIM).
[c]Period 3; a high cow diet from 31 to 72 DIM.

Table 2. Mean chemical composition and standard deviation of diet fed to cows from 21 days before calving until 30 days after calving, and from 31 to 72 days after calving.

	Period[1]			
Item	**1**	**2**	**3**	**SD[2]**
DM, %	47.1	47.9	47.1	0.57
CP, % of DM	18.0	17.6	18.3	0.27
ADF, % of DM	22.7	24.4	23.2	1.05
NDF, % of DM	35.6	37.3	36.3	1.71
Lignin, % of DM	4.53	4.00	3.80	0.29
NFC, % of DM	33.4	32.6	33.2	1.71
Starch, % of DM	22.3	21.4	23.6	1.73
Crude fat, % of DM	5.23	4.70	4.57	0.19
Ash, % of DM	7.79	7.77	7.59	0.25
TDN, % of DM	70.0	69.0	70.0	0.77
NE_L, Mcal/kg of DM[3]	1.63	1.63	1.65	0.03
Ca, % of DM	0.98	1.07	1.02	0.10
P, % of DM	0.44	0.45	0.45	0.01
Mg, % of DM	0.28	0.29	0.31	0.01
K, % of DM	1.51	1.55	1.41	0.05
Na, % of DM	0.30	0.38	0.32	0.01
S, % of DM	0.24	0.25	0.27	0.01
Fe, ppm	493	955	480	231
Zn, ppm	156	210	191	18.3
Cu, ppm	27.0	38.0	33.3	3.01
Mn, ppm	120	138	133	5.39
Mo, ppm	0.83	0.87	0.77	0.10

[1] Period 1: a close-up cow diet from 21 days before calving until parturition; period 2: a fresh cow diet from 0 to 30 days in milk (DIM); and period 3: a high cow diet from 31 to 72 DIM.
[2] Maximum value within periods.
[3] NRC, 2001.

Table 3. Least squares means and associated standard errors for embryo recovery, embryo quality, embryo stage, number of CL for cows receiving no supplementation (CON), rumen-protected methionine (MET), rumen-protected choline (CHO), rumen-protected methionine and rumen-protected choline (MIX) fresh cow diet from 21 days before calving until 30 days after calving; and no supplementation (CNT) or rumen-protected methionine (SMT) from 31 to 72 days after calving.

Items	Treatment					Group			*P*-Value		
	CON	MET	CHO	MIX	SEM	CNT	SMT	SEM	TRT[1]	GRP[2]	TRT×GRP[3]
Recovery rate, %	69.7	78.6	77.6	67.9	17	75.5	73.7	11	0.93	0.44	0.59
Embryos recovered, no.	6.62	9.9	8.3	5.5	1.25	6.95	8.25	1.59	0.44	0.36	0.31
CL number, no.	9.5	12.6	10.7	8.1	1.4	9.2	11.2	0.9	0.11	0.13	0.01
Embryo quality[4]	1.82	2.33	2.14	1.38	0.4	1.69	2.15	0.27	0.28	0.22	0.50
Embryo stage[4]	3.85	4.08	3.88	4.13	0.24	3.88	4.10	0.13	0.81	0.23	0.90

[1]TRT: Treatments, a close-up and a fresh cow diet from 21 days before calving until 30 days after calving.

[2]GRP: Group, a fresh cow diet from 31 to 72 days after calving.

[3]TRT × GRP: Treatment by group interaction.

[4]Classification according to the IETS. Manual of the International Embryo Transfer Society. Savoy, Illinois. 180 pp. 1999.

Table 4. Least squares means and associated standard errors for embryo methylation, lipid accumulation, and number of embryo cells for cows receiving no supplementation (CON), rumen-protected methionine (MET), rumen-protected choline (CHO), rumen-protected methionine and rumen-protected choline (MIX) from 21 days before calving until 30 days after calving; and no supplementation (CNT, evaluated embryos n= 38) or rumen-protected methionine (SMT) from 31 to 72 days after calving.

	Treatment					Group			*P*-Value		
Items	CON[5]	MET[6]	CHO[7]	MIX[8]	SEM	CNT[9]	SMT[10]	SEM	TRT[1]	GRP[2]	TRT×GRP[3]
Number of cells[4]	60.29	57.92	50.87	50.84	13.17	54.61	55.34	8.58	0.89	0.95	0.44
Methylation[4]	0.69	0.53	0.77	0.62	0.10	0.70	0.60	0.06	0.20	0.17	0.01
Lipid accumulation[4]	6.74	5.12	3.60	6.81	2.37	3.89	7.26	1.43	0.64	0.12	0.58

[1]TRT: Treatments, from 21 days before calving until 30 days after calving. [2]GRP: Group, from 31 to 72 days after calving. [3]TRT × GRP: Treatment by group interaction.[4]Fluorescent intensity. [5]CON, evaluated embryos n= 24. [6]MET, evaluated embryos n=35. [7]CHO, evaluated embryos n= 19. [8]MIX, evaluated embryos n= 34. [9]CNT, evaluated embryos n=38. [10]SMT, evaluated embryos n= 74.

4 Considerações Finais

A administração de somatotropina pré-parto em novilhas leiteiras aumentou o IGF-I sérico e intrafolicular, além de aumentar a expressão de genes relacionados a esteroidogênese em células foliculares do folículo dominante da primeira onda pós-parto. Essas mudanças foram associadas a um aumento da concentração sérica e intrafolicular de estradiol, que pode, potencialmente, aumentar a chance de ovulação da primeira onda folicular pós-parto. Apesar disso, a somatotropina não afetou outros parâmetros metabólicos e produtivos no período pós-parto recente.

A suplementação com metionina, colina ou ambas durante o período de transição parece não ter efeito sobre o desenvolvimento folicular pós-parto da primeira onda em vacas da raça Holandês. No entanto, a suplementação apenas com metionina durante o período de transição aumenta a expressão de *3B-HSD* nas células foliculares, o que poderia afetar a produção de progesterona. Além disso, a suplementação com metionina e colina induziu a uma *down regulation* dos genes pró-inflamatórios, indicando uma redução na intensidade de processos inflamatórios nas células foliculares.

A suplementação com metionina durante o período de transição de vacas da raça Holandês diminuiu a metilação global do DNA e aumentou o acúmulo de lipídios nos embriões. Além disso, a suplementação de metionina parece afetar o embrião de uma forma que aumenta a sua capacidade de sobrevivência, desde que há indícios de que as reservas lipídicas endógenas servem como um substrato energético.

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Anexos

UFPel
CEEA
Comissão de Ética em Experimentação Animal

UFPEL FLS. 19 CEEA

Pelotas, 11 de abril de 2011

De: Prof. Dr. Orlando Antonio Lucca Filho

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Prof. Marcio Nunes Correa

Faculdade de Veterinária

Senhor(a) Professor(a):

A CEEA analisou o projeto intitulado: **"Efeito do uso de rbST no período de pré-parto sobre os indicadores de balanço energético e potencial esteroidogênico do folículo da primeira onda pós-parto em vacas"**, processo n°23110.002562/2011-15, sendo de parecer **FAVORÁVEL** a sua execução considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste Projeto ser cadastrado junto ao Departamento de Pesquisa para posterior registro no COCEPE (Código para Cadastro n° **CEEA 2562**).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Orlando Antonio Lucca Filho

Presidente da CEEA

Ciente em: 12/04/2011

Assinatura do Professor Coordenador: