Universidade Federal de Minas Gerais
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Efeitos do tratamento de bezerras em aleitamento com somatotropina recombinante bovina sobre o eixo somatotrópico e parâmetros imunes

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“Great things never came from confort zone”

(autor desconhecido)

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Sumário

RESUMO

O desmame é um evento desafiador para bezerros leiteiros que deve ser enfrentado em um momento no qual os animais ainda são imunologicamente imaturos. O presente trabalho objetivou avaliar os efeitos do tratamento de bezerras leiteiras com somatotropina recombinante bovina (bST) sobre o eixo somatotrópico e parâmetros imunes. Para a realização do experimento foram utilizadas 36 bezerras fêmeas entre 21 e 63 dias, que foram distribuídas aleatoriamente entre os dois tratamentos: Controle = Salina (0,9%); bST = 1.5 to 1.8mg/Kg de bST a cada 7 dias. Concentrações de Hormônio da Crescimento (GH) ($P < 0.01$) e Fator de Crescimento Semelhante à Insulina do Tipo I (IGF-1) ($P < 0.01$) apresentaram aumento significativo nos animais tratados com bST. Nenhuma diferença foi encontrada quanto à atividade de neutrófilos, no que diz respeito à fagocitose ($P = 0.74$) e ao estresse oxidativo ($P = 0.71$). O desmame causou um impacto negative sobre os parametros imunes avaliados. O eixo somatotrópico e as análises metabólicas sugerem que os bezerros enfrentaram um perído de balanço energético negativo, com o possível desacoplamento do eixo somatotrópico. A estratégia de tratamento utilizada não foi suficiente para provocar melhora nos parâmetros imunes avaliados.

ABSTRACT

Weaning may be associated with negative energy balance and body weight loss when calves are still immunological incompetent, predisposing them to infectious diseases. The aim of the present experiment was to investigate the effects of treatment of pre-weaned dairy calves with recombinant bovine somatotropin (rbST) on the somatotropic axis, on selected immune parameters, and hematology of calves around weaning. Thirty-six Holstein female calves were randomly assigned to receive 1.5 to 1.8 mg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) per Kg of body weight or to receive injections of saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS), every 7 days, from 21 to 63 days of life. Calves were fed milk replacer ad libitum from birth to 38 d of age (d 0), when progressive weaning started, and calves were weaned at 49 d of age. Calves were weighed at birth and weekly from 21 to 63 d of age, when wither height was also measured. Calves were vaccinated with 0.5 mg of ovalbumin on study d -17 and 4. Blood samples were collected on d -17, -14, -10, 0, 11, 14, 18, and 25. Polymorphonuclear leukocytes were isolated and challenged ex vivo with E. coli to determine phagocytosis and oxidative burst capacity. Additionally, expression of CD62L and CD18 by granulocyte, lymphocyte, and monocyte CD14+ were determined. Blood samples were also used to determine hematological parameters and concentrations of growth hormone, insulin like growth factor-1, insulin, glucose, non-esterified fatty and beta-hydroxybutyrate, haptoglobin, and IgG anti-ovalbumin. Calves treated with rbST had greater concentrations of growth hormone and insulin-like growth factor 1 from d -14 to 25 than control calves, whereas insulin, non-esterified fatty and beta-hydroxybutyrate concentrations did not differ. On d 0, glucose concentration was greater for rbST treated calves. Treatment did not affect PMNL phagocytosis and oxidative burst, but intensity of expression of CD62L and CD18 by granulocytes tended to be increased by rbST treatment. Treatment did not

affect the IgG anti-ovalbumin concentration. Haptoglobin concentration was reduced in rbST treated calves on d 14 and there was a tendency for hematocrit to be lower in rbST treated calves. Treatment did not affect body weight, wither height and average daily gain, despite the fact that rbST treated calves had lower daily milk replacer intake. The relatively minor improvements in immune responses resulting from rbST treatment of weaning calves may not be sufficient to reduce the incidence of infectious diseases.

Key words: dairy calves, somatotropin, immune parameters

INTRODUÇÃO

Bezerros neonatos são imunologicamente imaturos e se apoiam nos mecanismos inatos e maternos, adiquiridos através do consumo de colostro, para se protegerem de doenças. As respostas conseguidas por estes animais são normalmente primárias e caracterizadas por longos intervalos e baixas concentrações de imunoglobulinas (Igs) (Barrington et al., 2001). O desenvolvimento pleno do sistema imune não ocorre rapidamente e sua maturidade deverá ser atingida apenas próxima aos seis meses de idade (Kampen et al., 2006).

Um fator agravante para o comprometimento da saúde dos neonatos é o fato de que os anticorpos e os fatores de proteção passiva adquiridos pela ingestão do colostro, começam a desaparecer da circulação por volta dos 16 aos 28 dias de vida. Isto cria a chamada "janela de susceptibilidade", um período no qual os níveis de proteção materna ainda interferem na resposta imune dos animais e estes ainda não tem mecanismos de proteção próprios desenvolvidos. Sendo assim, os animais se tornam mais predispostos ao desenvolvimento de doenças (Chase et al., 2008). Em muitas propriedades o desaleitamento pode conincidir ou ser bastante próximo ao período compreendido nesta janela, agravando ainda mais o desafio a ser enfrentado pelos animais.

Durante o período de transição vacas leiteiras apresentam depressão do sistema imune, que está diretamente relacionada à ocorrência de várias doenças no pós parto imediato (Crookenden et al. 2016). Essa condição de imunossupressão pode ser semelhante ao que ocorre com bezerras próximo e após o período de desaleitamento, um evento considerado estressante por vários autores. Hulbert (2011) mostrou que o desaleitamento causou em bezerras leiteiras um período transitório de menor atividade de neutrófilos, independente da idade em que os animais foram desaleitados. Ainda em 2011 o relatório publicado pelo *United States Department of Agriculture* (USDA)

intitulado *Heifer Raiser* mostrou que 4.2% de todas as bezerras em aleitamento morreram em 2010. As principais causas de óbito foram problemas digestivos e respiratórios (USDA, 2012).

Apesar de notadamente conhecido por seu poder de ação sobre o metabolismo dos mamíferos, o Hormônio do Crescimento (GH do *inglês growth hormone*) possui também papel significativo na regulação do sistema imune, segundo demonstrado por autores que trabalharam com humanos e roedores (Kiess et al., 1983; Weigent et al.,1996; Kelley et al., 2007). Weigent et al. (1992) demonstrou que além de poderem ser reguladas pelo GH, algumas células do sistema imune de roedores, como os leucócitos, tem a capacidade de produzir pequenas quantidades do hormônio, assim como do fator de crescimento semelhante a insulina do tipo I (IGF-1 do inglês *insulin-like growth fator – I*). Este último achado criou a hipótese de que não apenas o GH poderia atuar diretamente sobre o sistema imune, como também poderia atuar de forma indireta por meio de mecanismos mediados pelo IGF-1 (Tapson et al., 1988).

Alguns estudos já foram conduzidos em outras espécies animais com o intuito de demonstrar os efeitos do GH sobre o sistema imune. Leitões tratados com somatotropina recombinante suína próximos ao desmame apresentaram maiores concentrações de IGF-1, IgM e maior quantidade de neutrófilos um dia após o desmame nos animais tratados (Kojima et al., 2008). Vacas leiteiras também foram utilizadas em experimentos que visavam identificar os efeitos do bST sobre a imunidade durante o período de transição. Porém os protocolos de tratamento utilizados foram bastante diferentes quanto a dose utilizada, o tempo e a periodicidade, e também quanto aos resultados (Silva et al., 2015; Gulay et al., 2007; Gohary et al., 2014). Mas estudos visando a avaliação do GH sobre parâmetros imunes específicos ainda não foram executados em bezerros durante o período que compreende o desaleitamento. O objetivo do trabalho foi

determinar os efeitos do tratamento de bezerras leiteiras ao redor do desmame com bST sobre parâmetros imunes e sobre o eixo somatotrópico destes animais.

Revisão de literatura

Eixo Somatotrópico

O eixo somatotrópico é o sistema formado por hipotalamo, hipófise e fígado, responsável pela sinalização hormonal destas glândulas, que resulta na produção do GH, que por sua vez desencadeia a produção de IGF-1 no órgão hepático (Quik, 2012). Além destes hormônios, participam do eixo de forma direta, proteínas carreadoras e receptores específicos, e de forma indireta hormônios como a insulina, leptina e tireoidianos.

Hormônio do Crescimento (GH)

O hormônio do Crescimento (GH do inglês *Growth hormone*), também conhecido como Somatotropina, é produzido e secretado de forma pulsátil por células especializadas chamadas somatotrofos. Estas células estão localizadas no lobo anterior da glândula pituitária ou hipófise. Sua síntese e liberação são primeiramente controladas pelo hormônio hipotalâmico liberador de GH (GHRH do inglês *growth hormone releasing hormone*) e pela somatostatina (SST ou SRIF do inglês *somatotropin release inhibiting factor*). Estes por sua vez, são regulados em um mecanismo de feedback pelas concentrações sanguíneas do GH e do IGF-1 (Bartke, Sun e Longo, 2013).

A liberação do GH no organismo é fortemente estimulada pela hipoglicemia e inibida pelo consumo de alimentos em excesso, hiperglicemia e obesidade (Cornford et al., 2012). Nos ruminantes o aumento da atividade, o jejum e o estresse também estimulam a liberação do GH, assim como alguns hormônios esteroides, como a testosterona e o estrógeno. Estes hormôneos reprodutivos podem contribuir para a elevação prepubertal das concentrações de GH e contribui para o crescimento acelerado dos mamíferos durante este período (Bartke, Sun e Longo, 2013).

Uma quantidade significativa do GH total circulante é encontrada na circulação liagada à

proteínas ligantes que tem alta afinidade pelo hormônio, conhecidas como GH *binding proteins* (GHBP). Diferenças específicas entre espécies existem no que diz respeito a estrutura destas proteínas, porém um aspecto comum a todas é o fato de que são constituídas de fragmentos do domínio extracelular dos receptores de GH (Schilbach e Bidlingmaier, 2015).

O GH por si só é responsavél por regular uma série de processos fisiológicos, porém o mecanismo de ação pelo qual esses processos podem ser ativados depende da interação do hormônio com receptores específicos do tipo citocinas de classe I. Estes receptores possuem um domínio simples transmembranal e um domínio extra celular (Bazan, 1990). O domínio membranal proximal contém um padrão WSxWS, que é composto de uma dupla sequência de Trp-Ser-Xaa-Trp-Ser (sendo que Xaa representa qualquer aminoácido). Este padrão cria um arranjo aromático, que por sua vez tem grande importância para a expressão e estabilidade destes receptores (Dagil et al., 2011). O domínio extracelular é composto de dois sítios de ligação, sendo o sítio 1 o local pelo qual o GH tem maior afinidade. Ambos são sustentados por um terceiro sítio chamado domínio FNIII, caracterizado por fibronectinas do tipo III.

Uma outra estrutura importante dos recpetores de GH é o padrão rico em prolina BOX 1, que em conjunto com o padrão distal BOX 2 que possui sequências aromáticas e de resíduos ácidos, permitem a ligação coesa entre a membrana celular interna e a enzima tirosina quinase, chamadas *Janus Kinase 2* (JAK2). Estas proteínas por sua vez são necessárias para completar a sinalização intracelular e os processos regulados por ela compreendem o crescimento longitudinal pós-natal, reprodução e lactação, atravéz da regulação do metabolismo, da composição corporal, do controle de respostas inflamatórias, formação óssea e eritropoiese. Porém uma das principais ações promovidas pelo GH é o estímulo para expressão de gênica de IGF-1 no fígado (Bartke, Sun e Longo, 2013; Waters e Books, 2015).

O receptor de GH é ativado a partir da ligação de sua molécula bivalente ao sítio de maior afinidade do domínio extracelular. Esta ligação promove uma reorientação e uma rotação das subunidades do receptor, resultando em uma transição da forma paralela do domínio transmembranal para a forma aspiral e uma consequente separação das bases deste domínio no ponto de entrada do citoplasma celular. Essa mudança estrutural do receptor permite que o domínio inibitório de uma proteína pseudoquinase seja deslocado das duas enzimas JAK2. Esse movimento irá permitir que as JAK2s acopladas ao domínio transmembranal interajam e possam ser trans-ativadas. O resultado de todo esse processo é a fosforilação e a ativação dos caminhos de sinalização que irão regular todos os processos supracitados (Waters e Books, 2015).

Fator de Crescimento Semelhante à Insulina tipo 1 (IGF-1)

O hormônio Somatomedina C, ou IGF-1, é principalmente sintetizado e liberado pelo fígado, pelas células chamadas hepatócitos (Butler et al., 2002). Porém a produção local de IGF-1 em menor intensidade em outros tecidos, como nas gônadas e células imunes, já foi demonstrada em humanos e roedores (Weigent et al., 1992; Kelley et al., 2007). O IFG-1 é responsável por mediar várias funções atribuídas ao GH, sendo algumas destas estimular a proliferação celular e o crescimento. Além destas, o homônio também possue ações independentes como inibir a apoptose, reproduzir alguns efeitos da insulina e inibir a liberação de GH em um mecanismo de feedback negativo.

O IGF-1 exerce seus efeitos ao nível celular através de sua interação com receptores do Tipo I, que pertecem a família das tirosina kinases, sendo compostos por subunidades α e β, com estruturas peptídicas semelhantes aos receptores de insulina. Estes receptores estão presentes em quase todos os tecidos do organismo, localizando-se nas membranas das células. Sua sinalização primária é baseada em ativação via fosforilação de substratos de receptores de insulina (IRS-1 e IRS-2). A

somatomedina também possui a capacidade de se ligar a receptores do Tipo II, que tem maior afinidade pelo IGF-2, e a receptores de insulina (Cohick e Clemmons, 1993).

A maior parte do IGF-1 presente na circulação sanguínea e em espaços extracelulares se encontra na forma ligada a proteína de alta afinidade IGFBP (IGF *Binding Protein*). Existem seis principais tipos destas proteínas carreadoras, mas a maior parte do IGF-1 encontra-se ligado à IGFBP-3 (Renaville et al., 2002). Estas são responsaveis não apenas por carrear o hormônio, como também tem um papel importante na regulação de suas atividades. As IGFBPs são responsáveis por prolongar a meia vida e regular o clearance metabólico do IGF-1 e por modular a interação do hormônio com seus receptores (Jones e Clemmons, 1995).

Em um experimento conduzido gerando mutações em roedores a fim de extinguir a expressão gênica de receptores do GH no fígado, foi demonstrado que existe uma dependência do IGF-1 pelo GH, para sua síntese e liberação por este órgão. A consequência direta da ausência de expressão dos receptores de GH no fígado foi a redução drástica das concentrações de IGF-1 imunoreativos na circulação sanguínea. Porém, em outros tecidos como cérebro, rins e coração, a expressão de IGF-1 parece ser independente do estímulo do GH (Lupu et al., 2001). Estes achados caracterizam um quadro temporário de desacoplamento do eixo somatotrópico, que ocorre fisiológicamente em bovinos leiteiros de raças especializadas por exemplo, em período de transição, também conhecido como (Kim et al., 2014).

Imunidade

A imunidade se desenvolveu a partir da necessidade de eliminar patógenos infecciosos do corpo,

e esta pode ser compreendida em dois principais mecanismos nos mamíferos: a imunidade inata e a adquirida (Akira et al., 2006). A presença de um organismo desconhecido desencadeará uma série de processos executados pelo sistema imune com a intenção de proteger o hospedeiro.

A imunidade inata é normalmente a primeira linha de defesa do organismo e seus elementos já estão presentes no animal ao nascimento. São células com atividade fagocitária, como macrófagos, neutrófilos, eosinófilos e basófilos, assim como células dendríticas e as chamadas *Natural Killers*. A primeira resposta à esta invasão do organismo por um agente estranho ocorre normalmente pelo reconhecimento inespecífico de estruturas moleculares associadas ao patógeno, conhecidas pela sigla PAMP (do inglês *pathogen-associated molecular patterns*), mediado por receptores de expressos nos componentes do sistema imune inato (Kumar et al., 2011). Estas podem ser expressas na superfície das células, em compartimentos intracelulares, secretadas na corrente sanguínea ou em fluidos teciduais, podendo ser proteínas, lipídeos, carboidratos e ácidos nucleicos, dependendo do patógeno (Janeway Jr. and Medzhitov, 2002).

Além deste primeiro passo, após o estímulo das células fagocíticas pelos PAMPs, o organismo desencadeia o processo inflamatório, mediado por citocinas e quimiocinas, na tentativa de destruir o patógeno. Durante este processo, as células de defesa inata irão receber sinais para deixar a circulação com o intuito de migrar até o local da infecção. Isto é possível por meio da adesão da célula à parede dos vasos e da diapedese através destes, processos estes mediados por moléculas específicas, como a CD62-L (L-selectina) por exemplo (Bonilla et al., 2013). Ao atingir o local, as células do sistema imune inato exercem atividades de autofagia e fagocitose, sendo a primeira responsável por eliminar conteúdos intracelulares por meio de encapsulamento e degradação lisossomal, e a segunda por engolfar material extracelular e submetê-lo ao estressese oxidativo, promovendo a liberação de enzimas e a produção de radicais livres, como oxigênios

reativos, dentro da própria célula, com o intuito de destruir o antígeno (Eissa et al., 2013). Concluído este processo, as células podem entrar em apoptose para prevenir que as moléculas utilizadas para destruir os antígenos, sejam expostas aos tecidos adjacentes ou que caiam na circulação, causando danos ao organismo do animal. Porém, dependendo da severidade da infecção, algumas células podem se tornar necróticas, liberando estas moléculas (Hulbert e Moisá, 2016).

A ativação do mecanismo de imunidade adquirida ocorre de forma secundária, mediante ao estímulo do sistema imune inato a partir do reconhecimento do antígeno. (Bayne et al., 2003). A quebra da estrutura do corpo estranho pelas células fagocitárias pode liberar peptídeos bacterianos, estruturas reconhecidas como antígenos pelas células T. Estas células irão então estimular as células B a produzirem clones secretores de anticorpos, por meio de mecanismos que envolvem repetidas divisões e diferenciações celulares. Com isso, caso ocorra a invasão de um mesmo antígeno, este será rapidamente reconhecido e os anticorpos vão se ligar à sua membrana com o intuito de facilitar sua destruição pelas células brancas (Cunningham et al., 2016).

Este sistema é portanto capaz de promover o reconhecimento específico de antígenos e construir uma memória imunológica da infecção, com células com maior tempo de meia vida que as do sistema inato. Isto permite que posteriormente o organismo gere uma resposta mais rápida a novas infecções, auxiliando no controle da expansão da resposta inata e diminuindo assim a intensidade das respostas inflamatórias (Cunningham et al., 2016).

Imunidade do bezerro neonato

O sistema imune dos bovinos começa a se desenvolver logo após a fecundação, ainda dentro do útero materno, e lentamente atinge a capacidade plena próximo à puberdade.

Grande parte das células de defesa, específicas ou não, estão presentes ao nascimento, porém até por volta dos 130 dias de gestação, a maioria delas não é liberada dos locais onde são produzidas para o sangue. Ainda dentro do útero a contribuição dos neutrófilos, apesar de importante, é prejudicada, pois apesar destas células já serem capazes de exercer a fagocitose, até o final da gestação seu poder de destruição do antígeno é reduzida (Barrington e Parish, 2001).

Os dias que antecedem o parto são marcados por uma mudança no funcionamento do eixo hipotalamopituitário adrenal e inflamatório do feto. O mecanismo de *feedback* negativo que regula a liberação de glicocorticoide (GC) pelo córtex da glândula adrenal fica inibido, fazendo com que este hormônio aumente rapidamente na circulação fetal. Este é um processo fisiológico que indica que o animal esta em estresse dentro do útero e é necessário ao desencadeamento do parto. A elevação do GC na circulação também é de extrema importância para a maturação das mucosas digestivas e respiratórias do feto, principalmente dos pneumócitos tipo II (Teitelbaum e Walker, 2005). Um fator negativo desse evento sobre a imunidade do neonato é o fato de que o cortisol produzido pode permanecer em concentrações elevadas por aproximadamente uma semana após o nascimento. O GC irá então exercer função inibitória sobre componentes do sistema imune, comprometendo suas atividades (Barrington e Parish., 2001).

Após o nascimento a fagocitose exercida pelos neutrófilos passa a ser um dos principais mecanismos de defesa de que dispõe o neonato, apesar da atividade destas células ainda se encontrar diminuída durante os primeiros dias de vida. Além dos fatores supracitados, o desaleitamento também poderá determinar posteriormente redução da fagocitose, sendo este um

evento de grande impacto no sistema imune de bezerras leiteiras. A atividade dos neutrófilos não apresentará níveis adultos antes dos 90 dias de vida (Hulbert et al., 2011).

O sistema imune alcança a maturidade na medida em que este atinge a puberdade. Isso geralmente ocorre nos bovinos por volta dos cinco a nove meses de idade. Porém a imaturidade do sistema imune não significa que o animal não irá responder ao desafio provocado por um antígeno, mas que sua resposta será mais lenta e fraca (Cortese et al., 2009).

Eixo somatotrópico e o sistema imune

O cérebro é capaz de organizar interações entre o sistema neuroendócrino e o sistema imune, mas os hormônios do eixo somatotrópico detem a capacidade de interferir diretamente no funcionamento de componentes da imunidade de forma parácrina e ou autocrina (Kelly et al., 2007). Experimentos neste sentido começaram a ser conduzidos há mais de 30 anos em roedores e humanos, sendo que em 1983 já foi possível demonstrar que o uso de GH em meio de cultura contendo linfócitos isolados de sangue perférico humano, promoveu a proliferação destas células, ao passo que a adição de soro anti-GH ao meio bloqueou este efeito (Butenandt et al., 1983).

Na tentativa de elucidar os efeitos do GH sobre as células do sistema imune trabalhou-se com roedores hipofisectomizados. A retirada da glândula hipófise destes animais levou a deficiência de vários hormônios e não apenas do GH, como por exemplo da oxitocina, do hormônio adrenocorticotrófico e da prolactina. Na ausência da hipófise foram observados alguns “defeitos” em atividades do sistema imune celular e do adquirido, como a redução da resposta por anticorpos a infecções. Porém, a administração de GH nestes animais, conseguiu retomar o desenvolvimento

de parte do sistema imune humoral, no que diz respeito à proliferação linfocitária (Gala, 1991; Kelley et al., 2007).

Ainda nos anos 90, pesquisadores identificaram que o sistema imune também tem a habilidade de produzir GH, porém em taxas pequenas quando comparadas aos somatotrófos. Estudos com leucócitos de ratos indentificaram RNAm específicos para GH dentro destas células, e que estas podem secretar espécies de GH imunoreativos (Weigent, 1996). Foi demonstrado também que o GH por si só é capaz de melhorar a atividade dos linfócitos T e B, de macrófagos, neutrófilos e de células *Natural Killers* (NK) (Kelley et al. 2007; Hattori et al., 1990).

Na mesma década foi também demonstrado que o IGF-1 é capaz de exercer regulação direta do sistema imune através da modulação da proliferação e diferenciação de células mielóides, de células linfocitárias, e da prevenção da apoptose por células dependentes de interleucinas (IL-3) (Heemskerk et al., 1999). Além disto, outros trabalhos demonstraram que as mesmas células do sistema imune, e de outros tecidos, que produzem GH, o fazem para o IGF-1, o que passou então a sugerir haver um mecanismo de regulação autocrina destes hormônios (Weigent et al., 1992) e que o IGF-1 pudesse ser também um fator mediador, pelo qual o GH exerce seus efeitos sobre o sistema imune.

A somatotropina recombinante bovina foi aprovada em 1993 pelo *Food and Drug Administration* (FDA) do *United States Department of Agriculture* (USDA), com o intuito de promover aumento da produção de leite em bovinos. Apesar disso, o uso do bST em bovinos leiteiros adultos já foi realizado experimentalmente a fim de promover melhora no status imune destes animais, em situações nas quais estes se encontram normalmente imunossuprimidos, como durante o periodo de transição por exemplo.

Silva et al. (2015) utilizaram o bST em três diferentes doses, 0mg (controle), 87,5mg e 125mg, em animais em período de transição com escore de condição corporal (ECC) acima de 3,75 pontos (escala de 1 a 5, Ferguson et al. (1994)). O objetivo do trabalho foi avaliar os efeitos do bST sobre parâmetros imunes destes animais. Os resultados mostraram que os animais tratados com a maior dose apresentaram maior atividade de neutrófilos quanto a fagocitose e ao estress oxidativo, maior produção de anticorpos e expressão da molécula de adesão CD-62L (L-selectina), no dia do parto.

Os autores deste trabalho também relataram que houve menor incidência de metrite nos animais tratados. Em 2007 e 2014 outros autores também conduziram trabalhos com a idéia de avaliar o uso do bST sobre a imunidade de vacas leiteiras em período de transição, porém não foram identificadas melhoras em nenhum dos parâmetros imunes avaliados (Gulay et al., 2007; Gohary et al., 2014).

O conceito do uso de GH em animais leiteiros durante o período de transição, com a intenção de melhorar parâmetros de saúde, ainda é bastante discutido entre pesquisadores, principalmente no que diz respeito à dose e à periodicidade com a qual a aplicação dos tratamentos deve ser feita. Mas um objetivo comum tem sido aumentar o aporte de GH e consequentemente, aumentar a produção de IGF-1, para que estes exerçam sua influência em parâmetros imunes específicos, como a atividade de neutrófilos, expressão de moléculas de adesão e aumento do número de células leucocitárias.

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Effects of treatment of pre-weaned dairy calves with recombinant bovine somatotropin on immune responses and somatotropic axis

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INTRODUCTION

Neonatal calves are immunologically na.ve and rely heavily on passive transfer of immunoglobulins through colostrum and on innate immune mechanisms as their first line of defense against pathogens (Barrington et al., 2001). The development of the immune system until maturity is a slow process and lymphocyte populations reach complete differentiation and polymorphonuclear leukocytes complete function by approximately six months of life (Kampen, 2006). On the other hand, the immunoglobulins acquired from colostrum are practically extinguished from circulation by approximately 21 days of age (Chase et al., 2008; Hulbert and Mois., 2016), creating a window of susceptibility.

Weaning is another challenging period for dairy calves, which in many herds is also associated with regrouping (hutches to small groups, small groups to larger groups). Common metabolic changes associated with weaning are decreased circulating concentrations of glucose, insulin, and IGF-1 and increased circulating concentrations of beta-hydroxybutyrate (BHB) and, in some instances, non-esterified fatty acids (Omidi-Mirzaei et al., 2015; Sch.ff et al., 2016). Less is known about the associations between weaning and changes in immune parameters. Hulbert et al. (2011) demonstrated that calves weaned at 47 d of age, after being 70 fed 454 g/d of milk replacer (DM), had reduced percentage of PMNL positive for phagocytosis and oxidative burst and reduced intensity of phagocytosis ex-vivo from 45 to 66 d of age. Researchers have proposed that calves should be fed 15 to 20% of their live body weight of milk containing 20 to 25% of fat and 28% of protein (DM basis) to achieve maximum growth, with possible positive impacts on future weight gain, reproductive performance, and milk yield in the first lactation (Gelsinger et al., 2016; Soberon et al., 2012). A significant challenge for this strategy is the weaning period because calves may not consume enough solid feed (starter and forage) to compensate for the nutritional

needs in the absence of milk feeding (Miller-Cushon et al., 2013). Therefore, calves undergoing weaning may present negative energy balance (NEB) and consequently alterations in metabolic profile that could impair immune function.

Insulin like growth factor-1 is an important factor in proliferation, differentiation, and hypertrophy of various cell types, including immune cells (LeRoith and Roberts, 1991). Culture of human granulocytes in the presence of different concentrations of IGF-1 reduced the percentage of apoptotic granulocytes by 34 to 40% compared with culture of granulocytes in the absence of IGF-1 (Kooijman et al. 2002). Similarly, there was a 45% decrease in DNA fragmentation in granulocyte cultured with 6.5 nM IGF-1 compared with culture of granulocytes in the absence of IGF-1 (Kooijman et al. 2002). Culture of polymorphonuclear leukocyte (PMNL) with human growth hormone (GH; 100 to 1,000 ng/ml) or IGF-1 (100 to 1,000 ng/ml) increased the percentage of E. coli killed and increased the expression of CD11 (Inoue et al., 1998). Treatment of humans and rats deficient in GH with somatotropin (ST) resulted in increased circulating concentrations of IGF-1, PMNL, and antibodies (Kimata and Yoshida, 1994; Ibanez et al., 2005; Sohmiya et al., 2005). Thus, the reduced concentration of IGF-1 observed during the weaning period could partly explain the immunosuppression observed during this phase of life. Piglet treated with porcine ST (0.5 mg/kg) for 5 days and subjected to weaning and transportation had increased circulating concentrations of IGF-1, neutrophils, and IgM in the first 24 h after weaning compared with piglets not treated with ST and subjected to weaning and transportation (Kojima et al., 2008). In recent experiments, our group demonstrated that cows treated with 125 mg of rbST from -21 to 28 d relative to calving, a period characterized by intense NEB and immunosuppression, had greater intensity of phagocytosis and oxidative burst by PMNL, greater circulating concentration of anti-ovalbumin IgG, and reduced incidence of retained fetal membranes and metritis (Silva et al., 2015;

Silva et al., 2017).

The hypotheses of the current experiment were that treatment of weaning calves with rbST would increase GH and IGF-1 concentrations and consequently improve selected innate and adaptive immune parameters. Additionally, we hypothesized that treatment of weaning calves with rbST would only minimally affect metabolic parameters. Therefore, the objectives of the current experiment were to evaluate the effects treating weaning calves with rbST on GH and IGF-1 concentrations, on PMNL phagocytosis and oxidative burst, on expression of adhesion molecules by granulocytes, lymphocytes, and CD14+ monocytes, on concentration of IgG anti-ovalbumin, and on hematological parameters. The secondary objectives of the current experiment were to evaluate the effects treating weaning calves with rbST on metabolic parameters, growth, and intake of milk replacer.

MATERIALS AND METHODS

All procedures involving animals were approved by the animal care and use committee of the University of Florida (protocol #201609265).

Animals, Facilities, Nutrition and Management

The experiment was conducted in a commercial dairy farm located in northern Florida. Calves were born in a loose housing sand-bedded pen. Upon birth, calves were individually weighed using an electronic scale and were fed 2 L of colostrum within 30 min of birth and another 2 L of colostrum within 6 h of birth. At 48 h after birth, blood was sampled to determine serum total protein, a proxy for passive transfer of immunity. Only calves with total protein measured by

refractometer between 5.5 and 8.5 g/dL were used in this experiment. Throughout the experiment, calves were group housed (8 calves/pen from 24 h after birth to 20 days of age, 20 to 30 calves/pen from 21 to 63 d of age) in sand-bedded pens.

From 24 h after birth to 36 days of age, calves were fed milk replacer ad libitum. Starting at 38 d of age milk replacer allowance was progressively reduced until 49 d of age (weaning, d 0) when calves were completely weaned. From 21 to 49 d of age an automated milk replacer feeder recorded individual replacer intake. At 63 d of age, calves were moved to an open lot for approximately 60 calves. Calves had ad libitium access to starter, water, and mineral salt blocks starting at 24 h after birth. The chemical compositions of milk replacer and starter are described in Table 1.

Treatments

Calves (n = 36) were enrolled in the experiment in 3 consecutive days of the same week. Within each enrollment day, calves were paired for serum total solids at birth and average daily gain from birth to enrollment. Calves were allocated randomly to the control and rbST treatment. Calves assigned to the control treatment (n = 18) were treated with 0.5 mL of 0.9% of saline solution (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS), sub-cutaneously 139 in the neck area, weekly, from 21 to 63 days of age. Calves assigned to the rbST treatment (n = 18) were treated with 1.5 mg (21 to 35 d of age) and 1.8 mg (42 to 63 d of age) of rbST (Posilac, Elanco Animal Health, Greenfield, IN) per kg of body weight, sub-cutaneously in the neck area. Injections of saline and rbST were given in alternate sides of the neck area to prevent iatrogenic lesions of the neck. Injections were given using 1 mL tuberculin syringes and hypodermic 16 gauge and 5/8 inch long needles. On the day before treatment with rbST, rbST contained in commercially

available ready to use syringes of rbST (Posilac, Elanco Animal Health, Greenfield, IN) were dispensed into sterile containers and the appropriate volume of rbST was aspirated into sterile syringes that were kept refrigerated until treatment of calves on farm. The dose of rbST was chosen based on a pilot experiment with eight 21-d old calves that, under the same management conditions as the calves used in this experiment, were treated with 0 to 5 mg of rbST per kg of body weight. The resulting increase in IGF-1 concentration was linear. In previous experiments from our group, rbST-induced increases in GH and IGF-1 concentrations of approximately 30% above the concentrations of untreated cows resulted in improvements in PMNL function and anti-ovalbumin IgG concentration (Silva et al., 2015).

Blood Samples

Blood was sampled at -28, -25, -21, -11, 0 (49 d of age), 3, 7 and 14 d relative to weaning date by jugular venipuncture. In total approximately 23 mL of blood was sampled each day, two samples in 10 mL evacuated tubes containing Heparin (Vacutainer Systems, Becton Dickinson), one 10 mL evacuated tube without anticoagulant (Vacutainer Systems, Becton Dickinson), and one 3 mL evacuated tube containing EDTA (Vacutainer Systems, Becton Dickinson). Whole blood heparinized samples were used for PMNL function and PMNL, lymphocyte and CD14+ monocyte adhesion molecule assays. Plasma was used to determine the concentrations of GH, insulin, IGF-1 and haptoglobin. Serum was used to determine the concentrations of IgG anti-ovalbumin, glucose, non-esterified fatty acids, and beta-hydroxybutyrate (BHB). Samples collected in the 3 mL tubes containing EDTA were used for complete hemogram.

Tubes without anticoagulant were placed in ice immediately after sample collection. Heparinized tubes were kept at room temperature in a cooler without ice, protected from light,

until the whole blood for the PMNL function and adhesion molecule assays were collected, within 2 hours after sampling. Similarly, EDTA tube were also kept at room temperature, in a cooler without ice, until hemogram was conducted, within 2 h of sample collection. Tubes were centrifuged at 3,000 g for 10 min for plasma and serum separation. Eight aliquots were separated into 1.5 mL micro centrifuge tubes (Eppendorf, Enfield, CT) and kept frozen at -80 ÅãC until analysis.

Innate Immune Parameters and Hemogram

Polymorphonuclear leukocyte activity was determined following a challenge of the whole blood with intra-uterine pathogenic E. coli (E. coli; IUPEC, KCJ852) labeled with propidium iodide. Whole blood (100 µl) were incubated with 10 µl of dihydrorhodamine 123 (Sigma-Aldrich, St. Louis, MO), a free radical sensor, at 37 ˚C for 10 minutes. According to the individual's neutrophil count based on the hemogram, E. coli was added to the samples to result in a bacterium to neutrophil ratio of 40:1 and samples were incubated for 30 min at 37 ˚C. Thereafter, samples were kept on ice until the addition of Trypan Blue solution (Sigma-Aldrich, St. Louis, MO) to remove bacteria fluorescence and flow cytometry, which 184 was performed on a BD flow cytometer (BD Biosciences, San Jose, CA).

The adhesion molecule procedure determined the expression of L-selectin (CD62L; BAQ92A, Washington State University, Pullman, WA) and CD18 (BAQ30A, Washington State University, Pullman, WA), both labeled with a commercial kit (Alexa Fluor 488 antibody labeling kit, Thermo Fisher Scientific, Cleveland, OH), and CD14 (MHCD1418, Thermo Fisher Scientific, Cleveland, OH) through indirect immunofluorescence. Briefly, lyse and restore solutions were added to whole blood samples. Samples were then centrifuged at 450 g for 5 min and supernatant

decanted, in order to exclude red blood cells. The remaining cells were resuspended in 50 µl of antibody cocktail and incubated for 30 min in ice. Two mL of PBS were added to the samples, which were centrifuged for 650 g for 5 min, decanted, resuspended in 0.5 mL of PBS and analyzed by flow cytometer.

Results from both procedures were analyzed using FlowJow v.10.1software (Flow Jo, LLC, Ashland, OR) and data referent to phagocytosis, oxidative burst and adhesion molecules are expressed in median fluorescence intensity (MFI).

Blood samples collected with K2 EDTA tubes were analyzed within two hours after sampling using a Procyte hematology analyzer (IDEXX Laboratories, Westbrook, ME) for complete hemogram.

Hormone Analysis

Serum concentrations of GH were determined in duplicate in all samples by RIA using procedures described by Klindt et al (1983). Bovine GH (AFP-9884C, National Hormone and Pituitary Program) was used as the radioiodinated antigen and standard. Growth hormone antisera (AFPB55, National Hormone and Pituitary Program) was used at an initial dilution of 1:200,000. Inhibition curves of sera ranging from 50 to 400 mL were parallel to standard curves. Sensitivity of the assay was 19.9 pg/mL and the intra- and inter-assay CV were 7.3% and 8.1%, respectively.

Concentrations of insulin were determined using a commercial ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia AB, Winston-Salem, NC). Total IGF-1 concentrations were determined using a commercial ELISA kit (Quantikine ELISA Human IGF-1 immunoassay; R&D Systems, Minneapolis, MN) with 100% cross reactivity with bovine IGF-1.

Concentrations of Metabolites and Haptoglobin

Serum concentrations of non-esterified fatty acid, BHB, and glucose were measured using the RX Daytona bench clinical chemistry analyzer using commercial kits (Randox Laboratories Ltd., Charles Town, WV). Plasma haptoglobin concentration were determined by measuring haptoglobin/hemoglobin complex by the estimation of difference in peroxidase activity (Cooke and Arthington, 2013; Hulbert et al., 2011) and optical density was measured by a plate reader at 410 nm (Power Wave XS, BioTek, Winooski, VT). Results are described in absorbance multiplied per 100.

Ovalbumin vaccination and IgG anti-ovalbumin Concentration

Calves received two injections of 0.5 mg of chicken egg albumin (Type VII, Sigma-Aldrich Co., St. Louis, MO), diluted in 0.5 mL of phosphate buffer saline and emulsified in 0.5 mg of adjuvant Quil-A (Accurate Chemical & Scientific Corporation, Westbury, NY), previously diluted in 0.5 mL of PBS. The immunization was performed by subcutaneous injection in the neck area at 21 and 42 days of age.

Concentration of IgG anti-ovalbumin was determined by ELISA. Wells were coated with ovalbumin protein (#A5503; Sigma-Aldrich, Inc., St. Louis, MO) diluted in the same coating buffer (1.6 mg/L ovalbumin). Samples were added after residual coating ovalbumin protein was removed and plates were washed with TBST. Horseradish peroxidase conjugated antibody produced in sheep against bovine IgG heavy chain (#A10-118P; Bethyl Laboratories, Montgomery, TX) was added to each well, and following a 1 h incubation and subsequent wash procedure, 3,3′,5,5′-tetramethylbenzidine ELISA peroxidase substrate (Rockland Immunochemicals Inc., Gilbertsville, PA) was added to each well and allowed to react for 15 to

20 min. The enzyme reaction was ended by adding 0.2 M sulfuric acid, and absorbance was read at a wavelength of 450 nm (Victor 3 Multi-label Counter model 1420; PerkinElmer Life Sciences, Waltham, MA). Mean coefficients of variation for total IgG and ovalbumin-specific IgG assays for plasma were 4 and 3.6%, respectively.

Body Weight and Wither Height

Body weight was determined at birth, and at -29, -24, -20, -10, -1, 2, 6, and 13 d relative to weaning using an electronic scale that was calibrated at the start of the experiment. Wither height was determined at -29, -24, -20, -10, -1, 2, 6, and 13 d relative to weaning with a metric tape by the same person with the animal standing on plain hard soil.

Statistical Analysis

All statistical analyses were performed with SAS (9.3 SAS Institute 251 Inc. Cary, NC). Continuous variables were analyzed by ANOVA using the GLM procedure for pre-treatment values (baseline) and the MIXED procedure for repeated measures. The statistical models to compare baseline values between treatments included treatment and enrollment weight. The statistical models to evaluate the effects of treatment on repeated measure data (e.g. hormones of the somatotropic axis, immune parameters, metabolites, milk replacer intake, and average daily gain) included treatment, day, the interaction between treatment and day, base line value for the variable being analyzed, and enrollment weight. Calf was used as the random effect and was nested within treatment. Covariates with $P \geq 0.15$ were removed manually in a stepwise manner. Residuals were tested for normality and when normality was not met the dependent variable was transformed (log10 or square root). The following data were Log10 transformed for statistical

analysis: intensity of phagocytosis by PMNL; intensity of expression of CD62L and CD18 by granulocytes, lymphocytes, and CD14+ monocytes; leukocyte and neutrophil counts; and insulin, IGF-1, and BHB concentrations. Intensity of oxidative burst data was square root transformed. All data presented are referent to raw values. Concentrations of haptoglobin from - 24 to 0 d relative to weaning, at 3 d after weaning, and at 7 and 14 d after weaning were analyzed separately. Statistical significance was considered at $P \leq 0.05$ and tendency for statistical significance at $0.05 < P \leq 0.15$.

RESULTS

There were no differences between treatments regarding body weight at birth (Control = 43.8 $\pm$ 1.5 kg, rbST = 42.8 $\pm$ 1.5 kg; P = 0.65), body weight at enrollment (Control = 52.5 $\pm$ 1.9 kg, rbST = 53.1 $\pm$ 1.9 kg; P = 0.83), and wither height at enrollment (Control 273 = 80.4 $\pm$ 0.6 cm, rbST = 81.5 $\pm$ 0.6 cm; P = 0.22).

Effects of Treatment on Somatotropic Axis

At enrollment, there was no (P = 0.58) difference between treatments in GH concentration (Figure 2A). The interaction between treatment and day affected (P = 0.01) the GH concentration because on days -25, -11, 3, 7, and 14 relative to weaning GH concentrations were greater in rbST treated calves compared with control calves (Figure 2A). Concentration of IGF-1 was not (P = 0.72) different between treatments at enrollment. At enrollment, there was no (P = 0.73) difference between treatments regarding IGF-1 concentration (Figure 2B). There was a tendency (P = 0.08) for the interaction between treatment and day to affect the concentration of IGF-1. Despite the fact

that at -25, -21, -11, 0, 3, 7, and 14 d relative to weaning IGF-1 concentrations of rbST treated calves were ($P < 0.05$) greater than control calves, differences were greatest at -11 d relative to weaning (Figure 2B). Insulin concentration at enrollment was not ($P = 0.55$) different between treatments. Treatment did not ($P = 0.42$) affect concentrations of insulin from d -24 to 14 relative to weaning (Figure 2C).

Effects of Treatment on Phagocytosis and Oxidative Burst by PMNL, Expression of Adhesion Molecules and Haptoglobin Concentration

At enrollment, there were no differences between treatments regarding intensity of phagocytosis ($P = 0.25$) and oxidative burst ($P = 0.77$) by PMNL, percentage of PMNL positive for phagocytosis and oxidative burst ($P = 0.50$). Treatment did not affect the intensity of phagocytosis ($P = 0.74$; Figure 3A) and oxidative burst ($P = 0.71$; Figure 3B) by PMNL. Additionally, treatment did not affect the percentage of PMNL positive for 296 phagocytosis and oxidative burst ($P = 0.59$; Figure 3C).

At enrollment, there were no differences between treatments regarding the intensity of expression of CD62L ($P = 0.14$) and CD18 ($P = 0.18$) by granulocytes, expression of CD62L ($P = 0.38$) and CD18 ($P = 0.53$) by lymphocytes, and expression of CD62L ($P = 0.93$) and CD18 ($P = 0.46$) by CD14+ monocytes. Treatment with rbST tended to increase the intensity of CD62L ($P = 0.06$; Figure 4A) and CD18 ($P = 0.07$; Figure 4B) by granulocytes from d -25 to 14 relative to weaning. Intensity of expression of CD62L ($P = 0.13$; Figure 4C) and CD18 ($P = 0.18$; Figure 4D) by lymphocytes from d -25 to 14 relative to weaning were not affected by treatment. Treatment with rbST did not affect the intensity of expression of CD62L ($P = 0.86$; Figure 4E) and CD18 ($P = 0.95$; Figure 4F) by CD14+ monocyte.

Haptoglobin concentration at enrollment was not (P = 0.31) different between treatment (Figure 5). From -25 to 0 d relative to weaning treatment did not (P = 0.77) affect haptoglobin concentration, but on d 3 relative to weaning rbST calves had (P = 0.04) reduced haptoglobin concentration than control calves. From 7 to 14 d relative to calving, there were no (P = 0.56) differences in haptoglobin concentration between treatments (Figure 5).

Effects of Treatment on Hemogram Parameters and Metabolites

Results referent to hemogram are described in Table 2. At enrollment rbST calves had (P = 0.05) reduced leukocyte count and tended to have reduced lymphocyte (P = 0.09) and granulocyte (P = 0.11) count than control calves. Calves treated with rbST tended (P = 0.07) to have reduced hematocrit from d -25 to 14 relative to weaning. The interaction between treatment and day relative to weaning affected (P = 0.05) the platelet count from d -25 to 14 relative to weaning, because 3 d after weaning rbST treated calves tended (P = 0.10) to have greater platelet count than control calves (551.7 $\pm$ 32.6 vs. 470.2 $\pm$ 34.5). No other effects of rbST treatment on hemogram parameters were observed.

At enrollment, there were no differences between treatments regarding concentrations of glucose (P = 0.87), non-esterified fatty acids (P = 0.22), and BHB (P = 0.56). There was a tendency (P = 0.07) for the interaction between treatment and day relative to weaning to affect glucose concentration from -25 to 14 d relative to weaning (Figure 7A), because on d -11 relative to weaning rbST treated calves had (P = 0.03) greater glucose concentration than control calves (144.2 $\pm$ 6.6 vs. 123.8 $\pm$ 6.6 ng/dL). Treatment did not affect the concentrations of non-esterified fatty acids (P = 0.97) and BHB (P = 0.28) from -25 to 14 d relative to weaning (Figure 7B and 7C, respectively).

Effects of Treatment on Body Weight, Wither Height, ADG and Milk Replacer Consumption

Calves treated with rbST had (P = 0.02) reduced intake of milk replacer from d -28 to 0 relative to weaning (7.2 ± 0.3 vs. 8.1 ± 0.3 L/d). The interaction between treatment and day relative to weaning did not (P = 0.50) affect milk replacer intake (Figure 8A). Treatment did not (P = 0.81) affect the live body weight (control = 75.8 ± 1.2, rbST = 75.4 ± 1.2) from d -24 to 13 relative to weaning (Figure 8B). Similarly, there was no (P = 0.55) difference in average daily gain from d -24 to 14 relative to weaning between control (833.0 ± 41.1 g/d) and rbST (797.9 ± 338 41.1 g/d) calves.

DISCUSSION

The current experiment evaluated the use of rbST as an immunomodulatory 341 drug because of the resulting increase in GH concentration and GH-induced increase in IGF-1 concentration. As expected, rbST treated calves had significantly higher GH and IGF-1 concentrations compared with control calves throughout the experiment. The weaning-associated energy deficit was associated with a 62% decrease in IGF-1 concentration from d 0 to 3 d relative to weaning among control calves. Unexpectedly, rbST treated calves had a similar decrease in IGF-1 concentration (62%) from d -11 to 3 relative to weaning. This leads us to speculate that the metabolic challenges faced by the calves in the current experiment, evidenced by the subtle decreases in glucose and insulin concentrations and increases in non-esterified fatty acid and BHB concentrations around weaning, were sufficient to cause uncoupling of the somatotropic axis. In a series of experiments with periparturient dairy cows, Silva et al. (2015; 2017a,b) demonstrated that

rbST treatment increased GH concentration throughout the periparturient period, whereas rbST-induced increase in IGF-1 concentration was mainly observed prepartum and again only at approximately 21 to 28 d postpartum. The uncoupling of the somatotropic axis, characterized by the down-regulation of hepatic GH-1α receptors and the consequent low IGF-1 concentrations in the presence of high GH concentrations (Lucy et al., 2009; Silva et al., 2017b), may in part be explained by a subtle decrease in nutrient intake and the onset of negative energy balance. Similarly to the experiment conducted with periparturient dairy cows (Silva et al., 2015; Silva et al., 2017a,b), rbST treatment had no effect on insulin concentration and only marginally increased glucose -11 d relative weaning. These results suggest that weaning decisions regarding calves fed ad-libitum milk/milk replacer needs to be made on a calf-by-calf basis taking into consideration the individuals intake of starter, whenever possible.

Contrary to our hypotheses, rbST-induced increases in GH and IGF-1 363 concentrations did not lead to improvements in PMNL phagocytosis and oxidative burst. Additionally, treatment with rbST did not increase anti-ovalbumin IgG concentration. Treatment with rbST tended to upregulate the expression of CD62L and CD18 by granulocytes and CD62L by lymphocytes, particularly prior to weaning. Silva et al. (2015) demonstrated that treatment of periparturient cows with rbST increased PMNL phagocytosis and oxidative burst by approximately 30%, intensity of expression of CD62L by approximately 20%, and anti-ovalbumin IgG concentration by approximately 50% between -14 and 0 d relative to calving. The mechanisms of action of GH and IGF-1 on immune cells may be autocrine, paracrine, or endocrine. Mononuclear leukocytes from rats have the ability to produce GH (Weigent, 1992) and leukocytes express receptors for GH and IGF-1 (Tapson et al., 1988; Silva et a., 2017). The role of GH and IGF-1 on immune function has been studied at length in humans and rodents. Kiess (1983) demonstrated that human GH induces

proliferation of peripheral blood lymphocytes. Insulin like growth factor-1 is an important factor in proliferation, differentiation, and hypertrophy of various cell types, including immune cells (LeRoith and Roberts, 1991). Culture of human granulocytes in the presence of different concentrations of IGF-1 reduced the percentage of apoptotic granulocytes by 34 to 40% compared with culture of granulocytes in the absence of IGF-1 (Kooijman et al. 2002). Similarly, there was a 45% decrease in DNA fragmentation in granulocyte cultured with 6.5 nM IGF-1 compared with culture of granulocytes in the absence of IGF-1 (Kooijman et al. 2002). Culture of PMNL with human growth hormone (100 to 1,000 ng/ml) or IGF-1 (100 to 1,000 ng/ml) increased the percentage of E. coli killed and increased the expression of CD11, the alpha component of integrins that mediates adhesion of leukocytes to the endothelium, an important step in leukocyte transmigration (Inoue et al., 1998). Treatment of humans and rats deficient in GH with somatotropin increased circulating concentrations of 386 IGF-1, PMNL, and antibodies (Kimata and Yoshida, 1994; Ibanez et al., 2005; Sohmiya et al., 2005). Piglets treated with somatotropin before weaning and transportation had increased IgM concentrations (Kojima et al., 2008) and mice treated with somatotropin had increased IGF-1 and IgG, IgM, and IgA concentrations (Sohmiya et al. 2005). Smaniotto et al. (2004) demonstrated that treatment of mice with GH increased the number of CD62L+ T cells and the expression of CD62L (Smaniotto et al., 2004) and Ryu et al. (2000) demonstrated that treatment with GH enhanced adhesion of human neutrophils. Although culture of human PMNL in the presence of GH had no impact on expression of CD11b, culture of human PMNL in the presence of IGF-1 increased the intensity of expression of CD11b (Inoue et al., 1998). Questions still remain whether rbST induced upregulation of CD62L by granulocytes and lymphocytes and CD18 by granulocytes is sufficient to improve health of calves around weaning. Although the stress and metabolic challenge of weaning may

have blunted the positive effects of GH and IGF-1 on the immune parameters described previously, treatment of periparturient cows with rbST produced similar changes in GH and IGF-1 concentrations and increased PMNL function and IgG concentration in the presence of high cortisol, non-esterified fatty acid and BHB concentrations (Silva et al., 2015). It is possible that the GH and IGF-1 concentration of control calves in the current experiment was sufficient to assure proper function of immune cells.

In the current experiment nearly all immune parameters evaluated were reduced around weaning. While expression of CD62L decreased progressively from -28 to 0 d relative to weaning and expression of CD18 decreased progressively from -28 to 14 d relative to weaning, PMNL intensity of phagocytosis and oxidative burst decreased transiently at weaning and 7 d after weaning, respectively, mostly returning to pre-weaning levels 7 d later. Hulbert et al. (2011a) demonstrated that when liquid feed was consolidated into one feeding 409 per day early in life there was a tendency for the intensity of PMNL phagocytosis to decrease. Additionally, calves weaned at 27 d of age that reached 900 g/d of starter intake sooner (1 to 8 d after start of weaning) had greater intensity of expression of L-selectin and β2-integrin at weaning and 7 d later (Hulbert et al., 2011b). The transient changes in PMNL function and expression of adhesion molecules by granulocyte and lymphocyte may be insufficient to predispose calves to infectious diseases.

In the current experiment, weaning was associated with an increase in haptoglobin concentration. Calves treated with rbST, however, had reduced haptoglobin concentration at 3 d relative to weaning compared with control calves. Hulbert et al. (2011a) demonstrated that haptoglobin concentrations decreased from 45 to 66 d of age. Silva et al. (2015) demonstrated that rbST treated periparturient cows had reduced haptoglobin concentration 7 d postpartum compared with untreated cows. Haptoglobin is an acute phase protein produced mainly in the liver in

response to infectious diseases, particularly respiratory diseases (Sepp.-Lassila et al., 2015). The reduced haptoglobin concentration 3 d relative to weaning among rbST treated calves is an indication that rbST treatment reduced inflammation, but it is unclear whether this resulted from reduced incidence of diseases. Treatment of calves with rbST tended to reduce hematocrit throughout the experiment, but particularly 7 and 14 d after weaning. Growth hormone therapy decreased the haptoglobin concentrations of severely burned human patients (Jeschke et al., 2000). Growth hormone induced increase in IGF-1 may modulate acute phase response by decreasing interleukin-1 expression, leading to a decrease in type 1 acute phase proteins and increased in constitutive hepatic proteins (Jarrar et al., 1997; Jeschke et al., 2000). Steers challenged with in vivo with E. coli endotoxin had reduced TNF-α concentration from 1 to 3 h following challenge and greater packed cell volume from 1 to 4 h after challenge (Elsasser et al., 1994; Elsasser et al., 1996). Haeffner et al. (1997) demonstrated that pro-monocytic cells engineered by gene transfer to produce GH secreted less TNF-α in response to an LPS challenges and that GH treatment inhibited TNF-α secretion by human monocytes and macrophages. The effects of rbST treatment on the severity of inflammatory response and secretion of pro-inflammatory cytokines in weaning calves should be further evaluated because prolonged and uncontrolled inflammatory response might be detrimental to the individual and result in increased morbidity and mortality (Moshage, 1997).

Milk replacer intake of rbST treated calves from -27 to -1 d relative to weaning was approximately 11% lower than control calves. Nonetheless, body weight, average daily gain, and wither height were not affected by rbST treatment. In the current experiment it was not possible to determine individual starter intake because calves were group housed. Hammon et al. (2003) demonstrated that calves receiving 500 mg of rbST every 14 d from 3 to 42 d of age had greater

body weight at 6 weeks of age than control calves. Holzer et al. (1999, 2000) demonstrated that Holstein-Friesian bull calves treated with rbST from approximately 5 to approximately 8 months of age had greater average daily gain and greater conversion ratio than untreated calves. Several researchers have demonstrated that somatotropin treated animals have better feed efficiency, but this response is particularly dependent on nutritional status (Breier et al., 1988a,b; Peters, 1986). From the current experiment no conclusions regarding feed efficiency of female calves can be extrapolated because starter intake was not recorded. We speculate that the lack of difference in body weight between rbST treated calves and control calves despite the difference in milk replacer intake reflects either greater feed efficiency of rbST treated calves or compensatory consumption of starter by rbST treated calves.

CONCLUSION

Treatment of weaning calves with rbST led to a significant increase in GH and IGF-1 concentrations, but weaning calves demonstrated a pattern of GH and IGF-1 similar to that of periparturient cows that undergo uncoupling of the somatotropic axis. This is an important finding because it indicates that calves fed milk replacer ad libitum that are not consuming sufficient starter by the time complete weaning occurs could undergo significant metabolic challenge that could hamper their immune system. In fact, all calves in the current experiment had a significant decrease in immune parameters around weaning. The lack of effect of rbST treatment on PMNL phagocytosis and oxidative burst indicate that GH and IGF-1 concentrations are either not a limiting factor to immune function of weaning calves or that the inability of rbST treatment to

maintain a high and constant IGF-1 concentration during weaning impedes any improvements in immune parameters. In fact, expression of CD62L and CD18 by granulocytes and lymphocytes was greater for rbST treated claves than control calves, particularly up to -11 d relative to weaning when progressive weaning starter and IGF-1 concentrations started to decrease. Additional experiments are necessary to better elucidate the metabolic and immunological consequences ad libitum feeding of milk replacer.

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Table 1. Milk replacer and concentrate for starter feedlot compositions

Nutrient	Milk replacer	Concentrate starter
Crude protein, %	28	12
Crude fat, %	15	1
CF, %	0.15	26
Ca, %	>0.9 to <1.11	>0.45 to <0.95
P, %	>0.7	>0.4
K, %	-	>1.10
NaCl, IU/Kg	-	>0.25 to <0.75
Vitamin A, IU/Kg	>66,000	5,000
Vitamin D_3, IU/Kg	>11,000	-
Vitamin E, IU/Kg	>166	-
Chlortetracycline, ppm	-	70
Sulfamethazine, ppm	-	70

Table 2. Effect of rbST treatment on hemogram parameters

	Treatment[1]							
	Enrollment (-28d)			-25 to 14 relative to weaning		*P* - value		
Item (±SEM)	Control[1]	rbST[1]	*P* - value	Control[1]	rbST[1]	Trt[1]	Day	Trt x Day
Hematocrit, %	32.6 ± 1.4	31.5 ± 1.3	0.57	35.3 ± 0.7	33.5 ± 0.6	0.07	<0.01	0.23
Hemoglobin, g/dL	7.7 ± 0.9	7.9 ± 0.9	0.86	3.3 ± 0.08	3.2 ± 0.08	0.46	<0. 01	0.97
Red blood cell, $x10^6/\mu L$	8.4 ± 0.3	8.3 ± 0.3	0.72	9.3 ± 0.1	9.2 ± 0.1	0.48	<0.01	0.25
Patelet, $x10^3/\mu L$	514.8 ± 21.6	537.6 ± 20.4	0.45	533.3 ± 18.6	555.4 ± 17.6	0.40	<0.01	0.05
Leukocytes, $x10^3/\mu L$	10.72 ± 0.78	8.68 ± 0.63	0.05	11.96 ± 0.77	11.56 ± 0.73	0.51	<0.01	0.86
Lymphocytes, $x10^3/\mu L$	5.8 ± 0.3	5.0 ± 0.3	0.09	6.5 ± 0.2	6.2 ± 0.2	0.33	<0.01	0.74
Granulocytes,$x10^3/\mu L$	4.08 ± 0.55	3.02 ± 0.42	0.11	4.22 ± 0.55	4.38 ± 0.57	0.90	<0.01	0.90
Monocytes, $x10^3/\mu L$	0.54 ± 0.11	0.33 ± 0.11	0.19	1.04 ± 0.08	0.98 ± 0.07	0.58	<0.01	0.29

1 Treatments: Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health) every 7 days from 21 to 63 days of life.

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Figure 1. Schematic depiction of study design. Control = calves received injections 617 with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life.

Figure 2. Effect of rbST treatment on concentrations of (A) growth hormone, (B) insulin like growth factor-1, and (C) insulin. Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. Growth hormone: treatment ($P < 0.01$), day ($P < 0.02$) and interaction between treatment and day ($P = 0.01$). Insulin like growth factor-1: treatment ($P = 0.01$), day ($P < 0.01$) and interaction between treatment and day ($P = 0.08$). Insulin: treatment ($P = 0.42$), day ($P < 0.01$) and interaction between treatment and day ($P = 0.31$). *Within day, means with asterisk differ ($P \leq 0.05$).

Figure 3. Effects of rbST treatment on (A) polymorphonuclear leukocyte (PMNL) intensity of phagocytosis, (B) PMNL intensity of oxidative burst, and (C) percentage of PMNL positive for phagocytosis and oxidative burst. Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. At enrollment, there were no differences between treatments regarding intensity of phagocytosis ($P = 0.25$) and oxidative burst ($P = 0.77$) by PMNL and percentage of PMNL positive for phagocytosis and oxidative burst ($P = 0.50$). Intensity of phagocytosis: treatment ($P = 0.74$), day ($P < 0.01$) and interaction between treatment and day ($P = 0.75$). Intensity of oxidative burst: treatment ($P = 0.71$), day ($P < 0.01$), and interaction between treatment and day ($P = 0.36$). Percentage of PMNL positive for phagocytosis and oxidative burst: treatment 639 ($P = 0.59$), day ($P < 0.01$) and interaction

between treatment and day (P = 0.57).

Figure 4. Effect of rbST treatment on intensity of expression of L-selectin (CD62L) and CD18 by (A) granulocytes, (B) lymphocytes, and (C) CD14+ monocytes. Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. At enrollment, there were no differences between treatments regarding the intensity of expression of CD62L (P = 0.14) and CD18 (P = 0.18) by granulocytes, expression of CD62L (P = 0.38) and CD18 (P = 0.53) by lymphocytes, and expression of CD62L (P = 0.93) and CD18 (P = 0.46) by CD14+ monocytes. Granulocyte CD62L: treatment (P = 0.06), day (P < 0.01) and interaction between treatment and day (P = 0.15). Granulocyte CD18: treatment (P = 0.07), day (P < 0.01) and interaction between treatment and day (P = 0.34). Lymphocyte CD62L: treatment (P = 0.13), day (P < 0.01) and interaction between treatment and day (P = 0.58). Lymphocyte CD18: treatment (P = 0.18), day (P < 0.01) and interaction between treatment and day (P = 0.57). CD14+ monocyte CD62L: treatment (P = 0.86), day (P < 0.01) and interaction between treatment and day (P = 1.00). CD14+ monocyte CD18: treatment (P = 0.95), day (P < 0.01) and interaction between treatment and day (P = 0.99).

Figure 5. Effect of rbST treatment on haptoglobin concentration. Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. At enrollment, concentration of haptoglobin was not (P = 0.31) different between treatments. Day -25 to 0 relative to weaning: treatment (P = 0.77), day (P = 0.18) and interaction between treatment and day (P = 0.52). Day 3 relative to 662 weaning: treatment (P = 0.04). Day 7 to 14 relative to weaning: treatment (P = 0.56), day (P = 0.24) and interaction between

treatment and day (P = 0.33).

Figure 6. Effect of rbST treatment on hematocrit. Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. Baseline (-28 d): treatment (). Day -25 to 14 relative to weaning: treatment (P = 0.07), day (P < 0.01) and interaction between treatment and day (P = 0.23).

Figure 7. Effect of rbST treatment on concentrations of (A) glucose, (B) non-esterified fatty acid, and (C) beta-hydroxybutyrate. Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8 mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. At enrollment, there were no differences between treatments regarding concentrations of glucose (P = 0.87), non-esterified fatty acids (P = 0.22), and BHB (P = 0.56). Glucose: treatment (P = 0.20), day (P < 0.01) and interaction between treatment and day (P = 0.07). Non-esterified fatty acid: treatment (P = 0.97), day (P < 0.01) and interaction between treatment and day (P = 0.49). Beta hydroxybutyrate: treatment (P = 0.28), day (P < 0.01) and interaction between treatment and day (P = 0.84).

Figure 8. Effect of rbST treatment on (A) milk replacer intake and (B) live body weight. Treatments: Control = calves received injections with saline (Saline Solution 0.9%, Valley Vet Supply, Marysville, KS); rbST = calves received 1.5 to 1.8mg/Kg of rbST (Posilac, Elanco Animal Health, Greenfield, IN) every 7 days from 21 to 63 days of life. Milk replacer intake: treatment (P = 0.02), day (P < 0.01) and interaction between treatment and day (P = 0.50). Live body weight at birth (P = 0.65) and at enrollment (P = 0.83) were not 685 different between treatments. Live body weight: treatment (P = 0.81), day (P < 0.01) and interaction between treatment and day (P = 0.94).

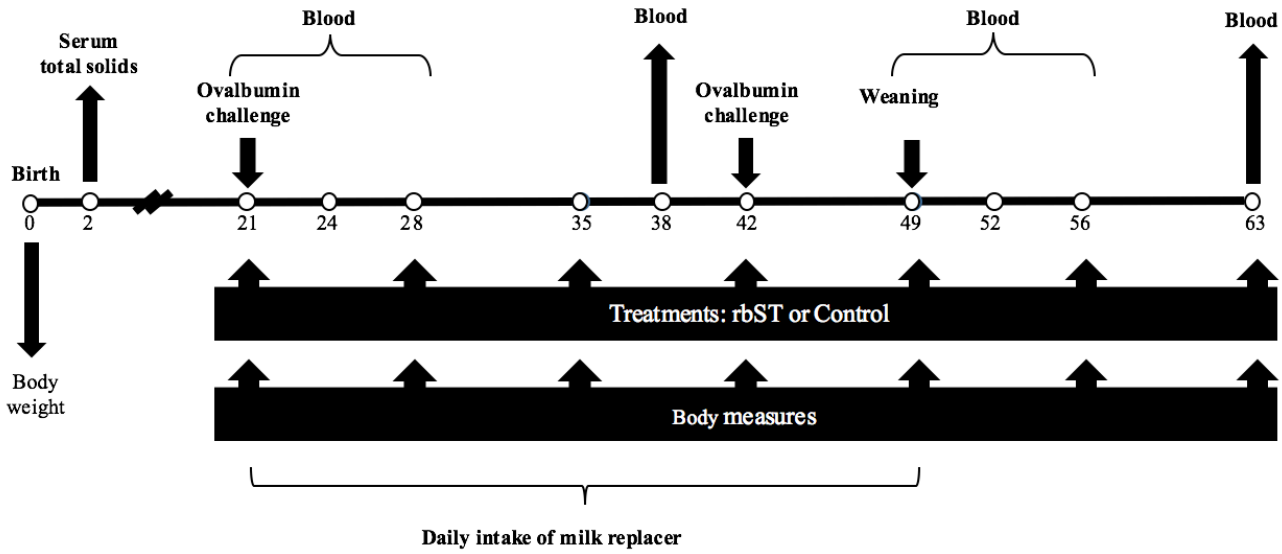
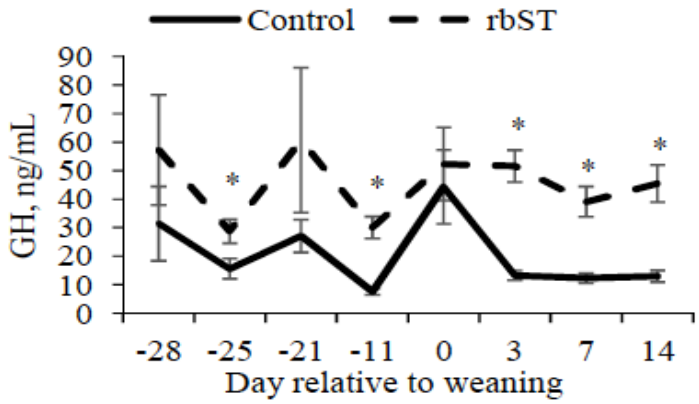


Figure 1. Belli et al.



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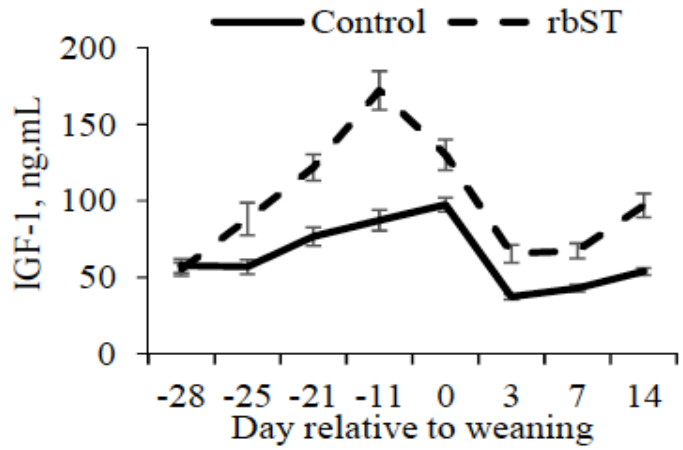

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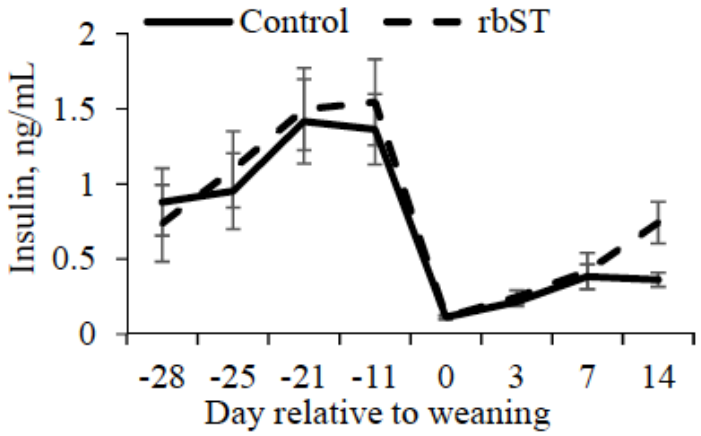
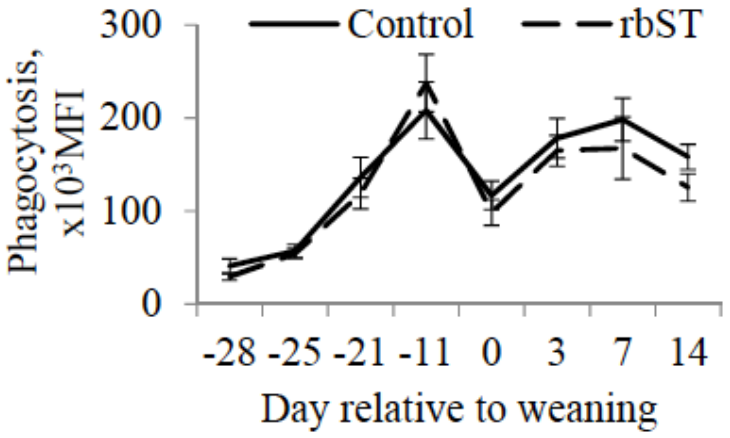


Figure 2C. Belli et al.



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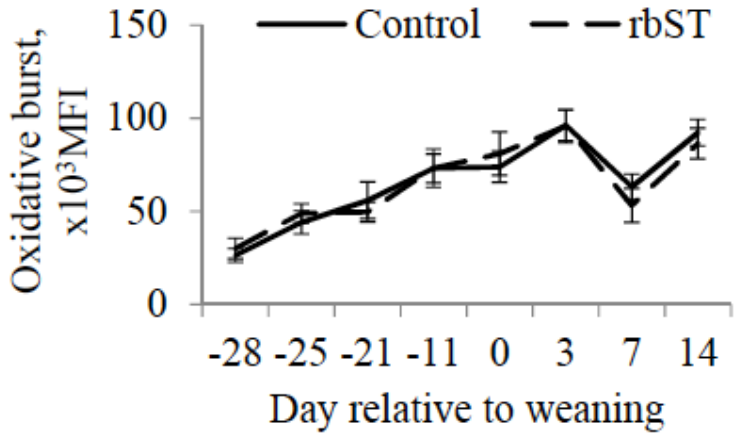

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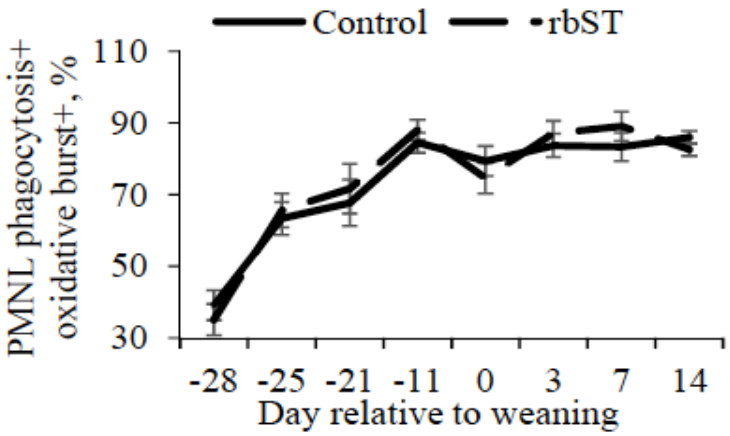

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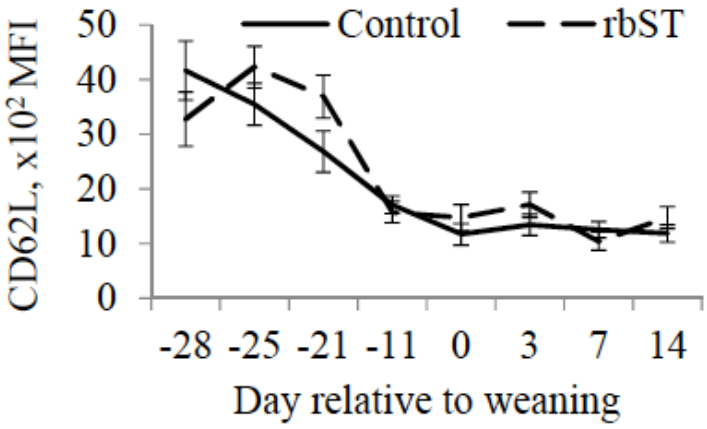

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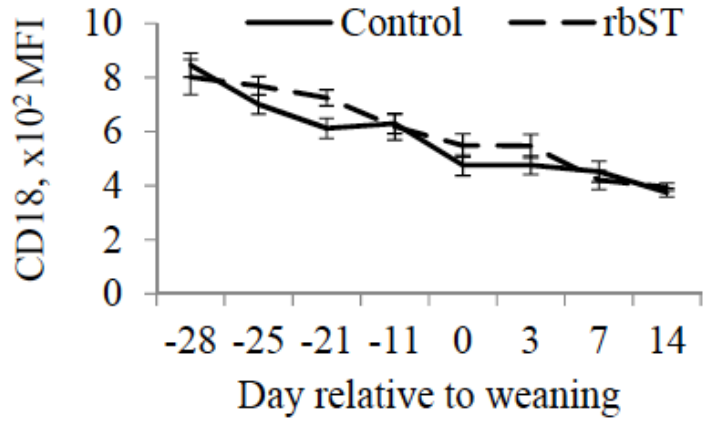

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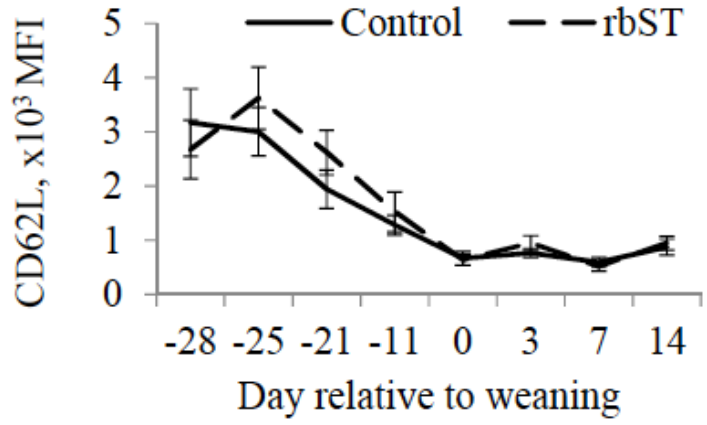

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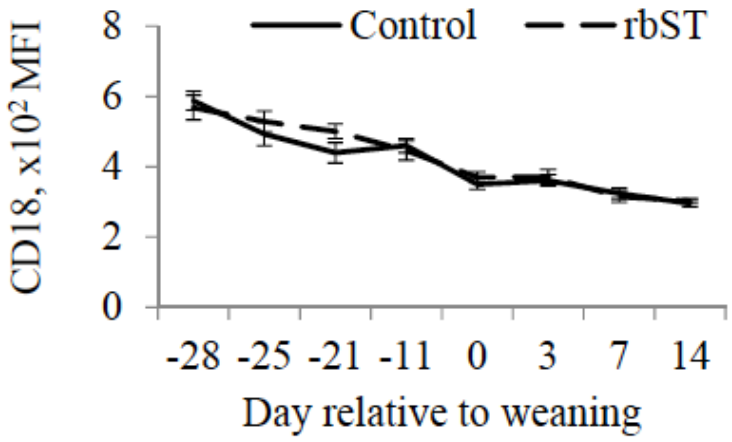

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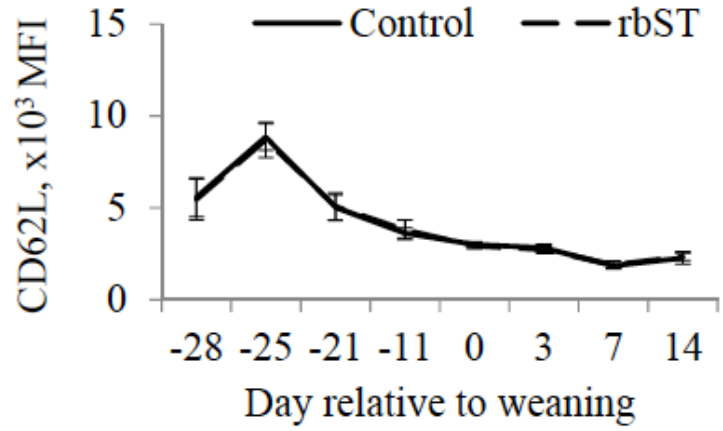

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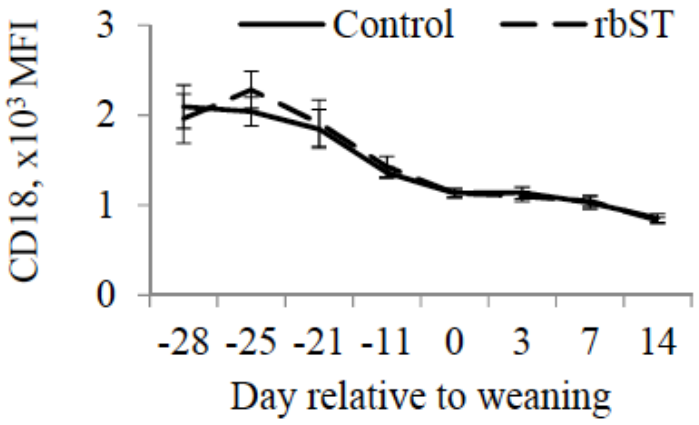

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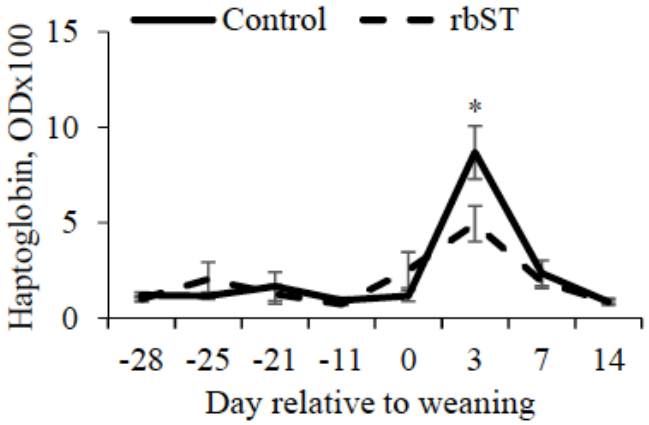

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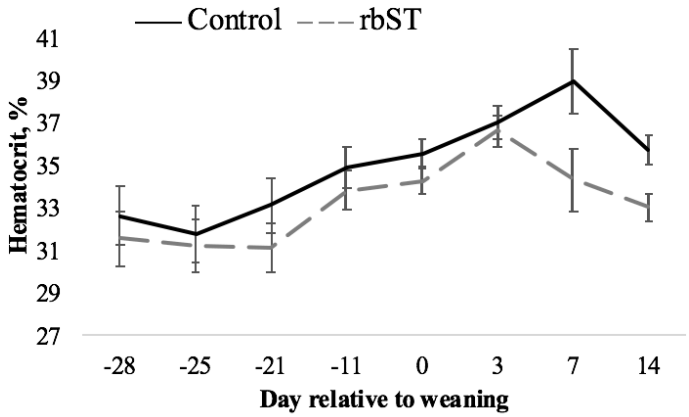

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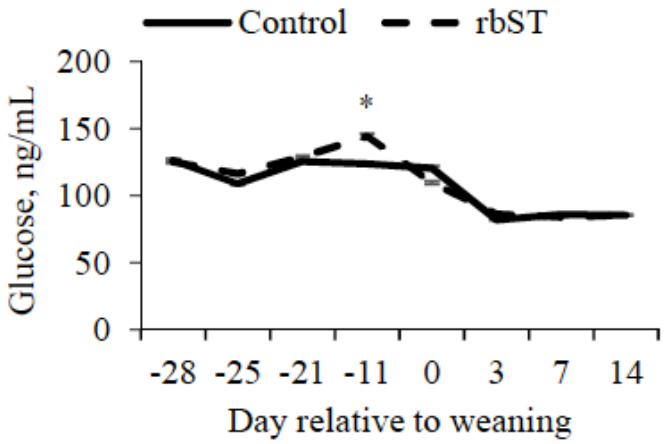


Figure 7A. Belli et al.

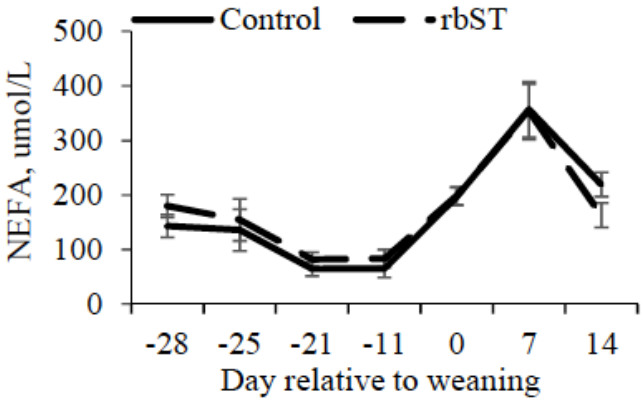
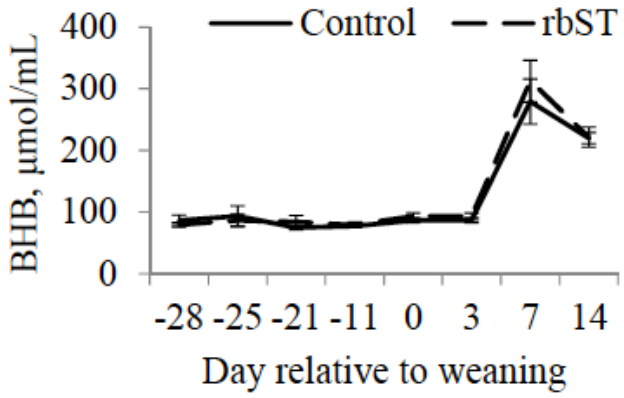


Figure 7B. Belli et al.



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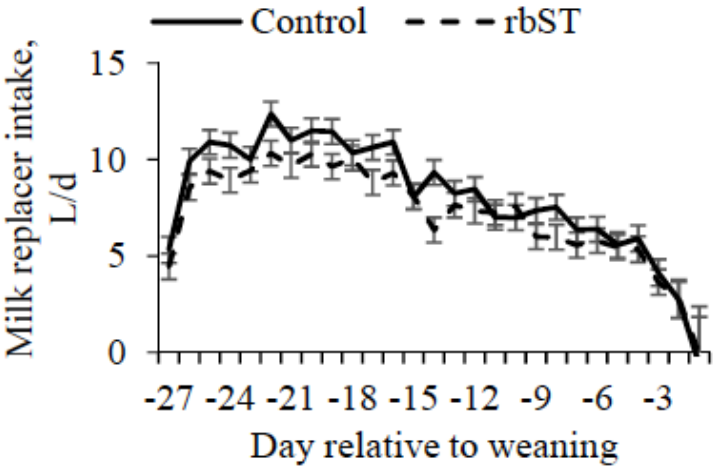

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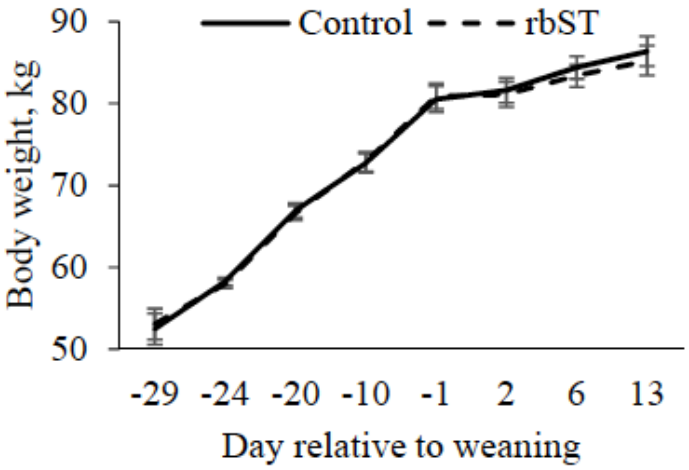

Figure 8B. Belli et al.