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Sistema GH/IGF-I nas características metabólicas e atividade reprodutiva pós-parto em bovinos

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Sistema GH/IGF-I nas características metabólicas e atividade reprodutiva pós-parto em bovinos

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Resumo

SCHNEIDER, Augusto. **Sistema GH/IGF-I nas características metabólicas e atividade reprodutiva pós-parto em bovinos**. 2011. 88f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A fertilidade em bovinos é muito influenciada por hormônios ligados ao metabolismo. Neste sentido, de especial interesse ao eixo reprodutivo são o hormônio do crescimento (GH) e o fator de crescimento semelhante a insulina I (IGF-I). Assim o objetivos desta tese foram 1) caracterizar a expressão de GHR e IGF-I no tecido hepático de vacas de corte lactantes e sua relação com a primeira ovulação; 2) caracterizar a expressão de GHR e IGF-I em células luteais e da granulosa; 3) avaliar o efeito da somatotropina exógena pré-parto sobre o retorno a atividade ovariana pós-parto; e 4) avaliar o efeito o polimorfismo *Alu*I no gene GHR sobre a fertilidade de vacas da raça Holandês. A expressão de GHR e IGF-I no tecido hepático de vacas de corte lactantes não foi diferente entre vacas que ovularam e não ovularam, bem como não variou sua expressão entre 0 e 40 dias pós-parto. Estes dados indicam que vacas de corte não sofrem a dissociação do eixo GH/IGF-I observada em gado leiteiro. Com relação a caracterização da expressão de GHR e IGF-I em células luteais e da granulosa, foi possível observar que a expressão de GHR, IGF-I e SOCS é claramente maior em células luteais, porém não difere entre folículos atrésicos ou estrogênio ativos. Além disso, o nível de GHR e IGF-I não foi correlacionado em células da granulosa ou luteais. Assim, partiu-se para o teste de estratégias em gado leiteiro e que não focassem na regulação da produção local de IGF-I. Neste sentido, o uso de somatotropina exógena no período pré-parto mostrou um benefício ao antecipar o momento da primeira ovulação pós-parto. Além do mais, as vacas tratadas aumentaram a produção de leite e reduziram o nível circulante de ácidos graxos não esterificados no período pós-parto recente. Por fim, a estratégia de genotipagem de vacas da raça Holandês para o polimorfismo GHR *Alu*I indicou que vacas portadoras do genótipo *Alu*I (-/-) tem menor produção leiteira e menor intervalo parto-concepção. Em geral, vacas que possuem ao menos um alelo *Alu*I(-) apresentaram menor intervalo parto-concepção e menor número de IA por concepção. Portanto, fica clara a importância do sistema GH/IGF-I para o desempenho tanto produtivo como reprodutivo de vacas de leite pós-parto, sendo que mais estudos com novas estratégias, assim como combinando diferente estratégias, podem trazer ainda mais benefícios aos sistemas de produção.

Palavras-chave: GH. IGF-I. bST. ovulação. polimorfismo. vacas.

Abstract

SCHNEIDER, Augusto. **GH/IGF-I system on the metabolic characteristics and postpartum reproductive activity in cattle.** 2011. 88f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Fertility in cattle is highly influenced by metabolic hormones. Of special interest for the reproductive axis are growth hormone (GH) and insulin-like growth factor (IGF-I). The aim of this thesis were 1) to characterize liver GHR and IGF-I expression in postpartum lactating beef cows and its relation to first ovulation; 2) to characterize GHR and IGF-I expression in granulosa and luteal cells; 3) to determine the effect of prepartum somatotropin on the postpartum resumption of ovarian activity; and 4) to determine the effect of the GHR *Alu*I polymorphism on the fertility of Holstein cows. The expression of GHR and IGF-I mRNA in the liver of postpartum lactating beef cows was not different between ovulatory and non-ovulatory cows, as well as it not change from 0 to 40 days postpartum. These data indicated that beef cows did not go through the same pattern of dissociation of the GH/IGF-I as observed for dairy cattle. Regarding the characterization of GHR and IGF-I expression in luteal and granulosa cells, it was possible to observe that GHR, IGF-I and SOCS expression were clearly higher in luteal than granulosa cells, but not different between atretic and estrogen active follicles. Moreover, GHR and IGF-I expression were not correlated in granulosa and luteal cells. With these results, we choose to focus on strategies in dairy cattle and not based on local regulation of GHR/IGF-I. In this sense, the use of prepartum somatotropin was benefitial in antecipate the resumption of postpartum ovarian activity. Further, treated cows had increased milk production and decreased concentrations of NEFA in the early postpartum. Also, the strategy based on the genotyping of Holstein cows for the GHR *Alu*I polymorphism indicated that cows carrying the *Alu*I(-/-) genotype had lower milk production and shorter calving conception interval. Cows carrying at least one *Alu*I(-) allele also had a shorter calving conception interval and less number of AI per conception. This way, it is clear the importance of the GH/IGF-I system to the reproductive and productive performance of postpartum dairy cows. More studies, with new strategies or combining different strategies can bring even more benefits to the production systems.

Keywords: GH. IGF-I. bST. ovulation. polymorfism. cows.

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Sumário

1. Introdução

O período de transição da gestação para a lactação é crítico para definir o desempenho reprodutivo de uma vaca. O retorno a atividade ovariana e a concepção em no máximo 100 dias após o parto são essenciais para a lucratividade de um sistema pecuário, seja de corte ou leite (ROCHE et al., 2000). Em vacas no terço final da gestação e início da lactação as demandas energéticas aumentam muito e ultrapassam a capacidade e/ou disponibilidade de alimento fazendo com que a mesma entre em balanço energético negativo (BEN) (BAUMAN AND CURRIE, 1980).

A duração e a intensidade do BEN está negativamente associada ao desempenho reprodutivo. Assim vacas em BEN tem um atraso no retorno a atividade ovariana pós-parto e consequentemente um atraso no momento da concepção (BUTLER & SMITH, 1989). Neste sentido, quanto mais ciclos estrais antes do momento da primeira inseminação, maior será a taxa de prenhez (THATCHER & WILCOX, 1973). Portanto, o uso de estratégias de seleção genética, tratamentos hormonais e manejo nutricional que antecipem o momento da primeira ovulação irão beneficiar também o momento da concepção. Durante o BEN muitos hormônios e metabólitos encontram-se alterados (WATHES et al., 2007). Apesar de todos terem importância para esta relação entre metabolismo e reprodução o objetivo desta tese é enfocar naquelas alterações ligadas ao eixo do hormônio do crescimento (GH, do inglês *growth hormone*) e fator de crescimento semelhante a insulina I (IGF-I, do inglês *insulin-like growth factor I*).

Logo após o parto, devido ao intenso BEN, vacas leiteiras tem uma redução drástica no nível sanguíneo de insulina, devido a drenagem de glicose pela glândula mamária para a síntese de lactose (BUTLER et al., 2003). Esta redução no nível de insulina leva a uma redução na expressão hepática do receptor do GH (GHR), especialmente GHR 1A (BUTLER et al., 2003), que compreende 50% do GHR hepático

(JIANG & LUCY, 2001a, b). Como o IGF-I é produzido em resposta a ativação do GHR pelo GH (JONES & CLEMMONS, 1995), nesta situação há uma dissociação do eixo GH/IGF-I, pois com a redução do GHR há uma redução na expressão hepática e nível circulante de IGF-I (FENWICK et al., 2008). Como consequência da redução do nível de IGF-I, o *feedback* negativo realizado pelo IGF-I na secreção de GH é diminuído também (MULLER et al., 1999) e há um aumento no nível circulante de GH (BUTLER et al., 2003). Esta elevação do nível de GH é benéfica para a produção de leite, pois estimula a lipólise e aumenta a disponibilidade de glicose para a síntese de leite pela glândula mamária (BELL, 1995). Devido ao aumento da lipólise neste período há um aumento nos níveis de ácidos graxos não esterificados (NEFA, do ingles, *non-esterified fatty acids*) e perda do escore de condição corporal (DOUGLAS et al., 2006). Em vacas de corte também é observada uma redução do nível circulante de IGF-I logo após o parto (SPICER et al., 2002), porém este mecanismo de dissociação do GH/IGF-I não foi demonstrado até o momento (JIANG et al., 2005).

O IGF-I circulante tem papel fundamental na reprodução (VELAZQUEZ et al., 2008). O nível sanguíneo de IGF-I está relacionado a idade ao primeiro parto (YIMAZ et al., 2006), retorno a atividade ovariana pós-parto (Butler et al., 2006) e sobrevivência embrionária (VELAZQUEZ et al., 2005). Além do mais, o IGF-I atua no crescimento e diferenciação de folículos antrais (RIVERA & FORTUNE, 2003). Neste sentido a queda do nível de IGF-I no período pós-parto leva a um atraso no desenvolvimento de folículos antrais e, consequentemente, da ovulação. Vários trabalhos demonstram que vacas que ovulam o folículo dominante da primeira onda pós-parto tem maiores níveis sanguíneos de IGF-I do que vacas anovulatórias (BUTLER et al., 2006, KAWASHIMA et al., 2007a, KAWASHIMA et al., 2007b). O IGF-I também atua aumentando a responsividade as gonadotrofinas hipofisiárias, estimulando a esteroidogênese (ARMSTRONG & WEBB, 1997) e reduzindo o nível de apoptose nas células da teca e granulosa (EL-ROEIY et al., 1994).

A maioria das ações do GH são mediadas pelo IGF-I, tanto em nível sistêmico como parácrino. No entanto, foi demonstrada que há expressão de GHR em células ovarianas em diversos estágios de crescimento (KOLLE et al., 1998, SHIMIZU et al.,

2008). Apesar disso, a expressão ovariana de IGF-I é controversa e diversos autores demonstraram que não há expressão de IGF-I nas células da granulosa (ARMSTRONG et al., 2000, PERKS et al., 1999, SUDO et al., 2007), apesar de outros demonstrarem que há (RHOADS et al., 2008, SCHAMS et al., 2002, SPICER et al., 1993), porém em níveis reduzidos quando comparados ao fígado e corpo lúteo (CL) (RHOADS et al., 2008). No entanto, desde a demonstração de que ratos com deleção hepato-específica do gene IGF-I conseguem reproduzir normalmente (YAKAR et al., 1999), a importância da expressão local de IGF-I tem sido revisada. Assim a resposta das células ovarianas ao GH é controversa (LUCY, 2000) e mais estudos são necessários visando melhor entender a regulação ovariana do eixo GH/IGF-I e possíveis implicações para seu uso em estratégias para maximizar o crescimento follicular.

A maioria das ações do IGF-I são exercidas através do IGFR-I e a ligação dos IGFs ao seu receptor é mediada pelas proteínas de ligação do IGF (IGFBP, do inglês *IGF binding proteins*) (SUDO et al., 2007). Nos folículos dominantes há um aumento da concentração intrafolicular de IGF-I livre com relação aos subordinados (BEG et al., 2001). A proteína A associada a prenhez (PAPP-A, do inglês *pregnancy-associated plasma protein-A*) é uma protease de IGFBPs (SUDO et al., 2007). Assim, a secreção de PAPP-A mRNA é estimulada pelo FSH e irá aumentar as concentrações de IGF-I e estradiol no fluído folicular do folículo dominante (RIVERA & FORTUNE, 2001; SANTIAGO et al., 2005).

Assim várias estratégias vem sendo utilizadas para melhorar a condição metabólica de vacas no período de transição e, consequentemente, o desempenho reprodutivo. Vários estudos demonstram que o desempenho no período pós-parto recente está muito relacionado com o manejo da vaca antes do parto (CURTIS et al., 1985). Neste sentido, estratégias nutricionais que visam adaptar a vaca aos desafios metabólicos do pós-parto são interessantes. Uma destas é o uso da restrição alimentar visando adaptar a vaca a alta taxa de lipólise que ocorre logo após o parto (DOUGLAS et al., 2006). Assim, o uso de somatotropina exógena do período pré-parto para estimular a lipólise tem função semelhante, acarretando em melhor ingestão de matéria

seca e produção de leite no pós-parto (GULAY et al., 2004, PUTNAM et al., 1999). Além do mais, a aplicação de somatotropina aumenta o número de folículos recrutados por até 21 dias após o fim das aplicações (KIRBY et al., 1996).

Outra estratégia interessante e cujo uso vem sendo incrementado com novas tecnologias que facilitam sua aplicação, é a seleção assistida por marcadores moleculares. Interessantemente, poucos estudos tem sido publicados visando endereçar o efeito de mutações em genes ligados ao eixo GH/IGF-I em bovinos com relação ao desempenho reprodutivo. No entanto, alguns trabalhos publicados com mutações no gene do GH não acharam benéfico sobre o momento da primeira ovulação pós-parto (BALOGH et al., 2009). Já polimorfismos localizados nos genes GHR e STAT5, um mediador intracelular do GH, foram positivamente associados ao momento da primeira ovulação pós-parto (SHIRASUNA et al., 2010). Assim, mais estudos são necessários visando identificar mutações nos genes do eixo GH/IGF-I que podem estar relcionadas ao mecanismo de aumento da produção leiteira e redução da fertilidade.

2. Objetivos

Os objetivos desta tese foram:

1. Avaliar o padrão de expressão dos genes GHR e IGF-I em vacas de corte lactantes e sua influência sobre o retorno a atividade ovariana pós-parto;
2. Avaliar a expressão dos genes GHR e IGF-I em folículos ovarianos de diferentes categorias e corpo lúteo de bovinos;
3. Avaliar o efeito da aplicação pré-parto de somatotropina sobre o metabolismo, produção de leite e retorno a atividade ovariana em vacas prímiparas da raça Holandês;
4. Avaliar o efeito do polimorfismo *Alu*I no gene GHR sobre a produção de leite e fertilidade de vacas da raça Holandês.

3. Artigo 1 – Insulin-like growth factor and growth hormone receptor in postpartum lactating beef cows

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Insulin-like growth factor and growth hormone receptor in postpartum lactating beef cows

Abstract – The objective of this study was to evaluate the plasma concentrations of insulin-like growth factor-I (IGF-I) and the mRNA hepatic expression of IGF-I and of the growth hormone receptors GHR and GHR 1A, in postpartum beef cows. Four Angus and four crossbred (Angus x Nelore) postpartum suckled beef cows were used. Liver samples for gene expression and blood samples for β-hidroxibutirate and IGF-I assay were collected each 10 days, from calving to 40 days postpartum, while samples for progesterone assay were collected every other day, from day 10 to 40 postpartum. Overall, three cows ovulated before 40 days postpartum. IGF-I concentration was higher in Angus x Nelore than in Angus cows. There was no difference in the expression of GHR, GHR 1A and IGF-I according to breed or ovulatory status. IGF-I concentrations are higher in crossbred cows, but have not changed according postpartum ovulatory status. Moreover, changes in postpartum IGF-I concentrations are not associated with changes in liver GHR, GHR 1A and IGF-I mRNA expression in both breeds.

Index terms: *Bos indicus*, *Bos taurus*, GHR, IGF-I, mRNA hepatic expression, ovulation.

Fator de crescimento semelhante à insulina e receptor do hormônio do crescimento em pós-parto de vacas de corte

Resumo – O objetivo deste estudo foi avaliar as concentrações plasmáticas do fator de crescimento semelhante à insulina tipo I (IGF-I) e a expressão hepática de mRNA de IGF-I e dos receptores do hormônio do crescimento GHR e GHR 1A, no pós-parto de vacas de corte. Quatro vacas Angus e quatro mestiças (Angus x Nelore) foram usadas durante o período de pós-parto. As coletas de tecido hepático, para avaliação da expressão gência, e de sangue, para análise de β-hidróxibutirato e IGF-I, foram realizadas a cada 10 dias, do parto até 40 dias pós-parto, enquanto as amostras para análise de progesterona foram coletadas a cada dois dias, dos 10 aos 40 dias

pós-parto. Três vacas ovularam antes dos 40 dias pós-parto. A concentração de IGF-I foi maior em vacas Angus x Nelore do que em vacas Angus. Não houve diferença na expressão hepática de GHR, GHR 1A e IGF-I de acordo com a raça ou com a ovulação. As concentrações de IGF-I são maiores em vacas cruzadas, e não há diferença entre vacas que tenham ovulado ou não. Além disso, as mudanças observadas na concentração de IGF-I durante o período pós-parto não estão associadas a alterações na expressão hepática de mRNA de GHR, GHR 1A e IGF-I, em ambas as raças.

Termos para indexação: *Bos indicus*, *Bos Taurus*, GHR, IGF-I, expressão hepática de mRNA, ovulação

Introduction

The major objective of cow-calf enterprises is to produce one calf per cow annually. Although the increased knowledge of cow reproductive biology (Santos et al., 2004), factors involved in the resumption of postpartum cyclicity in cows are still unclear. During the peripartum period, the stress of pregnancy, parturition, onset of lactation and suckling negatively affects the energy intake (Ciccioli et al., 2003). This condition induces a postpartum negative energy balance, which is associated with a prolonged interval from calving to first ovulation (Stagg et al., 1998). However, the underlying cause of prolonged postpartum anestrus is not the lack of dominant follicle development, as follicular growth restarts during the first 10 days after calving, but an ovulation failure (Diskin et al., 2003) due to reduced concentrations of metabolites that act directly on follicular growth and maturation (Beam & Butler, 1999).

The growth hormone receptor (GHR), which modulates insulin-like growth factor I (IGF-I) synthesis under GH control (Jones & Clemmons, 1995), is detected in greatest abundance in

the liver (Bornfeldt et al., 1989). The three most expressed liver variants are GHR 1A, 1B and 1C, responsible for 50, 35 and 15% of the total GHR mRNA, respectively (Jiang & Lucy, 2001). Although during the early postpartum period there is a simultaneous reduction in GHR 1A and IGF-I mRNA expression in the liver of dairy cows due the intense negative energy balance (Radcliff et al., 2003a), no similar condition was observed for beef cows (Jiang et al., 2005). The reduced expression of these genes in liver led to reduced plasma IGF-I concentration, which is restored about three weeks postpartum due to decreasing negative energy balance intensity (Kobayashi et al., 1999).

Apparently, the GH/IGF-I axis is also involved in the mechanisms of resumption of the postpartum cyclicity, since plasma concentrations of IGF-I during the postpartum period increased linearly up to the day of first ovulation (Stagg et al., 1998), and correlate with the length of anestrus in beef cows (Roberts et al., 2005). In addition, IGF-I concentration increased more in Angus x Brahman than Angus cows in the early postpartum period (Spicer et al., 2002), and Brahman cows had greater plasma IGF-I concentration than Angus cows during the entire postpartum period (Alvarez et al., 2000). The IGF-I acts on the ovary via the type-1 IGF receptor (Willis et al., 1998) and function as a modulator of gonadotrophin action, stimulating granulosa and theca cell proliferation and differentiation (Armstrong & Webb, 1997) and preventing follicular atresia (el-Roeiy et al., 1994).

Considering these evidences, it is important to evaluate if the increasing IGF-I concentrations in postpartum beef cattle is associated with higher hepatic expression of GHR/GHR1A/IGF-I mRNA, if increased plasma IGF-I concentration and expression of GHR/GHR1A/IGF-I mRNA in the liver are associated with early resumption of ovulation, and if expression of GHR/GHR1A/IGF-I mRNA during postpartum is higher in crossbred than in *Bos taurus* cattle. Therefore, the objective of this study was to evaluate the plasma concentrations of

insulin-like growth factor-I (IGF-I) and the mRNA hepatic expression of IGF-I and of the growth hormone receptors GHR and GHR 1A, in postpartum beef cows.

Materials and Methods

The Committee for Ethics in Animal Experimentation from the Universidade Federal de Pelotas has approved all procedures performed in this experiment (Protocol 23110.004382/2010-89).

Eight postpartum suckled beef cows, four Angus (*Bos taurus*) and four crossbreed cows [Angus x Nelore (*Bos taurus* x *Bos indicus*)] were used, in a Southern-Brazil (30º 36´ S and 51º 21´W, 6 m altitude) farm. All the parturitions occurred in a three-day interval (calving day was considered Day 0). The cows had a mean body condition score of 2.81±0.26 (ranging from 2.5–3.0; (Lowman et al., 1976) at the beginning of the experiment, and were maintained in a native pasture.

Blood samples were collected from coccygeal vein into heparinized 10-mL vacuttainer tubes (BD Diagnostics, São Paulo, Brazil). The tubes were immediately centrifuged (1,500 *g* for 15 min), and the plasma was harvested and stored at -80^{o}C until evaluation. Samples were collected every two days for progesterone analyses, from Day 10 to 40, and every 10 days, from Day 0 to 40, for IGF-I and β-hidroxibutirate analyses, respectively.

The β-hidroxibutirate assays were performed in a single batch. β-hidroxibutirate (Ranbut, Randox, Crumlin, United Kingdom) concentration was evaluated through final point enzymatic colorimetric reactions, quantified by a spectrophotometer, FEMTO 700 Plus, Femto Ind. e Com. de Instrumentos Ltda., São Paulo, Brazil (Velazquez et al., 2005). The detection limit of the assay was 1.04 mg dL^{-1}, and the intra-assay coefficient of variation was 3.7%.

The progesterone assays were performed in a single batch. Progesterone concentrations were measured using electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland) using Progesterone II kits, Roche Diagnostics, Manheim, USA (Bargouli et al., 2007). The detection limit of the assay was 0.03 ng mL^{-1} and the intra-assay coefficient of variation was 5.4%. A cow was considered ovulated when the blood concentration of progesterone rose above 1 ng mL^{-1} in two consecutives samples (Stevenson & Britt, 1979). Three cows (one Angus and two Angus x Nelore) ovulated before 40 days postpartum (mean 36.3±0.3 days) and were considered as the Ovulatory group (Ov). The other five cows did not ovulate in this period and were considered as the non-ovulatory group (Nov).

Plasma IGF-I concentrations were evaluated by radioimmunoassay, DSL-5600, Diagnostics Systems Laboratory, Webster, United States (Awawdeh et al., 2004), after an extraction step in which IGF-I was separated from its binding proteins, and had a minimum detection limit of 2.25 ng mL^{-1}. The intra-assay coefficients of variation were 5.14 and 9.15%, for low and high IGF-I concentrations, respectively. The inter-assay coefficients of variation were 1.06 and 0.66%, for low and high IGF-I concentrations, respectively.

On the same days of blood collections (Days 0, 10, 20, 30 and 40), liver biopsies were performed transcutaneously according Radcliff et al. (2003a). The liver samples were immediately stored in microtubes and frozen in liquid nitrogen until RNA extraction.

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The quality of the RNA was evaluated by calculating the ratio of absorbance at 260 and 280 nm, followed by electrophoresis on a 1.5% agarose gel stained with etidium bromide. Only RNA with intact 18S and 28S bands were used.

Total RNA was treated with DNAse I (DNAse Amp Grade, Invitrogen, Carlsbad, USA) to remove genomic DNA contamination and primed with oligo(dT)20 to synthesize single strand

cDNA (SuperScript III First-Strand Synthesis Supermix, Invitrogen, Carlsbad, USA). The PCR amplifications and fluorescence detection, using the cDNA obtained in the previous step, were performed in duplicate in the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, USA), using the SYBR Green detection chemistry (Platinum SYBR Green qPCR SuperMix-UDG kit, Invitrogen, Carlsbad, USA) as recommended by the manufacturer. The primer sequences were as follows: GHR (forward CCA GTT TCC ATG GTT CTT AAT TAT; reverse TTC CTT TAA TCT TTG GAA CTG G) (Pfaffl et al., 2002), GHR1A (forward AGC GAC ATT ACA CCA GCA GGA A; reverse AGG GGC CAG GGC AAT GTA CTT TT), IGF-I (forward TCG CAT CTC TTC TAT CTG GCC CTG T; reverse GCA GTA CAT CTC CAG CCT CCT CAG A) (Pfaffl et al., 2002) and β-actin (forward CTA GGC ACC AGG GCG TCA TG; reverse CTT AGG GTT CAG GGG GGC CT).

The PCR reaction efficiencies and cycle thresholds from the fluorescence readings of individual wells during the reaction were calculated using PCR Miner (Sheng & Russell, 2005). For each sample, a mean cycle threshold of two PCR reactions was calculated. Also, the expression of each target gene of interest was calculated relative to β-actin using the equation: relative target gene expression = $(1/E_{target}{}^{CTtarget})/(1/E_{\beta\text{-}actin}{}^{CT\beta\text{-}actin})$, where E was reaction efficiency and CT was cycle threshold (Cikos & Koppel, 2009). The mean coefficient of variation among sample CT´s was 0.71%. The specificity of each primer was verified by the detection of only one fluorescence peak at the dissociation curve for each replicate in the end of the PCR.

The statistical analyses were performed in SAS (SAS Institute Inc. Cary, NC, USA). The effects of age, parity, body condition score, and body weight had no effects in the model; therefore, they were excluded from the final statistical model. Analyses involving repeated measures over time were compared between breed and ovulatory status, by analysis of variance for repeated measures using the Mixed procedure to evaluate the main effects of breed, ovulatory

status, day and their interactions. When no effects of ovulatory status and breed over time were detected, they were removed from the model. Pearson's correlations were also determined. The analyses were carried out at 5% probability.

Results and Discussion

The most important observation of this study was the marked increase in plasmatic IGF-I concentration in postpartum crossbred cows Angus x Nelore compared to Angus cows despite no difference in the hepatic expression of GHR/IGF-I mRNA. In addition, no changes in the concentration of IGF-I or GHR/IGF-I mRNA expression were observed between ovulatory and non ovulatory cows.

There was no effect of days postpartum, ovulatory group, breed and its interactions on concentrations of β-hidroxibutirate (Table 1). Also, there was no effect of days postpartum or ovulatory group on IGF-I concentration. However, there were significant effects of breed (and breed-by-ovulation interaction on IGF-I plasma concentration. IGF-I concentration was higher in crossbred cows (Angus x Nelore, 53.8±4.6 ng mL^{-1}) than in Angus cows (39.8±1.5 ng mL^{-1}) during the period of study. In fact, IGF-I concentration was lower in both Ov and Nov Angus cows (42.4±7.5 and 38.6±5.1 ng mL^{-1}, respectively) when compared to Ov and Nov crossbred cows (50.6±20.5 and 57.0±13.7 ng mL^{-1}, respectively). In addition, there was no difference before and after ovulation in the concentrations of β-hidroxibutirate (7.9±1.3 vs. 5.9±1.4 mg dL^{-1}) and IGF-I (44.8±3.8 vs. 59.0±25.8 ng mL^{-1}) for cows on the Ov group.

The higher IGF-I concentration in crossbred compared to Angus cows observed in this study is consistent with previous observations (Alvarez et al., 2000). In addition, the increase of this hormone concentration was more evident at Day 40 postpartum. Similarly, Spicer et al.

(2002) observed a rise in IGF-I concentration from postpartum-week two to seven in Brahman x Angus cows, but not in Angus cows. The difference between the two breeds may be due to different lactational demand, since Nelore cows produce less milk than Angus cows (Jenkins et al., 2000), and there is a negative association between potential for milk production and circulating IGF-I concentration (Roberts et al., 2005).

Although difference between breeds was detected, plasmatic IGF-I concentration had not changed between Ov and Nov groups. However, previous data indicate that IGF-I was higher in both dairy (Kawashima et al., 2007) and beef cows (Kawashima et al., 2008) that ovulated earlier in the postpartum. Moreover, β-hidroxibutirate concentration had not changed between Ov and Nov groups in the current study, in agreement with results from Kawashima et al. (2008) in beef cattle, despite Taylor et al. (2003) had observed that dairy cows with delayed ovulation have higher levels of β-hidroxibutirate. In addition, there were no changes in the concentrations of IGF-I and β-hidroxibutirate before and after ovulation in the Ov group, contrasting with results from Kawashima et al. (2007), that observed a reduction in IGF-I concentrations after ovulation in postpartum dairy cows.

Hepatic expression of GHR, GHR 1A and IGF-I mRNA did not change from Days 0 to 40 postpartum (Table 1), as previously observed by Jiang et al. (2005) in Angus cows at the prepartum, partum and early postpartum period using ribonuclease protection assay. These data are contradictory with results from studies in Holstein cows, which had a simultaneously reduction in hepatic GHR 1A and IGF-I mRNA in the early postpartum period (Radcliff et al., 2003b), probably because dairy cows underwent a more severe negative energy balance at this period (Fenwick et al., 2008). Although it was detected an interaction between ovulation and day for IGF-I mRNA expression, no difference within days were observed. No difference (was detected in the expression of GHR (0.46±0.06 vs. 0.48±0.17), GHR 1A (0.29±0.04 vs.

0.23±0.06) and IGF-I (0.18±0.03 vs. 0.19±0.06) mRNA, when the periods before and after ovulationwere compared, in the three cows that ovulated (Ov Group). In spite of that, Rhoads et al. (2008) observed that there was no correlation between hepatic and ovarian expression of GHR or IGF-I mRNA in postpartum dairy cows, and the hypothesis that this gene is differentially regulated in the ovary of ovulatory cows could not be dismissed.

Plasma concentrations of IGF-I were not correlated with hepatic expression of its gene in the present study, contrasting with previous observations in dairy cows (Fenwick et al., 2008). Moreover, despite plasmatic concentration of IGF-I was higher in crossbreed cows, hepatic IGF-I mRNA expression did not follow the same pattern. This contradiction could be due to the fact that plasmatic concentration of IGF-I is also correlated to the hepatic expression of IGF binding proteins 1–6 and the IGF acid labile subunit (IGF ALS) (Fenwick et al., 2008), which regulates IGF-I plasma half-life and transport through the vascular endothelium (Thissen et al., 1994). Moreover, the decreased concentration of IGF-I in postpartum beef cows coincided with an increased concentration of circulating IGFBP-2 and decreased IGFBP-3 (Roberts et al., 1997). Therefore, more studies with a higher number of cows and considering the various intermediate steps of the somatotropic axis must be done in order to explain the source of serum IGF-I reduction in beef cattle.

A positive correlation between hepatic GHR and GHR1A mRNA expression was observed ($n = 24$, $r = 0.81$, $p<0.0001$). However, no correlation between GHR and IGF-I mRNA or GHR 1A and IGF-I mRNA was observed, except for crossbred cows that had a positive correlation between GHR 1A and IGF-I mRNA ($r = 0.55$, $p = 0.02$). This overall absence of correlation between GHR, GHR 1A and IGF-I mRNA was in agreement with previous data in dairy cattle (Butler et al., 2003). Although in that study there was no correlation between hepatic expression of GHR, GHR 1A and IGF-I mRNA in control cows, cows treated with insulin had a

positive correlation between GHR 1A and IGF-I mRNA. Similarly, in the current study a positive correlation was only observed between GHR 1A and IGF-I mRNA in crossbred cows. This observation may be linked to higher plasmatic concentration of IGF-I as a consequence of different lactational demand and negative energy balance intensity, resembling the condition in the study of Butler et al. (2003), in which insulin infusion induced a positive correlation between hepatic GHR 1A and IGF-I mRNA.

Conclusions

1. IGF-I concentrations are higher in crossbred cows, but do not change with postpartum ovulatory status.

2. Changes in postpartum serum IGF-I concentrations are not associated to liver GHR, GHR 1A and IGF-I mRNA expression in *Bos taurus* and *Bos taurus* x *Bos indicus* suckled beef cows.

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References

ALVAREZ, P.; SPICER, L.J.; CHASE JUNIOR, C.C.; PAYTON, M.E.; HAMILTON, T.D.; STEWART, R.E.; HAMMOND, A.C.; OLSON, T.A.; WETTEMANN, R.P. Ovarian and endocrine characteristics during an estrous cycle in Angus, Brahman, and Senepol cows in a subtropical environment. **Journal of Animal Science**, v.78, p.1291-1302, 2000.

ARMSTRONG, D.G.; WEBB, R. Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. **Reviews in Reproduction**, v.2, p.139-146, 1997.

AWAWDEH, M.S.; TITGEMEYER, E.C.; MCCUISTION, K.C.; GNAD, D.P. Effects of ammonia load on methionine utilization by growing steers. **Journal of Animal Science**, v.82, p.3537-3542, 2004.

BARGOULI, G.G.; TSANTARLIOTOU, M.P.; BROZOS, C.N.; KOKOLIS, N.A.; BOSCOS, C.M. Effect of norgestomet treatment on plasminogen activator activity in the cervical mucus and the endometrium in dairy cows. **Journal of Veterinary Medicine, Series A. Physiology, Pathology and Clinical Medicine**, v.54, p.393-397, 2007.

BEAM, S.W.; BUTLER, W.R. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. **Journal of Reproduction and Fertility**, v.54, p.411-424, 1999.

BORNFELDT, K.E.; ARNQVIST, H.J.; ENBERG, B.; MATHEWS, L.S.; NORSTEDT, G. Regulation of insulin-like growth factor-I and growth hormone receptor gene expression by diabetes and nutritional state in rat tissues. **Journal of Endocrinology**, v.122, p.651-656, 1989.

BUTLER, S.T.; MARR, A.L.; PELTON, S.H.; RADCLIFF, R.P.; LUCY, M.C.; BUTLER, W.R. Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy

cattle: effects on expression of IGF-I and GH receptor 1A. **Journal of Endocrinology**, v.176, p.205-217, 2003.

CICCIOLI, N.H.; WETTEMANN, R.P.; SPICER, L.J.; LENTS, C.A.; WHITE, F.J.; KEISLER, D.H. Influence of body condition at calving and postpartum nutrition on endocrine function and reproductive performance of primiparous beef cows. **Journal of Animal Science**, v.81, p.3107-3120, 2003.

CIKOS, S.; KOPPEL, J. Transformation of real-time PCR fluorescence data to target gene quantity. **Analytical Biochemistry**, v.384, p.1-10, 2009.

DISKIN, M.G.; MACKEY, D.R.; ROCHE, J.F.; SREENAN, J.M. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. **Animal Reproduction Science**, v.78, p.345-370, 2003.

EL-ROEIY, A.; CHEN, X.; ROBERTS, V.J.; SHIMASAKAI, S.; LING, N.; LEROITH, D.; ROBERTS JUNIOR, C.T.; YEN, S.S. Expression of the genes encoding the insulin-like growth factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. **Journal of Clinical Endocrinology and Metabolism**, v.78, p.1488-1496, 1994.

FENWICK, M.A.; FITZPATRICK, R.; KENNY, D.A.; DISKIN, M.G.; PATTON, J.; MURPHY, J.J.; WATHES, D.C. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. **Domestic Animal Endocrinology**, v.34, p.31-44, 2008.

JENKINS, T.G.; FERRELL, C.L.; ROBERTS, A.J. Lactation and calf weight traits of mature crossbred cows fed varying daily levels of metabolizable energy. **Journal of Animal Science**, v.78, p.7-14, 2000.

JIANG, H.; LUCY, M.C. Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: isolation, expression and effects on translational efficiency. **Gene**, v.265, p.45-53, 2001.

JIANG, H.; LUCY, M.C.; CROOKER, B.A.; BEAL, W.E. Expression of growth hormone receptor 1A mRNA is decreased in dairy cows but not in beef cows at parturition. **Journal of Dairy Science**, v.88, p.1370-1377, 2005.

JONES, J.I.; CLEMMONS, D.R. Insulin-like growth factors and their binding proteins: biological actions. **Endocrine Reviews**, v.16, p.3-34, 1995.

KAWASHIMA, C.; FUKIHARA, S.; MAEDA, M.; KANEKO, E.; MONTOYA, C.A.; MATSUI, M.; SHIMIZU, T.; MATSUNAGA, N.; KIDA, K.; MIYAKE, Y.; SCHAMS, D.; MIYAMOTO, A. Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave post-partum in high-producing dairy cows. **Reproduction**, v.133, p.155-163, 2007.

KAWASHIMA, C.; KIDA, K.; MATSUHASHI, M.; MATSUI, M.; SHIMIZU, T.; MATSUNAGA, N.; ISHII, M.; MIYAKE, Y.; MIYAMOTO, A. Effect of suckling on the reproductive performance and metabolic status of obese Japanese black cattle during the early postpartum period. **Journal of Reproduction and Development**, v.54, p.46-51, 2008.

KOBAYASHI, Y.; BOYD, C.K.; BRACKEN, C.J.; LAMBERSON, W.R.; KEISLER, D.H.; LUCY, M.C. Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of GHR 1A that is associated with decreased insulin-like growth factor I. **Endocrinology**, v.140, p.3947-3954, 1999.

LOWMAN, B.G.; SCOTT, N.A.; SOMERVILLE, S.H. **Condition scoring of cattle**. Edinburgh: Edinburgh School of Agriculture, 1976. 31p.

PFAFFL, M.W.; GEORGIEVA, T.M.; GEORGIEV, I.P.; ONTSOUKA, E.; HAGELEIT, M.; BLUM, J.W. Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3 in the bovine species. **Domestic Animal Endocrinology**, v.22, p.91-102, 2002.

RADCLIFF, R.P.; MCCORMACK, B.L.; CROOKER, B.A.; LUCY, M.C. Growth hormone (GH) binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. **Journal of Dairy Science**, v.86, p.3933-3940, 2003a.

RADCLIFF, R.P.; MCCORMACK, B.L.; CROOKER, B.A.; LUCY, M.C. Plasma hormones and expression of growth hormone receptor and insulin-like growth factor-I mRNA in hepatic tissue of periparturient dairy cows. **Journal of Dairy Science**, v.86, p.3920-3926, 2003b.

RHOADS, M.L.; MEYER, J.P.; KOLATH, S.J.; LAMBERSON, W.R.; LUCY, M.C. Growth hormone receptor, insulin-like growth factor (IGF)-1, and IGF-binding protein-2 expression in the reproductive tissues of early postpartum dairy cows. **Journal of Dairy Science**, v.91, p.1802-1813, 2008.

ROBERTS, A.J.; KLINDT, J.; JENKINS, T.G. Effects of varying energy intake and sire breed on duration of postpartum anestrus, insulin like growth factor-1, and growth hormone in mature crossbred cows. **Journal of Animal Science**, v.83, p.1705-1714, 2005.

ROBERTS, A.J.; NUGENT, R.A.; KLINDT, J.; JENKINS, T.G. Circulating insulin-like growth factor I, insulin-like growth factor binding proteins, growth hormone, and resumption of estrus in postpartum cows subjected to dietary energy restriction. **Journal of Animal Science**, v.75, p.1909-1917, 1997.

SANTOS, J.E.P.; THATCHER, W.W.; CHEBEL, R.C.; CERRI, R.L.; GALVAO, K.N. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. **Animal Reproduction Science**, v.82-83, p.513-535, 2004.

SAS INSTITUTE INC. SAS 9.1.3 Help and Documentation, Cary, NC: SAS Institute Inc., 2002-2004.

SHENG, Z.; RUSSELL, D.F. Comprehensive algorithm for quantitative real-time polymerase chain reaction. **Journal of Computational Biology,** v.12, p.1045-1062, 2005.

SPICER, L.J.; CHASE JUNIOR, C.C.; RUTTER, L.M. Relationship between serum insulin-like growth factor-I and genotype during the postpartum interval in beef cows. **Journal of Animal Science**, v.80, p.716-722, 2002.

STAGG, K.; SPICER, L.J.; SREENAN, J.M.; ROCHE, J.F.; DISKIN, M.G. Effect of calf isolation on follicular wave dynamics, gonadotropin and metabolic hormone changes, and interval to first ovulation in beef cows fed either of two energy levels postpartum. **Biology of Reproduction**, v.59, p.777-783, 1998.

STEVENSON, J.S.; BRITT, J.H. Relationships among luteinizing hormone, estradiol, progesterone, glucocorticoids, milk yield, body weight and postpartum ovarian activity in Holstein cows. **Journal of Animal Science**, v.48, p.570-577, 1979.

TAYLOR, V.J.; BEEVER, D.E.; BRYANT, M.J.; WATHES, D.C. Metabolic profiles and progesterone cycles in first lactation dairy cows. **Theriogenology**, v.59, p.1661-1677, 2003.

THISSEN, J.P.; KETELSLEGERS, J.M.; UNDERWOOD, L.E. Nutritional regulation of the insulin-like growth factors. **Endocrine Reviews**, v.15, p.80-101, 1994.

VELAZQUEZ, M.A.; NEWMAN, M.; CHRISTIE, M.F.; CRIPPS, P.J.; CROWE, M.A.; SMITH, R.F.; DOBSON, H. The usefulness of a single measurement of insulin-like growth factor-1 as a predictor of embryo yield and pregnancy rates in a bovine MOET program. **Theriogenology**, v.64, p.1977-1994, 2005.

WILLIS, D.S.; MASON, H.D.; WATSON, H.; FRANKS, S. Developmentally regulated responses of human granulosa cells to insulin-like growth factors (IGFs): IGF-I and IGF-II action

mediated via the type-I IGF receptor. **Journal of Clinical Endocrinology and Metabolism**, v.83, p.1256-1259, 1998.

Tables

Table 1. Mean±SE of β-hidroxybutirate (mg dL^{-1}) and insulin-like growth factor-I (IGF-I, ng mL^{-1}) blood concentrations, and of hepatic expression of growth hormone receptor GHR, GHR 1A and IGF-I mRNA in suckled beef cows, from 0 to 40 days postpartum, besides the statistical significance of the sources of variation.

Variable	Days postpartum					Sources of variation					
	0	10	20	30	40	Bred	Day	Bred vs. Day	Ovulatory staus	Ovulatory staus vs. Bred	Ovulatory staus vs. Day
β-hidroxibutirate	9.4±1.7	7.7±1.3	8.1±0.8	7.4±1.4	6.6±0.7	0.59	0.91	0.46	0.55	0.71	0.81
IGF-I	40.9±0.5	44.0±3.6	41.4±1.9	49.4±5.4	53.3±10.1	0.02	0.99	0.60	0.99	0.02	0.88
GHR mRNA	0.4±0.2	0.6±0.1	0.5±0.1	0.4±0.1	0.5±0.2	0.67	0.79	0.36	0.47	0.59	0.54
GHR 1A mRNA	0.3±0.1	0.4±0.1	0.3±0.0	0.3±0.1	0.2±0.1	0.48	0.43	0.52	0.48	0.28	0.52
IGF-I mRNA	0.2±0.1	0.2±0.1	0.1±0.0	0.1±0.1	0.2±0.1	0.17	0.12	0.12	0.06	0.47	0.06

4. Artigo 2 – Difference in the expression of components of the GHR/IGF-I axis in bovine granulosa and luteal cells

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Difference in the expression of components of the GHR/IGF-I axis in bovine granulosa and luteal cells

Abstract

This study has the objective to determine if E2 in follicular fluid (FF) can up-regulate SOCS-2 and -3 mRNA expression in granulosa cells and consequently reduce IGF-I mRNA expression when compared to luteal cells. Ovaries were collected from cows during slaughter. Follicles (7 estrogen-active follicles [EAF] and 7 atretic follicles [ATF]) were dissected from the stroma, FFL was aspirated and the granulosa cells recovered. CL (n = 7) were also dissected. Total RNA was isolated from granulosa and luteal cells and real-time PCR used to evaluate suppressor of cytokine signaling (SOCS-1, -2 and -3), GHR, IGF-I, IGF-II and ERα mRNA expression according to the ΔΔCt method. Estradiol (E2), progesterone (P4) and IGF-I were evaluated in FFL. In EAF and ATF, FFL E2 concentration was 137.3 ± 39.7 and 21.1 ± 6.0 ng/mL, with E2/P4 ratio of 2.06 ± 0.61 and 0.37 ± 0.11, respectively. IGF-I in FFL was 96 ± 18 and 85 ± 25 ng/mL for EAF and ATF, respectively. Expression of GHR, IGF-I, IGF-II, SOCS-1 and SOCS-2 mRNA was higher in the CL than EAF and ATF. GHR mRNA expression was 8 times higher, while IGF-I mRNA was 25 times higher in CL than in follicles. SOCS-3 and ERα expression was not different between CL and follicles. No difference between EAF and ATF for the genes studied was found. Regarding follicles, SOCS-2 was correlated to GHR ($r = 0.62$, $P<.05$), ERα ($r = 0.87$, $P<.0001$) and FFL E2 ($r = 0.55$, $P<.05$). In the CL, SOCS-2 was correlated to GHR ($r = 0.85$, $P<.05$) and ERα ($r = 0.85$, $P<.05$). The IGF-I and SOCS-2 to GHR ratio was lower ($P <.01$) in follicles than CL, which indicates that there is more IGF-I and SOCS-2 mRNA production per unit of GHR in the CL. In summary, the components of the GH/IGF-I axis were more expressed in the luteal than granulosa cells and no difference between ATF and EAF was found. SOCS-2

mRNA is regulated by E2 levels through ERα in granulosa and luteal cells, but we were not able to demonstrate its implication on disruption of GHR signaling.

Key Words: GHR, IGF-I, SOCS, ovary, bovine

Introduction

Plasma insulin-like growth factor I (IGF-I) is primarily produced in the liver under the control of growth hormone (GH) (Jones and Clemmons, 1995). IGF-I is a growth factor that stimulates growth and development within a variety of cell types (Jones and Clemmons, 1995). IGF-II is similar to IGF-I in structure and function, but GH does not control its secretion (Vicini et al., 1991). IGF-I and II are present in the follicular fluid, with IGF-II being observed in higher levels than IGF-I (Stewart et al., 1996).

Previous data indicate that intrafollicular IGF-I is mainly derived from the blood and its local production is not significant in cattle (Lucy, 2000). However, since the demonstration that mice with hepato-specific knockout of the IGF-I gene has normal reproductive function (Yakar et al., 1999), the importance of locally produced IGF-I has been reviewed. Although the expression of IGF-I in granulosa cells of the bovine follicle is controversial, with reports demonstrating both the presence (Rhoads et al., 2008a; Schams et al., 2002; Spicer et al., 1993) and absence (Armstrong et al., 2000; Perks et al., 1999; Sudo et al., 2007); its expression is higher and more easily detectable in the corpus luteum (CL) (Kirby et al., 1996; Neuvians et al., 2003).

Once GH binds to its receptor (GHR) it stimulates the Janus kinase (JAK) and signal transducer and activator of transcription (Stat) pathway (Herrington et al., 2000). Stat5 has been shown to have an essential role in GH signaling. Mice with a mutation in both the Stat5a and -5b genes have a 50% reduction in the plasma concentration of IGF-I (Teglund et al., 1998). On the other hand, the suppressor of cytokine signaling (SOCS) -1, -2, and -3 are proteins expressed in

response to several cytokines and hormones, including GH (Zhu et al., 2001). SOCSs proteins act as a negative feedback loop by subsequently inhibiting the JAK/STAT pathway either by direct interaction with activated JAKs or with cytokine receptors (Zhu et al., 2001). In humans, estradiol (E2) induces a dissociation of the GH/IGF-I axis leading to reduced circulating IGF-I (Huang and O'Sullivan, 2009). This effect is mediated by the estrogen receptor α (ERα) and increased SOCS-2 and -3 at the transcriptional level in the liver, thereby inhibiting JAK/STAT signaling (Leung et al., 2004). In cattle, data indicate that increased expression of SOCS-2 mRNA in liver also may impair the ability of GH to stimulate IGF-I (Winkelman et al., 2008).

Based on these evidences this study has the objective to determine if E2 in follicular fluid (FF) can up-regulate SOCS-2 and -3 mRNA expression in granulosa cells and consequently reduce IGF-I mRNA expression when compared to luteal cells. Moreover, we aim to characterize the pattern of mRNA expression for these genes between estrogen active follicles (EAF), atretic follicles (ATF) and CL, since there are no previous reports on this.

Materials and Methods

Tissue collection

Ovaries of Holstein cows were collected at a local slaughterhouse, located at Wyalusing, PA (USA) in a sterile flask containing saline on ice. After collection follicles were immediately dissected from surrounding stroma, the follicular fluid (FF) was aspirated and the follicular wall immersed in RNALater (Ambion Inc, Austin, USA). FF was stored at -80^{o}C for further analysis. In the laboratory, the follicle was cut in two halves, the antral cavity was scrapped and flushed with cold saline in a petri dish. Granulosa cells were recovered by centrifugation at 1200 *x g* for 2 min. The CL was also dissected from the ovary and immediately snap-frozen in liquid nitrogen and stored at −80°C in an aluminum foil bag. Follicular diameter was estimated from the weight

of the FF using the following equation $y=12.96x^{0.31}$, where y = the diameter of the follicle (mm) and x = the weight of the FF (g) (Murasawa et al., 2005).

Hormonal assays

E2 in FF was determined by radioimmunoassay (RIA) (Serono Maia, Cortlandt Manor, NY) by the procedure described by Butler et al. (2004). Progesterone (P4) in FF samples was also determined by RIA according to Elrod and Butler (1993). A follicle was considered to be an ATF when the E2:P4 ratio was less than 1 or an EAF, when E2:P4 ratio was higher than 1 (Ireland and Roche, 1982). IGF-I was quantified by RIA according to the method described by Butler et al. (2006). All assays were performed in a single batch and the intra-assay coefficient of variation (CV) was 14.1%, 5.1% and 10.9% for E2, P4 and IGF-I, respectively.

RNA isolation and quantitative real-time PCR

The samples (30 mg of CL or the pellet of granulosa cells) were homogenized with 1 mL of Qiazol (Qiagen, Valencia, USA). Total RNA was isolated and purified using RNeasy Mini columns and on-column RNase-free DNase treatment (Qiagen) following manufacturer's protocol. The quantity and integrity of RNA was determined using the BioAnalyzer and RNA Nano Lab Chip Kit (Agilent Technologies, Santa Clara, USA). Average quality score (RNA integrity number) was 7.9 for follicles and 9.2 for CLs. Reverse transcription reactions were performed with 1 μg of RNA using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, USA) in a 10 μL volume.

Real-time quantitative PCR using SYBR Green dye was used to evaluate GHR, IGF-I, IGF-II, SOCS-1, -2, -3 and ERα expression. Ribosomal protein S9 (RPS9) expression was used as an internal control. The primers sequences and references are shown (Table 1). The PCR reactions were performed in triplicate in a 25-μL volume using Power SYBR Green Mastermix (Applied Biosystems). The PCR reactions were performed and fluorescence was quantified with

the ABI Prism 7300 Sequence Detector (Applied Biosystems). For each assay, 40 PCR cycles were run and a dissociation curve was included to verify the amplification of a single PCR product in the end of the reaction. Analyses of amplification plots were performed with the Sequence Detection Software (Applied Biosystems). Data was analyzed using the ΔΔCT method (Livak and Schmittgen, 2001). A 10-point relative standard curve was generated using serial 10-fold dilutions of cDNA prepared from liver samples to calculate efficiency of each primer pair. Each assay plate included a negative control. The CV was below 10% for all the primer pairs used.

Statistical analyses

The results are presented as means ± standard error of the mean (SEM). All the statistical analyses were performed using SAS (SAS Institute Inc. Cary, NC, USA). Data (mRNA expression, follicle diameter, E2, P4 and IGF-I) was log transformed and analyzed through One-way ANOVA. Pearson correlation coefficient was determined for GHR and IGF-I, SOCS-2 and GHR, SOCS-2 and ERα, SOCS-2 and E2, GHR and ERα. A value of $P<0.05$ was considered statistically significant.

Results

For this study 7 EAF, 7 ATF and 7 CL were used. E2 concentration was lower in ATF (21.1 ± 6.0 ng/mL) than EAF (137.3 ± 39.7 ng/mL) ($P<0.01$). P4 concentration was not different between ATF (59.2 ± 7.7 ng/mL) and EAF (69.25 ± 5.42 ng/mL) ($P>0.05$). The E2:P4 ratio was lower in ATF (0.37 ± 0.11) than EAF (2.06 ± 0.61) ($P<0.01$). Concentration of IGF-I in FF was not different between ATF (85.7 ± 25.7 ng/mL) and EAF (96.2 ± 17.6 ng/mL) ($P>0.05$). Diameter was the same between ATF (13.0 ± 0.9 mm) and EAF (13.4 ± 0.9 mm) ($P>0.05$).

Expression of mRNA for the genes studied is summarized in Table 2. Briefly, expression of GHR, IGF-I, IGF-II, SOCS-1 and SOCS-2 mRNA was higher in luteal than granulosa cells, and none of the genes studied was different between ATF and EAF. In the follicle, SOCS-2 mRNA expression was correlated with E2 concentration in FFL ($r=0.56$, $P<0.05$). SOCS-2 was correlated to ERα and GHR in granulosa and luteal cells ($P<0.05$) (Figure 1). GHR was correlated to ERα in luteal ($P<0.01$) but not in granulosa cells ($P>0.05$) (Figure 2). GHR was not correlated to IGF-I in granulosa or luteal cells ($P>0.05$).

Discussion

To our knowledge this is the first report to quantify the levels of mRNA expression for GHR, IGF-I and SOCS, as well as its relationship, in granulosa cells from ATF and EAF and luteal cells. Our findings support that IGF-I is expressed in granulosa cells, although at lower levels than in luteal cells. Moreover, we demonstrated the pattern of expression for SOCS mRNA in the granulosa and luteal cells and how ERα and GHR synergistically affect its expression.

Although the levels of GHR mRNA expression in the granulosa and luteal cells were extremely similar to the described by Rhoads et al. (2008a), the levels of IGF-I mRNA in the luteal cells were much higher in the present study. In addition, the correlation between GHR and IGF-I mRNA found in luteal cells by Rhoads et al. (2008a) was not observed in the present study. We are not sure about the nature of these changes, but during luteolysis changes in GHR mRNA expression are not paralleled to changes in IGF-I mRNA (Neuvians et al., 2003), and can be one explanation for this difference. Despite that, Rhoads et al. (2008a) also found no correlation between GHR and IGF-I in granulosa cells.

There was a positive correlation between SOCS-2 mRNA and E2 concentration in FFL and SOCS-2 mRNA and ERα mRNA in granulosa and luteal cells. These findings are in

accordance to previous observations that E2 can up-regulate expression of SOCS-2 mRNA in a dose dependent way through ERα in hepatic cells (Leong et al., 2004). The same correlation between ERα and SOCS-2 mRNA was found in luteal and granulosa cells, even though ERα was not different and SOCS-2 mRNA was about 25 times higher in the luteal cells. This is due the higher expression of GHR mRNA in luteal cells and the synergistic action between GH and E2 to increase SOCS-2 mRNA expression (Leong et al., 2004). In addition, we were not able to detect any difference in the expression of SOCS-2 between ATF and EAF, even though E2 concentration in FFL was about 6 times higher in EAF than ATF. This is an indicative that E2 even in low levels is able to induce SOCS-2 mRNA expression and SOCS-2 expression is being more regulated by the amount of ERα than E2 in FFL. Consequently, we did not observe any changes in IGF-I mRNA expression between EAF and ATF. This is in accordance to Spicer and Chamberlain (2000), who demonstrated that in vitro granulosa cells did not change IGF-I production by E2 stimulus (0 and 300 ng/mL). In summary, although it is possible to observe an effect of E2 on SOCS-2 expression it was not possible to determine its involvement in the disruption of GHR signaling.

Although GHR expression was 8 times higher in luteal compared to granulosa cells, IGF-I expression was 25 times higher. Based on this, while the IGF-I production per unit of GHR was about 5 times higher in luteal cells, SOCS-2 production per unit of GHR was 2 times higher. In this way it is possible to speculate that GHR effectiveness in stimulate IGF-I mRNA is impaired in granulosa cells when comparing to luteal cells.

There was a high correlation between ERα and GHR mRNA in luteal cells, but not in granulosa cells even with the higher number of samples. It was previously demonstrated that E2 can up-regulate GHR mRNA expression in liver cells and that this effect is dependent on the

presence of the ER (Contreras and Talamantes, 1999). More studies are necessary to clarify the relation of ERα and GHR in CL growth and regression.

In summary, the components of the GH/IGF-I axis were more expressed in the luteal than granulosa cells and no difference between ATF and EAF was found. SOCS-2 mRNA is regulated by E2 levels through ERα in granulosa and luteal cells, but we were not able to demonstrate its implication on disruption of GHR signaling.

References

Armstrong, D.G., Gutierrez, C.G., Baxter, G., Glazyrin, A.L., Mann, G.E., Woad, K.J., Hogg, C.O., Webb, R., 2000, Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. J.Endocrinol. 165, 101-113.

Butler, S.T., Pelton, S.H., Butler, W.R., 2004, Insulin increases 17 beta-estradiol production by the dominant follicle of the first postpartum follicle wave in dairy cows. Reproduction 127, 537-545.

Butler, S.T., Pelton, S.H., Butler, W.R., 2006, Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. J Dairy Sci 89, 2938-2951.

Contreras, B., Talamantes, F., 1999, Growth hormone (GH) and 17beta-estradiol regulation of the expression of mouse GH receptor and GH-binding protein in cultured mouse hepatocytes. Endocrinology 140, 4725-4731.

do Amaral, B.C., Connor, E.E., Tao, S., Hayen, J., Bubolz, J., Dahl, G.E., 2010, Heat stress abatement during the dry period influences prolactin signaling in lymphocytes. Domest Anim Endocrinol 38, 38-45.

Elrod, C.C., Butler, W.R., 1993, Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. J Anim Sci 71, 694-701.

Herrington, J., Smit, L.S., Schwartz, J., Carter-Su, C., 2000, The role of STAT proteins in growth hormone signaling. Oncogene 19, 2585-2597.

Huang, D.S., O'Sullivan, A.J., 2009, Short-term oral oestrogen therapy dissociates the growth hormone/insulin-like growth factor-I axis without altering energy metabolism in premenopausal women. Growth Horm IGF Res 19, 162-167.

Ireland, J.J., Roche, J.F., 1982, Development of Antral Follicles in Cattle after Prostaglandin-Induced Luteolysis - Changes in Serum Hormones, Steroids in Follicular-Fluid, and Gonadotropin Receptors. Endocrinology 111, 2077-2086.

Janovick-Guretzky, N.A., Dann, H.M., Carlson, D.B., Murphy, M.R., Loor, J.J., Drackley, J.K., 2007, Housekeeping gene expression in bovine liver is affected by physiological state, feed intake, and dietary treatment. J Dairy Sci 90, 2246-2252.

Jones, J.I., Clemmons, D.R., 1995, Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16, 3-34.

Kirby, C.J., Thatcher, W.W., Collier, R.J., Simmen, F.A., Lucy, M.C., 1996, Effects of growth hormone and pregnancy on expression of growth hormone receptor, insulin-like growth factor-I, and insulin-like growth factor binding protein-2 and -3 genes in bovine uterus, ovary, and oviduct. Biol Reprod 55, 996-1002.

Leong, G.M., Moverare, S., Brce, J., Doyle, N., Sjogren, K., Dahlman-Wright, K., Gustafsson, J.A., Ho, K.K., Ohlsson, C., Leung, K.C., 2004, Estrogen up-regulates hepatic expression of suppressors of cytokine signaling-2 and -3 in vivo and in vitro. Endocrinology 145, 5525-5531.

Leung, K.C., Johannsson, G., Leong, G.M., Ho, K.K., 2004, Estrogen regulation of growth hormone action. Endocr Rev 25, 693-721.

Livak, K.J., Schmittgen, T.D., 2001, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Lucy, M.C., 2000, Regulation of ovarian follicular growth by somatotropin and insulin-like growth factors in cattle. Journal of Dairy Science 83, 1635-1647.

Murasawa, M., Takahashi, T., Nishimoto, H., Yamamoto, S., Hamano, S., Tetsuka, M., 2005, Relationship between ovarian weight and follicular population in heifers. J Reprod Develop 51, 689-693.

Neuvians, T.P., Pfaffl, M.W., Berisha, B., Schams, D., 2003, The mRNA expression of the members of the IGF-system in bovine corpus luteum during induced luteolysis. Domest Anim Endocrinol 25, 359-372.

Perks, C.M., Peters, A.R., Wathes, D.C., 1999, Follicular and luteal expression of insulin-like growth factors I and II and the type 1 IGF receptor in the bovine ovary. J Reprod Fertil 116, 157-165.

Pfaffl, M.W., Daxenberger, A., Hageleit, M., Meyer, H.H., 2002, Effects of synthetic progestagens on the mRNA expression of androgen receptor, progesterone receptor, oestrogen receptor alpha and beta, insulin-like growth factor-1 (IGF-1) and IGF-1 receptor in heifer tissues. J Vet Med A Physiol Pathol Clin Med 49, 57-64.

Rhoads, M.L., Meyer, J.P., Kolath, S.J., Lamberson, W.R., Lucy, M.C., 2008a, Growth hormone receptor, insulin-like growth factor (IGF)-1, and IGF-binding protein-2 expression in the reproductive tissues of early postpartum dairy cows. J Dairy Sci 91, 1802-1813.

Rhoads, M.L., Meyer, J.P., Lamberson, W.R., Keisler, D.H., Lucy, M.C., 2008b, Uterine and hepatic gene expression in relation to days postpartum, estrus, and pregnancy in postpartum dairy cows. J Dairy Sci 91, 140-150.

Schams, D., Berisha, B., Kosmann, M., Amselgruber, W.M., 2002, Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. Domest Anim Endocrin 22, 51-72.

Spicer, L.J., Alpizar, E., Echternkamp, S.E., 1993, Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. J.Anim Sci. 71, 1232-1241.

Spicer, L.J., Chamberlain, C.S., 2000, Production of insulin-like growth factor-I by granulosa cells but not thecal cells is hormonally responsive in cattle. J Anim Sci 78, 2919-2926.

Stewart, R.E., Spicer, L.J., Hamilton, T.D., Keefer, B.E., Dawson, L.J., Morgan, G.L., Echternkamp, S.E., 1996, Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137, 2842-2850.

Sudo, N., Shimizu, T., Kawashima, C., Kaneko, E., Tetsuka, M., Miyamoto, A., 2007, Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A (PAPP-A). Mol Cell Endocrinol 264, 197-203.

Teglund, S., McKay, C., Schuetz, E., van Deursen, J.M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G., Ihle, J.N., 1998, Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93, 841-850.

Vicini, J.L., Buonomo, F.C., Veenhuizen, J.J., Miller, M.A., Clemmons, D.R., Collier, R.J., 1991, Nutrient Balance and Stage of Lactation Affect Responses of Insulin, Insulin-Like Growth Factor-I and Factor-Ii, and Insulin-Like Growth Factor-Binding Protein-2 to Somatotropin Administration in Dairy-Cows. Journal of Nutrition 121, 1656-1664.

Winkelman, L.A., Lucy, M.C., Elsasser, T.H., Pate, J.L., Reynolds, C.K., 2008, Short communication: suppressor of cytokine signaling-2 mRNA increases after parturition in the liver of dairy cows. J Dairy Sci 91, 1080-1086.

Yakar, S., Liu, J.L., Stannard, B., Butler, A., Accili, D., Sauer, B., LeRoith, D., 1999, Normal growth and development in the absence of hepatic insulin-like growth factor I. P Natl Acad Sci USA 96, 7324-7329.

Zhu, T., Goh, E.L., Graichen, R., Ling, L., Lobie, P.E., 2001, Signal transduction via the growth hormone receptor. Cell Signal 13, 599-616.

Tables

Table 1 – Sequence and reference for the primers used in the experiment.

Primer	Sequence (5'- 3)	Reference
RPS9	F: CCTCGACCAAGAGCTGAAG R: CTCCAGACCTCACGTTTGTTC	(Janovick-Guretzky et al., 2007)
GHR	F: GGTATGGATCTCTGGCAGCTG R: CTCTGACAAGGAAAGCTGGTGTG	(Rhoads et al., 2008b)
IGF-I	F: TTGGTGGATGCTCTCCAGTTC R: GCACTCATCCACGATTCCTGT	(Rhoads et al., 2008b)
IGF-II	F: GACCGCGGCTTCTACTTCAG R: AAGAACTTGCCCACGGGGTAT	(Neuvians et al., 2003)
SOCS-1	F: CACAGCAGAAAAATAAAGCCAGAGA R: CTCGTACCTCCTACCTCTTCATGTT	(do Amaral et al., 2010)
SOCS-2	F: GGGATGCTTCCCTTCCTAAG R: GTGCTGGGACCTTTCACCTA	(do Amaral et al., 2010)
SOCS-3	F: GGCCACTCTCCAACATCTCTGT R: TCCAGGAACTCCCGAATGG	(do Amaral et al., 2010)
ERα	F: AGGGAAGCTCCTATTTGCTCC R: CGGTGGATGTGGTCCTTCTCT	(Pfaffl et al., 2002)

Table 2 – Summary of gene expression in granulosa cells from atretic (ATF) or estrogen active follicles (EAF) and corpus luteum (CL).

Variable	ATF (*n* = 7)		EAF (*n* = 7)		CL (*n* = 7)	
	Mean	SEM	Mean	SEM	Mean	SEM
GHR	1.13^{a}	0.16	1.13^{a}	0.23	8.99^{b}	1.83
IGF-I	1.17^{a}	0.26	2.83^{a}	1.48	55.58^{b}	8.17
IGF-II	2.74^{b}	1.95	5.54^{b}	3.98	154.68^{a}	30.48
ERα	1.27	0.31	1.39	0.11	1.45	0.28
SOCS-1	1.77^{b}	0.96	0.88^{b}	0.19	11.38^{a}	1.72
SOCS-2	1.39^{b}	0.39	1.38^{b}	0.22	33.43^{a}	9.35
SOCS-3	1.39	0.54	0.70	0.20	0.72	0.11
GHR/IGF-I	1.84^{b}	1.01	2.69^{b}	1.40	10.98^{a}	5.28
GHR/SOCS-2	1.24^{b}	0.28	1.29^{b}	0.11	3.65^{a}	0.57
GHR/SOCS-3	1.46^{a}	0.51	0.61^{a}	0.13	0.14^{b}	0.06

Figures

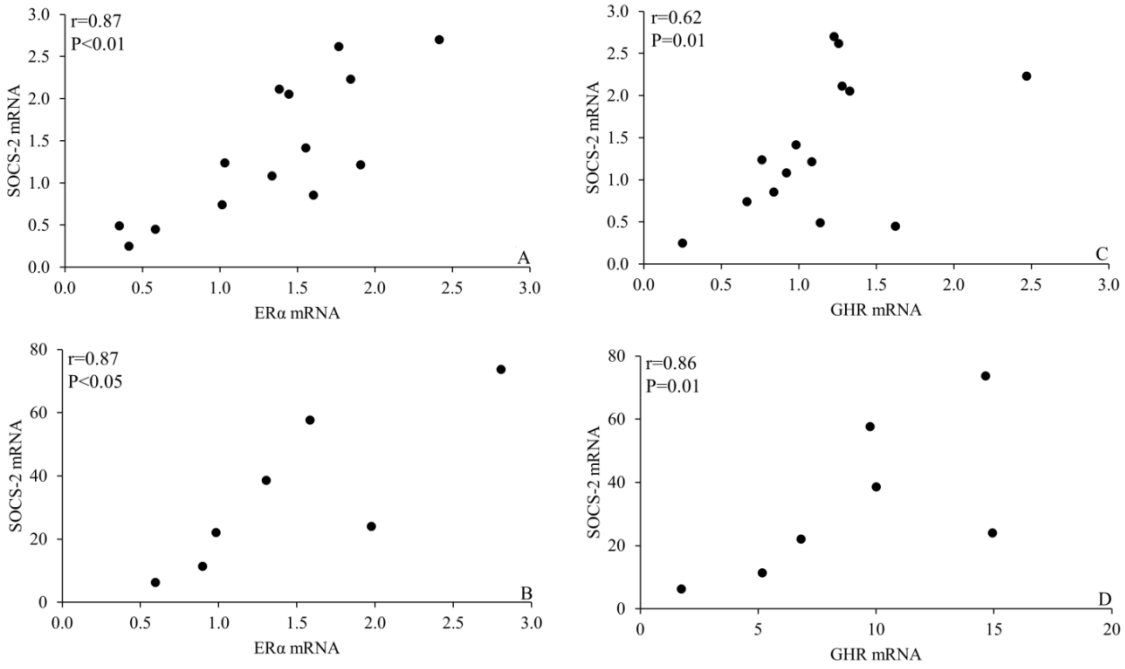

Figure 1 – Correlation between ERα and SOCS-2 mRNA in granulosa (A) and luteal cells (B) and between GHR and SOCS-2 mRNA in granulosa (C) and luteal cells (D).

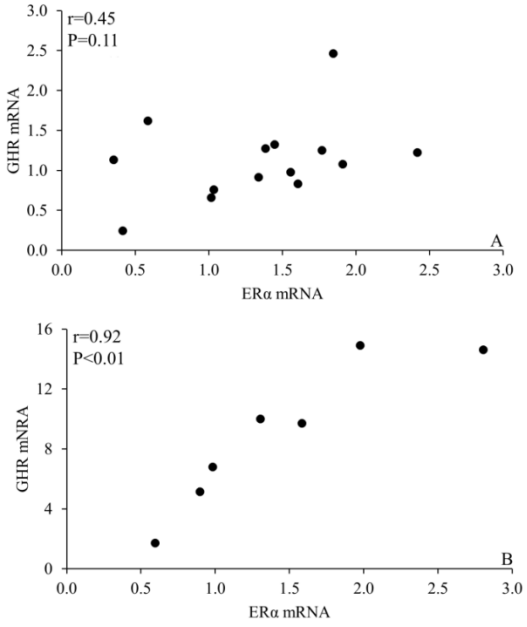

Figure 2 – Correlation between ERα and GHR mRNA in granulosa (A) and luteal cells (B) and between GHR and SOCS-2 mRNA in granulosa (C) and luteal cells (D).

5. Artigo 3 – Effect of prepartum somatotropin on metabolism, milk production and resumption of ovarian activity in postpartum primiparous Holstein cows

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Abstract

The aim of this study was to determine the effect of pre-partum somatotropin injection on metabolism, milk production and resumption of post-partum ovulation in primiparous Holstein dairy cows. For this study 31 primiparous Holstein cows were used. The cows were assigned randomly to two groups and given; 1) 500 mg sc injections of somatotropin (Somatotropin group, n = 15) at -32.2 ± 6.9 and -18.9 ± 6.9, and, if pertinent, at -6.6 ± 6.4 d from calving date and 2) no treatment (Control cows, n = 16). Blood samples were collected weekly from -35 to 49 d after calving. Cows with progesterone concentrations in plasma above 1 ng/mL in two consecutive samples were considered as having resumed ovarian activity. Somatotropin injection increased IGF-I and insulin ($P<0.01$) and tended to increase NEFA concentrations ($P=0.13$) during the pre-partum period. During the post-partum period, somatotropin reduced BCS loss ($P<0.01$) and NEFA concentration ($P=0.05$) and increased insulin ($P<0.01$). Somatotropin treatment also increased the average daily milk production ($P<0.01$) and reduced the SCC ($P<0.05$). Somatotropin treatment improved the number of cows resuming ovarian activity ($P<0.05$). In addition, the interval from calving to first ovulation was shorter for somatotropin injected cows ($P=0.05$). Calving-conception interval tented to be lower for treated cows ($P=0.13$). In conclusion, somatotropin treatment during the pre-partum period in primiparous Holstein cows increased milk production and the number of cows resuming ovarian activity. In addition, it improved the markers related to energy balance during the early post-partum period.

Key words: bST, dairy cows, IGF-I, ovarian activity, primiparous.

Introduction

During the last month of pregnancy and the early post-partum period, high producing cows experience a period of negative energy balance (NEB). It occurs as requirements for the final fetal growth and milk production exceed the energy obtained through feeding (Butler 2003). Often, the severity of the NEB is more pronounced in primiparous dairy cows, because besides the factors mentioned above they have energy requirements for their own growth (Bell 1995). In the early post-partum dairy cow, the duration and intensity of NEB, the level of body condition score (BCS) loss and the milk yield are strongly associated to the timing of first ovulation (Wathes et al. 2007, Butler 2003). As consequence, they have a negative impact on reproductive performance, since cows that ovulated earlier and had more estrous cycles before the first artificial insemination (AI) had higher conception rate (Thatcher and Wilcox 1973). In this way, strategies that minimize NEB and BCS loss in the early post-partum could have a positive impact on reproductive performance.

Supplementing cows with low doses of somatotropin during the periparturient period has been used due to possible beneficial effects on the physiological adaptations and liver function (Gulay et al. 2004, Gulay et al. 2003). Somatotropin has direct effects on the partitioning of nutrients to target tissues (Bauman and Vernon 1993), as well as indirect effects expressed in the mammary gland and other tissues that are mediated by IGF-I (Jones and Clemmons 1995). In this regard, somatotropin injection during the peripartum period is able to increase serum insulin-like growth factor I (IGF-I) concentration and milk production (Carriquiry et al. 2009b, Gulay et al. 2004, Putnam et al. 1999). Moreover, injection of somatotropin exclusively in the pre-partum period can also lead to increased dry matter intake (DMI) (Putnam et al. 1999). These evidences indicate that pre-partum injection of somatotropin is a potential tool to improve the performance of transition dairy cows.

Somatotropin (GH) plays a pivotal role in stimulating liver production of IGF-I (Jones and Clemmons 1995). Circulating levels of IGF-I are highly related to the extent of NEB (Wathes et al. 2007, Lucy et al. 2001), therefore its levels starts to decline in the third trimester of lactation reaching a nadir at calving (Knight 2001). Elevated concentrations of IGF-I are beneficial because in the ovary it acts as a modulator of gonadotrophin action, stimulating granulosa and theca cell proliferation and differentiation (Armstrong and Webb 1997), and preventing follicular atresia (el-Roeiy et al. 1994). In this way, dairy cows that ovulated the first post-partum follicular wave had higher serum IGF-I concentration in the peripartum period (Kawashima et al. 2007, Butler et al. 2006). Somatotropin injection starting at 10 days post-partum had no effect on the interval from calving to first ovulation (Carriquiry et al. 2009a). However, the number of recruited follicles is increased in dry cows or heifers injected with somatrotopin (Kirby et al. 1997, Gong et al. 1991, Gong et al. 1993), and its effect persisted for at least 21 d after termination of treatment (Kirby et al. 1997). These evidences indicate that pre-partum somatotropin injection could have some residual effects and might be beneficial for resuming post-partum ovarian activity, even if injected before calving.

Based on these evidences, the hypothesis of this study is that pre-partum somatotropin injection would improve milk production and anticipate post-partum resumption of ovarian activity in primiparous Holstein dairy cows during the transition period.

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).

Experimental design

For this study 31 primiparous Holstein cows in a commercial dairy herd in southern Brazil (32º 16' S, 52º 32' E) were used. The cows had a mean body weight of 568.8 ± 47.8 kg and BCS of 3.4 ± 0.4 (ranging from 2.5 to 4.0) at the beginning of the experiment. All calving occurred in a 30 d interval during the winter season.

The cows were assigned randomly to two groups, at -35 d from expected calving, and given; 1) 500 mg somatotropin (500 mg/2 mL, Boostin®, LG Life Sciences, Seoul, Korea) sc injections (Somatotropin group, n = 15) at -32.2 ± 6.9 and -18.9 ± 6.9, and, if pertinent, at -6.6 ± 6.4 d from calving date and 2) no treatment (Control cows, n = 16). All females were managed in the same conditions and nutritional regimen (Table 1).

Sample collection, measurement and analyses

Blood samples were collected weekly from -35 to 49 d after calving via venipuncture of the jugular vein into two tubes; one containing EDTA and potassium fluoride and one without anticoagulant. Samples were collected in the interval after milking and before feeding. Post-partum cows were milked twice daily at 0330 and 1530 and production was recorded daily (Alpro®, DeLaval, Kansas City, USA) to generate the weekly averages. Body weight was measured weekly using a chest tape and BCS was evaluated also weekly by subjective evaluation for a single technician.

Concentration of glucose and NEFA were measured colorimetrically using commercial kits, Glucose PAP Liquiform (Labtest Diagnostica, Lagoa Santa, Brazil) (Tabeleão et al., 2008) and NEFA-HR (Wako USA, Richmond, USA) using the micro-method described by Ballou et al. (2009). The intra and inter-assay coefficient of variation (CV) for glucose and NEFA were lower than 10%.

The procedure for IGF-I, insulin and progesterone analysis were followed as specified by the manufacturer. Total IGF-I and insulin concentrations in plasma were measured using

sandwich-type immunoassay commercial kits, DSL-10-2800 ACTIVE Non-extraction IGF-I ELISA (Diagnostic Systems Laboratories Inc., Webster, USA) (Obese et al. 2008) and DSL-10-1600 ACTIVE Insulin (Diagnostics Systems Laboratories Inc., Webster, USA) (Lee et al. 2002). The intra-CV was 4.2% and 4.0% and the inter-CV 8.5% and 13.0% for IGF-I and insulin, respectively.

Progesterone concentrations in plasma were measured using a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding commercial kit, Progesterone IB79105 (Immuno-Biological Laboratories Inc., Minneapolis, USA) (Colazo et al. 2008). The intra-CV was 9.9% and the inter-CV was 5.5%. Only samples collected after 7 days in milk (DIM) were analyzed. Cows with progesterone concentrations in plasma above 1 ng/mL in two consecutive samples were considered as having resumed ovarian activity (Stevenson and Britt 1979).

Milk samples also were collected weekly. Milk protein, fat and lactose assays were performed by infrared spectrophotometry (Bentley 20000, Bentley Instruments Inc., Chaska, USA). The somatic cell count (SCC) assay was performed by flow cytometry (Somacount 300, Bentley Instruments Inc., Chaska, USA).

Reproductive management

The voluntary waiting period used by the farm was 60 d. After that, estrus was detected by visual observation and all animals presenting visual signs of standing estrous behavior (cows staying still when mounted) were AI. Visual observation of estrous behavior and AI were performed by the same technician. Pregnancy diagnosis was performed by ultrasound 30 d after AI and re-checked 60 d later.

Statistical analyses

The results are presented as means ± standard error of the mean (SEM). All the statistical analyses were performed using SAS (SAS Institute Inc. Cary, NC, USA). Analyses involving repeated measures over time (e.g., BCS, body weight, glucose, NEFA, milk production and quality, insulin and IGF-I) were compared between treatments by analysis of variance for repeated measures using the MIXED procedure to evaluate the main effects of time, treatment, and their interactions (Littell et al. 1998). The statistical models and data analyses were designed and performed, respectively, separately for the pre- and post-partum period. When the interaction between treatment and time was significant ($P<0.05$) pair-wise comparison of individual means was carried out with the Tukey–Kramer test. Calving-conception interval was compared between groups by one–way analysis of variance (ANOVA). The interval between calving and week of the first ovulation was compared by the Kruskal-Wallis non-parametric test. Proportion of cows ovulating was analyzed by chi-square. Pregnancy and ovulation rate were evaluated through survival analysis using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). A value of $P<0.05$ was considered statistically significant.

Results

Data regarding BCS and plasma variables is summarized in Figure 1. Overall, somatotropin treatment increased IGF-I and insulin secretion during the pre-partum period ($P<0.01$). During the post-partum period, somatotropin treatment led to decreased BCS loss ($P<0.01$), and increased ($P<0.01$) insulin concentration. Somatotropin treatment tended to increase NEFA pre-partum ($P=0.13$) and reduced its concentration in the post-partum period ($P=0.05$). There was no difference in body weight during the pre- (560.3 ± 9.2 kg) or post-partum

(530.3 ± 5.7 kg) period. There was no effect of group or time for pre-partum glucose (3.1 ± 0.1 mmol/L) and there was only an effect of time for post-partum glucose (3.1 ± 0.1 mmol/L)

Data for milk production and composition is summarized on Table 2. Briefly, somatotropin treatment increased the average daily milk production ($P<0.01$) and reduced the SCC ($P<0.05$).

More cows treated with somatotropin resumed the ovarian activity in the first 7 weeks post-partum ($P<0.05$, Figure 2). In addition, the interval from calving to first post-partum ovulation was shorter for somatotropin treated cows ($P=0.05$; Table 3). A higher number of cows in the somatotropin group ovulated the first post-partum dominant follicle (53.3%) compared to control group (12.5%, $P<0.05$), as indicated by number of cows ovulating before 21 DIM. Although the number of cows pregnant before 150 DIM was not different between groups (Figure 3; $P=0.30$), the calving conception interval tended to be lower for treated cows ($P = 0.13$).

Discussion

Pre-partum somatrotopin treatment seems to be a potential strategy that could improve the post-partum energy balance, as observed by the lower BCS loss, lower NEFA and higher insulin concentration during this period. Previous studies have shown that cows treated with a low dose of somatotropin in the peripartum period, had a better recovery of BCS during early lactation, despite they produced more milk (Gulay et al. 2003, Putnam et al. 1999). Although we have used a full dose and only in the pre-partum period, our results agree with studies previously described. Putnam et al. (1999) observed that the higher post-partum BCS was associated to increased DMI during this period for treated cows. Although not measured, higher DMI could explain why somatotropin treated cows had better energy balance related parameters in the present study,

including higher insulin and lower NEFA concentrations in the early post-partum. This finding further supports the interpretation of Gulay et al. (2004) that the extra energy to support increased milk yield arose from greater DMI rather than from greater mobilization of body reserves in somatotropin treated cows.

Cows in the somatotropin group tented to have higher NEFA than control cows during the pre-partum due to the lipolityc effects of GH (Dietz and Schwartz 1991). Despite that, treated cows had lower circulating NEFA than control cows in the post-partum. This condition resembles the proposed by Douglas et al. (2006), which restricted intake of pre-partum cows and observed high and low NEFA concentration on pre- and post-partum, respectively. In this sense, cows with chronically elevated pre-partum NEFA have alterations in metabolism that better adapted the liver to contend with the marked increases in NEFA in the early post-partum period (Grum et al. 1996). These alterations occur due to enhanced hepatic capacity for β-oxidation and decreased esterification of long chain fatty acids, thereby leading to less accumulation of lipid and TG in the liver (Douglas et al. 2006). The controlled and previous exposition to a catabolic condition could decrease the metabolic challenges that occur around calving (Loor 2010). Nevertheless, Putnam et al. (1999) found no difference on NEFA concentrations for cows receiving somatotropin in the pre-partum. In contrast, Vallimont et al. (2001) found a reduction in post-partum NEFA concentration without observing changes during the pre-partum period. It is important to take into account that the lipolytic effects of somatotropin are observed only when cows are in negative energy balance (Bauman 1992).

Milk production was increased by pre-partum somatotropin treatment, in agreement to previous observation (Putnam et al. 1999). In the same way, pre-partum injection of somatotropin in ewes led to higher milk production and mammary cell proliferation (Stelwagen et al. 1993). This effect is mainly mediated by IGF-I, as showed in studies in cultured mammary cells

obtained from both pregnant and lactating cows (Baumrucker and Stemberger 1989). Furthermore, according to the SCC data, apparently, the health of mammary gland has also improved in cows injected with somatotropin. The decreased SCC in dairy cows receiving somatotropin is already showed elsewhere (Gulay et al. 2004, Putnam et al. 1999). Somatotropin may protect the blood-milk barrier and restore the integrity of mammary epithelial tight junctions of an inflamed mammary gland (Burvenich et al. 1999), thus reducing infection in the early post-partum period. Reduced SCC in the early post-partum can also have contributed to increased milk production (Hortet et al. 1999).

Somatotropin treatment increased the number of cows resuming ovarian activity. In addition, more cows in this group have ovulated the follicle from the first post-partum follicular wave as indicated by the rise in progesterone concentration before 21 DIM (Kawashima et al. 2006). In the present study cows treated with somatotropin had higher IGF-I concentration in the pre-partum and it is well established that cows ovulating the first dominant follicle wave have higher serum IGF-I concentration in the pre and post-partum period (Kawashima et al. 2007, Butler et al. 2006). In addition to that, somatotropin treated cows had higher insulin concentrations. Circulating insulin concentration is proposed as a key factor in mediating the effect of dietary intake on ovarian function (Armstrong et al. 2002). In this sense, dairy cows fed diets designed to increase circulating insulin and IGF-I concentrations had increased ovarian function (Armstrong et al. 2001) and earlier onset of estrous cycles (Gong et al. 2002). Cows that ovulated the first post-partum follicular wave had higher circulating insulin concentrations than anovulatory cows (Butler et al. 2006). In summary, the higher proportion of cows resuming ovarian activity earlier in this study was probably due to the combined effects of both factors stimulating post-partum follicle development. Another factor is that the number of recruited follicles is increased in cattle injected with somatotropin (Kirby et al. 1997, Gong et al. 1991,

Gong et al. 1993), and this effect persisted for at least 21 d after termination of treatment (Kirby et al. 1997). This could be other pathway by which pre-partum somatotropin can influence the fate of the first post-partum follicular wave.

Despite the positive effects in the resumption of ovarian activity, we could not see any significant increase in the pregnancy rate for treated cows, probably due to low statistical power and number of cows enrolled in this study. Nonetheless, calving conception interval tended to be lower for treated cows. This is in accordance with previous observations (Thatcher and Wilcox 1973, Kawashima et al. 2006), which indicates that earlier resumption of estrous cycles is associated to increased pregnancy rate in the first services.

In conclusion, somatotropin injection during the pre-partum period in primiparous Holstein cows is able to increase milk production and the number of cows resuming ovarian activity. In addition, it also improved parameters that indicate that these cows had a less severe NEB during the early post-partum period.

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References

Armstrong D. G.; Gong J. G.; Gardner J. O.; Baxter G.; Hogg C. O.; Webb R., 2002: Steroidogenesis in bovine granulosa cells: the effect of short-term changes in dietary intake. Reproduction, 123 371-378.

Armstrong D. G.; McEvoy T. G.; Baxter G.; Robinson J. J.; Hogg C. O.; Woad K. J.; Webb R.; Sinclair K. D., 2001: Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol Reprod, 64 1624-1632.

Armstrong D. G.; Webb R., 1997: Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. Rev Reprod, 2 139-146.

Ballou M. A.; Gomes R. C.; Juchem S. O.; DePeters E. J., 2009: Effects of dietary supplemental fish oil during the peripartum period on blood metabolites and hepatic fatty acid compositions and total triacylglycerol concentrations of multiparous Holstein cows. J Dairy Sci, 92 657-669.

Bauman D. E., 1992: Bovine somatotropin: review of an emerging animal technology. Journal of Dairy Science, 75 3432-3451.

Bauman D. E.; Vernon R. G., 1993: Effects of exogenous bovine somatotropin on lactation. Annu Rev Nutr, 13 437-461.

Baumrucker C. R.; Stemberger B. H., 1989: Insulin and insulin-like growth factor-I stimulate DNA synthesis in bovine mammary tissue in vitro. J Anim Sci, 67 3503-3514.

Bell A. W., 1995: Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci, 73 2804-2819.

Burvenich C.; Paape M. J.; Hoeben D.; Dosogne H.; Massart-Leen A. M.; Blum J., 1999: Modulation of the inflammatory reaction and neutrophil defense of the bovine lactating mammary gland by growth hormone. Domest Anim Endocrinol, 17 149-159.

Butler S. T.; Pelton S. H.; Butler W. R., 2006: Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. J Dairy Sci, 89 2938-2951.

Butler W. R., 2003: Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livestock Science, 211-218.

Carriquiry M.; Dahlen C. R.; Weber W. J.; Lamb G. C.; Crooker B. A., 2009a: Postpartum ovarian activity in multiparous Holstein cows treated with bovine somatotropin and fed n-3 fatty acids in early lactation. J Dairy Sci, 92 4876-4888.

Carriquiry M.; Weber W. J.; Dahlen C. R.; Lamb G. C.; Baumgard L. H.; Crooker B. A., 2009b: Production response of multiparous Holstein cows treated with bovine somatotropin and fed diets enriched with n-3 or n-6 fatty acids. J Dairy Sci, 92 4852-4864.

Colazo M. G.; Ambrose D. J.; Kastelic J. P.; Small J. A., 2008: Comparison of 2 enzyme immunoassays and a radioimmunoassay for measurement of progesterone concentrations in bovine plasma, skim milk, and whole milk. Can J Vet Res, 72 32-36.

Dietz J.; Schwartz J., 1991: Growth hormone alters lipolysis and hormone-sensitive lipase activity in 3T3-F442A adipocytes. Metabolism, 40 800-806.

Douglas G. N.; Overton T. R.; Bateman H. G., 2nd; Dann H. M.; Drackley J. K., 2006: Prepartal plane of nutrition, regardless of dietary energy source, affects periparturient metabolism and dry matter intake in Holstein cows. J Dairy Sci, 89 2141-2157.

el-Roeiy A.; Chen X.; Roberts V. J.; Shimasakai S.; Ling N.; LeRoith D.; Roberts C. T., Jr.; Yen S. S., 1994: Expression of the genes encoding the insulin-like growth factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. J Clin Endocrinol Metab, 78 1488-1496.

Gong J. G.; Bramley T.; Webb R., 1991: The effect of recombinant bovine somatotropin on ovarian function in heifers: follicular populations and peripheral hormones. Biol Reprod, 45 941-949.

Gong J. G.; Bramley T. A.; Wilmut I.; Webb R., 1993: Effect of recombinant bovine somatotropin on the superovulatory response to pregnant mare serum gonadotropin in heifers. Biol Reprod, 48 1141-1149.

Gong J. G.; Lee W. J.; Garnsworthy P. C.; Webb R., 2002: Effect of dietary-induced increases in circulating insulin concentrations during the early postpartum period on reproductive function in dairy cows. Reproduction, 123 419-427.

Grum D. E.; Drackley J. K.; Younker R. S.; LaCount D. W.; Veenhuizen J. J., 1996: Nutrition during the dry period and hepatic lipid metabolism of periparturient dairy cows. J Dairy Sci, 79 1850-1864.

Gulay M. S.; Hayen M. J.; Liboni M.; Belloso T. I.; Wilcox C. J.; Head H. H., 2004: Low doses of bovine somatotropin during the transition period and early lactation improves milk yield, efficiency of production, and other physiological responses of Holstein cows. J Dairy Sci, 87 948-960.

Gulay M. S.; Hayen M. J.; Teixeira L. C.; Wilcox C. J.; Head H. H., 2003: Responses of Holstein cows to a low dose of somatotropin (bST) prepartum and postpartum. J Dairy Sci, 86 3195-3205.

Hortet P.; Beaudeau F.; Seegers H.; Fourichon C., 1999: Reduction in milk yield associated with somatic cell counts up to 600 000 cells/ml in French Holstein cows without clinical mastitis. Livest Prod Sci, 61 33-42.

Jones J. I.; Clemmons D. R., 1995: Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev, 16 3-34.

Kawashima C.; Fukihara S.; Maeda M.; Kaneko E.; Montoya C. A.; Matsui M.; Shimizu T.; Matsunaga N.; Kida K.; Miyake Y.; Schams D.; Miyamoto A., 2007: Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave post-partum in high-producing dairy cows. Reproduction, 133 155-163.

Kawashima C.; Kaneko E.; Amaya Montoya C.; Matsui M.; Yamagishi N.; Matsunaga N.; Ishii M.; Kida K.; Miyake Y.; Miyamoto A., 2006: Relationship between the first ovulation within three weeks postpartum and subsequent ovarian cycles and fertility in high producing dairy cows. J Reprod Dev, 52 479-486.

Kirby C. J.; Smith M. F.; Keisler D. H.; Lucy M. C., 1997: Follicular function in lactating dairy cows treated with sustained-release bovine somatotropin. J Dairy Sci, 80 273-285.

Knight C. H., 2001: Lactation and gestation in dairy cows: flexibility avoids nutritional extremes. Proc Nutr Soc, 60 527-537.

Lee S. H.; Engle T. E.; Hossner K. L., 2002: Effects of dietary copper on the expression of lipogenic genes and metabolic hormones in steers. J Anim Sci, 80 1999-2005.

Littell R. C.; Henry P. R.; Ammerman C. B., 1998: Statistical analysis of repeated measures data using SAS procedures. J Anim Sci, 76 1216-1231.

Loor J. J., 2010: Genomics of metabolic adaptations in the peripartal cow. Animal, 4 1110-1139.

Lucy M. C.; Jiang H.; Kobayashi Y., 2001: Changes in the somatotrophic axis associated with the initiation of lactation. J Dairy Sci, 89 E113-E119.

NRC, 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Press, Washington, DC.

Obese F. Y.; Humphrys S.; Macmillan K. L.; Egan A. R., 2008: Measuring concentrations of insulin-like growth factor-I with an enzyme-linked immunosorbent antibody assay in plasma samples from Holstein cows. J Dairy Sci, 91 160-168.

Putnam D. E.; Varga G. A.; Dann H. M., 1999: Metabolic and production responses to dietary protein and exogenous somatotropin in late gestation dairy cows. J Dairy Sci, 82 982-995.

Stelwagen K.; Grieve D. G.; Walton J. S.; Ball J. L.; Mcbride B. W., 1993: Effect of Prepartum Bovine Somatotropin in Primigravid Ewes on Mammogenesis, Milk-Production, and Hormone Concentrations. J Dairy Sci, 76 992-1001.

Stevenson J. S.; Britt J. H., 1979: Relationships among luteinizing hormone, estradiol, progesterone, glucocorticoids, milk yield, body weight and postpartum ovarian activity in Holstein cows. J Anim Sci, 48 570-577.

Tabeleão V. C.; Goulart M. A.; Schwegler E.; Weiser M. A.: Moura S. V.; Silva V. M.; Pereira V S.; Del Pino F. A. B.; Corrêa M. N., 2008: Ruminal and metabolic evaluation of bovines maintained in semi-confinement system. Arch Zootec 57 147-154.

Thatcher W. W.; Wilcox C. J., 1973: Postpartum estrus as an indicator of reproductive status in the dairy cow. J Dairy Sci, 56 608-610.

Vallimont J. E.; Varga G. A.; Arieli A.; Cassidy T. W.; Cummins K. A., 2001: Effects of prepartum somatotropin and monensin on metabolism and production of periparturient Holstein dairy cows. J Dairy Sci, 84 2607-2621.

Wathes D. C.; Fenwick M.; Cheng Z.; Bourne N.; Llewellyn S.; Morris D. G.; Kenny D.; Murphy J.; Fitzpatrick R., 2007: Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. Theriogenology, 68 Suppl 1 S232-241.

Tables

Table 1 – Composition of the feeds offered to the cows during the experiment

	Pre-partum		Post-partum
Feeds	Up to -21 DIM	-21 to 0 DIM	0 to 60 DIM
Native pasture	*Ad libitum*	*Ad libitum*	-
Ryegrass pasture	-	-	*Ad libitum*
White clover pasture	-	-	*Ad libitum*
Sorghum grain, kg	-	1.20	4.60
Soybean meal, kg	-	1.00	2.30
Soybean hulls, kg	-	1.50	0.80
Rice Bran, kg	-	-	1.70
Urea, kg	-	0.05	0.10
Premix, kg	-	0.25	0.50
	Feed composition		
Crude protein*, %	12.0	16.3	19.0
Fat*, %	1.8	3.0	4.1
Neutral detergent fiber*, %	74.2	50.9	26.5
Acid detergent fiber*, %	37.5	30.8	15.1

* Estimated based on feed analyses and NRC (2001)

Table 2 – Milk production and composition in control and somatotropin treated groups.

	Treatment			P-values		
Variable	Control	Somatotropin	SEM	Treat	Time	Treat*Time
Milk[1], kg/cow/day	22.3	25.1	0.4	<.01	0.01	0.71
Lactose[2], %	4.7	4.7	0.01	0.42	0.60	0.57
Fat[2], %	3.1	3.1	0.04	0.46	0.16	0.13
Protein[2], %	3.0	3.0	0.01	0.34	0.27	0.58
SCC* x 1,000[2], cells/mL	123.5	64.4	9.7	0.01	0.58	0.85

[1] Weekly average from daily measurements taken from 8 to 45 days post-partum

[2] Average of samples collected weekly from 8 to 45 days post-partum

* Somatic Cell Count

Table 3 – Reproductive variables for control and somatotropin treated groups.

	Treatment		
Variables	Control	Somatotropin	P-value
Cows with ovarian activity at 45 DIM, %	37.5 (6/16)	73.3 (11/15)	0.04
Week of first progesterone rise (> 1 ng/mL), weeks	4.8 ± 0.8	3.0 ± 0.4	0.05
Days to first AI, days	81.4 ± 2.3	76.9 ± 4.3	0.36
Calving conception interval, days	212.5 ± 27.7	138.8 ± 34.7	0.13

Figures

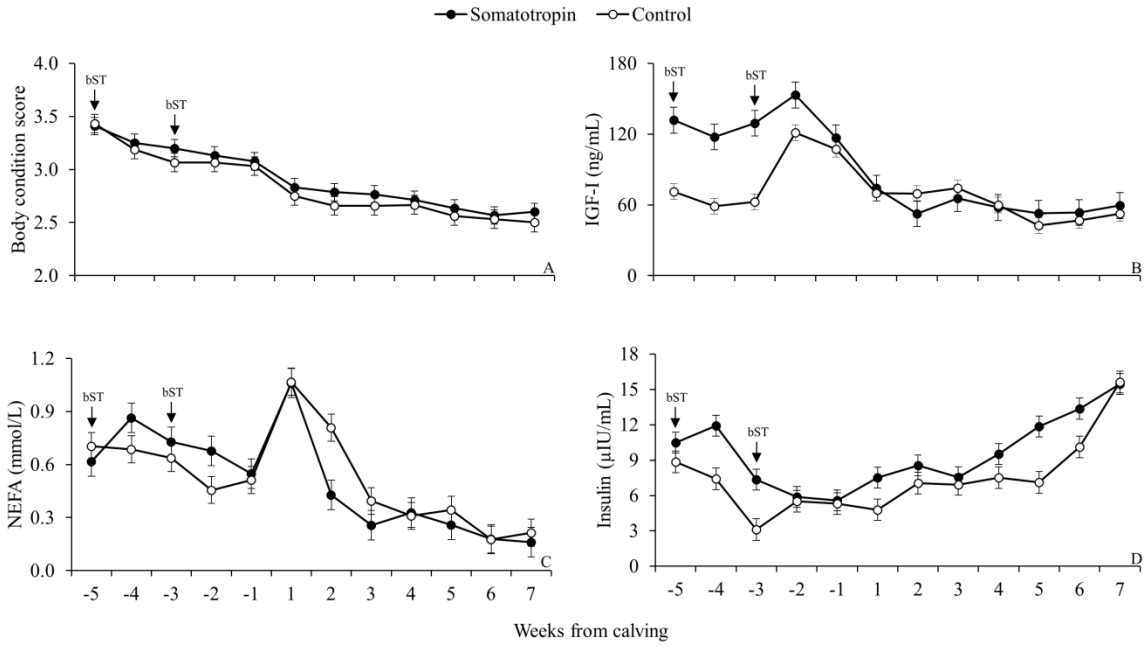

Figure 1 – Body condition score (A), IGF-I (B), NEFA (C), and insulin (D) from -5 to 7 weeks from calving for somatrotopin treated (black, ●) or control cows (white, ○). There was no effect of group and time interaction for any of the variables (P>0.05). In the pre-partum there was a group effect for insulin and IGF-I (P<0.1) and a tendency for NEFA (P=0.13). In the post-partum there was a group effect for BCS, NEFA and insulin (P<0.05). There was a time effect for BCS, NEFA and insulin in the pre- and post-partum period (P<0.05) and for IGF-I in the pre-partum period (P<0.05).

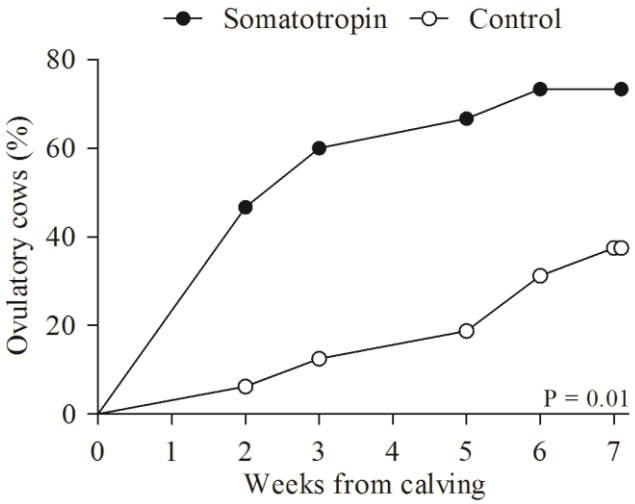

Figure 2 – Interval from calving to first ovulation determined by the first rise in progesterone (> 1 ng/mL) sustained for at least two consecutive weeks for somatrotopin treated (black, ●) or control cows (white, ○).

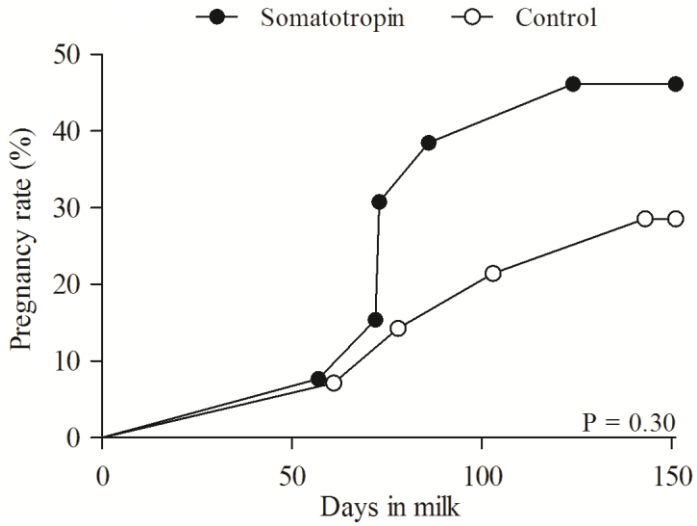

Figure 3 – Interval from calving to conception up to 150 days in milk for somatotropin treated (black, ●) or control cows (white, ○).

6. Artigo 4 – Effect of GHR *Alu*I polymorphism on milk production and fertility of Holstein cows

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Abstract

The aim of this work was to determine the effect of the GHR *Alu*I polymorphism on milk production and calving-conception interval in Holstein cows. Beginning on -21 days from expected calving, 94 lactating Holstein cows were provided with free access to a TMR. Cows were milked twice daily, whereas milk yield was determined weekly. For determination of GHR genotype, blood samples were collected by coccygeal venipuncture, DNA was extracted, the PCR for the GHR region performed and the alleles of determined by digestion with the *Alu*I enzyme. Of the 94 cows analyzed, 35 (37.2%) were from the *Alu*I(+/+) genotype, 48 (51.1%) from the *Alu*I(-/+) genotype and 11 (11.7%) from the *Alu*I(-/-) genotype. Allele frequency was 0.62 and 0.37, for the *Alu*I(+) and *Alu*I(–) alleles respectively. *Alu*I(-/-) cows produced 2 kg less than *Alu*I(+/+) and *Alu*I(+/-) cows. The calving conception interval was shorter for *Alu*I(-/-) cows (81.7 ± 4.9 days) than *Alu*I(+/+) cows (113.0 ± 9.1 days) ($P<0.05$). *Alu*I(+/-) cows (94.7 ± 5.3 days) tended to have a shorter calving conception interval than *Alu*I(+/+) cows ($P<0.10$). In summary, cows from the *Alu*I(-/-) genotype produced less milk and conceived earlier. Moreover, the GHR *Alu*I polymorphism can be one of the genes involved in the loss of fertility of Holstein cattle observed over the past 60 years.

Key words: GHR, IGF-I, polymorphism, reproduction, dairy cow

Introduction

During the last month of pregnancy and the early postpartum period, high producing dairy cows experience a period of negative energy balance (NEB). It occur as requirements for the final fetal growth and milk production exceed the energy obtained through feeding (Butler, 2003). In the early postpartum dairy cow the duration and intensity of NEB, the level of body condition

score (BCS) loss and the milk yield are strongly associated to the timing of first ovulation (Butler, 2003; Wathes et al., 2007). As a consequence, they have a negative impact on reproductive performance, since cows that ovulated earlier and had more estrous cycles before the first artificial insemination (AI) had higher conception rate (Thatcher and Wilcox, 1973).

Several hormones and metabolites convey the effects of NEB on reproductive function. Insulin-like growth factor (IGF-I), which is produced by the liver in response to growth hormone (GH) (Jones and Clemmons, 1995), is highly related to the extent of NEB (Lucy et al., 2001; Wathes et al., 2007). During the early postpartum period there is a simultaneous reduction in growth hormone receptor (GHR) and IGF-I mRNA expression in the liver of dairy cows due to the intense NEB (Radcliff et al., 2003), suggesting an uncoupling of the GH/IGF-I axis, therefore reducing dramatically the IGF-I synthesis in the early postpartum period (Fenwick et al., 2008). The reduced expression of GHR genes and its alternatives transcripts, mainly GHR 1A, led to reduced plasma IGF-I concentration, which is restored about 3 weeks postpartum when NEB intensity start to decrease (Kobayashi et al., 1999). In the ovary IGF-I act as a modulator of gonadotrophin action, stimulating granulosa and theca cell proliferation and differentiation (Armstrong and Webb, 1997), and preventing follicular atresia (el-Roeiy et al., 1994). In this sense, it is well established that cows that ovulated the first postpartum follicular wave had higher serum IGF-I concentration in the peripartum period (Butler et al., 2006; Kawashima et al., 2007).

The GHR/*Alu*I polymorphism was identified by (Aggrey et al., 1999) and is a point mutation (A→T) located at position - 1182 in the regulatory region of the GHR. This region is referent to the promoter region of GHR 1A, a liver specific variant (Heap et al., 1995) responsible for 50% of the total GHR in liver (Jiang and Lucy, 2001a, b). One of the GHR/*Alu*I polymorphism genotypes is associated to increase in liver expression and circulating concentration of IGF-I in Holstein bulls and heifers (Maj et al., 2008).

Based on the evidences previously described, the ability of the cow to restore postpartum hepatic GHR expression could be associated with the ability to restore the GHR/IGF-I axis, and consequently, resume postpartum ovulation. Considering this, cows with a specific genotype that may enhance the fertility and reduce the detrimental effects of NEB may be interesting for the milk industries and farmers. Thus, the aim of this work is to determine the effect of the GHR *Alu*I polymorphism on milk production and calving-conception interval in Holstein cows.

Material and Methods

Experimental design

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Beginning on -21 days from expected calving, 94 lactating Holstein cows were provided with free access to a TMR (Table 1). Cows were feed twice daily with *ad libitum* access to water. Weekly samples of the feed offered were composited on a monthly basis for nutrient analysis (Dairy One Cooperative, Ithaca, NY, USA). Feed refusals were weighed and discarded to estimate feed intake. Cows were milked twice daily, whereas milk yield was determined weekly. Milk samples were collected weekly for compositional analysis (Dairy One Cooperative). Body condition score was evaluated by the same technician on days -21 from expected calving, and then at 0, 7, 21 and 60 days in milk (DIM).

DNA analysis

For determination of GHR genotype, blood samples were collected by coccygeal venipuncture into evacuated plastic tubes containing EDTA and were stored at 4^{o}C until further analysis. DNA was extracted from 300 µL aliquot using the Wizard Genomic DNA Purification

Kit (Promega Corporation, Madison, USA) and quantified by measuring the absorbance at 260 nm on a spectrophotometer.

For determination of the alleles of the GHR gene, an 836-bp fragment was amplified using the following primers: Forward: TGCGTGCACAGCAGCTCAACC and Reverse: AGCAACCCCACTGCTGGGCAT. PCR reactions were performed in a final volume of 25 µL (Taka Ex Taq, Takara Bio Inc, Otsu, Japan) using 50 ng of genomic DNA. The annealing temperature for the primer was 66^{o}C. The amplified fragments were digested in a reaction mixture containing 5 µL of the PCR product and 3 U of the restriction enzyme Alu*I* (Invitrogen Corporation, Carlsbad, USA). The digestion mixtures were incubated in a thermocycler at 37^{o}C for 2 h. After digestion of the amplified products, the DNA fragments were separated on a 2% agarose gel (Invitrogen Corporation). A 100-bp molecular weight standard (New England Biolabs Inc, Ipswich, USA) was applied to each gel to permit the calculation of the size of the digested fragments. The DNA fragments were stained with ethidium bromide and visualized on an agarose gel by exposure to ultraviolet light. The gels were photographed for later analysis of the data using a digital photodocumentation system. The genotypes of the individuals were determined by analyzing the size of the fragments reported as base pairs (bp). The following genotypes were identified: *Alu*I(+/+): 602 bp, 145 bp, 75 bp; *Alu*I(-/-): 747 bp, 75 bp; and *Alu*I(+/-): 747 bp, 602 bp, 145 bp, 75 bp (Aggrey et al., 1999).

Reproductive management

For first postpartum services, cows were presynchronized with 2 injections of PGF2α (25 mg im; Lutalyse, Pfizer Animal Health, New York, USA) given 14 d, apart at 28 and 44 DIM. Ten days after the second injection of PGF2α, all cows initiated Ovsynch program (Pursley et al., 1995). The initial GnRH dose (100 µg im; Cystorelin, Merial Ltd, Iselin, USA) was followed 7 d

later by an injection of PGF2α (25 mg im; Lutalyse) and 48 h later cows received the second dose of GnRH (100 μg im; Cystorelin) followed by timed AI 12 hours later. Cows that were previously inseminated, but showed visual signs of estrous behavior before the pregnancy diagnosis were reinseminated. Additionally, open cows at the time of pregnancy diagnosis (32 d post-AI) were re-enrolled in an Ovsynch program. Reconfirmation of pregnancy was made twice at 42 and 60 d post-AI. The reproductive performance of the cows enrolled in the trial was followed until 200 DIM.

Definition of diseases

Cows were inspected for development of diseases signs in the postpartum period. The definitions used by the farm were the following: retained placenta: retention of fetal membranes for more than 24 h; metritis: abnormal vaginal discharge for 2 days in the first 3 weeks postpartum; displaced abomasum: presence of abdominal ping and required surgical correction; ketosis: no appetite and presence of ketone bodies in the urine; feet problems: difficult to walk plus visual inspection; mastitis: abnormal milk and/or high SCC; milk fever: subnormal body temperature and recumbent. The cows were also grouped by the development of 1, 2 or 3 episodes of the diseases listed above.

Statistical analyses

The results are presented as means ± standard error of the mean (SEM). All the statistical analyses were performed using SAS (SAS Institute Inc. Cary, NC, USA). Analyses involving repeated measures over time (e.g. milk production, energy corrected milk, fat, protein, lactose and total solids) were compared between treatments by analysis of variance for repeated measures using the MIXED procedure to evaluate the main effects of time, genotype, and their interactions (Littell et al., 1998). When the interaction between genotype and time was significant ($P<0.05$), pair-wise comparison of individual means was carried out. Cows that were tagged as

"do not breed" were exclude from all reproductive analysis (14 cows) and the ones that not conceived before 200 DIM were not included in the calving conception interval and number of AI per conception analysis (22 cows). The calving conception interval was analyzed through One-way ANOVA. Pregnancy rate was evaluated through survival analysis using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). Disease incidence was evaluated through Chi-square. A value of $P<0.05$ was considered statistically significant.

Results

Of the 94 cows analyzed for the GHR *Alu*I polymorphism, 35 (37.2%) were from the *Alu*I(+/+) genotype, 48 (51.1%) from the *Alu*I(-/+) genotype and 11 (11.7%) from the *Alu*I(-/-) genotype. Allele frequency was 0.62 and 0.37, for the *Alu*I(+) and *Alu*I(–) alleles respectively. The Chi-square (P=0.90) test indicated that the population was in Hardy-Weinberg equilibrium (expected frequencies were *Alu*I(+/+) = 38%, *Alu*I(-/+) = 46% and *Alu*I(-/-) = 14%).

Milk production and composition is summarized in Table 2. Briefly, *Alu*I(-/-) cows had the lowest milk production. There was no difference in BCS evaluated from -21 to 60 DIM (P=0.20). However, postpartum BCS loss from 0 to 60 DIM was higher for *Alu*I(+/-) (-0.38 ± 0.05) than *Alu*I(+/+) (-0.24 ± 0.06) and *Alu*I(-/-) (-0.23 ± 0.08) ($P<0.05$).

Incidence of retained placenta, metritis, dislocated abomasum, ketosis, feet problems, mastitis, milk fever was not different between genotypes ($P>0.05$). The proportion of cows that had none, 1, 2 or 3 episodes of the diseases listed above was also not different ($P>0.05$) between genotypes. The proportion of cows discarded from reproduction was also not different ($P>0.05$) between genotypes.

Pregnancy rate for cows carrying at least one *Alu*I(-) allele or two *Alu*I(+) alleles is represented in Figure 1. The calving conception interval was shorter for *Alu*I(-/-) cows (81.7 ± 4.9 days) than *Alu*I(+/+) cows (113.0 ± 9.1 days) ($P<0.05$). *Alu*I(+/-) cows (94.7 ± 5.3 days) tended to have a shorter calving conception interval than *Alu*I(+/+) cows ($P<0.10$).

Discussion

In the present study we observed that cows with the *Alu*I(-/-) genotype had a shorter calving conception interval and decreased milk production. It is well known that there is an inverse relationship between milk production and conception rate in Holstein cows in the period from 1950 to 2000 (Butler, 2003). In the same way, comparison of allele frequencies between Holstein bulls born between 1950 and 1970 and bulls of the 1980s indicated that the frequency of the *Alu*I(+) allele had increased and consequently the *Alu*I(-) allele had decreased (Aggrey et al., 1999). These evidences are in accordance to our data, since the frequency of the *Alu*I(-) allele described in this study was similar to the reported for bulls born in the 1980s (Aggrey et al., 1999). In addition, these data indicates that the GHR *Alu*I polymorphism can be one of the genes involved in the negative interrelationship between milk production and pregnancy rate on the last 60 years observed by (Butler, 2003).

Based on the results of pregnancy rate for cows with the *Alu*I(-) allele, it is possible to infer that these cows had increased circulating IGF-I concentration. Higher circulating IGF-I concentration is positive associated to interval from calving to first ovulation (Butler et al., 2006; Kawashima et al., 2007), pregnancy rate at first service (Patton et al., 2007) and embryo development (Velazquez et al., 2005) in cattle. Since cows that ovulate earlier also had a shorter calving conception interval (Kawashima et al., 2006; Thatcher and Wilcox, 1973), this would

explain why more cows with the *Alu*I(-) allele were pregnant earlier and thus this group had shorter calving conception interval.

Despite the good fertility, cows with the *Alu*I (-/-) genotype produced in average 2.1 kg/day less milk than cows from *Alu*I(+/-) and *Alu*I(+/+) genotypes. The uncoupling of the GH/IGF-I axis during early lactation supports a facilitatory role for the indirect actions of GH on lipolysis and gluconeogenesis (Bell, 1995), and attenuated growth promoting actions and support by IGF-I in peripheral tissues (Thissen et al., 1994), thus increasing milk production. In the *Alu*I(-/-) cows plasmatic IGF-I concentration could be increased, thus it is possible that these cows had lower GH secretion, since IGF-I inhibits GH secretion at the pituitary level (Muller et al., 1999). In this sense, perhaps these cows had lower circulating GH and less support to milk production. Despite that, BCS loss was not different for *Alu*I(-/-) and *Alu*I(+/+) genotypes.

In summary, cows from the *Alu*I(-/-) genotype produced less milk and conceived earlier. However, cows from the *Alu*I(+/-) genotype are of special economic interest, since they kept the same levels of milk production as *Alu*I(+/+) cows and tended to have shorter calving conception interval. Moreover, the GHR *Alu*I polymorphism can be one of the genes involved in the loss of fertility of Holstein cattle observed over the past 60 years.

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References

Aggrey, S.E., Yao, J., Sabour, M.P., Lin, C.Y., Zadworny, D., Hayes, J.F., Kuhnlein, U., 1999, Markers within the regulatory region of the growth hormone receptor gene and their association with milk-related traits in Holsteins. J Hered 90, 148-151.

Armstrong, D.G., Webb, R., 1997, Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. Rev Reprod 2, 139-146.

Bell, A.W., 1995, Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci 73, 2804-2819.

Butler, S.T., Pelton, S.H., Butler, W.R., 2006, Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. J Dairy Sci 89, 2938-2951.

Butler, W.R., 2003, Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livestock Science, 211-218.

el-Roeiy, A., Chen, X., Roberts, V.J., Shimasakai, S., Ling, N., LeRoith, D., Roberts, C.T., Jr., Yen, S.S., 1994, Expression of the genes encoding the insulin-like growth factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. J Clin Endocrinol Metab 78, 1488-1496.

Fenwick, M.A., Fitzpatrick, R., Kenny, D.A., Diskin, M.G., Patton, J., Murphy, J.J., Wathes, D.C., 2008, Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. Domest Anim Endocrinol 34, 31-44.

Heap, D., Lucy, M.C., Collier, R.J., Boyd, C.K., Warren, W.C., 1995, Rapid communication: nucleotide sequence of the promoter and first exon of the somatotropin receptor gene in cattle. J Anim Sci 73, 1529.

Jiang, H., Lucy, M.C., 2001a, Involvement of hepatocyte nuclear factor-4 in the expression of the growth hormone receptor 1A messenger ribonucleic acid in bovine liver. Mol Endocrinol 15, 1023-1034.

Jiang, H., Lucy, M.C., 2001b, Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: isolation, expression and effects on translational efficiency. Gene 265, 45-53.

Jones, J.I., Clemmons, D.R., 1995, Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16, 3-34.

Kawashima, C., Fukihara, S., Maeda, M., Kaneko, E., Montoya, C.A., Matsui, M., Shimizu, T., Matsunaga, N., Kida, K., Miyake, Y., Schams, D., Miyamoto, A., 2007, Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave post-partum in high-producing dairy cows. Reproduction 133, 155-163.

Kawashima, C., Kaneko, E., Amaya Montoya, C., Matsui, M., Yamagishi, N., Matsunaga, N., Ishii, M., Kida, K., Miyake, Y., Miyamoto, A., 2006, Relationship between the first ovulation within three weeks postpartum and subsequent ovarian cycles and fertility in high producing dairy cows. J Reprod Dev 52, 479-486.

Kobayashi, Y., Boyd, C.K., Bracken, C.J., Lamberson, W.R., Keisler, D.H., Lucy, M.C., 1999, Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of GHR 1A that is associated with decreased insulin-like growth factor I. Endocrinology 140, 3947-3954.

Littell, R.C., Henry, P.R., Ammerman, C.B., 1998, Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 76, 1216-1231.

Lucy, M.C., Jiang, H., Kobayashi, Y., 2001, Changes in the somatotrophic axis associated with the initiation of lactation. J Dairy Sci 89, E113-E119.

Maj, A., Snochowski, M., Siadkowska, E., Rowinska, B., Lisowski, P., Robakowska-Hyzorek, D., Oprzadek, J., Grochowska, R., Kochman, K., Zwierzchowski, L., 2008, Polymorphism in genes of growth hormone receptor (GHR) and insulin-like growth factor-1 (IGF1) and its association with both the IGF1 expression in liver and its level in blood in Polish Holstein-Friesian cattle. Neuro Endocrinol Lett 29, 981-989.

Muller, E.E., Locatelli, V., Cocchi, D., 1999, Neuroendocrine control of growth hormone secretion. Physiol Rev 79, 511-607.

Patton, J., Kenny, D.A., McNamara, S., Mee, J.F., O'Mara, F.P., Diskin, M.G., Murphy, J.J., 2007, Relationships among milk production, energy balance, plasma analytes, and reproduction in Holstein-Friesian cows. Journal of Dairy Science 90, 649-658.

Pursley, J.R., Mee, M.O., Wiltbank, M.C., 1995, Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology 44, 915-923.

Radcliff, R.P., McCormack, B.L., Crooker, B.A., Lucy, M.C., 2003, Growth hormone (GH) binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. J.Dairy Sci. 86, 3933-3940.

Thatcher, W.W., Wilcox, C.J., 1973, Postpartum estrus as an indicator of reproductive status in the dairy cow. J Dairy Sci 56, 608-610.

Thissen, J.P., Ketelslegers, J.M., Underwood, L.E., 1994, Nutritional Regulation of the Insulin-Like Growth-Factors. Endocrine Reviews 15, 80-101.

Velazquez, M.A., Newman, M., Christie, M.F., Cripps, P., Crowe, M.A., Smith, R.F., Dobson, H., 2005, The usefulness of a single measurement of insulin-like growth factor-1 as a predictor of embryo yield and pregnancy rates in a bovine MOET program. Theriogenology 64, 1977-1994.

Wathes, D.C., Fenwick, M., Cheng, Z., Bourne, N., Llewellyn, S., Morris, D.G., Kenny, D., Murphy, J., Fitzpatrick, R., 2007, Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. Theriogenology 68 Suppl 1, S232-241.

Tables

Table 1 – Feed composition and DMI during the experimental period.

Variable	Prepartum	Postpartum
DMI, kg/d	13.8	17.6
Dry Matter %	43.14	49.5
Crude Prot. %	10.0	16.6
Soluble Protein %	47.8	44.0
ADF %	28.6	21.9
NDF %	45.4	32.6
Lignin %	4.6	3.8
NFC %	35.4	39.1
Starch %	26.8	24.4
Crude Fat %	3.4	4.9
Ash %	5.8	6.9
TDN %	67.2	72.8
NEL, Mcal/kg	1.56	1.72

Table 2 – Milk production and composition for the *Alu*I(+/+), *Alu*I(+/-) and *Alu*I(-/-) genotypes.

	GHR *Alu*I Allele		GHR *Alu*I Genotype			*P*	
	(+)	(-)	(+/+)	(+/-)	(-/-)	Allele	Genotype
Milk, kg/day	36.2	36.3	36.3[a]	36.7[a]	34.4[b]	0.89	0.01
ECM	41.4	41.0	41.4	41.1	40.4	0.79	0.90
Fat, %	4.7	4.5	4.7	4.5	4.6	0.22	0.20
Protein, %	3.0	3.1	3.0	3.1	3.1	0.32	0.60
Lactose, %	4.72	4.76	4.72	4.75	4.75	0.03	0.26
Total solids, %	13.4	13.4	13.4	13.3	13.5	0.92	0.80

There was an effect of Day (P<0.0001) and no effect of Group*Day for any of the variables studied.

Table 3 – Reproductive variable for cows with the *Alu*I(-) and *Alu*I(+) alleles and genotypes.

	GHR *Alu*I Allele		GHR *Alu*I Genotype			*P*	
	(+)	(-)	(+/+)	(-/+)	(-/-)	Allele	Genotype
Cows, n	32	49	32	40	9	NA	NA
Days to 1^{o} AI, days	69.9 ± 0.4	69.8 ± 0.3	69.9 ± 0.4	69.6 ± 0.4	70.8 ± 0.8	NA	NA
Number of AI/conception, n	2.5 ± 0.3[a]	1.7 ± 0.1[b]	2.5 ± 0.3[a]	1.8 ± 0.1[b]	1.4 ± 0.2[b]	0.4	0.8
Pregnancy rate at 1^{o} AI, %	21.9	34.7	21.9	32.5	44.4	NS	NS
Days to conception, days	113.0 ± 9.0[a]	92.3 ± 4.5[b]	113.0 ± 9.0[a]	94.7 ± 5.3[ab]	81.7 ± 4.9[b]	0.02	0.07

Figures

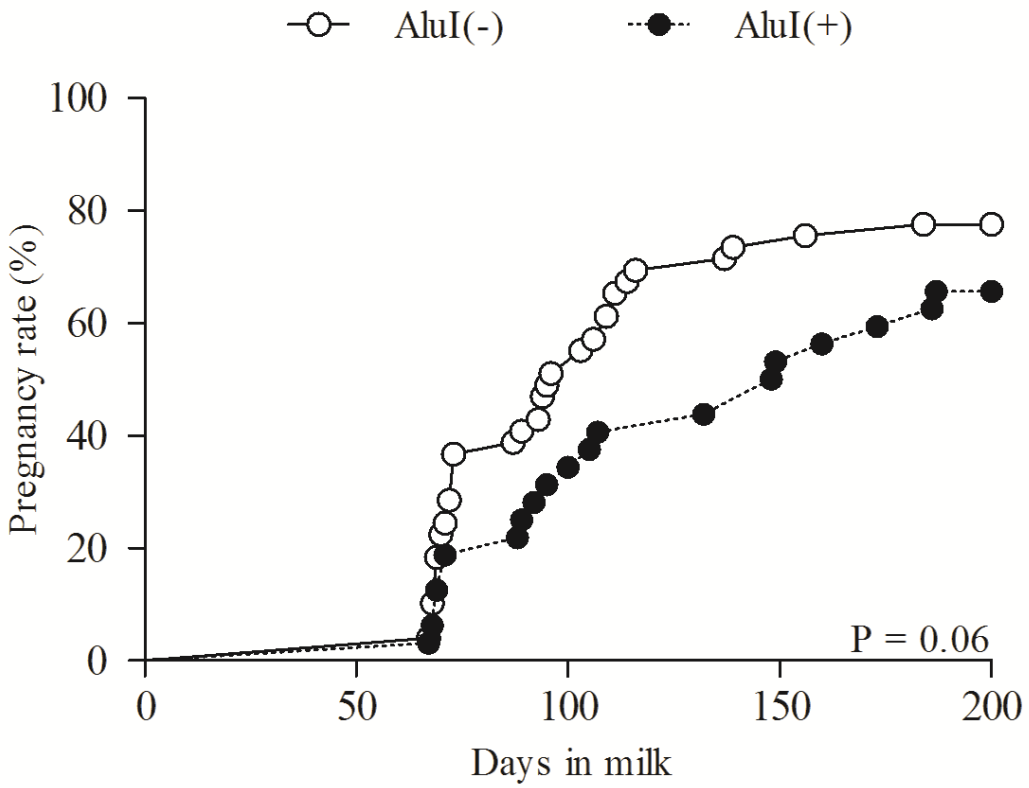

Figure 1 – Survival analysis for pregnancy rate up to 200 DIM in cows with at least one *Alu*I(-) allele in comparison to cows with two *Alu*I(+) alleles. Pregnancy rate tended to be higher for cows carrying the *Alu*I(-) allele.

7. Conclusão Geral

Nas condições estudadas a expressão de GHR/IGF-I não foi alterada no pós-parto de vacas de corte. Baseado neste resultado entende-se que não há um grande desafio de adaptação como há na vaca leiteira e, portanto, estudos de estratégias visando aumentar o desempenho reprodutivo baseadas no eixo GH/IGF-I não apresentariam um benefício tão grande quanto se aplicadas em vacas leiteiras. Em um segundo momento foi avaliada a expressão dos genes GHR e IGF-I em folículos ovarianos de diferentes categorias e corpo lúteo de vacas leiteiras. Apesar das óbvias diferenças entre folículo e corpo lúteo, não foi possível identificar correlação alguma entre produção de GHR e IGF-I nestas células. Em um terceiro momento foi avaliado o efeito da aplicação pré-parto de somatotropina sobre o metabolismo, produção de leite e retorno a atividade ovariana em vacas prímiparas da raça Holandês. Neste estudo observou-se que o GH pré-parto é capaz de adpatar o metabolismo da vaca para o início da lactação, aumentando a produção leiteira e melhorando o BEN. Além do mais, a exposição a altos níveis de IGF-I associado a melhora do BEN, ocasionaram um maior número de vacas ovulatórias no grupo tratado com GH pré-parto. Outra estratégia visou avaliar o efeito do polimorfismo *Alu*I no gene GHR sobre a produção de leite e taxa de concepção de vacas da raça Holandês. Como é sabido que a produção de leite e taxa de conepção são inversamente proporcionais, o gene GHR mostrou-se um excelente candidato neste sentido. Esta hipótese foi confirmada pela observação de que vacas com o genótipo GHR *Alu*I(-/-) apesar de produzirem menos leite tem um menor intervalo parto-concepção. Este conjunto de resultados deixa clara a importância do sistema GH/IGF-I para o desempenho tanto produtivo como reprodutivo de vacas de leite pós-parto, sendo que mais estudos com novas estratégias, assim como combinando diferente estratégias, podem trazer ainda mais benefícios aos sistemas de produção.

8. Referências Bibliográficas

ARMSTRONG, D. G., C. G. GUTIERREZ, G. BAXTER, A. L. GLAZYRIN, G. E. MANN, K. J. WOAD, C. O. HOGG, R. WEBB. Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. **Journal of Endocrinology,** v. 165(1), p. 101-113. 2000.

ARMSTRONG, D. G, R. WEBB. Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. **Reviews of Reproduction,** v. 2(3), p. 139-146. 1997.

BALOGH, O., K. KOVACS, M. KULCSAR, A. GASPARDY, A. ZSOLNAI, L. KATAI, A. PECSI, L. FESUS, W. R. BUTLER, G. HUSZENICZA. AluI polymorphism of the bovine growth hormone (GH) gene, resumption of ovarian cyclicity, milk production and loss of body condition at the onset of lactation in dairy cows. **Theriogenology,** v. 71(4), p. 553-559. 2009.

BAUMAN, D. E, W. B. CURRIE. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. **Journal of Dairy Science,** v. 63(9), p. 1514-1529. 1980.

BEG, M.A., BERGFELT, D.R., KOT, K., WILTBANK, M.C., GINTHER, O.J. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. **Biology of Reproduction**, v. 64, p. 432–441. 2001.

BELL, A. W. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. **Journal of Animal Science,** v. 73(9), p. 2804-2819. 1995.

BUTLER, S. T., A. L. MARR, S. H. PELTON, R. P. RADCLIFF, M. C. LUCY, W. R. BUTLER. Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy cattle: effects on expression of IGF-I and GH receptor 1A. **Journal of Endocrinology,** v. 176(2), p. 205-217. 2003.

BUTLER, S. T., S. H. PELTON, W. R. BUTLER. Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. **Journal of Dairy Science,** v. 89(8), p. 2938-2951. 2006.

BUTLER, W. R, R. D. SMITH. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. **Journal of Dairy Science,** v. 72(3), p. 767-783. 1989.

CURTIS, C. R., H. N. ERB, C. J. SNIFFEN, R. D. SMITH, D. S. KRONFELD. Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. **Journal of Dairy Science,** v. 68(9), p. 2347-2360. 1985.

DOUGLAS, G. N., T. R. OVERTON, H. G. BATEMAN, 2ND, H. M. DANN, J. K. DRACKLEY. Prepartal plane of nutrition, regardless of dietary energy source, affects periparturient metabolism and dry matter intake in Holstein cows. **Journal of Dairy Science,** v. 89(6), p. 2141-2157. 2006.

EL-ROEIY, A., X. CHEN, V. J. ROBERTS, S. SHIMASAKAI, N. LING, D. LEROITH, C. T. ROBERTS, JR., S. S. YEN. Expression of the genes encoding the insulin-like growth

factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. **Journal of Clinical and Endocrinology Metabolism,** v. 78(6), p. 1488-1496. 1994.

FENWICK, M. A., R. FITZPATRICK, D. A. KENNY, M. G. DISKIN, J. PATTON, J. J. MURPHY, D. C. WATHES. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. **Domestic Animal Endocrinology,** v. 34(1), p. 31-44. 2008.

GULAY, M. S., M. J. HAYEN, M. LIBONI, T. I. BELLOSO, C. J. WILCOX, H. H. HEAD. Low doses of bovine somatotropin during the transition period and early lactation improves milk yield, efficiency of production, and other physiological responses of Holstein cows. **Journal of Dairy Science,** v. 87(4), p. 948-960. 2004.

JIANG, H, M. C. LUCY. Involvement of hepatocyte nuclear factor-4 in the expression of the growth hormone receptor 1A messenger ribonucleic acid in bovine liver. **Molecular Endocrinology,** v. 15(6), p. 1023-1034. 2001a.

JIANG, H, M. C. LUCY. Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: isolation, expression and effects on translational efficiency. **Gene,** v. 265(1-2), p. 45-53. 2001b.

JIANG, H., M. C. LUCY, B. A. CROOKER, W. E. BEAL. Expression of growth hormone receptor 1A mRNA is decreased in dairy cows but not in beef cows at parturition. **Journal of Dairy Science,** v. 88(4), p. 1370-1377. 2005.

JONES, J. I, D. R. CLEMMONS. Insulin-like growth factors and their binding proteins: biological actions. **Endocrine Reviews,** v. 16(1), p. 3-34. 1995.

KAWASHIMA, C., S. FUKIHARA, M. MAEDA, E. KANEKO, C. A. MONTOYA, M. MATSUI, T. SHIMIZU, N. MATSUNAGA, K. KIDA, Y. MIYAKE, D. SCHAMS, A. MIYAMOTO. Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave post-partum in high-producing dairy cows. **Reproduction,** v. 133(1), p. 155-163. 2007a.

KAWASHIMA, C., M. SAKAGUCHI, T. SUZUKI, Y. SASAMOTO, Y. TAKAHASHI, M. MATSUI, A. MIYAMOTO. Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. **Journal of Reproduction and Development,** v. 53(1), p. 113-120. 2007b.

KIRBY, C. J., W. W. THATCHER, R. J. COLLIER, F. A. SIMMEN, M. C. LUCY. Effects of growth hormone and pregnancy on expression of growth hormone receptor, insulin-like growth factor-I, and insulin-like growth factor binding protein-2 and -3 genes in bovine uterus, ovary, and oviduct. **Biology of Reproduction,** v. 55(5), p. 996-1002. 1996.

KOLLE, S., F. SINOWATZ, G. BOIE, D. LINCOLN. Developmental changes in the expression of the growth hormone receptor messenger ribonucleic acid and protein in the bovine ovary. **Biology of Reproduction,** v. 59(4), p. 836-842. 1998.

LUCY, M. C. Regulation of ovarian follicular growth by somatotropin and insulin-like growth factors in cattle. **Journal of Dairy Science,** v. 83(7), p. 1635-1647. 2000.

MULLER, E. E., V. LOCATELLI, D. COCCHI. Neuroendocrine control of growth hormone secretion. **Physiology Reviews,** v. 79(2), p. 511-607. 1999.

PERKS, C. M., A. R. PETERS, D. C. WATHES. Follicular and luteal expression of insulin-like growth factors I and II and the type 1 IGF receptor in the bovine ovary. **Journal of Reproduction and Fertility,** v. 116(1), p. 157-165. 1999.

PUTNAM, D. E., G. A. VARGA, H. M. DANN. Metabolic and production responses to dietary protein and exogenous somatotropin in late gestation dairy cows. **Journal of Dairy Science,** v. 82(5), p. 982-995. 1999.

RHOADS, M. L., J. P. MEYER, S. J. KOLATH, W. R. LAMBERSON, M. C. LUCY. Growth hormone receptor, insulin-like growth factor (IGF)-1, and IGF-binding protein-2 expression in the reproductive tissues of early postpartum dairy cows. **Journal of Dairy Science,** v. 91(5), p. 1802-1813. 2008.

RIVERA, G. M, J. E. FORTUNE. Proteolysis of insulin-like growth factor binding proteins -4 and -5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. **Endocrinology,** v. 144(7), p. 2977-2987. 2003.

ROCHE, J. F., D. MACKEY, M. D. DISKIN. Reproductive management of postpartum cows. **Animal Reproduction Science,** v. 60-61, p. 703-712. 2000.

SCHAMS, D., B. BERISHA, M. KOSMANN, W. M. AMSELGRUBER. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. **Domestic Animal Endocrinology,** v. 22(1), p. 51-72. 2002.

SHIMIZU, T., C. MURAYAMA, N. SUDO, C. KAWASHIMA, M. TETSUKA, A. MIYAMOTO. Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. **Animal Reproduction Science,** v. 106(1-2), p. 143-152. 2008.

SHIRASUNA, K., C. KAWASHIMA, C. MURAYAMA, Y. AOKI, Y. MASUDA, K. KIDA, M. MATSUI, T. SHIMIZU, A. MIYAMOTO. Relationships between the First Ovulation Postpartum and Polymorphism in Genes Relating to Function of Immunity, Metabolism and Reproduction in High-producing Dairy Cows. **Journal of Reproduction and Development,** v., p. 2010.

SPICER, L. J., E. ALPIZAR, S. E. ECHTERNKAMP. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. **Journal of Animal Science,** v. 71(5), p. 1232-1241. 1993.

SPICER, L. J., C. C. CHASE, JR., L. M. RUTTER. Relationship between serum insulin-like growth factor-I and genotype during the postpartum interval in beef cows. **Journal of Animal Science,** v. 80(3), p. 716-722. 2002.

SUDO, N., T. SHIMIZU, C. KAWASHIMA, E. KANEKO, M. TETSUKA, A. MIYAMOTO. Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A (PAPP-A). **Molecular and Cellular Endocrinology,** v. 264(1-2), p. 197-203. 2007.

THATCHER, W. W, C. J. WILCOX. Postpartum estrus as an indicator of reproductive status in the dairy cow. **Journal of Dairy Science,** v. 56(5), p. 608-610. 1973.

VELAZQUEZ, M. A., M. NEWMAN, M. F. CHRISTIE, P. CRIPPS, M. A. CROWE, R. F. SMITH, H. DOBSON. The usefulness of a single measurement of insulin-like growth factor-1 as a predictor of embryo yield and pregnancy rates in a bovine MOET program. **Theriogenology,** v. 64(9), p. 1977-1994. 2005.

VELAZQUEZ, M. A., L. J. SPICER, D. C. WATHES. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. **Domestic Animal Endocrinology,** v. 35(4), p. 325-342. 2008.

WATHES, D. C., M. FENWICK, Z. CHENG, N. BOURNE, S. LLEWELLYN, D. G. MORRIS, D. KENNY, J. MURPHY, R. FITZPATRICK. Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. **Theriogenology,** v. 68 Suppl 1, p. S232-241. 2007.

YAKAR, S., J. L. LIU, B. STANNARD, A. BUTLER, D. ACCILI, B. SAUER, D. LEROITH. Normal growth and development in the absence of hepatic insulin-like growth factor I. **Proceedings of the National Academy of Sciences of the United States of America,** v. 96(13), p. 7324-7329. 1999.

YIMAZ, A., M. E. DAVIS, R. C. M. SIMMEN. Analysis of female reproductive traits in Angus beef cattle divergently selected for blood serum insulin-like growth factor I concentration. **Theriogenology,** v. 65(6), p. 1180-1190. 2006.