UNIVERSIDADE FEDERAL DE PELOTAS

Programa de Pós-Graduação em Biotecnologia Agrícola

Dissertação

Efeito do jejum e da insulina exógena sobre parâmetros metabólicos e expressão gênica do receptor do hormônio do crescimento (GH) e fator de crescimento semelhante à insulina tipo I (IGF-I), no folículo pré-ovulatório de ovelhas

Augusto Schneider

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Resumo

SCHNEIDER, Augusto. **Efeito do jejum e da insulina exógena sobre parâmetros metabólicos e expressão gênica do receptor do hormônio do crescimento (GH) e fator de crescimento semelhante à insulina tipo I (IGF-I), no folículo pré-ovulatório de ovelhas**. 2008. 45 f. Dissertação (Mestrado). Programa de Pós-Graduação em Biotecnologia Agrícola. Universidade Federal de Pelotas, Pelotas.

Em tecidos não ovarianos a expressão do receptor do hormônio do crescimento (GHR) é regulada pelas concentrações de insulina e está correlacionada a expressão de fator de crescimento semelhante a insulina tipo I (IGF-I). No ovário a expressão de IGF-I também está relacionada aos níveis de insulina, porém ainda não foi demonstrada sua correlação com a expressão de GHR. O objetivo deste estudo foi investigar o efeito do jejum ou administração de insulina nos níveis de glucose, ácidos graxos não esterificados, uréia, IGF-I, insulina e estradiol e na expressão folicular de RNAm para GHR e IGF-I em ovelhas. Quinze ovelhas receberam um dispositivo intravaginal liberador de progesterona, que foi removido após seis dias (Dia 0). No Dia -2 as ovelhas foram divididas em: grupo controle, que recebeu uma dieta de manutenção; grupo insulina, que recebeu injeções de insulina a cada 12 horas do Dia -2 ao Dia 2 e grupo jejum, que foi submetido à dieta hídrica do Dia -2 ao Dia 2. Os resultados revelaram que a administração de insulina ou o jejum durante o desenvolvimento de uma onda folicular não influenciaram (P=0,22) o diâmetro folicular, no entanto a administração de insulina aumentou (P=0,02) a produção de estradiol. Não houve diferença entre os grupos para a expressão de RNAm para GHR e IGF-I nas células da granulosa(P=0,62; 0,43) ou teca (P=0,92; 0.43). Para o grupo jejum houve uma correlção positiva entre glucose e estradiol (r=0,97, P=0,006) e RNAm IGF-I nas células da granulosa (r=0,96, P=0,03). Para o grupo insulina o estradiol foi positivamente correlacionado ao diâmetro folicular (r=0,93, P=0,02) e expressão de RNAm para GHR (r=0,87, P=0,05) e IGF-I (r=0,79, P=0,10)nas células da granulosa. Em conclusão, a insulina exógena ou o jejum não afetaram o diâmetro folicular e a expressão de mRNA para GHR e IGF-I no folículo pré-ovulatório, apesar da insulina exógena ter aumentado a produção de estradiol.

Palavras chave: insulina, IGF-I, GHR, ovário, ovelha.

Abstract

SCHNEIDER, Augusto. **Effects of fasting and exogenous insulin on metabolic parameters and gene expression of growth hormone receptor (GHR) and insulin like growth factor I (IGF-I), in the pre-ovulatory follicles of ewes**. 2008. 45 f. Dissertation (Master). Programa de Pós-Graduação em Biotecnologia Agrícola. Universidade Federal de Pelotas, Pelotas.

In non-ovarian tissues GHR expression is regulated by insulin concentrations and is correlated to IGF-I expression. In the ovary IGF-I expression is also related to insulin levels, however no correlation with GHR was demonstrated until now. The aim of this study was to investigate the effect of fasting or insulin administration on blood concentrations of glucose, nonsterified fatty acids, insulin, insulin like growth factor I (IGF-I) and estradiol and on follicular expression of growth hormone receptor (GHR) and IGF-I mRNA in ewes. Fifteen ewes received an intravaginal progesterone releasing device that was removed 6 days later (Day 0). In Day -2 the ewes were divided in: control group, which received maintenance diet, insulin group, which received insulin injections every 12 hours from Day -2 to 2 and fasting group, which was submitted to fasting from Day -2 to 2. The results of the current study revealed that insulin administration or fasting during the development of a follicular wave did not affect (P=0.22) follicular diameter, although insulin injection increased (P=0.02) estradiol production. There was no difference among groups for GHR or IGF-I mRNA expression in granulosa (P=0.62, 0.43) or theca cells (P=0.92, 0.43). For fasting group there was a positive correlation between glucose and estradiol (r=0.97, P=0.006) and granulosa cell IGF mRNA (r=0.96, P=0.03). For insulin group estradiol was positive correlated to follicular diameter (r=0.93, P=0.02) and granulosa cell GHR (r=0.87, P=0.05) and IGF-I mRNA (r=0.79, P=0.10). In conclusion, exogenous insulin or fasting did not affect follicular diameter and expression of GHR and IGF-I mRNA in the pre-ovulatory follicle, although exogenous insulin increased estradiol production.

Keywords: insulin, IGF-I, GHR, ovary, ewe.

Sumário

1. Introdução Geral

A ingestão inadequada de nutrientes resulta em diminuição da eficiência reprodutiva em ovinos e bovinos (para revisão BUTLER e SMITH, 1989; SCARAMUZZI et al., 2006). Vários estudos tentam identificar os fatores metabólicos e nutricionais ligando a nutrição ao desenvolvimento folicular. O fator de crescimento semelhante a insulina tipo I (IGF-I) é um potencial candidato a mediador desta interação (MUNOZ-GUTIERREZ et al., 2004), sendo sintetizado primariamente no fígado, onde sua transcrição é regulada positivamente pelo estímulo hormônio do crescimento (GH) mediado por seu receptor (GHR) (BUTLER et al., 2003).

Bovinos em bom estado nutricional contêm nove classes de transcritos do GHR no fígado (JIANG et al., 1999; JIANG e LUCY, 2001b; a), sendo os principais referidos como GHR 1A, GHR 1B e GHR 1C e reponsáveis por 50%, 35% e 15% do RNAm total para GHR, respectivamente (KOBAYASHI et al., 1999; JIANG e LUCY, 2001a). A expressão hepática de GHR e o nível de síntese de IGF-I são regulados pelo nível de insulina (BUTLER et al., 2003), com elevados níveis de insulina induzindo aumento da transcrição do gene do GHR e IGF-I, evidenciando porque animais em condições nutricionais inadequadas podem apresentar uma dissociação do eixo GH/IGF-I endócrino. No entanto, em tecidos extra-hepáticos a relação entre insulina e GHR é inversa, estabelecendo um mecanismo de compensação ao sistema endócrino, pois em osteoblastos (LEUNG et al., 1996) e no tecido adiposo (BUTLER et al., 2003) níveis altos de insulina e IGF-I reduzem a transcrição do gene do GHR, impedindo que o GH, que em períodos de hipoinsulinemia encontra-se aumentado (BUTLER et al., 2003), aumente a transcrição de IGF-I nos tecidos locais.

A importância do IGF-I endócrino em relação ao parácrino/autócrino tem sido motivo de debate (VELAZQUEZ et al., 2008). O IGF-I de origem hepática, ou endócrino, foi correlacionado com a idade ao primeiro parto (YILMAZ et al., 2006), taxa de concepção no primeiro serviço (PATTON et al., 2007), múltiplas ovulações (ECHTERNKAMP et al., 2004) e desenvolvimento embrionário (VELAZQUEZ et al., 2005). No entanto, desde a demonstração de que ratos com *knockout* hepato

específico para o gene do IGF-I tem função reprodutiva normal (YAKAR et al., 1999), a importância do IGF-I produzido em tecidos locais tem sido revista. Apesar disto, alguns autores observaram que os níveis de expressão de RNAm para IGF-I em folículos de diferentes estágios de desenvolvimento em ovinos e bovinos é muito baixo ou mesmo inexistente (PERKS et al., 1995; ARMSTRONG et al., 2000). No entanto, outros autores demonstraram que há expressão de IGF-I em células da teca e granulosa de e que esta sofre regulação por hormônios metabólicos (SPICER e CHAMBERLAIN, 2000). Evidenciando a necessidade de mais estudos relacionados à expressão de IGF-I em nível ovariano e sua relação com o *status* energético do indíviduo, buscando entender os mediadores metabólicos que podem influenciar sua expressão em diferentes momentos do desenvolvimento folicular.

O IGF-I, juntamente com seus receptores e as seis proteínas de ligação, possui um importante papel no desenvolvimento folicular. O IGF-I estimula a produção de estradiol pelas células da granulosa (GUTIERREZ et al., 1997) e a proliferação e diferenciação de células da teca e granulosa (SPICER et al., 1993). Além do mais, sua expressão local é aumentada no período final de maturação folicular em células da teca e granulosa (SCHAMS et al., 2002).

O GH também possui ação na regulação da função ovariana, tendo como mediador a expressão de GHR em nível folicular (ZHAO et al., 2000). O tratamento com GH aumenta significativamente a população de pequenos folículos em bovinos (GONG et al., 1991; 1993a; 1993b), além de possuir efeito estimulatório sobre o desenvolvimento de folículos antrais em camundongos (LIU et al., 1998). Estudos demonstram que há síntese de RNAm para GHR em células da teca e granulosa de folículos antrais (SHIMIZU et al., 2008) e em folículos primordiais (KOLLE et al., 1998). No entanto, estes estudos não avaliaram sua correlação com a expressão de mRNA para IGF-I e sua importância no processo de desenvolvimento e atresia folicular. Apesar de Cohick et al. (1996) afirmarem que não há correlação entre o GH endócrino e a produção ovariana de IGF-I, seu estudo avaliou a expressão em ovários inteiros, não avaliando a resposta individual de células da teca e/ou granulosa, ou mesmo diferentes categorias de folículos, que podem contribuir com a alteração dos níveis intrafoliculares dos níveis de IGF-I.

Deste modo a origem do IGF-I intrafolicular permanece motivo de debate, pois além de não encontrada correlação com o GH ou GHR, o nível de produção local de IGF-I no folículo também é dependente de hormônio luteinizante (SPICER e

CHAMBERLAIN, 2000), hormônio folículo estimulante (KHALID et al., 2000), insulina (SPICER e CHAMBERLAIN, 2000) e proteínas de ligação do IGF (PERKS e WATHES, 1996). Este fato é evidenciado pela correlação positiva observada entre o nível de IGF-I sanguíneo e intrafolicular para folículos médios (SPICER et al., 1992), porém não observada para folículos grandes (SCHOPPEE et al., 1996), indicando a existência de um mecanismo de compensação, conforme o momento da ovulação se aproxima.

2. Objetivos

Os objetivos desta dissertação foram:

1) Avaliar o efeito do jejum ou administração de insulina sobre parâmetros metabólicos de ovelhas submetidas à sincronização de cios;
2) Avaliar o efeito do jejum ou administração de insulina sobre o diâmetro do folículo pré-ovulatório e número de folículos em ovelhas submetidas à sincronização de cios;
3) Avaliar o efeito do jejum ou administração de insulina sobre a expressão de RNAm para GHR e IGF-I no folículo pré-ovulatório de ovelhas;
4) Avaliar a relação entre a expressão de RNAm para GHR e IGF-I no folículo pré-ovulatório de ovelhas.

3. Artigo

Effect of exogenous insulin or fasting on growth hormone receptor and insulin-like growth factor I gene expression in the pre-ovulatory follicle of ewes

Formatado segundo as normas da revista *Animal Reproduction Science*

Abstract

The aim of this study was to investigate the effect of fasting and insulin injections on blood concentrations of glucose, nonsterified fatty acids, insulin, insulin like growth factor I (IGF-I) and estradiol and on expression of growth hormone receptor (GHR) and IGF-I mRNA in ovarian follicles of ewes. Fifteen ewes received an intravaginal progesterone releasing device that was removed 6 days later (Day 0). In Day -2 the ewes were divided in: 1) control group (n = 5) that received maintenance diet; 2) insulin group (n = 5) and 3) fasting group (n = 5), that received insulin injections every 12 hours and fasting, respectively, from Day -2 to 2. Insulin injection increased (P=0.02) estradiol production. No difference among groups was detected for GHR or IGF-I mRNA expression in granulosa (P=0.62, 0.43) or theca cells (P=0.92, 0.43). For fasting group there was a positive correlation between glucose and estradiol (r=0.97, P=0.006) and granulosa cell IGF mRNA (r=0.96, P=0.03). For insulin group estradiol was positive correlated to follicular diameter (r=0.93, P=0.02) and granulosa cell GHR (r=0.87, P=0.05) and IGF-I mRNA (r=0.79, P=0.10). In conclusion, insulin injection increased estradiol production without any change in the expression of GHR and IGF-I mRNA in the pre-ovulatory follicle.

Keywords: IGF-I, GHR, ovary, mRNA, ewe.

Introduction

Ovarian follicular growth is controlled by several systemic and local intraovarian factors (Spicer et al., 1992). Physiologically, various metabolic messengers, including insulin and glucose, function to convey nutritional status to other systems within the body (Whitley et al., 2000). In this way, limited food resources can reduce reproductive efficiency to an extent dependent upon the degree of food restriction (Mackey et al., 2000) and the reproductive status at the time of the restriction (Smith, 1988). Many changes in metabolic profile, such as those associated with energy restriction (e.g. lower circulating levels of insulin, insulin-like growth factor-I (IGF-I), leptin and glucose; and higher concentrations of nonesterified fatty acids (NEFA), β-hydroxybutyrate and growth hormone (GH)), negatively affect follicular development (Butler et al., 2004).

The GH/IGF-I axis plays a role in vertebrate growth and has been responsible for the control of reproductive processes (Duan, 1998). The GH receptor (GHR), which modulates IGF-I synthesis under GH control (Jones and Clemmons, 1995), is found in greatest abundance in the liver (Bornfeldt et al., 1989; Edens and Talamantes, 1998). However, GHR is also found in many tissues (Lucy et al., 1998), including the ovary, where it is expressed in corpus luteum (Lucy et al., 1993) and theca and granulosa cells of ovarian follicles (Shimizu et al., 2008). Higher insulin plasmatic concentrations increases hepatic GHR expression (Baxter et al., 1980; Butler et al., 2003), however, this relationship in extrahepatic tissues is the opposite (Leung et al., 1997; Butler et al., 2003), establishing a compensatory mechanism. The GHR expression affects indirectly the follicular development and fertility by mediating GH actions, which include local (Khalid et al., 2000) and systemic IGF-I

synthesis (Butler et al., 2003), increasing androstenedione secretion by theca cells (Spicer and Stewart, 1996) and *in vitro* oocyte maturation (Izadyar et al., 1996).

Although hepatic IGF-I production is controlled by GH secretion (Jones and Clemmons, 1995), there is conflicting evidence that ovarian IGF-I production is regulated similarly (Cohick et al., 1996; Juengel et al., 1997; Khalid et al., 2000). Therefore, intrafollicular IGF-I origin remains unclear, since luteinizing hormone (LH) (Spicer and Chamberlain, 2000), follicle stimulating hormone (FSH) (Khalid et al., 2000), insulin (Spicer and Chamberlain, 2000) and IGF binding proteins (Perks and Wathes, 1996), also directly regulate its local production and availability. This is more clear in large follicles, where a systemic decrease of IGF-I is not followed by intrafollicular decrease of its levels (Spicer et al., 1992), as observed for medium follicles (Schoppee et al., 1996). This indicates the presence of a compensatory mechanism, probably modulated by insulin, but also dependent on LH and FSH receptors, preventing the negative effects of short term starvation on follicular selection and final development. IGF-I acts in synergism with gonadotropins, increasing gonadotropin receptor numbers and activity of gonadotropin receptor second messenger systems (Adashi, 1994; 1998).

Considering these points, the present study was designed to investigate the effect of fasting and insulin administration on metabolic profile and expression of GHR and IGF-I mRNA in theca and granulosa cells of the ewe pre-ovulatory follicle. In this study, 2 hypotheses were tested, 1) the fasting decreases follicular GHR/IGF-I mRNA expression and development; and 2) exogenous insulin injection increases follicular GHR/IGF-I mRNA expression and development.

Materials and Methods

Animals and treatments

Multiparous crossbreed mature ewes (*Ovis aries*, Corriedale *vs* Texel, *n* = 15), 2.5 years of age, non lactating, weighing 53.40 ± 6.10, 56.40 ± 3.91 and 52.80 ± 5.21 kg for fasting, control and insulin groups, and with 3.10 ± 0.22 (3.0 – 3.5) of body condition score (1=lean and 5=obese) (Russel, 1991) for the three groups, were used during the breeding season in southern Brazil. The diet was based on tifton hay (neutral detergent fiber 77.34%, acid detergent fiber 59.78%, ether extract 1.26%, crude protein 7.96%) and concentrate (crude fiber 13.90%, ether extract 5.68%, crude protein 14.80%) according to NRC (NRC, 1985), and *ad libitum* access to water. The animals were housed grouped (5 ewes) at indoor stalls (2.00 X 3.50 m).

The ewes were submitted to pre-synchronization protocol with intravaginal sponges containing medroxiprogesterone acetate (60 mg) and observed for estrus. In the 11th day of the estrous cycle all ewes received an injection of 125 µg of sodium cloprostenol (i.m., Ciosin®, Fort Dodge, Campinas, Brazil) and 36 hours after, 100 µg of gonadorelin (i.m., Fertigen®, Tortuga, Sto. Amaro, Brazil) in order to induce the ovulation 24 hours later (Rubianes et al., 1997). Twenty four hours after gonadorelin injection an intravaginal progesterone releasing device (CIDR, Eazi-Breed CIDR®, InterAg, Hamilton, New Zealand) was inserted and remained in the ewes for 6 days, when 125 µg of sodium cloprostenol (i.m., Ciosin®, Fort Dodge, Campinas, Brazil) was injected. The moment of CIDR removal was considered the Day 0 of the experiment.

On Day -2 the ewes were assigned by body condition score and weight to one of the experimental groups; 1) control group (*n* = 5), received a maintenance diet during the entire experimental period; 2) fasting group (*n* = 5), from which food was completely removed from Day -2 to Day 2 and had only access to water; and 3)

insulin group (*n* = 5), which received the same diet as control group plus injections of human recombinant insulin (s.c., 0.25 IU/kg, Novolin® N, Novo Nordisk, Bagsvaerd, Denmark) twice daily from Day -2 to Day 2.

Blood sampling

Blood samples were taken daily from Day -2 to Day 2 by jugular venipuncture before the first morning meal (12 hours of fasting). Samples were collected on two 10 mL vacuttainer tubes (BD Diagnostics, São Paulo, Brazil) one containing EDTA and one with EDTA plus potassium fluoride. Tubes were centrifuged and plasma separated and stored in microtubes at −80^{o} C until assayed for glucose, urea, NEFA, insulin, IGF-I, estradiol and progesterone.

Metabolite and hormone assays

Concentrations of glucose, urea and NEFA were evaluated daily from Day -2 to 2 through final point enzymatic colorimetric reactions quantified by a spectrophotometer (FEMTO 700 Plus, Femto Ind. e Com. de Instrumentos Ltda., São Paulo, Brazil). The reactions to measure glucose (Glicose PAP Liquiform, Labtest®, Lagoa Santa, Brazil), urea (Uréia CE, Labtest®, Lagoa Santa, Brazil) and NEFA (Wako NEFA-HR, Wako Chemicals USA®, Richmond, United States) were performed as recommended by the manufacturers, and had a minimum detection limit of 0.02 mmol/L, 0.33 mmol/L and 0.01 mmol/L, respectively

Plasma insulin concentrations, from Day -2 to 2, were evaluated by radioimmunoassay (KIP1254, BioSource Europe®, Nivelles, Belgium) with a minimum detection limit of 0.009 µg/L. The intra-assay coefficients of variation were 5.22% and 2.44%, for low and high insulin concentrations, respectively. The inter-assay coefficients of variation were 0.89% and 1.86%, for low and high insulin concentrations, respectively.

Plasma IGF-I concentrations, on Days -2, 0 and 2, were also evaluated by radioimmunoassay (DSL-5600, Diagnostics Systems Laboratory®, Webster, United States) (Miles et al., 1974), after a extraction step in which IGF-I was separated from its binding proteins, with a minimum detection limit of 2.25 ng/mL. 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.

Estradiol (Day 2) and progesterone (Day -6 and 0) concentrations were measured using electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland) using Estradiol II and Progesterone II kits (Roche Diagnostics, Manheim, USA) (Bargouli et al., 2007). The detection limit of the assay was 0.03 ng/mL and 5.00 pg/mL for progesterone and estradiol, respectively.

Follicle dissection

On Day 2, approximately 32 hours after CIDR removal, all ewes were killed at a local slaughterhouse and ovaries were collected in individual sterile flasks containing saline and transported to the laboratory on ice. The procedures for follicular dissection were adapted from Cao et al. (2006). The diameter of dominant follicles was measured with calipers and those larger than 4 mm were considered as pre-ovulatory (Meza-Herrera et al., 2008). The follicular fluid was aspirated and the antral cavity was flushed repeatedly with cold saline, which was collected into microtubes. Granulosa cells were recovered from the fluid by centrifugation at 1200 g for 1 minute. After, the follicle was sliced in two halves, allowing access to the theca layer, which was removed with fine forceps and washed in saline by passing repeatedly through a 3 mL syringe. The samples were collected into microtubes

containing Trizol (Invitrogen®, Carlsbad, USA) and frozen at -80^{o} C until RNA extraction.

All the follicles of the pair of ovaries were counted and classified according their diameter in small (< 2 mm), medium (2 – 4 mm) and pre-ovulatory (> 4 mm) (Sarath et al., 2008).

Real Time RT-PCR analysis

Total RNA was extracted using Trizol (Invitrogen®, Carlsbad, USA) according to manufacturer's instructions. Integrity of the extracted RNA was determined by staining the samples of total RNA with ethidium bromide, followed by electrophoresis on a 1.5% agarose gel.

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 cDNAs (SuperScript III First-Strand Synthesis Supermix, Invitrogen®, Carlsbad, USA). The PCR amplifications and fluorescence detection, using cDNAs 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 (For CCA GTT TCC ATG GTT CTT AAT TAT, Rev TTC CTT TAA TCT TTG GAA CTG G) (Pfaffl et al., 2002), IGF-I (For TCG CAT CTC TTC TAT CTG GCC CTG T, Rev GCA GTA CAT CTC CAG CCT CCT CAG A) (Pfaffl et al., 2002) and β-actin (For CTA GGC ACC AGG GCG TCA TG, Rev CTT AGG GTT CAG GGG GGC CT).

Real-time fluorescence raw data was taken and a cycle threshold (CT) value for each sample and efficiency for each primer pair was calculated using Real Time

PCR Miner (Zhao and Fernald, 2005), using β-actin as endogenous control to standardize the amount of target mRNA in the reaction and estimate the initial sample (R0) mRNA concentrations (Zhao and Fernald, 2005). The specificity of each primer was detected at the dissociation curve for each replicate. The mean coefficient of variation among sample CT´s was 1.71%.

Statistical analysis

The results are presented as means ± standard error of the mean (SEM). Data were log scaled when they were not normally distributed. All the statistical analyses were performed with SAS 9.0 (SAS Institute Inc. Cary, NC, USA). The single point measurements (e.g., estradiol, progesterone, gene expression, follicular and luteal diameter) were compared among the groups by one–way analysis of variance (ANOVA). Hormone and metabolite data were analyzed as repeated measures within ewe using compound symmetry structure, when the interaction between treatment and time was significant ($P<0.05$) pair-wise comparisons of individual means were carried out with the Tukey–Kramer test. Comparison among number of small, medium, pre-ovulatory and total follicles per group were carried out through Kruskall-Wallis nonparametric one-way ANOVA. The correlation among parametric or nonparametric variables within each group was assessed using Pearson or Spearman correlation coefficient (r), respectively. A value of $P<0.05$ was considered statistically significant.

Animal welfare

The Committee for Ethics in Animal Experimentation from the Federal University of Pelotas has approved all procedures performed in this experiment.

Results

Estrous synchronization

We considered that all ewes ovulated with the injection of gonadorelin on Day -7, since they presented a corpus luteum at Day 2. The progesterone concentrations at CIDR insertion were bellow 1 ng/mL (0.87 ± 0.06 ng/mL), which indicates a luteolytic response to the cloprostenol injection and that the ewes did not have a functional corpus luteum at the moment of gonadorelin injection and CIDR insertion.

Metabolite and hormone concentrations

Daily hormonal concentrations of glucose, urea, NEFA and insulin are shown in Figure 1. There was a group by day effect for the four parameters that are further detailed in Figure 1. Also, a group by day effect was observed for IGF-I concentrations, which were higher in control than insulin group on Day 2 (Figure 2).

The insulin group had higher estradiol concentrations on Day 2 (53.70 ± 1.82 pg/mL, $P<0.05$) than fasting group (29.97 ± 6.96 pg/mL), but was not different from control group (35.92 ± 5.72 pg/mL).

The progesterone concentration at CIDR removal (Day 0) was higher ($P<0.001$) in fasting group (8.80 ± 1.88 ng/mL) than control (3.60 ± 0.24 ng/mL) and insulin groups (3.60 ± 0.40 ng/mL).

Ovarian morphology

The diameter of the pre-ovulatory follicle on Day 2 was not different among groups, 7.40 ± 0.37, 7.10 ± 0.55 and 8.60 ± 0.87 mm for fasting, control and insulin groups, respectively. The corpus luteum diameter, induced on Day -7, also was not different among groups (4.78 ± 0.47 mm).

There was no difference in the number of total follicles (12.66 ± 2.28) per animal for the three groups, neither in the number of small (10.73 ± 2.19), medium (0.8 ± 0.2) and pre-ovulatory (1.13 ± 0.09) follicles.

Follicular GHR and IGF-I mRNA expression

There was no difference among groups for GHR mRNA expression in granulosa or theca cells and IGF-I mRNA expression in granulosa or theca cells of the pre-ovulatory follicle (Figure 3). No difference was detected in the expression of GHR and IGF-I mRNA between theca and granulosa cells.

Gene expression, metabolite and hormone interaction

On Day 2 there was a positive correlation between glucose and estradiol (r=0.97, P<0.01), glucose and granulosa cell IGF mRNA (r=0.96, P<0.05) and a tendency (r=0.90, P<0.1) for a correlation between estradiol and granulosa cell IGF mRNA for fasting group only.

There was a positive correlation between estradiol and follicular diameter (r=0.93, P<0.05), estradiol and granulosa cell GHR mRNA (r=0.87, P<0.05) and a tendency for IGF-I mRNA (r=0.79, P<0.1) in the insulin group on Day 2. Furthermore granulosa cell GHR and IGF-I mRNA (r=0.98, P<0.05) were positively correlated, but theca cell IGF-I mRNA and granulosa cell GHR (r=-0.88, P<0.05) and IGF-I (r=-0.90, P<0.05) were negatively correlated.

The diameter of the pre-ovulatory follicle and number of medium follicles were negatively correlated (r=-0.96, P<0.01) in the insulin group. The number of small follicles was negatively correlated with estradiol (r= 0.90, P<0.05) and follicular diameter (r=-0.89, P<0.05) on Day 2.

Discussion

The results of the current study showed that: (1) insulin injection and fasting did not affect follicular diameter, although insulin injection increased estradiol production; (2) exogenous insulin and fasting did not affect the expression of GHR or

IGF-I mRNA in the pre-ovulatory follicle; (3) glucose, estradiol and IGF mRNA were positive correlated in fasting group and; (4) estradiol was positive correlated to follicular diameter and granulosa cell GHR and IGF-I mRNA in insulin group.

Although expression of GHR mRNA was detected in the granulosa and theca cells of primordial and small antral follicle of ewes (Eckery et al., 1997), this is the first work that observed expression of this gene in the pre-ovulatory follicle of ewes. Moreover, expression of IGF-I mRNA was detected in theca and granulosa cells of the pre-ovulatory follicle, as already reported by Khalid et al. (2000) for granulosa cells, but in contrast, Spicer et al. (1995) reported that only 5% of the ovine pre-ovulatory follicles express IGF-I mRNA. Nonetheless, there was no difference in GHR and IGF-I mRNA expression in the pre-ovulatory follicle as a result of fasting or insulin administration. As previously reported in *in vitro* studies, lower insulin levels enhance IGF-I expression in bovine granulosa cells (Spicer and Chamberlain, 2000), however in the current study, as no difference between groups for insulin concentrations was observed, no alterations in IGF-I mRNA expression were detected.

Insulin administration did not increase the follicular diameter, contrasting with the described for cattle (Simpson et al., 1994) and buffaloes (Ramoun et al., 2007). However, insulin administration improved estradiol production, as reported by Butler et al. (2004), and was correlated to pre-ovulatory follicular diameter in insulin group, which had the largest follicles, although not statistically significant. Insulin acts through its own receptor to modulate the response of granulosa cells to gonadotrophins (Willis et al., 1996) and enhances estradiol production by granulosa cells (Willis et al., 1996). Furthermore, increased expression of insulin receptor gene

in the pre-ovulatory follicle is associated with increased estradiol production (Shimizu et al., 2008), showing the importance of insulin in the modulation of steroidogenesis.

The GHR and IGF-I mRNA expression in granulosa cells were positively correlated in insulin group, indicating that GHR could modulate the effects of GH in ovarian IGF-I production, as described by Juengel et al. (1997) for the ovine corpus luteum. Similar results were reported by Butler et al. (2003), which observed that GHR and IGF-I mRNA were positive correlated in the hepatic cells of insulin treated cows but not in no treated cows. In contrast, Whates et al. (1995) did not observe a positive effect of GH on IGF-I mRNA expression in ewes granulosa cells. On the other hand, the negative effect of theca cells IGF-I mRNA on granulosa cell GHR and IGF-I mRNA could be attributed to the combined effects of systemic insulin and theca cells IGF-I secretion, that acting together through their specific receptors decrease GHR expression in extrahepatic tissues, as previously reported (Leung et al., 1996).

Glucose concentrations in plasma and follicular fluid are closely correlated (Leroy et al., 2004) and the follicle supplement its need for ATP by anerobic glycolysis, with lactate as the end product (Rabiee et al., 1997). Since, LH and FSH stimulates lactate production (Boland et al., 1993) and there is a positive correlation between ATP and pregnenolone synthesis in ovarian mitochondria (Robinson et al., 1970), we could expected that glucose concentrations in combination with higher LH pulsatility from progesterone device removal (Menegatos et al., 2003) were correlated with estradiol production. However, for unknown reasons, this correlation was only observed for the fasting group, which had the lowest estradiol levels. Also, in pigs there is evidence of a correlation between expression of IGF-I mRNA in granulosa cells and estradiol production (Samaras et al., 1993), in the current study similar results were found in fasting and insulin group, where IGF-I mRNA was

correlated to estradiol production. This hypothesis explain the positive correlation between estradiol and IGF-I mRNA in fasting and insulin group, and also the positive correlation between glucose, estradiol and IGF-I mRNA in fasting group.

Insulin injection or fasting did not affect the ovarian follicular population observed on Day 2, but in insulin group the pre-ovulatory follicular diameter was negatively correlated to the number of small and medium follicles. Since FSH peaks preceding follicular waves in the ewe are critical for the genesis of these waves (Barrett et al., 2006), the reduced number of small and medium follicles could be due to inhibin suppression of FSH secretion (Mann et al., 1992a; Barrett et al., 2006). Furthermore, there was a negative correlation between estradiol and number of small follicles, as most inhibin and estradiol are produced from the largest follicles of a wave (Mann et al., 1992b) and are positively correlated (Souza et al., 1997).

The increased insulin concentrations after CIDR removal is in agreement with data from Kawashima *et al.* (2007) and may serve to support follicular selection and final development, probably due to stimulatory effects of estradiol on insulin secretion by the pancreas (Morimoto et al., 2001). Moreover, the increased IGF-I concentrations observed after CIDR removal were previously reported (Armstrong et al., 2002) and could be a response to the increased GH secretion induced by estradiol. However, extremely higher concentrations of estradiol are not stimulatory and even may inhibit GH secretion. This could be translated to an *in vivo* function related to estrogen negative feedback as observed in this study, as long as control and fasting groups presented increased IGF-I secretion after CIDR removal. Nonetheless, insulin group (the highest estradiol concentrations) had lower IGF-I levels, indicating a possible threshold level for the positive effects of estradiol on GH and IGF-I secretion.

There was a twofold increase in progesterone concentrations in fasting group, due reduced hepatic cholesterol clearance induced by reduced feed intake (Parr et al., 1993b; a). However, it was not correlated to follicular diameter or estradiol production, as reported by Kyima et al. (2004), which observed a delay in the pre-ovulatory surge of estradiol and LH, as a result of high progesterone concentrations induced by fasting. Although, this mechanism, lower or delayed estradiol production, could be responsible for the reduced estradiol levels observed for fasting group.

Further studies are needed to more precisely address the relationship between insulin and estradiol production *in vivo*, including the investigation of more genes related to steroidogenic enzymes and LH and FSH receptors, to establish what dose and moment are critical for insulin addition in a progestin based synchronization protocol, including in nutrient deprived animals, that had a reduction in estradiol production.

In conclusion, insulin injection increased estradiol production without any change in the expression of GHR and IGF-I mRNA or diameter of the pre-ovulatory follicle.

Acknowledgements

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Figures

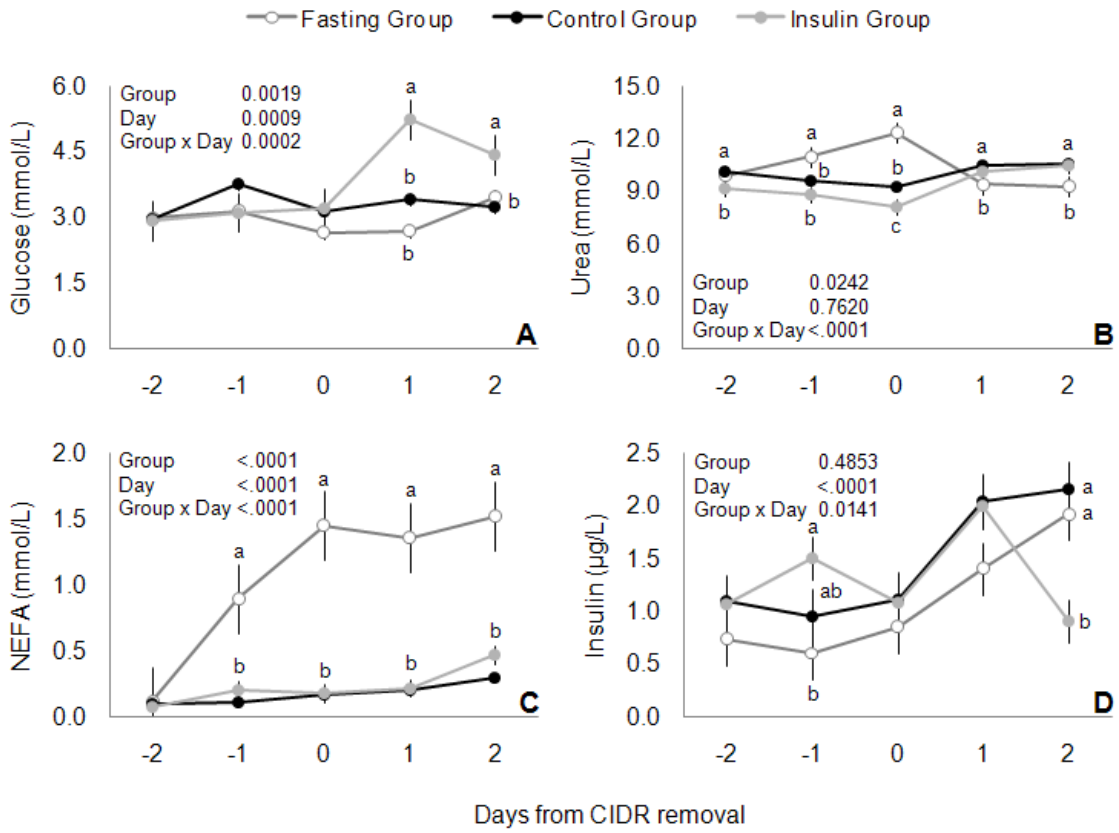

Figure 1 – Plasma glucose (mmol/L) (A), urea (mmol/L) (B), nonsterified fatty acids (NEFA) (mmol/L) (C) and insulin (µg/L) (D) ± SEM for ewes during the experiment in fasting, control and insulin groups from Day -2 through 2. Different superscripts show significantly different values (P<0.05).

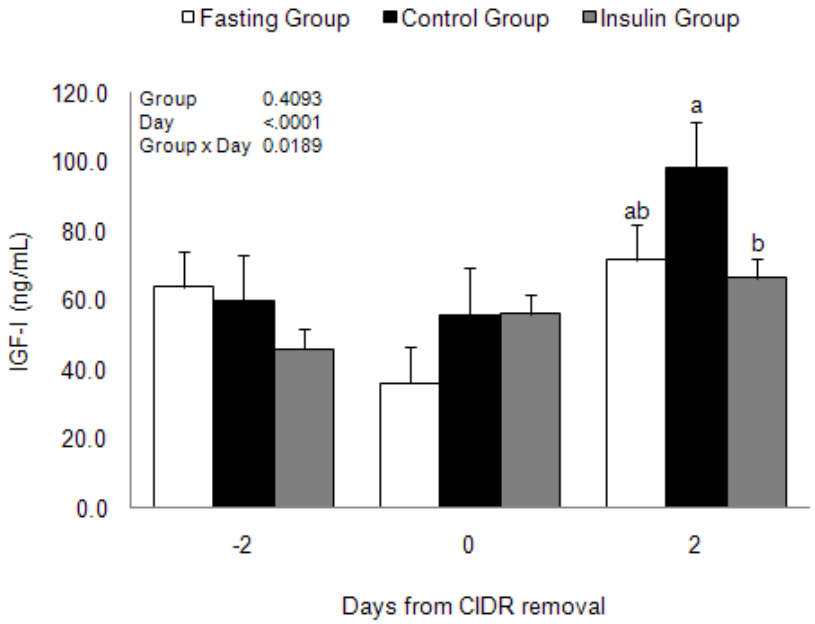

Figure 2 - Plasma IGF-I (ng/mL) ± SEM for ewes during the experiment in fasting, control and insulin groups on Day -2, 0 and 2. Different superscripts show significantly different values (P<0.05).

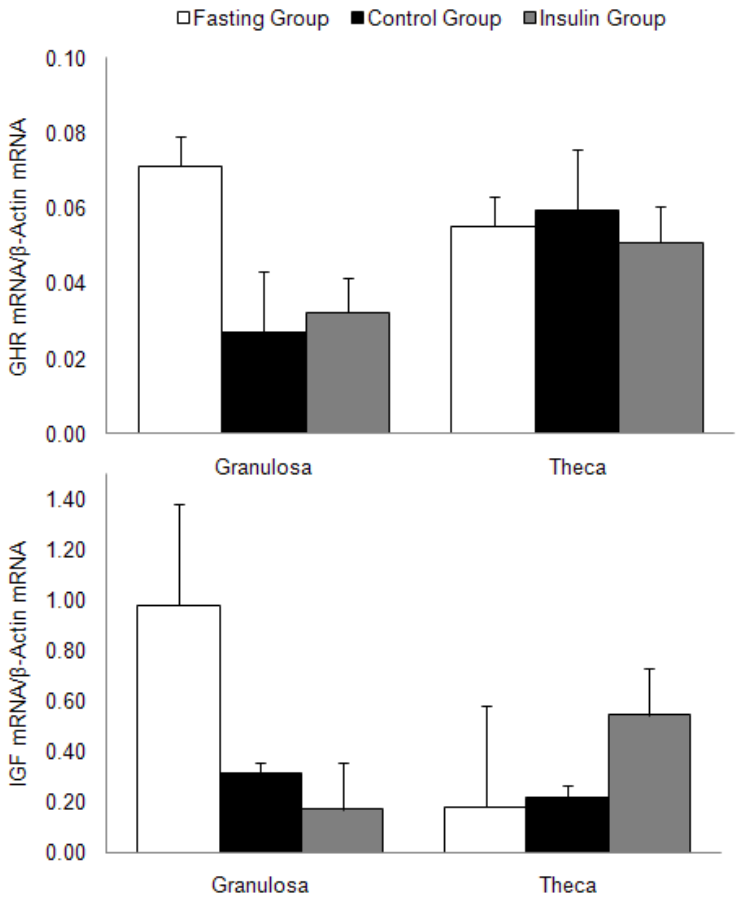

Figure 3 - Expression of GHR and IGF-I mRNA (relative amount) ± SEM in granulosa and theca cells of the pre-ovulatory follicle on Day 7 of the experiment in fasting, control and insulin groups. There were no differences among means (P>0.05).

4. Conclusão geral

Em conclusão, a insulina exógena ou o jejum não influenciaram o diâmetro folicular e a expressão de RNAm para GHR e IGF-I no folículo pré-ovulatório, apesar da insulina exógena ter aumentado a produção de estradiol.

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