UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Veterinária

Tese

Efeito da Somatotrofina Suína (pST) sobre o desenvolvimento testicular, idade à puberdade e qualidade espermática de machos suínos

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Pelotas, 2012

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

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Pelotas, 2012

Dados de catalogação na fonte:
(Marlene Cravo Castillo – CRB-10/744)

R113e Rabassa, Viviane Rohrig

Efeito da somatotrofina suína (pST) sobre o desenvolvimento testicular, idade à puberdade e qualidade espermática de machos suínos / Viviane Rohrig Rabassa ; orientador Marcio Nunes Corrêa ; co-orientadores Ivan Bianchi, Eduardo Schmitt, Francisco Augusto B. Del Pino e Luiz Francisco Machado Pfeifer. Pelotas,2012.63f. : il..- Tese (Doutorado) –Programa de Pós-Graduação em Ciências.Área de conhecimento Clínica Médica. Faculdade de Veterinária. Universidade Federal de Pelotas. Pelotas, 2012.

1.GH 2.IGF-I 3.Suíno 4.Puberdade 5.Testículo I.Corrêa, Marcio Nunes(orientador) II. Título.

CDD 636.4.082 41

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Agradecimentos

Ao Programa de Pós-graduação em Veterinária pela oportunidade de realizar o doutorado.

A CAPES pelo período de fornecimento de bolsa de doutorado e a FAPERGS e CNPq pelos recursos que auxiliaram na execução deste estudo.

Ao meu orientador Dr. Marcio Nunes Corrêa agradeço de forma muito especial pelos quase dez anos de orientação e amizade.

Aos meus co-orientadores Dr. Ivan Bianchi, Dr. Eduardo Schmitt, Dr. Luiz Franscisco Machado Pfeifer e Dr. Francisco Augusto Burkert Del Pino e ao Dr. Augusto Schneider pela amizade e auxílio na elaboração das idéias e confecção dos artigos.

Aos integrantes do Núcleo de Pesquisa, Ensino e Extensão em Pecuária (NUPEEC) pelo auxílio nas discussões e execução dos experimentos, no qual foram fundamentais.

Ao Hospital de Clínicas Veterinária, Centro Agropecuário da Palma, Laboratório de Bioquímica Clínica, Centro de Biotecnologia, Departamento de Patologia Animal e Laboratório de Reprodução Animal (REPROPEL) pela disponibilidade de pessoal, infra-estrutura e materiais para condução das atividades.

A minha amiga Elizabeth Schwegler pelo companheirismo e amizade sincera durante todos estes anos.

A minha mãe Naimar Beatriz Gonçalves Rohrig pelo apoio e incentivo em todas as horas.

Ao meu filho Arthur Rohrig Rabassa pelo amor incondicional e por ser a alegria da minha vida.

Resumo

RABASSA, Viviane Rohrig. **Efeito da Somatotrofina Suína (pST) sobre o desenvolvimento testicular, idade à puberdade e qualidade espermática de machos suínos**. 2012. 63f. Tese (Doutorado) - Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

A somatotrofina (ST), a qual tem muitos de seus efeitos mediados pelo fator de crescimento semelhante à insulina I (IGF-I), tem efeito positivo sobre a gametogênese e a esteroidogênese, sendo utilizada como alternativa de tratamento em homens com infertilidade. Quanto ao seu efeito sobre a idade à puberdade, em humanos com deficiência endógena, a sua suplementação causa maior desenvolvimento testicular, gametogênese e esteroidogênese e antecipação da puberdade. Porém, não há estudos que determinem os mecanismos de ação deste hormônio sobre a função testicular e idade à puberdade em suínos sadios. Dessa forma as hipóteses deste estudo foram: 1) machos suínos pré-púberes recebendo ST exógena tem seu crescimento testicular acelerado, devido à ação mitogênica do IGF-I; 2) incremento na gametogênese e esteroidogênese, com consequente antecipação da idade à puberdade; e 3) melhora da qualidade espermática após o estabelecimento da puberdade. Para confirmar estas hipóteses foram realizados quatro experimentos. O Experimento 1 teve o objetivo de determinar o intervalo entre aplicações de ST suína (pST), a partir da determinação dos níveis de IGF-I. O Experimento 2 teve o objetivo de avaliar a proliferação de células testiculares em animais submetidos à administração de pST, bem como seu efeito sobre o metabolismo de leitões. No Experimento 1, os níveis séricos médios de IGF-I do Grupo pST foram superiores ($P<0,05$) aos do Grupo Controle, com diferença entre grupos até o 3º dia após aplicação do hormônio, determinando o intervalo entre doses a ser utilizado nos próximos experimentos. No Experimento 2 houve um aumento no peso testicular ($P=0,06$) no Grupo pST, porém uma diminuição do número de túbulos seminíferos e células de Sertoli neste grupo ($P<0,05$). O Experimento 3 teve o objetivo de determinar o efeito da administração de pST durante o período pré-puberdade sobre o perfil metabólico, desenvolvimento testicular e início do desenvolvimento sexual de suínos jovens. O grupo pST apresentou maiores níveis circulantes de testosterona a partir dos cinco meses de idade ($P<0,05$), o qual coincidiu com o início da atividade reprodutiva destes animais, além de apresentar maior libido ($P<0,05$). O Experimento 4 teve o objetivo de determinar o efeito do pST sobre a qualidade espermática após o estabelecimento da puberdade. Na avaliação da qualidade espermática foi observado aumento do vigor, volume espermático, da concentração espermática total e do número de doses inseminantes no grupo pST ($P<0,05$). Ainda, o grupo pST apresentou maior peso testicular aos 12 meses de idade em relação ao grupo controle ($P<0,05$). Concluindo, o uso de pST em suínos jovens apresenta efeitos positivos sobre o desenvolvimento testicular, níveis de testosterona, libido e qualidade espermática após o estabelecimento da puberdade.

Palavras-chave: GH. IGF-I. suíno. puberdade. testículo. sêmen.

Abstract

RABASSA, Viviane R. **Effect of porcine somatotropin (pST) on male pig testicular development, age to puberty and semen quality**. 2012. 63. Tese (Doutorado) - Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

The somatotropin (ST), which has its effects mediated by insulin-like growth factor I, has positive effects on gametogenesis and steroidogenesis, and can be used as an alternative treatment for infertile men. Exogenous somatotropin supplementation improves testicular development, gametogenesis and steroidogenesis, anticipating onset of puberty. However, no studies aiming to determine the mechanisms of action of this hormone on testicular function and age at puberty in healthy young boars were performed. Thus the hypothesis of this study were: 1) prepubertal male pigs receiving exogenous ST have accelerated testicular growth due to the mitogenic actions of IGF-I; 2) an increase in gametogenesis and steroidogenesis with consequent antecipation of age at puberty and 3) improvement of semen quality after the establishment of puberty. To confirm this hypothesis four experiments were performed. The Experiment 1 aimed to determine the interval between applications of porcine ST (pST), based on the determination of IGF-I. Experiment 2 aimed to measure the proliferation of testes cells in boars subjected to pST administration, as well as its effect on the metabolism. In Experiment 1, the mean concentration of serum IGF-I for pST Group was higher ($P<0.05$) than for Control Group, with differences between groups within the first three days after injection, determining the interval between injection to be used in the following experiments. In Experiment 2 there was a increase in the testicular weight ($P=0.06$) in pST Group, but a decrease in the number of seminiferous tubules and sertoli cells in this group ($P<0.05$). Experiment 3 aimed to determine the effect of pST administration during the prepubertal period on the metabolic profile, testicular development and anticipation of the sexual maturity. The pST Group had higher circulating concentration of testosterone after five months of age ($P<0.05$), which was concomitant with the onset of reproductive activity. These boars also had higher libido ($P<0.05$). The Experiment 4 aimed to determine the effect of pST on the semen quality after the establishment of puberty. It was observed an increase in vigor, volume, total sperm concentration and total number of inseminating doses ($P<0.05$). Still, the pST Group had a higher testicular weight at 12 months of age ($P<0.05$). In conclusion, the use of pST in young pigs is present positive effects on the testicular development, testosterone levels, libido and semen quality after the establishment of puberty.
an alternative to increase reproductive efficiency, increasing the semen quality and the number of inseminating doses produced from one ejaculate.

Keywords: GH. IGF-I. pig. puberty. testicle. semen.

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1. Introdução

O hormônio do crescimento (GH) ou somatotrofina (ST) é um hormônio peptídico, composto de 191 aminoácidos, o qual é produzido, estocado e secretado pela adeno-hipófise (BAUMANN, 1991). Este hormônio é amplamente utilizado na produção animal, devido ao seu efeito anabólico. O GH tem sido empregado na suinocultura como promotor de crescimento em vários países, como Estados Unidos, México e Austrália (ETHERTON et al. 1987; KLINDT et al., 1995), porém não sendo permitido o seu uso no Brasil para esta finalidade. Ainda, apresenta um efeito galactopoiético, sendo utilizado em vacas leiteiras a fim de aumentar a produção de leite e a persistência do pico da lactação, sem alterar a composição do leite (SANTOS, 2001).

Além destas aplicabilidades na produção animal, o GH tem efeito sobre a gametogênese e a esteroidogênese, sendo usado em fêmeas submetidas a protocolos de superovulação (COCHRAN et al., 1999; FOLCH et al., 2001; MOREIRA et al., 2002), atuando também sobre o desenvolvimento placentário e crescimento fetal (STERLE et al., 1995). Estudos em humanos têm sido realizados com o objetivo de determinar a influência do GH sobre a reprodução como alternativa em homens com problemas de infertilidade (OVESEN et al., 1996; MAURAS et al., 2005). Porém, em animais de produção, com adequada fertilidade, os efeitos da suplementação de GH não estão elucidados, havendo alguns estudos realizados em roedores (ARSENIJEVIC et al., 1989; SPITERI-GRECH & NIESCHLAG, 1990), cães (SJOGREN et al., 1998), garanhões (STORER et al.,

2005) e touros (SAUERWEIN et al., 2000, VIEIRA et al., 2010), que demonstram efeitos sobre o perfil hormonal e a qualidade espermática, mas não elucidam os seus mecanismos de ação.

O GH estimula a produção de fator de crescimento semelhante à insulina I (IGF-I), o qual é um peptídeo com função mitogênica, sintetizado em vários tecidos, entre eles os testículos (LACKEY et al., 2000). Muitos dos efeitos do GH sobre a reprodução são mediados pela ação do IGF-I, como por exemplo, a síntese de testosterona (LARON & KLINGER, 1998), indicando a importância de se manter níveis elevados de IGF-I, a partir da administração de GH, para que sejam obtidos efeitos sobre parâmetros reprodutivos.

Quanto ao efeito do GH sobre a idade à puberdade, em humanos com deficiência de GH endógeno, foi observado maior desenvolvimento testicular, antecipação do estabelecimento da puberdade, gametogênese e esteroidogênese (MAURAS et al., 2005). Porém, não há estudos que determinem os mecanismos de ação deste hormônio sobre a função testicular e idade à puberdade em suínos sadios, quando suplementados durante a fase de crescimento testicular. Um indicativo do efeito do GH e IGF-I sobre o desenvolvimento testicular é a capacidade do IGF-I de estimular a proliferação das células de Sertoli (ROSER, 2001), bem como, do GH atuar no desenvolvimento dos túbulos seminíferos (SWANLUND et al., 1995) em machos pré-púberes. Também, a diminuição na concentração sérica de IGF-I, em novilhos submetidos a deficiência nutricional, foi associada ao atraso da produção de testosterona previamente à puberdade (BRITO et al., 2007).

Além dos efeitos já citados do GH e IGF-I sobre o desenvolvimento testicular, o aumento nos níveis de hormônio luteinizante (LH) no período pós-natal também é responsável pela maturação e diferenciação das células de Leydig o que está

relacionado ao aumento na produção de testosterona, como observado em terneiros por BAGU et al. (2006). Neste estudo, um período de rápido crescimento testicular coincidiu com o período de aumento na concentração e afinidade de receptores para gonadotrofinas nos testículos. Esta mudança nos receptores foi capaz de manter o crescimento testicular mesmo durante o período de diminuição nos níveis séricos de LH e do hormônio folículo estimulante (FSH), o qual ocorre próximo ao início da puberdade. Ainda, através da administração de GH exógeno, é possível aumentar os níveis circulantes de LH e a concentração de seus receptores (SIROTKIN, 2005) e associado ao aumento nas concentrações de IGF-I, pode-se ter um aumento na proliferação celular em nível testicular e, consequentemente maior produção espermática. Se esse efeito for confirmado, poderia refletir na otimização do uso do reprodutor, antecipando a idade à puberdade, permitindo seu uso mais precoce. Porém, este efeito benéfico do GH sobre a função testicular parece ter diferença entre espécies, visto que em ratos (OHYAMA et al., 1999; SIROTKIN, 2005), mas não em ruminantes (FOLCH et al., 2001), foi obtido efeito sobre os níveis circulantes das gonadotrofinas, não sendo conhecido seu mecanismo de ação em suínos.

Outra importante ação do GH, sendo um hormônio metabólico, é o seu efeito sobre o perfil energético e protéico. O GH estimula a síntese protéica, aumentando a retenção de nitrogênio e fósforo para a produção de adenosina tri-fosfato (KOLB, 1984), podendo, com isto, melhorar a sua disponibilidade para o metabolismo espermático. Assim, com estas alterações metabólicas e consequente incremento nos níveis plasmáticos de glicose para a taxa metabólica basal, a função gonadal pode ser favorecida através de um melhor metabolismo espermático, efeito este que ainda não foi avaliado em suínos.

Dessa forma a hipótese deste estudo é que machos suínos pré-púberes recebendo GH exógeno tenham seu crescimento testicular acelerado, devido à ação mitogênica do IGF-I, bem como tenham um incremento na gametogênese e esteroidogênese, com consequente antecipação da idade à puberdade e melhora da qualidade espermática após o estabelecimento da puberdade.

2. Objetivos

Objetivo Geral

Avaliar o efeito da administração de GH exógeno (somatotrofina suína - pST) sobre o desenvolvimento testicular e idade à puberdade de suínos pré-púberes, bem como sobre a qualidade espermática destes machos após a puberdade.

Objetivos Específicos

- Determinar o efeito da administração do GH exógeno em machos suínos pré-púberes, sobre o desenvolvimento testicular;
- Determinar o perfil hormonal (IGF-I e testosterona), energético (glicose, colesterol e ácidos graxos não-esterificados - NEFA), protéico (uréia e albumina), mineral (fósforo) e enzimas hepáticas (AST e GGT) de suínos tratados com pST;
- Verificar o tempo para estabelecimento da puberdade dos suínos tratados com pST.
- Avaliar a expressão gênica testicular de receptores para GH (GHR), IGF-I e PCNA (proliferating cell nuclear antigen), bem como a expressão hepática de GHR e IGF-I.
- Avaliar o efeito do pST sobre parâmetros bioquímicos do plasma seminal (proteína total, frutose e colesterol).

- Avaliar o efeito do pST sobre a qualidade espermática (volume, vigor, motilidade, morfologia, concentração espermática, integridade de membrana, integridade de acrossoma e funcionalidade de mitocôndria).

3. Artigo 1

Artigo formatado de acordo com as normas da revista Reproduction in Domestic Animals (ISSN 0936-6768 impresso; 1439-0531 *on line*)

Federal University of Pelotas, Brazil

EFFECT OF PORCINE SOMATOTROPIN (pST) ON METABOLISM AND TESTICULAR CHARACTERISTICS OF PREPUBERTAL PIGS

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ABSTRACT

The aim of this study was to determine the effect of pST on the testicular characteristics and metabolism of prepubertal pigs. For this two studies were designed. Experiment 1 aimed to determine the interval between applications of pST based on the concentrations of circulating IGF-I. Experiment 2 aimed to evaluate testicular characteristics in prepubertal pigs submitted to pST administration. In Experiment 1 twelve piglets (pST n=6; Control n=6) with 30 days of age were used. The pST Group was submitted to one i.m. injection of pST and Control to one placebo injection. Blood collections were performed until d 7 post-application of pST, to determine IGF-I concentration and metabolic profile. In Experiment 2 twelve piglets (22 to 52 days of age) were used. The pST Group was submitted to pST injections every three days, and Control Group received placebo doses every three days. Blood collections were performed every 3 days until d 30 to determinate IGF-I concentration and metabolic profile. Samples of liver and testicle were collected to determine gene expression and testicular characteristics. In Experiment 1 IGF-I concentration was higher for pST Group (126.8±8.9 ng/mL) than Control Group (93.4±6.9 ng/mL) (P=0.02), nevertheless we established the 3 days window between applications as safe for the purpose of this study. In Experiment 2 the pST Group had higher body weight and testicular weight (pST: 9.7±1.0 g; Control: 7.4±0.5 g) (P=0.06), which can be explained by the increase in gene expression of GHR, IGF-I and PCNA in testes (P<0.05), but reduced number of seminiferous tubules and sertoli cells per mm^2 (P<0.05). These results indicate that pST administration increase body development and consequently testis development of prepubertal pigs, however reduces the number of seminiferous tubules and sertoli cells.

INTRODUCTION

Growth hormone (GH) or somatotropin (ST) is largely used in animal production and it is used as a growth promoter in the swine industry (KLINDT et al., 1995). GH stimulates

the production of insulin-like growth factor I (IGF-I), which is a peptide with mitogenic functions mainly synthesized in the liver, although it can be synthesized in various tissues, even in the testicles (LACKEY et al., 2000).

Exogenous GH administration has positive effects on testicular development in growth hormone-deficient humans (TATÒ et al., 1996) and its ability to increase IGF-I circulating levels are a critical factor in determining its effects in leydig cell number and steroidogenic capacity in mouse (WANG and HARDY, 2004). Despite this, the results in domestic livestock are still variable. Exogenous porcine ST (pST) treatment promoted tubular and sertoli cell maturation in prepubertal pigs (SWANLUND et al., 1995); however, it also down-regulates somatotropic function in young pigs (MATTERI et al., 1997). The variation observed in these studies suggests that more studies are still needed.

In prepubertal pigs two main phases of testicular tissue growth are observed: from birth to 30 days of age and from four months of age to close to puberty establishment (FRANÇA et al., 2000), in which the presence of GH receptors is higher in the testis (N'DIAYE et al., 2002), indicating that these periods would be a good window for exogenous GH administration. These are important periods for the manipulation of reproductive function in male pigs. Thus, due to mitogenic effect of IGF-I on testicular components (WANG and HARDY, 2004), as well as the effect of GH on the maturation of sertoli cells and seminiferous tubules (SWANLUND et al., 1995), the hypothesis of this study is that through the administration of exogenous GH during the period of high proliferation of testicular tissue, it is possible to increase the production of testicular IGF-I and consequently, increase the number of sertoli cells and seminiferous tubules, increasing the testicular volume of prepubertal pigs.

Based on these evidences, two experiments were designed. Experiment 1 aimed to determine the interval between applications of pST based on the concentrations of circulating

IGF-I. Experiment 2 aimed to evaluate metabolism and testicular characteristics in prepubertal pigs submitted to pST administration.

MATERIALS AND METHODS

All procedures performed in this experiment were approved by the Ethics Committee on Animal Experimentation of Federal University of Pelotas (CEEA 6574).

Experiment 1

Twelve piglets (Landrace x Large White), housed in collective pens, with 30 days of age were used. The animals were randomly assigned to one of two groups: pST Group (n=6) and Control Group (n=6). The pST Group was submitted to one i.m. injection of pST (Reporcin, OzBioPharm Pty Ltd), corresponding to 90 µg/kg of body weight (SWANLUND, et al., 1995) while Control Group received a placebo (sodium chloride 0.9%) dose at the same route and volume as pST group. Piglets were weighed in Day 0 and in the end of the experiment.

Blood Sampling and Metabolic Analysis

Blood collections were performed by venipuncture every 6 hours in the first 36 hours, followed by daily collections until d 7 post-application of pST. Serum was collected after centrifugation at 3000g for 15 min and frozen at - 70ºC until analysis.

IGF-I concentrations were determined using an IGF-I ELISA commercial kit (DSL-10-2800; Diagnostic Systems Laboratories Inc., Webster, USA) according to the manufacturer instructions. The intra-assay coefficient of variation was 5.64%.

The glucose, cholesterol, urea, albumin, phosphorus, aspartate amino transferase (AST) and gamma glutaryl transferase (GGT) serum concentration was determined by

colorimetric methods according to manufacturer's instructions (Labtest Diagnóstica S.A., Brazil). The coefficient of variation for all analysis was below 10%.

Experiment 2

Twelve piglets (Landrace x Large White), housed in collective pens, with 22 to 52 days of age were used. The animals were randomly assigned to one of two groups: pST Group (n=6) and Control Group (n=6). The pST Group was submitted to i.m. injection of the pST (Reporcin, OzBioPharm Pty Ltd) every three days (based on results from Experiment 1). The dose and via of pST and placebo were the same as in Experiment 1. Piglets were weighed at d 0 and then every 3 days until d 30.

The piglets had free access to the diet (Table 1) and water in both experiments.

Blood Sampling and Metabolic Analysis

Blood samples were collected every 3 days after pST application until d 30. Glucose, cholesterol, urea, albumin, phosphorus, AST, GGT (Labtest Diagnóstica S.A., Brazil), not esterified fatty acid (NEFA - Wako Diagnostics, USA) and β-hydroxy butyrate (BHBA - Ranbut, Randox Laboratories, UK) were determined by colorimetric methods. The coefficient of variation was below 10%.

Tissue collection

At d 17 and d 30 of the Experiment 2 hepatic biopsies were performed with a semi-automatic biopsy needle (Tru-cut 16G x 15 cm – Biomedical, Delebrio, Italy). First, the skin of the 6th intercostal space on the right side of the piglets was anesthetized with lidocaine 2% (Anestésico L Pearson – Laboratório Pearson Ltda, Brazil) and cut then the needle was introduced at an approximately vertical position relative to the plane of the skin and liver

sample was collected. The samples were snap frozen in liquid nitrogen and stored at -70ºC for RNA extraction.

At d 17 the piglets were submitted to testicular biopsy in the right testicle, with the same biopsy needle used in hepatic biopsies. The scrotum skin was anesthetized with lidocaine 2% (Anestésico L Pearson – Laboratório Pearson Ltda, Brazil), the needle was introduced and testicle sample was collected. At d 30 the animals were submitted to orchiectomy for testicular weight and collection of tissue sample from the left testicle. Sedation was realized with 4 mL/20 kg of azaperone (Stresnil, Janssen Animal Healthy, Belgium) by i.m. injection. The scrotum was anesthetized with lidocaine 2% (Anestésico L Pearson – Laboratório Pearson Ltda, Brazil) and two scrotal incisions were performed to testicles removal. The left testicle was separated from the epididymis, weighed, and longitudinally cut. Tissue samples were obtained from testicular parenchyma. One sample was stored in the fixative solution (formalin 10% buffered) for immunohistochemistry analysis. The other sample was snap frozen in liquid nitrogen and stored at -70ºC for RNA extraction.

Real Time RT-qPCR analysis

Total RNA was extracted using Trizol (Invitrogen®, Carlsbad, USA) according to manufacturer's instructions. Additional purification was performed according to the Qiagen RNeasy manual (RNeasy Mini Kit, Qyagen, Valencia, CA, USA). The RNA was reconstituted in 30 µL of nuclease-free water and the concentration was determined spectrophotometrically at 260 and 280 nm. Integrity of the extracted RNA was determined by staining the samples of total RNA by electrophoresis on a 1% agarose gel. Only RNA with intact 18S and 28S bands was used. Total RNA was used for cDNA synthesis using SuperScript III First-Strand Synthesis Supermix (Invitrogen®, Carlsbad, USA). The

quantitative real-time PCR using the cDNA obtained in the previous step, was performed with the Stratagene MX3005P Real Time PCR machine (Agilent Technologies UK Ltd, Stockport, UK), using the SYBR Green detection chemistry (Platinum SYBR Green qPCR SuperMix-UDG kit, Invitrogen®, Carlsbad, USA) as recommended by the manufacturer. The PCR parameters were 5 min at 50° C and 10 min at 95° C followed by 40 cycles of 95° C for 30 sec, 58° C and 75° C for 1 min each. Specific primers for GH receptor (GHR), IGF-I, proliferating cell nuclear antigen (PCNA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; which was used as a control gene) were used. The primer sequences were as follows: GHR (For TGG TGG GAC TGT GGA TCA AA; Rev GGT TGC ACT ATT TCC TCA ACG G) (Gen Bank accession NM 214254.2), IGF-I (For CTT CAG TTC GTG TGC GGA GA; Rev CCC TGT GGG CTT GTT GAA AT) (Gen Bank accession NM 214256.1), PCNA (For ATA ATG CAG ACA CCT TGG CAC TAG; Rev CCT TTT CCT GAT TTG GAG CTT C) (Gen Bank accession XM 003359889.1) and GAPDH (For GTT TGT GAT GGG CGT GAA C; Rev ATG GAC CGT GGT CAT GAG T) (WILLING and VAN KESSEL, 2007). The PCR reaction efficiency and cycle thresholds from the fluorescence readings of individual wells during the reaction were analysed using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen (2001) and the handbook of *Chemistry guide of applied biosystems.*

Immunohistochemistry

The cells marking were performed by immunohistochemistry using monoclonal antibody vimentin for quantification of sertoli cells and seminiferous tubules and to measure the diameter of the tubules. Sections of 5 μm of paraffin-embedded tissues were deparaffinised by immersion in xylene and rehydrated with graded alcohols. After deparaffinization, tissue sections were quenched with 3% hydrogen peroxide and rinsed in water. The antigenic sites were exposed by microwaving in a citrate buffer solution (pH 6,0)

for 10 minutes (4′-4′-3′ intervals), followed by blocking in skim milk solution (5%) for 20 minutes. Primary antibody (Monoclonal Mouse Anti-Vimentin Clone V9 Dako, Carpenteria, CA) at a 1:100 dilution was incubated overnight at 4°C followed by two 20-minute room temperature incubations with biotinylated link antibody (LSAB2 kit) and horseradish peroxidase–conjugated streptavidin (LSAB2 kit). The reaction was revealed in diaminobenzidine peroxidase substrate (Dako, Carpenteria, CA) for no longer than 5 minutes. The slides were counterstained with Mayer's hematoxylin and coverslipped with Permount. The quantification of structures was performed using the ImageJ 1.44 software (National Institutes of Health, Bethesda, EUA).

Statistical Analysis

Data was analyzed separately for each experiment. Statistical analysis was conducted using the Statistical Analysis System (SAS Institute Inc. Cary, NC, USA). Analyses of variance for repeated measures were used to examine the effects of the pST on metabolic profile (levels of glucose, cholesterol, albumin, urea, phosphorus, AST, GGT, BHBA, NEFA and IGF-I) and body weight gain, with a Tukey test adjustment. The testicular morphological and functional characteristics (concentration of sertoli cells and seminiferous tubules and diameter of tubules) were evaluated by Fatorial ANOVA. Testicular (GHR, IGF-I and PCNA) and hepatic gene expression (GHR and IGF-I) were evaluated by *t* test.

RESULTS

Experiment 1

IGF-I concentration was higher for pST Group (126.8±8.9 ng/mL) than Control Group (93.4±6.9 ng/mL) (P=0.02). There was also an effect for the group x collection interaction (P=0.01) for IGF-I concentration (Figure 1).

The concentration of cholesterol (pST: 47.5±1.5 mg/dL; Control: 48.8±1.6 mg/dL), urea (pST: 14.6±0.8 mg/dL; Control: 14.3±0.9 mg/dL), albumin (pST: 2.7±0.1 g/dL; Control: 2.7±0.1 g/dL), AST (pST: 37.1±1.5 UI/L; Control: 38.7±1.6 UI/L), GGT (pST: 81.8±1.5 UI/L; Control: 77.5±1.6 UI/L) and phosphorous (pST: 7.9±0.1 mg/dL; Control: 7.7±0.1 mg/dL) were not different between groups (P>0.05).

Experiment 2

The pST Group had higher body weight (Figure 2) in the end of the experiment compared to Control Group (P=0.06). pST Group also had higher testicular weight (pST: 9.7±1.0 g; Control: 7.3±0.5 g; P=0.06). It was observed a reduced number of sertoli cells (P=0.0004) and seminiferous tubules (P=0.002) in the pST Group (Table 2). The diameter of the seminiferous tubules was not influenced by the use of pST.

With regard to metabolic parameters, between-groups difference was observed only for serum GGT, which was higher in the Control Group (pST: 51.6±3.6 UI/L; Control: 67.7±3.9 UI/L) (P=0.005). The levels of NEFA (pST: 0.5±0.1 mmol/L; Control: 0.5±0.1 mmol/L), BHBA (pST: 0.5±0.6 mmol/L; Control: 1.0±0.6 mmol/L), glucose (pST: 85.0±1.7 mg/dL; Control: 86.4±1.8 mg/dL), cholesterol (pST: 73.6±2.0 mg/dL; Control: 71.5±2.1 mg/dL), urea (pST: 34.3±1.4 mg/dL; Control: 33.5±1.6 mg/dL), albumin (pST: 3.1±0.1 g/dL; Control: 3.0±0.1 g/dL), AST (pST: 31.5±0.9 UI/L; Control: 32.9±0.9 UI/L) and phosphorous (pST: 9.3±0.1 mg/dL; Control: 9.3±0.1 mg/dL) were not different between groups (P>0.05).

The expression of GHR, IGF-I and PCNA in testicles were increased in pST Group at d17 (P<0.05), however were not different at d30 (P>0.05) (Figure 3). The pST Group had higher IGF-I expression in liver than Control Group (3.9±1.5 vs. 1.0±0.2; P=0.10). GHR expression in liver was not different between groups (Control: 1.0±0.1; pST: 1.7±0.5; P=0.26).

DISCUSSION

The commercial injectable pST for pigs has a short half-life. The recommended schedule for application is one injection every 24 hours when used as growth promoter. However, when used for other purposes, such as for manipulating reproductive functions, their injection can be performed at longer intervals, which is dependent of the time that it remains stimulating IGF-I production. The IGF-I concentration remained high for at least 3 days after pST injection in this study. The abrupt reduction in serum IGF-I on the fifth day (Figure 1) is probably due to a change in the interval of feeding, which was higher than normal, leading to a stress condition. During stress, there is a decrease in the expression of IGF-I in liver, muscle and adipose tissue, triggering a fall in serum levels (BRAMELD et al., 1996). Nevertheless we established the 3 days window between applications as safe for the purpose of this study.

The increased body and testicular weight in Experiment 2 indicates that pST administration was effective in increasing body development of prepubertal pigs, and consequently testis development. This effect may be explained by the increase in gene expression of GHR, IGF-I and PCNA in testes, which is indicative of tissue proliferation (WILLING & VAN KESSEL, 2007). Although this hormone has reduced the number of seminiferous tubules and sertoli cells per mm^2, testicular volume was higher. The consequence this effect on semen production only can be verified in other experiments, in which pigs are evaluated until the establishment of puberty. Still, these results were contrary to the obtained by Roser (2001), in which IGF-I was able to stimulate proliferation of sertoli cells. On the other hand, the results observed for the diameter of the seminiferous tubules was similar to the obtained by Swanlund et al., (1995), which demonstrated that pST was not able to increase the diameter, but anticipate the maturation of the seminiferous tubules in prepubertal pigs.

Moreover, based on the results of metabolic profiling, it was determined that the dose of 90 µg/kg of pST can be used without changes in the metabolic balance of prepubertal pigs, avoiding the liver overload which could be generated if the diabetogenic effect of pST was exacerbated (EVOCK-CLOVER et al., 1992). These results were similar to observed by Klindt et al. (2007), which demonstrated that prepubertal animals treated with pST show less change in metabolic and hormonal parameters, when compared to pubertal or adult pigs undergoing the same treatment.

Thus, these results indicate that pST administration increase body development and consequently testis development, without changing the metabolic balance of prepubertal pigs. However, this hormone reduces the number of seminiferous tubules and sertoli cells, further studies should focus on determine its effect on other testicular structures.

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Table 1. Composition and analysis of the diet utilized during the experimental period.

Composition	Diets		
	Preinitial[a]	Initial 1[b]	Initial 2[c]
Ingredients			
Sugar (%)	2.0	2.0	3.0
Corn (%)	43.6	50.9	61.6
Soybean meal (%)	24.4	27.0	32.4
Colistin sulphate (%)	0	0.05	0
Vitamin-mineral premix (%)	30.0	20.0	3.0
Nutritional values			
Crude protein (%)	19.6	20.2	19.0
Metabolizable energy (MCal/kg)	3.3	3.3	3.2
Crude fiber (%)	2.9	3.2	3.2
Ether extrate (%)	2.5	2.6	2.6
Mineral matter (%)	5.5	4.5	4.8
Calcium (%)	0.8	0.7	0.6
Phosphorus (%)	0.6	0.5	0.6
Lactose (%)	8.1	5.0	0
Lysine (%)	1.5	1.4	1.2

[a]Diet fed the piglets from 22 to 30 days of age; [b]Diet fed the piglets from 31 to 42 days of age; [c]Diet fed the piglets from 43 to 52 days of age.

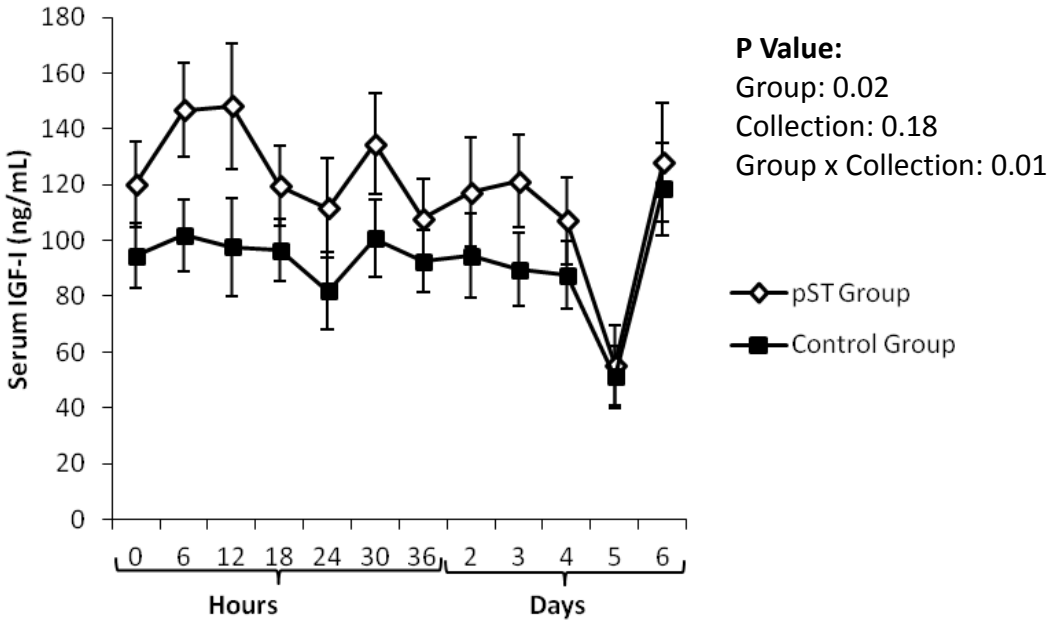

Figure 1. Serum IGF-I in prepubertal male pigs submitted to one dose of porcine somatotropin (pST). pST was injected at hour 0 in pST Group.

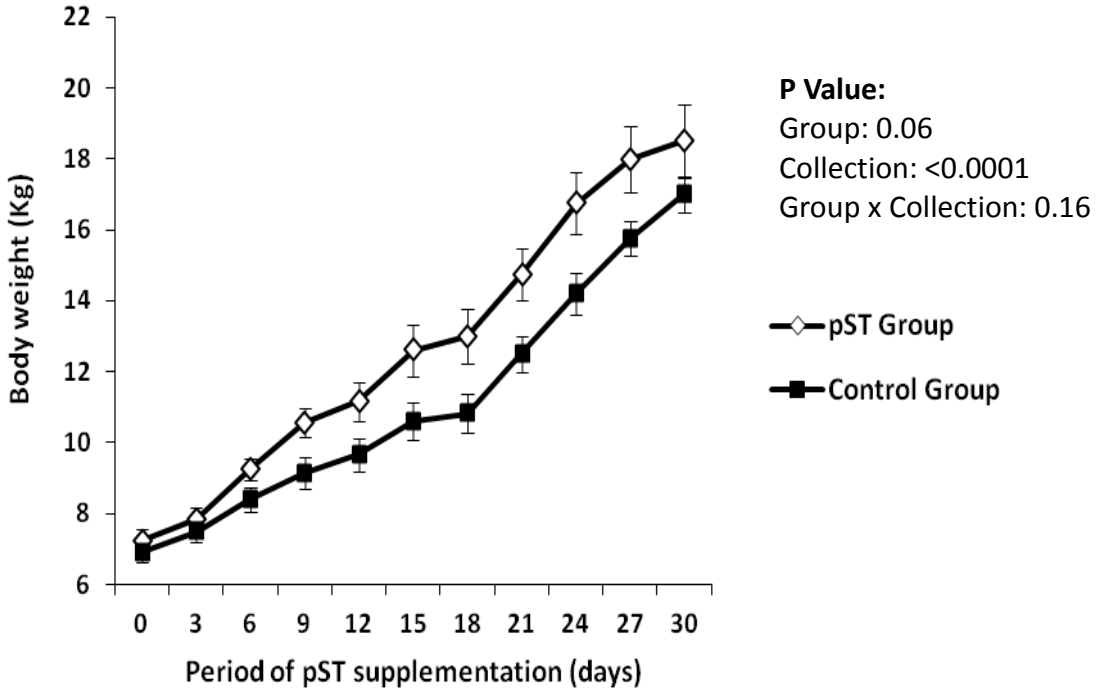

Figure 2. Body weight of male pigs submitted to porcine somatotropin (pST) administration during prepubertal period.

Table 2. Testicular morphological and functional characteristics after 30 days of porcine somatotropin (pST) administration during the prepubertal period of pigs.

Parameter	pST (± SEM)	Control (± SEM)	P value
Sertoli cells, n/mm^2	209.5 (12.3)^a	372.0 (41.6)^b	0.0004
Seminiferous tubules, n/mm^2	28.7 (1.5)^a	35.1 (1.3)^b	0.002
Seminiferous tubules diameter, µm	202.8 (1.8)	199.1 (1.8)	0.15

Different superscripts within the same row indicate significant differences ($P<0.05$).

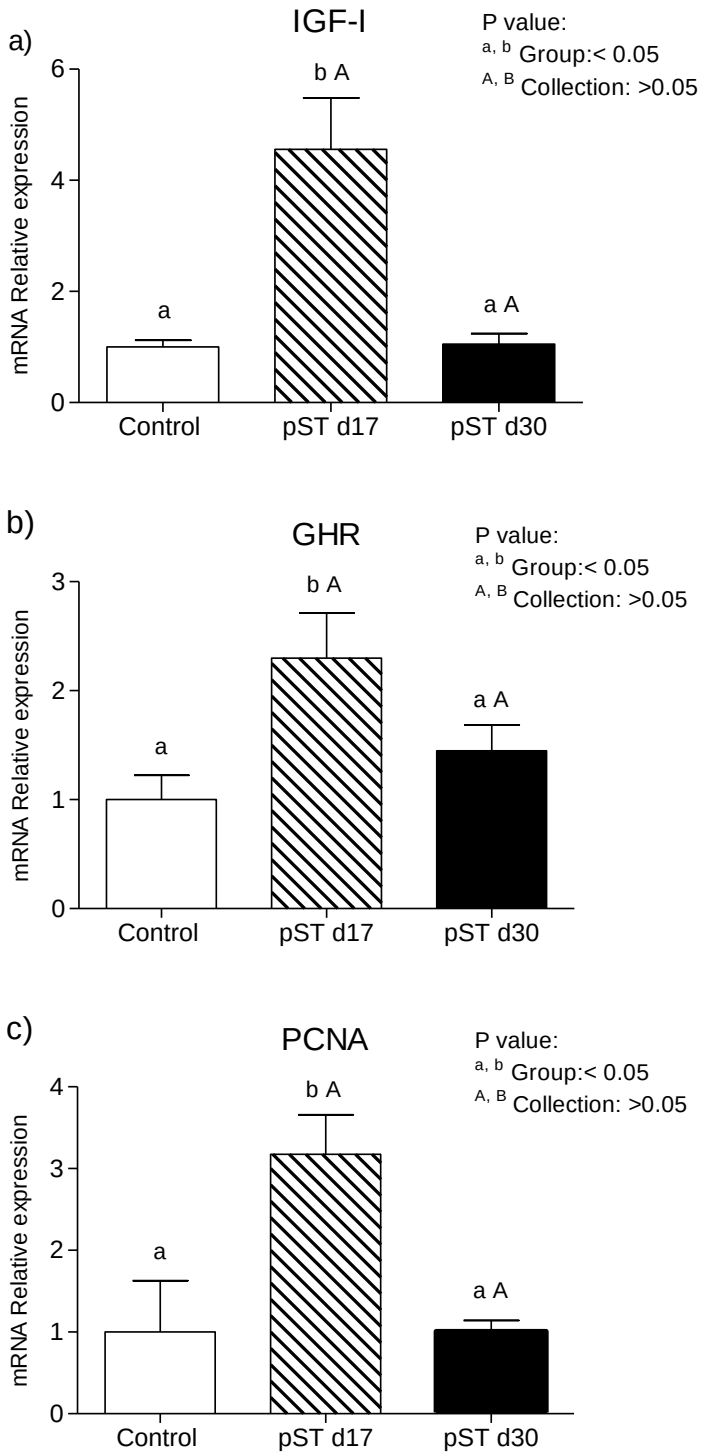

Figure 3. Gene expression of growth hormone receptor (GHR), IGF-I and proliferating cell nuclear antigen (PCNA) in testicle of pigs after 30 days of porcine somatotropin (pST) administration during the prepubertal period.

4. Artigo 2

Artigo formatado de acordo com as normas da revista Reproduction (ISSN 1470-1626 impresso; 1741-7899 *on line*)

EFFECT OF PORCINE SOMATOTROPIN (pST) ON METABOLISM, TESTICULAR DEVELOPMENT AND ANTECIPATION OF SEXUAL MATURITY IN YOUNG BOARS

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ABSTRACT

The aim of this study was to determine the effect of pST injection on blood metabolites, testicular development and early sexual maturity in young boars. Sixty piglets Landrace x Large White, at 22 days of age, were assigned to one of two groups: pST Group (n=30) and Control Group (n=30). The pST Group was submitted to pST injections (90 µg/kg BW) every three days until 210 days of age. Control Group received placebo in the same way as pST Group. Blood collections were performed for evaluation of testosterone, glucose, cholesterol, urea, albumin, phosphorous, AST and GGT. At d 0, 60, 120 and 180 of experiment 6 boars from each group were submitted to orchiectomy to verify testicular development and weight. The evaluation of libido and semen characteristics (volume, motility and vigor) began at 150 days of age. Testosterone concentration was higher in pST Group (2.3±0.2) than Control Group (1.0±0.2), as well as libido score. Average time of interest in the artificial sow was higher for pST treated boars (88.0±6.4 sec) than control boars (63.0±3.9 sec). There was no difference between groups for testicular weight (P=0.59), but pST increase the number of seminiferous tubules per mm^2. In conclusion, the pST injection increase the testosterone concentration and anticipate sexual maturity in prepubertal pigs, without changing the metabolic balance. In testicle, pST present effect on number of seminiferous tubules per mm^2, but had no effect on testicular weight and semen quality.

INTRODUCTION

Exogenous growth hormone (GH) supplementation is positive associated with improved testicular development, early onset of puberty, gametogenesis and steroidogenesis (MAURAS et al., 2005). It has been demonstrated that GH replacement therapy can anticipate age at puberty in humans with endogenous GH deficiency (KAMP et al., 2002). However, there are no studies that aimed to determine the effect of this hormone on testicular function and sexual maturity in healthy pigs supplemented during the prepubertal period.

Most of the positive actions of GH are mediated by IGF-I (SIROTKIN, 2005). IGF-I has the ability to stimulate the proliferation of sertoli cells (ROSER, 2001), as well as to stimulate the development of the seminiferous tubules in prepubertal males (SWANLUND et al., 1995). In addition, the reduced serum IGF-I concentration in steers subjected to poor nutrition is associated with a delayed testosterone production prior to puberty (BRITO et al., 2007). The administration of

exogenous GH can increase circulating concentration of luteinizing hormone (LH) and its receptors in target tissues (CHATELAIN et al., 1991; SIROTKIN, 2005). Increased LH secretion in the postnatal period is responsible for the maturation and differentiation of leydig cells and increased testosterone production (BAGU et al., 2006). Therefore, the combined effects of GH on LH and IGF-I secretion can increase testicular cell proliferation and consequently sperm production. If confirmed it could reduce the necessity of males by anticipating the age at puberty and increased sperm production. However, species specific effects of GH on testicular function appears to exist. For example, GH is positive associated with circulating concentrations of gonadotropins in rats (OHYAMA et al., 1999; SIROTKIN, 2005) but not in ruminants (FOLCH et al., 2001), and still to be determined its effect on the pig gonadotropin concentration.

Based on these evidences, the aim this study was to determine the effect of pST injection on blood metabolites, testicular development and anticipation of sexual maturity in young boars.

MATERIALS AND METHOD

Use of animals was approved by the Ethics Committee on Animal Experimentation of Federal University of Pelotas (CEEA 6574).

Animals and Experimental Groups

Sixty piglets (Landrace x Large White) with 22 days of age were used and randomly assigned to one of two groups: pST Group (n=30) and Control Group (n=30). The pST Group was submitted to i.m. injections of 90 µg/kg of body weight (BW) of pST (Reporcin, OzBioPharm Pty Ltd) every three days until 210 days of age. Control Group received i.m. placebo injections (sodium chloride 0.9%) at the same frequency. The pigs were weighed weekly until 210 days of age.

Blood Sampling and Analysis

Blood collections were performed by venipuncture of the jugular vein every 7 days until 210 days of age. Serum was collected after centrifugation at 3000g for 15 min and frozen at - 80ºC until analysis.

Testosterone (Testosterone, Human GmBh, Germany) concentration was determined using commercial ELISA kits according to the manufacturer instructions, with intra-assay coefficient of variation (CV) of 10.65%. Inter-assay CV was 7.26%.

The glucose, cholesterol, urea, albumin, aspartate amino transferase (AST) and gamma glutaryl transferase (GGT) serum concentration was determined by colorimetric methods according to manufacturer's instructions (Labtest Diagnóstica S.A., Brazil). The CV for all analysis was below 10%.

Testicular tissue collection

At 0, 60, 120 and 180 days of experimental period 6 animals of each group were submitted to orchiectomy. Animals were sedated by i.m. injection of 4 mL/20 kg of BW of 40% azaperone (Stresnil, Janssen Animal Healthy, Belgium). The scrotum was anesthetized with 2% lidocaine (Anestésico L Pearson – Laboratório Pearson Ltda, Brazil) and two scrotal incisions were performed for testicles removal. The left testicle was separated from the epididymis, weighed, and longitudinally cut. Tissue samples were obtained from the testicular parenchyma and stored in a fixative solution (10% buffered formalin) for immunohistochemistry analysis.

Immunohistochemistry

Immunohistochemistry was performed to quantify sertoli cells and seminiferous tubules and to measure the diameter of the tubules. Sections of 5 µm of paraffin-embedded tissues were deparaffinised by immersion in xylene and rehydrated with graded alcohols. After rehydratation, tissue sections were quenched with 3% hydrogen peroxide and rinsed in water. The antigenic sites were exposed by microwaving in a citrate buffer solution (pH 6,0) for 10 minutes (4′-4′-3′ intervals), followed by blocking in skim milk solution (5%) for 20 minutes. Primary antibody (Monoclonal Mouse Anti-Vimentin Clone V9 Dako, Carpenteria, CA) at a 1:100 dilution was incubated overnight at 4°C followed by two 20-minute room temperature incubations with biotinylated link antibody (LSAB2 kit) and horseradish peroxidase–conjugated streptavidin (LSAB2 kit). The reaction was revealed in diaminobenzidine peroxidase substrate (Dako, Carpenteria, CA) for no longer than 5 minutes. The slides were counterstained with Mayer's hematoxylin and coverslipped with Permount. The quantification of structures was performed using the software ImageJ 1.44 (National Institutes of Health, Bethesda, EUA).

Libido and semen evaluation

The evaluation of libido and semen characteristics began after boars were adapted to the semen collection management, which began at 150 days of age. Boars were moved individually three times per week to a semen collection room equipped with an artificial sow. Each individual training session lasted at most 10 min. During each training session, boars were classified for their libido in an

1 – 4 scale, similar the described by Kozink et al. (2002): 1 – boars showed no interest in artificial sow; 2 – slight interest in artificial sow but did not attempt to mount; 3 – mounted the artificial sow but did not allow semen collection; 4 – mounted the artificial sow and allowed semen collection. The duration of the interest in the artificial sow interest was recorded in seconds. The number of attempts needed for the first mount and for first ejaculation were also evaluated.

Whole ejaculates were collected using the gloved-hand technique. Semen (without the gelatinous fraction) was evaluated for appearance (samples with urine or blood were discarded), volume (mL), motility (%) and vigor (0-5 score).

Statistical Analysis

Statistical analysis was conducted using the Statistical Analysis System (SAS Institute Inc. Cary, NC, USA). Analysis of variance for repeated measures was used to examine the effects of the pST on testicular parameters (weight, quantification of sertoli cells and seminiferous tubules and diameter of seminiferous tubules), metabolic profile (testosterone, glucose, cholesterol, urea, albumin, AST and GGT), body weight gain and time of artificial sow interest with a Tukey test adjustment. The number of attempts needed for first mount and number of attempts needed for first ejaculation were evaluated by one-way ANOVA. The libido score was evaluated by Qui-squared test.

RESULTS

The testosterone concentration was higher in the pST Group, with a group x collection effect after 150 days of age (Figure 1). Regarding metabolic parameters, serum albumin had greater levels in the pST Group (pST: 3.5±0.1 g/dL; Control: 3.3±0.1 g/dL; P=0.09). The levels of glucose (pST: 91.7±1.3 mg/dL; Control: 90.2±1.2 mg/dL), cholesterol (pST: 73.0±1.7 mg/dL; Control: 74.1±2.1 mg/dL), urea (pST: 30.8±0.7 mg/dL; Control: 32.9±0.7 mg/dL), AST (pST: 21.7±1.1 UI/L; Control: 21.9±1.0 UI/L) and GGT (pST: 115.2±7.5 UI/L; Control: 126.9±8.8 UI/L) were not different between groups (P>0.05).

pST Group had a greater occurrence of mount with semen collection compared to Control Group (score 4 of libido; P=0.003) (Figure 2). Average time of interest in the artificial sow was higher for pST treated boars (87.9±6.4 sec) than Control Group (63.0±3.9 sec) (P=0.0007). The number of attempts needed for the first mount was of 6.3±1.4 for pST Group and 6.2 ±1.0 for Control Group (P=0.98), and the number of attempts needed for first ejaculation was of 7.9±0.8 for pST Group and 6.4±1.3 for Control Group (P=0.32).

There was higher body weight in the pST Group (pST: 67.1±1.0 kg; Control: 64.2±1.0 kg; P=0.07). However, there was no difference between groups for testicular weight (pST Group: 125.2±34.8 g; Control Group: 120.6±31.5 g; P=0.59). Higher number of seminiferous tubules was observed in pST Group (P=<0.0001), however Control Group has greater number of sertoli cells (P=0.0024) and higher diameter of seminiferous tubules (P=0.0001) (Table 1).

Spermatic motility (pST: 58.4±5.8 %; Control: 48.0±8.1 %; P=0.78), vigor (pST: 2.1±0.2; Control: 2.0±0.3; P=0.77) and volume (pST: 25.6±9.6 mL; Control: 10.5±12.6 mL; P=0.35) were not different between groups.

DISCUSSION

Testicular steroidogenic activity is influenced by the physiologic development, as well as the endocrine status in testes (SINCLAIR et al., 2001) and it is associated to sexual maturity. Based on the testosterone concentration results, sexual maturity occurred about 12 weeks before in pST Group (Figure 1), as indicated by the time of the first testosterone peak (KUMARESAN et al., 2011). This anticipation of sexual maturity is supported by the results observed in the sexual behavior of the males, since boars showed more interest in the artificial sow and more mounts with ejaculation (Figure 2). The effect on testosterone concentration probably is due to an increase in expression of LH receptors in leydig cells, which is influenced by GH and IGF-I (pig: PERRARD-SAPORI et al., 1987; mice: CHATELAIN et al., 1991).

Despite that, differently of El-Gohary et al. (2011) that observed increased sperm motility and ejaculate volume in rams treated with rbST during the establishment of puberty, in this study there was no positive effect on semen quality. Still, our results agree with the absence of effect on testicular size. A positive factor in our study, despite the lack of effect on testicular weight, was the increased number of seminiferous tubules, which probably compensate the decreasing in its diameter and number of sertoli cells, since there diameter of the seminiferous tubules is positively correlated with testicular size (FORD & WISE, 2011).

In conclusion, the pST injection increase the testosterone concentration and anticipate sexual maturity in prepubertal pigs, without changing the metabolic balance. In testicle, pST had effect on number of seminiferous tubules per mm^2, but no effect was observed on testicular weight and semen quality.

FUNDING

This work was supported by CNPq (grant number 478385/2009-9) and FAPERGS (grant number 0904231).

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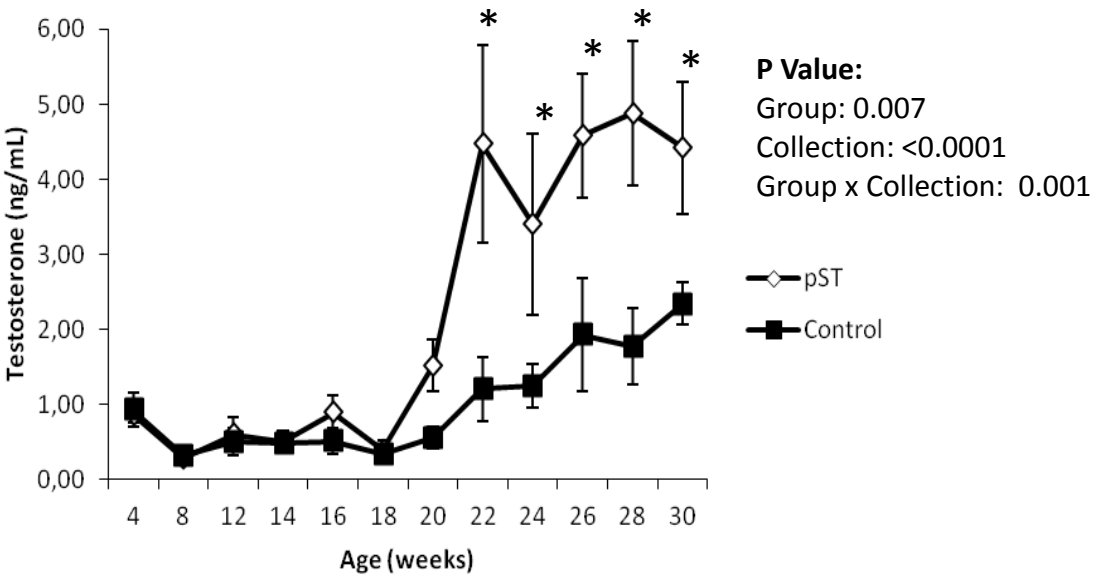

Figure 1. Testosterone concentration (ng/mL; mean ± SEM) of male pigs submitted to administration of porcine somatotropin (pST).

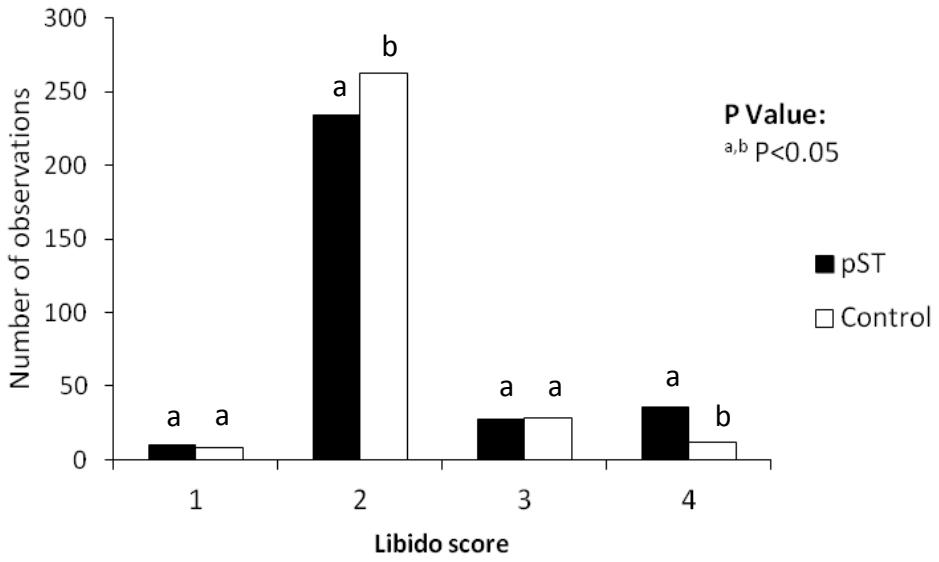

Figure 2. Effect of porcine somatotropin (pST) on libido score of the young boars from 150 to 210 days of age. Libido score: 1 – boars showed no interest in artificial sow; 2 – slight interest in artificial sow but did not attempt to mount; 3 – mounted the artificial sow but did not allow semen collection; 4 – mounted the artificial sow and allowed semen collection.

Table 1. Testicular characteristics of prepubertal pigs from 22 to 210 days of age submitted to somatotropin (pST) injection.

Parameter	pST (± SEM)	Control (± SEM)	P value		
			Group	Collection	Collection x Group
Testicular weight	125.2 (34.8)	120.6 (31.5)	0.59	<0.0001	0.24
Sertoli cells, n/mm^2	1254.1 (35.8)^a	1368.5 (44.2)^b	0.0024	<0.0001	0.03
Seminiferous tubules, n/mm^2	45.8 (2.2)^a	39.0 (1.7)^b	<0.0001	<0.0001	0.04
Seminiferous tubules diameter, µm	250.3 (7.5)^a	269.0 (7.6)^b	0.0001	<0.0001	<0.0001

Different superscripts within the same row indicate significant differences (P<0.05).

5. Artigo 3

Artigo formatado de acordo com as normas da revista Reproduction (ISSN 1470-1626 impresso; 1741-7899 *on line*)

EFFECT OF PORCINE SOMATOTROPIN (pST) INJECTION ON BLOOD METABOLITES, TESTICULAR SIZE AND SPERM CHARACTERISTICS OF YOUNG PIGS

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ABSTRACT

The aim of this study was to determine the effect of pST injection on blood metabolites, testicular size, sperm characteristics and biochemical constituents in seminal plasma of young pigs. Twenty four pigs at 22 days of age were assigned to one of two groups: pST Group (n=12) and Control Group (n=12). The pST Group was submitted to pST injections (90 µg/kg BW) every three days until 330 days of age. Blood collections were performed weekly to evaluation of IGF-I, testosterone, glucose, cholesterol, urea, albumin, phosphorous, AST, GGT and non-esterified fatty acids concentration. Semen collection was performed for evaluation of the volume, pH, motility, vigor and concentration. Semen was also evaluated during the 72 hs of storage (15ºC) for vigor, morphology, plasma and acrosomal membrane integrity and mitochondrial function. Cholesterol, total protein and fructose concentration were also determined in seminal plasma. Testicular weight was evaluated at 365 days of age. There was an effect of pST treatment on spermatic vigor in fresh semen (3.0 vs 2.6, P=0.02). The pST Group had lower sperm concentration (346.5 vs. 608.4, P=0.001), but had higher volume (174.5 vs. 40.1, P=< 0.0001), total sperm count (63.1 vs. 22.3, P=0.047) and number of inseminating doses (20.9 vs. 7.4, P=0.047). During 72 hs storage, the pST Group had lower number of morphological alterations (P=<0.0001). The pST Group had greater testicular weight than the Control Group (479.0 vs. 381.5, P=0.0005). Thus, we conclude that the pST increases the reproductive efficiency of young boars, by increasing the sperm quality.

INTRODUCTION

Growth hormone (GH) treatment has been widely used in human reproduction as an alternative for treatment of men infertility (OVESEN et al., 1996; MAURAS et al., 2005). However, in animals or humans with normal reproductive function the effects of GH supplementation are not clear, with some studies in rodents (ARSENIJEVIC et al., 1989; SPITERI-GRECH & NIESCHLAG, 1990), horses (STORER et al., 2005) and bulls (HAFEZ et al., 2005, VIEIRA et al., 2010) showing positive effects on the hormonal profile and sperm quality. While others show adverse effect on testicular development (dogs: SJOGREN et al., 1998; men: BERTELLONI et al., 1999).

Insulin-like growth factor I (IGF-I) has its production stimulated by GH mainly in liver (JONES & CLEMMONS, 1995). IGF-I is a mitotic factor and can improve testicular development and size (SWANLUND et al., 1995). Increased testicular size can be associated to higher sperm production (BORG et al., 1993). Thus injecting exogenous GH in farm animals could bring benefits such as increased number of inseminating doses produced from each ejaculate, decreasing male:female ratio in commercial herds (ROCA et al., 2011). In addition, the presence of IGF-I was detected in seminal plasma, thus the effect of local IGF-I production should not be disregarded as demonstrated before that it is positive associated to sperm motility (HENRICKS et al., 1998).

GH is a major metabolic hormone and has other effects in protein and energy metabolism. GH stimulates protein synthesis, increasing the retention of nitrogen and phosphorus for the production of ATP (TRYFONIDOU & HAZEWINKEL, 2004). Since ATP is essential for sperm motility (LAMIRANDE & GAGNON, 1992), exogenous GH injection can have a positive effect in this variable. So with these metabolic changes, spermatic function can be favored by better seminal metabolism (SAUERWEIN et al., 2000), as demonstrated in bulls, in that exogenous bovine somatotropin injection increased fructose concentration in seminal plasma (HAFEZ et al., 2005).

Based on these evidences, the aim of this study was to determine the effect of pST injection on blood metabolites, testicular size, sperm characteristics and biochemical constituents of seminal plasma from young pigs.

MATERIALS AND METHODS

All procedures performed in this experiment were approved by the Ethics Committee on Animal Experimentation of Federal University of Pelotas (CEEA 6574).

Animals and Experimental Groups

Twenty four pigs (Landrace x Large White) with 22 days of age were used. The animals were randomly assigned to one of two groups: pST Group (n=12) and Control Group (n=12). The pST Group was submitted to i.m. injections of 90 µg/kg of body weight of pST (Reporcin, OzBioPharm Pty Ltd, Australia) every three days (SWANLUND, et al., 1995).Control Group received i.m. placebo

injections (sodium chloride 0.9%) in the same way as pST group. Hormone applications were performed from 22 to 330 days of age. Pigs were weighed weekly from birth to 365 days of age (end of the experiment).

Blood Sampling and Metabolic Analysis

Blood collections were performed by venipuncture of the jugular vein weekly during the period of hormone injection and continued for five weeks after the last injection. Serum was collected after centrifugation at 3000g for 15 min and frozen at - 80ºC until analysis. Testosterone concentration (Testosterone, Human GmBh, Germany) was performed by ELISA, with an intra-assay coefficient of variation (CV) of 10.65%, respectively; and inter-assay CV of 7.26%. Glucose, cholesterol, urea, albumin, phosphorous, AST, GGT (Labtest Diagnóstica S.A., Brazil) and non esterified fatty acids (NEFA - Wako Diagnostics, USA) were evaluated by colorimetric method. The CV for these assays was below 10%.

Semen collection and evaluation

Semen evaluation started when pigs were at 210 days of age and after adaptation to the semen collection management. Whole ejaculates (without the gelatinous fraction) were collected weekly, using the gloved-hand technique, starting at 210 days of age and continued for another 35 days after the last pST injection, corresponding to the period of spermatogenesis in swine (SWIERSTRA, 1968). After initial evaluation of appearance (samples with urine or blood were discarded), volume (mL), pH, motility (%) and vigor (0-5 score) were evaluated. After that the semen was diluted 1:1 with BTS extender (Beltsville Thawing Solution, BTS; Minitüb, Tiefenbach, Germany). The spermatic concentration was performed in a Neubauer chamber to determine concentration ($x10^6$/mL), total count ($x10^9$/ejaculate) and the semen volume required to obtain a dose with three billion of spermatozoa. The ejaculate was brought to a final volume of 100 mL again with BTS. The semen doses were then maintained at room temperature and low light for 2 hours and stored at 15-18ºC in a refrigerator for 72 hs.

During the 72 hs of storage semen doses were evaluated every 24 hours (0, 24, 48 and 72 hours) for motility, vigor and morphology (%). For this assessment a mixture of semen and 3% formol-

citrate was made and evaluated under a phase contrast microscope (1000x). The tail abnormalities, isolated head, presence of proximal and distal cytoplasmatic drops and total amount of morphologically normal cells were calculated. The integrity of plasma and acrosomal membrane and mitochondrial function was also evaluated by fluorescents probes. Sperm membrane integrity was evaluated using the markers carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) according to the technique described by Harrison & Vickers (1990). To evaluate acrosomal integrity Lectin from *Arachis hypogaea* FITC Conjugate and PI were used as fluorescence markers (KAWAMOTO et al., 1996). Mitochondrial function was evaluated by the method decribed by Fraser et al. (2002) using PI and rhodamine 123 (R123). The evaluations were performed under an epifluorescent microscope (Olympus BX 51, America Inc., Sapporo, Japan) at 400x magnification (filter wave length of 525 nm). Two hundred sperm were counted in each slide and the membranes (acrosomal and plasmatic) were classified as intact or damaged. All evaluations were performed by the same technician.

Seminal plasma assays

Ejaculates aliquots were separated immediately after collection and centrifuged at 3000 x g for 15 min at room temperature to separate spermatozoa and seminal plasma. Concentrations of cholesterol, total protein (Labtest Diagnóstica S.A., Brazil) and fructose were evaluated by colorimetric methods. For fructose evaluation 500 µL of seminal plasma was diluted in 950 µL of distilled water. After the sample was deproteinized by the addition of 2% zinc sulphate and 0.4% sodium hydroxide and centrifugation at 2000g for 5 min. The supernatant was then removed and 1 mL of 0.1% resorcinol and 3 mL of 30% HCl were added and the solution was heated for 20 min at 85ºC. This final solution was read at 450 nm in a spectrophotometer.

Testicular weight

To determine the testicular weight 12 pigs (six animals from each group) were orchiectomyed at 210 days of age and of six pigs (three animals from each group) at the end of the experiment (365 days of age) to determine testicular weight. The sedation was performed with 4 mL/20 kg of azaperone 40% (Stresnil, Janssen Animal Healthy, Belgium) by i.m. injection. The scrotum was anesthetized with lidocaine 2% (Anestésico L Pearson – Laboratório Pearson Ltda, Brazil). Two

scrotal incisions were made and the testicles removed, separated from the epididymis and the left testicle was weighed.

Statistical Analysis

Statistical analysis was conducted using the Statistical Analysis System (SAS Institute Inc. Cary, NC, USA). Analyses of variance with repeated measures were used to examine the effects of the pST on metabolic profile (testosterone, glucose, cholesterol, urea, albumin, AST, GGT and phosphorus) body weight gain, sperm characteristics (volume, motility, vigor, concentration, spermatic pathologies, integrity of plasma and acrosomal membrane and mitochondrial function) and seminal plasma constituents (fructose, cholesterol, total protein and IGF-I), with a Tukey test adjustment. Semen analyses and seminal plasma constituents were grouped every two weeks. To evaluate the residual effect after the period of hormone injection, the data were separated into Period 1 (period during hormone injection) and Period 2 (period of five weeks after the end of hormone injection). Testicular weight was evaluated by one-way ANOVA.

RESULTS

The characteristics of fresh and stored semen were improved by treatment with pST. The spermatic vigor was higher in the pST Group in fresh semen (Table 1), but no effect during storage time was observed (Table 2). Regarding sperm motility, pST treatment had no effect on motility of fresh semen (Table 1); however, when semen was stored for 72 hs, a greater motility decrease was observed in the Control Group after 48 hs. pST Group kept the motility stable during the 72 hs storage (Table 2). During the 72 hs storage, pST Group also had a lower amount of morphological alterations and lower plasmatic membrane damage (P=0.07) (Table 2). The pST Group had lower sperm concentration, but had a higher volume and increased total sperm output. Considering this, pST Group had increased the number of inseminating doses per ejaculate (Table 1).

Regarding the biochemical constituents of seminal plasma, there was no difference between groups for the concentration of total protein (pST: 3.4±0.6 g/dL; Control: 3.4±1.0 g/dL; P=0.96), cholesterol (pST: 8.8±1.5 mg/dL; Control: 11.5±2.4 mg/dL; P=0.40) and fructose (pST: 5.8±1.2 mg/mL; Control: 6.5±1.8 mg/mL; P=0.78).

In addition, it was observed that the number of semen doses (P=0.03) and sperm output (P=0.03) was increased in the pST Group in Period 2 (mean of 38.9±8.9 doses and 117.0±26.8 $x10^9$/ejaculate) compared to Period 1 (mean of 14.0±1.6 doses and 42.3±4.8 $x10^9$/ejaculate), i.e. during the five weeks of evaluation after the end of pST injection.

Testosterone levels were similar between groups (pST: 2.4±0.3 ng/mL; Control: 1.7±0.3 ng/mL; P=0.14). With regard to metabolic parameters, between-groups difference was observed only for serum albumin (pST: 4.0±0.1 g/dL; Control: 3.6±0.1 g/dL; P=0.0006) and phosphorus (pST: 7.5±0.1 mg/dL; Control: 7.0±0.1 mg/dL; P=0.06), which was higher in the pST Group. Concentrations of glucose (pST: 74.9±1.4 mg/dL; Control: 74.4±1.4 mg/dL), cholesterol (pST: 77.5±2.0 mg/dL; Control: 74.5±2.0 mg/dL), urea (pST: 30.1±1.2 mg/dL; Control: 29.5±1.2 mg/dL), AST (pST: 24.2±1.5 UI/L; Control: 26.8±1.5 UI/L) and GGT (pST: 95.7±14.0 UI/L; Control: 87.6±14.0 UI/L) were not different between groups (P>0.05).

The pST Group had higher testicular weight than the Control Group (P=0.0005), with expressive differences at 365 days of age (Figure 1). Body weight was not different between groups (pST: 115.4±2.5 kg; Control: 110.3±2.5 kg; P=0.17).

DISCUSSION

The pST injection was able to increase testicular size after the puberty, allowing an increase in reproductive efficiency in the post-pubertal period. Several studies (HAHN et al., 1969; BORG et al., 1993) indicate that testis size is positively correlated with sperm count per ejaculate. This was further confirmed by the current study since increased semen volume, total sperm count and number of semen doses per ejaculate were observed. Our observations agree with HAFEZ et al. (2005), who observed increased ejaculate volume, mass motility and total number of spermatozoa in ejaculates and decrease percentage of abnormal sperm cells of bulls treated with rbST. We did not observed difference in testosterone concentration after puberty, so pST did not act by this pathway as proposed by LANGFORD et al. (1987). In this way, it is possible that a direct effect of pST on local IGF-I occurred, which is significantly correlated with the percentage of morphologically normal spermatozoa and motility (GLANDER et al., 1996). Sertoli, leydig and peritubular cells also secrete IGF-I

(CAILLEAU et al., 1990), which stimulate DNA synthesis in spermatogenic cells, acting directly as a stimulator of cellular proliferation (trout: LOIR, 1994; rat: SÖDER et al., 1992), increasing the number of spermatic cells in the ejaculate. Still, its effect on the spermatic motility is cited as being due to increased energy metabolism (SELVARAJU et al., 2009). However, in this study mitochondrial function in spermatozoa and energetic parameters in seminal plasma have not changed. Probably the effect of the pST on motility is due to direct action of the IGF-I in spermatozoa, since its receptors were detected in spermatozoa, with related action on motility (HENRICKS et al., 1998). Other important action of the IGF-I is as antioxidant in seminal plasma (SELVARAJU et al., 2009), protecting the spermatozoa against damage in the plasmatic membrane during the storage, as demonstrated in this study.

In this study, GH influence proteic and mineral metabolism. The effect of pST on concentration of albumin is due to influence of the GH on the proteic turnover in liver, as demonstrated by the decreasing in albumin levels in hypophysectomised rats and its partial restoration after GH supplementation (FELDHOFF et al., 1977). The increase in phosphorus levels is due to antiphosphaturic effect of GH (SAGGESE et al., 1993), which is mediated by effect of IGF-I on renal phosphorus reabsorption (KOPPLE et al., 1995). This effect contributes with the influence of the GH on energy metabolism, through the retention of phosphorus for the ATP production (TRYFONIDOU & HAZEWINKEL, 2004), which can explain the increase in sperm motility during storage in this study (LAMIRANDE & GAGNON, 1992).

In conclusion, the pST injection increase the reproductive efficiency of young boars, by increasing the testicular weight and sperm quality, with positive effects on the characteristics of fresh (vigor, volume and total sperm concentration) and stored semen (motility, amount of morphological alterations and integrity of plasmatic membrane).

FUNDING

This work was supported by CNPq (National Council for Research and Development, Brazil, grant number 478385/2009-9) and FAPERGS (Foundation for the Support Research in the State of the Rio Grande do Sul, Brazil, grant number 0904231).

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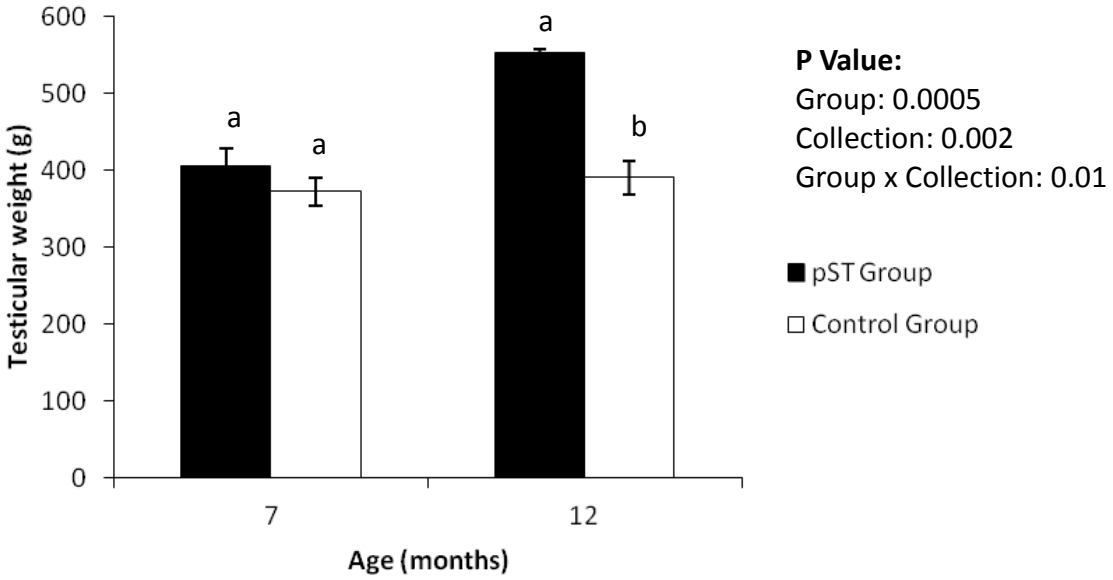

Figure 1. Testicular weight (mean ± standard error of mean) of the young pigs submitted to somatotropin (pST) injection during prepubertal and pubertal period.

Table 1. Fresh semen quality (mean ± standard error of mean) of the young pigs submitted to somatotropin (pST) injection during prepubertal and pubertal period.

Parameters	**pST (± SEM)**	**Control (± SEM)**	**P Value**
Ejaculate volume (mL)	174.5 (18.7)[a]	40.1 (11.8)[b]	< 0.0001
Sperm motility sperm (%)	74.7 (3.3)	67.5 (3.6)	0.15
Spermatic vigor (score: 0-5)	3.0 (0.1) [a]	2.6 (0.1) [b]	0.02
Spermatic concentration ($x10^6$/mL)	346.5 (39.9)[a]	608.4 (69.6)[b]	0.001
Sperm output ($x10^9$/ejaculate)	63.1 (11.2)[a]	22.3 (9.3)[b]	0.047
Number semen doses ($3x10^9$ sperm/dose)	20.9 (3.7)[a]	7.4 (3.1)[b]	0.047
pH	7.6 (0.1)	7.5 (0.1)	0.58

Different superscripts within the same row indicate significant differences (P<0.05).

Table 2. Effect (mean ± standard error of mean) of somatotropin (pST) injection during prepubertal and pubertal period on semen quality of young pigs during 72 hs of storage at 15ºC.

Parameter	0h		24h		48h		72h		P Value
	pST	Control	pST	Control	pST	Control	pST	Control	Group
Sperm motility	58.6 (5.1)^{a}	60.0 (0.0)^{ab}	48.1 (3.1)^{abc}	42.3 (3.8)^{bc}	34.6 (2.8)^{b}	28.4 (5.0)^{d}	36.1 (2.8)^{b}	24.6 (4.9)^{d}	0.28
Sperm vigor	2.7 (0.3)^{a}	3.0 (0.0)^{ad}	2.1 (0.1)^{ac}	2.1 (0.1)^{ad}	1.9 (0.1)^{bcd}	1.8 (0.3)^{bcd}	1.8 (0.1)^{bcd}	1.6 (0.2)^{bcd}	0.53
Morphology									
Normal cells	88.6 (4.6)^{a}	39.6 (5.6)^{b}	86.1 (4.6)^{a}	43.9 (5.6)^{b}	87.9 (4.6)^{a}	55.2 (5.6)^{b}	86.9 (4.3)^{a}	42.5 (5.2)^{b}	<0.0001
Isolated head	0.1 (0.1)^{ab}	0.8 (0.2)^{c}	0.1 (0.1)^{a}	0.6 (0.2)^{bc}	0.1 (0.1)^{ab}	0.2 (0.2)^{a}	0.2 (0.1)^{a}	0.6 (0.2)^{c}	0.0001
Proximal drop	0.6 (0.8)^{a}	4.0 (1.0)^{bc}	0.9 (0.8)^{a}	2.2 (1.0)^{ac}	0.7 (0.8)^{a}	3.0 (1.0)^{c}	0.6 (0.8)^{a}	3.1 (0.9)^{c}	0.0009
Distal drop	4.7 (2.0)^{a}	26.2 (2.4)^{b}	3.7 (2.0)^{a}	16.8 (2.4)^{c}	3.5 (2.0)^{a}	18.4 (2.4)^{c}	5.5 (1.8)^{a}	24.8 (2.2)^{bc}	<0.0001
Wrapped tail	0.1 (0.1)^{ac}	0.3 (0.1)^{abd}	0.2 (0.1)^{abc}	0.3 (0.1)^{bd}	0.1 (0.1)^{abc}	0.2 (0.1)^{acd}	0.1 (0.1)^{c}	0.5 (0.1)^{d}	0.0009
Tucked tail	5.6 (4.0)^{a}	29.1 (4.9)^{b}	8.7 (4.0)^{a}	36.2 (4.9)^{b}	7.2 (4.0)^{a}	31.4 (4.9)^{b}	6.5 (3.8)^{a}	28.6 (4.5)^{b}	<0.0001
Intact membrane (%)	66.7 (3.1)	63.2 (4.6)	62.4 (3.1)	58.9 (4.6)	62.7 (3.1)	53.6 (4.6)	61.7 (3.1)	49.9 (4.6)	0.07
Intact acrosoma (%)	57.2 (4.1)	64.8 (6.1)	50.4 (4.1)	57.0 (6.1)	46.6 (4.1)	42.7 (6.1)	51.9 (4.1)	40.8 (6.1)	0.95
Normal mitochondrial function (%)	69.4 (4.1)^{a}	64.9 (5.7)^{a}	64.0 (4.1)^{a}	59.7 (5.7)^{a}	57.8 (4.1)^{a}	48.1 (5.7)^{a}	52.6 (4.1)^{b}	43.9 (5.7)^{b}	0.17

Different superscripts within the same row indicate significant differences (P<0.05).

6. Conclusão Geral

Nas condições estudadas, o uso de pST em suínos jovens foi eficiente em antecipar a maturidade sexual, bem como apresentou efeitos benéficos sobre a qualidade espermática do sêmen fresco e durante a sua conservação a 15ºC. Assim, este hormônio mostrou ser uma alternativa para a antecipação do uso de machos suínos em sistemas de produção, por aumentar o número de montas com ejaculação durante o período de treinamento para coleta de sêmen, além de aumentar a eficiência reprodutiva destes machos, através do aumento do volume e concentração espermática total, gerando assim maior número de doses inseminantes produzidas a partir de um ejaculado, com isto diminuindo os custos de produção. Porém, os mecanismos envolvidos no efeito do pST sobre o desenvolvimento testicular ainda precisam ser elucidados. A ação mitogênica do IGF-I ficou demonstrada pelo aumento do tamanho testicular após o estabelecimento da puberdade, porém se tornam necessários novos estudos para determinar o porquê deste efeito não ter ocorrido durante os períodos de maior proliferação tecidual testicular, os quais seriam anteriores ao estabelecimento da puberdade. Assim, este conjunto de resultados deixa clara a influência do sistema GH/IGF-I sobre a fisiologia reprodutiva de machos suínos, indicando este hormônio como uma alternativa para a manipulação da função reprodutiva de animais jovens, além de levantar novos pontos que devem ser elucidados sobre a fisiologia do período de estabelecimento da puberdade em suínos.

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