

Effect of porcine somatotropin on metabolism and testicular characteristics of prepubertal pigs

Efeito da somatotrofina suína sobre o metabolismo e características testiculares de suínos pré-púberes

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Abstract

The effect of pST on the testicular characteristics and metabolic parameters of prepubertal pigs was evaluated. Experiment 1 aimed to determine the interval between applications of pST based on the concentrations of circulating IGF-I. Experiment 2 aimed to evaluate the effect of pST on metabolic parameters, testicular characteristics, and expression of GHR, IGF-I and PCNA. In Experiment 1 twelve piglets with 30 days of age were used. The pST Group (n = 6) was submitted to one i.m. injection of pST and the Control Group (n = 6) to one placebo injection. Blood collections were performed until day 7 post pST application to determine IGF-I concentration and metabolic profile. In Experiment 2 twelve piglets with 22 days of age were used. The pST Group was submitted to pST injections every three days, and the Control Group received placebo doses during 30 days. Blood collections were performed every 3 days. Samples of liver and testicular tissue were collected to determine gene expression and testicular characteristics. In Experiment 1 IGF-I concentration was higher for the pST Group (P = 0.02). In Experiment 2 the pST Group had higher body and testicular weight (P=0.06) and increased gene expression of PCNA in testes (P < 0.05). However, a reduction in the number of seminiferous tubules, and Sertoli cells, and in GHR expression (P < 0.05) was observed. Thus, pST administration increased body and testis development in prepubertal pigs, however it reduced the density of seminiferous tubules and Sertoli cells.

Keywords: Swine. Growth hormone. IGF-I. Testicle.

Resumo

Foi investigado o efeito da pST sobre características testiculares e metabolismo de suínos pré-púberes. O Experimento 1 determinou o intervalo entre aplicações de pST, baseado nas concentrações de IGF-I. O Experimento 2 avaliou o efeito da pST sobre o metabolismo, características testiculares e expressão gênica de GHR, IGF-I e PCNA. No Experimento 1, foram usados 12 leitões com 30 dias de idade. O grupo pST (n = 6) foi submetido a uma injeção IM de pST e o grupo Controle (n = 6) a uma injeção de placebo. Coletas de sangue foram realizadas até o dia sete após a aplicação de pST para determinação dos níveis de IGF-I e parâmetros metabólicos. No Experimento 2, foram usados 12 leitões com 22 dias de idade. O grupo pST foi submetido às aplicações de pST a cada 3 dias, e o grupo Controle, às doses de placebo, durante 30 dias. Coletas de sangue foram realizadas a cada três dias. Amostras de fígado e testículo foram coletadas para determinar a expressão gênica e características testiculares. No Experimento 1, a concentração de IGF-I foi maior no grupo pST (P = 0,02). No Experimento 2, o grupo pST teve maior peso corporal e testicular (P = 0,06) e aumento na expressão de PCNA no testículo (P < 0,05). Contudo, foi observada uma redução no número de túbulos seminíferos, células de Sertoli e GHR (P < 0,05). Assim, a administração de pST aumentou o desenvolvimento testicular e corporal de suínos pré-púberes, porém reduziu a densidade de túbulos seminíferos e células de Sertoli.

Palavras-chave: Suíno. Hormônio do crescimento. IGF-I. Testículo.

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Introduction

Growth hormone (GH) or somatotropin (ST) is a pituitary hormone with important effects on the reproductive function, acting as an endocrine and autocrine/paracrine regulator (SIROTKIN, 2005; LUCY, 2011). 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; GRAY; HENRICKS, 2000).

As a metabolic hormone, GH has various regulatory effects on the energetic, mineral and protein metabolism, and body development (TRYFONIDOU; HAZEWINKEL, 2004; VIJAYAKUMAR et al., 2010). Some of the GH effects include peripheral insulin resistance with consequent increase in circulating levels of non-esterified fatty acids (NEFA) and glucose (WRAY-CAHEN et al., 2012). Still, GH and IGF-I act on renal reabsorption of phosphorus, which is directed to bone metabolism and soft tissues to produce adenosine tri-phosphate (SAGGESE et al., 1993). However, these effects are dependent on the level/dose of GH and physiological individual condition (KLINDT et al., 1996). When circulating levels of GH are too high some metabolic disorders may be triggered and the endogenous GH production can be down-regulated (MATTERI et al., 1997).

Regarding the GH function in reproductive processes, 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 is a critical factor in determining its effects on Leydig cell number and

steroidogenic capacity in the mouse (WANG; HARDY, 2004). However, 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, chronic administration down-regulated somatotrophic function in young pigs (MATTERI et al., 1997). The variation observed in these studies suggests that more studies are still needed to elucidate GH effects on reproductive function.

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 the establishment of puberty (FRANÇA et al., 2000). At puberty the presence of GH receptors (GHR) in the testis increases (N'DIAYE et al., 2002), indicating that those would be important periods for GH actions on the reproductive function in male pigs. In addition to the mitogenic effects of IGF-I on testicular cells (WANG; HARDY, 2004), GH acts on the maturation of Sertoli cells and seminiferous tubules (SWANLUND et al., 1995). This lead us to the hypothesis 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 this evidence, 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, testicular characteristics, and testicular and liver gene expression 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 the 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, Oz BioPharm Pty Ltd, Knoxfield, Australia), corresponding to 90 µg/kg of body weight (SWANLUND et al., 1995) while the Control Group received a placebo (sodium chloride 0.9%) dose at the same route and volume as the pST Group. The piglets were weighed at Day 0 and at the end of the experiment. The piglets had free access to the diet (Table 1) and water.

Blood collections were performed by venipuncture every 24h until day 7 post-application of pST. Serum was collected after centrifugation at 3000g for 15 min and frozen at -70°C 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's instructions. This commercial kit has been previously validated for use in swine samples (KOJIMA et al., 2008). Analyses were performed in a single batch and the intra-assay coefficient of variation (CV) was 5.6%. The glucose, cholesterol, urea,

albumin, phosphorus, aspartate amino transferase (AST) and gamma glutaryltransferase (GGT) serum concentration were determined by colorimetric methods according to the manufacturer's instructions (Labtest Diagnóstica S.A., Brazil). The intra and inter-assay CV for all analyses 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 an i.m. injection of pST (Reporcin) every three days (based on results from Experiment 1) during 30 days. The dose and via of pST and placebo were the same as in Experiment 1. The piglets were weighed at day 0 and then every 3 days until d 30.

Blood samples were collected every 3 days after pST application from 22 until 52 days of age. Glucose, cholesterol, urea, albumin, phosphorus, AST, GGT (Labtest Diagnóstica S.A., Brazil), NEFA (Wako Diagnostics, USA) and β-hydroxybutyrate (BHBA - Ranbut, Randox Laboratories, UK) were determined by colorimetric methods. The intra and inter-assay CV was below 10%.

Table 1 – Composition and analysis of the diet utilized during the experimental period – Pelotas – 2013

Composition	Diets		
	Pre-initial ^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 extract (%)	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

^aDiet fed to piglets from 22 to 30 days of age; ^bDiet fed to piglets from 31 to 42 days of age; ^cDiet fed to piglets from 43 to 52 days of age

At 39 and 52 days of age in Experiment 2, hepatic biopsies were performed with a semi-automatic biopsy needle (Tru-cut 16G x 15 cm – Biomedical, Delebri, 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 a liver sample was collected. The samples were snap frozen in liquid nitrogen and stored at -70°C for RNA extraction.

At 39 days of age the piglets were submitted to testicular biopsy in the right testicle, for RNA extraction, with the same biopsy needle used for hepatic biopsies. The scrotum skin was anesthetized with lidocaine 2%, the needle was introduced and a testicle sample was collected. At 52 days of age the animals were submitted to orchietomy 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% 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 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 -70°C for RNA extraction.

Total RNA from testis and liver samples was extracted using Trizol (Invitrogen®, Carlsbad, USA) according to the 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 by spectrophotometry 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. All RNA samples with intact 18S and 28S bands were

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 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 TGGTGGGACTGTGGATCAAA; Rev GGTTCGACTATTTTCCTCAACGG) (Gen Bank NM 214254.2), IGF-I (For CTTCAGTTTCGTGTGCGGAGA; Rev CCCTGTGGGCTTGTGAAAT) (Gen Bank NM 214256.1), PCNA (For ATAATGCAGACACCTTGGCACTAG; Rev CCTTTTCCTGATTTGGAGCTTC) (Gen Bank XM 003359889.1) and GAPDH (For GTTTGTGATGGGCGTGAAC; Rev ATGGACGTGGTCATGAGT) (WILLING; VAN KESSEL, 2007). The PCR reaction efficiency and cycle thresholds from the fluorescence readings of individual wells during the reaction were analyzed using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen (2001) and the handbook of *Chemistry guide of applied biosystems*.

The cell marking process was performed by immunohistochemistry using monoclonal antibody vimentin for quantification of Sertoli cells and seminiferous tubules and to measure the diameter of the tubules (DUFOUR; RAJOTTE; KORBUTT, 2002). Sections of 5 µm of paraffin-embedded tissues were deparaffinized 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. Evaluations were performed in five slides per testicle, being analyzed by light microscope with a magnification of 400 x. The quantification of structures was performed using the ImageJ 1.44 software (National Institutes of Health, Bethesda, EUA).

Statistical Analysis

Data were 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 (glucose, cholesterol, albumin, urea, phosphorus, AST, GGT, BHBA, NEFA and IGF-I), testicular (GHR, IGF-I and PCNA) and hepatic (GHR and IGF-I) gene expression and body weight gain, with the Tukey test adjustment. The testicular morphological and functional characteristics (concentration of Sertoli cells and seminiferous tubules and diameter of tubules) were evaluated by One Way ANOVA. All data are presented as mean \pm standard error of the mean (SEM).

Results

Experiment 1

The mean IGF-I concentration was higher for the pST Group (126.8 ± 8.9 ng/mL) than the Control Group (93.4 ± 6.9 ng/mL), during the seven-day period evaluated ($P = 0.02$). On d 3 the IGF-I levels

were 124.1 ± 7.3 ng/mL in the pST Group and 94.6 ± 6.2 ng/mL in the Control Group ($P < 0.05$). On d 3 the difference in IGF-I concentration was of 30 ng/mL ($P < 0.05$) between treated and control animals; however, on d 4 there was no difference ($P > 0.05$) between the groups, indicating that after 3 days IGF-I concentrations returned to basal levels.

The mean concentrations 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 phosphorus (pST: 7.9 ± 0.1 mg/dL; Control: 7.7 ± 0.1 mg/dL) were not different between the groups ($P > 0.05$) and there was also no interaction between group and day after injection.

Experiment 2

The pST Group showed higher body weight (Figure 1) at the end of the experiment compared to the Control Group ($P = 0.06$). The pST Group also had higher testicular weight (pST: 9.7 ± 1.0 g; Control: 7.3 ± 0.5 g; $P = 0.06$). A reduced number of Sertoli cells ($P = 0.0004$) and seminiferous tubules ($P = 0.002$) was observed in the pST Group (Table 2). The diameter of the seminiferous tubules was not influenced by the use of pST ($P = 0.15$).

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) ($P = 0.005$). Serum concentrations 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 phosphorus (pST: 9.3 ± 0.1 mg/dL;

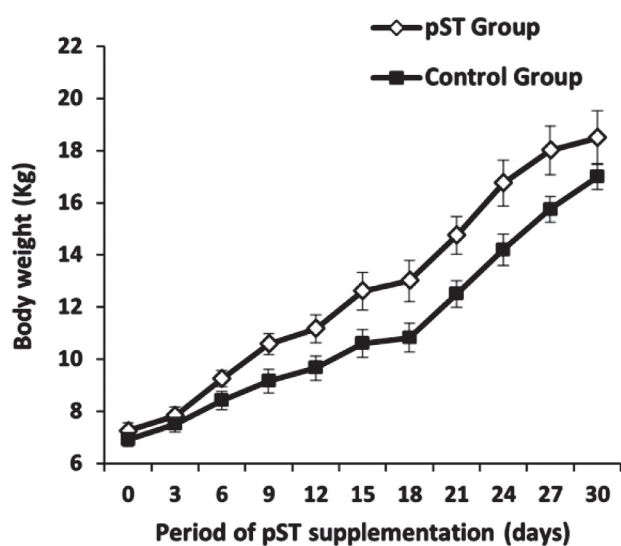


Figure 1 – Body weight of male pigs submitted to porcine somatotropin (pST) administration during the prepubertal period. Pelotas, 2013

Source: (RABASSA, 2014)

Control: 9.3 ± 0.1 mg/dL) were not different between the groups ($P > 0.05$).

The expression of PCNA in testicles was increased in the pST Group (pST: 0.82 ± 0.02 ; Control: 0.76 ± 0.02 ; $P = 0.03$); however, the expression of GHR was decreased in the pST Group in comparison to the Control Group (pST: 1.67 ± 0.06 ; Control: 1.93 ± 0.07 ; $P = 0.01$). The expression of IGF-I in the testicles did not differ between the groups (pST: 1.04 ± 0.01 ; Control: 1.05 ± 0.01 ; $P = 0.09$). The pST and Control Groups did not differ in the liver expression of GHR (Control: 4.50 ± 0.47 ; pST: 5.76 ± 0.60 ; $P = 0.12$) and IGF-I (1.13 ± 0.02 vs. 1.15 ± 0.02 ; $P = 0.51$).

Discussion

The commercial injectable pST for pigs has a short half-life. The recommended schedule for application is one injection every 24h when used as a growth promoter (KHAN et al., 2010). However, when used for other purposes, such as manipulating reproductive functions, pST injection can be performed at longer intervals, depending on how long it remains stimulating IGF-I production. The IGF-I concentration remained high for at least 3 days after pST injection in this study, determining the interval between hormone doses utilized in Experiment 2, since the applications every 24h could exacerbate other metabolic effects of pST, such as peripheral insulin resistance and lipolysis (WRAY-CAHEN et al., 2012).

Based on the results of metabolic profiling, it was determined that the dose of $90 \mu\text{g/kg}$ of pST every three days 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). However, other positive effects of GH on metabolism, such as increase in phosphorus and protein metabolism (SAGGESE et al., 1993; TRYFONIDOU; HAZEWINDEL, 2004) were not confirmed in this study. These results are similar to those observed by Klindt et al. (1996), which demonstrated that prepubertal pigs treated with pST show fewer alterations in metabolic and hormonal parameters when compared to pubertal or adult pigs undergoing the same treatment.

Table 2 – Testicular morphological and functional characteristics of pigs after 30 days of porcine somatotropin (pST) administration during the prepubertal period – Pelotas – 2013

Parameter	pST (\pm SEM)	Control (\pm 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 ($P < 0.05$).

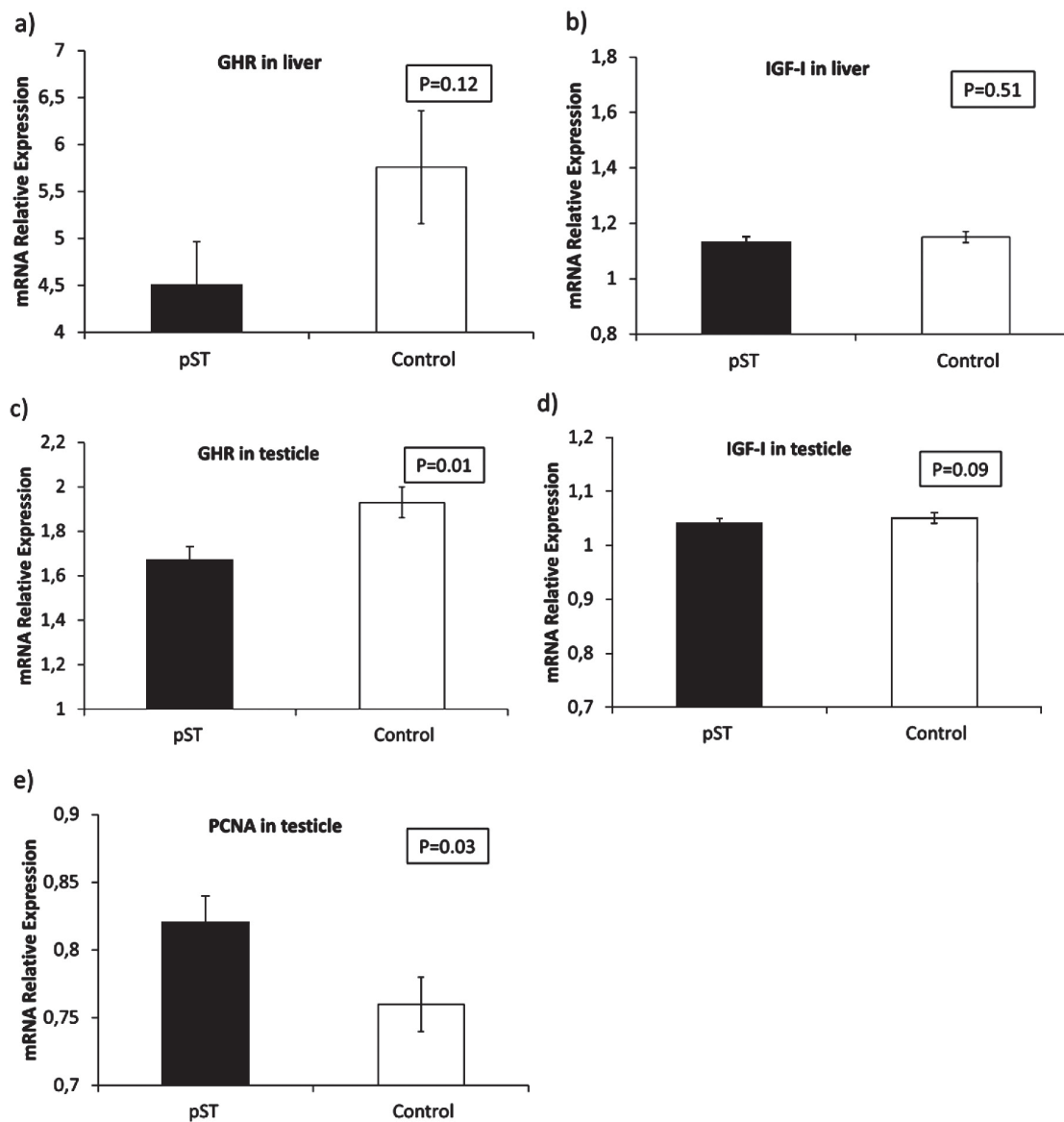


Figure 2 – Relative gene expression in testicle (IGF-I, GHR and PCNA) and liver (IGF-I and GHR) of male pigs submitted to porcine somatotropin (pST) administration during the prepubertal period. a) mRNA relative expression of GHR in liver; b) mRNA relative expression of IGF-I in liver; c) mRNA relative expression of GHR in testicle; d) mRNA relative expression of IGF-I in testicle; e) mRNA relative expression of PCNA in testicle. Pelotas, 2013
Source: (RABASSA, 2014)

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 is further confirmed by the increased expression of PCNA mRNA in testes, which is an indicative of tissue proliferation (WILLING; VAN KESSEL, 2007). Although pST administration reduced the density of seminiferous tubules and Sertoli cells, testicular

volume was higher, probably due to increased proliferation of testicular stroma. Still, these results were contrary to those described by Roser (2001), in which IGF-I stimulate proliferation of Sertoli cells. On the other hand, the results observed for the diameter of the seminiferous tubules were similar to those obtained by Swanlund et al., (1995), which demonstrated that pST was not able to increase the diameter, but anticipated the maturation of the

seminiferous tubules in prepubertal pigs, determined by increased tubule diameter, lumen formation, and initiation of spermatogenesis.

However, the decrease in GHR mRNA expression in testicle indicates that a down-regulation occurred at the testicular levels, due to increased circulating IGF-I as demonstrated for other tissues (LEUNG et al., 1996). The observed effects on testicular tissue proliferation probably were due to higher circulating

IGF-I rather than its increased local production.

Thus, these results indicate that pST administration of 90 µg/kg of body weight every 3 days increases body development and consequently testis development, without changing the metabolic balance of prepubertal pigs. However, it also reduces the density of seminiferous tubules and Sertoli cells and further studies should focus on determining its effect on other testicular structures.

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