Animal Reproduction Science 207 (2019) 44–51

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Are testicular cortisol and WISP2 involved in estrogen-regulated Sertoli cell proliferation?

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ARTICLE INFO

Keywords:
Glucocorticoid
Boar
Sertoli cell
Estradiol
Testis development

ABSTRACT

The number of Sertoli cells has a major effect on adult testis size and sperm production capacity. Mechanisms that regulate the number of Sertoli cells in livestock are at best nebulously understood; however, with lesser testicular estrogen production, proliferation of Sertoli cells is prolonged compared with vehicle-treated littermates. Decreased *WISP2* gene expression in testes as a result of less endogenous estrogen is similar to altered *WISP2* gene expression following corticosteroid treatment of some cultured cells. Taken together, these findings indicate decreased testicular cortisol might be in the signaling pathway between reduced endogenous estrogens and the prolonged interval of Sertoli cell proliferation. Hence, in these studies, potential actions of testicular corticosteroid on Sertoli cell numbers were evaluated. Testicular cortisol concentrations were reduced at 6.5 weeks of age ($P < 0.05$) in littermates treated with the aromatase inhibitor, letrozole, compared with littermates treated with vehicle. Letrozole treatment leads to reduced testicular estradiol and greater Sertoli cell numbers during the early juvenile interval in pigs. The inverse relationship between testicular glucocorticoid and Sertoli cell proliferation was also tested by increasing local testicular glucocorticoids using the synthetic compound, dexamethasone. Local administration beginning at 1.5 weeks of age (osmotic pump and catheter ($n = 3$) or a silastic implant ($n = 5$)) reduced Sertoli cell numbers at 6.5 weeks of age compared with littermates that received the vehicle treatment ($P \leq 0.05$). In summary, testicular glucocorticoid concentration was inversely correlated with Sertoli cell numbers during the first wave of Sertoli cell proliferation.

1. Introduction

Sertoli cells support male germ cell development during spermatogenesis; the number of Sertoli cells is believed to be a major determinant of sperm production capacity and testicular size (Berndtson et al., 1987a, b; Berndtson and Thompson, 1990). Prolonged proliferation of Sertoli cells occurs in the early neonatal period in boars in response to reduced endogenous testicular estradiol induced by the aromatase inhibitor letrozole (Berger et al., 2012). This prolonged interval of proliferation leads to an increase in Sertoli cell numbers. An initial analysis of gene expression suggested *WISP2* gene expression decreased during this period of reduction in endogenous estrogen. *In vitro*, treatment with dexamethasone resulted in an increased *WISP2* gene expression (Ayala-Sumuano et al., 2008; Pantoja et al., 2008; Ferrand et al., 2012). These observations together with the reduced expression of the *WISP2* gene following letrozole treatment raised the possibility that decreased testicular cortisol concentrations during testicular development

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https://doi.org/10.1016/j.anireprosci.2019.05.014
Received 25 December 2018; Received in revised form 12 May 2019; Accepted 28 May 2019
Available online 29 May 2019
0378-4320/

were involved in the pathway leading from reduced testicular estradiol to increased Sertoli cell number. Conversely, we hypothesized that treatment with the synthetic glucocorticoid, dexamethasone, would reduce Sertoli cell numbers in neonatal pigs. This would be consistent with a role for *WISP2* gene expression in the signaling cascade regulating duration of initial Sertoli cell proliferation in the neonatal pig although correlation does not confirm causation. Reduced anogenital distance and reduced testicular size were reported in rat pups following imposition of late gestation stress (Dahlof et al., 1978a,b). Furthermore, treatment with corticosteroids during late pregnancy also resulted in reduced testicular size, a possible indicator of reduced Sertoli cell numbers given the role of Sertoli cells in regulating testis size although a mid-gestational injection of glucocorticoid in sheep did not immediately (5 days post injection) alter Sertoli cell number in ram fetuses (Pedrana et al., 2008). The glucocorticoid receptor has been detected in Sertoli cells from rodents, sheep, and humans during early testicular development (Levy et al., 1989; Pedrana et al., 2008; Hazra et al., 2014; Nordkap et al., 2017).

To further understand mechanisms regulating Sertoli cell proliferation in the boar, testicular cortisol concentration was evaluated in 6.5 week old boars with reduced testicular estrogen signaling and increased Sertoli cell number and in peripuberal boars with reduced testicular estrogen signaling. Next, the effect of dexamethasone, a synthetic cortisol, on Sertoli cell numbers during the first postnatal wave of Sertoli cell proliferation was examined in two experiments. Lastly, *WISP2* gene expression was evaluated to examine its potential involvement in regulating the number of Sertoli cells in boars.

2. Materials and methods

All animal experiments were conducted in accordance with the Guide for the Use and Care of Agricultural Animals in Research. Animal protocols were approved by the UC Davis Institutional Animal Care and Use Committee.

2.1. Direct interference with estrogen signaling

Cortisol was analyzed in testicular tissue and plasma from animals that were treated to reduce estrogen synthesis or estrogen interaction with nuclear receptors and their littermate controls in experimental designs described previously (Berger et al., 2013; Berger and Conley, 2014). In the first design, one member of littermate pairs of boars was treated weekly with the aromatase inhibitor, letrozole, beginning at 1 week of age and the remaining littermate was treated with the canola oil vehicle with testes being collected for experimental evaluations at 6.5 weeks of age (five littermate pairs). In the second design, one littermate was treated with the estrogen receptor blocker, fulvestrant, delivered using an osmotic pump with the littermate receiving the PBS: ethanol vehicle, again with the testes being collected at 6.5 weeks of age for tissue analysis (three littermate pairs). In the third experimental design, boars were treated with letrozole from 11 to 16 weeks of age (and littermates treated with canola oil vehicle) with tissues being collected for evaluations at 16 and 20 weeks of age (Berger et al., 2019). All these animals were derived from PIC genetic lines and testicular weights and Sertoli cell numbers have been reported.

2.2. Testicular infusion of dexamethasone

Littermate pairs of boars were anesthetized with telazol® (Fort Dodge Animal Health, Overland Park, KS, USA) at 1.5 weeks of age with one member randomly assigned to vehicle treatment (1:25 ethanol:PBS) and the other to dexamethasone (0.1 μg/day, #9184, Sigma Aldrich, St. Louis, MO, USA) treatment. A sterile needle was used to make an incision in the tunica albuginea of the testis and one end of a silicone catheter was inserted and sutured. The other end of the catheter had been attached to an Alzet® osmotic pump (#1004, Durect Corp., Cupertino, CA) filled with vehicle or dexamethasone solution on the previous day to allow equilibration. The osmotic pump was placed between the tunica albuginea and the visceral tunica vaginalis and incisions sutured with Vicryl®, (Ethicon, Inc., Somerville, NJ, USA). Boars were crossbred with a Yorkshire, Hampshire and Duroc genetic background. Testes and a blood sample were recovered from the three littermate pairs of boars at 6.5 weeks of age.

2.3. Silastic dexamethasone implant

Littermate pairs of boars were anesthetized with telazol® at 1.5 weeks of age. One member received a silastic implant containing dexamethasone and the remaining littermate received an implant containing only silastic (Conley and Ford, 1989). A 14 g needle was used to create the incision site in the scrotum for the silastic implant, which was inserted into the testis. Boars were crossbred with a Yorkshire, Hampshire, Duroc, and Spot genetic background. Testes and a blood sample were recovered from the five littermate pairs of boars at 6.5 weeks of age.

2.4. Letrozole-treated boars and WISP2

One member of eight littermate pairs of boars were treated weekly with 0.1 mg letrozole/kg bw administered orally, beginning at 1 week of age. The other member of each littermate pair was treated weekly with canola oil vehicle. At 5 weeks of age, testes were recovered, either after the littermate pair was euthanized or after the littermate pair was castrated and testicular parenchyma was immediately frozen on dry ice. Boars were derived from PIC genetic lines (Hendersonville, TN, USA). Frozen testicular parenchyma from four of these littermate pairs of boars were processed for RNASeq analysis and expression of the *WISP2* gene was estimated using the Sus Scrofa Ensembl Genome Assembly 10.2 (Oberbauer et al., 2014; Ayuso et al., 2015) in a preliminary trial. Tissues from six

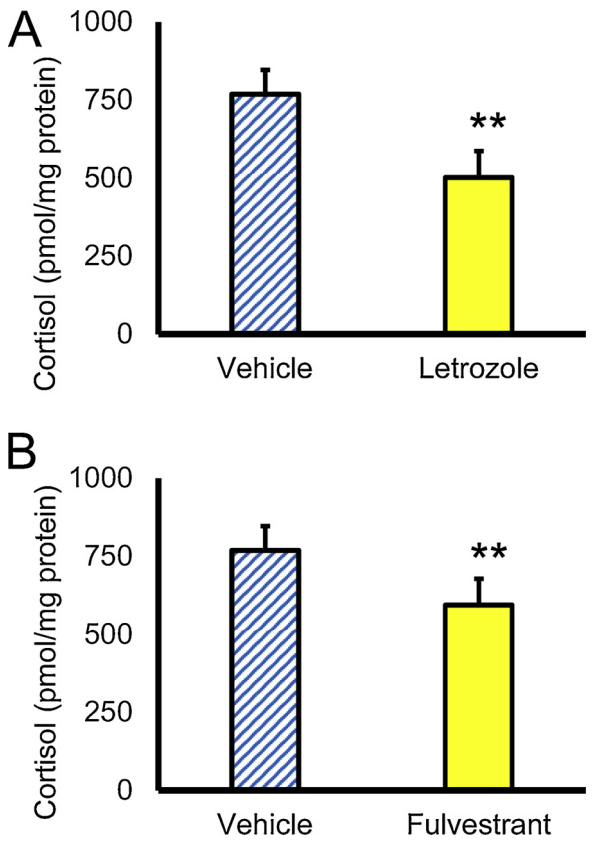

Fig. 1. Testicular cortisol was reduced in 6.5 week boars in response to reduced estrogen signaling; A. Testicular cortisol (pmol/mg protein) was reduced in boars treated with the aromatase inhibitor, letrozole, compared with littermates receiving the vehicle; Bars represents the mean of five boars; B. Testicular cortisol was reduced in boars treated with the estrogen receptor blocker, fulvestrant, compared with littermates receiving the vehicle; Bars represents the mean of three boars; ** $P < 0.01$.

littermate pairs of boars including two of the initial littermate pairs were analyzed for *WISP2* gene expression by qPCR and tissues from these same littermate pairs of boars assessed for WISP2 protein. Tissues from seven littermate pairs of boars treated with vehicle or letrozole from 1 week of age (Berger et al., 2013; Berger and Conley, 2014) were assessed for *WISP2* gene expression at 6.5 weeks of age.

2.5. Tissue processing and analyses

Testes were trimmed free of epididymis and additional tissue and weighed. A central slice (2–3 mm thick) of one testis was fixed in 4% paraformaldehyde in PBS overnight. Tissue was subsequently rinsed in PBS overnight and dehydrated in a graded ethanol series prior to paraffin embedding. The remaining testicular parenchyma was separated from the tunica and mediastinum and frozen on dry ice. Blood was cooled to 4 °C, centrifuged at 1000 x *g*, and plasma stored at −17 °C until analysis.

Thick (30 μm) sections were cut and placed on slides. Sertoli cells were labeled with GATA-4 antibody (sc 1237, Santa Cruz Biotechnology, Inc, Dallas, Texas, USA) as previously described (At-Taras et al., 2006). A minimum of 200 Sertoli cells were counted in 35 fields, each approximately 17,000 μm^2 x 17 μm thick with an optical fractionator approach using design-based stereology to determine Sertoli cell density. Density was multiplied by weight of testis to determine total Sertoli cell number. Thin sections (5 μm) were stained with hematoxylin and eosin; eight randomly selected frames were captured using a 10X objective, QImaging Micropublisher 3.3 digital camera and QCapture Pro software (QImaging Corporation, Burnaby, BC, Canada). The smallest tubule cross-section was visually identified in each frame and measured; the coefficient of variation (CV) for means determined from eight frames was 2.5%. Percentage of testicular parenchyma occupied by seminiferous tubule was determined in eight frames from littermates receiving the osmotic pumps with a CV for means from eight frames of 3.5%. For cortisol assays, frozen testes stored at −80 °C were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) containing 5 mM β-mercaptoethanol and 0.5 mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) buffer for 5 min and sonicated for 3 s. The homogenate was extracted with ethyl acetate, dried, and re-suspended in sample buffer provided with the enzyme immunoassay (#K003, Arbor Assays, Ann Arbor, MI). Plasma and testis homogenate samples were analyzed for cortisol in duplicate and the mean value used for subsequent analysis. The standard curve had an R-squared value of 0.99. The average CV was 9.7% for tissue samples and 10.2% for plasma samples. The

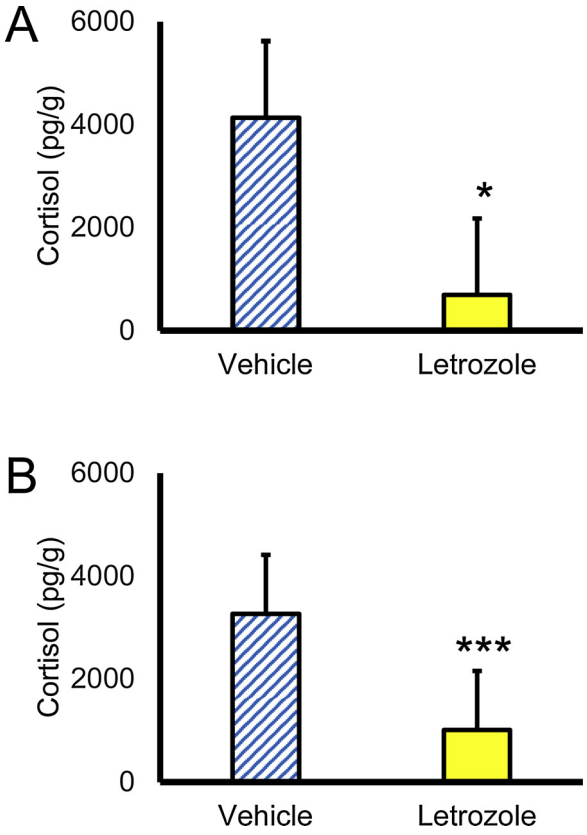

Fig. 2. Testicular cortisol was reduced in response to reduced estrogen signaling; A. Testicular cortisol (pg/g tissue) was reduced in 16 week old boars treated with the aromatase inhibitor, letrozole, from 11 to 16 weeks of age compared with littermates receiving the vehicle; B. Testicular cortisol (pg/g tissue) was reduced in 20 week old boars treated with the aromatase inhibitor, letrozole, from 11 to 16 weeks of age compared with littermates receiving the vehicle; Bars represents the mean of four boars; * $P < 0.05$; *** $P < 0.001$.

extraction efficiencies for the tissue samples and plasma samples were 75.5% and 86%, respectively.

The WISP2 protein was analyzed in frozen tissue (-80 °C) samples after homogenization in PBS (10 mg tissue: 100 µl PBS) followed by centrifugation at 1500 x *g*. The relative abundances of WISP2 protein in the supernatant were determined following manufacturer's direction with a porcine specific quantitative sandwich ELISA kit (#MBS076868, MyBioSource, San Diego, CA, USA). Total protein of each sample was determined using the Bradford assay (Thermo Fisher Scientific, Waltham, USA) and relative abundance of WISP2 protein was expressed as a proportion of total protein.

2.6. qPCR

The RNA was isolated from tissue samples using Qiazol (Qiagen, Hilden, Germany); cDNA was then synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The forward primer pair for *WISP2* (XM_003134453.4) was 5′-GGC GTC TTC TGG TCC ATT CA-3 and the reverse primer was 5′-TAT CTG TGC CCC ACT TCA TCG-3′, providing a product of 146bp; efficiency was 107.6%. Arginyl t-RNA synthetase (*RARS)* was used as the reference gene (Hughes and Berger, 2018). Threshold cycle (Ct) was determined with qPCR using a QuantStudio instrument and software with SYBR Green Reagent (Applied Biosystems, Foster City, CA, USA). The ΔCt was calculated as Ct *WISP2* - Ct *RARS* and used as an indicator of relative abundance of mRNA for the *WISP2* gene.

2.7. Statistical analysis

Data were analyzed using a mixed model with litter as a random factor and treatment as a fixed factor using R statistical programs (R_Core_Team, 2014) and the lmer procedure.

3. Results

Reduced testicular cortisol concentration ($P < 0.05$) was associated with reduced estrogen signaling. This was the case in 6.5 week old animals treated with letrozole from 1 to 5 weeks of age (Fig. 1A) and in 6.5 week old animals treated with fulvestrant to

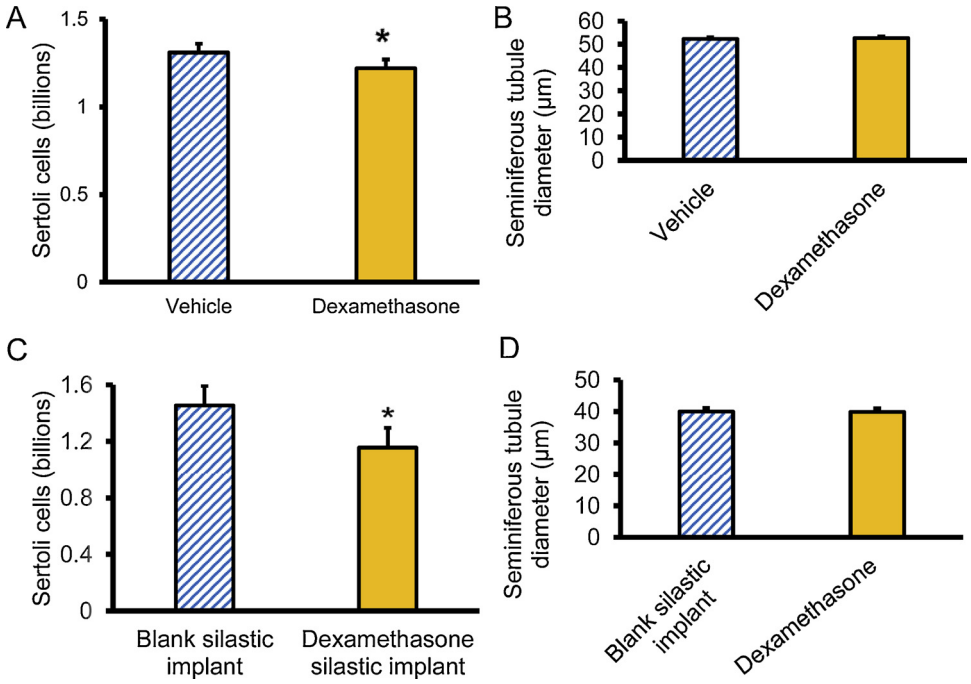

Fig. 3. Sertoli cell numbers were reduced in juvenile boars in response to dexamethasone but seminiferous tubule diameter was not altered; A. Dexamethasone introduced via an osmotic pump beginning at 1.5 weeks of age reduced Sertoli cell numbers at 6.5 weeks of age compared with littermates receiving osmotic pumps loaded with vehicle; Bars represent means of three boars; B. Diameter of seminiferous tubules in boars receiving osmotic pumps; C. A silastic implant in the testis releasing dexamethasone reduced Sertoli cell numbers compared with littermates receiving a silastic only implant; Bars represent means of five boars (6.5 weeks of age); D. Diameter of seminiferous tubules in boars receiving silastic implants; * $P \leq 0.05$.

block signaling via the nuclear estrogen receptors (Fig. 1B). Importantly, concentration of testicular cortisol was not correlated with systemic concentrations of cortisol ($r = 0.04$). Reduced testicular cortisol concentrations were also a characteristic of 16 and 20 week old boars (peripuberal boars) treated with letrozole from 11 to 16 weeks of age to reduce testicular estrogen (Fig. 2A, B).

Infusion of dexamethasone from the osmotic pump into the testis decreased Sertoli cell numbers slightly but significantly compared with the littermates receiving only vehicle infused into the testis ($P \leq 0.05$; 1.22 compared with 1.31 billion, SEM = 0.05; Fig. 3A). Neither tubule diameter (Fig. 3B) nor percentage of testicular parenchyma occupied by seminiferous tubules (67%) were significantly affected by treatment at 6.5 weeks of age. Boars receiving dexamethasone from a silastic implant in the testis had fewer Sertoli cells at 6.5 weeks of age compared with littermates receiving only the silastic implant ($P < 0.05$; 1.09 compared with 1.46 billion; Fig. 3C). Testicular weights were not altered by dexamethasone treatment compared with littermates (7.83 compared with 9.14; SEM = 0.7, $P > 0.10$). Tubular diameter was similar in animals receiving the dexamethasone silastic implant and the silastic only implant (Fig. 3D).

The relative abundance of *WISP2* mRNA was less in testes from 5-week old boars treated with the aromatase inhibitor, letrozole, compared with littermates treated with the vehicle (Fig. 4A) consistent with the approximate five-fold difference in preliminary RNAseq findings (4.36 RPKM compared with 0.85 RPKM). In contrast to the reduced relative abundance of *WISP2* mRNA in letrozole-treated boars, relative abundance of WISP2 protein was greater in the letrozole-treated littermates (Fig. 4B). Although relative abundance of *WISP2* mRNA was numerically greater (about 50%) in 6.5 week old boars that had received the dexamethasone containing silastic implant (Δ_{CT} of 2.12 compared with 2.71); this was not significant nor was relatively abundance of WISP2 mRNA altered in 6.5 week old boars receiving letrozole compared with littermates receiving only vehicle (Fig. 4C).

4. Discussion

The finding in the present study that there was an absence of correlation between systemic cortisol and testicular cortisol indicates testicular concentration of cortisol is being regulated by the local testicular endocrine and enzymatic milieu. Others have suggested enzymatic mechanisms within the boar testis might modify cortisol intra-testicular concentrations (Claus et al., 2005; Sharp et al., 2009; Cabrera-Sharp et al., 2013) consistent with modification of testicular cortisol independent of systemic concentrations of this hormone.

Decreased testicular cortisol in the current study was associated with decreased estrogen signaling that was previously reported to increase Sertoli cell numbers (Berger et al., 2012, 2013). Because testicular cortisol was decreased in juvenile boars when estrogen production was decreased and when binding of estrogens to the nuclear receptors was blocked, local estrogen signaling may influence

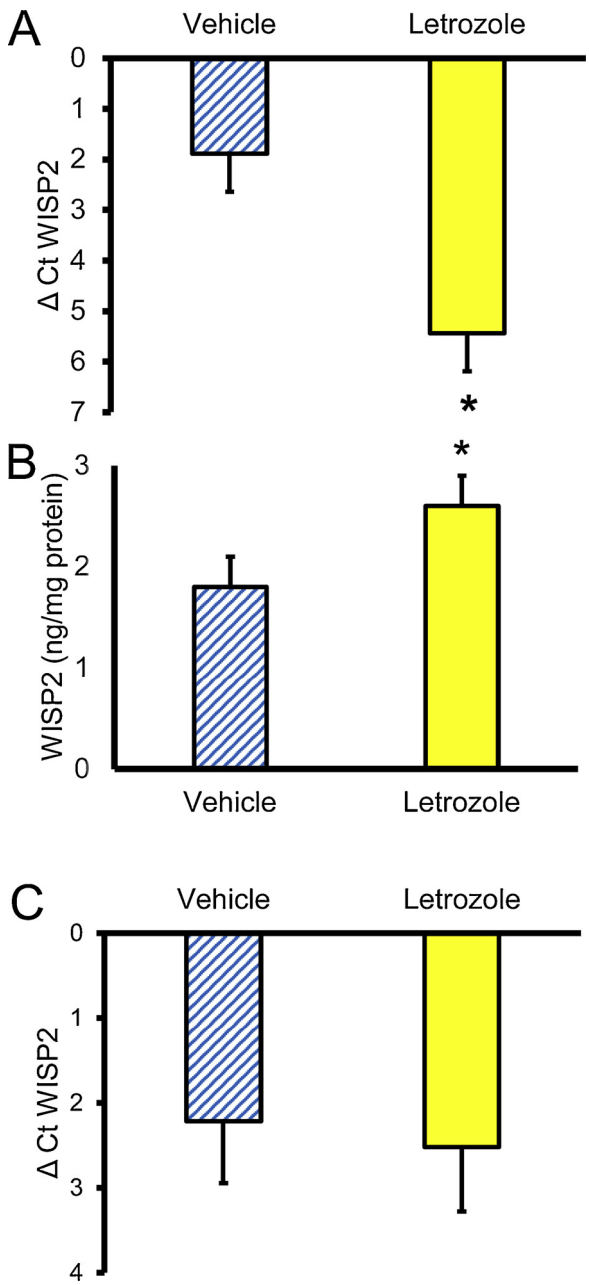

Fig. 4. Relative abundance of *WISP2* in response to letrozole; A. Boars receiving letrozole from 1 week of age had a lesser relative abundance of *WISP2* mRNA at 5 weeks of age compared with littermates treated with vehicle. Bars represent means of six boars; * $P < 0.05$; B. These same boars had increased relative abundance of testicular WISP2 protein; Bars represent means of six boars; * $P < 0.05$; C. Relative abundance of *WISP2* mRNA was similar in letrozole-treated boars and the vehicle-treated littermates at 6.5 weeks of age; Bars represent means of seven boars.

testicular cortisol concentrations. Boars treated with the aromatase inhibitor during the late prepuberal interval (11 to 16 weeks of age) had decreased testicular estrogens (Berger et al., 2019) and reduced concentrations of testicular cortisol when a mixed model analysis was used to assess these interactions although Sertoli cell numbers were not affected by treatment during this period. This finding is consistent with a relationship between estradiol and cortisol concentrations and indicates the cortisol response was not mediated by events downstream from Sertoli cell proliferation. A potential regulatory relationship between cortisol and estrogen concentrations may exist in multiple tissues and organisms. (Mattsson and Olsson, 2007; Tagawa et al., 2009; Andersson et al., 2010; McInnes et al., 2012; van Lier et al., 2014).

Increasing testicular glucocorticoid concentration is achievable using the synthetic cortisol, dexamethasone. We hypothesize that if a reduction in signaling from testicular cortisol is part of the mechanism for prolonging Sertoli cell proliferation and increasing

Sertoli cell numbers, treatment of the testis with dexamethasone would result in a decrease in Sertoli cell numbers. In the initial experiment, a small quantity of dexamethasone delivered to the testis via an osmotic pump resulted in a small but statistically significant decrease in Sertoli cell numbers at 6.5 weeks, indicating testicular cortisol may have a functional role in regulation of Sertoli cell proliferation. The treatment response was credible considering the small CV for Sertoli cell density (and testis weight) but not compelling. Static placement of the osmotic pumps is not possible due to the rapid growth of juvenile boars, therefore, a second experiment was conducted utilizing a dexamethasone-containing silastic implant in the testis. This experiment was conducted to confirm response to increased testicular concentration of this synthetic glucocorticoid. In this second dexamethasone experiment, treatment again reduced Sertoli cell numbers. The rate of testicular development did not appear to be affected by dexamethasone treatment based upon seminiferous tubule diameter or proportion of parenchyma occupied by seminiferous tubules; however, the experiments were designed to evaluate Sertoli cell numbers and number of replicates would have been insufficient to detect small differences due to treatment or parameters with larger variation (testis weight) or larger CV. The question of whether increased stress to the animal during this juvenile interval would increase testicular cortisol and modulate Sertoli cell proliferation remains unanswered although some evidence of stress-related impairment in rat testis size does exist (Dahlof et al., 1978a,b). These observations support a functional role for testicular cortisol in regulating Sertoli cell numbers during the juvenile interval. Although proliferation during the second wave of Sertoli cell multiplication immediately preceding the peripuberal interval could hypothetically be affected by the magnitude of proliferation during the first wave of Sertoli cell development, previous observations do not indicate number of cell divisions during the second wave of Sertoli cell development is affected by the magnitude of the proliferative response during the first wave of development (At-Taras et al., 2006; Berger et al., 2008, 2013). If the magnitude of the second wave of Sertoli cell development is not affected by alterations during the first developmental wave, a reduction in Sertoli cell numbers due to increased testicular glucocorticoid during the first wave of Sertoli cell proliferation would lead to fewer Sertoli cells in the adult, smaller adult testis size and decreased postpuberal sperm production.

Potential involvement of testicular cortisol was initially suggested by the apparent change in abundance of *WISP2* mRNA based on the preliminary RNAseq data. Reduction in expression of the *WISP2* gene following reduced estrogen production was confirmed by relative abundances of WISP2 mRNA as determined by qPCR in testes from 5 week old boars in the present study. The simultaneous observation of decreased *WISP2* gene expression and increased WISP2 protein at 5 weeks may reflect the time lag between mRNA synthesis and protein synthesis/turnover. The interval between 5 and 6.5 weeks of age is the period of proliferation induced by a previously prolonged reduction in estrogen signaling (Berger et al., 2012), therefore, changes in WISP2 protein abundance may contribute to the signaling cascade. Alternatively, changes in WISP2 protein might be induced by events in the pathway leading to prolonged Sertoli cell proliferation but changes in abundance of WISP2 protein might be superfluous to the signaling cascade. Because expression of the *WISP2* gene was not different between letrozole-treated boars and vehicle-treated littermates at 6.5 weeks of age, the numerically greater but statistically equivalent gene expression in the dexamethasone-treated boars compared with littermates receiving silastic alone at 6.5 weeks of age is not surprising.

In conclusion, a reduction in testicular cortisol concentration is associated with an increase in Sertoli cell numbers. The inverse relationship between testicular cortisol and Sertoli cell numbers was present in the dexamethasone studies, reinforcing the idea that local testicular cortisol can inhibit Sertoli cell proliferation. Hence, cortisol may have a role in mediating the prolonged proliferation of porcine Sertoli cells following a reduction in concentrations of testicular estrogens.

Conflicts of interest

None.

Acknowledgements

The authors thank Jennifer Hughes for assistance with surgical insertion of the osmotic pumps and Barbara Nitta-Oda for enumeration of Sertoli cells and assistance with the qPCR analysis. The research was partially supported by MSP 3171 from USDA, by USDA NIFA NRICGP2008-35203-19082, a W.K. Kellogg Endowment, Henry A. Jastro Research Awards to PS and ST, and the infrastructure support of the Department of Animal Science, College of Agricultural and Environmental Sciences, and the California Agricultural Experiment Station of the University of California, Davis

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