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Nursing supports neonatal porcine testicular development

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ABSTRACT

The lactocrine hypothesis suggests a mechanism whereby milk-borne bioactive factors delivered to nursing offspring affect development of neonatal tissues. The objective of this study was to assess whether nursing affects testicular development in neonatal boars as reflected by: (1) Sertoli cell number and proliferation measured by GATA-4 expression and proliferating cell nuclear antigen immunostaining patterns; (2) Leydig cell development and steroidogenic activity as reflected by *insulin-like factor 3* (*INSL3*), and P450 side chain cleavage (scc) enzyme expression; and (3) expression of *estrogen receptor-alpha* (*ESR1*), *vascular endothelial growth factor* (*VEGF*) *A*, and *relaxin family peptide receptor* (*RXFP*) *1*. At birth, boars were randomly assigned (n = 6–7/group) to nurse ad libitum or to be pan fed porcine milk replacer for 48 h. Testes were collected from boars at birth, before nursing and from nursed and replacer-fed boars at 50 h on postnatal day (PND) 2. Sertoli cell proliferating cell nuclear antigen labeling index increased ($P < 0.01$) from birth to PND 2 in nursed, but not in replacer-fed boars. Sertoli cell number and testicular GATA-4 protein levels increased ($P < 0.01$) from PND 0 to PND 2 only in nursed boars. Neither age nor nursing affected testicular *INSL3*, P450scc, *ESR1*, or *VEGFA* levels. However, testicular *relaxin family peptide receptor 1* (*RXFP1*) levels increased ($P < 0.01$) with age and were greater in replacer-fed boars on PND 2. Results suggest that nursing supports neonatal porcine testicular development and provide additional evidence for the importance of lactocrine signaling in pigs.

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1. Introduction

Maternal effects on offspring development do not end at birth (postnatal day = PND 0) [1], but continue thereafter when biologically active factors present in milk, such as growth factors, antimicrobial peptides, and hormones [2] are communicated to neonates via nursing [3,4]. Thus, the lactocrine hypothesis was proposed to describe the mechanism by which milk-borne bioactive factors, exemplified by the hormone relaxin (RLX) in the pig, might affect development of neonatal somatic tissues [4]. It is known that nursing promotes differentiation and function of anterior pituitary mammotropes in rats [5], forestomach development in marsupials [6], and immune system maturation and function in humans [7]. In the pig, nursing for 2 d from birth supports endometrial development, including cell compartment-specific expression of *estrogen receptor-α* (*ESR1*), *vascular endothelial growth factor A* (*VEGFA*), and *relaxin family peptide receptor 1* (*RXFP1*) [8]. Further, imposition of the lactocrine-null condition for 2 d from birth by feeding milk replacer in lieu of colostrum altered patterns of uterine gene expression at transcriptional and translational levels by PND 2 [8]. These effects persisted to PND 14, even after replacer-fed gilts were returned to nursing on PND 2, such that uterine gland development was markedly reduced by PND 14 [8]. Thus, effects of lactocrine signaling on the reproductive tract, evident as early as PND 2, can indicate changes in the organizational program that affect tissue developmental trajectory [1].

In the boar, Sertoli cell number, established during the prepubertal period in pigs [9], determines adult testis size

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and capacity for sperm production [10]. The first phase of Sertoli cell proliferation occurs during the first 2 wk of porcine neonatal life [9,10]. Leydig cell development, reflected by insulin-like factor 3 (INSL3) expression [11], is also significant during the first mo of neonatal life in the pig [9]. Accordingly, testicular steroidogenesis, marked by P450 side-chain cleavage (scc) enzyme expression [12], increases markedly during this period [13].

Lactocrine-sensitive endometrial gene products, including *ESR1*, *VEGFA*, and *RXFP1* [8], also affect testicular development, structure, and function. Localized to Sertoli, Leydig, peritubular myoid, and germ cells in the neonatal boar [14], ESR1 is required to sustain germ cells and stabilize seminiferous tubule structure [15,16]. Similarly, VEGFA is important for seminiferous tubule development and vascularization, as well as for maintenance of germ cells in developing testes [17,18]. Signaling through RXFP1, localized in Sertoli, Leydig, and germ cells of neonatal boars [19], supports sperm maturation and fertility [20,21]. Factors affecting neonatal porcine testis development, including Sertoli cell proliferation, Leydig cell development, and associated steroidogenic activity, can determine reproductive capacity in adulthood.

Whether lactocrine signaling is required to: (1) establish the neonatal testicular developmental program; or (2) determine testicular developmental trajectory has yet to be determined. To address the first of these questions, objectives were to determine effects of neonatal age and nursing from birth on: (1) Sertoli cell number and proliferation as reflected by GATA-4 expression [22] and proliferating cell nuclear antigen (PCNA) immunostaining patterns [23,24]; (2) Leydig cell development and steroidogenic activity indicated by *INSL3* and P450scc expression; and (3) *ESR1*, *VEGFA*, and *RXFP1* expression in testes collected from boars immediately after birth and on PND 2.

2. Materials and methods

2.1. Animals

Boars (*Sus scrofa domesticus*) were born from an established herd of crossbred (Duroc, Hampshire, Yorkshire, and Landrace) sows and raised at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. All procedures involving animals were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee (Protocol #88-709) and conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [25]. Care was taken to ensure that treatments were balanced for potential effects of litter (n = 16) by placing littermates into each of the treatment groups and insuring that sows were nursing litters of similar size. Based on a study suggesting that pigs of lower birth weight display below average postnatal development [26], boars were weighed at birth and included in the experiment if birth weight was 1.3 kg or greater. Body weights were recorded daily through PND 2. Pigs were randomly selected to be fed a nutritionally complete commercial pig milk replacer (Advance Liqui-Wean MSC Specialty Nutrition; Carpentersville, IL, USA) and housed in a pen adjacent to the sow and littermates.

2.2. Experimental design and tissue collection

At birth, crossbred boars were randomly assigned to treatment groups in which pigs were either: (1) euthanized for tissue collection at birth, before nursing (PND 0; n = 7); (2) nursed ad libitum for 48 h (PND 2N; n = 6); or (3) pan-fed milk replacer ad libitum (PND 2R; n = 6) for 48 h. Figure 1 depicts the experimental design. Boars were euthanized and testes were obtained on either PND 0, immediately after birth, or on PND 2 at 50 h of age after nursing or replacer feeding. Testes were trimmed of associated tissues and testicular wet weights (mg) were recorded. Each testis was cut into half and the halves were immersed in either RNA-Later and stored at −80°C, or fixed in Xpress Universal Molecular fixative (Sakura; Torrance, CA, USA).

2.3. Protein extraction and evaluation of P450scc and GATA-4 levels

Whole testicular tissue cross-sections (20 to 50 mg) were homogenized in 200-μL lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 300 mM NaCl, 1 mM $MgCl_2$, pH 7.5). Samples were then centrifuged (12,000 *g*, 4°C) for 15 min. Supernatant was removed and stored at −80°C. Protein concentration of supernatant was determined using the DC Protein Assay kit (Bio-Rad Laboratories; Hercules, CA, USA). To evaluate relative tissue levels of targeted proteins, testicular proteins (20–30 μg) were resolved on 12.0% total monomer, Bis-Tris-HCl-buffered polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membranes (Bio-Rad Laboratories; Hercules, CA, USA). In addition to sample testicular protein from neonatal boars representative of each day and treatment, protein isolated from adult porcine ovary was included on each gel and/or blot as a positive control for GATA-4 [27] and P450scc [28]. After blocking in 10.0% non-fat dry milk in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris [pH 7.5], 0.14 mM NaCl, 3 mM KCl, and 0.05% Tween-20), membranes were probed with either goat anti-mouse GATA-4 antibody (1:500; sc-1237; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat P450scc antibody (1:2,000; Ab-1244; Millipore, Darmstadt, Germany), or goat anti-human actin antibody (1:1,000; sc-1615; Santa Cruz Biotechnology; Santa Cruz, CA, USA) overnight at 4°C. After washing with TBST, blots were incubated with either HRP-conjugated rabbit anti-goat secondary antibody (GATA-4/actin; 1:2,000; Zymed;

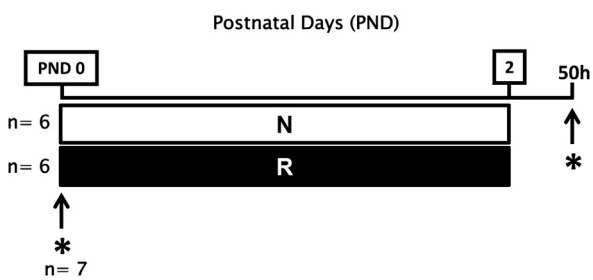

Fig. 1. Experimental design. Boars were assigned at birth (PND 0) to either nurse ad libitum (N) or to receive milk replacer by pan-feeding (R). Testes were collected on PND 0 or PND 2, as indicated by asterisks.