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Timing and duration of nursing from birth affect neonatal porcine uterine matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1

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ABSTRACT

Nursing for 2 d from birth supports neonatal porcine uterine and cervical development. However, it is not clear how timing or duration of lactocrine signaling from birth (postnatal day = PND 0) affects development of neonatal female reproductive tract tissues. Therefore, studies were conducted to determine effects of age at first nursing and duration of nursing from birth on specific elements of the matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) system in uterine and cervical tissues at PND 2. When nursing was initiated at 0 h or 30 min of age, targeted proteins, including proMMP9 and MMP9, were detected in uterine and cervical tissues on PND 2, as was uterine TIMP1. However, these proteins were undetectable when nursing was delayed for 12 h and when gilts were fed milk replacer for 48 h from birth. Increasing the duration of nursing from 30 min to 12 h from birth increased uterine ($P < 0.05$) and cervical ($P < 0.001$) MMP9 levels to those observed in gilts nursed for 48 h. Similarly, uterine TIMP1 levels increased with duration of nursing. Uterine MMP2 levels were detectable but unaffected by age at first nursing or duration of nursing from birth. Uterine MMP2 and MMP9 activities, monitored by zymography, reflected immunoblotting data. Results provide evidence for the utility of MMP9 and TIMP1 as markers of age- and lactocrine-sensitive porcine female reproductive tract development.

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1. Introduction

Nursing supports neonatal porcine reproductive tract development by delivery of milk-borne bioactive factors from mother to offspring as proposed in the lactocrine hypothesis [1,2]. Imposition of the lactocrine-null condition for 2 d from birth (postnatal day = PND 0), by feeding gilts a commercial milk replacer instead of colostrum, altered patterns of cervical [3] and uterine [4,5] gene expression at transcriptional and/or translational levels on PND 2. These effects persisted to PND 14 even after gilts were returned to nursing on PND 2 [4]. In the absence of lactocrine signaling for 2 d from birth, endometrial growth and uterine gland development were markedly retarded by PND 14 [4]. Thus, porcine uterine and cervical development is supported by lactocrine signals during the first 2 d of neonatal life. Whether a critical window for lactocrine signaling supportive of female reproductive tract (FRT) development exists within this 48 h neonatal period remains to be determined.

Colostrum is rich in milk-borne bioactive factors, and the composition of porcine colostrum, like that of other mammals [6], changes throughout the course of lactation [7,8]. Further, the composition of colostrum and milk can vary from anterior to posterior mammary glands [9], and

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consumption of colostrum by newborn pigs can be delayed if sows fail to initiate lactation or to produce sufficient colostrum for the litter [10,11]. In pigs, a window for transmission of colostral milk-borne bioactive factors to offspring is open before gut closure, which is estimated to occur within the first 48 h after birth [12]. Data indicating that lifetime fecundity of adult female pigs is markedly reduced when their colostrum consumption is minimal on the first day of neonatal life [7] emphasizes the importance of these issues for reproductive development. Whether timing of colostrum consumption relative to birth or duration of nursing from birth influences lactocrine signaling in the neonatal uterus is unknown.

A recent analysis of the neonatal porcine uterine transcriptome on PND 2 showed that expression of multiple matrix metalloproteinase (MMP) and associated genes was affected by age and lactocrine signaling between birth and PND 2 [5]. Matrix metalloproteases are proteolytic enzymes that facilitate extracellular matrix (ECM) remodeling and tissue growth during development and disease [13]. The activity of MMPs is regulated tightly by coexpression of specific tissue inhibitors of metalloproteinases (TIMPs) [14]. Uterine [15] and cervical [3] MMP9 protein levels increased in nursed gilts as compared to gilts fed milk replacer for 2 d from birth. Whether the MMP/TIMP system is affected by the timing or duration of nursing from birth in developing porcine FRT tissues is unknown. Objectives of this study were to determine the effects of: (1) age at first nursing and (2) duration of nursing from birth on specific elements of the cervical and uterine MMP/TIMP system at PND 2.

2. Materials and methods

2.1. Animals

Gilts (*Sus scrofa domesticus*) born from an established herd of crossbred (Duroc, Hampshire, Yorkshire, and Landrace) sows were 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 and conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010; Federation of Animal Science Societies, Champaign, IL, USA). Gilts were weighed at birth and included in the experiment if body weight was 1.3 kg or greater, based on studies suggesting that lower weight pigs display below average postnatal development [16]. Body weights were recorded daily until PND 2. Gilts that nursed remained with their dams, whereas those fed milk replacer (Advanced Liqui-Wean; Carpentersville, IL, USA) were housed in a separate pen in the same environment and in close proximity to their dams and littermates. All pens were equipped with heat lamps. Care was taken to insure that treatments were balanced for potential effects of litter ($n = 17$), and that sows were nursing litters of similar size.

2.2. Experimental design and tissue collection

Two experiments were conducted. To determine the effects of age at first nursing on uterine levels of targeted proteins (MMP2, MMP9, and TIMP1) and on cervical MMP9 at PND 2, crossbred gilts were assigned randomly at birth to treatment groups ($n = 5$/group) in which gilts were either: (1) nursed ad libitum for 48 h; (2) gavage-fed milk replacer from birth and switched to nursing at 0.5 h of age; (3) gavage-fed milk replacer from birth and switched to nursing at 12 h of age; or (4) gavage-fed milk replacer (30 mL/kg BW/2 h) for 48 h from birth (Fig. 1A). To determine effects of the duration of nursing from birth on the same endpoints, gilts ($n = 5$–6/group) were assigned randomly at birth to be either: (1) gavage-fed milk replacer for 48 h; (2) nursed ad libitum for 0.5 h from birth; (3) nursed for 12 h from birth and then gavage-fed milk replacer through 48 h; or (4) nursed ad libitum for 48 h from birth (Fig. 2A). In all experiments, pigs were euthanized at 48 h after birth when uterine and cervical tissues were removed, trimmed free of associated tissues, immersed in RNALater and stored at −80°C.

2.3. Western blot analyses

Whole cervical and uterine tissues (20 mg) were homogenized in lysis buffer (1% Triton X-100, 10% glycerol, 150-mm Tris-HCl, 300-mm NaCl, and 1-mm $MgCl_2$; pH 7.5) and incubated at 4°C for 30 min. Individual homogenates representing each tissue sample were centrifuged (12,000*g* at 4°C for 10 min), and protein supernatants were stored at −20°C. Total protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Tissue proteins (20 μg) were resolved on 12% (total monomer) NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) under reducing conditions followed by transfer onto nitrocellulose membranes (0.45-μm pore size; Bio-Rad Laboratories). Individual samples from each animal for each treatment were run in duplicate. Treatment effects on tissue MMP9 (pro 92 kDa, active 84 kDa), MMP2 (pro 72 kDa, active 66 kDa), and TIMP1 (28 kDa) protein levels were evaluated after immunoblotting using actin (43 kDa) as a loading control.

Nitrocellulose membranes were blocked with 10% nonfat milk powder (NFMP) in TBST buffer (25-mm Tris [pH 7.5], 0.14-mm NaCl, 3-mm KCl, and 0.05% Tween 20) for 1 h at room temperature. Membranes were then incubated with either mouse antihuman MMP2 (1:1000; IM33; EMD Millipore, Billerica, MA, USA), mouse antihuman MMP-9 (1:100; IM09L; EMD Millipore), rabbit antihuman TIMP1 (1:1000; C20; Santa Cruz Biotechnology, Dallas, TX, USA), or goat antihuman actin antibody (1:3000, C11; Santa Cruz Biotechnology) in TBST-5% NFMP overnight at 4°C. Human MMP family proteins are well conserved across species, and homology between human and pig proteins is high (96% for MMP2, 80% for MMP9, and 84% for TIMP1; BLAST, US National Center for Biotechnology Information). Antibodies directed against human MMP2, MMP9, and TIMP1 were validated for use in the pig uterus [17,18].

Membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat antimouse secondary antibody (1:2000 for MMP2 and MMP9; Invitrogen, Grand Island, NY, USA), Trueblot HRP mouse antirabbit IgG (1:2000 for TIMP1; Rockland, Limerick, PA, USA), or rabbit antigoat IgG (1:2000 for actin; Invitrogen) in