Cellular proliferation rate and insulin-like growth factor binding protein (IGFBP)-2 and IGFBP-3 and estradiol receptor alpha expression in the mammary gland of dairy heifers naturally infected with gastrointestinal nematodes during development

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ABSTRACT

Mammary ductal morphogenesis during prepuberty occurs mainly in response to insulin-like growth factor-1 (IGF-1) and estradiol stimulation. Dairy heifers infected with gastrointestinal nematodes have reduced IGF-1 levels, accompanied by reduced growth rate, delayed puberty onset, and lower parenchyma-stroma relationship in their mammary glands. Immunohistochemical studies were undertaken to determine variations in cell division rate, IGF-1 system components, and estradiol receptors (ESR) during peripubertal development in the mammary glands of antiparasitic-treated and untreated Holstein heifers naturally infected with gastrointestinal nematodes. Mammary biopsies were taken at 20, 30, 40, and 70 wk of age. Proliferating cell nuclear antigen immunolabeling, evident in nuclei, tended to be higher in the parenchyma of the glands from treated heifers than in those from untreated. Insulin-like growth factor binding proteins (IGFBP) type 2 and type 3 immunolabeling was cytoplasmic and was evident in stroma and parenchyma. The IGFBP2-labeled area was lower in treated than in untreated heifers. In the treated group, a maximal expression of this protein was seen at 40 wk of age, whereas in the untreated group the labeling remained constant. No differences were observed for IGFBP3 between treatment groups or during development. Immunolabeling for α ESR (ESR1) was evident in parenchymal nuclei and was higher in treated than in untreated heifers. In the treated group, ESR1 peaked at 30 wk of age and then decreased. These results demonstrate that the parasite burden in young heifers negatively influence mammary gland development, affecting cell division rate and parameters related to estradiol and IGF-1 signaling in the gland.
Key words: prepubertal mammary gland, proliferating cell nuclear antigen, insulin-like growth factor binding protein, estradiol receptor alpha

INTRODUCTION

Milk production is a function of the number of mammary epithelial cells and their secretory activity. The bulk of the mammary epithelium, which constitutes the parenchyma of the gland, grows allometrically (faster than the rest of the body) during the peripubertal period and pregnancy. It has been stated that future milk yield potential depends on the optimum development of the tissue that is established around the time of puberty (Akers et al., 2005). Mammary development during peripuberty consists of growth and branching of ducts of parenchymal cells, from the nipple to the inner of the gland, filling up the mesenchymal fat pad. This ductal morphogenesis occurs in response to specific hormones and growth factors that stimulate growth and differentiation in the different maturational stages of the gland (McNally and Martin, 2011). Both IGF-1 and estradiol are the main promoters of ductal growth during puberty and synergize in their action, whereas progesterone mainly acts during pregnancy, and also requires IGF-1 presence (Kleinberg and Barcellos-Hoff, 2011). This growth factor strongly stimulates parenchymal cell division during early mammary development and synergizes with estradiol for ductal growth and branching promotion (Hinck and Silberstein, 2005). Within the gland, paracrine interactions between stromal and parenchymal cells take place, which involve growth factors and their receptors, whose actions are finely tuned by external hormones (Forsyth, 1991; Akers et al., 2005; Kleinberg and Barcellos-Hoff, 2011). Particularly, the temporal expression of IGF-1, its re-

ceptors, and binding proteins has been demonstrated to change within the gland according to physiological state (development, lactation, and involution of the gland) in a highly regulated manner (Plath-Gabler et al., 2001).

In animals fed on pastures, natural infections with gastrointestinal nematodes are common. Gastrointestinal parasitism is a major constraint to animal health, productivity, and profitability in grazing livestock production systems. In Argentine farms, replacement dairy heifers are mostly grown outdoors and allowed free grazing on pastures when they are as young as 60 d of age, therefore they are vulnerable to high infection threat. Dairy heifers with gastrointestinal nematodes have reduced growth rate, delayed age at puberty and at production onset, and increased culling rate during the following first lactation (Mejía et al., 1999; Mejía et al., 2009). Altered profiles of serum IGF-1, leptin, and luteinizing hormone pulsatile secretion were associated with the detrimental effect of the nematodes (Lacau-Mengido et al., 2000; Díaz-Torga et al., 2001). Particularly, IGF-1 levels during the whole prepubertal and pubertal period were significantly reduced in the parasitized when compared with antiparasitic-treated age-matched animals (Lacau-Mengido et al., 2000). The IGF-1 is a key factor for mammary gland development during the peripubertal period, and, consequently, a detrimental effect of parasites on parenchymal development was recently demonstrated (Perri et al., 2013). In the present work, to define the mechanisms involved in differential gland development, we investigated whether anthelmintic treatment in naturally infected dairy heifers during early development could modify the cellular division rate of mammary parenchymal and stromal cells as well as the expression pattern of modulators in the gland, such as IGF-1 system components and estradiol receptor α (**ESR1**).

MATERIALS AND METHODS

The experiment was conducted at the dairy farm of the Experimental School of Inchausti, 25 de Mayo, Province of Buenos Aires, Argentina (35°36′S, 60°32′W). Forty-four female Holstein calves were randomly assigned to an untreated (**U**) or to a treated group (**T**) at birth. The T heifers received monthly, from birth to 18 mo of age, different anthelmintic drugs to minimize parasite burden avoiding drug resistance generation. The following drugs were used: ivermectin (0.63 mg/kg), fenbendazol (7.5 mg/kg), and levamisol (10 mg/kg; Perri et al., 2013).

All the heifers were raised together with the reposition herd of the dairy farm, outdoors on infected pastures. At birth, calves were placed in individual cages, directly on the pastures, which were moved when the floor turned dirty or humid. They stayed in cages during the first 2 mo of life, and during this time they were fed 2 L of warm milk twice a day and had ad libitum access to balanced supplement. Then, they were included in the grazing herd and grazed on alfalfa and (or) ryegrass pastures, in a rotational grazing system (stocking density: 8 animals/ha), with ad libitum access to the supplement. At 160 kg of BW, the supplement was changed to corn (2 kg/animal per day) and the stocking density was reduced to 2 animals/ha.

At 20, 30, 40, and 70 wk of age, mammary biopsies were taken from 6 heifers in each group (Perri et al., 2013). Briefly, heifers were sedated with 1% acepromazine (0.15 mg/kg, Holliday Scott S.A., Buenos Aires, Argentina) and immobilized in a supine position. Udders were cleaned with soap and water, then rinsed and disinfected with iodinated solution. The biopsy was taken 2 cm away from the nipple in the rear right quarter, under local anesthesia (5 mL of lidocaine 2% sc). Each biopsy (at 20, 30, 40, and 70 wk) was obtained in a different place avoiding previous cicatrix. A Tru-Core I fully automatic biopsy gun provided with a Tru-Core 14 gauge × 20-cm needle (Medical Device Technologies Inc., Gainesville, FL) was used. The needle directly pierced the skin and took a gland sample 3-mm wide × 1-cm long at approximately 2.5 cm of depth with minimal lesion. Mammary gland samples were immediately fixed in buffered 4% formaldehyde and transferred 24 h later to 70% ethanol until processed.

At the same moment, jugular blood and fecal samples were individually taken from all the heifers in the study for serum IGF-I determination by RIA and nematode egg counting [eggs per gram (**EPG**)] in feces. Blood samples were centrifuged and sera stored at −20°C for IGF-1 determination. Fecal samples were maintained at 4°C until examination. Extra blood samples for progesterone determination by RIA were obtained weekly from 20 wk of age until the end of the experiment, to establish age of puberty onset. All the procedures were consistent with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Histological and Immunohistochemical Procedures

Samples were fixed in buffered formaldehyde for 8 h at 4°C, then rinsed abundantly with tap water and PBS (pH 7.4; 0.01 *M*), subjected to dehydration in increasing concentrations of ethanol, washed in xylol, and embedded in paraffin. Serial sections, 5-μm thick, were cut with a manual microtome and mounted on glass slides previously coated with 3-aminopropyl-triethoxysilane (Sigma-Aldrich, St. Louis, MO). Slides